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METABOLIC AND NEUROCHEMICAL STUDIES ON SOME SEROTONINERGIC ANTIDEPRESSANTS

BY

LIANA J. URICHUK 

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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: METABOLIC AND NEUROCHEMICAL STUDIES ON SOME SEROTONINERGIC ANTIDEPRESSANTS hereby submitted by LIANA J. URICHUK in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

This thesis is dedicated to my parents, Orest and Joanne Urichuk, who never lost faith in me, and to my husband, Regan Wolansky, who stood by me through it all.

ABSTRACT

A number of studies pertaining to metabolic and neurochemical issues of some serotoninergic antidepressants have been addressed in this thesis. The first series of experiments investigates and compares the effects of a nonselective, irreversible monoamine oxidase (MAO) inhibitor, phenelzine(PLZ) to the selective, reversible inhibitors of MAO-A, moclobemide (MOC) and brofaromine (BROF). Data in these experiments revealed that all 3 drugs produced a down-regulation of tryptamine receptors in brain, with PLZ and MOC producing a decrease in the density of receptors and BROF producing an apparent decrease in the affinity of the radioligand for the receptor. In contrast, however, only PLZ produced a significant down-regulation of serotonin $(5\text{-HT})_{2A}$ receptors despite the fact that all three drugs significantly increased brain levels of 5-HT. Other neurochemical investigations on γ-aminobutyric acid (GABA), urinary levels of tryptamine, 3-methoxy-4-hydroxy-phenylglycol and β-phenylethylamine and on levels of MAO-A and -B activity in rat brain revealed marked differences between both MOC and BROF and PLZ on aminergic and GABAergic mechanisms in rat brain.

Attention was then directed towards investigation of the formation of some novel metabolites of fluoxetine (FLU; Prozac®). These studies resulted in the development of two novel analytical techniques that allowed for the identification and quantitation of several novel metabolites of FLU [namely trifluoromethylphenol (TFMP), γ-hydroxyphenylpropylamine (HPPA) and N-methyl-γ-hydroxyphenyl-propylamine (NMHPPA) in rat and human biological samples. These studies also

revealed that both HPPA and NMHPPA can be deaminated by MAO, a finding which supports an alternative metabolic pathway that has been proposed for FLU.

Preliminary experiments utilizing cDNA-expressed human CYP isozymes and human liver microsomes were conducted to determine which cytochrome P450 (CYP) isozymes might mediate the metabolism of FLU. Analyses revealed that multiple CYP isozymes may play a role in the formation of metabolites of FLU *in vitro* including CYP1A2, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, CYP2D6 and CYP3A4.

A series of experiments were also conducted to examine potential drug-drug interactions between MOC and FLU using human liver microsomal preparations. The data from these experiments suggest that there may be a potential for pharmacokinetic MOC/FLU interactions if they are administered concomitantly and this may be due to the inhibition of CYP3A4, CYP2C19 and CYP1A2.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere gratitude towards my supervisor, Dr. Glen B. Baker and my co-supervisor, Dr. Ronald T. Coutts. The guidance and support you have given me has been invaluable and the extent of your knowledge will forever hold me in awe. I would also like to thank the Alberta Heritage Foundation for Medical Research (Mental Health) for providing financial support for the duration of my program.

The technical assistance I received from Jordyce van Muyden, Gail Rauw, Carolyn Kuefler and Rick Strel was excellent and I hope that they will continue to provide me with insight in my future endeavors.

I will always remember my time spent at the Neurochemical Research Unit where I acquired not only knowledge, but also wonderful friendships, many "coffee room conversation" memories and hundreds of recipes.

To my family, even though you were never exactly sure of what I was trying to accomplish (or what I would be when I finished), you always supported me. For this, I thank you. And to Regan, you were my light at the end of a very long tunnel. I will never forget your unwaivering support, the sacrifices you made for me and your unconditional love. I am looking forward to moving on with the rest of our lives.

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LIST OF ABBREVIATIONS

AAAD	aromatic amino acid decarboxylase
b.i.d.	*bis in die;* twice a day
B_{max}	density of receptors
BROF	brofaromine
Ca^{2+}	calcium ion
cDNA	complementary deoxyribonucleic acid
Ci	curies
CI	chemical ionization
CNS	central nervous system
CYP	cytochrome P450
DA	dopamine
DAG	diacylglycerol
DMI	desipramine
DMSO	dimethyl sulfoxide
DSP4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
ECD	electron capture detector
EI	electron impact
EM	extensive metabolizer
FID	flame ionization detector
FLU	fluoxetine
g	gram
GABA	γ-aminobutyric acid
GABA-T	γ-aminobutyric acid transaminase
GC	gas chromatography
GI	gastrointestinal
h	hour
5-HIAA	hydroxyindole-3-acetic acid
H_2O_2	hydrogen peroxide
5-HT	5-hydroxytryptamine, serotonin

HP	Hewlett Packard
HPLC	high performance liquid chromatography
HPPA	γ-hydroxyphenylpropylamine
HVA	homovanillic acid
i.d.	internal diameter
IAA	indole-3-acetic acid
IC_{50}	concentration producing 50% inhibition
IP_3	inositol triphosphate
K^+	potassium ion
K_d	equilibrium dissociation constant or affinity of a receptor for a ligand
K_M	dissociation constant
L	litre
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
mCi	millicurie
MHPG	3-methoxy-4-hydroxyphenylglycol
ml	millilitre
mmol	millimole
min	minutes
MOC	moclobemide
MS	mass spectrometry
NA	noradrenaline
Na^+	sodium ion
NADPH	β-nicotinamide adenine dinucleotide phosphate
NCI	negative chemical ionization
NFLU	norfluoxetine
ng	nanogram
nm	nanometer
nM	nanomolar
NMHPPA	N-methyl-γ-hydroxylphenylpropylamine
NPD	nitrogen-phosphorus detector

ODS	octadecylsilane
p.o.	*per os* (oral) administration
PCPA	p-chlorophenylalanine
PCR	polymerase chain reaction
PEA	β-phenylethylamine
PFBC	pentafluorobenzoyl chloride
PFBSC	pentafluorobenzenesulfonyl chloride
PIP_2	phosphatidylinositol-4,5-bisphosphate
PLZ	phenelzine
PM	poor metabolizer
r^2	correlation coefficient
RFLP	restriction fragment length polymorphism
RIMA	reversible inhibitor of MAO-A
s	seconds
SCOT	support-coated open tubular
SIM	single ion monitoring
SSRI	selective serotonin reuptake inhibitor
T	tryptamine
TCA	tricyclic antidepressant
TCD	thermal conductivity detector
TCP	tranylcypromine
TFMP	*p*-trifluoromethylphenol
TIM	total ion monitoring
TRP	tryptophan
$t_{½}$	elimination half-life
UV-Vis	ultraviolet-visible
WCOT	wall-coated open tubular
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar

V_{max} maximum rate of reaction

CHAPTER 1

General Introduction

1.1 INTRODUCTION

Major depression is one of the most prevalent psychiatric diseases, with an incidence of 4-5% in the general population (Boyd and Weissman, 1982; Holden, 1986). Current antidepressant therapy is effective in treating about 70% of depressed patients (Quitkin et al., 1984). The remaining 30% of depressed patients who do not respond to the usual antidepressants at normal doses are termed refractory depressives. Strategies that have been employed for this latter group of depressed patients have included high doses and/or combinations of antidepressant drugs even though information may be scarce on the metabolism of the drugs utilized and the possibility of drug-drug interactions.

Monoamine oxidase (MAO) inhibitors were shown many years ago to have antidepressant effects and to increase brain levels of the biogenic amines, including noradrenaline (NA) and serotonin (5-hydroxytryptamine; 5-HT) [Zellar et al., 1952; Brodie et al., 1956]. These observations, together with the discovery that the tricyclic antidepressant (TCA) imipramine inhibited the reuptake of NA back into the presynaptic nerve terminal after it was released (Glowinski and Axelrod, 1964; Iversen, 1971), formed the basis for the biogenic amine theory of depression. Imipramine was later shown to inhibit the reuptake of 5-HT as well. Simply stated, the "biogenic amine theory" indicates that depression may be the result of a functional deficiency of NA and/or 5-HT at certain brain synapses (Schildkraut, 1965; Lapin and Oxenkrug, 1969). The role of NA in the pharmacotherapy of depression became questionable, however, as more and more evidence implicated

5-HT as being the biogenic amine most involved in antidepressant therapy (Smith et al., 1997). For example, the impairment of 5-HT synthesis led to a transient reappearance of depressive symptoms in patients who had been successfully treated with an antidepressant (Shopsin et al., 1975) and tryptophan, a precursor to 5-HT, was reported to have mood elevating effects (Coppen et al., 1963; Pare, 1963; Glassman and Platman, 1969). Several studies found that tryptophan (TRP) depletion resulted in a considerable lowering of mood in both depressed patients who had been successfully treated with antidepressants (Delgado et al., 1990) and in normal male subjects who were considered to be at risk for developing depression (Young et al., 1985; Benkelfat et al., 1994). In addition, the tremendous success of the selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine (FLU; Prozac®), for the treatment of depression further implicated 5-HT as being important to the etiology and pharmacotherapy of depression.

Other problems with the biogenic amine theory of depression became apparent when trying to explain the following contradictions:

1. Biochemical changes such as inhibition of MAO or inhibition of NA and 5-HT uptake occur soon after antidepressant administration, yet clinical improvement requires several days or weeks (Oswald et al., 1972; Quitkin et al., 1986).
2. There are several "novel" antidepressants which are effective even though they do not inhibit MAO or affect NA or 5-HT uptake (Freeman and Sulser, 1972; Sulser et al., 1978).
3. Using amino acid precursors of NA and/or 5-HT as antidepressant

agents produces inconsistent effects on depressed patients (Coppen et al., 1972;Sharp et al., 1992).

In 1972, Ashcroft et al. proposed a modified amine hypothesis suggesting that interactions among neurochemical systems and effects on postsynaptic receptors were important to the treatment of affective disorders. Indeed, it was soon discovered that chronic treatment with drugs from various classes of antidepressants led to a down-regulation of cortical β-adrenoceptors (due to an increased availability of NA) or 5-HT_2 receptors (due to increased availability of 5-HT) [Charney et al., 1981; Enna et al., 1981; Baker and Greenshaw, 1989]. It was suggested that the length of time required for these receptor changes to occur correlated with the delay in clinical response. Electrophysiological studies have shown that acute administration of an SSRI or MAO inhibitor (MAOI) decreases the firing of 5-HT neurons in the dorsal raphé nucleus of the rat brain (Aghajanian et al., 1990; Blier et al., 1987). With longer term treatment, however, firing activity recovers. The mechanism responsible for this is thought to be the desensitization of somatodendritic 5-HT_{1A} autoreceptors (whose role is to regulate neuronal firing) and, perhaps, of terminal autoreceptors, which would result in an increase in the amount of 5-HT released per action potential (Blier and de Montigny, 1994; Artigas et al., 1996). Thus, during the initial phases of antidepressant treatment, the reduction in firing of 5-HT neurons would likely diminish or dampen the increase in 5-HT concentrations in the synaptic cleft that was produced by the inhibition of either MAO or 5-HT reuptake (Artigas et al., 1996). Together, these phenomena

are consistent with the delay in antidepressant response. Recently, however, there has been an interest in combining an antidepressant that increases 5-HT availability with a 5-HT_{1A} autoreceptor antagonist, such as pindolol, to reduce the latency in the onset of action of antidepressant response by preventing the negative feedback at the somatodendritic level (Artigas et al., 1996; Maes et al., 1996; Romero et al., 1996; Berman et al., 1997).

The knowledge that 5-HT_{1A} agonists have been shown to be effective antidepressants (Robinson et al., 1989; Schweizer et al., 1986) and that 5-HT_{1A} receptors have been implicated in anxiety (Deakin, 1988) warrants some discussion. There is increasing epidemiological evidence supporting the comorbidity of depression and anxiety (Deakin, 1988; Nutt and Glue, 1991; Weissman, 1992). In addition, there is also an abundance of research supporting functional interactions between 5-HT_{1A} and 5-HT_2 receptors (Darmani et al., 1990; Araneda and Andrade, 1991; Deakin et al., 1991; Dursun and Handley, 1993). This literature may help explain the following observations:

1. Antidepressants of various types increase postsynaptic 5-HT_{1A} function (de Montigny et al., 1989, 1990; Blier et al., 1985; Newman et al., 1993).
2. 5-HT_{1A} agonists, although indicated for anxiety disorders, are apparently effective antidepressants (Robinson et al., 1989; Schweizer et al., 1986).
3. 5-HT_2 antagonists have significant anxiolytic properties (Deakin and Wang, 1990; Deakin, 1988).

4. Chronic treatment with 5-HT_{1A} agonists has been reported to produce a down-regulation of 5-HT_2 receptors in rat brain (Benjamin et al., 1990; Schechter et al., 1990; Taylor and Hyslop, 1991; Frazer et al., 1991; Dursun and Handley, 1993), suggesting a common 5-HT_2 receptor mechanism of action involved in both anxiety and depression (Schreiber and DeVry, 1993).

5. Chronic administration of antidepressants has also been shown to decrease anxiety (Klein, 1964; Bodnoff et al., 1988; Kahn et al., 1986).

Because the symptoms (Goldberg et al., 1987; Kellar, 1992) and the treatments for anxiety and depression overlap, it has been suggested that they are manifestations of a single disorder (Deakin, 1988). It is also possible that imbalances between 5-HT_{1A} and 5-HT_2 receptors could lead to either anxiety or depression, and the most potent therapeutic agents may prove to be those which can effectively restore the balance (Deakin et al., 1991; Schreiber and DeVry, 1993).

1.2 ANTIDEPRESSANT DRUG CLASSES

1.2.1 *Tricyclic Antidepressants*

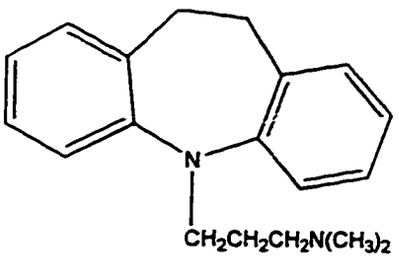
The tricyclic compounds were first developed in the 1950s and remained the cornerstone of antidepressant therapy for about 30 years (Preskorn, 1994). Most tricyclic antidepressants (TCAs) consist of two benzene rings joined by a seven-member ring [Figure 1-1]. The prototypical TCA, imipramine, is a structural analogue of chlorpromazine (a neuroleptic) and was originally developed in the

search for better neuroleptic agents (Hyman et al., 1995). The antidepressant properties of imipramine were discovered serendipitously in the late 1950s while investigating the actions of phenothiazine derivatives in depressed patients (Kuhn, 1970; Khan et al., 1986).

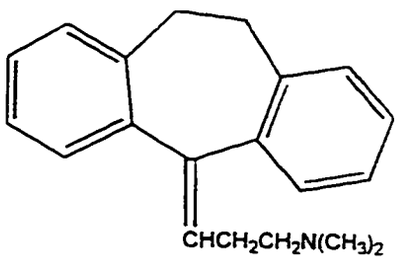
TCAs are rapidly and completely absorbed from the gastrointestinal (GI) tract after oral administration. Because they are also highly lipophilic, the free fraction can pass into the brain and several other tissues (Baldessarini, 1985) where they strongly bind to proteins (including plasma proteins). Most of the oral dose of a TCA undergoes extensive first-pass metabolism in the liver, so that only 50-60% of an orally administered dose reaches the systemic circulation (Preskorn, 1993; Hyman et al., 1995). Some of the active or inactive metabolites may be reabsorbed in the gut and eventually taken into the systemic circulation. Metabolism occurs *via* four main routes:

1. Desmethylation of the side chain - the tertiary amine tricyclics undergo side chain desmethylation to yield active metabolites.
2. N-oxidation of the side chain - may result in weakly active compounds.
3. Hydroxylation at various positions of the ring structure - may result in active metabolites that are more cardiotoxic than the parent drugs.
4. Conjugation with glucuronic acid - produces inactive compounds (Rudorfer and Potter, 1989; Hyman et al., 1995).

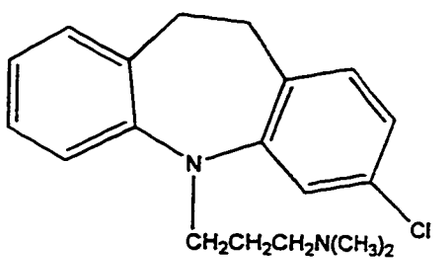
Tertiary Amines:



Imipramine

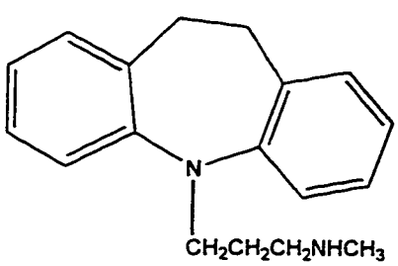


Amitriptyline

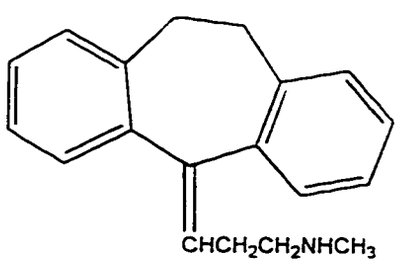


Clomipramine

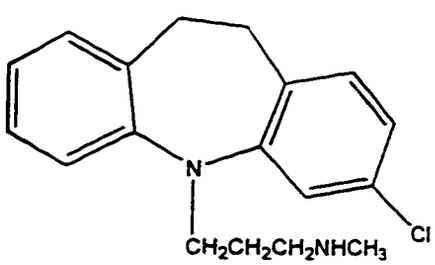
Corresponding Secondary Amines:



Desipramine



Nortriptyline



N-desmethylclomipramine

Figure 1-1: Structures of some tertiary tricyclic antidepressants and their corresponding secondary amine tricyclic metabolites (desipramine and nortriptyline are also marketed as antidepressants).

It should be acknowledged at this point that although the metabolites of specific antidepressants may be deemed inactive from the perspective of antidepressant effect, they may not be devoid of inhibitory effects on enzymes that may ultimately affect further metabolism (Riesenman, 1996).

There are wide interindividual variabilities in the metabolism of TCAs. Much of this variability is due to genetically determined differences in biotransformation rates, as will be discussed later. However, concurrent disease states can affect clearance rates of the TCAs and concomitant drug use may result in induction or inhibition of the hepatic enzymes involved in metabolizing the TCAs; thus these factors may also contribute to interindividual variation in metabolism (Preskorn, 1993). The mechanism believed to be responsible for the antidepressant effect of TCAs is inhibition of the neuronal uptake pumps for NA and 5-HT, an effect which ultimately increases concentrations of these biogenic amines at their receptor sites (Carlsson et al., 1969, Shaskan and Snyder, 1970; Ross et al., 1972; Hyman et al., 1995). The TCAs, however, possess different uptake inhibition profiles. The tertiary amine tricyclics (imipramine, amitryptiline, and clomipramine) inhibit both NA and 5-HT uptake, but are relatively more potent at inhibiting the latter. The secondary amine tricyclics (desipramine, nortriptyline and desmethylclomipramine) are more potent inhibitors of NA uptake than their corresponding tertiary amine parent drugs (Hyttel, 1982; Baker and Greenshaw, 1989).

TCAs have multiple mechanisms of action and, thus, people taking them may experience multiple effects. Some potential undesired effects of TCAs include the inhibition of sodium (Na^+) fast channels and the Na^+:potassium (K^+) uptake pump,

which could potentiate cardiovascular difficulties such as hypo/hypertension, tachycardia and arrhythmias. In addition, the blockade of several neurotransmitter receptors may lead to a wide array of side effects. Those commonly seen due to muscarinic cholinergic receptor blockade include blurred vision, constipation, urinary retention, dry mouth and excessive sweating (Snyder and Yamamura, 1977). α-Adrenergic receptor blockade can cause tachycardia, hypo/hypertension, arrhythmias and sedation (U'Prichard et al., 1978) and H_1-histaminergic receptor blockade can contribute to sedation and orthostatic hypotension (Diffley et al., 1980). The tertiary amine tricyclics tend to be more potent at blocking these neurotransmitter receptors than the secondary amines (Potter and Manji, 1990). Potentially lethal side effects may also occur when TCAs are given concomitantly with other drugs (such as MAOIs). These interactions are discussed in more detail in section 1.4.3.

1.2.2 *Monoamine Oxidase Inhibitors*

The discovery of the MAOIs was made in the 1950s when searching for antitubercular drugs. During this time it was noted clinically that iproniazid not only alleviated tuberculosis symptoms, but also caused euphoria in some patients (Selikoff et al., 1952). Subsequently, iproniazid was shown to be an inhibitor of the enzyme MAO. Soon after iproniazid was introduced as an antidepressant in 1959 it had to be withdrawn because it was found to cause serious liver damage, but other MAOIs were soon developed to replace it.

MAO is found primarily on the outer membrane of mitochondria and is

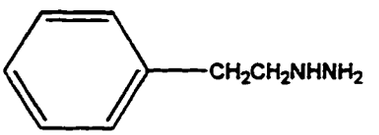
responsible for the oxidative deamination of endogenous and exogenous amines (Fowler and Ross, 1984; Tiller, 1993). In presynaptic nerve terminals, MAO metabolizes free biogenic amines, while in the liver and gut MAO metabolizes bioactive amines that are ingested in foods (Hyman et al., 1995). In 1968 it was discovered that there were two forms of MAO: A and B (Johnson, 1968; Fitton et al., 1992; Saura et al., 1992). These two forms are encoded by two different genes (Bach et al., 1988; Powel et al., 1988) and clearly differ in their tissue and cellular distribution and their substrate specificities (Johnson, 1968). In terms of distribution, most tissues, including the brain (Murphy and Donnelly, 1974) and the blood-brain barrier (Yu, 1984), contain a mixture of both MAO-A and -B. Exceptions to this would be the lining of the gut wall, GI tract and the placenta where MAO-A is found almost exclusively and in the platelets where MAO-B is the only isoform found (Kanazawa, 1994; Mayersohn and Guentert, 1995). In addition, human liver contains more MAO-B than -A (Mayersohn and Guentert, 1995). As previously mentioned, both forms of MAO are found in the brain in humans, but they are distributed differentially. Although MAO-B is more predominant overall (i.e. about 70-75% MAO-B: 25-30% MAO-A), the relative distribution of the two isoforms varies throughout the brain (Mayersohn and Guentert, 1995; Luque et al., 1996). Autoradiographic procedures confirmed the abundance of MAO-A in the locus ceruleus and dorsal vagus nucleus while MAO-B was abundant in the raphé nucleus (Saura Marti et al., 1990). As opposed to the human, in the rat MAO-A is the predominant isoform found in brain. In terms of substrate specificity, MAO-A preferentially deaminates NA and 5-HT whereas the preferential substrates for

MAO-B are β-phenylethylamine (PEA) and benzylamine (Freeman, 1993; Hyman et al., 1995). Selective inhibitors of MAO-A and -B are clorgyline and (-)-deprenyl, respectively (Johnson, 1968; Finberg and Youdim, 1983).

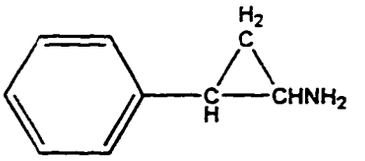
Both neurons and glia contain MAO. MAO-A is found predominantly in catecholaminergic neurons while MAO-B is prevalent in the glia (Leonardi and Azmitia, 1994). It is interesting to note that even though MAO-A has a higher affinity for 5-HT than does MAO-B (99 μmol/L and 1170 μmol/L, respectively), MAO-B is the major molecular form found in serotonergic neurons (Leonardi and Azmitia, 1994; Luque et al., 1996).

The MAOIs can be classified according to chemical structure (i.e. hydrazine *vs.* non-hydrazine), relative selectivity for subtypes of MAO (i.e. A or B), or by degree of affinity for enzyme inhibitor sites (i.e. reversible or irreversible) [Thase et al., 1995]. For many years, the most commonly prescribed MAOIs for psychiatric disorders have been phenelzine (PLZ) and tranylcypromine (TCP) [Figure 1-2], both of which are nonselective and irreversible (Dubovsky, 1987). These MAOIs can affect many other systems in the body in addition to MAO, including receptors and uptake mechanisms for neurotransmitters and enzymes other than MAO (Baker et al., 1991) and, thus, have many potential side effects. The most common side effect of MAOIs is orthostatic hypotension (Murphy et al., 1985). Other side effects include anticholinergic effects (Baldessarini , 1985), weight gain, sexual dysfunction, edema, insomnia, daytime sedation, monoclonus, decreased rapid eye movement (REM) sleep, possible stimulant effects (Cooper et al., 1991) and the tyramine pressor effect (Murphy et al., 1985). Because the MAOIs PLZ and TCP are struc-

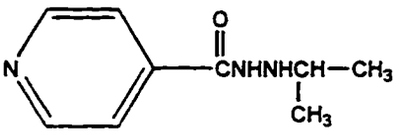
Irreversible MAOIs:



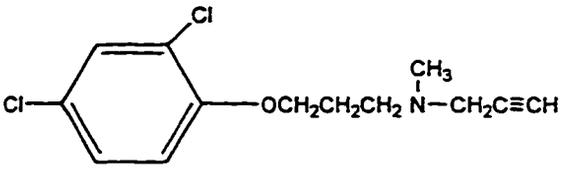
Phenelzine



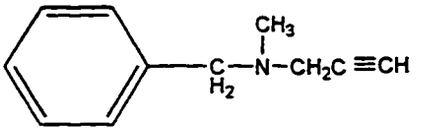
Tranylcypromine



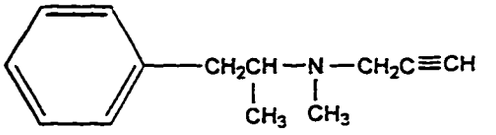
Iproniazid



Clorgyline

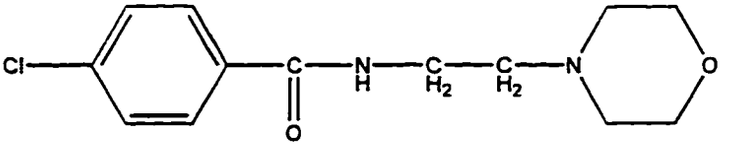


Pargyline

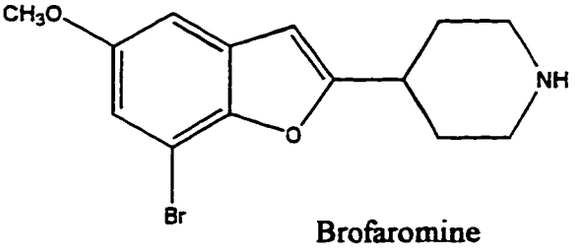


Deprenyl

RIMAs:



Moclobemide



Brofaromine

Figure 1-2: Structures of selected monoamine oxidase inhibitors.

turally similar to the catecholamines and psychostimulants such as amphetamine. some investigators have proposed that the parent compounds or active metabolites may be responsible for some of the stimulant effects that have been observed in some patients taking MAOIs (Cooper et al., 1991). The tyramine pressor effect or the "cheese effect" is rare, but is the most serious toxic reaction to MAOIs. This side effect, with symptoms ranging from headaches to hypertensive crisis to stroke, may occur when patients taking an MAOI consume food (i.e. aged cheese, red wine, chocolate, yeast products) rich in sympathomimetic amines such as *p*-tyramine. The "cheese effect" occurs as a result of the inhibition of intestinal MAO-A, which allows tyramine to reach pharmacologically active plasma concentrations and to be taken into noradrenergic neurons *via* an uptake mechanism. Within the cytosol, *p*-tyramine has access to the synaptic vesicles and can displace the stored NA which will subsequently leave the neuron and cause an over-stimulation of adrenergic receptors and a dramatic elevation in blood pressure (Haefly et al., 1992; Sandler, 1981). Health care professionals can help patients avoid this potentially toxic side effect by giving them a list of foodstuffs to avoid.

A potentially life-threatening drug interaction involves the concomitant use of MAOIs and other agents that are serotonergic, including certain tricyclics (i.e. especially clomipramine), SSRIs and buspirone (Hyman et al., 1995). In combination with MAOIs, these agents may result in a "serotonin syndrome" which may be mild (i.e. tachycardia, hypertension, fever) or severe (i.e. hyperthermia, coma, convulsions, death) [Baldessarini, 1989].

The threat of serious side effects and the imposed dietary restrictions greatly reduced the clinical use of MAOIs in most countries (Freeman, 1993). However, some researchers proposed that MAOIs may be more effective than other antidepressants in treating "atypical" depression (characterized by irritability more than dysphoria and by hypersomnia, hyperphagia and psychomotor agitiation) [Preskorn, 1993]. In addition, as reviewed by Murphy et al. (1987), the MAOIs may be more effective against major depressive disorder than originally thought. Thus, drugs that would inhibit only one form of MAO have been developed in the hope that they would be safer clinically. Clorgyline is an irreversible MAO-A inhibitor that has been reported to have good antidepressant properties, but also the ability to produce the "cheese effect" (Murphy et al., 1985). (-)-Deprenyl is an irreversible inhibitor of MAO-B that is used extensively in the treatment of Parkinson's Disease, but is not a particularly successful antidepressant (Mann et al., 1989). Moclobemide (MOC; Manerix®) [Figure 1-2] is a selective, reversible inhibitor of MAO-A which has been used successfully as an antidepressant and has produced encouraging results as an antipanic agent (see Fulton and Benfield, 1996). Brofaromine (BROF) [Figure 1-2] is also a selective, reversible inhibitor of MAO-A and, although it has produced encouraging results as an antidepressant and as an effective treatment for social phobia (van Vliet et al., 1996; Volz et al., 1996; van Vliet et al., 1993), BROF has recently been dropped as a potential drug for clinical use due to patent protection issues. MOC and BROF do not cause the characteristic problems of the mixed MAO-A/B inhibitors (Da Prada et al., 1990; Volz et al., 1994). To help distinguish these newer agents from the older, more problematic ones, a new acronym was

introduced: **R**eversible **I**nhibitor of **M**onoamine oxidase type **A** (**RIMA**) [Thase et al., 1995).

The basic mechanism of action of the MAOIs is to bind to and deactivate MAO, which ultimately results in a potentiation of the effects of the biogenic amine neurotransmitters (NA, DA and 5-HT), thus alleviating depression. In addition to inhibiting the metabolism of 5-HT, NA, and DA, MAOIs also inhibit the metabolism of trace amines (i.e. β-phenylethylamine, octopamine, tryptamine, tyramine and N-methylhistamine) and may cause profound elevations in brain levels of these amines which may contribute to the antidepressant action of this class of drugs (Philips and Boulton, 1979; Phillips et al., 1980; Baker et al., 1985).

Although MAOIs and RIMAs are used predominantly for atypical depression (Himmelhoch et al., 1982; Thase et al., 1992; Quitkin et al., 1994), they have also been proposed to be effective in other psychiatric disorders such as seasonal affective disorder (Rosenthal et al., 1987), obsessive compulsive disorder (Jenike et al., 1983), phobic disorders and panic attacks (Sheehan et al., 1980; Nutt and Glue, 1989; Gitow et al., 1994; Johnson et al., 1994), general anxiety (Sheehan et al., 1980), bulimia (Walsh et al., 1984; Kennedy and Goldbloom, 1994) and migraine (Anthony and Lance, 1969). As mentioned above, they may also be more effective in treating major depressive disorder than was originally thought (Murphy et al., 1987; Johnson et al., 1994).

Two RIMAs, MOC and BROF, are of specific interest to this thesis and are discussed in detail below.

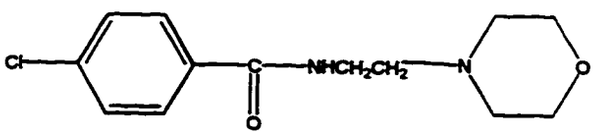
1.2.2.1 *Moclobemide*

The antidepressant efficacy of MOC, *p*-chloro-N-[2-morpholino-ethyl]benzamide (experimental name Ro 11-1163), has been well established, and the drug is used in over 50 countries worldwide (Dingemanse et al., 1995). MOC is structurally different from the classical MAOIs as it is a benzamide derivative [see Figure 1-2]. Single, oral doses of MOC given to healthy volunteers produced an increase in levels of NA, DA, 5-HT and some O-methylated catecholamine metabolites, a decrease in levels of homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) which are metabolites of DA, 5-HT and NA, respectively [Kettler et al., 1990; Freeman, 1993], and virtually no effect on/or affinity for muscarinic, dopaminergic, serotonergic, adrenergic, opioid or benzodiazepine receptors (Freeman, 1993). MOC is only about 50% protein bound and is almost completely absorbed from the GI tract following oral administration, with approximately 95% of the drug cleared renally within 24 hours (Mayersohn and Guentert, 1995). Neither advanced age nor impaired renal function changes plasma concentrations after a MOC dosage, but liver disease drastically decreases the elimination capacity of the liver for MOC (so dosage must be decreased in liver-impaired subjects).

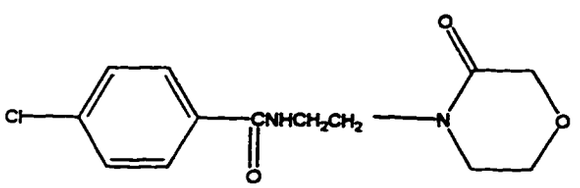
The results of several tyramine interaction studies with MOC clearly indicate that the 'cheese effect' is greatly reduced with this reversible and selective inhibitor relative to the nonselective MAO-A inhibitors (Waldmeier, 1985; Youdim et al., 1988). The weak potentiation of the tyramine pressor effect may be attributed to the ability of tyramine to displace MOC from the enzyme's active site due to competitive

interaction (Mayersohn and Guentert, 1995; Waldmeier et al., 1994; Waldmeier, 1985). Because both clorgyline (an irreversible MAO-A inhibitor) and high doses of (-)-deprenyl (an irreversible MAO-B inhibitor which also inhibits MAO-A at high doses) markedly potentiate the tyramine pressor response, reversibility may be a major contributing factor for the weak tyramine pressor effect of MOC (Mayersohn and Guentert, 1995). The most frequent adverse effects associated with MOC are increased anxiety or restlessness, dizziness, nausea, dry mouth and insomnia (Mayersohn and Guentert, 1995).

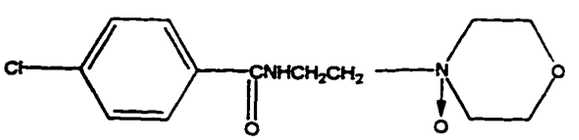
The primary routes of metabolism of MOC involve oxidative attack on the morpholine moiety where both C- and N-oxidation and deamination may occur (Jauch et al., 1990; Mayersohn and Guentert, 1995) [Figure 1-3]. Aromatic ring hydroxylation is another primary route of metabolism of MOC. Among the known metabolites of MOC, two have been found in human plasma: Ro 12-5637 and Ro 12-8095. Ro 12-5637, an N-oxide, is generally present only in trace amounts, but does retain some MAO-A inhibitory activity. Ro 12-8095 , although also present in plasma, is inactive (Mayersohn and Guentert, 1995). It is important to note that after multiple doses, MOC appears to inhibit its own metabolism. This may be due to inhibition of metabolism by a metabolite of MOC that is formed during its biotransformation (Mayersohn and Guentert, 1995). *In vivo*, MOC is a highly selective inhibitor of MAO-A, but this inhibition is less pronounced *in vitro* (Waldmeier et al., 1994), suggesting that a more active, so far unidentified metabolite, might exist in the plasma (DaPrada et al., 1989; Waldmeier et al., 1994). This also follows from the fact that, in the rat, MOC is more potent following oral *vs.*



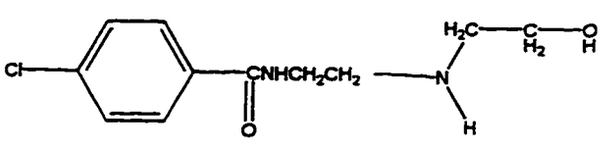
Moclobemide (Ro 11-1163)
[< 1%]



Ro 12-8095
[< 1%]



Ro 12-5637
[35%]



Ro 16-3177
[2%]

Figure 1-3: Some metabolites of moclobemide that were pertinent to this thesis. Detailed metabolic pathways for moclobemide can be found in Waldmeier et al., 1994.

subcutaneous administration (DaPrada et al., 1989).

Extensive biotransformation of MOC leading to opening of the morpholine ring is much more pronounced in rats than in humans (Waldemeier et al., 1994). This is consistent with the observation that the metabolism of morpholine derivatives of several xenobiotics is markedly different between different species (Waldmeier et al., 1994).

1.2.2.2 *Brofaromine*

BROF is a tight-binding, reversible inhibitor of MAO-A and has a longer duration of action and considerably more activity *in vitro* than MOC. The major differences between BROF and MOC are that the former is more highly protein bound and also has concomitant 5-HT uptake-inhibiting properties (Waldmeier and Stöcklin, 1989; Waldmeier et al., 1993). It has been suggested that this 5-HT uptake inhibition may play a role in the clinical effects of BROF because the dose required to obtain antidepressant action is 2-3 times higher than the dose required for MAO inhibition (Waldmeier et al., 1993), indicating that some other mechanism may be playing a role. Orally administered tyramine can displace ^{3}H-brofaromine from intestinal MAO (Waldmeier and Stöcklin, 1992), and this is consistent with the observation that BROF only weakly potentiates tyramine (Da Prada et al., 1984).

The major metabolic route for BROF is O-demethylation to O-desmethylbrofaromine *via* the action of CYP2D6 (Jedrychowski et al., 1993) [Figure 1-4], then subsequent conjugation and excretion. O-Desmethylbrofaromine is an

active metabolite and it is 6 times more potent than BROF at inhibiting 5-HT uptake, but 100 times less potent at inhibiting MAO-A (Bieck et al., 1993). This metabolite accounts for about 40% of the administered dose of BROF, with another 40% of the dose attributed to unchanged BROF and 5% to conjugated BROF (Waldmeier et al., 1994). Total renal excretion accounts for 76% of an oral dose of BROF (Bieck et al., 1993).

1.2.3 *Selective Serotonin Reuptake Inhibitors*

The SSRIs are rationally developed psychotropic medications that have launched a new era in drug development (Preskorn, 1996). The basis for rational drug development is having some knowledge or theory about what mechanisms underlie specific psychopathologic conditions and then designing a drug to target that specific mechanism and avoid others (Preskorn et al., 1995). The goal, of course, is to produce pharmacological agents that are more efficacious and have fewer side effects so that they are better tolerated and safer than older agents. Drugs with this profile might also be less likely to produce adverse drug-drug interactions. Prior to the SSRIs, which became available in the late 1980s, serendipidity played a major role in the discovery of psychotropic medications.

The SSRIs were developed based on years of research on the beneficial and adverse mechanisms of action of existing drugs, namely TCAs (Thompson, 1993). The diversity in structure of the TCAs led to the search for other compounds that were more selective for 5-HT uptake sites without also inhibiting NA uptake sites

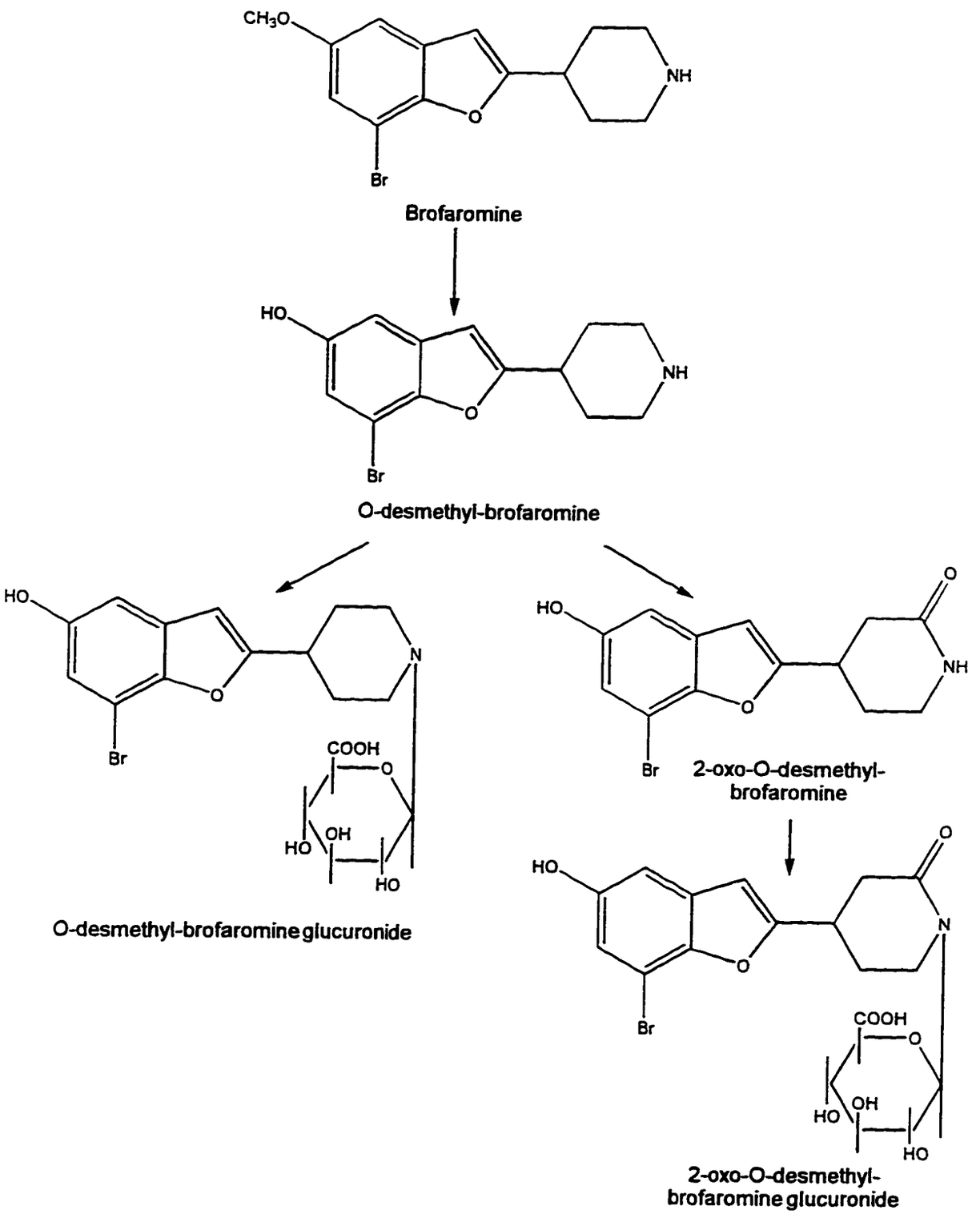


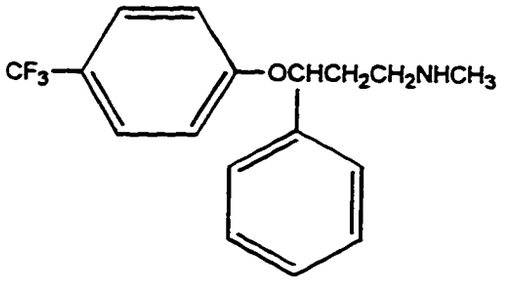
Figure 1-4: Structures and proposed metabolism of some metabolites of brofaromine in humans. Adapted from Waldmeier et al. (1994).

(Wong et al., 1974). In the last ten years, this search has resulted in the marketing of five SSRIs [Figure 1-5] in many countries around the world, each SSRI developed by a different pharmaceutical company:

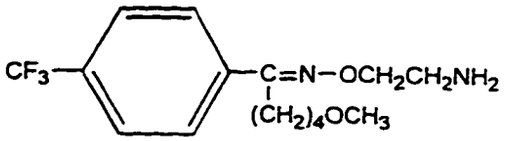
1. Fluoxetine by Eli Lilly
2. Fluvoxamine by Solvay-Duphar
3. Citalopram by Lundbeck
4. Paroxetine by Smith Kline-Beecham
5. Sertraline by Pfizer

The SSRIs have had an extraordinary impact on the treatment of depression, with more than ten million people prescribed fluoxetine (FLU) after only six years on the market (Hyman et al., 1995; Levin and DeVane, 1993). As a class, the SSRIs have no anticholinergic, antihistaminergic, anti-α-adrenergic or cardiotoxic effects and they also do not produce weight gain (Hyman et al., 1995). The side effect profile of the SSRIs is usually mild, with symptoms including nausea, initial anxiety, headaches and possibly longer term sexual dysfunction (Preskorn, 1996). The SSRIs all inhibit the hepatic isoenzyme cytochrome P450 2D6 (CYP2D6; see section 1.4.2) which is responsible for the oxidative metabolism of many drugs (Otton et al., 1993; Hyman et al., 1995), although the potency of that inhibition varies markedly among these drugs. Because of this, the combination of SSRIs and other drugs that are also substrates for CYP2D6 may result in increased therapeutic or toxic effects due to competition for the enzyme. The SSRIs are also highly protein-bound which means that they may displace concomitantly administered drugs from carrier proteins, resulting in higher levels of free drug and

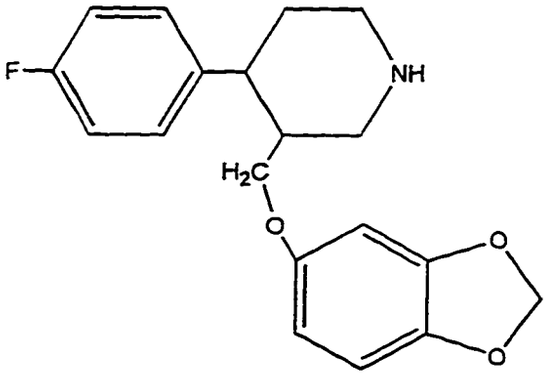
SSRIs:



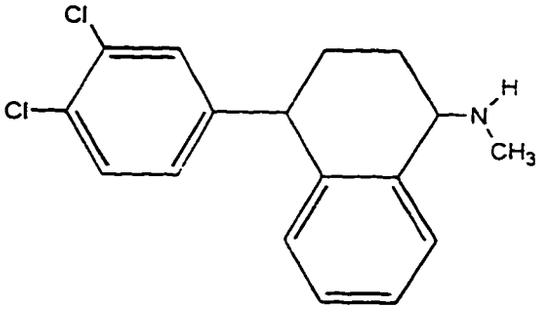
Fluoxetine



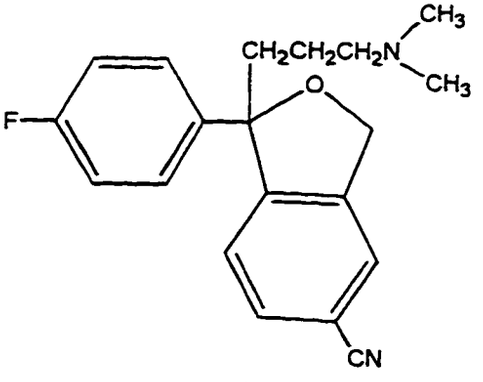
Fluvoxamine



Paroxetine



Sertraline



Citalopram

Figure 1-5: Structures of selective serotonin reuptake inhibitors.

possible changes in the therapeutic or toxic effects of one or both of the coadministered agents (Hyman et al., 1995).

The SSRI, FLU is of particular interest to this thesis and is described in detail below.

1.2.3.1 *Fluoxetine*

FLU was the first of the class of SSRIs to be approved for clinical use as an antidepressant, and it was marketed in 1987 (Brøsen and Skjelbo, 1991). FLU hydrochloride is marketed as 20 mg (base equivalent) capsules under the proprietary name Prozac® (Risley and Bopp, 1990), although generic forms have been made available recently with the end of Lilly Pharmaceutical's patent protection. Since its introduction, FLU has generated more money in sales than all other antidepressants combined (Greenburg et al., 1994). Much of FLU's worldwide popularity can be attributed to the overwhelming attention the drug has received from the lay press. FLU has been the subject of major articles in Newsweek and the Washington Post, has been the topic on TV talk shows such as Donahue and Larry King Live, and has been the subject of bestselling books such as "Listening to Prozac" by Dr. Peter Kramer. Because the major neurochemical effect of FLU is to decrease 5-HT turnover (Fuller et al., 1991), this drug became a popular treatment option for diseases where 5-HT dysfunction is implicated (i.e. major depression, obsessive compulsive disorder, panic disorder, obesity, bulimia, alcoholism, cataplexy) [Dubovsky, 1994]. Indeed, since its introduction, FLU has been touted as everything from a 'wonder drug' (Greenburg et al., 1994) to a

'suicide promoter ' (Freemantle, 1994).

FLU, N-methyl-3-phenyl-3-(4-trifluoromethylphenoxy)propylamine, is derived from a chemical series of phenoxypropylamines (Fuller et al., 1991). Subtle modifications of the phenoxyphenylpropylamine skeleton can result in dramatic alterations in selectivity of the molecule for either the 5-HT or NA uptake carrier. The trifluoromethyl substituent (in the para position of the phenoxy ring) is a fundamental aspect of the molecule which lends lipophilic character to FLU and is responsible for much of the potency and selectivity of FLU as a 5-HT reuptake inhibitor (Fuller et al., 1991).

Because FLU has an asymmetric carbon atom, as does its major metabolite norfluoxetine (NFLU), commercially available FLU is a racemate. Although both stereoisomers (of FLU and NFLU) inhibit 5-HT uptake, their time courses and potencies of 5-HT uptake inhibition are significantly different (Wong et al., 1985; Gram, 1994). After a single dose of S-FLU, 5-HT uptake inhibition lasts up to 24 hours, whereas inhibition lasts only 8 hours after a single dose of R-FLU (Wong et al., 1985). This observation is consistent with the finding that S-FLU is more tightly associated with serum proteins than R-FLU (Peyton et al., 1991). Research has also shown that S-FLU is slightly more potent than R-FLU at inhibiting ^{3}H-5-HT uptake in cortical synaptosomes *in vitro*, at inhibiting ^{3}H-FLU binding (indicative of binding to the 5-HT carrier) in cortical membranes, at inhibiting 5-HT uptake *ex vivo* in synaptosomes from brain stem and cortex, and at antagonizing *p*-chloroamphetamine-induced depletion of brain 5-HT (Fuller and Snoddy, 1986;

Fuller et al., 1991; Robertson et al., 1988; Wong et al., 1985).

As mentioned above, NFLU, the major metabolite of FLU, is also a chiral compound. However, the differences in potencies observed between the enantiomers of NFLU are more pronounced than those observed between the enantiomers of FLU. S-NFLU is considerably more potent than R-NFLU at inhibiting uptake of 5-HT in rat brain synaptosomes *in vitro*, at antagonizing *p*-chloroamphetamine-induced depletion of rat brain 5-HT (Fuller et al., 1991), and at inhibiting ^{3}H-paroxetine binding to 5-HT uptake sites (Wong et al., 1993). Interestingly, it has also been reported that the S-enantiomers of both FLU and NFLU were 5 to 6 times more potent than the R-enantiomers as competitive inhibitors of CYP2D6-mediated bufuralol 1'-hydroxylation *in vitro*.

Despite the extensive use of FLU, it is estimated that as much as 50% of its metabolism is still unaccounted for (Lemberger et al., 1985; Bergstrom et al., 1988). This could prove to be very important because metabolites may contribute not only to the mechanism of action of FLU, but also to its side effect profile (van Harten, 1993). As noted, NFLU, the major metabolite of FLU identified to date, is also a potent and selective inhibitor of 5-HT uptake. The elimination half-life ($t_{½}$) for FLU is 1-4 days and for NFLU is 7-15 days (Altamura et al., 1994). Because of its long $t_{½}$ and its ability to inhibit 5-HT reuptake, NFLU can significantly influence the clinical efficacy of FLU (Hyttel, 1994). As shown in Figure 1-6, after FLU is N-demethylated to NFLU, it can be conjugated with glucuronic acid and excreted or can be O-dealkylated to form *p*-trifluoromethylphenol (TFMP) and γ-hydroxy-γ-phenylprop-

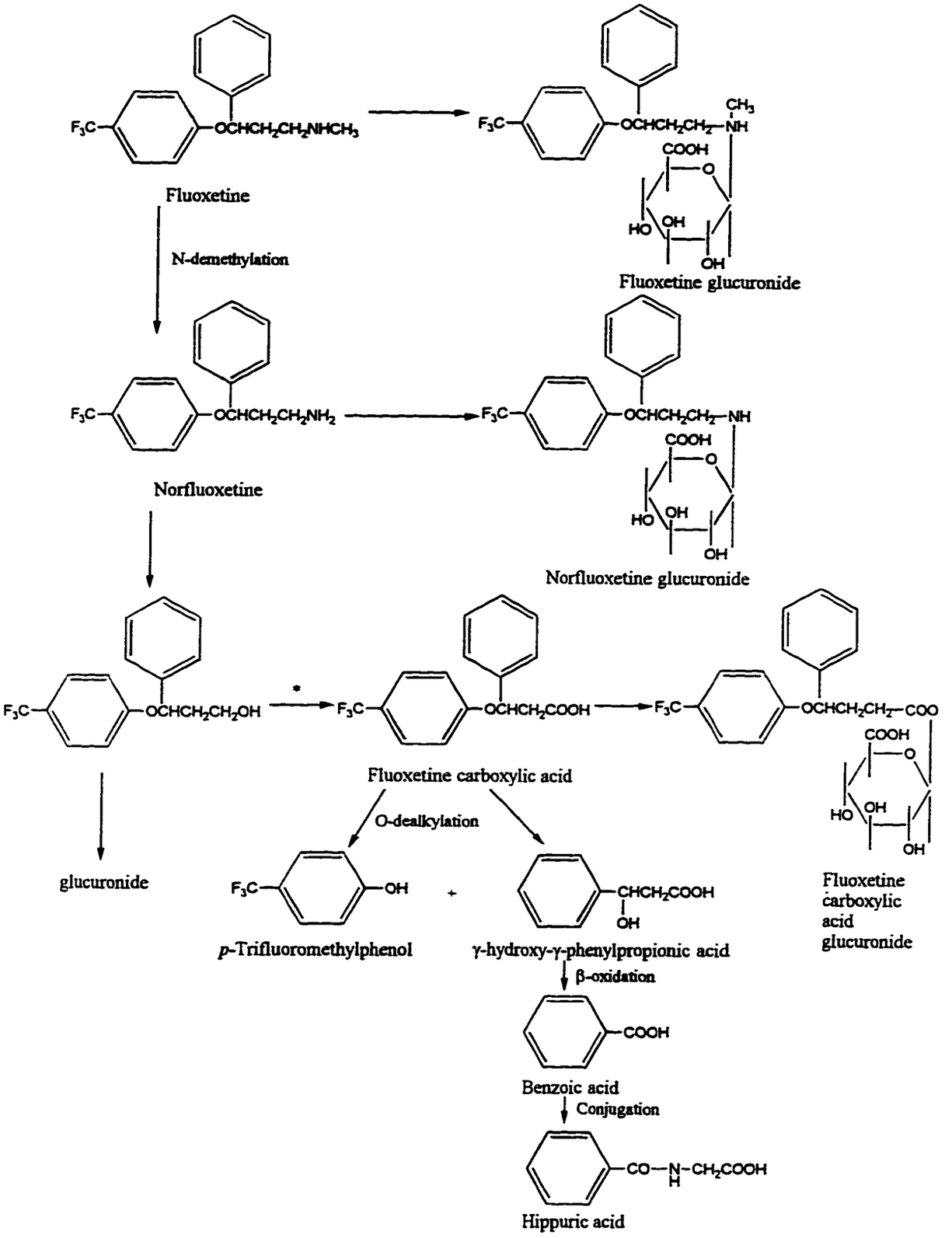


Figure 1-6: Proposed pathway for the metabolism of fluoxetine. Adapted from Altamura et al. (1994). * indicates *via* the aldehyde.

ionic acid. This last metabolite may be further metabolised to hippuric acid, a glycine conjugate of benzoic acid, and excreted (Benfield et al., 1986). In a study examining urinary elimination of metabolites of FLU, healthy volunteers were administered FLU (60 mg) every day for 45 days, but on day 39 (when steady-state was thought to be achieved) the volunteers received a single oral ^{14}C-radiolabelled dose of FLU (60 mg) and their urine was collected and analysed. The results indicated that about 11% of the administered radiolabelled dose was excreted as FLU, 7% as NFLU, 7 and 8% as FLU and NFLU glucuronides, respectively, and more than 20% of the radioactivity was excreted as hippuric acid (Lemberger et al., 1985; Bergstrom et al., 1988). It was also determined that about 80% of the administered dose was eliminated in the urine and 15% of the dose was excreted in the feces. Using a 30 mg radiolabelled dose of FLU, only 2-5% of the drug excreted in the urine was unchanged (Lemberger et al., 1985), which suggests that FLU undergoes extensive hepatic metabolism. Both FLU and NFLU are also potent, competitive inhibitors of CYP2D6. Consequently, FLU may impair the hepatic metabolism of many other drugs (e.g. MAOIs and TCAs), leading to marked elevations in their plasma levels and possibly to the development of toxicity (Schoerlin et al., 1990; Beasley et al., 1993; Dingemanse, 1993; Gram et al., 1995; Lane, 1996).

Attempts to establish a relationship between plasma concentrations of FLU and its effects have been unsuccessful (Gram, 1994; Amsterdam et al., 1997). This may be partly due to the non-linear pharmacokinetic profile of FLU which means that increasing the dosage of FLU results in disproportionately higher plasma

concentrations of the drug.

FLU's primary clinical indication is for treatment of major depression (Spencer, 1993). Meta analyses comparing FLU to other antidepressants have shown that although FLU is not superior to TCAs for the treatment of depression, compliance is better in those patients taking FLU due to less frequent adverse effects (LaPia et al., 1992). FLU has been tried with varying degrees of success in the treatment of several other disorders since its introduction. Among the more frequent uses are obsessive-compulsive disorder, obesity and bulimia nervosa. Patients treated with FLU for these disorders had varying degrees of response depending on the dosage used (Gram, 1994), which was anywhere from 5 to 100 mg/day. Preliminary studies have suggested that FLU may be effective in treating various forms of pain and studies have also indicated that FLU is efficacious for panic disorder (see Wong et al., 1995). Some of the minor clinical indications for FLU include anorexia nervosa, post-traumatic stress disorder, alcohol abuse, Tourette's syndrome, migraine, headache, premenstrual syndrome, trichotillomania (hair pulling), borderline personality disorder, and social phobia (Dubovsky, 1994; Gram, 1994; Messiha, 1993; Wong et al., 1995). Because these indications were reported while FLU was in its post-marketing surveillance phase, most of the studies were uncontrolled, small, and inconclusive. Finally, there have also been rare indications reported for FLU that are not yet substantiated, including controlling narcotic withdrawal symptoms (Polson et al., 1992), the treatment of ritualistic behavior in autism (Todd, 1991), exhibitionism, pathologic jealousy, fibrositis (Messiha, 1993), depersonalization disorder (Fichtner et al., 1992), sexual

dysfunction, and even ciguatera fish poisoning (Berlin et al., 1992). Despite the fact that many therapeutic uses for FLU have been suggested, FLU's efficacy in most of them requires further study and understanding.

In therapeutic trials the most common adverse effects from FLU administration were nausea, nervousness, and insomnia; these effects occurred in 20-25% of the patients (see Wong et al., 1995 for review; Benfield et al., 1986). The adverse effects of FLU generally increased with higher doses to include dizziness, anxiety, headache, anorexia, tremor, and diarrhea (Beasley et al., 1990). Rarer effects that have been reported in post-marketing surveillance reports include akathisia, anorgasmia, skin rash, suicidal preoccupation, self harm (Lebegue, 1992), spontaneous bleeding (Aranth and Lindberg, 1992), and sexual dysfunction (Patterson, 1993). FLU is not associated with the risk of cardiovascular or central nervous system (CNS) toxic effects (Gram, 1994; Steinberg et al., 1986). It should be noted that the rarer adverse effects that have been reported in these studies have often only occurred in 1 or 2 patients and, in many instances, FLU was not the only drug the person was taking. Therefore, it is possible that FLU is not the direct or the only cause of some of these rarer toxicities.

Because FLU received extensive media attention over the possibility that it induced suicidal ideation in a number of patients, a summary of this literature will be presented briefly here.

An important clinical message about depression and suicide is that suicidal thoughts and acts are part of the depressive syndrome and all clinicians must be alert for the emergence of suicidal ideation during treatment (Nakielny, 1994;

Massica et al., 1992). The idea that FLU enhanced or provoked suicidal thoughts stemmed from case reports on a mere six people (Teicher et al., 1990). In Teicher's paper it was claimed that FLU promoted suicidal ideation in severely depressed patients by enhancing drive and counteracting psychomotor retardation. When the lay press obtained this information a literal 'media circus' began. Psychiatrists and depressed patients were interviewed on TV and radio talk shows and became the subjects of major newspaper and magazine articles. What was not mentioned in the media, however, was that reports of suicidal ideation have also been associated with other antidepressants (Levin and DeVane, 1993). Several studies have been conducted since Teicher's original report in 1990 which have discredited his claim that FLU is a promoter of suicidal thoughts (Morton et al., 1993; Burrows and Norman, 1994; Massica et al., 1992; Ashleigh and Fesler, 1992). A meta-analysis of over 3000 patients comparing FLU with TCAs and placebo showed no causal link between FLU and suicidal ideation (Nakielny, 1994). In fact, this analysis demonstrated that FLU may even decrease suicidal ideation. Therefore, since suicide is one of the cardinal features of major depression, it is likely unjustified to attribute this emergent finding soley to the medication that has been taken during the illness.

1.2.4 *"Novel" Antidepressants*

This class of antidepressants includes antidepressants which do not fit into the aforementioned categories. Iprindole, mianserin, alprazolam, and trazodone are

novel antidepressants [Figure 1-7] which do not appreciably inhibit neuronal uptake of amine neurotransmitters or MAO activity. These drugs are effective in treating depression, however, and in some cases are popular for use in the elderly due to a lower incidence of anticholinergic side effects and cardiotoxicity compared to the TCAs (Damlouji et al., 1985).

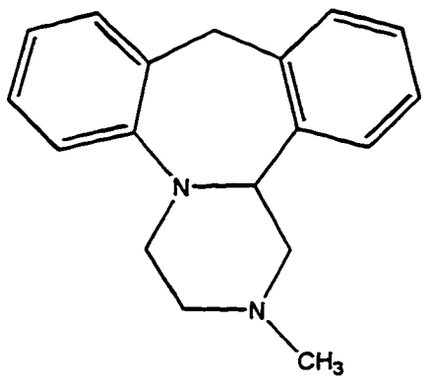
Venlafaxine and nefazodone are novel antidepressants that have recently been introduced. Venlafaxine inhibits the uptake of both NA and 5-HT and lacks anticholinergic, antihistaminergic and α_1-adrenergic effects (Hyman et al., 1995; Preskorn et al., 1995). Nefazodone is chemically similar to trazodone, but has less α_1-adrenergic and sedative side effects (Hyman et al., 1995). Nefazodone is a combination 5-HT uptake inhibitor and 5-HT_2 receptor blocker (Preskorn et al., 1995; Hyman et al., 1995).

1.3 SEROTONIN AND TRYPTAMINE: RELATIONSHIP TO DEPRESSION AND ANTIDEPRESSANT DRUG THERAPY

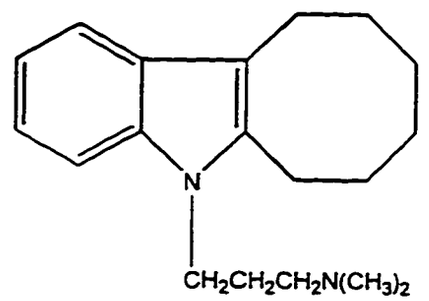
1.3.1 *Synthesis and Metabolism of Serotonin and Tryptamine*

The synthesis and metabolism of both 5-HT and tryptamine (T) utilize some common pathways and enzymes (Figure 1-8). Both pathways ultimately depend on tryptophan (TRP), which is an essential amino acid that can be obtained from the diet (Olendorf, 1971) and readily crosses the blood-brain barrier. Brain concentrations of T and 5-HT are maintained at physiological levels by the affinity of aromatic amino acid decarboxylase (AAAD) and TRP-hydroxylase, respectively,

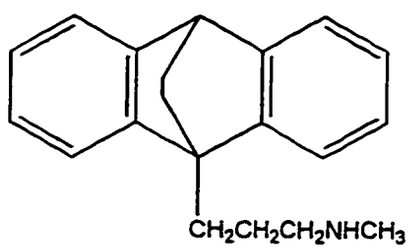
Novel Antidepressants:



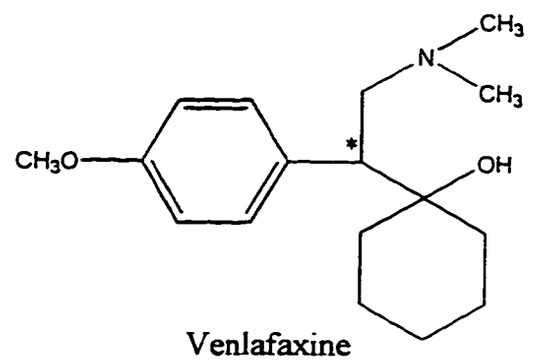
Mianserin



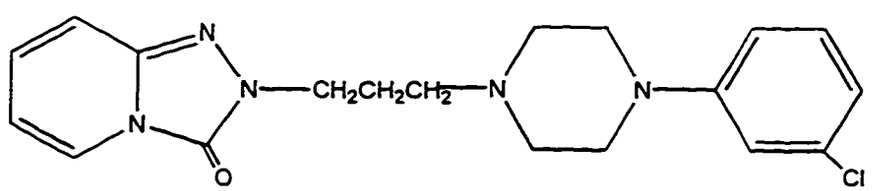
Iprindole



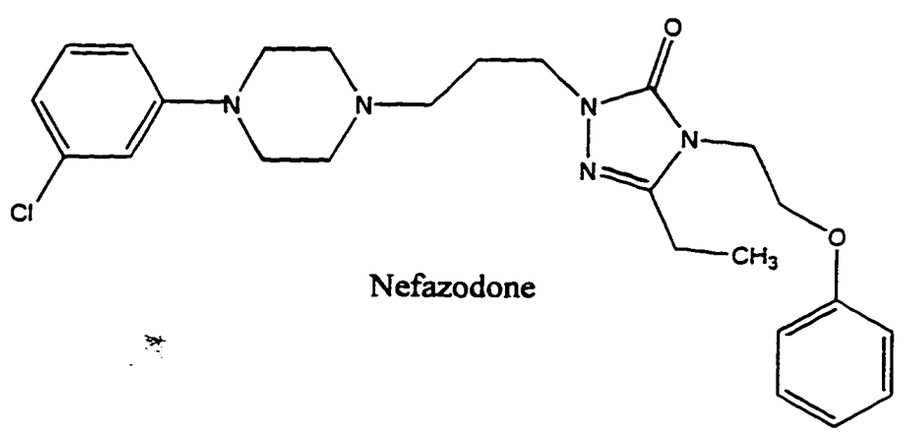
Maprotiline



Venlafaxine



Trazodone



Nefazodone

Figure 1-7: Structures of selected novel antidepressants.

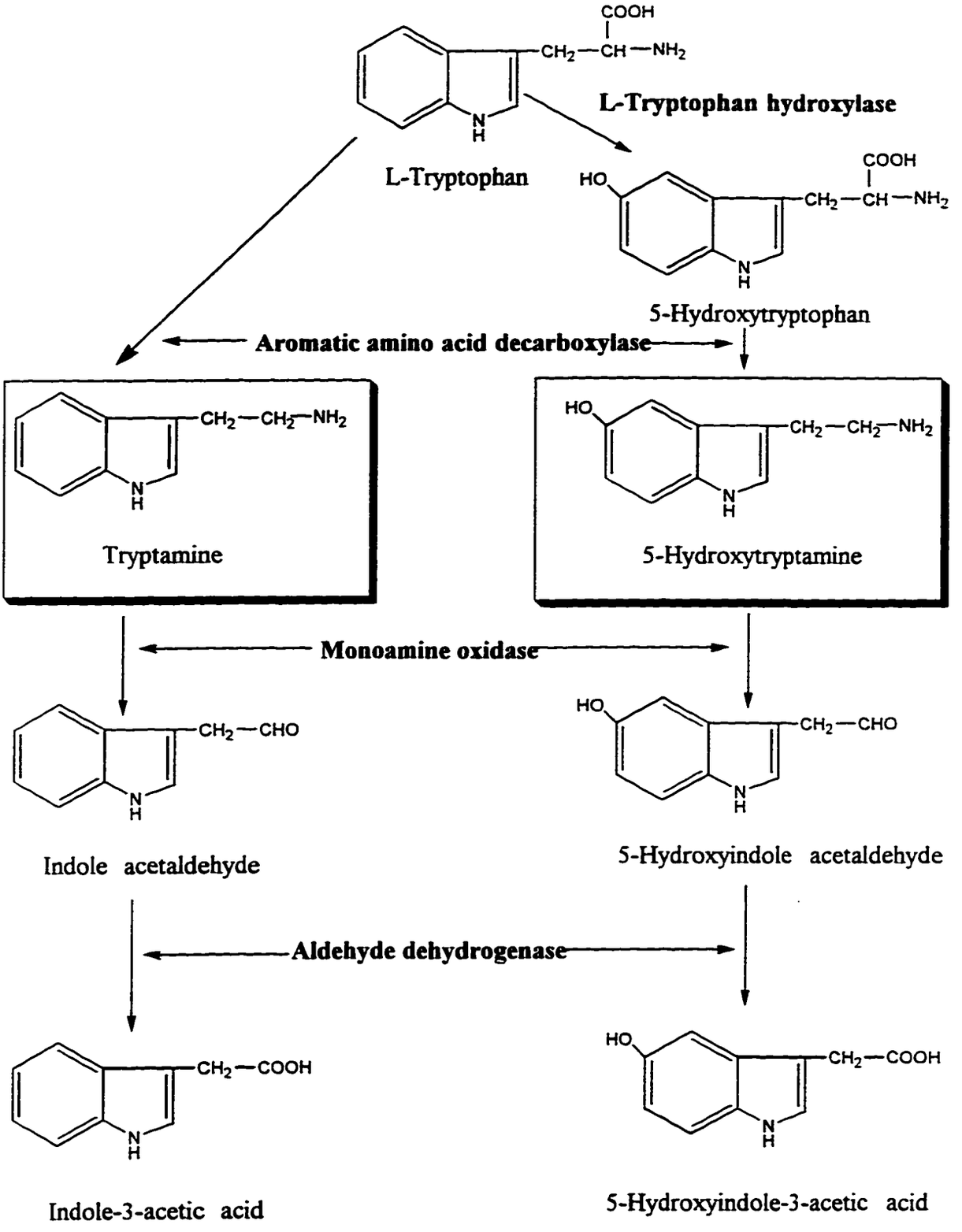


Figure 1-8: Biosynthesis and degradation of tryptamine and 5-hydroxytryptamine. Adapted from Mousseau (1993).

for TRP (Christensen et al., 1970; Warsh et al., 1979). Under normal physiological conditions, AAAD has a very low affinity for TRP and this results in brain concentrations of T that are much lower than those of 5-HT (Artigas and Gelpi, 1979). This low endogenous concentration of T (i.e. less than 10 ng/g in whole brain) led to the label of "trace amine" (Usdin and Sandler, 1976). The synthesis of both 5-HT and T can be blocked by AAAD inhibitors (Saavedra and Axelrod, 1973; Juorio and Durden, 1984).

The major route of catabolism for 5-HT and T is enzymatic deactivation *via* sequential action by MAO and aldehyde dehydrogenase (see Figure 1-8) to result in formation of 5-HIAA and indole-3-acetic acid (IAA), respectively (Wu and Boulton, 1973; Mousseau, 1993). The form of MAO that appears to be most involved in 5-HT metabolism is MAO-A, while both forms may be involved in T metabolism (Neff and Yang, 1974; Sullivan et al., 1986). Substantial increases in brain T concentrations following MAO inhibition have been unequivocally demonstrated (Boulton and Baker, 1975; Philips and Boulton, 1979; Durden and Philips, 1980; Baker et al., 1985). The proportional increase in T levels after MAO inhibition is much greater than that of 5-HT (Boulton and Baker, 1975; Baker et al., 1985).

Despite the structural similarities between T and 5-HT, T does not appear to act as a precursor for 5-HT synthesis (Sasse et al., 1982; Courtois et al., 1988).

1.3.2 *Serotonin Receptors*

Prior to the introduction of molecular biological techniques, the classification of 5-HT receptors was predominantly based on receptor pharmacology utilizing

selective agonists and antagonists (Peroutka, 1993; Sleight et al., 1995). Molecular biology has not only confirmed previous classification in molecular terms, but has also revealed the existence of several novel 5-HT receptors for which little or no pharmacological data exist (Peroutka, 1995; Sleight et al., 1995; Dubovsky and Thomas, 1995). The result of the rapid advance of molecular cloning techniques is that 5-HT receptor subtype classifications are constantly being revised. In addition, as new receptor subtypes are identified, researchers are faced with the ominous task of determining their physiological roles, identifying selective agents for them and determining possible therapeutic indications (Sleight et al., 1995).

Currently, seven distinct families of 5-HT receptors, designated 5-HT_1 - 5-HT_7, have been proposed (Shen et al., 1993; Dubovsky and Thomas, 1995; Peroutka, 1995) [see Table 1-1]. Members of the same family share 30-50% sequence homology and less than 30% sequence identity with members of other families (Peroutka, 1993). 5-HT receptors differ with respect to nervous system distribution, structure, location on the neuron, pharmacology and second messenger systems (Dubovsky and Thomas, 1995). The majority of the research has been conducted on the first three families which follows from the fact that they were the first ones characterized. For the purpose of this thesis the 5-HT_2 family is of primary interest and will, therefore, be discussed in more detail below.

The 5-HT_2 family of receptors now consists of three receptor subtypes: 5-HT_{2A}, $_{2B}$, and $_{2C}$. These receptor subtypes were only recently identified and some confusion may be generated from reading literature written prior to the reclassification of the 5-HT_2 receptor. What is now known as the 5-HT_{2A} receptor

Table 1-1: 5-HT receptor subtypes.

RECEPTOR	LOCATION ON NEURON	PREDOMINANT EFFECTORS
5-HT_{1A}	Presynaptic (somatodendritic and terminal) autoreceptor Postsynaptic (terminal)	↓ cAMP ↑ K^+ conductance *via* a G protein
5-HT_{1B}	Presynaptic Postsynaptic 〉autoreceptor?	↓ cAMP
$5\text{-HT}_{1D\alpha}$	Presynaptic (terminal) autoreceptor Postsynaptic?	↓ cAMP
$5\text{-HT}_{1D\beta}$	unknown	↓ AC
5-HT_{1E}	Presynaptic (terminal) autoreceptor?	↓ cAMP
5-HT_{1F} (5-HT_6)	Presynaptic (terminal) autoreceptor? Postsynaptic	↓ cAMP
5-HT_{2A} (5-HT_2)	Postsynaptic	IP_3/DAG (↑ PI turnover)
5-HT_{2B}	Postsynaptic	↓ K^+ conductance IP_3/DAG
5-HT_{2C} (5-HT_{1C})	Postsynaptic	IP_3/DAG ↑ Ca^{2+} influx ↓ K^+ conductance
5-HT_3	Presynaptic Postsynaptic 〉autoreceptor?	IP_3/DAG ↑ cation conductance
5-HT_4	Postsynaptic	↑ cAMP
5-HT_{5A}	Postsynaptic	unknown
5-HT_{5B}	Postsynaptic	unknown
5-HT_6	Postsynaptic	↑ cAMP activates AC
5-HT_7	Presynaptic Postsynaptic	↑ cAMP

(Data from Dubovsky and Thomas, 1995; Peroutka, 1993; Shih et al., 1995)

Abbreviations: cAMP = cyclic adenosine 3', 5'-monophosphate, K^+ = potassium ion, Ca^{2+} = calcium ion, PI = phosphoinositol, IP_3 = inositol triphosphate, DAG = diacylglycerol, AC = adenylate cyclase

Symbols: ↑ = increases, ↓ = decreases, ? = possible

was previously referred to simply as the 5-HT_2 receptor. In addition, the 5-HT_{2C} receptor was previously referred to as the 5-HT_{1C} receptor. The reason for classifying the latter receptor into a completely different family is that with the advent of more specific and accurate tools, it was determined that the '5-HT_{1C}' receptor more closely resembled 5-HT_2 sites (Peroutka, 1993). The 5-HT_{2A} receptor is the particular serotonin receptor that was studied in some of the projects mentioned in this thesis, although it should be pointed out that there is the possibility that ketanserin, the selective 5-HT_2 antagonist used, may have some affinity for other as yet unidentified 5-HT_2 receptor subtypes. Therefore, for the purpose of this thesis, when 5-HT_2 receptors are referred to, 5-HT_{2A} receptors are being discussed, with the recognition that other 5-HT_2 receptor subtypes may inadvertanty be involved.

As discussed in section 1.1, it is recognized that several different 5-HT receptors may play a role in the pharmacotherapy and etiology of psychiatric disorders. However, evidence suggests that 5-HT_2 receptors may play a pivotal role in depression, and because the tools to measure these receptor changes were readily available, these receptors were focussed on for the purpose of this thesis. The 5-HT_2 receptor is active after a nerve impulse only when concentrations of 5-HT reach sufficient levels to elicit a response. Many antidepressants have been shown to down-regulate 5-HT_2 receptors (Peroutka and Snyder, 1980; Eison et al., 1991; Baker and Greenshaw, 1989). The mechanism responsible for this reduction in density differs depending on what type of antidepressant is being used. For example, TCAs act as direct antagonists at the 5-HT_2 site and have been shown to

decrease both 5-HT_2-mediated behavior and receptor density in rats even after the presynaptic neurons were lesioned with either 5,7-dihydroxytryptamine or N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) [Eison et al., 1991]. MAOIs act to increase 5-HT levels, but after prolonged stimulation the 5-HT_2 receptor ultimately becomes desensitized, followed by subsequent receptor down-regulation (Goodnough and Baker, 1994; Peroutka and Snyder, 1980; Goodwin et al., 1984; Sherry-McKenna et al., 1992; Mousseau et al., 1992; Blier et al., 1987). Finally, SSRIs may desensitize somatodendritic and terminal autoreceptors which could eventually result in receptor down-regulation (Blier et al., 1987), although inconsistent receptor effects have been reported for some SSRIs (e.g. FLU) [Klimek et al., 1994].

At present, a specific agonist for 5-HT_2 receptors appears to be α-methyl-5-HT whereas ketanserin, LY53857, and ritanserin are thought to be relatively specific antagonists. There may, however, be some overlap in the receptor subtype that is labelled depending on the concentration of the agonist or antagonist and the affinity the receptor has for it.

The 5-HT_2 receptor either increases or decreases membrane excitability depending on whether protein kinase C activation or potassium (K^+) channel closing predominates (Dubovsky and Thomas, 1995). 5-HT_2 receptors are linked to the inositol phosphate second messenger system in that phospholipase C is bound to their membranes and is activated when the receptor is occupied. This leads to the catabolism of phosphotidylinositol-4,5-bisphosphte (PIP_2) into two second messengers: inositol triphosphate (IP_3) and diacylglycerol (DAG) (Fowler and Tiger,

1991). IP_3 can release intracellular calcium (Ca^{2+}) stores which can initiate other reactions such as Ca^{2+}-calmodulin-dependent protein phosphorylation. In addition, activation of the 5-HT_2 receptor results in the inhibition of K^+ conductance which may result in a slow depolarization of the neuron (Vandermaelen and Aghajanian, 1980, 1982).

1.3.3 *Tryptamine Receptors*

The presence of specific binding sites for T was demonstrated in the 1980s (Kellar and Cascio, 1982; Perry et al., 1982; Wood et al., 1984; Greenshaw and Dewhurst, 1987; van Nguyen et al., 1989), but was alluded to as early as the 1950s (Gaddum, 1953; Wooley and Shaw, 1957). There is generally good agreement that T receptors are characteristically saturable, high affinity sites that are unevenly distributed in the brain (Juorio and Paterson, 1990; Mousseau, 1993). The highest densities of T receptors are found in the cortex, striatum and hippocampus in rat brain (Mousseau, 1993; Juorio and Paterson, 1990) while in human brain the highest densities are in the hippocampus and thalamus, followed by the caudate nucleus and cortex (Mousseau and Butterworth, 1994). T binding sites in the human and rat brain have similar kinetics and distribution, thus suggesting that a homologous receptor is being studied (Mousseau and Butterworth, 1994).

1.3.4 *Relevance to Psychiatry*

5-HT contributes to the regulation of many psychobiological functions that are disrupted in psychiatric disorders such as mood, appetite, anxiety, arousal,

vigilance, irritability, thinking, sleep/wake cycle, circadian and seasonal rhythms, nociception and neuroendocrine functions (Dubovsky and Thomas, 1995). This indolealkylamine has been implicated in depression since the early 1950s when it was discovered that iproniazid inhibited MAO and, thus, increased brain levels of the biogenic amines (Zellar et al., 1952; Brodie et al., 1956). 5-HT has been further implicated in depression due to the following observations:

1. TRP, the precursor to 5-HT, has been noted to have mood elevating effects (Coppen et al., 1963, Pare, 1963; Glassman and Platmen, 1969; Benkelfat et al., 1994).
2. TRP depletion studies resulted in a considerable lowering of mood in depressed patients who had been successfully treated with anti-depressants (Delgado et al., 1990) and also in normal male subjects who were considered to be at risk for developing depression (Young et al., 1985; Benkelfat et al., 1994; Smith et al., 1997).
3. Administration of *p*-chlorophenylalanine (PCPA), a compound known to deplete 5-HT, resulted in a reversal of the antidepressant effects established by both imipramine and tranylcypromine (Shopsin et al., 1975, 1976).
4. The sensitivity of postsynaptic neurons to 5-HT following chronic antidepressant treatment was increased (deMontigny and Aghajanian, 1978).
5. SSRIs have been found to be effective antidepressants (Benfield et al., 1986; Levin and DeVane, 1993).

6. Antidepressants of several types have been shown to decrease 5-HT_2 receptor density following chronic administration (Peroutka and Snyder, 1980; Eison et al., 1991).

5-HT has also been implicated in schizophrenia since 5-HT_2 receptor antagonists (i.e. "atypical" antipsychotics and also antidepressants) have proven to be effective at improving negative symptoms (i.e. flattened affect, alogia and avolition) of schizophrenia (see Abi-Dargham et al., 1997; Sharma et al., 1997 for reviews). In addition, as discussed in section 1.1, 5-HT_{1A} receptors may play a role in the production and amelioration of anxiety disorders that often co-exist with depression (Artigas et al., 1996; Khan et al., 1986; Deakin, 1988; Bodnoff et al., 1988, 1989). Due to its contribution to vast psychobiological functions, 5-HT may, indeed, be implicated in most psychiatric disorders. What must be remembered, however, is that the activity of 5-HT cannot be easily separated from the action of the other transmitters with which it coexists.

Disturbances in the synthesis and metabolism of T have implicated T in the etiology of psychiatric disorders such as depression, Parkinson's Disease, schizophrenia and Tourette's Syndrome (see Mousseau, 1993, for review). Urinary T concentrations have been reported to be altered in the general psychiatric population (Slingsby and Boulton, 1976). In schizophrenic patients, dramatic increases in urinary T levels have been shown to coincide with the manifestation of psychosis (Brune and Himwhich, 1962; Herkert and Keup, 1969). Similarly, in depressed patients, decreased concentrations of urinary T were correlated with depressed mood (Coppen et al., 1965).

T has been shown to inhibit the uptake (Jones, 1982; Baker et al., 1977, 1980) and increase the release (Robinson and Marsden, 1984) of 5-HT at nerve endings and has also been shown to have moderate affinity for 5-HT receptors (Bennett and Snyder, 1976; Seeman et al., 1980). It has also been demonstrated that T concentrations are dramatically elevated after MAOI treatment (see Philips and Boulton, 1979 and Juorio and Paterson, 1990). In addition, 5-HT receptor antagonists have been shown to block some physiological responses that are normally induced by T (i.e. thermoregulation, neuronal firing rate and vasoconstriction: see Mousseau, 1993, for review). Because of these known interactions between T and 5-HT and the knowledge that both contribute to the etiology of depression, both of these transmitters were of interest in experiments reported in this thesis.

1.4 GABA IN DEPRESSION AND PANIC DISORDER

As mentioned in section 1.1, there is increasing evidence supporting the comorbidity of anxiety and depression (Deakin, 1988; Nutt and Glue, 1991; Weissman, 1992) and for a high frequency of both disorders in the same family (Andrade et al., 1994). Panic disorder is a distinct form of anxiety disorder that is characterized by a rapid onset of autonomic symptoms (i.e. panic attacks), including palpitations, chest pain, sweating, nausea, dizziness, hot or cold flashes, and fear of dying or of losing control. It has been estimated that panic disorder may occur in up to 3% of the general population and that patients suffering from

depression and atypical depression commonly suffer from panic attacks, with a prevalence of 15 - 30% in major depressive disorder and 37 - 53% in atypical depression (Kayser et al., 1988; Lydiard, 1991).

The involvement of GABA in mood disorders is supported by clinical studies which revealed decreased GABA levels in the cerebrospinal fluid (Gold et al., 1980; Kasa et al., 1981) and plasma (Petty et al., 1990) of depressed patients. When patients were treated with antidepressants, the cerebrospinal fluid levels of GABA were found to recover to normal levels (Berrettini et al., 1980). Other studies have indicated that the density of brain $GABA_A$ receptor sites was significantly greater in the frontal cortex of depressed suicide victims compared to controls (Cheetham et al., 1988). Animal models of depression also support a role for GABA in mood disorders (Lloyd et al., 1985), although there is considerable controversy in this area (McManus and Greenshaw, 1991a,b).

Benzodiazepines, which are commonly used for the treatment of anxiety, facilitate GABAergic transmission by interacting with specific recognition sites on the $GABA_A$ receptor. It has also been proposed that a GABAergic abnormality may be involved in panic disorder because drugs which increase the activation of the GABAergic system have also been found to be effective antipanic drugs (Breslow et al., 1989). The fact that many antidepressant drugs, for example PLZ (an MAOI) and imipramine and desipramine (TCAs), are effective in the treatment of panic disorder (Sheehan et al., 1980; Suryani-Cadotte et al., 1990; Zitrin et al., 1983; Johnson et al., 1994) suggests that there may be a common biochemical abnormality between depression and panic disorder. Indeed, PLZ produces

increased brain levels of GABA in the rat (Baker et al., 1991; McManus et al., 1992; McKenna et al., 1994) and alter steady-state levels of mRNA for isoforms of $GABA_A$ receptor subunits following long-term exposure (Tanay et al., 1996). Imipramine and desipramine have also been linked to the GABAergic system in that imipramine increases calcium-dependent GABA release in rat thalamus (Korf and Venema, 1983) and after chronic administration, imipramine and desipramine have been reported to up-regulate $GABA_B$ receptors (Breslow et al., 1989), although this latter effect is still controversial. In addition to the above mentioned antidepressant drugs, BROF (a RIMA) has been demonstrated to be relatively potent at inhibiting GABA uptake (this thesis) and also to be effective in the treatment of panic disorder (Özdaglar et al., 1989; Garcia-Borreguero et al., 1992; Saxena et al., 1992; Den Bohr et al., 1992; van Vliet et al., 1992).

Evidence for interactions between 5-HT and GABA also exist in the literature. For example, 5-HT may increase the sensitivity of the $GABA_A$ receptor to GABA (Akasu, 1988) and sites of interaction between GABA and 5-HT may exist (Bosler, 1989). It has also been demonstrated that $5\text{-}HT_3$ receptor antagonists, such as ondansetron, inhibited ^{3}H-flunitrazepam and ^{35}S-TBPS binding to mouse brain membranes, decreased $^{36}Cl^-$ flux in mouse cortical microsacs, and reduced GABA-gated chloride currents in Xenopus oocytes expressing human $\alpha 1$, $\beta 1$ and $\gamma 2$ $GABA_A$ receptor subunits (Klein et al., 1994). These data indicate that 5-HT antagonists may have effects on the benzodiazepine receptor-$GABA_A$ binding site-chloride ionophore supramolecular complex. In addition, a recent report indicates

that the 5-HT_3 receptor is expressed in a subpopulation of GABAergic neurons in rat neocortex and hippocampus (Moralse et al., 1996). Together, these findings lend further support for the involvement of GABA and 5-HT in antidepressant and/or antipanic drug therapies.

Because there appears to be a commonality among many of the antidepressant/antipanic drugs in their ability to augment GABA transmission, it was of interest to examine the effects of some MAO inhibitors on GABA, in addition to 5-HT, in some of the experiments reported in this thesis.

1.5 ANTIDEPRESSANT DRUG METABOLISM

Almost all known drugs and xenobiotics are metabolised to some extent within the body before they are excreted (Coutts et al., 1994). The main purposes of drug metabolism are:

1. To convert drugs into products that are less active pharmacologically and
2. To convert drugs into products that are more polar, thus more water soluble, so they can be excreted more readily and rapidly.

There are cases, however, where metabolism leads to the production of metabolites that have pharmacological activity the same as, greater than, or different from the parent drug. These metabolites are termed active metabolites (reviews: Rudorfer and Potter, 1985; Young, 1991; Baker et al., 1994). In addition, some metabolites may be more lipophilic than the parent compounds and, thus, be less readily

excreted.

Drug metabolism takes place primarily in the liver, but may also occur in the kidney, blood, brain, lungs, GI tract, and skin (Gibson and Skett, 1994). Drug metabolism is normally divided into two phases, phase I and phase II, which will be discussed in more detail below. In addition, a third phase of metabolism has recently been postulated which involves metabolism of conjugates by the intestinal microflora, re-absorption and then further metabolism (Gibson and Skett, 1994).

1.5.1 *Phase I and Phase II Metabolism*

Most drugs undergo biotransformation *via* phase I and phase II metabolic reactions. The most important metabolic reaction is drug oxidation which is a phase I reaction and occurs mainly in the liver. Oxidation reactions in the liver take place in hepatocytes which have oxidase enzymes (i.e. the mixed-function oxidase system) contained in the smooth endoplasmic reticulum (i.e. microsomes). The introduction of oxygen into a drug molecule is a complex reaction which is catalyzed by the mixed-function oxidase system (Gibson and Skett, 1994), otherwise known as the cytochrome P-450 system (CYP) (Coutts et al., 1994). The CYP450 system will be considered separately as it is a diverse system which deserves more attention. Another enzyme which deserves consideration, however, is the microsomal flavin-containing monooxygenase (F.M.O.). This enzyme was originally termed "microsomal mixed function amine oxidase" because many tertiary amines were found to be N-oxidised by it (Gibson and Skett, 1994). It has now been shown

that F.M.O. also catalyses the S-oxidation of several organic compounds. F.M.O. has a broad substrate specificity including many drugs and xenobiotics such as the phenothiazines, ephedrine, N-methylamphetamine, norcocaine and some pesticides (Gibson and Skett, 1994). It uses either NADH or NADPH as the source of reducing equivalents for the oxidation reaction, with NADPH being the preferred cofactor. F.M.O. is found in many tissues, with the highest concentrations found in the liver, and is thought to occur in multiple forms (i.e. have several isozymes) [Gibson and Skett, 1994].

Many phase I reactions involve oxidations (i.e. dealkylation, deamination, and aromatic ring hydroxylation), but other reactions include reductions, hydrolysis and hydrations (Gibson and Skett, 1994). The main purpose of phase I metabolism is to prepare the drug molecule for phase II metabolism. In most cases, this is achieved by introducing a new chemical group (i.e. -OH, $-NH_2$, -SH, -COOH) into the molecule that will react with phase II enzymes (Gibson and Skett, 1994).

Phase II reactions are generally conjugations that yield water-soluble products which can be excreted in the bile or urine. The most important phase II reaction in humans involves conjugation with glucuronic acid. Substrates for glucuronide formation include any number of compounds containing one or more of the following groups: -OH, $-NH_2$, -NHR, -SH, -COOH (Gibson and Skett, 1994) which correspond to many of the products of phase I metabolism. Other phase II reactions include sulfate formation, acetate formation, methylation, acetylation, glycine conjugation, glutamine conjugation and other conjugations. Quantitatively,

the liver is also the main organ responsible for phase II metabolism (Gibson and Skett, 1994).

1.5.2 *Cytochromes P-450*

The existence of CYP enzymes/isozymes stems back over one billion years when they occurred in single-celled organisms (reviews: Gonzalez, 1990; Gonzalez and Gelboin, 1992). Initially, CYP isozymes were responsible for the synthesis of steroids and other substrates necessary for cell wall maintenance. With time, however, xenobiotic CYP isozymes (i.e. those able to metabolize foreign substances) evolved from the former CYP isozymes and enabled animals to eat plants and other substances that would have previously been toxic to them (see review: Gonzalez, 1990).

CYP isozymes are haemoproteins which comprise the terminal oxidase component of an electron transfer system in the endoplasmic reticulum that is responsible for the oxidation of literally hundreds of structurally diverse drugs and chemicals (Gibson and Skett, 1994). The name cytochrome P-450 derives from: **C**ytochrome or colored pigment, apo**P**rotein that binds the haem, and **450** nanometers, which is the wavelength of absorption of the complex when it is reduced (Garfinkel, 1958; Omura and Sata, 1962). The haem iron undergoes cyclic oxidation and reduction and is an absolute requirement for the catalytic activity of CYP enzymes. Also of essence is one atom of molecular oxygen and NADPH for the following oxidation reaction to proceed:

$$NADPH + H^+ + O_2 + R\text{-}H - NADP^+ + H_2O + R\text{-}OH$$

where R-H is the drug and R-OH is the oxidized drug. During the above reaction, one atom of molecular oxygen is incorporated into the substrate, the other is reduced to water, and the reducing equivalents derived from NADPH + H^+ are consumed (Gibson and Skett, 1994).

Before the advent of molecular genetics, there was no agreed-upon nomenclature system for the CYP isozymes. More than 150 CYP isozymes have been characterized in various plants and animals (for reviews, see Coon et al., 1992; Wrighton and Stevens, 1992; Daly et al., 1993; Nelson et al., 1993; Coutts et al., 1994). The human CYP isozymes have been allocated to ten different gene families based on the degree of similarity in their amino acid sequences [Table 1-2]. Each gene family displays less than 40% amino acid sequence similarity with all other families and members of the same family have 40% or greater amino acid sequence identity. Some families contain subfamilies (designated by a different capital letter) where members contain greater than 59% amino acid sequence similarity. Each CYP within a subfamily is distinquished by a terminal arabic number. Families 1-4 are involved in the metabolism of numerous drugs and xenobiotics while families 7-27 are involved in endogenous steroidal synthesis (Gonzalez and Gelboin, 1992). The human CYPs that have received the most attention are 1A1, 1A2, 2C8/9, 2D6 and 3A4. The CYPs that are the most relevant to the work described in this thesis are 2D6 and 3A4, and these will be discussed in more detail below.

CYP2D6 is the most extensively studied of all the cytochrome P450 isozymes

Table 1-2: Cytochromes P450.

FAMILY	SUBFAMILY	COMMENTS
CYP1	CYP1A1 CYP1A2*	CYP1A1 can be induced by cigarette smoke and may be involved in carcinogenic activation. CYP1A2 is responsible for the 3-demethylation of caffeine; may be induced by cigarette smoke, physical exercise, and ingestion of charbroiled meats and cruciferous vegetables; and may increase the bioactivation of carcinogens.
CYP2	CYP2A3 CYP2B6, CYP2B7, CYP2B8 CYP2C6, CYP2C8*, CYP2C9*, CYP2C10, CYP2C19 CYP2D6*, CYP2D7, CYP2D8 CYP2E1 CYP2F1	CYP2C19 is also known as mephenytoin hydroxylase (CYP_{MP}) and exhibits polymorphism. CYP2D6 is involved in the oxidation of numerous drugs and xenobiotics and exhibits polymorphism. CYP2E1 metabolizes many low molecular weight chemicals, many of which are suspected carcinogens.
CYP3	CYP3A3, CYP3A4*, CYP3A5	CYP3A4 is also known as nifedipine oxidase.
CYP4	CYP4B1	
CYP7	isolated, but not yet characterized	
CYP11	CYP11B1	
CYP17	CYP17A1	Families 7 - 27 are all involved in endogenous steroidal synthesis.
CYP19	CYP19A1	The family number reflects the site of oxidation on the steroid nucleus.
CYP21		
CYP27	isolated, but not yet characterized	

* indicates the human CYP isozymes that have received the most attention (Coutts, 1994).

(Riesenman, 1996). It is the source of the debrisoquine/sparteine polymorphism and has also been called debrisoquine 4-hydroxylase, sparteine dehydrogenase, or debrisoquine/sparteine oxidase (Eichelbaum, 1982; Nakamura et al., 1985; Coutts, 1994). The discovery of polymorphism at this isozyme was serendipitous (see Eichelbaum, 1982 for review), but led to the classification of individuals as extensive metabolizers (EMs) or poor metabolizers (PMs) of certain drugs. Individuals lacking CYP2D6 (i.e. possessing two defective alleles) are called PMs due to their inability to efficiently metabolize drugs that require this isozyme for biotransformation (Riesenman, 1996; Coutts, 1994). Individuals who are either homozygous for the normal wild-type allele or are heterozygous (i.e. one active and one defective allele) are called EMs (Coutts, 1994; Riesenman, 1996). It has been determined that approximately 5-10% of Caucasians and 2% of Orientals are PMs of substrates of CYP2D6 (Bertilsson et al., 1992; Relling et al., 1991; Steiner et al., 1988). There is a wide interindividual variability of CYP2D6 activity within the EM phenotype which has led to the suggestion that further divisions such as slow, fast and ultra-rapid EM may be clinically useful (Brøsen et al., 1993; Riesenman, 1996). The molecular basis for ultra-rapid metabolism has been determined to be gene amplification. It should also be pointed out that some of these isozymes can be induced. The process of induction involves increasing the rate of transcription of the genes encoding a particular P450 species by an inducer (i.e. another drug, an environmental chemical or pollutant) (Coutts, 1994). Most inducers not only stimulate the production of more than one CYP, but also their own metabolism as well as the metabolism of other chemicals.

CYP2D6 catalyses the oxidation of numerous drugs [Table 1-3]. Generally, CYP2D6 catalyses the metabolism of lipophilic, aromatic compounds that possess a basic centre which is protonated at physiological pH (Coutts, 1994). For metabolic oxidation to occur, the drug molecule is oriented so that the N^+ atom points toward an anionic location (COO- group) of the active site while the aromatic ring is aligned to a planar region of the protein [Figure 1-9] (Guengerich et al., 1986). It has been proposed that the distance between the site of metabolic oxidation on the P450 active site and the protonated atom on the substrate must be between 0.5 and 0.7 nm for oxidation to take place (Guengerich et al., 1986; Meyer et al., 1986). It has been suggested that CYP2D6 is involved in hydroxylation, deamination and possibly N-dealkylation reactions (Riesenman, 1996). From the model suggested above, however, metabolic N-dealkylation and deamination should be catalysed by a CYP other than CYP2D6 since the distance between the protonated N atom and the site of oxidation (i.e. the adjacent C atom) is only about 0.15 nm. CYP2D6 has, nevertheless, been confirmed to be involved in certain N-dealkylations (Coutts et al., 1994). The explanation for this may, in part, be that although many drugs have a principal CYP enzyme that is responsible for the bulk of its metabolism, it would be an oversimplification to suggest that one specific isozyme is responsible for all of its biotransformation (Preskorn, 1996; Riesenman, 1996; Coutts et al., 1994; Coutts, 1994). The isozyme(s) involved in metabolic reactions can be identified by various molecular modeling techniques and mathematical models of *in vitro* data (see Koymans et al., 1992; Von Moltke et al., 1994; Heim and Meyer, 1990). The literature resulting from the application of these tech-

Table 1-3: Some of the drugs whose metabolism is catalyzed by CYP2D6.

ajmaline	flecainide	oxprenalol
alprenolol	chlorpromazine	oxycodone
amiflamine	fluoxetine	paroxetine
amitriptyline	fluperlapine	penicillamine
aprindine	fluphenazine	perhexiline
brofaromine	flurazepam	perphenazine
bufarolol	guanoxan	phenacitin
bunitrol	haloperidol	phenformin
bupranolol	hydrocone	pindolol
captopril	4-hydroxyamphetamine	pipamperone
cinnarizine	imipramine	propafenone
citalopram	indoramine	propranolol
clomipramine	labetolol	N-propylajmaline
clonidine	lidocaine	remoxipride
clozapine	lobeline	risperidone
codeine	maprotiline	sparteine
debrisoquin	*p*-methoxyamphetamine	temazepam
deprenyl	methoxyphenamine	thebain
desipramine	metiamide	thioridazine
desmethylcitalopram	metoprolol	timolol
N-desmethylcitalopram	mexiletine	tomoxetine
N-desmethylclomipramine	minaprine	trifluperidol
dextromethorphan	norcodeine	trimipramine
dihydrocodeine	nortriptyline	tropisetrone
domperidone	omeprazole	venlafaxine
encainide	ondansetron	yohimbine
ethylmorphine	oxazepam	

[data extracted from Kroemen and Eichelbaum (1995) and Spatznegger and Jaegar (1995)].

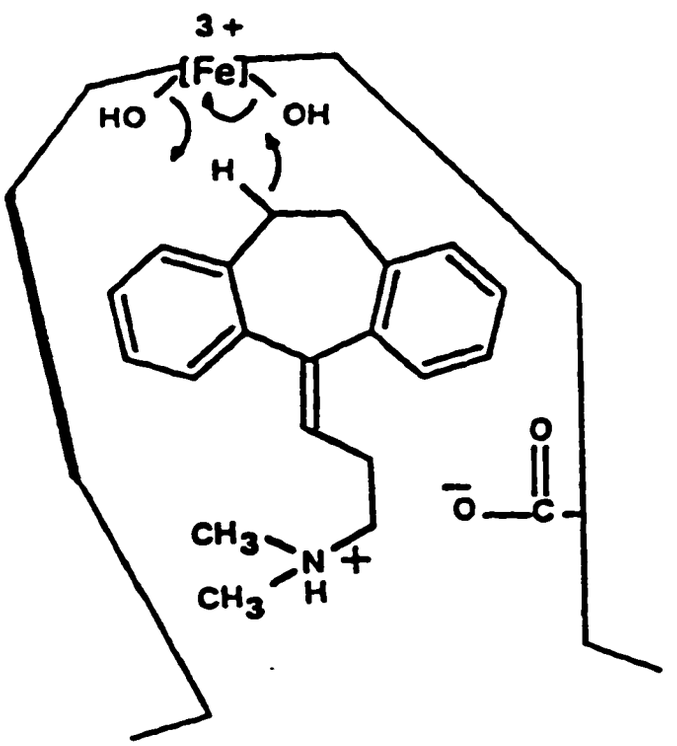


Figure 1-9: Proposed model for the active site of CYP2D6 in humans where amitriptyline is appropriately oriented for metabolic oxidation to occur. Reprinted with permission from Coutts (1994), p. 33.

niques is often confusing and contradictory. What is clear, however, is that the same drug may be metabolized by more than one isozyme. Some clarification is necessary here as it is often erroneously thought that one isozyme could take over for another in cases where one isozyme is inhibited or unable to act on the drug. However, since both the affinity of an enzyme for a particular substrate and the inherent activity of that enzyme are constant, an alternative enzymatic pathway will become active only when the concentration of substrate has reached sufficient levels for this pathway to be meaningful (Preskorn, 1996).

CYP2D6 is synthesized on the long arm of chromosome 22 and is believed to be expressed only in humans (Coutts, 1994). In the rat, CYP2D1 appears to have a similar, but not identical, substrate specificity to human CYP2D6 (Gonzalez, 1992). It is important to note that drugs that are identified as substrates for CYP2D6 may also be competitive inhibitors of this isozyme, depending on the affinity of the drug for the isozyme and its concentration at the active site (Riesenman, 1996). The converse of this, i.e. that an inhibitor of CYP2D6 may also be a substrate for it, is not necessarily true. For example, quinidine is the most potent inhibitor of CYP2D6 identified to date, but it is metabolized by CYP3A4 (Guengerich et al., 1986).

CYP3A4 may possibly be the most clinically important CYP enzyme, making up 25% (Watkins, 1994) to 60% (Guengerich et al., 1986) of the total liver P450 content. CYP3A4 is also known as nifedipine oxidase. The substrates for CYP3A4 are structurally diverse and among others include cyclosporin, nifedipine, quinidine, lidocaine, triazolam, alprazolam, terfenadine, and testosterone (Coutts, 1994;

Riesenman, 1996). Unlike CYP2D6, CYP3A4 does not display genetic polymorphism, but there is large interindividual variability in its activity (Watkins, 1994; Riesenman, 1996). In rats, the P450 enzyme most closely resembling human CYP3A4 appears to be CYP3A2 (Imaoka et al., 1990).

1.5.3 *Pharmacogenetics and Drug-Drug Interactions*

Pharmacogenetics refers to the study of genetically determined variations in drug metabolism and plays an important role in the extent to which individuals can metabolize certain drugs and whether drug-drug interactions will occur upon the coadministration of drugs. As previously noted, CYP2D6 is polymorphic (as is CYP2C19). The clinical consequences of polymorphic drug oxidation may be as follows:

1. PMs may achieve higher levels of the parent drug and, if it is active, may experience exaggerated or toxic effects.
2. PMs may not achieve the desired therapeutic action of a drug if biotransformation is necessary for the formation of an active metabolite.
3. EMs may not achieve the desired therapeutic effects if they rapidly convert active parent compound to inactive metabolites, which could then, in turn, contribute to adverse effects (Riesenman, 1996; Coutts, 1994).

In order to determine if an individual is a PM or an EM involves the application of phenotyping or genotyping. Phenotyping involves using various probe

drugs such as debrisoquine, dextromethorphan or sparteine. The individual takes the drug and then gives a urine sample (at some predetermined time interval) which is then analysed for levels of the drug and its metabolites. EMs would have low levels of parent compound and high concentrations of its metabolites, whereas PMs would be the opposite [Figure 1-10]. The advantages of phenotyping are that it is non-invasive, rapid and inexpensive. Disadvantages are that a pharmacologically active compound must be administered (which raises legal issues), and if the subject is already receiving other drugs then there is potential for drug-drug interactions. This latter concern also raises the issue of phenocopying which is the apparent transformation of an EM to a PM due to concomitant drug administration (e.g. administration of quinidine, a potent inhibitor of CYP2D6, would convert EMs of the drugs listed in Table 1-3 to PMs if they were coadministered) (Coutts, 1994). Genotyping involves utilization of allele-specific polymerase chain reaction (PCR)-based techniques (Bertilsson and Dahl, 1996) alone or in combination with restriction fragment length polymorphism (RFLP) analysis (Gonzalez and Idle, 1994). The major advantage of this method is that it can be performed using blood samples from patients irrespective of drug treatment. The disadvantages are that it is more invasive and expensive than phenotyping and requires more specialized laboratory technology.

Several of the drugs used in psychiatry (e.g. neuroleptics, TCAs, SSRIs) are substrates and/or inhibitors of CYP isozymes; thus these drugs may not only be affected by levels of coadministered drugs, but may also themselves influence the metabolism of these other drugs. It should also be recognized that because the

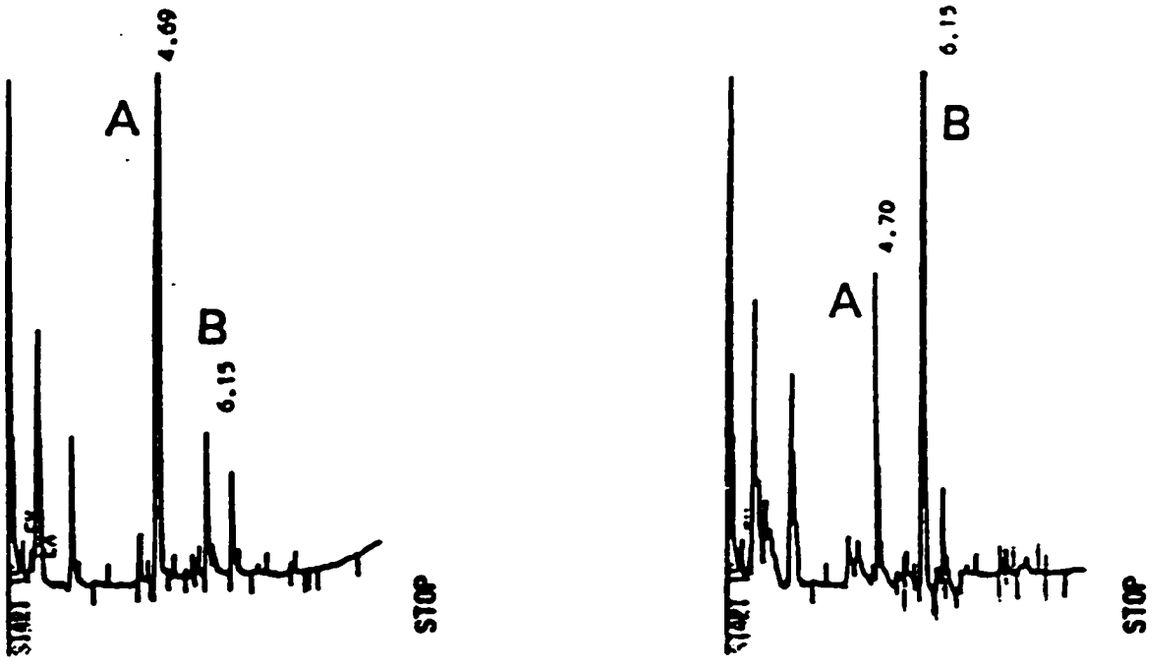


Figure 1-10: Examples of urine extracts from poor (left) and extensive (right) metabolizers of dextromethorphan. A= dextromethorphan, B= dextrorphan. Reprinted with permission from Coutts (1994), p. 35.

metabolism of certain drugs is controlled by genetic factors, large interindividual differences in plasma concentration may occur. This could result in large variations in the effective therapeutic dosage of a particular drug between different individuals (Coutts, 1994). And, to reiterate, two or more drugs that are given concomitantly may compete for CYP isozymes involved in their metabolism, thus further altering plasma levels of one or more of the drugs.

The SSRIs are known to inhibit several P450 isozymes (Baumann and Rochat, 1995; Harvey and Preskorn, 1996 a,b; Preskorn, 1997; Lane, 1996) and, as indicated in Table 1-4, all inhibit CYP2D6 to some extent. Because the SSRIs are often given in combination with other drugs that may be metabolized by the same CYP isozymes, many potential drug-drug interactions can occur. The first well-published reports of drug-drug interactions and the newer antidepressants involved FLU and desipramine (DMI) [review: Riesenman, 1996]. The major metabolic pathway for DMI is *via* CYP2D6 to form 2-OH-DMI. However, the fact that FLU is a potent inhibitor of CYP2D6 was not known for some time after FLU was marketed. Thus, when FLU was initially introduced, many clinicians switched patients from DMI to FLU antidepressant therapy or had patients on both drugs (Riesenman, 1996). Subsequently, there were many reports of increased DMI levels and decreased levels of its hydroxylated metabolites (Aranow et al., 1989; Fuller and Perry, 1989; Weilburg et al., 1989; Vandel et al., 1992; Wilens et al., 1992; Bergstrom et al., 1992; Aspeslet et al., 1994; Goodnough and Baker, 1994; Preskorn et al., 1994). FLU has been extensively studied with regard to possible drug-drug interactions since it became known that it potently inhibits CYP2D6 (see

Table 1-4: Selective serotonin reuptake inhibitors and the CYP isozymes with which they interact .		
	Isozyme(s) Inhibited	Isozyme(s) Involved in Metabolism
Fluoxetine	2D6, 3A4, 2C19	2D6 (partially), rest unknown
Fluvoxamine	1A2, 3A4, 2C19	unknown
Paroxetine	2D6	2D6
Sertraline	2D6	3A4 for demethylation
Citalopram	2D6 (mild)	2C19 and 2D6

Adapted from data presented in Preskorn (1996).

Table 1-3). Indeed, there are now literally volumes of literature describing drug-drug interactions between FLU and other drugs such as neuroleptics, antidepressants, anxiolytics, hypnotics, antimanics, drugs of abuse, anticonvulsants, antiarrhythmics, diuretics, calcium channel blockers and anticoagulants among others [see Messiha (1993) and Harvey and Preskorn (1996a,b) for reviews]. For the purpose of this thesis, however, drug-drug interactions involving FLU and other antidepressants (in particular MAOIs) are of interest and will be discussed in more detail below.

Both FLU and NFLU inhibit multiple CYP isozymes at clinically relevant concentrations and, consequently, also inhibit their own metabolism (Altamura et al., 1994; Preskorn, 1996). There are likely several CYP isozymes responsible for the metabolism of FLU at different concentrations, which would account for the non-linear pharmacokinetic profile of FLU (Preskorn, 1996). As discussed previously, when higher affinity enzymes are inhibited, lower affinity enzymes may become relevant and, thus, active as the drug concentration rises.

The coadministration of FLU and MAOIs can result in serious side effects (Ciraulo and Shader, 1990; Messiha, 1993). In general, MAOIs increase the functional activity of 5-HT by increasing the amount stored and, thus, the amount released upon the depolarization of 5-HT- containing neurons (Beasley et al., 1993). When this increase in 5-HT neurotransmission is further augmented by FLU, the functional levels of 5-HT can be dramatically increased (Aranow et al., 1989). The combination of FLU and TCP, PLZ, or deprenyl has resulted in adverse events such as the serotonin syndrome, autonomic depressive states and hypertensive crisis (Levinson et al., 1991; Ciraulo and Shader, 1990; Beasley et al., 1993). In humans

the coadministration of FLU and MAOIs has been used for refractory depression (Dingemanse, 1993; Power et al., 1995; Bakish et al., 1995; Liedenberg et al., 1996), but this combination may be toxic because: 1) irreversible MAOIs can inhibit the MAO enzyme and, consequently, the metabolism of biogenic amines, for as long as 10-14 days; 2) the 5-HT levels in the synapse could remain elevated for a prolonged period of time because of the long half-lives of FLU and NFLU; and 3) the inhibition of CYP2D6 by FLU can result in the maintenance of increased plasma drug levels for a prolonged period of time (Messiha, 1993).

The combination of FLU and MOC has demonstrated good efficacy in cases of refractory depression, but has created controversy as to whether or not toxic side effects such as the serotonin syndrome result from the combination (Bakish et al., 1995; Liebenberg et al., 1996; Dingemanse, 1993). Seven fatal cases of the serotonin syndrome have been reported after an overdose of combined antidepressant therapy with MOC and another antidepressant, but in these cases the drugs used were citalopram and clomipramine (Neuvonen et al., 1993; Spigset et al., 1993; Kuisma, 1995; Hernandez et al., 1995; Power et al., 1995; Liebenberg et al., 1996). Out of thirteen post-marketing surveillance cases reported to Hoffmann La-Roche of concomitant MOC/FLU treatment, headache was the only adverse event reported (see Dingemanse et al., 1995). When this combination was given to healthy volunteers, the adverse events were similar to those seen when each compound was given separately (Dingemanse, 1993) and, again, there was no indication of the development of a serotonin syndrome. Despite the apparent safety of combination therapy with FLU and MOC, many clinicians are still cautious

in prescribing it. This caution may be especially warranted in cases where the patient is taking several medications for comorbid medical conditions (which is often the case). In addition, the recent finding that MOC is an inhibitor of CYP2D6, CYP2C19 and CYP1A2 (Gram et al., 1995) further implicates possible drug-drug interactions after FLU/MOC treatment, since it is possible that one or all of these CYP isozymes may play a role in the metabolism of FLU. Another factor to consider is that after a week of multiple dosing the clearance of MOC decreases, presumably due to inhibition of its own metabolism (Hyman et al., 1995). If MOC reaches high enough concentrations to inhibit the CYP isozyme(s) involved in FLU metabolism, then levels of FLU will also increase and, in turn, toxic side effects could result. This explanation was recently used to account for a latent adverse reaction seen after four weeks of concomitant use of FLU and MOC (see Liedenberg et al., 1996). The issue of drug-drug interactions is further complicated if the individual is a PM. Thus, genetic factors should be considered before drug dosage regimens are established.

1.6 CHIRALITY

As indicated in Figure 1-6 , both FLU and NFLU contain a chiral center. To appreciate the importance of this, a discussion of chirality follows.

In 1815 Jean-Baptiste Biot first described optical activity as the ability of a substance to rotate plane polarized light (Krstulovic, 1989). Later that century, in 1848, Louis Pasteur proposed that optical activity was the result of a molecular asymmetry which produced non-superimposable mirror images (Drayer, 1988).

These concepts of chirality and optical activity laid the groundwork for the general theory of organic structure in three dimensions put forth by Van't Hoff and Le Bel (Richardson, 1901).

The term chirality derives from the Greek work "cheiros" which means handedness. If an object has 'handedness', it has a mirror image which is identical, but cannot be superimposed or interconverted without breaking and reforming a bond. These mirror images are called enantiomers and arise when a tetravalent atom, whose valencies are directed to the four corners of a regular tetrahedron, carries four different substituents (Caldwell, 1992). Of the various atoms that can contribute to chiral centres, the most common is carbon, but may also be nitrogen, sulfur, phosphorous and others. Enantiomers have different optical activities in that one rotates plane polarized light to the right [(+) or dextrorotatory] and the other to the left [(-) or levorotatory]. In a nonchiral environment, these enantiomers have identical physical (melting point, boiling point, refractive index, solubility) and chemical properties.

1.6.1 *Nomenclature*

As mentioned, (+) and (-) are indications of the direction in which the enantiomer rotates plane polarized light. The enantiomers are thus prefixed by (+) and (-), or *dextro-* and *levo-* (or more simply, *d* and *l*). It should be noted that optical rotation caused by an enantiomer can be changed by a number of factors such as the solvent in which the enantiomer is dissolved, the temperature at which the measurement was taken, the sample concentration, the light wavelength, and the

pH of the aqueous solutions. Changes in optical activity that may occur due to alterations in one of these variables are not the result of a change in the spatial orientation of the atoms (i.e. the breaking and remaking of bonds).

Enantiomers may also be named according to their 3-dimensional structure, or their absolute configuration (Klyne and Buckingham, 1978). Unlike optical rotation, the configuration of the enantiomers can only be altered by breaking and reforming bonds. Absolute configuration is expressed using the Cahn-Ingold-Prelog method (Cahn et al., 1956) which uses upper case R (rectus) and S (sinister) as an indication of the location of the substituents around the chiral center. Not all optically active compounds are designated R or S: for example, amino acids and carbohydrates are still being referred to as L and D according to the Fischer Convention (Horn, 1984). It is important to note that R and S or L and D give no indication as to the effect the enantiomer would have on plane polarized light and that, at present, there is no way to connect absolute configuration with optical rotation without additional experimentation plus the use of a polarimeter.

Target sites for drug action such as receptors, enzymes and ion channels often interact differently with stereoisomers and thus the enantiomers may differ in terms of their biological effects (Caldwell, 1992). The more active enantiomer is called the "eutomer" and the less active enantiomer is called the "distomer". The ratio of activity, the eudismic ratio (activity of eutomer/activity of distomer), is an indication of the degree of stereoselectivity and very much depends on the optical purity of the enantiomers (Lehman, 1976; Caldwell, 1992).

1.6.2 *The Relevance of Chirality in Pharmaceuticals*

The fact that many psychiatric drugs have one or more chirai centers is often not considered in basic or clinical studies, the assumption being that the enantiomers do not have different actions (Jamali et al., 1989; Coutts and Baker, 1989; Baker et al., 1994). However, the enantiomers of a racemic drug often differ in pharmacodynamic and pharmacokinetic properties when they are introduced into a chiral environment, such as the human body (Eichelbaum, 1988; Jamali et al., 1989; Testa, 1990). Unfortunately, many researchers and clinicians do not realize, or do not acknowledge, the importance of discriminating between enantiomers when examining pharmacologic, pharmacokinetic, pharmacodynamic and toxic effects of a drug that is administered as a racemic mixture (Jamali et al., 1989; Caldwell, 1992; Mehvar, 1992). This poses a problem when total blood levels of the drug are being evaluated for the following reasons:

1. Only one enantiomer may actually be active and thus a portion of the total drug does not contribute to therapeutic effect (Drayer, 1988; Ariëns, 1989).
2. One enantiomer may cause the majority of the side effects (Jamali et al., 1989).
3. The enantiomers may be absorbed, metabolised and excreted at different rates (Testa, 1986; Eichelbaum, 1988; Coutts and Baker, 1989).
4. The enantiomers may compete with each other for protein binding sites, receptors or enzyme active sites, which will alter metabolism

kinetics (Jamali et al., 1989; Caldwell, 1992).

Chirality of pharmaceuticals is becoming an important issue with regard to drug development, drug approval and clinical use (Campbell, 1990; Testa and Trager, 1990). Drugs of natural origin which contain a chiral center are usually optically active (which means they exist as only one of the two possible enantiomers), whereas drugs of synthetic origin that contain a chiral center are usually optically inactive, so they contain an equal mixture of the enantiomers (Kondepudi and Nelson, 1985; Mason, 1986). Because, until recently, there was no easy way to separate enantiomers (Davies, 1990), it is estimated that approximately 90% of chiral drugs in the world are marketed as racemic mixtures (Lennard, 1991) and up to 75% of currently used synthetic drugs are chiral (Caldwell, 1992). Although it is possible that both enantiomers contribute equally to the therapeutic effect, the issues mentioned above must also be considered when developing or using chiral agents.

1.6.3 *Using Gas Chromatography to Analyze Racemic Mixtures*

As mentioned above, when racemic mixtures are studied, it is often of interest to measure the two enantiomers separately. Conventional analytical methods cannot distinguish between enantiomers, but the advent of optically pure reagents and chiral chromatographic columns has made it relatively easy to measure the enantiomers individually (Pasutto, 1992; Wozniak et al., 1991). Stereoisomers that are not enantiomers (i.e. not mirror images of each other) are termed diastereomers and, in contrast to enantiomers, they have different physical

and chemical properties (Wright and Jamali, 1993). The general approach to chromatographic separation of enantiomers is to form diastereomers either irreversibly through covalent reaction with an optically pure reagent, or reversibly through interaction with an optically pure chiral stationary phase (i.e. column) [Wright and Jamali, 1993].

Chromatographic separation of enantiomers can be performed either indirectly (Gal, 1988; Pasutto, 1992) or directly (Allenmark, 1984; Pasutto, 1992). The indirect method involves chiral derivatization techniques that are based on the formation of a covalent bond between the enantiomers and the optically pure reagent to yield diastereomers. Because the diastereomers have different properties, they can be analysed on a conventional column. Some potential problems with this method are the possibility of enantiomeric impurity of the chiral derivatizing reagent (Gal, 1988; Pasutto, 1992) and the possibility of unequal rates of reaction between the enantiomers and the derivatizing reagent (Krstulovic, 1989; Wright and Jamali, 1993).

The direct method of chromatographically analyzing racemic mixtures involves separating the enantiomers on a chiral stationary phase with or without previous derivatization with a conventional derivatizing agent (Wright and Jamali, 1993). The problem of enantiomeric impurity is avoided with this method (Pasutto, 1992). The disadvantages of the direct method are that chiral columns are more expensive than conventional columns (Pasutto, 1992), they do not last as long (Wright and Jamali, 1993), and there is often much variability between columns from different batches (Geisslinger et al., 1989; Straka et al., 1990; Pasutto, 1992).

1.7 GENERAL ANALYTICAL TECHNIQUES APPLICABLE TO THIS THESIS

1.7.1 *Receptor Binding*

Studies of receptor-ligand interactions have greatly enhanced our knowledge of how various neurotransmitters and drugs act on specific receptor sites in different target tissues (Hrdina, 1986). The first step in receptor-ligand interactions is the binding of the ligand to a particular receptor. An inverse relationship between levels of the neurotransmitter and the number of receptor sites may occur after a prolonged exposure to the neurotransmitter. For example, if there is a massive decrease in the levels of an active neurotransmitter, a functional supersensitivity may develop, paralleled by an up-regulation of postsynaptic receptors. Conversely, prolonged increases of a neurotransmitter may result in subsensitivity and a reduction in receptor density. Since, to a certain extent, "everything binds to everything" (Hrdina, 1986), the binding of the radiolabelled ligand to the biological preparation in a receptor-binding assay must meet the following criteria in order for the binding site to be considered a specific receptor site:

1. Saturable - "Specific" binding is to a finite number of sites that become saturated when the preparation is exposed to increasing concentrations of the ligand. In contrast, "nonspecific" binding is to other tissue components, glassware, filters, etc., and is nonsaturable.
2. Specific - The receptor should be present only in cells known to respond to the particular transmitter being investigated.
3. Reversible

4. Stereoselective

The saturation binding assay is the cornerstone of all binding assays and is the basis for K_d (affinity) and B_{max} (receptor density) determinations. The assay essentially involves the incubation of a fixed amount of tissue with increasing concentrations of the radioligand in the presence (nonspecific binding) or absence (total binding) of an excess amount of unlabelled ligand which will compete for the receptor. Specific binding at each concentration of radioligand is then calculated as the difference between total and nonspecific binding [Figure 1-11].

Many radioligands are available to reversibly bind to the receptor in a saturation binding assay. Suitable ligands must be pure, stable, have biological activity, have high specific activity (i.e. tritium) and be specific for the given receptor. In reality, however, this last criterion has been difficult to achieve because many ligands bind to more than one site. The choice of a ligand ultimately depends on the following:

1. Receptor density - Receptors present in low density require a ligand with high specific activity.
2. Receptor affinity - Only high affinity receptors are measurable.
3. Receptor specificity - The ligand should be highly specific for the receptor of interest.
4. Cost
5. Safety

Binding assays employed in most direct studies of ligand-receptor interaction involve incubation of the selected tissue preparation with an isotopically labelled

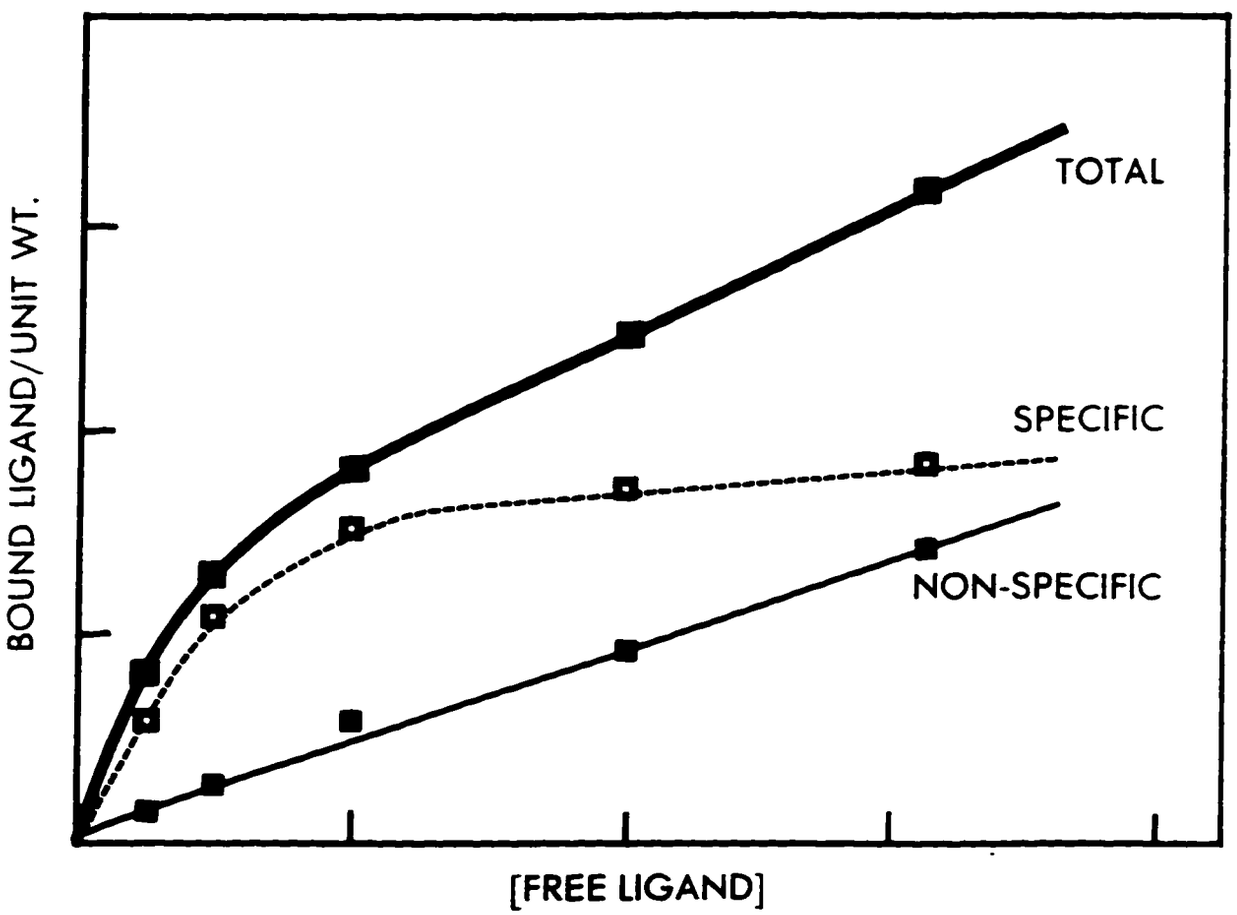


Figure 1-11: Typical binding curves from a direct binding assay. Reprinted with permission from Humana Press from *Neuromethods, Volume 4, Receptor Binding,* Boulton A.A., Baker G.B. and Hrdina P. [eds.] (1986), p.8.

ligand, separation of the bound from the free ligand (e.g. *via* filtration), and determination of the bound radioactivity (Hrdina, 1986). The analysis of the kinetics of ligand-receptor interactions is based on the law of mass action and Michaelis-Menten analysis of enzyme-substrate interactions according to the following equations:

$$(1) \qquad [R] + [L] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [RL]$$

where [R] is the concentration of the free receptor, [L] is the concentration of the ligand, [RL] is the concentration of the receptor-ligand complex, k_1 is the association constant , and k_2 is the dissociation constant. This reversible reaction is assumed to obey the law of mass action at equilibrium. K_d is the equilibrium dissociation constant for the receptor-ligand interaction (or the affinity of the receptor for the ligand) and is also defined by the law of mass action:

$$(2) \qquad K_d = \frac{[R][L]}{[RL]} = \frac{k_2}{k_1}$$

Derivation of the above equation, followed by subsequent linearization, yields the Scatchard equation:

$$(3) \qquad \frac{[RL]}{[L]} = \frac{[B_{max}]}{K_d} - \frac{1}{K_d}[RL]$$

where $\frac{[RL]}{[L]}$ is $\frac{Bound}{Free}$ (or $\frac{B}{F}$), B_{max} is the density of receptors, and K_d is the affinity of the receptor for the ligand. So, to simplify the equation,

$$\textbf{(Scatchard Equation)} \qquad \frac{B}{F} = \frac{B_{max} - B}{K_d}$$

By measuring B and knowing the concentration of F in the incubation mixture, $\frac{B}{F}$ can be plotted and a best line can be fitted by linear regression to determine B_{max} (x-intercept) and K_d (-the reciprocal of the slope) values (see Figure 1-12).

1.7.2 *Gas Chromatography*

Gas Chromatography (GC) is a physical technique that separates two or more compounds based on their differential distribution between a stationary and a mobile phase. Theoretically, any compound that can be vaporized or converted to a volatile derivative can be analyzed by GC. In reality, however, GC is most commonly applied to the analysis of organic compounds in their neutral, nonionic forms (Poklis, 1989). The stationary phases can be liquid or solid, but are most commonly high-boiling nonvolatile liquids coated or bonded on inert supports. The mobile phase is a stream of inert carrier gas such as helium, nitrogen, argon or hydrogen which functions to carry the vaporized components over the stationary phase. A mixture of compounds is separated into its constituent components by moving the mobile phase over the stationary phase. Actual separation is achieved by injecting the sample into a heated inlet block so it is immediately vaporized and swept by a stream of carrier gas through the column (which contains the stationary phase). The components are adsorbed onto the stationary phase at the head of the column and are gradually desorbed by fresh carrier gas, based on their partition coefficients between the stationary and the mobile phase (Baker and Greenshaw, 1989). Partitioning of the components between the two phases occurs repeatedly as carrier gas sweeps them toward the column outlet.

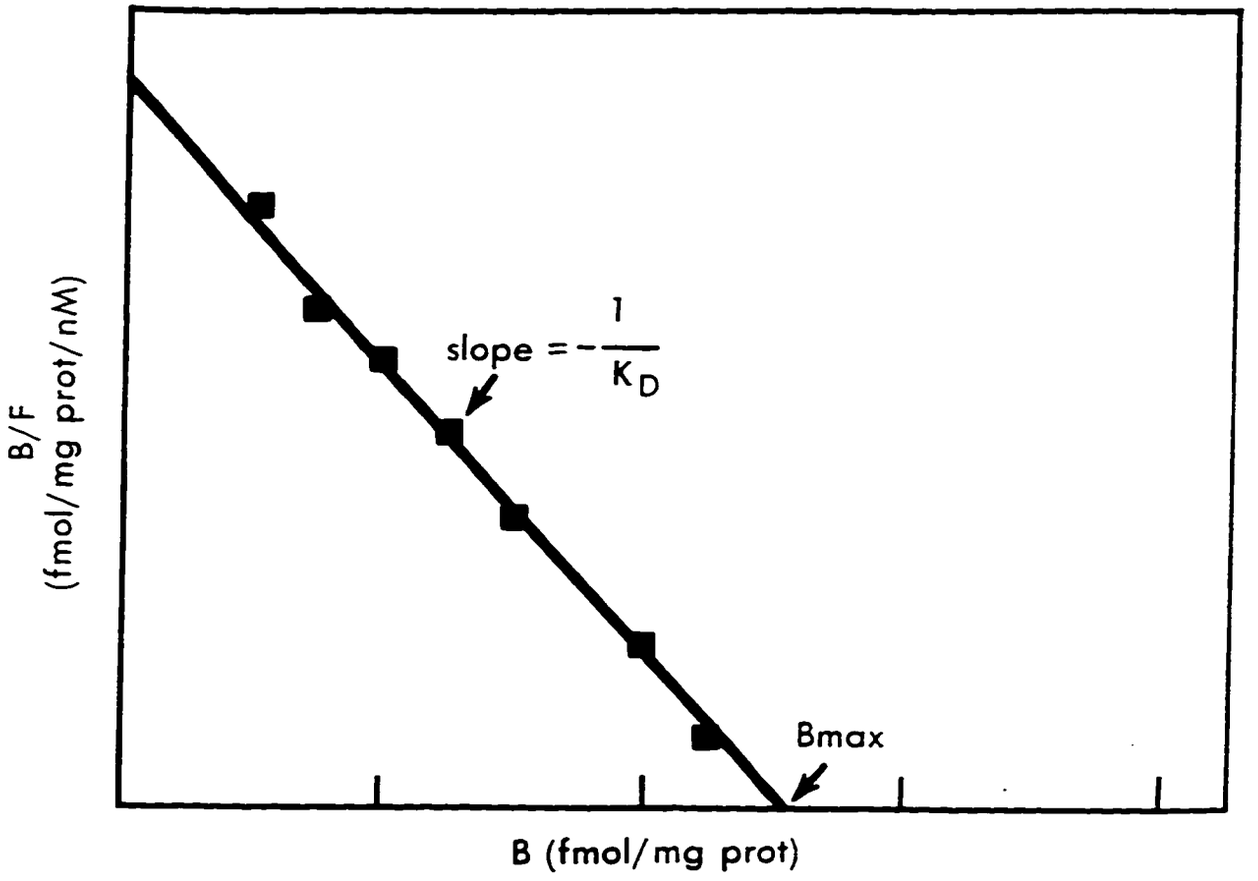


Figure 1-12: A typical Scatchard plot of binding data. Reprinted with permission from Humana Press from *Neuromethods, Volume 4, Receptor Binding,* Boulton A.A., Baker G.B. and Hrdina P. [eds.] (1986), p.67.

A basic GC system consists of a heated inlet or sample injection port to allow entry and vaporization of the sample and entry of the inert carrier gas, an oven in which the column is contained with a variable temperature range (i.e. 20-300°C), a detector that detects each component as it elutes from the column, and an integrator or recorder that records and integrates the signal received by the detector into a series of peaks *versus* time (Coutts and Baker, 1982; Burtis et al., 1987). Some of these GC components will be discussed in more detail below.

The two main types of injection systems available for GC are the splitless and split systems (Coutts et al., 1985). The splitless system is recommended for analysis of very dilute and wide boiling-range samples and involves introducing and vaporizing the sample in a glass-lined tube that extends from the septum to the head of the column. The split system is recommended for the analysis of concentrated samples and involves splitting the carrier gas stream such that only a small proportion of the injected sample enters the column. The splitless system was used in the GC analysis described in this thesis.

GC columns are commercially available in materials such as borosilicate glass fused silica, stainless steel, nickel, copper, aluminum, and nylon (Baker et al., 1982; Coutts and Baker, 1982; Coutts et al., 1985). The experiments described in this thesis used fused silica capillary columns, so only this type of column will be discussed. The two types of capillary columns available are the wall-coated open tubular (WCOT) and the support-coated open tubular (SCOT). The WCOT contains a liquid phase which is deposited directly onto the inner glass of the column whereas the SCOT includes a thin layer of solid support material which is coated

with the liquid phase (Poklis, 1989). WCOT columns were used in the experiments described in this thesis because they are suited better for relatively low analyte concentrations as they permit the analysis of larger volumes of sample.

The detectors that are generally employed for GC include the thermal conductivity detector (TCD), the flame ionization detector (FID), the nitrogen-phosphorus detector (NPD), the electron capture detector (ECD), and the mass spectrometer (MS) as a detector. The ECD and MS were utilized for this thesis, but only the ECD will be described here (see section 1.6.4 for MS). In the ECD, a radioactive isotope (i.e. ^{63}Ni or ^{3}H) releases beta particles (high-energy electrons) that collide with the carrier gas molecules to produce many low-energy electrons which are collected on electrodes to produce a small standing current (Baker et al., 1982; Coutts and Baker, 1982; Coutts et al., 1985). As sample components containing chemical groups with high electron affinity are eluted from the column, they capture the low-energy electrons generated by the isotope to form negatively charged ions. The detector measures the loss of cell current which is amplified by the integrator and recorded as a peak. The ECD can detect and quantitate as little as 1 picogram of analyte and is particularly sensitive for compounds containing halogen atoms or ketone or nitro groups (i.e. electronegative compounds) [Coutts and Baker, 1982; Baker et al., 1982].

Before GC analysis can be conducted, derivatization of the compound of interest is often necessary (see Coutts and Baker, 1982; Coutts et al., 1985 for reviews). The main reasons for derivatization include:

1. To increase volatility.

2. To increase stability.
3. To decrease the polarity of the analytes in order to improve chromatographic properties.
4. To improve extraction efficiency from aqueous solutions.
5. To introduce a functional group that is sensitive to the detector being employed.

Derivatizations usually involve replacement of the active hydrogen in polar groups (i.e. -NH, -OH, -SH) *via* acylation, alkylation, silylation or condensation.

1.7.3 *High Performance Liquid Chromatography*

High performance liquid chromatography (HPLC) is a form of separation science where a liquid phase is percolated through a column containing the stationary phase to promote the separation of sample components by a differential distribution between these two phases. A basic HPLC system involves pumping solvents from a reservoir at a set rate and set percent composition and blending them into a homogenous mixture with the mobile phase. The mobile phase flows through an injection valve that has an associated sample loop before entering the column. Guard columns are often placed in line ahead of the main analytical column to trap particulates and bind strongly retained compounds. These columns usually have the same stationary phase as the analytical column. Individual components that are separated in the column pass into the detector where an electronic signal corresponding to the amount of compound present is recorded as

a peak in a chromatogram (Bowers, 1989). To reiterate, the basic components of an HPLC system include a reservoir, a pump, an injector system, a column, a detector and an integrator and/or recorder. Some of these components will be discussed in more detail below.

Several kinds of solvent-delivery systems or pumps are available for HPLC, including pneumatic amplifiers, syringe, diaphragm and small displacement reciprocating pumps. The most common kind of pump and the one used for HPLC analysis described in this thesis was the reciprocating pump. This type of pump uses two or more pump heads that are out of phase so that there is a constant delivery of solvent while keeping 'pump noise' at a minimum.

The types of injector systems available for HPLC include the syringe and the sample loop injector. The sample loop injector was used for the HPLC analysis described herein and involves loading a sample aliquot into an external loop of stainless steel tubing, rotating the valve so the sample loop is flushed onto the column by the mobile phase, and then returning the valve so the next sample can be loaded. This type of sample injector can also be automated to allow for reliable, precise and unattended HPLC operation.

In comparison to GC, HPLC has relatively few standard stationary phases (Bowers, 1989). However, changing solvent strength, pH, and composition of the mobile phase greatly adds to the versatility of HPLC. The most common type of HPLC is bonded-reversed phase HPLC and this method was employed in the HPLC analysis described in this thesis. This mode of HPLC utilizes a nonpolar, bonded stationary phase such as octadecylsilane (ODS or C-18) and a polar mobile phase

such as acetonitrile. With this mode of HPLC polar molecules are eluted first and nonpolar last.

The detectors most commonly used for HPLC include the absorbance or ultraviolet-visible wavelength (UV-Vis) detector, the fluorescence detector, the electrochemical detector, and the MS as a detector. The type of detector utilized for HPLC work in this thesis was the UV-Vis detector, so it will be described in more detail. Drug molecules that contain functional groups that absorb UV-Vis radiation can be analyzed and quantitated by UV-Vis detection, with quantitation based on Beer's law. Three types of photometers are available, including: 1) fixed wavelength which monitors absorbance at a predetermined wavelength, 2) variable wavelength which can be adjusted to monitor different wavelengths in the same sample mixture, and 3) photodiode array which continually monitors the mobile phase effluent over a wide range of wavelengths (i.e. 200-300 nanometers; nm). The photometer used in this thesis was the variable wavelength photometer with a tungsten-halide lamp used to emit bands. This unit is capable of detection between 190 and 700 nm, although its primary use is in the UV range between 190 and 380 nm.

1.7.4 Mass Spectrometry

Mass spectrometry (MS) is a powerful analytical system capable of chemical structure elucidation. The coupling of a GC to a mass spectrometer provides a highly specific identification technique. Historically, there were problems combining GC and MS because while the GC operates at positive pressure, MS operates under a high vacuum. This problem has been overcome by the introduction of large

vacuum pumps and a separator which removes most of the carrier gas, thus concentrating the analyte and decreasing the total volume of gas entering the mass spectrometer. The mass spectrometer functions to generate ions from volatized compounds, separate the ions according to their mass to charge (m/z) ratios and then develop a mass spectrum of the relative abundance of the ions *versus* the m/z ratio. Generally, the production of a spectrum by MS involves ionization, mass filtration and detection. There are three modes of ionization commonly used in MS, including electron impact (EI), chemical ionization (CI), and negative chemical ionization (NCI) [Poklis, 1989]. The EI mode was used for all MS analysis conducted for this thesis, so only this mode will be discussed here.

In the EI mode, the sample under investigation is vaporized in a vacuum chamber and then leaked into the ionization chamber where some of the sample molecules are bombarded with a beam of high energy electrons (Baker et al., 1982; Coutts et al., 1985; Poklis, 1989). Upon impact, fragmentation occurs to form positive, negative and neutral fragments, and this compound fragmentation is very reproducible. The charged fragments (usually the positive ones) are separated according to their respective masses by accelerating them out of the ion source and toward an ion collector. Electrical signals are produced by the ions striking the collector and these are amplified and recorded to produce the mass spectrum.

Two analyzing modes are available for MS: total ion monitoring (TIM) and single or selective ion monitoring (SIM). In SIM, preselected ions can be focussed at the detector by holding the magnetic field constant (Coutts et al., 1985). This greatly increases the sensitivity of MS (i.e. picogram quantities) for targeted

compounds.

1.8 THESIS OBJECTIVES

The following objectives dealing with serotoninergic antidepressants formed the basis for this thesis:

A. To determine the effects of chronic administration of the selective, reversible MAO-A inhibitors MOC and BROF on:

   i) tryptamine (T) and 5-HT_2 receptors.

   ii) excretion of T, 5-HT, MHPG and β-PEA

   iii) brain levels of T and 5-HT

   iv) levels of MAO-A and -B inhibition

   and to compare these effects to the nonselective, irreversible MAOI, PLZ.

B. To determine the effects of MOC and BROF on GABAergic mechanisms and compare them to PLZ.

C. To investigate some novel metabolites of FLU by:

   i) developing a TFMP assay.

   ii) developing an amine metabolite assay.

   iii) performing GC-MS on some potential metabolites of FLU.

   iv) determining if the above metabolites effect 5-HT uptake and/or are substrates for MAO.

D. To investigate potential metabolic pathways for FLU in humans by:

i) analyzing some of the metabolites found in human urine and/or plasma after FLU treatment.

ii) utilizing cDNA-expressed human CYP450 isozymes to investigate the formation of some novel metabolites from both FLU and NFLU.

E. To investigate potential drug-drug interactions between MOC and FLU using human liver microsomal preparations and cDNA expressed isozymes.

The studies relevant to each of these objectives are presented in separate chapters to permit a brief, but specific, introduction on the rationale for each.

1.9 CHEMICALS AND INSTRUMENTATION

1.9.1 *Chemicals Used*

Table 1-5: Chemicals used for the experiments described in this thesis.

Chemicals	Suppliers
acetic acid - glacial	BDH Chemicals (Toronto, ON)
acetic anhydride	Caldron Laboratories (Georgetown, ON)
acetonitrile, HPLC grade distilled in glass	BDH Chemicals
alanine	Aldrich (Milwaukee, WI)
γ-aminobutyric acid	Aldrich
^{3}H-γ-aminobutyric acid	Dupont, NEN Products (Boston, MA)
2-aminoethylsothiouronium bromide	Sigma (St. Louis, MO)

Chemicals	Suppliers
aminooxyacetic acid	Sigma
ammonium acetate	Fisher Scientific (Edmonton, AB)
ammonium hydroxide	Fisher Scientific
ascorbic acid	Fisher Scientific
brofaromine HCl	Ciba-Geigy Pharmaceuticals (Summit, NJ)
calcium chloride	Fisher Scientific
chloroform, reagent grade	Fisher Scientific
p-chloro-N-[2-[2-(hydroxyethyl)amino]-ethyl]benzamide (Ro 16-3177)	Hoffmann-La Roche
p-chloro-N-(2-morpholinoethyl)benz-amide N-oxide (Ro 12-5637)	Hoffmann-La Roche
p-chloro-N-[2-(3-oxomorpholino)ethyl-benzamide (Ro 12-8095)	Hoffmann-La Roche
p-chlorophenylethylamine	Sigma
copper sulfate	Fisher Scientific
dimethylsulfoxide (DMSO)	BDH Chemicals
2,4-dichlorophenol	Sigma
di-(2-ethylhexyl)phosphate (DEHPA)	Sigma
ethyl acetate	BDH Chemicals
ethylenediaminetetraacetic acid (EDTA)	Sigma
rac-fluoxetine HCl	Lilly Research Laboratories (Indianapolis, IN)
fluvoxamine maleate	Solvay Duphar Pharmaceuticals (Hanover, Germany)
folin-phenol reagent	Sigma

Chemicals	Suppliers
glucose	Fisher Scientific
D-glucose-6-phosphate	Sigma
D-glucose-6-dehydrogenase	Sigma
β-glucuronidase/aryl sulfatase (glusalase)	Sigma
glutathione	Sigma
glycerol	Sigma
gum tragacanth	Fisher Scientific
haloperidol	Sigma
hydrochloric acid	Fisher Scientific
p-hydroxybenzyl alcohol (PHBA)	Fisher Scientific
γ-hydroxyphenylpropylamine (HPPA)	Lilly Research Laboratories
^{14}C-5-hydroxytryptamine	Dupont, NEN Products
^{3}H-5-hydroxytryptamine	Dupont, NEN Products
p-iodo-N-(2-morpholinoethyl)benzamide (Ro 11-9900)	Hoffmann-La Roche
isopentane	BDH Chemicals
^{3}H-ketanserin	Dupont, NEN Chemicals
ketoconazole	RBI Chemicals (Wayland, MA)
α-ketoglutarate	Sigma
magnesium chloride	Fisher Scientific
magnesium sulfate	Fisher Scientific
methanol, high grade	BDH Chemicals
3-methoxy-4-hydroxyphenylglycol (MHPG)	Calbiochem (San Diego, CA)
N-methyl-γ-hydroxyphenylpropylamine (NMHPPA)	Lilly Research Laboratories

Chemicals	Suppliers
5-methyltryptamine (5-MT)	Aldrich
mianserin	Sigma
moclobemide	Hoffmann-La Roche Limited (Mississauga, ON)
nialamide	Sigma
β-nicotinamide adenine dinucleotide phosphate (NADP)	Sigma
nipecotic acid	RBI Chemicals
rac-norfluoxetine	Lilly Research Laboratories
octyl sodium sulfate	Aldrich
pargyline HCl	Sigma
pentafluorobenzenesulfonyl chloride (PFBS)	Aldrich
pentafluorbenzoyl chloride (PFBC)	Aldrich
pentafluorpropionic anhydride (PFPA)	Aldrich
perchloric acid	Fisher Scientific
phenelzine sulfate	Sigma
β-phenylethylamine HCl	Sigma
^{14}C-β-phenylethylamine	Dupont, NEN Products
poly(ethylenimine)	Aldrich
potassium bicarbonate	Fisher Scientific
potassium carbonate anhydrous	Fisher Scientific
potassium chloride	Fisher Scientific
potassium phosphate dibasic	Fisher Scientific
potassium phosphate monobasic	J.T. Baker Canada (Toronto, ON)
pyridoxal phosphate	Sigma

Chemicals	Suppliers
quinidine sulfate	McArthur Chemical Company (Montreal, ON)
scintillation fluid (Ready Safe™)	Beckman Instruments Inc. (Edmonton, AB)
sodium bicarbonate	Fisher Scientific
sodium chloride	Fisher Scientific
sodium hydroxide	Fisher Scientific
sodium phosphate, monobasic	Fisher Scientific
sodium borate	Fisher Scientific
S-(-)-N-(trifluoroacetyl)prolyl chloride	Aldrich
sodium dodecyl sulfate	Scwarz/Mann (Cambridge, MA)
sodium carbonate	Fisher Scientific
sodium potassium tartrate	Allen & Hanbury's (Toronto, ON)
sodium phosphate, dibasic, anhydrous	Fisher Scientific
sodium phosphate monobasic	Fisher Scientific
sodium acetate	J.T Baker Canada
toluene, distilled in glass	BDH Chemicals
toluene, reagent grade	BDH Chemicals
trifluoroacetic anhydride (TFAA)	Aldrich
p-trifluoromethylphenol	Aldrich
tri-n-octylamine	Sigma
tris(hydroxymethyl)aminomethane (TRIS)	Fisher Scientific
Triton X-100®	Terochem Lab. Ltd. (Edmonton, AB)
^{3}H-tryptamine	Dupont, NEN Products
tryptamine HCl	Sigma

1.9.2 ANALYTICAL INSTRUMENTATION AND APPARATUS

1.9.2.1 *Gas Chromatography (GC)*

For determination of trifluoromethylphenol (TFMP), NMHPPA, HPPA and MHPG a Hewlett Packard (HP) Model 5880 gas chromatograph equipped with a 15 mCi ^{63}Ni linear electron capture detector (ECD), an HP 7673A automatic sample injector and an HP 5880A integrator was used. The chromatographic column was a 25 m x 0.3 mm internal diameter (i.d.) HP-5 narrow-bore fused-silica column (1.05 µm film of 5% phenylmethylsilicone as the stationary phase; Hewlett-Packard, Palo Alto, CA, USA). The carrier gas was ultra-pure helium (Linde, Union Carbide) at a flow rate of 3 ml/min and the make-up gas was argon-methane (95:5; Linde, Union Carbide) at a flow-rate of 30 ml/min. The injector port and detector temperatures were 200°C and 325°C, respectively.

Determination of the enantiomers of both FLU and NFLU was performed on an HP 5880 gas chromatograph equipped with a 15mCi ^{63}Ni ECD, an HP 7673A automatic sampler and an HP 5880 A integrator. The chromatographic column was a 15 m x 0.25 mm i.d. DB-5 cross-linked fused-silica capillary column (0.22 µm thickness; J&W Scientific, Palo Alto, CA, USA). The carrier gas, helium (Linde, Union Carbide), was set at a flow rate of 1 ml/min. The make-up gas was argon-methane (95:5; Linde, Union Carbide) at a flow rate of 30 ml/min. The injection port temperature was 270°C and the detector temperature was 325°C. A splitless injection system was employed.

1.9.2.2 *High Pressure Liquid Chromatography (HPLC)*

Levels of MOC and three of its metabolites were examined using an HPLC system consisting of a Waters 510 solvent delivery system (Waters Associates, Milford, MA) coupled to a Waters WISP 710B automatic injector (Waters Associates, Milford, MA). The components were separated with a Hypersil CN (4.6 mm x 250 mm i.d.; 5 µm particle size) column coupled to a guard column which was packed with the same material as the analytical column. Eluants from the column were detected by a Waters 481 ultraviolet detector (Milford, MA) set to measure at a wavelength of 240 nm. Chromatographic peaks were recorded and integrated using an HP 3392 A integrator. The mobile phase, pumped at a flow rate of 1.0 ml/min, consisted of a combination of acetonitrile (50%) and ammonium acetate buffer (final concentration 10 mM) adjusted to pH 5.4 with acetic acid. The solvent was delivered at a flow-rate of 1 ml/min.

1.9.2.3 *Mass Spectrometric (MS) Detection*

Confirmation of the chemical structures of all the derivatives utilized in the development of GC assays for this thesis were obtained by combined GC-MS. The GC-MS system utilized an HP 5840 A GC inlet coupled to an HP 5985 A MS with dual electron impact/chemical impact sources and an HP 7920 data system. The system also included an HP 2648 A graphics terminal, and HP 9876 A printer, HP 7920 disc drive (software) and HP 21 MX series E computer (hardware). Operating conditions for the MS were as follows: ion source temperature, 200°C; interface temperature, 275°C; column pressure, 34.5 kPa; accelerating voltage, 2200eV;

ionization voltage, 70eV; scan speed, 100 amu/second and dwell time, 200 msec. The same column and oven conditions described in section 1.8.2.1 were used.

1.9.2.4 *Liquid Scintillation spectrometry*

A Beckman LS 6000 liquid scintillation spectrometer coupled to a Datamax 43 printer was used for counting radioactivity in all receptor binding assays, in procedures for analysis of MAO activity and in GABA- and 5-HT- uptake studies.

1.9.2.5 *Ultraviolet Spectrophotometer*

A Hitachi U2000 spectrophotometer (Tokyo, Japan) was used for determination of protein concentrations in homogenates used in receptor binding studies.

1.9.2.6 *Filtration*

Filtration in radiochemical binding assays was carried out with a 48 sample Brandel Cell Harvester (Gathersburg, MD) filtered with Whatman GF/C filters.

Filtration in uptake studies was conducted with a 12 sample Millipore vacuum filter apparatus (Millipore Ltd., Nepean, Ontario, Canada) filtered with Whatman GF/A filters.

1.9.2.7 *Centrifuges*

A Sorvall GLC-2b or Sorvall GLC-1 General Laboratory Centrifuge (Dupont

Instruments, Wilmington, DE, USA) was used for low-speed (up to 1500 g), small volume centrifugations. Higher speed centrifugations were carried out in a Beckman Model J-21B refrigerated preparative centrifuge (Palo Alto, CA, USA) or a Beckman Model L7-55 refrigerated preparative ultracentrifuge (Palo Alto, CA, USA).

1.9.2.8 *Savant Evaporator*

A Savant Speed Vac SS1 (Savant Instruments, Inc., Farmington, NY, USA) was used for evaporating samples. The instrument consists of a concentrator (a rotor chamber with a heater), a chemical trap that accepts disposable cartridges, a refrigerated condensation trap and a vacuum pump. The process of drying involves combining centrifugal force and vacuum.

1.9.2.9 *Tissue Homogenizer*

A combination of a TRI-R S63C (TRI-R Instruments, Rockville Center, NY, USA) variable speed laboratory motor with a Teflon™ glass pestle and a glass grinding tube was used for homogenizing all tissue samples for GC and HPLC analysis. For the sake of consistency, a setting of 7 was used at all times.

For the receptor binding assays an Ultra-Turrax T-25 (Janke & Kunkel Instruments, Staufen, Germany) tissue homogenizer was used.

1.9.2.10 *Tissue Chopper*

Prisms of tissue for uptake studies were obtained with a McIlwain tissue chopper (The Mickel Laboratory Engineering Company, Gomshall, Surrey, U.K.).

1.9.2.11 *Shaker-Mixer*

Two types of vortex-shakers were used: Ika-Vibrex VXR2 Shaker™ (Janke and Kunkel Instruments, Staufen, Germany) and a Thermolyne Maxi Mix™ vortex mixer (Sybron/Thermolyne Instruments, Dubuque, IO, USA).

1.9.2.12 *Weighing Balances*

A Mettler AE 160 electronic balance (Zurich, Switzerland) was used for weighing chemicals and biological samples.

1.9.2.13 *Glassware Cleaning*

All glassware was rinsed with tap water and then washed with biodegradable Sparkleen™ (Fisher Scientific Co.) in a dishwasher (Miele Electronic 6715). Non-radioactive test tubes were sonicated (ultra-sonic cleaner, Mettler Electronics) in a 2-5% solution of Decon 75 concentrate (BDH Chemicals) before being washed in the dishwasher (without Sparkleen™). Radioactive glassware was allowed to soak for a minimum of 24 h in a 2-5% solution of Decon 75, and cleaned as described above. After removal from the dishwasher, all glassware was air-dried in a mechanical convection oven (Model 28, Precision Scientific Group, Chicago, USA).

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CHAPTER 2

Comparison of neurochemical effects of the irreversible, nonselective MAO inhibitor phenelzine, and the reversible, selective MAO-A inhibitors moclobemide and brofaromine in the rat after acute and chronic administration.

2.1 INTRODUCTION

When the MAOIs were first introduced, most of them soon fell from favor because of apparent toxic side effects (Blackwell and Marley, 1966) and unfavorable reports of their efficacy (Raskin et al., 1974). It was later observed, however, that the earlier trials in which MAOIs appeared to be less effective than tricyclics suffered from poor patient selection and also that the doses of MAOIs used in these early trials would now be considered sub-therapeutic (Hyman et al., 1995). This observation, together with the recognition that the "cheese effect" in patients taking MAOIs has been greatly exaggerated, helped renew interest in MAOI therapy (Coutts et al., 1986). Even with the renewed interest, however, clinicians were still cautious in prescribing irreversible MAOIs due to their delayed onset of action, their prolonged duration of action, the possibility of hepatotoxicity, and the impending possibility of hypertensive crisis if patients did not comply to the diet restrictions set out for them. The development of reversible, selective inhibitors of MAO-A (i.e. RIMAs) introduced a new generation of MAOIs that were found to have antidepressant efficacy with less risk of producing serious hypertensive crisis (Nair et al., 1993). The RIMAs were also found to differ from the older MAOIs in that, since they do not irreversibly inhibit the MAO enzyme, they have a relatively brief pharmacological action (Priest, 1990). Despite the apparent safety of the RIMAs, however, the nonselective, irreversible MAOIs PLZ and tranylcypromine (TCP) have been the mainstays of MAOI therapy for many years and are the only two MAOIs available in the United States for the treatment of psychiatric disorders

(Hyman et al., 1995). In Canada, PLZ, TCP and MOC are the MAOIs currently available. Encouraging results have also been obtained using BROF as a treatment for major depression (Volz et al., 1994), but BROF has recently been dropped as an experimental drug due to patent protection issues. However, because of its properties, BROF continues to be a useful pharmacological tool in animal studies.

Although the MAOIs are not used as extensively as the other classes of antidepressants in the treatment of major depressive disorder, they are still often the drugs of choice for atypical depression and depression associated with anxiety, agitation and phobias (Tyrer, 1976; Sheehan et al., 1980; Jenike, 1984; Murphy et al., 1984). However, a survey conducted by Murphy et al. in 1987 revealed that PLZ, TCP and isocarboxazid are all equally as effective as TCAs in treating not only atypical, but also major depression. Subsequent studies have substantiated this view (see Martin et al., 1994 for review). In addition, trials comparing MOC with imipramine and other antidepressants (see Priest, 1990) indicated that MOC is equally as effective as other antidepressants for a wide range of depressive illnesses, including major depression. Thus, MAOIs and RIMAs may be more useful in treating major depressive disorders than is generally realized and greater knowledge of these drugs will be useful to regulatory bodies in determining which drugs to consider for the treatment of depression.

Despite the fact that the non-selective MAOIs (e.g. PLZ) have been used clinically for many years, much is still unknown about their mechanisms of action. It is well known that, when administered acutely, MAOIs and TCAs act through

separate neurochemical mechanisms. For example, because TCAs block the reuptake of 5-HT and NA at nerve terminals, they effectively delay the inactivation of these neurotransmitters, thereby increasing their availability at post-synaptic receptors. In contrast, MAOIs decrease the intracellular metabolism of neurotransmitters in the pre-synaptic nerve terminal by inhibiting the enzyme responsible for their catabolism and, thus, ultimately increasing the availability of amines that can be released from the nerve terminal (Nair et al., 1993). In both cases, however, it is likely that there are much more complex actions occurring to produce antidepressant effects than a simple elevation of brain catecholamine and 5-HT levels. More specifically, the inhibition of MAO by drugs such as PLZ and TCP is known to often dramatically elevate levels of a number of trace amines (e.g. β-phenylethylamine, tryptamine, tyramine) in the brain (Philips and Boulton, 1979; Boulton and Juorio, 1982; Baker et al., 1992), and several studies have also shown that in the rat, brain levels of γ-aminobutyric acid (GABA) significantly increase following PLZ administration (Perry and Hansen, 1973; Baker et al., 1991; McManus et al., 1992). In addition, there is a growing body of evidence suggesting GABAergic deficiencies in depression and panic disorder and efficacy of GABA-mimetic drugs of various types in treating these disorders (Breslow, 1989; Petty, 1995; Bourin et al., 1997).

The indolealkylamines tryptamine (T) and 5-HT have both been implicated in the etiology and pharmacotherapy of depression (Saavedra and Axelrod, 1972; Slingsby and Boulton, 1976; Philips and Boulton, 1979; Baker and Dewhurst, 1985;

Juorio and Paterson, 1990; Mousseau et al., 1993). There is general agreement that long-term administration of many antidepressants, including TCAs and MAOIs, results in a down-regulation of 5-HT_{2A} receptors (Baker and Greenshaw, 1989). T is normally found in trace amounts in the brain (i.e. <1 ng/g in rat brain), but because oxidative deamination is the principal catabolic route for T, MAOIs act to increase its concentration, often dramatically (Philips and Boulton, 1979; Sullivan et al., 1986). Because T can markedly alter the uptake and/or release of 5-HT at nerve endings (Baker et al., 1977) and can also have a direct effect on 5-HT receptors, it is often presumed that T acts as a neuromodulator of 5-HT systems in the brain (Juorio and Paterson, 1990). However, the presence of specific, high affinity binding sites (receptors) for T in brain (Cascio and Kellar, 1983; Wood et al., 1984; Greenshaw and Dewhurst, 1987; van Nguyen et al., 1989) suggests that it may also be a neurotransmitter in its own right in some brain areas. Production of down-regulation of these T receptors is a characteristic common to various types of irreversible MAOIs (Wood et al., 1984; Martin et al., 1987; Sherry-McKenna et al., 1992; Mousseau et al., 1993; Goodnough et al., 1994), but there is a paucity of information available on the effects of reversible MAO-A inhibitors on T receptors. In this investigation, we examined the effects of treatment with PLZ (a nonselective, irreversible MAOI) on binding of ^{3}H-T and ^{3}H-ketanserin (radioligand for 5-HT_{2A} receptors) to membrane enriched fractions from rat brain regions and these results were compared to those obtained after treatment with MOC and BROF (selective, RIMAs).

In addition to their well-known effects on brain levels of the catecholamines

(McKenna et al., 1993; Philips and Boulton, 1979; Philips et al., 1980), 5-HT and T, MAOIs have been reported to dramatically increase brain levels of other trace amines such as β-phenylethylamine (PEA) that have been implicated in the etiology and pharmacotherapy of depression (see Mousseau, 1993, for review). In the present study, a series of acute and chronic experiments were conducted in an effort to better understand the neurochemical similarities and differences between non-selective, irreversible MAOIs (such as PLZ) and selective, RIMAs of MAO-A (such as MOC and BROF). Specifically, activities of MAO-A and MAO-B as well as the levels of a number of amines or their metabolites, including T, 5-HT, MHPG (major metabolite of NA in the CNS) and PEA were analysed in rat brain and/or urine after acute and chronic administration of the drugs. In addition, MOC, BROF and PLZ were compared for their effects on rat brain GABA levels in acute studies and on GABA-T activity and GABA uptake *in vitro* in rat brain tissue.

2.2 MATERIALS AND METHODS

2.2.1 Animals and Dosing

Male Sprague-Dawley rats (Ellerslie, Biosciences, Edmonton, Canada), 200-300 g, were housed in pairs in an environmentally controlled room using a 12 h light - 12 h dark cycle. MOC (15 mg/kg, p.o., b.i.d.), BROF•HCl (15 mg/kg, p.o., b.i.d.), PLZ sulfate (15 mg/kg, p.o., once daily), or vehicle (0.05% solution of gum tragacanth, p.o., b.i.d.) were administered for 4, 10, 14 or 30 days. Urine samples (24 h) were collected on days 4, 14 and 28 from the rats which received vehicle,

PLZ, or BROF for 30 days by placing them singly into metabolic cages following their morning drug administration and housing them there for 24 h. Due to limitations on availability of metabolic cages, urines were not collected for animals treated with MOC. The volumes of the collected urines were measured and the samples were frozen at -20°C until the time of analysis. Rats were sacrificed by guillotine decapitation either 1 or 6 h after the final dosing in each of the studies and their brains removed. These short time intervals for sacrifice were chosen because of the short elimination half-lives of BROF and MOC in the rat (Waldmeier et al., 1993). Cortex and hippocampus were dissected out from the rest of brain tissue for the purpose of receptor binding studies and for determination of brain levels of MOC and BROF. The rest of brain tissue was used for measuring levels of T and 5-HT and for MAO-A and -B activity. All tissue samples were frozen at -80°C until the time of analysis.

2.2.2 *Receptor Binding Assays*

^{3}H-T binding was performed on a membrane fraction prepared from the cortex using the procedure of Mousseau et al.(1993) using ^{3}H-T as the radioligand with the modification that a pargyline concentration of 30 μM was used in the incubation procedure. A membrane preparation was made by homogenizing the tissue in 10 volumes of washing buffer (50 mM TRIS-HCl buffer, pH 7.4) and centrifuging two times at 22, 000 x g for 17 min. The resulting tissue pellet was resuspended in 10 volumes of incubation buffer (50 mM TRIS, 30μM pargyline, and

100 µM ascorbic acid) as per original weight of cortex, incubated for 30 min at 37°C and used for the binding experiment. In triplicate, test tubes containing TRIS buffer or cold T (10 µM; cold competitor used to determine nonspecific binding of ^{3}H-T), ^{3}H-T (specific activity 25 Ci/mmol; final concentration of 0.125 nM to 10 nM to produce an 8 point saturation curve) and tissue for a final volume of 1 ml were incubated at 0°C for 1 h. Subsequently, the tissue was rapidly filtered and washed with ice-cold washing buffer (5 ml). Filters were placed into scintillation vials with 5 ml Ready Safe™ scintillation fluid. The vials were vortexed and incubated for 12 h at room temperature before the radioactivity was counted in the scintillation counter for 5 min. An outline of the ^{3}H-T binding parameters is shown in Table 2-1. Figure 2-1 displays a typical 8-point saturation curve, ranging from 0.125 nM to 10 nM, produced from the assay. Single-point binding studies on T receptors followed the same procedure as above with the exception that only one concentration of ^{3}H-T (2 nM) was incubated with the tissue and either TRIS buffer or cold T.

Single-point binding studies on 5-HT_{2A} receptors were performed on membrane fractions from the hippocampus using ^{3}H-ketanserin (specific activity 77.1 Ci/mmol, 0.5 nM) as the radioligand (Eison et al., 1991; Goodnough and Baker, 1994). A membrane preparation was made by homogenizing the tissue in 10 volumes of 50 mM Tris-HCl buffer (pH 7.5) and centrifuging twice at 22, 000 x g for 17 min. The resulting tissue pellet was resuspended in 10 volumes as per original weight and used for the binding experiment. Whatman filters were presoaked for a minimum of 3 h in 2 g/L polyethylenimine. In triplicate, test tubes

TABLE 2-1: ASSAY CONDITIONS USED IN THE DETERMINATION OF ^{3}H-TRYPTAMINE BINDING TO THE TRYPTAMINE RECEPTOR

Tubes	Buffer (µl)	Cold ligand (µl)	Hot ligand (µl)	Tissue (µl)	Concentration of hot stock solution (nM)	Final concentration of ligand in the tube (cold and hot)
1-3/4-6	700/0	0/700	100	200	1.25	0.125nM/ 10 µM
7-9/10-12	700/0	0/700	100	200	2.5	0.25nM/ 10 µM
13-15/16-18	700/0	0/700	100	200	5	0.5nM/ 10 µM
19-21/22-24	700/0	0/700	100	200	10	1nM/ 10 µM
25-27/28-30	700/0	0/700	100	200	20	2 nM/ 10 µM
31-33/34-36	700/0	0/700	100	200	30	3 nM/ 10 µM
37-39/40-42	700/0	0/700	100	200	50	5 nM/ 10 µM
43-45/46-48	700/0	0/700	100	200	100	10nM/ 10 µM

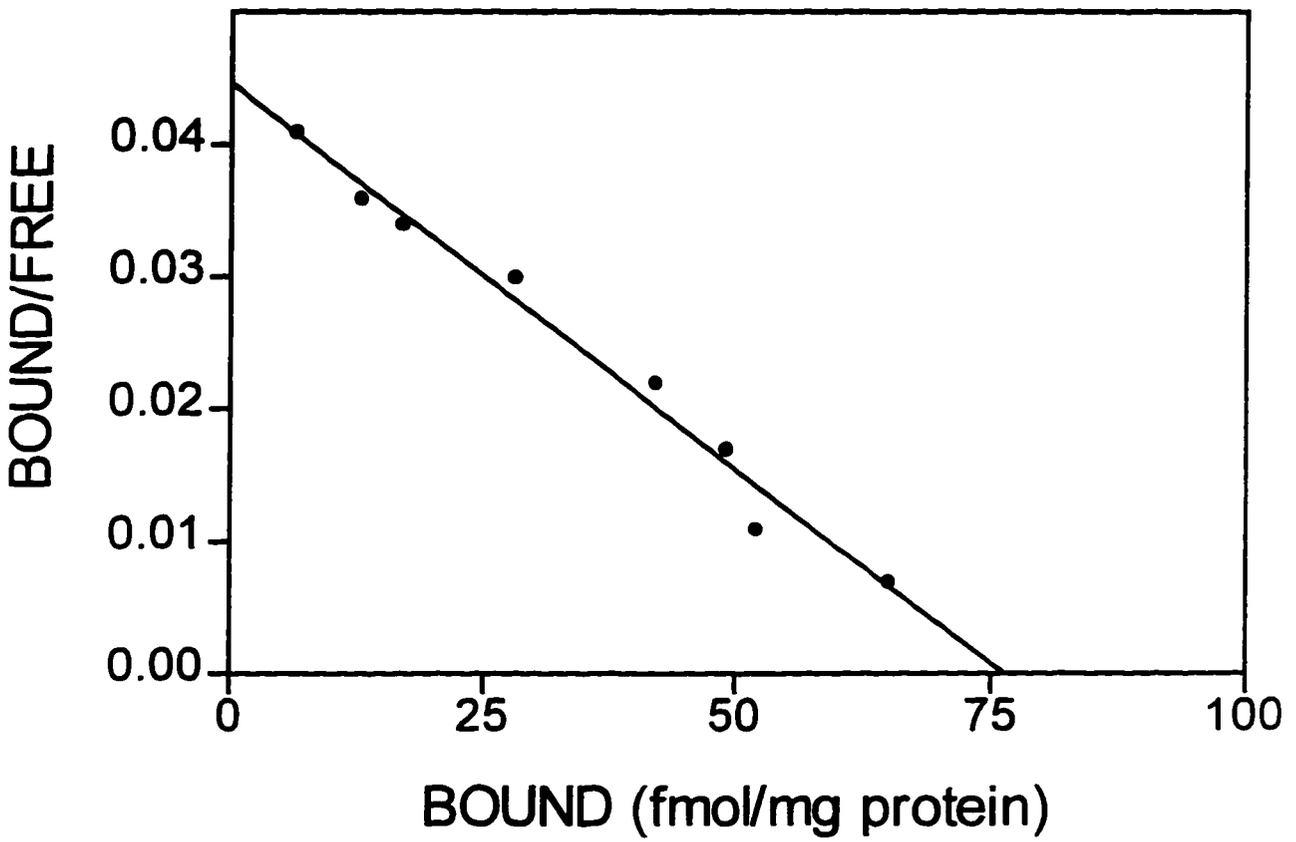


Figure 2-1: Full 8 point saturation curve for the ^{3}H-tryptamine binding assay.

containing TRIS buffer or mianserin (1.0 µM; cold competitor used to determine nonspecific binding of ^{3}H-ketanserin), ^{3}H-ketanserin (1 nM; this is near the K_d value determined previously; see Goodnough and Baker, 1994), and tissue for a final volume of 1 ml were incubated at 37°C for 15 min. Subsequently, the tissue was rapidly filtered and washed with ice-cold TRIS buffer (5 ml). Filters were placed into scintillation vials with 5 ml Ready Safe™ scintillation fluid. The vials were vortexed and incubated for 12 h at room temperature before the radioactivity was counted in the scintillation counter for 3 min. An outline of the ^{3}H-ketanserin binding parameters is shown in Table 2-2.

For both ^{3}H-T and ^{3}H-ketanserin binding, a triplicate set of vials, containing the total amounts of radioligand solution added, were run for each concentration to measure total radioactivity for the corresponding total and non-specific sets of tubes. The EBDA program (McPherson, 1987) was employed to determine the binding parameters (i.e. B_{max} and K_d).

2.2.3 Protein Determination

Protein content in rat brain homogenates was determined using a modification of the Lowry Folin Reagent Assay (Lowry et al., 1951). A volume of tissue homogenate containing between 25 µg and 75 µg of protein was added to test tubes and the volume was then made up to 200 µl by adding distilled water. Sodium dodecyl sulphate (100 µl, 2% w/v in distilled water) was added to the tubes and the mixtures were vortexed. Next, 800 µl of Solution A (1/.01/.01 v/v/v 2% sodium carbonate, 1% cupric sulfate and 2% sodium potassium tartrate) was added

TABLE 2-2: ASSAY CONDITIONS USED IN THE DETERMINATION OF ^{3}H-KETANSERIN BINDING TO THE 5-HT$_2$ RECEPTOR

Tubes	Buffer (µl)	Cold ligand (µl)	Hot ligand (µl)	Tissue (µl)	Final concentration of ligand in the tube (cold and hot)
1 - 3	700	0	100	200	1 nM
4 - 6	0	700	100	200	1 µM

and the tubes were vortexed and incubated for 10 min. Folin Ciocalteau's reagent (100 µl, 1:1 v/v 2 N Folin and distilled water) was added and the tubes were vortexed and incubated for a minimum of 10 min in the dark. Protein concentrations were measured using the Hitachi U2000 spectrophotometer. The tissue samples were run in parallel with a standard curve of wide range that is stored in the memory of the spectrophotometer.

2.2.4 Analysis of MAO Activity

MAO activity in brain tissue was analysed using a modification of the radiochemical procedure of Wurtman and Axelrod (1963), with ^{14}C-5-HT and ^{14}C-PEA used as substrates for MAO-A and -B, respectively. Tissue for this assay was homogenized in 5 volumes of 0.2 M potassium phosphate buffer (4°C, pH 7.8). To each tube 50 µl of tissue homogenate and 50 µl of appropriate radiolabelled substrate (i.e. ^{14}C-5-HT or ^{14}C-PEA) were added to the tubes and they were kept on ice. To blank tubes 10 µl HCl (3M) was added to prevent any reaction from occurring. Samples were briefly flushed with oxygen and the tubes were quickly stoppered. The tubes were then incubated for 10 min at 37°C. The enzyme reaction was terminated with the addition of 10 µl of 3 M HCl to all samples, except the blanks. The metabolites formed in the reaction were extracted into 1 ml of ethyl acetate/toluene (1:1 v/v, water-saturated) by vortex-mixing twice and centrifuging at 1600 rpm for 30 s. Following extraction, 700 µl of the organic (top) layer was carefully transferred into scintillation vials and 4 ml of Ready Safe™ scintillation fluid was added to each. Radioactive content per tube was measured using a liquid

scintillation spectrophotometer. Percent MAO inhibition was calculated by applying the following equation:

$$\textbf{\% inhibition} = 100\text{-}[100 \times \frac{(\text{sample - blank})}{(\text{sample - control})}]$$

2.2.5 Analysis of Some Amine and Metabolite Levels

Brain and urine levels of T, 5-HT, and PEA were measured by electron-capture gas chromatographic methods developed in our laboratories (Baker et al., 1981, 1986a, 1986b). Specifically, rat brain tissue was homogenized in 5 volumes of ice-cold perchloric acid (0.1 N), centrifuged at 12,000 x g for 20 min and 3 ml portions of each supernatant used for analysis. When analysing urine, 1 ml aliquots were used. The internal standard, 5-methyltryptamine, was added to each supernatant at a concentration of 250 ng and the samples were then basified with solid potassium bicarbonate to a pH of 7.8. The mixtures were each transferred to another set of tubes and 300 µl of sodium phosphate buffer (pH 7.8) was added to each tube prior to extraction with 5 ml of the liquid ion-pairing compound di-(2-ethylhexyl)phosphate (2.5% v/v DEHPA in chloroform). The mixtures then underwent shaking for 5 min and were centrifuged for 5 min at 1,000 x g before the aqueous (top) layer was aspirated to waste. The organic layers were transferred to another set of clean tubes and then back-extracted with 2.5 ml HCl (0.5 N) by shaking for 5 min and centrifuging (1,000 x g) for 5 min. The aqueous (top) layers were transferred to another set of tubes and neutralized with solid sodium bicarbonate. Acetylation was then carried out by adding 300 µl of acetic anhydride

to each tube, shaking and maintaining basic conditions with solid sodium bicarbonate as described by Martin and Baker (1977). The acetylated products were extracted by shaking with ethyl acetate for 5 min. After a brief centrifugation, the organic phases were transferred to another set of tubes and each was washed by adding 800 µl of double-distilled water, vortex-mixing for 10 sec and centrifuging for 2 min (1,000 x g). The organic (top) layers were transferred to another set of tubes and dried under a stream of nitrogen gas. The remaining residues were each redissolved in 25 µl of ethyl acetate and derivatized by adding 75 µl of pentafluoropropionic anhydride (PFPA) and reacting for 30 min at 60°C. Each reaction mixture was then mixed with 200 µl of toluene and washed by adding saturated sodium borate (3 ml), mixing for 15 sec and centrifuging for 2 min. Each organic (top) layer was transferred to a microfuge tube and aliquots of these solutions were injected onto a gas chromatograph equipped with an HP-5 fused silica capillary column (25 m x 0.3 mm i.d. x 1.05 µm film of phenylmethylsilicone), a 15 mCi ^{63}Ni linear ECD, an automatic sample injector and a printer-integrator. Separation of the compounds of interest was accomplished using the following automatic oven temperature program: initial temperature of 80°C for 0.5 min, increasing at a rate of 15 °C/min to 250°C where it was held for 10 min.

Urine levels of MHPG were also measured by electron capture gas chromatographic methods developed in our laboratories (see Baker et al., 1986b). In this case 1 ml of urine was used for analysis, after 24 h incubation with glusalase to free conjugated MHPG. To each urine sample was added 22 µl of sodium

acetate (1N, pH 6.0), 110 µl EDTA (2%) and 22 µl β-glucuronidase/aryl sulfatase (glusalase) ; the samples were placed in a shaking water bath at 37°C. Following incubation, 75 µl of perchloric acid (4 N) was added to each sample and the mixtures were vortex-mixed. The mixtures were transferred to microfuge tubes and centrifuged for 1 min, then transferred to a set of tubes with the internal standard *p*-hydroxybenzyl alcohol added. The mixtures were then basified with solid potassium bicarbonate (pH 7.8) prior to centrifuging for 5 min (1,000 x g). The mixtures were transferred to another set of tubes and acetylated by adding 225 µl acetic anhydride under basic conditions and shaking (see Martin and Baker, 1977). The mixtures were removed to another set of tubes and then extracted with 4 ml of ethyl acetate by shaking for 2 min and centrifuging for 10 min (1,000 x g). Each ethyl acetate (top) layer was transferred to another tube and taken to dryness under a stream of nitrogen gas. The residue was reacted in each case with 25 µl of ethyl acetate and 75 µl of trifluoroacetic anhydride (TFAA) for 30 min at room temperature. The derivatized compounds were then mixed with 300 µl of toluene and washed with 3 ml of saturated sodium borate by vortex-mixing for 15 s and centrifuging briefly. Each toluene (top) layer was transferred to a microfuge tube and aliquots of these solutions were injected onto a gas chromatograph equipped with an HP-5 fused silica capillary column (25 m x 0.32 mm i.d. x 1.05 µm film of phenylmethylsilicone, cross-linked 5% phenylmethylsilicone phase), a 15 mCi ^{63}Ni linear ECD, an automatic sample injector and a printer-integrator. Separation of the compounds of interest was accomplished using the following automatic oven

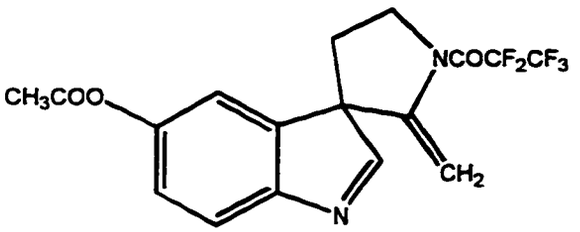
temperature program: initial temperature of 105°C for 0.5 min, increasing at a rate of 15°C/min to 230°C where it was held for 5.0 min.

In all GC assays conducted for this thesis, a calibration curve was run concomitantly with the biological samples under investigation. The curves were prepared by adding known, varying amounts of authentic standards and a fixed amount of the appropriate internal standard to a series of tubes and carrying these tubes through the assay procedure in parallel with the sample tubes. Each curve was analysed using the appropriate tissue homogenate or biological fluid obtained from control animals. The ratio of the peak height of the derivatized drug to that of the derivatized internal standard was calculated and plotted against the concentration of the drug. The final calibration concentration ranges were as follows: 20 ng to 800 ng T and 5-HT per volume of rat urine, 10 ng to 1 µg 5-HT per volume of rat brain homogenate, 0.5 ng to 25 ng T per volume of rat brain homogenate, 75 ng to 1.2 µg PEA per volume of rat urine and 500 ng to 3 µg MHPG per volume of rat urine. Structures of the GC derivatives of the above compounds are indicated in Figure 2-2.

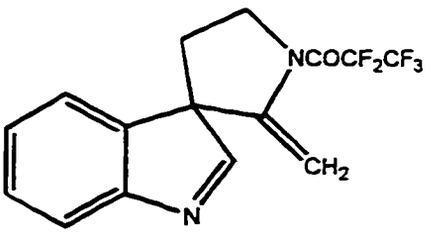
2.2.6 Analysis of Tissue Levels of Brofaromine

Rat brain tissue was weighed and homogenized in five volumes of ice-cold double-distilled water as better recoveries of BROF were found out of water than out of 0.1 N perchloric acid. Aliquots of rat brain homogenate (1 ml) were placed in a screw-cap culture tube (Fisher Scientific) and diluted to a final volume of 2 ml with distilled water. The internal standard (desipramine) was added to this solution. The

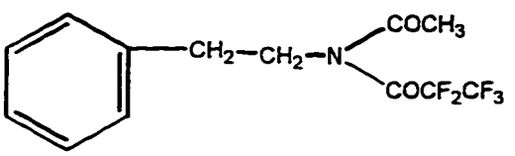
GC Derivatives:



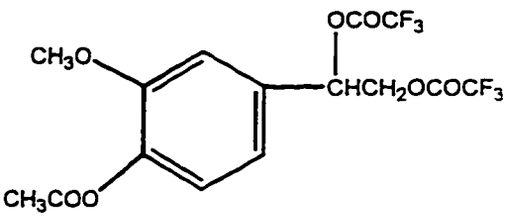
Spirocyclic N-PFP, O-acetyl derivative of 5-hydroxytryptamine



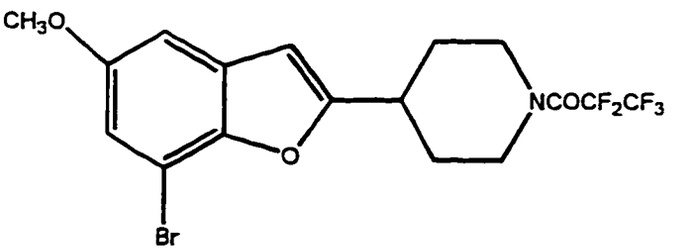
Spirocyclic N-PFP derivative of tryptamine



N-acetyl, N-PFP derivative of β-phenylethylamine



O,O-di-TFA 3-methoxy-4-acetoxyphenylglycol: a derivative of MHPG



N-PFP derivative of brofaromine

Figure 2-2: Derivatives of tryptamine, 5-HT, β-phenylethylamine, 3-methoxy-4-hydroxyphenyl glycol and brofaromine formed for GC analysis. Formation of spirocyclic derivatives of indolealkylamines under the reaction conditions used have been confirmed previously by nuclear magnetic resonance studies (Blau et al., 1977).

final calibration concentration range was 20 ng to 2.0 µg BROF per volume of tissue homogenate. The samples were then basified by adding 25% potassium carbonate (500 µl) and briefly vortex-mixing. Ethyl acetate (4 ml) was added to each sample and they were mixed for 5 min in an Ika Vibrex VXR vortex-mixer (Janke and Kunkel, Staufen, Germany) and centrifuged for 5 min at 1,000 x g in a benchtop centrifuge (Sorvall GLC-2B general laboratory centrifuge, Du Pont, Wilmington, DE, USA). The upper ethyl acetate layers were retained and transferred to another set of screw-cap culture tubes (160 mm x 15 mm) before they were back-extracted with 0.5 N HCl (2.5 ml). These mixtures were then vortex-mixed for 5 min and centrifuged for 5 min at 1,000 x g. The upper organic layers were retained and transferred to another set of tubes. They were then basified with solid potassium carbonate in addition to 200 µl of 25% potassium carbonate. Ethyl acetate (4 ml) was added to each sample and they were shaken for 5 min and centrifuged for 5 min. The upper organic layers were then transferred to glass drying tubes and the solvent was evaporated using a Savant Speed Vac SSI (Savant Instruments Inc., Farmington, NT, USA). To the dry residue was added 25 µl of ethyl acetate and 75 µl of the derivatizing reagent, pentafluoropropionic anhydride (PFPA). The tubes were briefly vortex-mixed and the reaction was allowed to proceed at 60°C for 30 min. The samples were cooled to room temperature and toluene (300 µl) and sodium borate (3 ml) were added to each tube. These mixtures were briefly vortexed and then centrifuged for 5 min at 1,000 x g. The upper (toluene) layers were retained and an aliquot (1 µl) of this solution was injected on a GC-ECD

equipped with a 25 m x 0.32 mm i.d. HP-5 narrow-bore fused-silica column (1.05 µm film of 5% phenylmethylsilicone as the stationary phase; Hewlett-Packard, Palo Alto, CA, USA). Chromatographic separation was achieved using the following oven temperature program: the initial oven temperature of 105°C was held for 0.5 min then increased at a rate of 20°C/min to a final temperature of 270°C. A Hewlett-Packard 5880A integrator was used to measure peak areas. The derivative of BROF formed and analyzed by GC is indicated in Figure 2-2.

2.2.7 Analysis of Tissue Levels of Moclobemide

Rat brains were weighed and homogenized in 5 volumes of 0.1 N perchloric acid containing EDTA (10 mg/100 ml) and ascorbic acid (0.88 mg/100 ml). The homogenates were centrifuged for 15 min at 12,000 x g and 1 ml of the resulting supernatant was added to each tube. Next, 1 ml of distilled water was added to each tube to increase the volume and 250 ng of the internal standard (Ro 11-9900; dissolved in methanol) was added. The final calibration concentration range was 50 ng to 2.0 µg MOC per volume of tissue homogenate. The samples were basified using 600 µl sodium phosphate buffer (pH~11), 5 ml ethyl acetate was added and the samples were then shaken for 15 min and centrifuged for 10 min (3000 x g). The samples were dried under nitrogen and reconstituted in 200 µl methanol. Aliquots (20 µl) were injected onto the HPLC system described in section 1.8.2.2 of this thesis. This procedure resulted in the detection of MOC as well as three of its metabolites, Ro 12-5637, Ro 12-8095 and Ro 16-3177. These metabolites were

evaluated for experiments conducted in Chapter 5 of this thesis and will be discussed further there.

2.2.8 GABA Analysis in Rat Brain

GABA levels in whole rat brain were measured using the procedure of Wong et al. (1990). Rat brains were weighed and homogenized in 5 volumes of ice-cold 0.1 N perchloric acid and centrifuged (15 min at 12,000 x g and -4°C) to remove the protein precipitate. A 10 µl portion of the supernatant was removed for the assay and norleucine (250 ng) was added as the internal standard. Next, 1 ml of 2.5% w/v potassium carbonate was added to the tubes, followed by the addition of 1 ml of an isobutyl chloroformate solution (5 µl isobutyl chloroformate in 1 ml acetonitrile:toluene, 1:9, v/v). This mixture was then vortex-mixed for 15 min at room temperature. After a brief centrifugation, the top (organic) layer was aspirated and discarded. To the bottom (aqueous) phase was added 1.5 ml 2M sodium phosphate buffer, pH 5.3., followed by the sequential addition of 2.5 ml chloroform ($CHCl_3$), 200 µl dicyclohexylcarbodiimide solution (5 µl in 1 ml $CHCl_3$) and 200 µl pentafluorophenol solution (5 µl in 1 ml $CHCl_3$). These solutions were vortex-mixed for 15 min at room temperature and after a brief centrifugation, the top (aqueous) layer aspirated and discarded. The bottom $CHCl_3$ layer was then evaporated (at 60°C) to dryness under a gentle stream of nitrogen. The residue was reconstituted in 300 µl toluene, which was then washed briefly with 1 ml distilled water. An aliquot of the toluene layer was injected on a GC-ECD equipped with a 25 m x 0.32 mm i.d.

fused-silica column (1.05 µm film of 5% phenylmethylsilicone as the stationary phase; Hewlett-Packard, Palo Alto, CA, USA). Chromatographic separation was achieved using the following oven temperature program: the initial oven temperature of 100°C was held for 0.5 min then increased at a rate of 25°C/min to a final temperature of 200°C. After maintaining at 200°C for 0.5 min, the temperature was increased to 230°C at a rate of 3°C/min. A Hewlett-Packard 5880A integrator was used to measure peak areas.

2.2.9 GABA Uptake Analysis

^{3}H-GABA uptake into cortical prisms was measured using the procedure of Iversen and Neal (1968), with ^{3}H-GABA as the substrate and nipecotic acid or fluspirilene as known inhibitors of GABA uptake. Specifically, the cortex was dissected from the rest of naive rat brain tissue and was chopped into 0.1 x 0.1 x 2 mm prisms using a tissue chopper and resuspended in 5 volumes of incubation mix [composition as follows: 123 mM sodium chloride, 5 mM potassium chloride, 2.7 mM calcium chloride, 1.2 mM magnesium sulfate, 20 mM TRIS-HCl buffer (pH 7.5), 1 mM ascorbic acid, 10 mM glucose and 0.1mM aminoxyacetic acid (an inhibitor of GABA-transaminase)]. This suspension, which contained 1 mg of tissue per ml of incubation medium, was stored out of direct light and buried in ice. Aliquots (1 ml) of the tissue suspension were added to flasks containing 4 ml of incubation mix and this mixture was incubated for 15 min at 37°C. Appropriate inhibitors (at a variety of concentrations) were added to each tube except the blanks and controls, followed by the addition of 10 µl of ^{3}H-GABA (final GABA concentration 100 µM).

These radioactive mixtures were incubated for 5 min at 37°C, then quickly filtered with a Millipore vacuum filter apparatus, rinsing twice with 5 ml warm (37°C) incubation mix. Filters were transferred to scintillation vials and 10 ml of Ready Safe™ scintillation fluid was added to each. Radioactive content per tube was measured using a liquid scintillation spectrophotometer. Percent GABA uptake inhibition was calculated by applying the following equation:

$$\textbf{\% inhibition} = 100\text{-}\left[\frac{(\text{mean of sample - mean of blank}) \times 100}{(\text{mean of controls - mean of blank})}\right]$$

The resulting percentage of inhibition was plotted against drug concentrations (on a logarithmic scale) to determine the mean concentration giving 50% inhibition of uptake (IC_{50}).

2.2.10 Analysis of GABA-Transaminase Activity

In vitro GABA-T activity was analysed by homogenizing naive, quick frozen rat brain in 20 volumes of a medium described by Palfreyman et al. (1978) with the composition modified as follows: glycerol (20% v/v), Triton X-100 (0.13% v/v), reduced glutathione (100 µM), pyridoxal 5-phosphate (1 µM), Na_2EDTA (1mM), dipotassium monophosphate (5mM) and sufficient glacial acetic acid to bring the pH value to 7.2-7.4. A 10 µl aliquot of this mixture was then utilized to assay for GABA-T activity using a modification of the radiochemical procedure described by Sterri and Fonnum (1978). Nineteen µl of the incubation medium [prepared by combining 40 µl 50mM α-ketoglutarate, 40 µl 10mM nicotinamide adenosine dinucleotide, 40

µl 10mM 2-aminoethylisothiouronium bromide, 160 µl distilled water and 100 µl 50 mM Tris buffer (pH 7.9)] was added to tubes on ice containing a 10 µl aliquot of tissue homogenate (or 10 µl distilled water for the blanks). The tubes were incubated at 37°C for 10 min, then placed on ice for 5 min. Five µl of a ^{3}H-GABA mixture [containing 2.4 µl ^{3}H-GABA (specific activity 40 Ci/mmol) plus 18 µl 100mM GABA and 63.6 µl distilled water] and 10 µl of inhibitor were added to each tube. This mixture was incubated at 37°C for 30 min with the tubes uncapped. The tubes were placed on ice and 100 µl of tri-*n*-octylamine was added. The mixture was vortexed briefly and centrifuged at 1,000 x *g* for 2 min. After centrifugation to separate the layers, a 35 µl aliquot of the top layer was removed in each case and added to a scintillation vial containing 4 ml scintillation fluid and counted for 5 min in a liquid scintillation counter.

2.2.11 Statistical Analysis

Data were analysed by two-way Analysis of Variance (ANOVA) followed, where necessary, by the Newman Keuls test for multiple comparisons between the groups. A p value of <0.05 was used to determine significance.

2.3 RESULTS

2.3.1 ^{3}H-Tryptamine Receptor Binding

A reduction in single-point ^{3}H-T binding was evident by day 4 of the study, and by day 10, binding was already reduced to 62% and 72% (n=8) of control

values with BROF and PLZ, respectively. By day 30, all three drugs had caused a significant reduction in ^{3}H-T (2nM) binding to membrane-enriched fractions in cortex. Binding was reduced to 48, 37 and 39% (*n*=8) of control (vehicle-treated) values (119 ± 5 fmol/mg protein) in the rats treated with MOC, BROF and PLZ, respectively (see Figure 2-3). Scatchard analysis indicated that the reduction in ^{3}H-T binding was the result of a reduction in B_{max} (i.e. a decrease in density) in the case of PLZ and MOC, but largely due to an increase in K_d (i.e. a decrease in affinity) in the case of BROF after both 10 and 30 days of administration [Figures 2-4 and 2-5]. The results for BROF were unexpected because normally a change in affinity is not responsible for the down-regulation of receptors. It was thought that the reason for the decreased affinity might be due to the presence of some residual drug in the binding preparations due to the short length of time after the last dosage and the time of death. To test this, some wash-out experiments were conducted where the animals were decapitated either 1 or 6 hours after the last drug administration after 10 or 14 days of chronic administration. As shown in Figure 2-6, however, a similar decrease in affinity was shown for BROF after a 6 hour washout.

2.3.2 ^{3}H-Ketanserin (5-HT$_2$-Receptor) Binding

The ^{3}H-ketanserin binding data are summarized in Figure 2-7. PLZ caused a significant down-regulation of 5-HT$_2$ receptors after both 10 and 30 days of administration while MOC and BROF had no effect.

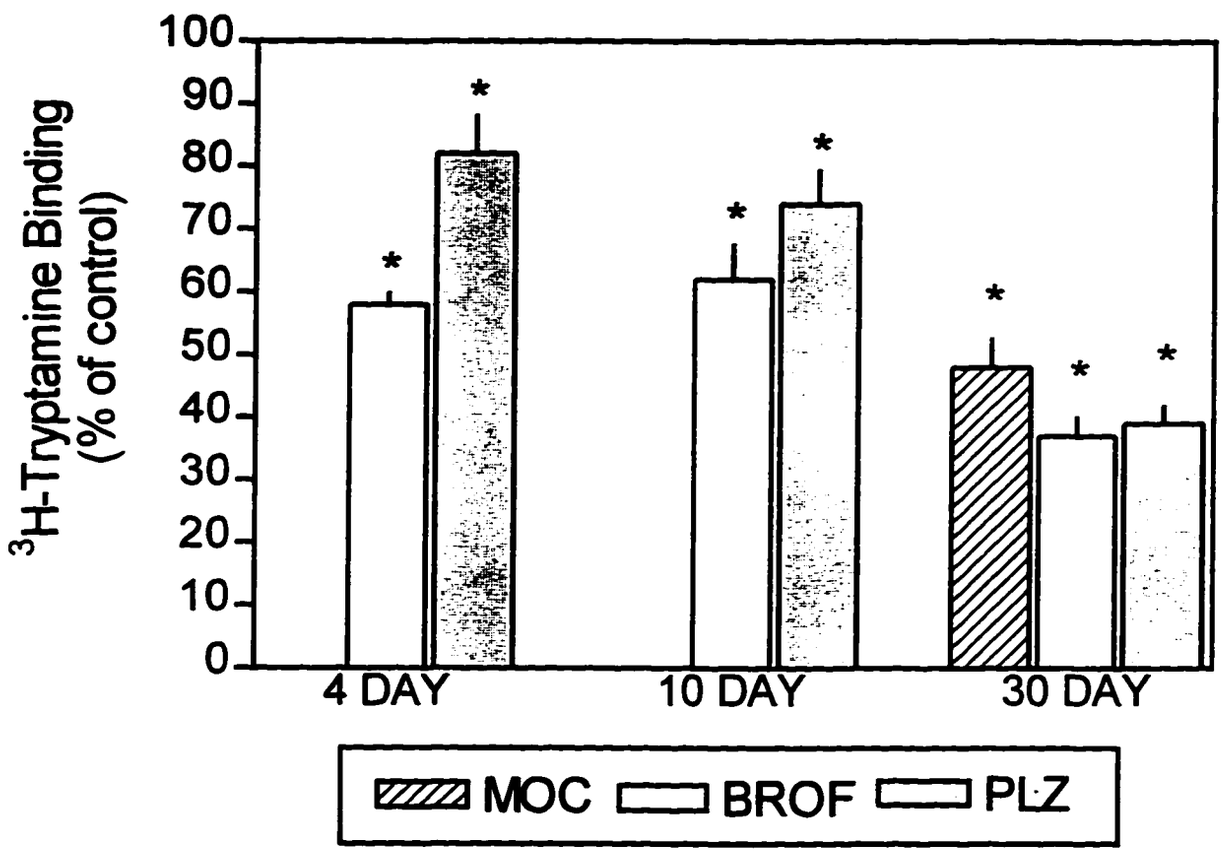


Figure 2-3: Summary of single-point ^{3}H-tryptamine binding data in rat cortex after 4-, 10- and 30-day administration of MAO inhibitors. MOC was studied only at 30 days in this initial study. * indicates $p<0.05$ compared to control (vehicle-treated) values.

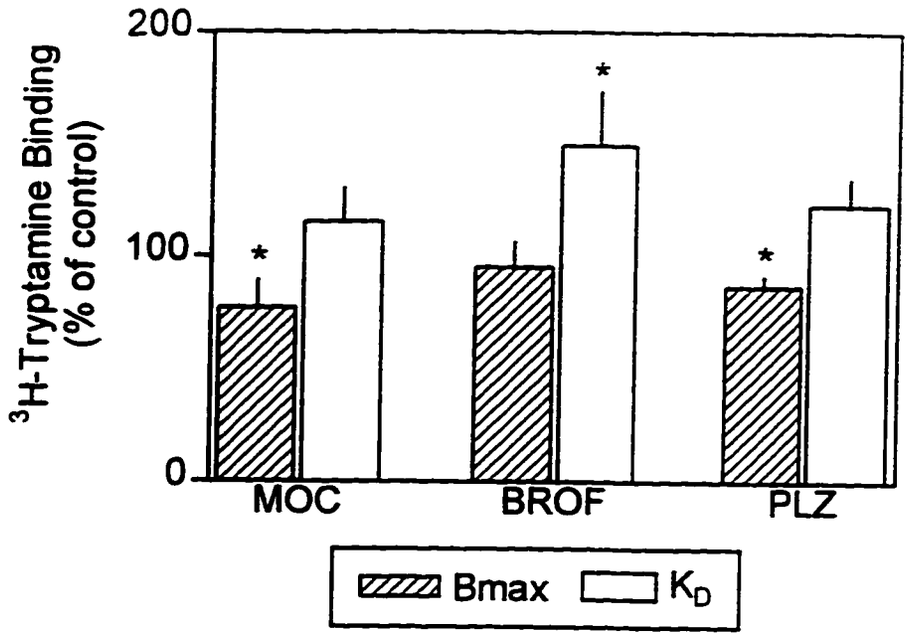


Figure 2-4: Scatchard analysis of ^{3}H-tryptamine binding in rat cortex after 10 day administration of MAO inhibitors where animals were sacrificed 1 h after the last drug administration. * indicates $p<0.05$ compared to control (vehicle-treated) values.

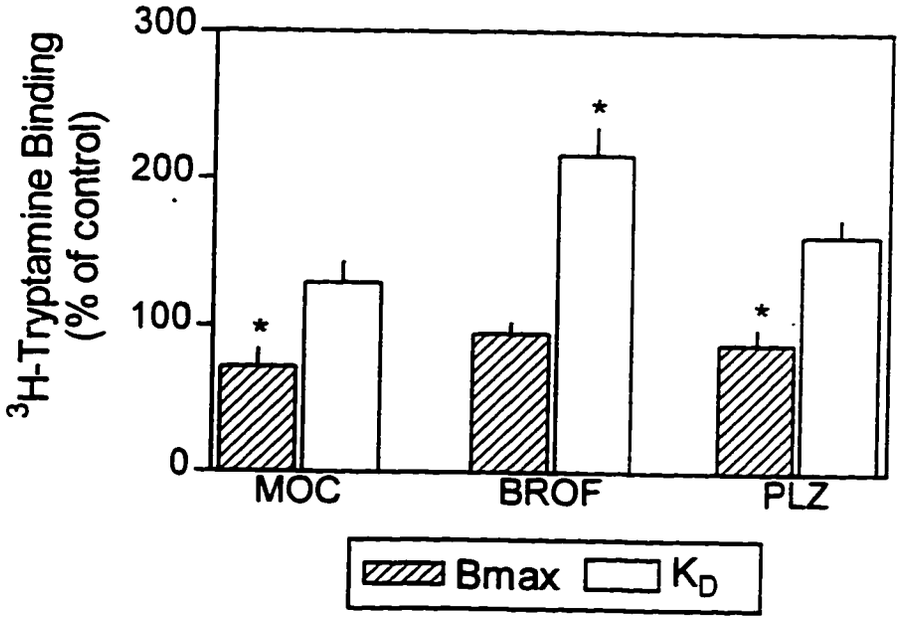


Figure 2-5: Scatchard analysis of ^{3}H-tryptamine binding in rat cortex after 30 day administration of MAO inhibitors where animals were sacrificed 1 h after the last drug administration. *indicates $p<0.05$ compared to control (vehicle-treated) values.

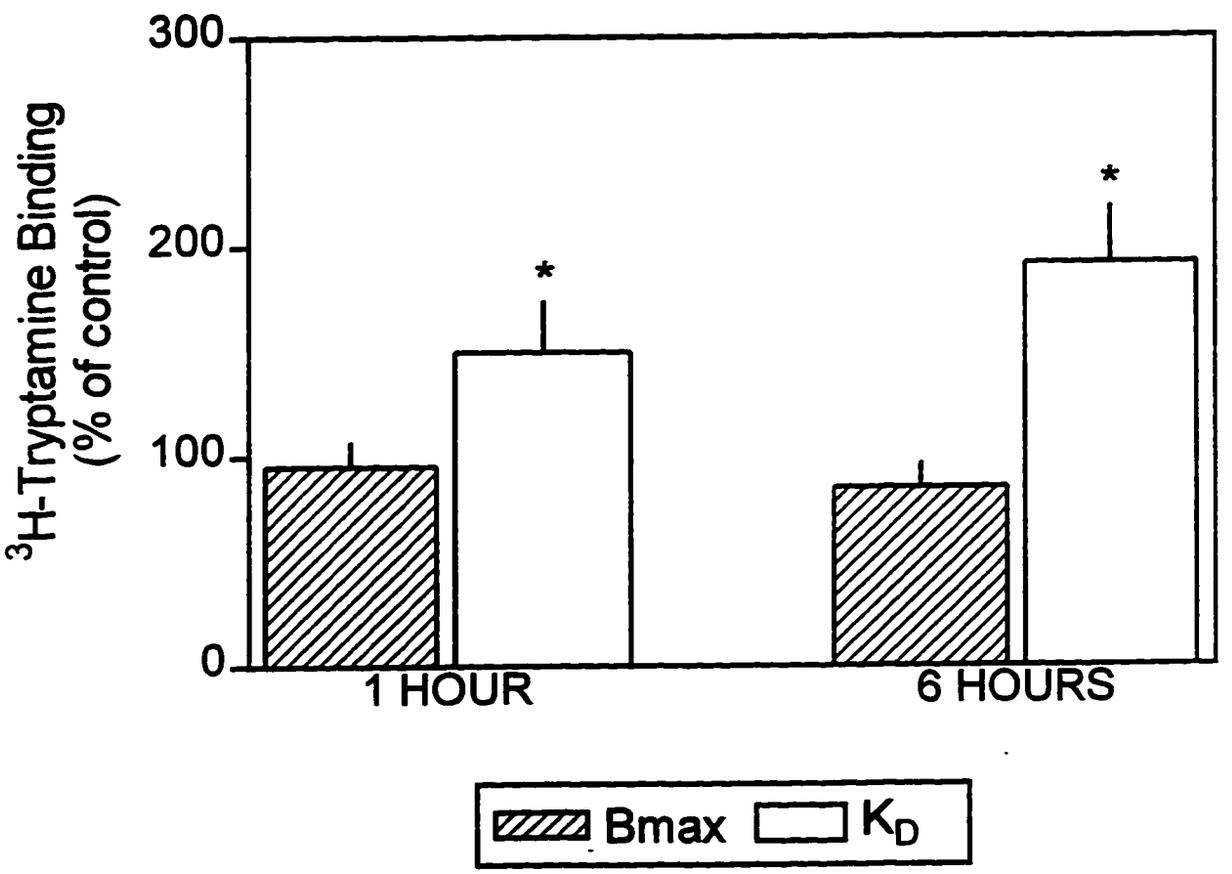


Figure 2-6: Scatchard analysis of ^{3}H-tryptamine binding in rat cortex after 10 day administration of brofaromine and either a 1- or a 6-hour washout. *indicates $p<0.05$ compared to control (vehicle-treated) values.

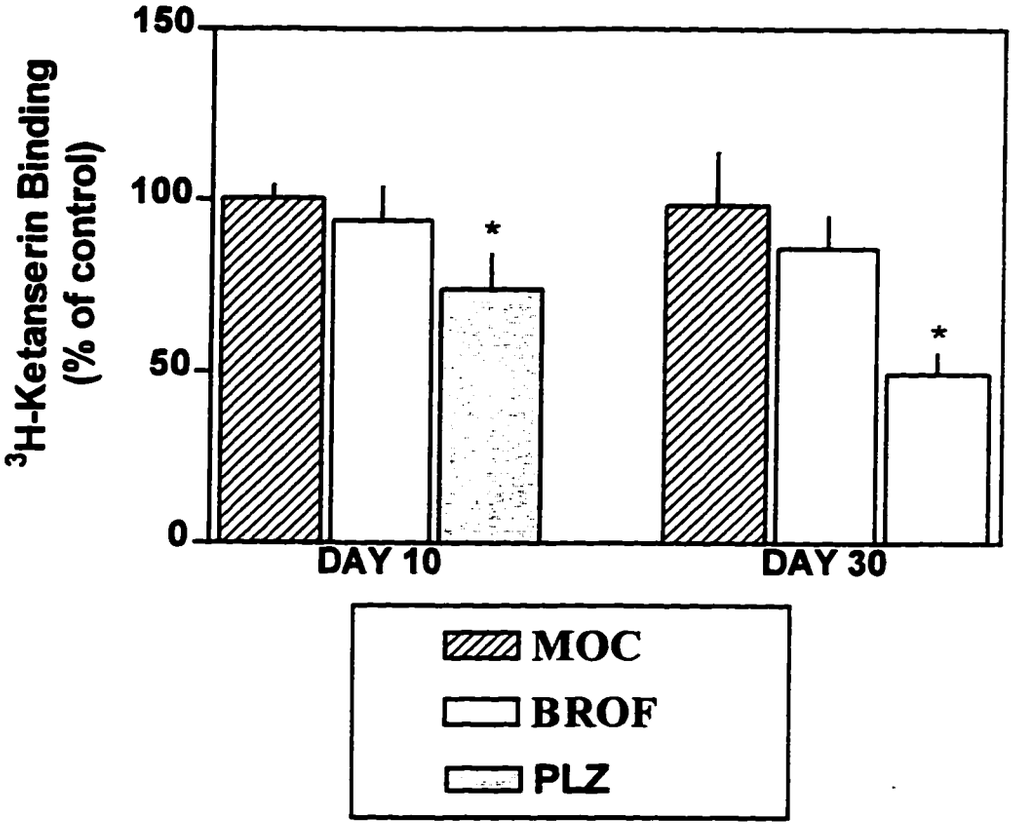


Figure 2-7: Summary of single-point ^{3}H-ketanserin binding data in rat hippocampus after 10- and 30-day administration of MAO inhibitors. *indicates $p<0.05$ compared to control (vehicle-treated) values.

2.3.3 *Inhibition of Monoamine Oxidase*

The levels of MAO-A and MAO-B inhibition are shown in Figure 2-8. Chronic (30 day) administration of MOC and BROF inhibited MAO-A by approximately 80% and MAO-B by less than 20%. PLZ, however, inhibited both MAO-A and -B by over 85%. Inhibition of MAO after 14 days of administration (data not shown) were comparable to the above results.

2.3.4 *Concentrations of Amines*

The concentrations of T and 5-HT in rat brain after chronic (14 day) drug treatment are shown in Table 2-3. MOC, BROF and PLZ all significantly increased levels of 5-HT, while only MOC and PLZ significantly increased T concentrations. The levels of 5-HT in the PLZ-treated rats were approximately twice those in the BROF- or MOC-treated animals.

The urinary levels of T are shown in Figure 2-9. By day 4 of the study, urine levels of T were already elevated to 650% and 1,000% of control values for BROF and PLZ, respectively. The urinary level of T continued to rise in BROF-treated animals and reached 900% of control values by day 28. In the PLZ-treated rats, urinary T levels appeared to reach a maximum (i.e. 1200% of control values) by day 14 where it remained until day 28. At all time intervals, T levels were higher in the PLZ-treated than in the BROF-treated rats.

The urinary levels of MHPG are shown in Figure 2-10. BROF and PLZ caused significant decreases in the urine level of MHPG throughout the study with levels already decreased to 52% and 34% of control values for BROF and PLZ,

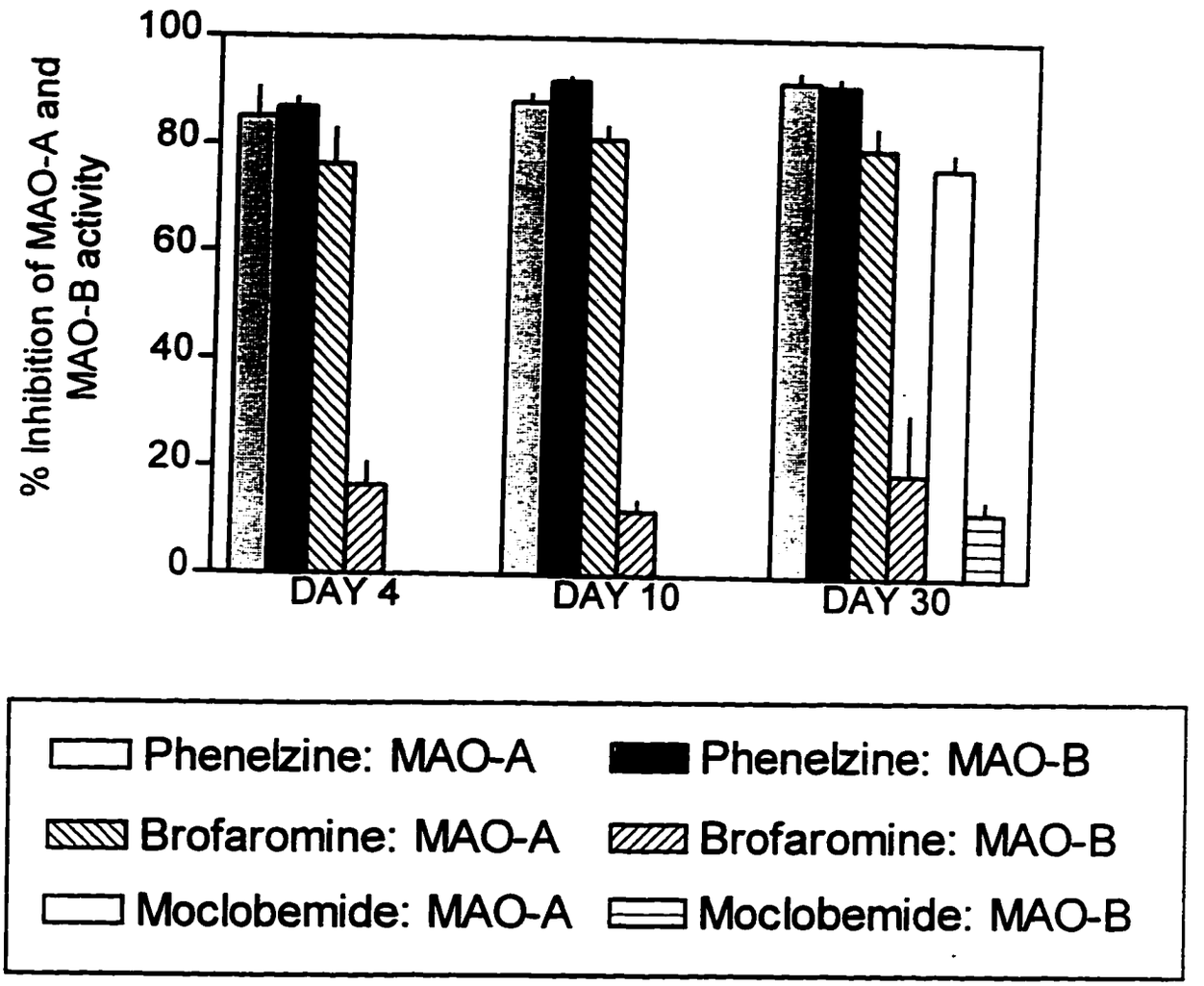


Figure 2-8: Levels of MAO-A and MAO-B inhibition in rat brain (minus cortex and hippocampus) 4, 10 or 30 day administration of moclobemide, brofaromine and phenelzine. Values represent means ± S.E.M. (*n*=3). All values were significantly different from control values and inhibition of both MAO-A and -B produced by phenelzine was significantly different from that produced by either moclobemide or brofaromine.

	Moclobemide	Brofaromine	Phenelzine	Vehicle
T (ng/g)	*3.3 ± 1.6	1.6 ± 1.3	*6.5 ± 0.5	1.5 ± 0.85
5-HT (ng/g)	*1118.4 ± 65.1	*1040.0 ± 62.6	*2314.9 ± 204.7	413.4 ± 44.8

Table 2-3: Concentration of tryptamine and 5-hydroxytryptamine in rat brain (minus cortex and hippocampus) following 14 day administration of MAO inhibitors. Values represent means ± S.E.M. (*n*=6). *Indicates $p<0.05$ compared to control (vehicle-treated) values.

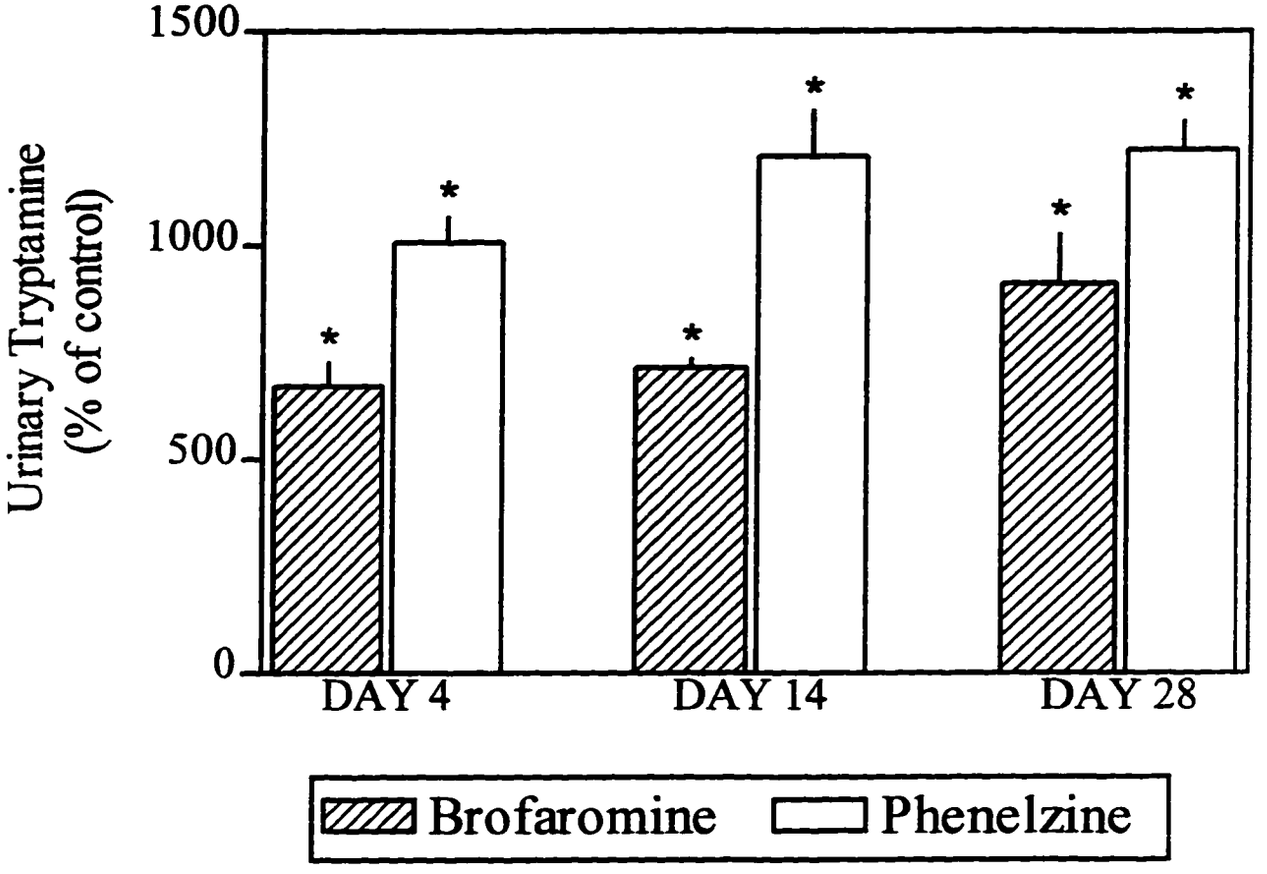


Figure 2-9: Urinary levels (24 h) of tryptamine following administration of brofaromine and phenelzine. Values represent means ± S.E.M. *Indicates $p<0.05$ compared to control values. The absolute levels of tryptamine in control rat urines were 1113 ± 93, 1676 ± 360 and 1177 ± 197 ng/24 h for 4-, 14- and 28-day urines, respectively.

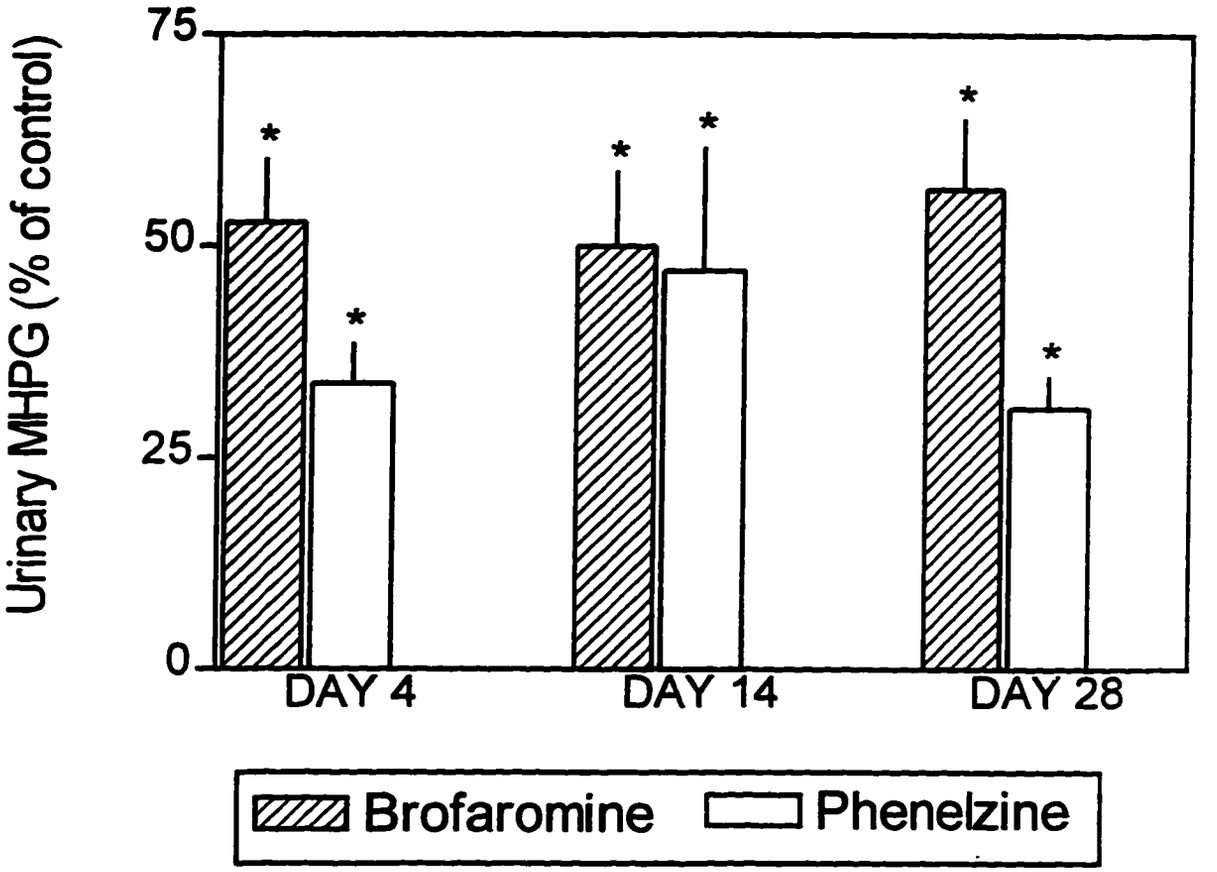


Figure 2-10: Urinary levels (24 h) of MHPG after administration of brofaromine and phenelzine. Values represent means ± S.E.M. * indicates $p<0.05$ compared to control values. The absolute control values for MHPG were 42 ± 23, 41 ± 20 and 37 ± 19 µg/24 h for 4-, 14- and 28-day urines, respectively.

respectively, by day 4. By day 28, however, this effect was slightly more pronounced for PLZ, with values being decreased to 31% of control values compared to 57% of control for BROF.

The urinary levels of PEA are shown in Figure 2-11. In this case only PLZ increased urinary PEA levels, with the maximum increase appearing by day 14 (i.e. 470% of control values). Urinary levels of PEA in BROF-treated rats were not significantly different from control values at any of the time intervals.

2.3.5 Tissue Levels of Moclobemide and Brofaromine

Table 2-4 shows the cortical levels of MOC and BROF that were attained following 14 days of chronic administration. Levels of BROF were approximately 15 times greater than those of MOC.

2.3.6 GABA Levels

Acute studies conducted in our laboratory indicated that at 3 h after an intraperitonial (i.p.) injection to rats of MOC, BROF, or PLZ (each at 30 mg/kg), PLZ produced a three-fold increase in brain levels of GABA while neither MOC nor BROF had any effect. This time interval was chosen because extensive previous studies in our laboratories had demonstrated that at this time GABA levels had peaked in rat brain after PLZ administration (Baker et al., 1991; McKenna et al., 1994).

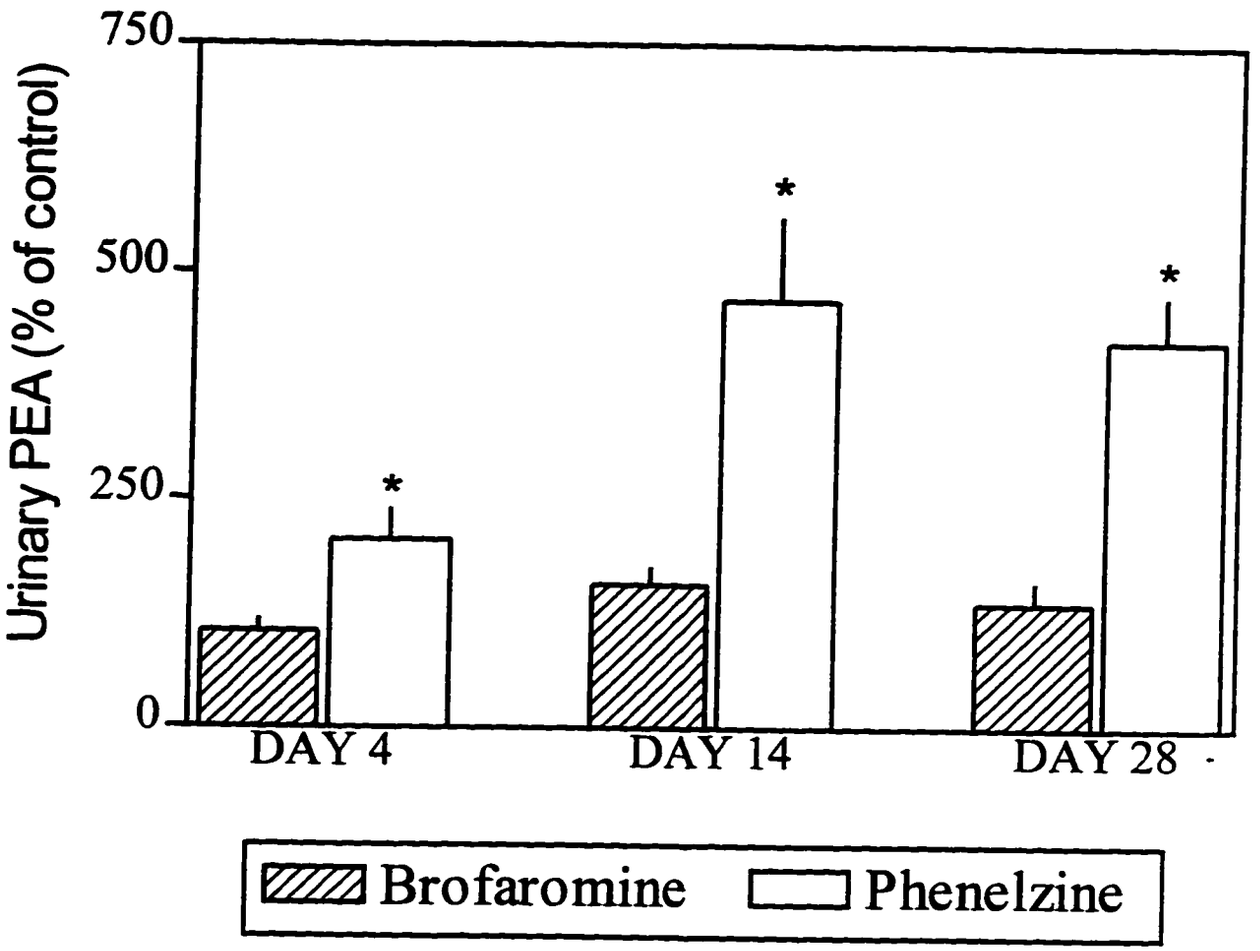


Figure 2-11: Urinary levels (24 h) of β-phenylethylamine (PEA) after administration of brofaromine and phenelzine. Values represent means ± S.E.M. *Indicates $p<0.05$ compared to control values. The absolute control values for PEA were 4723 ± 902, 4733 ± 733 and 4702 ± 758 ng/24 h for 4-, 14- and 28-day urines, respectively.

Moclobemide	Brofaromine
142.49 ng/g (0.53 µM)	2512.07 ng/g (7.6 µM)

Table 2-4: Levels of moclobemide and brofaromine in rat cortex after 14 days chronic administration (15 mg/kg, p.o., b.i.d.). Rats were sacrificed 6 h after drug administration.

2.3.7 GABA Uptake

A preliminary screening comparing two known inhibitors of GABA uptake to the MAO inhibitors MOC, BROF and PLZ showed that at a 1 x 10^{-4} M concentration, fluspirilene, an antipsychotic with known GABA uptake inhibiting properties, caused 64.0 ± 2.6% inhibition of GABA uptake while nipecotic acid, a frequently employed inhibitor of GABA uptake, at the same concentration inhibited uptake by 85.1 ± 2.4%. BROF at 1 x 10^{-4} M produced a 66.7 ± 3.1 % inhibition, but neither MOC nor PLZ had a significant effect on GABA uptake. Following this preliminary screening, further uptake studies were then performed utilizing a range of concentrations to compare the effects of nipecotic acid and BROF on GABA uptake. A summary of these results is presented in Tables 2-5 and 2-6. These results indicate that BROF is a moderately potent inhibitor of GABA uptake, being only slightly weaker than nipecotic acid in this regard.

2.3.8 GABA-T Analysis

Table 2-7 shows a summary of the effects of MOC, BROF and PLZ on the inhibition of GABA-T. In this case, PLZ was ~10 times stronger an inhibitor of GABA-T than MOC or BROF.

2.4 DISCUSSION

These studies were conducted to gain further insight into differences in neurochemical effects between different types of MAOIs. For this purpose, brain levels of 5-HT (biogenic amine; indolealkylamine) and T (trace amine; indolealkyl-

Concentration (M)	% Inhibition produced by brofaromine	% Inhibiton produced by nipecotic acid
5×10^{-4}	97.1 ± 1.5	91.7 ± 1.5
1×10^{-4}	66.7 ± 3.1	85.1 ± 2.4
5×10^{-5}	36.3 ± 5.0	54.9 ± 8.2
1×10^{-5}	5.0 ± 4.7	33.7 ± 7.4

Table 2-5: % inhibition of GABA uptake produced by brofaromine and nipecotic acid. Values represent means ± S.E.M. (*n*=6).

Drug	IC_{50} (M)
Brofaromine	$7.2 (\pm 1.5) \times 10^{-5}$
Nipecotic Acid	$4.8 (\pm 2.0) \times 10^{-5}$

Table 2-6: IC_{50} values for GABA uptake produced by brofaromine and nipecotic acid. Values represent means ± S.E.M. (*n*=6).

Drug	IC_{50} (M)
Moclobemide	5.4 (±1.3) x 10^{-5}
Brofaromine	2.9 (±1.3) x 10^{-5}
Phenelzine	4.3 (±3.5) x 10^{-6}

Table 2-7: IC_{50} values for *in vitro* GABA-transaminase activity produced by moclobemide, brofaromine and phenelzine. Values represent means ± S.E.M. (*n*=3).

amine; mixed substrate for MAO-A and -B), and urinary levels of T, PEA (trace amine; selective MAO-B substrate) and MHPG (major metabolite of NA in the central nervous system; marker for MAO-A) were analysed. MHPG was measured instead of NA because NA is relatively unstable in aqueous solutions. The amines analysed in this study have all been suggested to be involved in the etiology and pharmacotherapy of psychiatric disorders, including depression (Baker and Dewhurst, 1985; Sandler et al., 1979). In addition, levels of MAO-A and -B activity in the brain were analysed to ensure that the drugs and the route of administration were, indeed, effective and that adequate levels of MAO inhibition were achieved. It has been suggested that about 80% inhibition of MAO is required before antidepressant effects occur in the clinical situation (Preskorn, 1993). Acute studies were also conducted on GABA levels, on GABA-T and on GABA uptake inhibition because of increasing evidence in recent years that GABAergic mechanisms may contribute to the overall therapeutic profile of PLZ (Breslow, 1989; Baker et al., 1991; McKenna et al., 1993; Bourin et al., 1994; Glue et al., 1994; Petty, 1995; Tanay et al., 1996; Lai et al., 1997; Paslawski et al., 1996). GABA has also been implicated in the etiology of disorders such as panic disorder in which the three MAOIs of interest here have been reported to be effective (Breslow, 1989; Petty, 1995; Bourin et al., 1997).

The data from these studies indicate that the selective MAO-A inhibitors MOC and BROF and the nonselective MAO inhibitor PLZ all cause a down-regulation of T receptors in brain. The down-regulation produced by PLZ was comparable to that obtained previously in our laboratories (Mousseau, 1991).

However, in the case of BROF, this effect seems to be largely the result of an affinity (K_d) change , while in the case of MOC and PLZ a decrease in the density of receptors occurs. The decrease in affinity was still apparent when a 6 h washout was given between the last drug administration and the time of death. This suggests that the affinity change may not simply be the result of BROF still being present in the membrane preparations, although BROF is known to bind more tightly to proteins and have a longer duration of action than MOC (Waldmeier and Stöcklin, 1989; Waldmeier et al., 1993). The fact that both PLZ and MOC produced significant increases in brain levels of T is consistent with their ability to cause a reduction in the density of T receptors. The T level produced by PLZ was very similar to that obtained previously in our laboratories (Baker et al., 1984) when it was given for a similar length of time. BROF seemed to cause a down-regulation of T receptors by decreasing the affinity of the radioligand for the receptor. This effect on affinity may have been the result of a direct interaction of this drug with receptors; particularly since BROF was present in such a high concentration (Table 2-4) in the cortex compared to MOC. More comprehensive studies of BROF (e.g. with washout periods of longer than 6 h) on T receptors would be informative, but because ^{3}H-T is no longer commercially available, these studies could not be conducted here.

Production of down-regulation of 5-HT_{2A} receptors is a characteristic common to many antidepressants after chronic administration (Baker and Greenshaw, 1989; Eison et al., 1991). MOC and BROF had no effect on 5-HT_{2A}-receptor binding in the hippocampus, even after 30 days of administration, while

PLZ caused a significant reduction. All 3 drugs produced significant increases in brain levels of 5-HT, but the levels produced by PLZ were significantly greater than those produced by either MOC or BROF. The 5-HT values obtained with PLZ are very similar to those obtained with this drug in previous studies at a similar time interval in our laboratories (Baker et al., 1984). It may be that the 5-HT levels in brain produced by MOC and BROF never reached sufficient concentrations to produce down-regulation of 5-HT_{2A} receptors, although to my knowledge a comprehensive study comparing 5-HT levels and 5-HT_{2A} down-regulation by MAOIs has not been reported in the literature.

It is interesting to note that, although the mechanisms may not be the same, all 3 drugs produced a down-regulation of T receptors in cortex, but only PLZ produced a down-regulation of 5-HT_{2A} receptors in hippocampus. The data also demonstrated that MOC, BROF and PLZ all inhibited the oxidative deamination of 5-HT in the brain, thus significantly increasing its levels. These results were expected because all of these drugs inhibit MAO-A and 5-HT is a selective substrate for this isoenzyme. Even at the relatively high doses studied, BROF and MOC did not inhibit MAO or elevate 5-HT to the same extent as did PLZ. BROF attained substantially higher tissue concentrations than MOC, which is consistent with the finding that BROF binds more tightly to proteins and has a longer duration of action than MOC (Waldmeier and Stöcklin, 1989; Waldmeier et al., 1993).

T has been proposed to be a nonspecific substrate for both MAO-A and MAO-B in the literature, but there is controversy among some researchers who suggest that it may be a specific substrate for MAO-A (see Mousseau, 1993 for

review). In the present study, only PLZ significantly increased the brain levels of T and the increase in urinary T was greater with PLZ than with BROF. These data suggest that MAO-B may play an important role in the catabolism of T in rat (i.e. because BROF does not significantly inhibit MAO-B, while PLZ does). It may also be possible that MAO-A was not sufficiently inhibited to produce a measurable increase in brain T levels in the BROF- or MOC-treated animals (i.e. there was sufficient MAO-A activity remaining to metabolize T). There may also be central *versus* peripheral differences in the contribution of MAO-B to the catabolism of T. It must be noted, however, that the analysis of brain tissue concentrations of T were conducted on the rest of brain tissue remaining after the removal of cortex and hippocampus which were required for receptor binding studies. This fact could prove to be important because it has been suggested that the subtypes of MAO involved in T metabolism might simply depend on the location of the two forms, which differ throughout the brain (see Mousseau, 1993).

As previously mentioned, MHPG is the major metabolite of NA in the CNS, and because NA is a selective substrate for MAO-A, MHPG can be considered to be a marker for this isoform of MAO. BROF and PLZ both caused significant decreases in urinary MHPG which would be expected from drugs that are potent inhibitors of MAO-A. There is considerable debate in the literature regarding the validity of using urinary metabolites as measures of CNS MAO activity because only a portion of the monoamine metabolites excreted in the urine may be derived from central sources (Ravindran et al., 1994; Baker et al., 1990). A study conducted by Maas et al. (1979) in humans, however, suggests that the relative contribution of the

CNS to urinary MHPG is quite considerable.

PEA is a trace amine that may play an important role in depression [although the relative ineffectiveness of the MAOI (-)-deprenyl as an antidepressant at doses at which it selectively inhibits MAO-B (Gerlach et al., 1996) does not support such a role for PEA] and may also effect functional aspects of the catecholamines (Hampson et al., 1988; McKenna et al., 1993). As expected, only PLZ caused an increase in urinary PEA levels because PEA is a selective substrate for MAO-B.

Levels of MAO-A and -B activity in rat brain were consistent with the selectivity of MOC and BROF for MAO-A and the lack of selectivity of PLZ. MOC and BROF caused about 80% inhibition of MAO-A while PLZ inhibited both MAO-A and -B by more than 85% in brain (the % inhibition of both MAO-A and -B by PLZ was significantly greater than that of both MOC and BROF).

The present acute data indicate that PLZ, MOC and BROF may all effect GABA in the brain, but that they may do so by different means. PLZ increases brain levels of GABA three-fold, has no significant effects on GABA uptake *in vitro*, but, as has been shown previously, is a relatively potent inhibitor of GABA-T *in vitro* and *ex vivo* (Popov and Mathies, 1969; Baker et al., 1991). In contrast to PLZ, MOC was shown to have no significant effect on GABA levels after acute administration or on GABA uptake *in vitro* (about one tenth as potent as PLZ), but it was found to be a weak inhibitor of GABA-T activity *in vitro*. BROF had no effect on brain GABA levels and was found to be a weak inhibitor of GABA-T activity *in vitro* (with similar activity to MOC), but was found to be a much more potent inhibitor of GABA uptake

in vitro than PLZ or MOC. If the effects of MOC and BROF on GABA-T are the result of direct competition, the brain levels for these two MAOIs shown in Table 2-4 suggest that they would not be present in high enough concentrations to be having a notable effect on GABA-T *in vivo*. This could probably account for MOC and BROF's lack of effect on brain GABA levels. Similarly, BROF would probably not be having a marked effect on GABA uptake at these doses. However, several animal studies in the literature (e.g. Waldmeier and Baumann, 1983; Cicardo et al., 1986; Keller et al., 1987; Da Prada et al., 1990; Colzi et al., 1992; Moreau et al., 1993; Haefley et al., 1992; Waldmeier, 1993; Gerardy, 1994; Celada et al., 1994; Bel and Artigas, 1995; Guo et al., 1996) use higher doses of BROF and MOC than used here, and at such doses, effects on GABA should be considered. Such GABAergic effects are of interest given the increasing evidence in the literature indicating that several types of drugs that augment GABA's actions have anxiolytic and/or antipanic actions (Breslow, 1989; Ontiveros and Fontaine, 1992; Keck et al., 1993; Sherif et al., 1994; Paslawski et al., 1996; Marazziti and Cassano, 1996; Singh et al., 1996; Davis et al., 1996; Bennett et al., 1997). It is also of interest that long-term administration of PLZ to rats has been reported to cause an increase in steady-state levels of mRNAs for isoforms of $GABA_A$ receptor subunits and the GABA transporter GAT-1 (Tanay et al., 1996; Lai et al., 1997). Thus, future studies on such mechanism changes might be worthwhile with MOC and BROF.

In summary, comprehensive studies were conducted to investigate differences between selective, RIMAs of MAO-A (MOC and BROF) and a non-selective, irreversible MAOI (PLZ) with regard to some of their neurochemical

actions. The findings from these studies indicate marked differences between MOC, BROF and PLZ on aminergic and GABAergic mechanisms in the rat.

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3.0 CHAPTER 3

Investigation of the formation of some novel metabolites of fluoxetine *in vitro* and *in vivo*.

(Parts of the work presented in this chapter were included in a manuscript in press in *Journal of Chromatography: Biomedical Applications*).

3.1 INTRODUCTION

The lack of direct effects of the SSRI, FLU, on noradrenergic, cholinergic or histaminergic systems at therapeutic doses (Bowden et al., 1993) is likely associated with FLU's mild side effect profile, and, hence, its popularity with clinicians. Although FLU appears to be selective for inhibiting the reuptake of serotonin, recent studies have revealed that FLU also interacts with various CYP isozymes (Glue and Banfield, 1996), a factor which may contribute to side effects and drug-drug interactions involving FLU.

FLU is N-demethylated to NFLU (Altamura et al., 1994), which is also a potent SSRI and inhibitor of CYP isozymes (Preskorn, 1996; Glue and Banfield, 1996). Both FLU and NFLU, as well as their glucuronide conjugates, have been detected in human urine following administration of radiolabelled FLU (Altamura et al., 1994). In addition, a substantial proportion of the radioactivity is excreted in urine as hippuric acid, a glycine conjugate of benzoic acid (see figure 1-6). It was proposed in the paper by Altamura et al. (1994) that the first steps in the formation of hippuric acid would be deamination of FLU and/or NFLU by MAO followed by O-dealkylation, yielding *p*-TFMP and a precursor of benzoic acid. While TFMP has been identified as a urinary metabolite in humans (Benfield et al., 1986), we have shown that neither FLU nor NFLU are substrates for MAO (Holt et al., 1995). Thus, an alternative pathway must exist through which a benzoic acid precursor might be produced.

The pathway we propose for the formation of hippuric acid involves direct O-

dealkylation of either FLU to TFMP and N-methyl-γ-hydroxy-γ-phenylpropylamine (NMHPPA), or of NFLU to TFMP and γ-hydroxy-γ-phenylpropylamine (HPPA) [see figure 3-1]. Both of these amines have been identified in the urine of depressed patients receiving FLU (Urichuk et al., 1996 and this thesis). The common aldehyde derived from both compounds (presumably by the action of MAO), γ-hydroxy-γ-phenylpropionaldehyde, would then undergo dehydrogenation and β-oxidation to benzoic acid. In order to confirm this hypothesis, it was necessary to demonstrate that both phenylpropylamine derivatives were substrates for MAO.

The present chapter includes the gas chromatographic procedures that were developed to detect and quantitate the potential metabolites of FLU in tissue or urine from rats that were administered FLU and also in the urine of depressed patients who had been receiving FLU for three weeks. In addition, the procedures utilized to determine if potential metabolites of FLU are substrates for MAO and if they inhibit the reuptake of 5-HT are described and the results are discussed.

3.2 MATERIALS AND METHODS

3.2.1 Part 1 - Metabolite Investigation in Rat Brain, Liver and Urine.

3.2.1.1 Drug Administration

Male Sprague-Dawley rats (Ellerslie Biosciences, Edmonton, Canada), 200-300 g, were housed in pairs in an environmentally controlled room using a 12 h light-12 h dark cycle. At the appropriate time animals were injected intraperitoneally with either FLU•HCl (10 mg/kg; dissolved in 20% DMSO in double-distilled water)

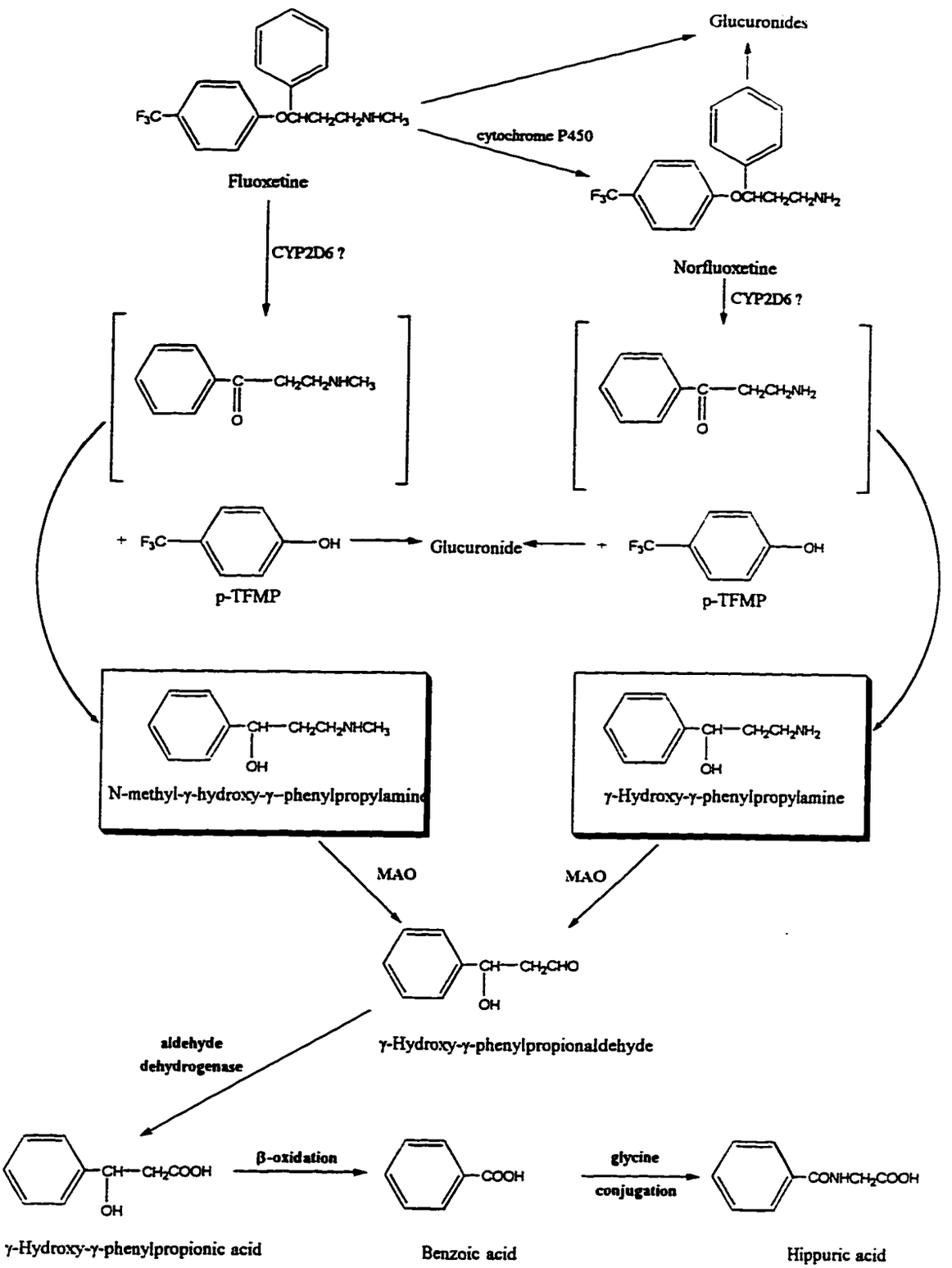


Figure 3-1: Proposed pathway for the metabolism of fluoxetine *via* γ-phenyl-propylamine intermediates in the urine.

or vehicle (20% DMSO in double-distilled water). Animals were placed in metabolic cages immediately after injection in order to collect 24-h urine samples. The volume of urine collected was measured and the samples were frozen at -20 °C until the time of analysis. Following 10 drug-free days, rats were reinjected with the drugs and sacrificed by decapitation 5 h post-injection; brains and livers were removed and immediately frozen in dry ice-cooled isopentane or on dry ice, respectively.

Patients diagnosed with major depressive disorder were asked to collect a 24-h urine sample 3 weeks after commencing treatment with FLU•HCl (Prozac®; 10-20 mg, once daily). A blood sample (16 ml) was collected into vacutainers (with EDTA as the anticoagulant) and centrifuged at 1,000 x g for 10 min to separate the red cells from the plasma. Plasma and urine samples were collected from these subjects before FLU treatment began and again after 3 weeks of treatment. Urine was collected for the 24 h prior to the day of blood collection. The plasma and urine samples were frozen at -20 °C until analyzed.

3.2.1.2 Analysis of the Enantiomers of Fluoxetine and Norfluoxetine

The enantiomers of free FLU and NFLU were analyzed using the procedure of Torok-Both et al. (1992). Rat brain or liver was weighed and homogenized in five volumes of ice-cold 0.1 N perchloric acid. An aliquot (600 µl) of the homogenate was removed and used in the procedure. For the analysis of rat urine, aliquots (1 ml) were used in the analytical procedure. The appropriate aliquot was placed into a clean glass test tube and diluted to a final volume of 2 ml with distilled water. To

this solution was added 25 µg of the internal standard, *p*-chlorophenylethylamine. The samples were basified by adding 500 µl of a 25% potassium carbonate solution (to bring the pH to ~11.5) and were briefly vortex-mixed. To this mixture 5 ml of toluene was added and the samples were mixed for 15 min in an Ika Vibrex vortex-mixer (Janke and Kunkel, Staufen, Germany) and centrifuged for 10 min at 1,000 x g to separate the aqueous and organic phases. The upper toluene layer was transferred to a second glass tube and back-extracted by adding 2 ml of 0.5 M HCl, shaking for 10 min and centrifuging for 5 min at 1,000 x g. The upper toluene layer was aspirated to waste and the remaining aqueous layer was basified with 1 ml of 25% potassium carbonate in addition to a small amount of solid potassium carbonate (pH = ~10.5-11.5). Toluene (4 ml) was added to this basic solution and the samples were shaken for 15 min and centrifuged for 10 min at 1,000 x g. The toluene layer was retained and transferred to a glass drying tube. The organic solvent was evaporated using a Savant Speed Vac SSI (Savant Instruments Inc., Farmington, NT, USA). To the dry residue was added 100 µl of toluene and 4 µl of the derivatizing reagent, S-(-)-N-(trifluoroacetyl)prolyl chloride (because this derivatizing reagent is prone to racemization after certain periods of time, it was used within six months after its arrival). The tubes were briefly vortex-mixed and the reaction was allowed to proceed at 60°C for 60 min. The samples were again evaporated to dryness in the Savant Speed Vac SSI and the residue was reconstituted in 150 µl of toluene. An aliquot (1 µl) of this solution was injected on a GC-ECD equipped with a fused-silica capillary column (0.22 µm thickness).

Chromatographic separation was achieved using the following oven temperature program: the initial oven temperature of 105°C was held for 0.5 min then increased at a rate of 30°C/min to 207°C where it was maintained for 4 min. The temperature was then increased at a rate of 1°C/min to 225°C and then at a rate of 25°C/min to 300°C where it was held for 2 min. A Hewlett-Packard model 5880 A integrator was used to measure peak areas.

In order to analyse total levels of the enantiomers of FLU and NFLU in human urine, the sample preparation was boiled with 1N HCl for 30 min, then cooled to room temperatue, prior to the initial basification step with 500 µl of a 25% potassium carbonate solution. Extraction was then obtained by following the steps outlined above. Conjugated levels of the enantiomers of FLU and NFLU were calculated by applying the following equation:

$$\% \text{ Conjugated} = \frac{(\text{Total} - \text{Free})}{(\text{Total})} \times 100$$

3.2.1.3 Development of an Assay for p-Trifluoromethylphenol

Sample preparation

Aliquots (1 ml) of human plasma and human and rat urine were used for the analytical procedure. Rat brain and liver tissue were weighed and homogenized in five volumes of ice-cold double-distilled water. An aliquot (2 ml) was removed from the homogenized sample and used in the analytical procedure. Appropriate calibration standards of TFMP were prepared along with the samples by diluting standard solutions of TFMP in control biological samples prepared from drug-naive

rats or in plasma or urine from drug-naive human subjects. The final calibration concentration ranges were as follows: 50 ng to 2.0 µg TFMP per volume of human or rat urine; 1.0 ng to 500 ng TFMP per volume of human plasma; and 5 ng to 500 ng TFMP per volume of tissue homogenate. In all studies, the volumes of biological sample used in the calibration curves were the same as those used from the FLU-treated subjects. Calibration curves consisting of known, varying amounts of TFMP and a known, fixed amount of internal standard were included with each assay run.

Sample extraction

An aliquot of human plasma (1 ml), human or rat urine (1 ml), or rat tissue homogenate (2 ml) was placed in a screw-cap culture tube (Fisher Scientific) and diluted, if required, to a final volume of 2 ml with distilled water. The internal standard (250 ng of 2,4-dichlorophenol) was added to this solution. The samples were then basified by adding excess potassium bicarbonate (400 mg) and briefly vortex-mixed. The mixture was then decanted into clean screw-cap culture tubes (160 mm x 15 mm) and 4.5 ml of ethyl acetate containing acetonitrile (10%v/v) and the derivatizing reagent, pentafluorobenzenesulfonyl chloride (PFBSC; 0.1% v/v), was added to each sample. The two phases were mixed for 20 min in an Ika Vibrex VXR vortex-mixer (Janke and Kunkel, Staufen, Germany) and centrifuged for 2 min at 1,000 x g in a benchtop centrifuge (Sorvall GLC-2B general laboratory centrifuge, Du Pont, Wilmington, DE, USA). The upper ethyl acetate layer was retained and transferred to another screw-cap culture tube (160 mm x 15 mm) and washed briefly

by adding 600 µl distilled water, vortex-mixing for 5 s and centrifuging for 2 min. The upper organic layer was retained and transferred to a glass drying tube. The excess reagent was evaporated using a Savant Speed Vac SSI (Savant Instruments Inc., Farmington, NT, USA) and the residue was reconstituted in 200 µl toluene. A final wash included adding 200 µl 1N ammonium hydroxide to the samples, vortex-mixing for 5 s, transferring to 400 µl microfuge tubes and centrifuging for 10 s. The upper toluene layer was retained for gas chromatographic (GC) analysis.

The above sample extraction permitted the measurement of free levels of TFMP in biological samples. It was also of interest, however, to analyse total levels of TFMP in humans and rats that were treated with FLU to determine whether or not TFMP is conjugated. In order to analyse total TFMP levels, the sample preparation was boiled with 300 µl of I N HCl for 30 min, then cooled to room temperature, prior to the initial basification with excess potassium bicarbonate. Extraction was then obtained by following the steps outlined above. Conjugated levels of TFMP were calculated by applying the following equation:

$$\% \text{ Conjugated TFMP} = \frac{(\text{Total TFMP} - \text{Free TFMP})}{(\text{Total TFMP})} \times 100$$

Gas chromatography

Samples were analysed using a chromatographic system consisting of a Hewlett-Packard (HP) 5880 gas chromatograph equipped with a 15 mCi ^{63}Ni linear electron capture detector and a Hewlett-Packard model HP 7673 A automatic

sample injector. The chromatographic column was a 25 m x 0.32 mm i.d. HP-5 narrow-bore fused-silica column (1.05 µm film of 5% phenylmethylsilicone as the stationary phase; Hewlett-Packard, Palo Alto, CA, USA). The carrier gas was ultra-pure helium (Praxair, Edmonton, Canada) at a flow-rate of 3 ml/min and the make-up gas was argon-methane (95:5; Praxair, Edmonton, Canada) at a flow-rate of 30 ml/min. The injector port and detector temperatures were 200°C and 325 C, respectively. The oven temperature was set initially at 80°C which was maintained for 0.5 min and then increased at a rate of 10°C/min to a final temperature of 270°C which was maintained for 10 min. A Hewlett-Packard 5880 A integrator was used to measure peak areas.

3.2.1.4 Development of an Amine Metabolite Assay

Sample preparation

Aliquots (1 ml) of human and rat urine were used for the analytical procedure. Appropriate calibration standards of NMHPPA and HPPA were prepared along with the samples by diluting standard solutions of these amines in control urines from drug-naive rats or drug-naive individuals. The final calibration concentration range was 50 ng to 2.0 µg NMHPPA and HPPA per volume of human or rat urine. In all studies, the volumes of biological sample used in the calibration curves were the same as those used from the FLU-treated subjects. Calibration curves consisting of known, varying amounts of NMHPPA, HPPA and a known, fixed amount of internal standard (2,4-dichlorophenol in distilled water) were included with each assay run.

Sample extraction

An aliquot (1 ml) human or rat urine was placed in a screw-cap culture tube (Fisher Scientific) and diluted, if required, to a final volume of 2 ml with distilled water. The internal standard (250 ng of 2,4-dichlorophenol) was added to this solution. The samples were then basified by adding excess potassium bicarbonate (400 mg) and briefly vortex-mixed. The mixture was then decanted into clean screw-cap culture tubes (160 mm x 15 mm) and 4.5 ml of ethyl acetate containing acetonitrile (10%v/v) and the derivatizing reagent, pentafluorobenzoyl chloride (PFBC; 0.1% v/v), was added to each sample. The two phases were mixed for 20 min in an Ika Vibrex VXR vortex-mixer (Janke and Kunkel, Staufen, Germany) and centrifuged for 2 min at 1,000 x g in a benchtop centrifuge (Sorvall GLC-2B general laboratory centrifuge, Du Pont, Wilmington, DE, USA). The upper ethyl acetate layer was retained and transferred to another screw-cap culture tube (160 mm x 15 mm) and washed briefly by adding 600 µl distilled water, vortex-mixing for 5 s and centrifuging for 2 min. The upper organic layer was retained and transferred to a glass drying tube. The excess reagent was evaporated using a Savant Speed Vac SSI (Savant Instruments Inc., Farmington, NT, USA) and the residue was reconstituted in 200 µl toluene. A final wash included adding 200 µl 1N ammonium hydroxide to the samples, vortex-mixing for 5 s, transferring to 400 µl microfuge tubes and centrifuging for 10 s. The upper toluene layer was retained for gas chromatographic (GC) analysis.

Gas chromatography

Samples were analysed using a chromatographic system consisting of a Hewlett-Packard (HP) 5880 gas chromatograph equipped with a 15 mCi ^{63}Ni linear electron capture detector and a Hewlett-Packard model HP 7673 A automatic sample injector. The chromatographic column was a 15 m x 0.25 mm i.d. DB-5 cross-linked fused-silica capillary column (0.22 µm thickness; J & W Scientific, Palo Alto, CA, USA). The carrier gas, helium (Linde, Union Carbide), was set at a flow-rate of 1 ml/min. The make-up gas was argon-methane (95:5; Linde, Union Carbide) at a flow-rate of 30 ml/min. The injector port temperature was 270°C and the detector temperature was 325°C. The oven temperature was set initially at 105°C, maintained for 0.5 min and then increased at a rate of 10 °C/min to a final temperature of 270°C which was maintained for 10 min. A Hewlett-Packard 5880 A integrator was used to measure peak areas.

3.2.1.5 Gas Chromatography-Mass Spectrometry

Confirmation of the chemical stuctures of the derivatives of TFMP, NMHPPA, HPPA and the internal standard, 2,4-dichlorophenol, was obtained by combined gas chromatography-mass spectrometry (GC-MS). The GC-MS system utilized a HP 5840 A GC inlet coupled to an HP 5985 A MS with dual EI/CI sources and an HP 7920 data system. The system also included an HP 2648 A graphics terminal, an HP 9876 A printer, HP 7920 disc drive (software) and HP 21 MX series E computer (hardware). Operating conditions for the MS were as follows: ion source temperature, 200°C; interface temperature, 275° C; column pressure, 34.5 kPa;

accelerating voltage, 2200 eV; ionization voltage, 70 eV; scan speed, 100 amu/sec and dwell time, 200 msec. The specific column and temperature programs that were utilized for the GC-electron-capture detection of TFMP, NMHPPA and HPPA were also used for GC-MS analysis.

3.2.2 Part 2 - Investigation of 5-HT Uptake Inhibiting Properties of TFMP, NMHPPA and HPPA, Novel Metabolites of Fluoxetine.

3.2.2.1 5-HT Uptake Analysis

^{3}H-5-HT uptake into striatal prisms was measured using the procedure of Martin et al. (1978); FLU was included as a known inhibitor of 5-HT uptake. Specifically, untreated rats were killed by guillotine decapitation, their brains removed, placed on an ice-cooled plate and the striatum was dissected out from the rest of the brain. The striatum was then chopped into 0.1 x 0.1 x 2 mm prisms using a McIlwain tissue chopper (The Mickle laboratory Engineering Company, Gomshall, Surrey, U.K.) and suspended in 5 volumes of incubation medium containing 123 mM sodium chloride, 5 mM potassium chloride, 2.7 mM calcium chloride, 1.2 mM magnesium sulfate, 20 mM TRIS-HCL buffer (pH 7.5), 1 mM ascorbic acid, 10 mM glucose and 12.5 µM nialamide. This suspension was stored out of direct light and buried in ice. Aliquots (1 ml) of the tissue suspension were added to flasks containing 4 ml of incubation medium and this mixture was incubated for 15 min at 37°C in a Heto Denmark shaking water bath (Bach-Simpson Ltd., London, Ontario, Canada). Next, 10 µl of appropriate inhibitor was added to each tube except the

blanks and controls, followed by the addition of 10 µl of ^{3}H-5-HT (final concentration 0.1 µM). These radioactive mixtures were incubated for 5 min at 37°C, then quickly filtered with a Millipore vacuum filter apparatus [Millipore (Canada) Ltd., Nepean, Ontario, Canada] and rinsed two times with 5 ml warm (37°C) incubation medium. Filters were transferred to scintillation vials and 10 ml of Ready Safe™ scintillation fluid was added to each. Radioactive content per tube was counted (in dissassociations per minute) using a liquid scintillation spectrophotometer. Percent 5-HT uptake inhibition was calculated by applying the following equation:

$$\textbf{\% inhibition} = 100-\left[\frac{(\text{mean of sample - mean of blank})}{(\text{mean of controls - mean of blank})} \times 100\right]$$

3.2.3 Part 3 - Use of a New Spectrophotometric Amine Oxidase Assay to Determine if Two Amine Metabolites of Fluoxetine are Substrates for Monoamine Oxidase.

3.2.3.1 Mitochondrial preparation

The preparation of hepatic MAO for spectrophotometric studies involved homogenizing liver tissue (5g) from drug-naive rats in 1:40 (w/v) of 0.32 M ice-cold sucrose, with a Polytron mechanical homogeniser. Homogenates were centrifuged at 1,000 x *g* and 4°C for 10 min and the supernatant, carefully withdrawn by syringe, was retained and centrifuged at 10,000 x *g* and 4°C for 30 min to obtain a crude mitochondrial pellet. The supernatant was then discarded, and the resulting pellet was resuspended in 4 ml of 0.32 M sucrose, with a Polytron mechanical

homogeniser. This suspension was then carefully layered (using a Pasteur pipette) onto the top of 1.2 M sucrose, such that the volume ratio of 0.32 M sucrose/1.2 M sucrose was 10:1, and the mixture was centrifuged at 53,000 x *g* and 4°C for 2 h. The supernatant was discarded and the resulting mitochondrial pellet was gently washed with a small amount of potassium phosphate buffer (pH 7.2 - 7.8) to remove any of the remaining sucrose. The pellet was then resuspended in 10 - 20 ml potassium phosphate buffer. This mixture was centrifuged at 10,000 x *g* and 4°C for 30 min to wash the mitochondria. The supernatant was discarded and the pellet was resuspended with a Polytron mechanical homogeniser for a final time in 1 - 2 ml potassium phosphate buffer and immediately frozen at -70°C. This mitochondrial preparation was used as the source of MAO in spectrophotometric amine oxidase studies.

3.2.3.2 Amine oxidase assay and analysis

Analysis of two novel amine metabolites of FLU were performed using the procedure developed by Holt et al. (1997). The basis for the spectrophotometric amine oxidase assay is that, because hydrogen peroxide (H_2O_2) is a common product to MAO-mediated deamination reactions, it can be used to demonstrate amine turnover by MAO (see Figure 3-2). In this assay, 4-aminoantipyrine acts as the proton donor in the peroxidase reaction and then condenses with vanillic acid (4-hydroxy-3-methoxybenzoic acid) to yield a red quinoneimine dye with an absorbance maximum at 498 nm and a molar absorption coefficient of $4700 M^{-1}cm^{-1}$.

Overall reaction:

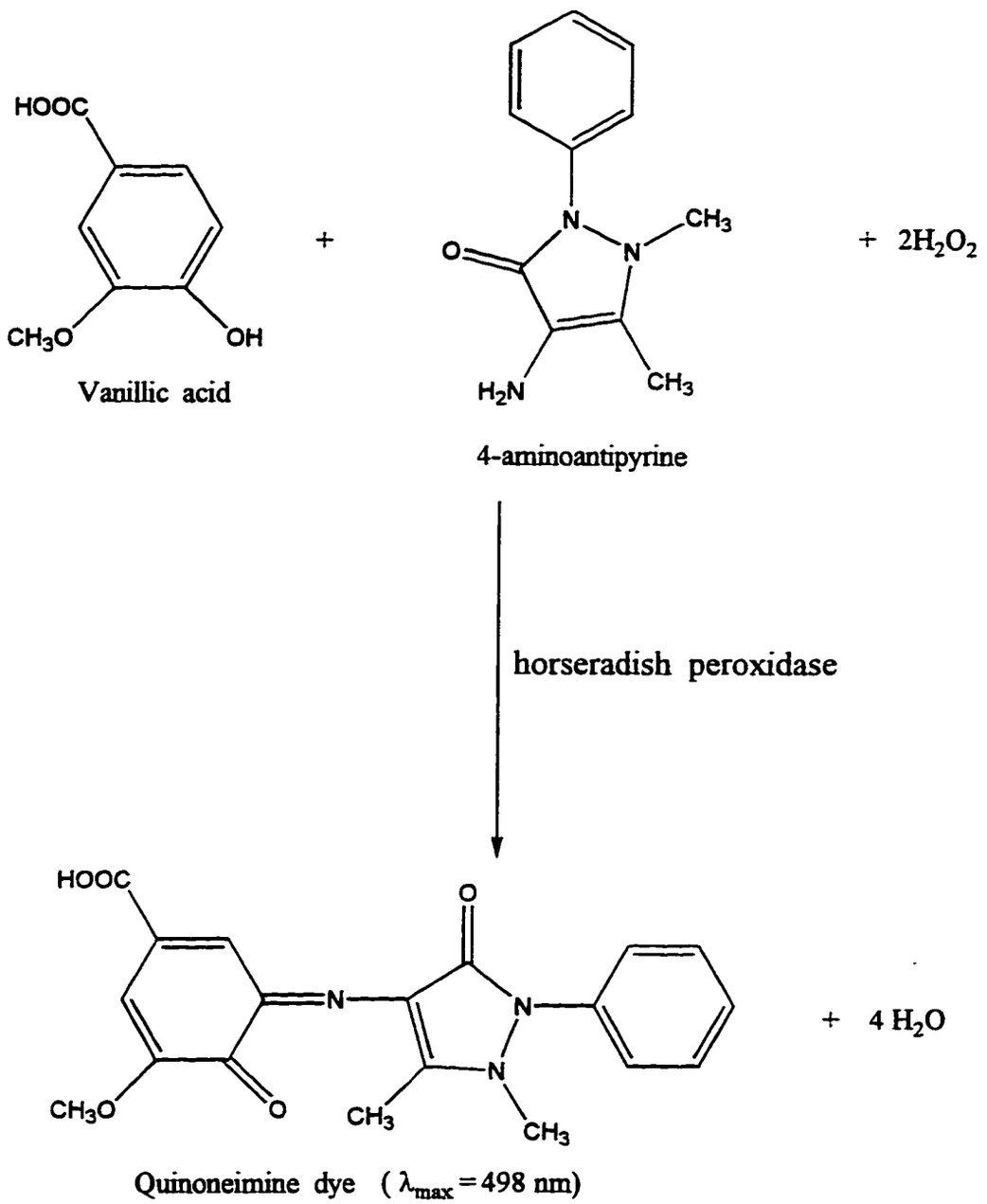


Figure 3-2: Overall reaction for the peroxidase-linked assay for deamination of fluoxetine metabolites by monoamine oxidase, as described in Holt et al. (1997).

The molar absorbance was calculated from the slope of a standard curve that was obtained by standardising a H_2O_2 stock solution by titration with potassium permanganate. The absorbance at 498 nm is proportional to the amount of H_2O_2 released by the amine oxidase reaction. The assay was conducted in 96-well microtitre plates (Corning, NY) and each well contatined 50 µl of rat mitochondria (as described above) as the source of MAO, 50 µl of a chromogenic solution containing 4-aminoantipyrine (500 µM), vanillic acid (1 mM) and horseradish peroxidase (type II, 4 U ml^{-1}) in potassium phosphate buffer (0.2 M, pH 7.6), and 200 µl of aqueous amine substrate. Blank wells had buffer added in place of substrate. Thus, the final assay mixture had a volume of 300 µl and a pathlength of 9 mm. The reactions in the plate were followed continuously in a THERMOmax microplate reader (Molecular Devices, Menlo Park, CA) with a 490 nm filter and a plate chamber temperature of 37°C. Absorbance readings were taken every 15 s for 30-40 min and initial (maximum) reaction velocities were determined by linear regression of the data (Graph Pad Prism, version 1.03).

To obtain kinetic constants for turnover of the amine metabolites of FLU by MAO-A and MAO-B, homogenates were first treated with selective MAO inhibitors before incubating with a range of concentrations of the amine substrates. Homogenates were preincubated at 37°C for 30 min with clorgyline (500 nM) to inhibit MAO-A, or with pargyline (500 nM) to inhibit MAO-B. Metabolism by MAO-A was then examined by incubating pargyline-treated homogenates with NMHPPA (150 µM - 1.5 mM) or with HPPA (450 µM - 1.5 mM) in the presence of chromogenic solution, as described above. Similarly, metabolism by MAO-B was examined by

incubating clorgyline-treated homogenates with NMHPPA (200 μM - 450 μM) or with HPPA (30 μM - 100 μM). These concentrations were chosen from preliminary screening experiments with the phenylpropylamines. Four replicate assays were made in microtitre plate wells, as described above. Data were analysed using the linear regression facility of GraphPad Prism, version 1.03.

3.3 RESULTS

3.3.1 Part 1 - Metabolite Investigation in Rat and Human Biological Samples.

The procedures described for analysing levels of TFMP and NMHPPA are rapid and sensitive and the GC-MS derivatives formed are stable. Unfortunately, however, an interfering peak in control urine extracts from both rats and humans did not allow for the quantitation of HPPA. Typical chromatograms of derivatized extracts of human and rat biological samples from both drug-naive and FLU-treated subjects are shown in Figures 3-3 and 3-4.

Combined GC-MS was utilized to analyse the structures of the derivatives for TFMP, NMHPPA, HPPA and the internal standard, 2,4-dichlorophenol. The fragmentation patterns obtained for TFMP and 2,4-dichlorophenol (Figures 3-5 and 3-6, respectively) confirmed their structures. In the spectrum for NMHPPA, the formation of the ions with m/z 340 (0.37%), m/z 327 (14.8%), m/z 326 (22.6%), m/z 164 (0.4%), m/z 195 (100%), m/z 167 (19.5%), m/z 239 (86.9%) and m/z 253 (39.8%) could be readily interpreted based on its proposed structure (see Figure 3-7). Other ions in the spectrum (e.g. m/z 168; 11.7%), however, were not easily

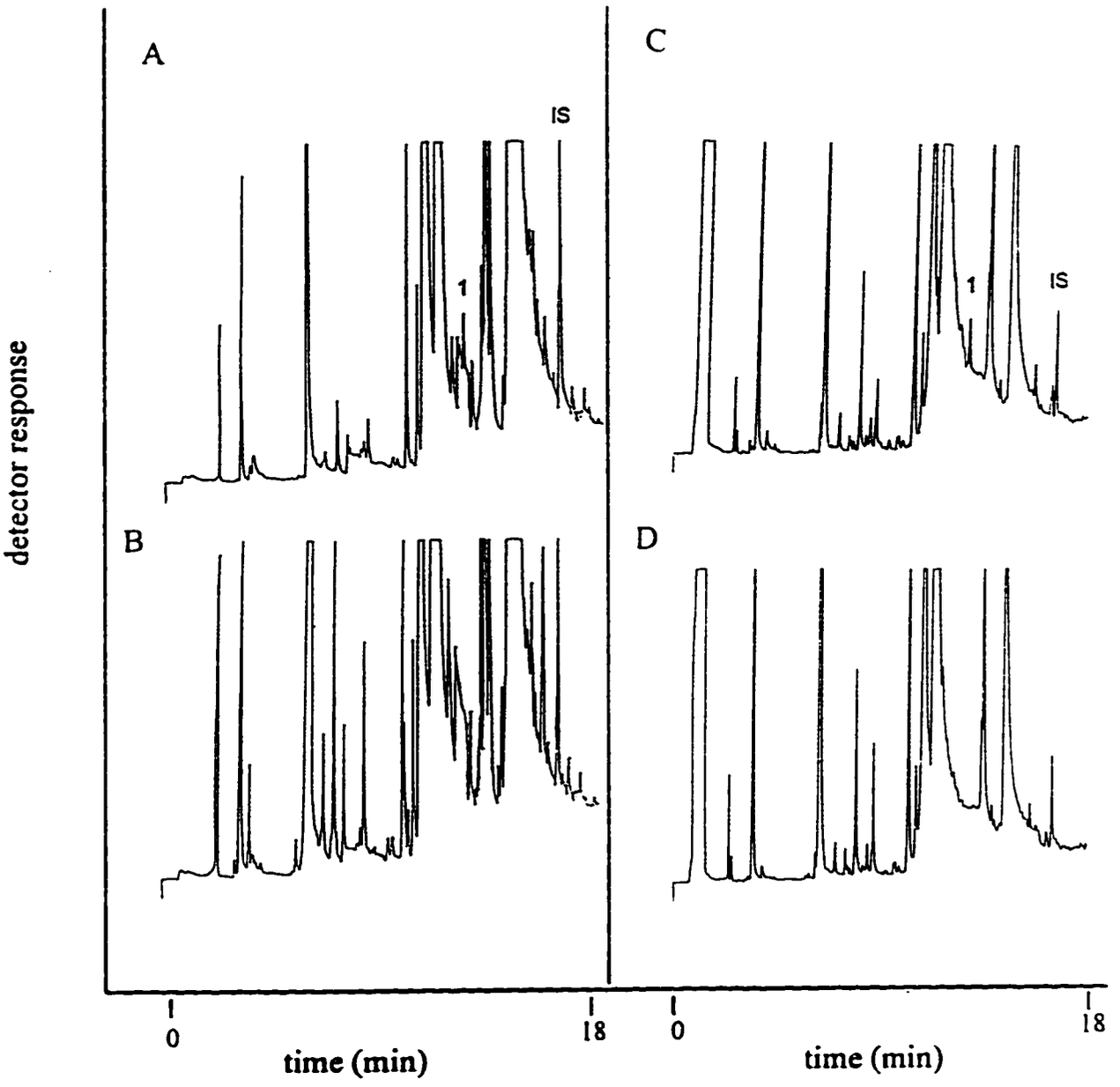


Figure 3-3: Derivatized extracts of (A) brain and (C) liver from rats injected with fluoxetine (10 mg/kg) and sacrificed 5 h post-injection or of (B) brain and (D) liver from drug-naive rats injected with 20% DMSO in water. The GC peaks are identified as follows: 1= TFMP and IS= internal standard. The amount of IS added to brain samples (500 ng) was twice the amount of that added to liver samples (250 ng). The GC retention times of these peaks were 12.74 and 16.76 min, respectively.

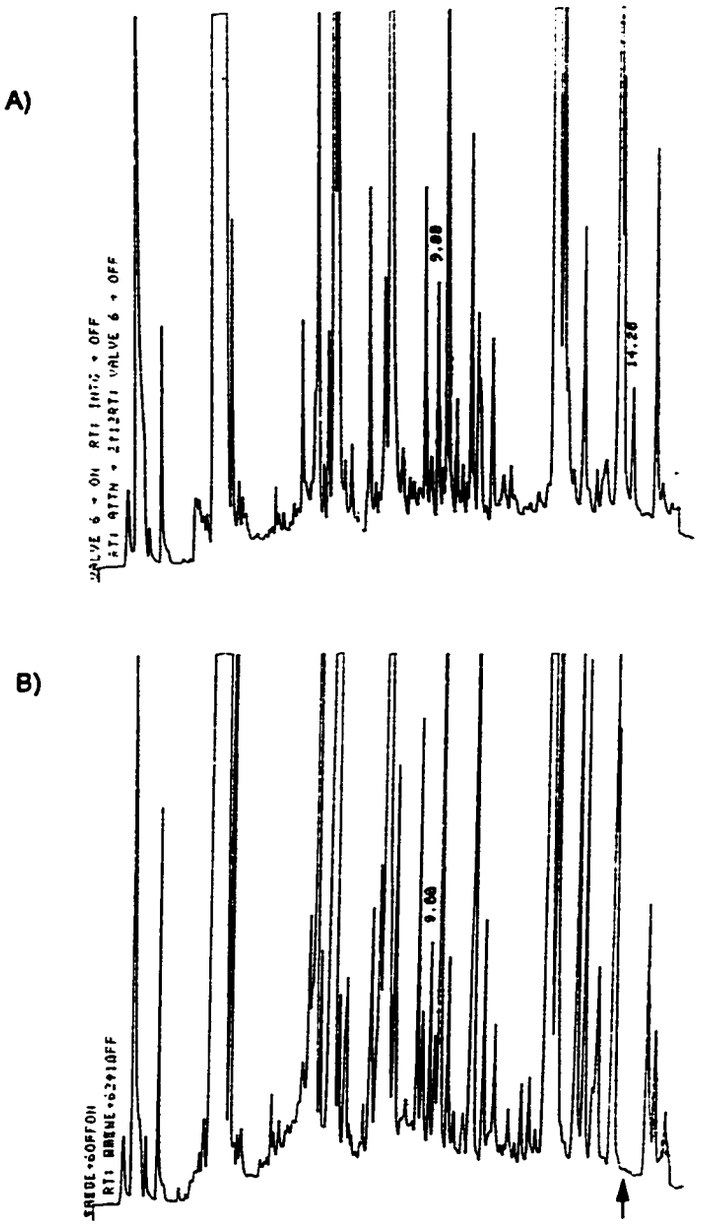


Figure 3-4: Derivatized extracts of NMHPPA from (A) urine obtained from depressed patients treated with fluoxetine HCl (Prozac®; 10-20 mg, once daily) for three weeks or (B) urine from drug-naive individuals. The GC peaks are identified as follows: 9.00 min= internal standard and 14.20 min= NMHPPA.

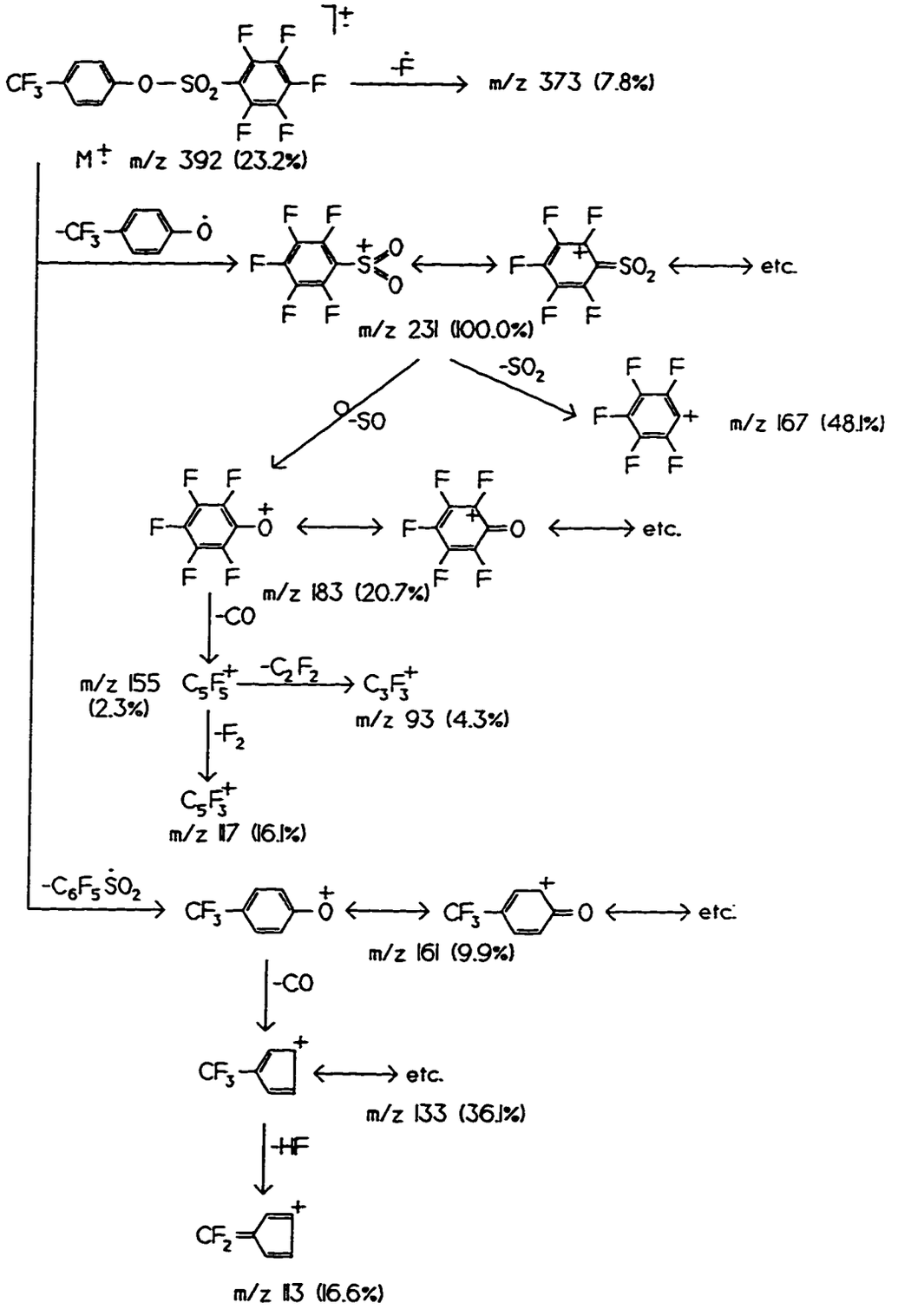


Figure 3-5: Proposed electron impact mass spectrometric fragmentation pattern for the pentafluorobenzenesulfonyl derivative of TFMP. The % relative abundance values are shown in parentheses.

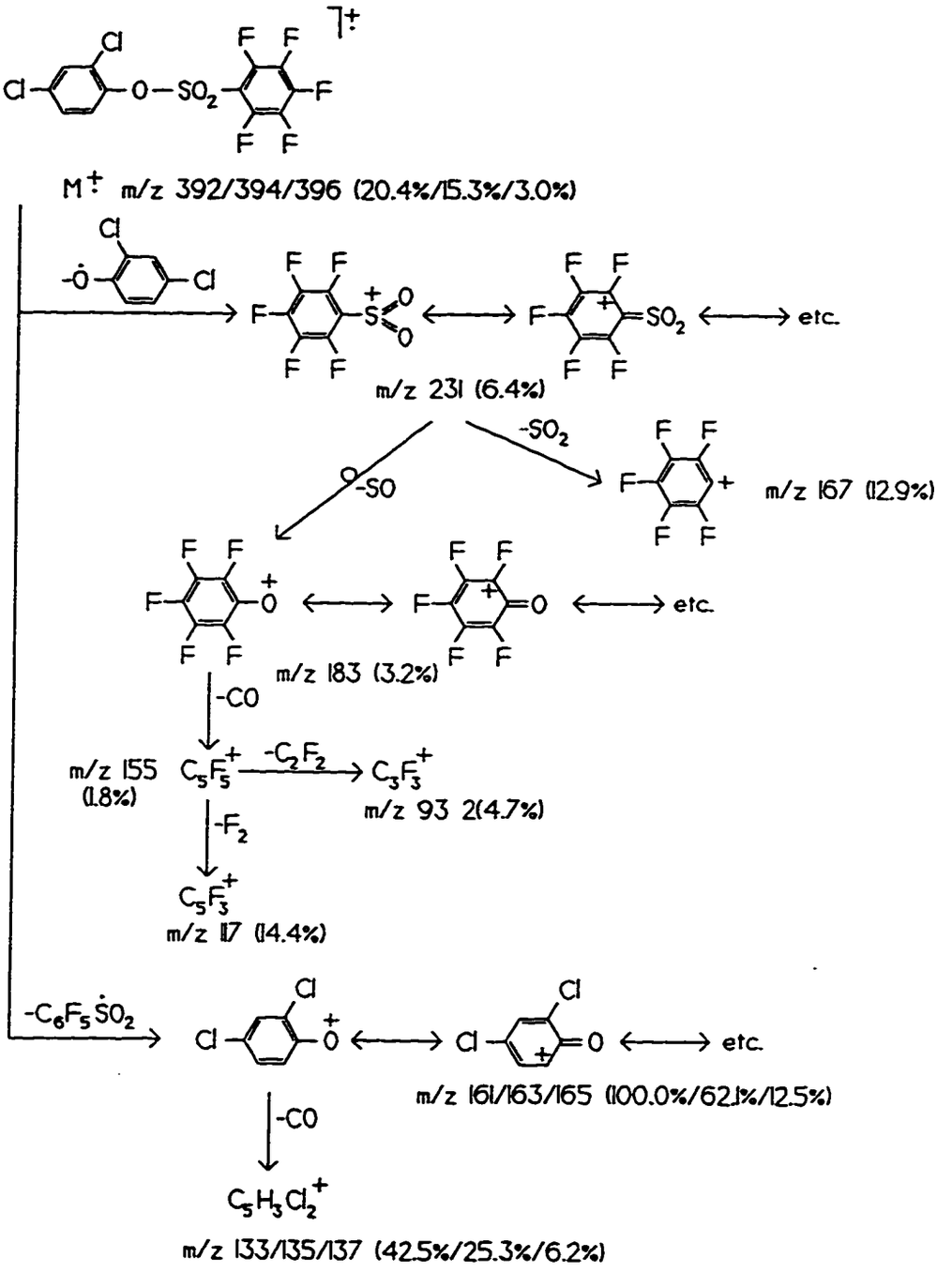


Figure 3-6: Proposed electron impact mass spectrometric fragmentation pattern for the pentafluorobenzenesulfonyl chloride derivative of the internal standard, 2,4-dichlorophenol. The % relative abundance values are shown in parentheses.

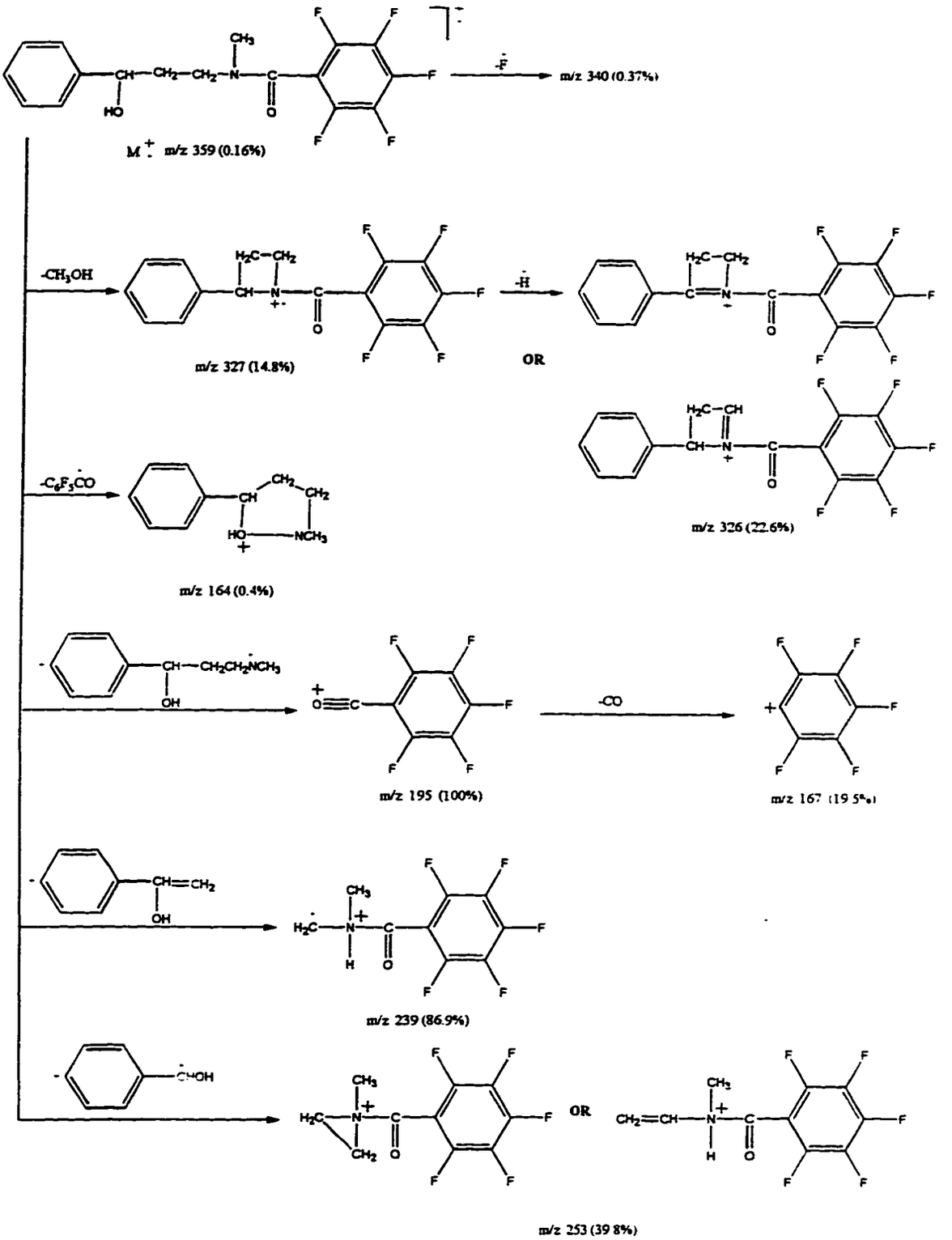


Figure 3-7: Proposed electron impact mass spectrometric fragmentation pattern for the pentafluorobenzoyl derivative of NMHPPA. The % relative abundance values are shown in parentheses.

interpreted. In the case of HPPA, the following ions were identified and agreed with the proposed structure for its derivative: m/z 345 (10.4%), m/z 343 (69.2%), m/z 326 (12.1%), m/z 150 (1.1%), m/z 195 (64.6%) and m/z 167 (22.7%) [see Figure 3-8]. Other ions, especially m/z 250 (100%), m/z 325 (78.4%) and m/z 168 (35.3%), could not be easily identified following usual fragmentation pathways. Thus, although the spectrum obtained for the derivative of HPPA provided some evidence for its proposed structure, the verification of the structure for HPPA was not unequivocal.

Calibration curves were obtained by analyzing standards prepared in parallel with the samples for each assay run. Regression analysis of the relationship between standard concentrations of TFMP and the chromatographic peak/area ratio of TFMP/internal standard yielded a linear relationship over the concentration range analyzed (1.0 ng to 2.0 µg of metabolite per volume of biological sample), with typical r^2 values of >0.99. Likewise, the chromatographic peak/area ratio of NMHPPA/internal standard yielded a linear relationship over the concentration range analyzed (5.0 ng to 2.0 µg of metabolite per volume of biological sample), with typical r^2 values of ~ 0.99. Calibration curves generated on different days were reproducible. The mean interassay coefficients of variation for TFMP were as follows: 7.4% (*n*=5) for 500 ng in extracts of rat urine, 9.7% (*n*=4) for 250 ng in samples of rat liver, 3.1% (*n*=4) for 50 ng in samples of rat brain, 6.9% (*n*=5) for 500 ng in extracts of human urine and 7.7% (*n*=4) for 25 ng in extracts of human plasma. The mean interassay coefficient of variation for NMHPPA was 10.78%

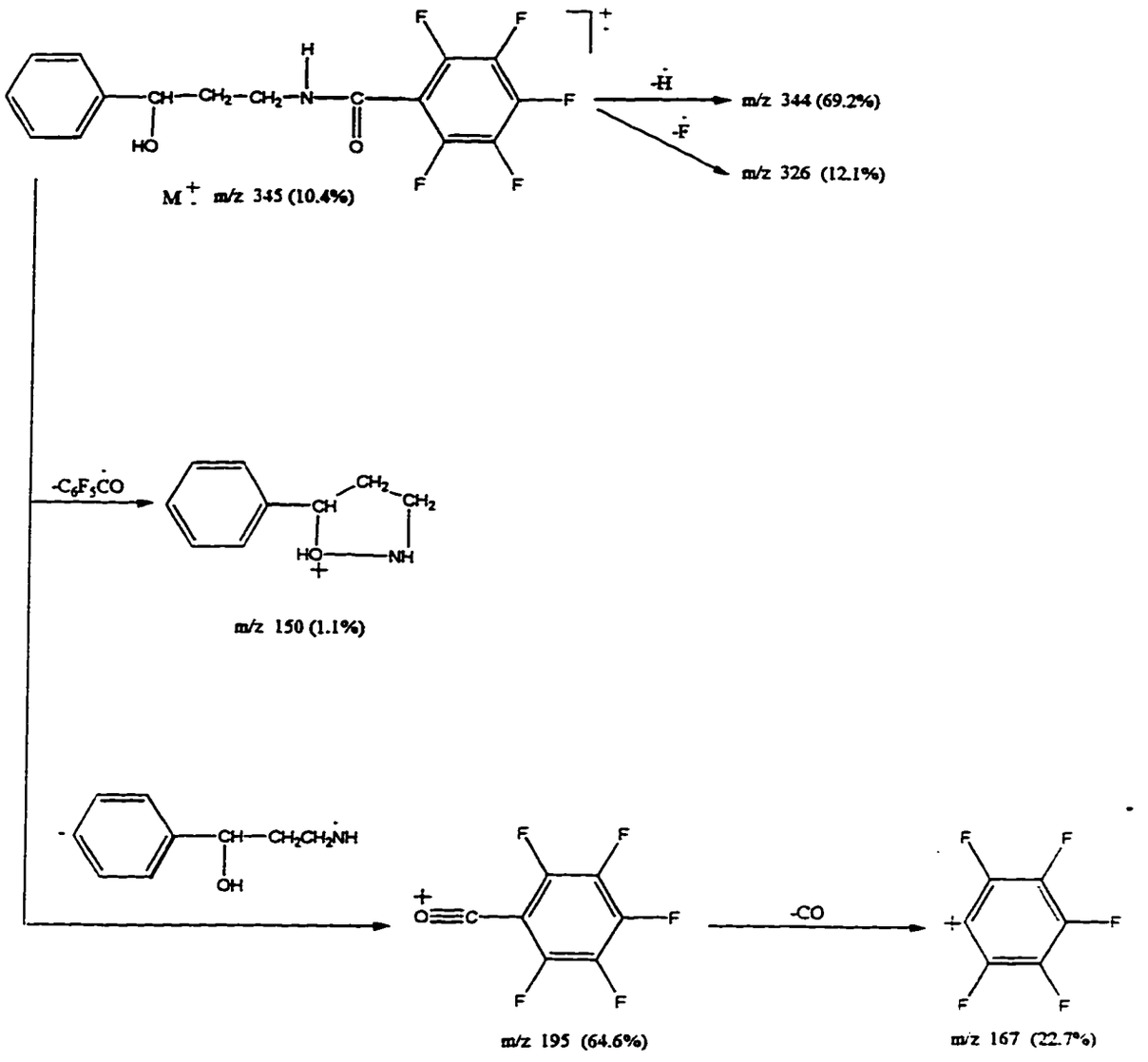


Figure 3-8: Proposed electron impact mass spectrometric fragmentation pattern for the pentafluorobenzoyl derivative of HPPA. The % relative abundance values are shown in parentheses.

(*n*=3) for 1.0 µg in extracts of human urine. The practical limit of sensitivity (signal:noise ratio >3) for TFMP was <5 ng/ml (<25 pg "on-column") in human plasma samples, <10 ng/g of rat brain tissue, <25 ng/g of rat liver tissue and <25 ng/ml in human and rat urine extracts. For NMHPPA, the practical limit of sensitivity was <25 ng/ml in human and rat urine extracts. The mean percent recovery of TFMP from rat urine extracts was 85.6 ± 2.9% (*n*=5), 89.4 ± 3.6% (*n*=6) from rat liver samples, 86.2 ± 1.9% (*n*=5) from rat brain samples, 86.3 ± 3.9% (*n*=5) from human urine extracts and 87.5 ± 4.6 % (*n*=5) from human plasma samples. For NMHPPA, the mean percent recovery was 68 ± 10% (*n*=3) from rat urine extracts and 77 ± 18% (*n*=3) from human urine extracts.

The mean free TFMP levels in brain and liver samples obtained from FLU-treated rats are shown in Table 3-1. Table 3-2 shows 24-h urine levels of free TFMP and NMHPPA in rats following a single injection of FLU. Table 3-2 also indicates total levels of TFMP, S- and R-FLU and S- and R-NFLU in rat urine and the percentage of each that is conjugated.

3.3.2 Part 2 - Investigation of 5-HT Uptake Inhibiting Properties of TFMP, NMHPPA and HPPA, Novel Metabolites of Fluoxetine.

A preliminary screen showed that at a concentration of 100 µM, TFMP inhibited ^{3}H-5-HT uptake into striatal prisms by 26.25 ± 3.96% (*n*=6), HPPA inhibited uptake by 28.72 ± 3.21% and NMHPPA had no discernable effect on uptake (*n*=4). As expected, however, FLU (which has an IC_{50} of <1 µM in this system), caused

Tissue or body fluid	Concentration TFMP	Sample size (*n*)	FLU Dosage
Rat brain	17.0 ± 4.1 ng/g	6	10 mg/kg
Rat liver	236 ± 85 ng/g	8	10 mg/kg

Table 3-1: Concentrations of free TFMP in rat brain and liver (values represent means ± S.E.M.). Rats were sacrificed 5 h post-injection following a single i.p. injection of FLU.

Metabolite	Total (µg/24 h urine)	Free (µg/24 h urine)	Conjugated (µg/24 h urine)	% Conjugated
TFMP	8.33 ± 2.05	1.1 ± 0.77	7.22 ± 1.57	86.8 ± 4.55
NMHPPA	n/d	7.59 ± 0.84	n/d	n/d
S-FLU	9.53 ± 0.68	4.98 ± 0.81	3.4 ± 0.78	36.8 ± 8.2
R-FLU	10.16 ± 0.92	5.51 ± 0.53	4.02 ± 0.89	39.8 ± 8.7
S-NFLU	3.85 ± 0.97	2.88 ± 1.2	2.42 ± 0.73	62.03 ± 18.2
R-NFLU	2.42 ± 0.21	1.87 ± 0.61	1.57 ± 0.19	62.77 ± 6.6

Table 3-2: 24 h urine levels of total and free TFMP, FLU and NFLU and free levels of NMHPPA in the rat. Rats were given a single i.p. injection of FLU•HCl (10 mg/kg). Values represent means ± S.E.M. (*n*=6). n/d = not determined.

complete inhibition of 5-HT uptake in this study.

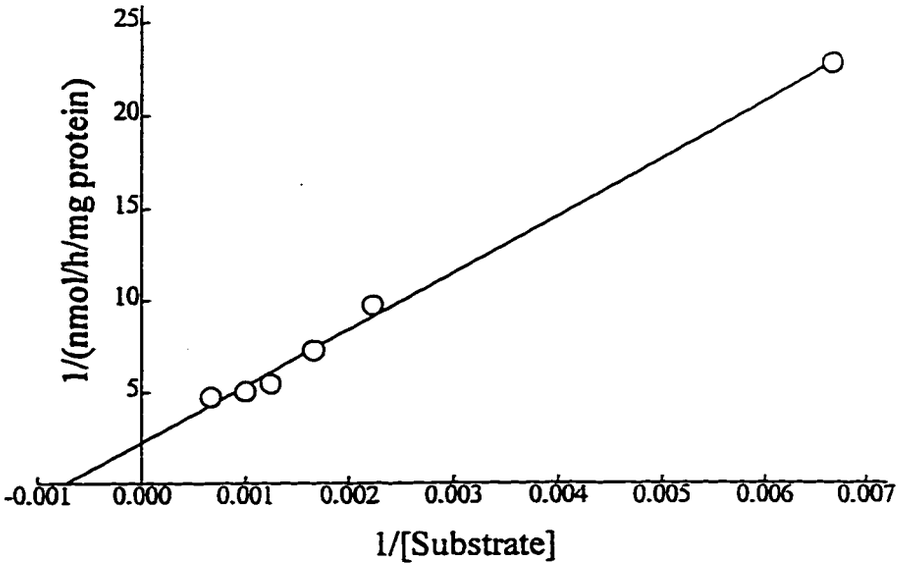
3.3.3 Part 3 - Use of a New Spectrophotometric Amine Oxidase Assay to Determine if two Amine Metabolites of Fluoxetine are Substrates for MAO.

Figure 3-9 shows a Lineweaver-Burk plot for the metabolism of NMHPPA by MAO-A (upper panel) and MAO-B (lower panel). Figure 3-10 shows a Lineweaver-Burk plot for the metabolism of HPPA by MAO-B. Kinetic constants obtained from these data, the means from 4 assays, are shown in Table 3-3. The rate of turnover of HPPA by MAO-A was below the detection limits of the assay.

3.4 DISCUSSION

3.4.1 Part 1 - Metabolite Investigation in Rat and Human Biological Samples.

Extractive derivatization with PFBSC under aqueous conditions permitted the identification and quantitation of TFMP as a metabolite of FLU in human plasma, human and rat urine and in rat brain and liver samples. The first report of TFMP being a proposed metabolite of FLU was by Benfield et al., (1986), but the first reported quantitative measurement of this metabolite was from our laboratory (Aspeslet, 1994). Aspeslet also determined that when µg/24 h levels of TFMP and levels of FLU and NFLU in human urine were converted to µmol/24 h, the mean TFMP level excreted in the urine was 79.5% of the level of FLU and 56.3% of the level of NFLU. These data indicated that TFMP may be an important metabolite of



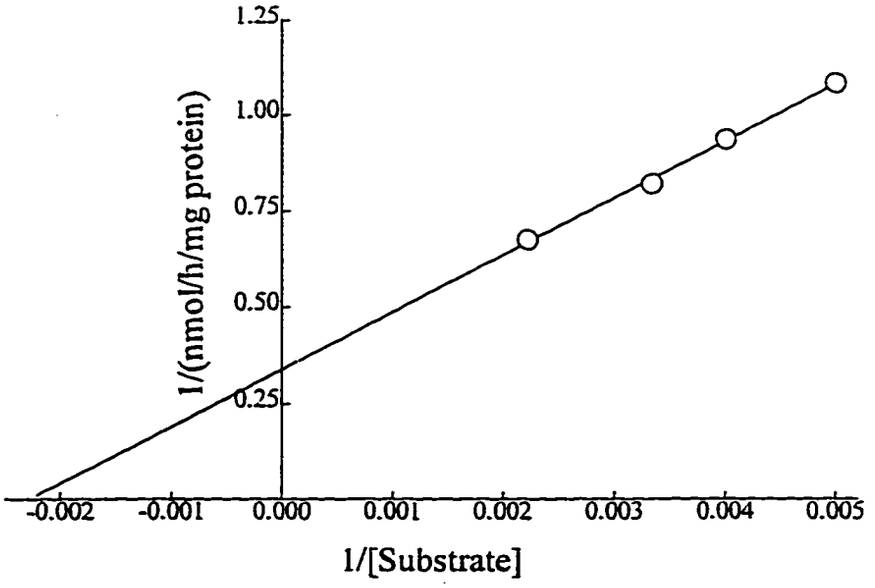


Figure 3-9: Lineweaver-Burk plot for the metabolism of NMHPPA by MAO-A (upper panel) and MAO-B (lower panel).

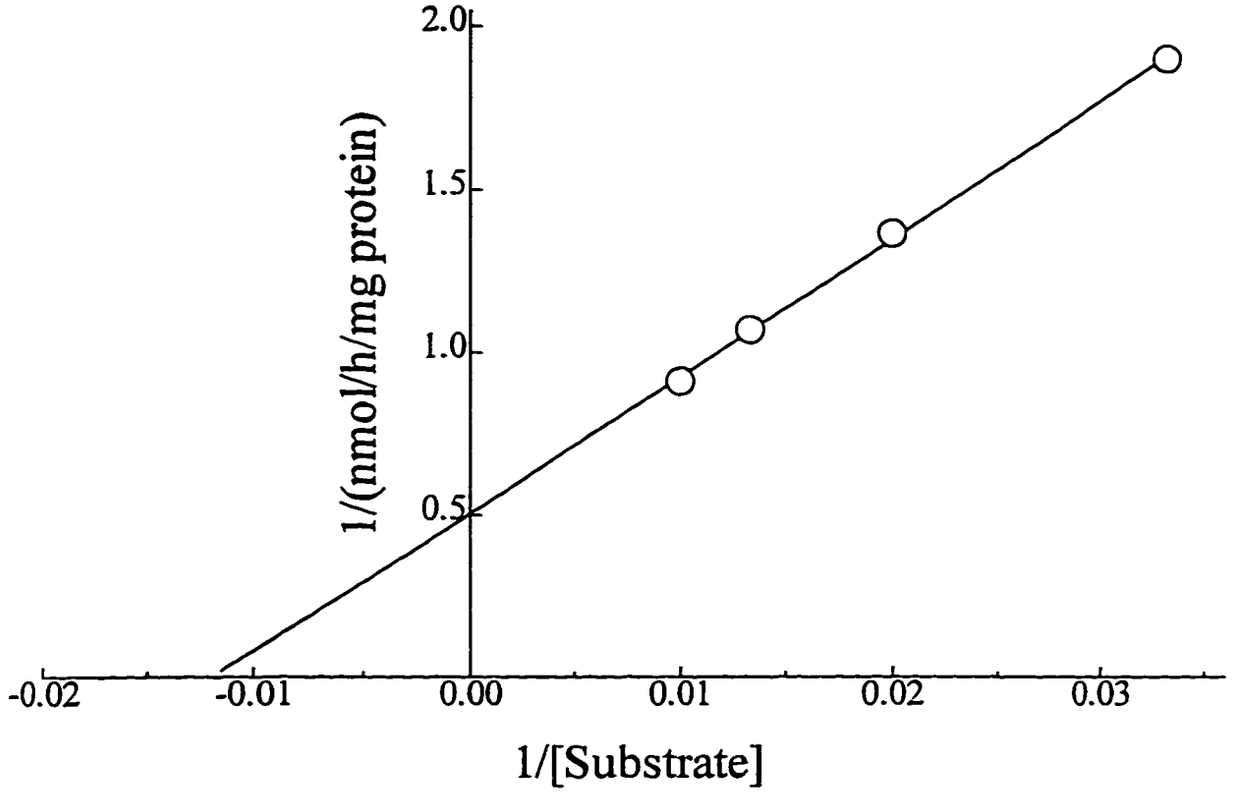


Figure 3-10: Lineweaver-Burk plot for the metabolism of HPPA by MAO-B.

	K_m (μM)	V_{max} (nmol/h/mg protein)	r^2
MAO-A *with* NMHPPA	1370	5.2	0.996
MAO-A *with* HPPA	n/d	n/d	n/d
MAO-B *with* NMHPPA	436	34.5	0.997
MAO-B *with* HPPA	84	23.3	0.999

Table 3-3: Kinetic constants for the metabolism of the novel amine metabolites of FLU by the MAO subtypes. Values are the means from 4 assays. n/d = could not be determined.

FLU. Aspeslet quantitated TFMP in human and rat urine samples (Aspeslet, 1994), but investigations of TFMP reported in the present thesis extended analysis to include rat brain and liver and this required some modifications to the original assay procedure. In addition, TFMP in human plasma was now also quantitated. It is interesting, but not surprising based on its phenolic structure, that TFMP was also found to be extensively conjugated (~87%).

A novel phenylpropylamine metabolite of FLU, namely NMHPPA, was identified and quantitated in human and rat urine using extractive derivatization with PFBC under aqueous conditions. HPPA, the potential corresponding metabolite of NFLU, was not measurable in urines because of an interfering peak, but, as shown later in this thesis, was shown to be a metabolite of NFLU after incubation with human liver microsomes. The identification of these novel metabolites of FLU is important because, as previously mentioned, about 50% of an administered dose of FLU is still unaccounted for (Bergstrom et al., 1988; Lemberger et al., 1985). In addition, these metabolites seem to fit well in an alternative pathway that is proposed for the formation of a benzoic acid precursor (see figure 3-1). Aspeslet (1994) suggested, from an examination of the structure of NFLU, that TFMP is a potential metabolite of it as well as FLU. The pathway proposed here agrees with this proposal and will be discussed further in Chapter 4.

3.4.2. Part 2 - Investigation of 5-HT Uptake Inhibiting Properties of TFMP, NMHPPA and HPPA, Novel Metabolites of Fluoxetine.

The data obtained from 5-HT uptake studies on novel metabolites of FLU indicate that TFMP, NMHPPA and HPPA are weak inhibitors, at best, of 5-HT uptake, even at high concentrations (i.e. 100 μM). In the present study the levels of TFMP in rat brain and liver were 0.11 μM and 1.46 μM, respectively. Thus, TFMP is unlikely to contribute to the development of adverse effects such as the serotonin syndrome, even if its levels were increased dramatically due to drug-drug interactions. It is possible, however, that these novel metabolites may contribute to side effects that have been reported after FLU administration that are not normally attributed to increased serotonin levels such as extrapyramidal symptoms and stimulant effects (Gram, 1994), but further study is needed to examine this issue.

3.4.3 Part 3 - Use of a New Spectrophotometric Amine Oxidase Assay to Determine if Two Amine Metabolites of Fluoxetine are Substrates for MAO.

The investigations of the two amine metabolites of FLU, NMHPPA and HPPA, indicate that both amines can be deaminated by MAO. The lower K_M values measured for both substrates with MAO-B suggest that this enzyme may be predominantly responsible for their metabolism *in vivo*. Although V_{max} values estimated for substrate turnover by MAO-A were significantly lower than those

measured for MAO-B, this may reflect a loss in MAO-A activity during preparation of tissue homogenates. The activity (V_{max}) ratio of MAO-A to MAO-B, measured with the common substrate, tyramine, was 0.1. This ratio *in vivo* is approximately 0.7, indicating that approximately 80% of MAO-A activity was lost during homogenate preparation, and V_{max} values with MAO-A would reflect this loss. Rapid loss of MAO-A activity, but not MAO-B activity, is a frequent occurrence following homogenisation (Holt and Baker, 1996). Thus, these analyses support the metabolic pathway for FLU shown in figure 3-1, where CYP450-mediated formation of the amines, probably *via* a ketone intermediate, is followed by deamination by MAO (largely MAO-B), dehydrogenation of the aldehyde, then β-oxidation and conjugation to hippuric acid.

The finding that these novel amine metabolites of FLU are substrates for MAO is important because it gives new information about the potential metabolic pathways for FLU. This pathway may also have relevance to possible pharmacokinetic interactions with coadministered MAOIs. Drugs such as PLZ or TCP, which inhibit both MAO-A and MAO-B, may prevent deamination of NMHPPA and HPPA. Since NMHPPA and HPPA do not have potent effects on 5-HT reuptake, such accumulation would probably not contribute directly to the serotonin syndrome. However, by blocking this metabolic route through inhibition of further metabolism of NMHPPA and HPPA, PLZ and TCP may result in an increased accumulation of FLU and NFLU, with possible adverse consequences. The RIMAs, such as MOC and BROF, with much reduced effects on MAO-B, might be less likely

to cause an accumulation of NMHPPA and HPPA.

Although the catabolism of FLU that is now proposed seems to correlate with the metabolites that are found in the urine, there is some discrepancy with metabolites that have been found in the plasma. For example, fluoxetine carboxylic acid was found to be a major circulating metabolite in rat, dog and mouse plasma, whereas this metabolite was not found in 24 h rat urine (Schmalz et al., 1995). These data agree with the pathway proposed by Altamura et al. (1994) for the metabolism of FLU (see figure 1-6), where fluoxetine carboxylic acid is O-dealkylated to form TFMP and γ-hydroxy-γ- phenylpropionic acid. The involvement of fluoxetine carboxylic acid in metabolism is not clear at this time, but the examination of plasma from patients treated with FLU for NMHPPA and HPPA may provide further information on possible metabolic routes of FLU.

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4.0 CHAPTER 4

Investigation of potential metabolic pathways for fluoxetine in humans.

(Parts of the work presented in this chapter were presented at the 19th annual meeting of the Canadian College of Neuropsychopharmacology in Toronto, Ontario).

4.1 INTRODUCTION

The SSRIs have a high affinity for many CYP isozymes and are also extensively metabolized by them (Harvey and Preskorn, 1996). The knowledge that different CYP enzymes mediate the metabolism of different SSRIs is important as it helps explain many of the pharmacokinetic differences between the SSRIs. The identification of the CYPs reponsible for the metabolism of individual SSRIs is also important clinically for predicting whether concomitantly administered drugs can affect their clearance, and thus also their efficacy and tolerability (Ciraulo and Shader, 1990; Altamura et al., 1994; Preskorn, 1996a,b). Although the metabolism of citalopram, paroxetine and sertraline is characterized quite well, there is little clinically relevant information on the metabolism of fluvoxamine and FLU (Preskorn, 1996a). This is surprising given the extensive use of FLU since its introduction (Brösen and Skjelbo, 1991; Wong et al., 1995) and the increasing popularity of fluvoxamine.

The difficulty in characterizing the metabolism of FLU is several fold. For example, FLU is marketed as a racemic compound which essentially means that two separate compounds are being metabolized. This metabolism may occur at different rates *via* different pathways and enzymes. To further complicate the matter, NFLU is not only active, but also chiral. Other factors which complicate metabolic investigations are that FLU, and particularly NFLU, have long half-lives and both inhibit several CYPs, to varying degrees, at clinically relevant dosages. In addition, not only can FLU and NFLU inhibit their own metabolism by inhibiting

the CYPs responsible for it, but the potency of this inhibition may vary between the respective enantiomers of both FLU and NFLU (Preskorn, 1996a). Thus, when characterizing the metabolism of FLU, one must essentially account for the biotransformation of four separate drugs and the involvement of multiple CYP isozymes.

The following is a brief summary of what is known about the CYPs that mediate the metabolism of FLU. It is thought that the N-demethylation of R-and S-FLU is likely mediated, at least in part, by the same CYP isozyme(s), even though the rate of N-demethylation differs between the enantiomers *in vitro* in human liver microsomes (Stevens and Wrighton, 1993). The enzyme(s) responsible for N-demethylation have not yet been unequivocally characterized, although it is thought that CYP2D6 plays, at the least, a minor role (Stevens and Wrighton, 1993; Baumann and Bertschy, 1993). It has been suggested that there are likely multiple CYP isozymes involved in the metabolism of FLU at different concentrations (Preskorn, 1996a), which would account for FLU's nonlinear pharmacokinetic profile. For example, lower affinity enzymes would not be relevant until the drug concentration is sufficiently high to inhibit the higher affinity enzymes. Indeed, in a study utilizing isoform-specific inhibitors and human liver microsomes, Wang and Unadkat (1995) determined that the isozymes involved in the N-demethylation of R-FLU included CYP2C9, CYP2E1, CYP2D6, CYP3A4, CYP2B6, CYP2C19 and CYP1A2 while the isoforms involved in the N-demethylation of S-FLU included CYP2E1, CYP2D6, CYP3A4, CYP2B6, CYP2C19 and CYP1A2, with some forms being major, and other forms minor, contributors.

The present investigation utilized human urine and/or plasma samples, cDNA-expressed human CYP isozymes and human liver microsomes to investigate potential metabolic pathways for FLU and NFLU in humans. Specific interest was in the enzymes involved in the N-demethylation of R- and S-FLU to R- and S-NFLU and in those that might play a role in the O-dealkylation of FLU and NFLU to *p*-TFMP. Preliminary experiments were also conducted to investigate potential isoforms responsible for the production of NMHPPA and HPPA from FLU and NFLU, respectively.

4.2 MATERIALS AND METHODS

4.2.1 Part 1 - Drug and Metabolite Levels in Human Urine and/or Plasma

4.2.1.1 Drug Administration and Analysis

Patients diagnosed with major depressive disorder were asked to collect a 24 h urine sample 3 weeks after commencing treatment with FLU•HCl (Prozac®; 10-20 mg, once daily). A blood sample (16 ml) was collected into vacutainers (with EDTA as the anticoagulant) and centrifuged at 1,000 x g for 10 min to separate the red cells from the plasma. Plasma and urine samples were collected from these subjects before FLU treatment began and again after 3 weeks of treatment. Urine was collected for the 24 h prior to the day of blood collection. The plasma and urine samples were frozen at -20°C until analyzed.

4.2.1.2 Analysis of the Enantiomers of Fluoxetine and Norfluoxetine

The enantiomers of free FLU and NFLU were analyzed using the procedure of Torok-Both et al. (1992). See section 3.2.1.2 of this thesis for the specific method.

4.2.1.3 Analysis of Metabolites of Fluoxetine/Norfluoxetine

Levels of TFMP, NMHPPA and HPPA were analyzed using the procedure described in Chapter 3 of this thesis. As indicated in Chapter 3, however, HPPA could not be quantitated in untreated, control human urine extracts due to an interfering peak.

4.2.2 Part 2 - Investigation of the Metabolism of Fluoxetine and Norfluoxetine Using cDNA-Expressed Human CYP450 Isozymes and Human Liver Microsomes.

4.2.2.1 Isozymes and Microsome Preparations

Complementary (c) DNA-expressed human CYP450 isozymes were prepared by individually transfecting metabolically competent derivatives of the human AH H-1 $TK^{+/-}$ cell line with human CYP450 cDNA for CYP1A2, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, CYP2D6, CYP3A4 and CYP2E1. The individual isozyme preparations were prepared by and purchased from Gentest Co (Woburn, MA). Control preparations were from cells transfected with the vector alone. The microsomes contained adequate NADPH-P450 reductase and cytochrome b_5 for the

P450 form-specific metabolic assays.

Human liver microsomal preparations were obtained from the International Institute for the Advancement of Medicine (Exton, PA).

4.2.2.2 Analysis

Racemic FLU (100 μM) was incubated with human liver microsomes for 5, 10, 20, 30 or 60 min to determine the time course of metabolite formation and, thus, the incubation time required for subsequent experiments. Racemic FLU (100 μM) was then incubated with microsomes prepared from a panel of 16 human livers that were characterized for their catalytic activity for each of the following isozymes: CYP1A2 (characterized using phenacetin O-deethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2C19 (mephenytoin 4-hydroxylation), CYP2D6 (dextromethorphan O-demethylation), CYP2E (chlorzoxazone 6-hydroxylation), CYP3A4 ([^{14}C]-testosterone β-hydroxylation) and CYP4A ([^{14}C]-lauric acid omega-hydroxylation). The rate of formation of S-NFLU, R-NFLU, HPPA, NMHPPA and TFMP was correlated with the activities of the specific isozymes for each of the 16 livers. Any isozyme(s) producing a significant correlation was (were) implicated in the production of the metabolite of interest. The correlations were analyzed by simple linear regression and slopes were tested for linearity with an F test (Graph Pad Prism, version 2.0).

In order to determine which individual isozymes might play a role in the metabolism of racemic FLU to S-NFLU, R-NFLU, TFMP and NMHPPA or of NFLU to TFMP and HPPA, FLU or NFLU (100 μM, added in a volume of 50 μl) was

incubated with 10 µl of a 10 mg/ml microsomal preparation expressing one of the following: CYP1A1, CYP1A2, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, CYP2D6, CYP3A4 or CYP2E1. The incubation time was determined from previous experiments and varied depending on which metabolite was being investigated.

Isoform-specific inhibitors were used to further assess the role of a specific isoform in the metabolism of FLU or NFLU. The inhibitor was added in several different concentrations while the substrate concentration was held constant to determine if a concentration-dependent inhibition of the metabolite was produced by the isoform specific inhibitor. These studies were conducted in both human liver microsomes and human cell lines expressing the isozyme of interest.

The substrate of interest in all experiments mentioned above was incubated in the following reaction mixture: 25 µl of a NADPH generating system consisting of β-nicotinamide adenine dinucleotide phosphate (1.3 mM), glucose 6-phosphate (3.3 mM), glucose 6-phosphate dehydrogenase (0.4 U/ml) and $MgCl_2$ (3.3 mM); 10 µl of microsomal enzyme preparation, 50 µl of substrate (100 µM final concentration), and enough potassium phosphate buffer (0.1 M, pH 7.4) to bring the final volume to 100 µl. The incubations were conducted in 1.5 ml polypropylene microcentrifuge tubes (Fisher, Ottawa, ON) at 37°C for the pre-determined length of time. Following incubation, the tubes were placed on ice and basified with the appropriate base (depending on the assay protocol for the metabolite of interest). Basification of the reaction mixture stops the metabolic reactions so that the assay can proceed. Before continuing with the assay, however, the volume of the mixture was made up to 1 ml with distilled water. The specific assay protocols for the

metabolites of FLU are given in Chapter 3.0 of this thesis. Despite problems in quantitating HPPA in human urine extracts due to interfering peaks in the chromatogram (discussed in Chapter 3), there was no interference in control microsomal preparations. Thus, analysis of HPPA was possible in the microsome and individual isozyme experiments discussed in this chapter.

4.3 RESULTS

4.3.1 Part 1 - Drug and Metabolite Levels in Human Urine and/or Plasma

After three weeks of treatment with FLU, plasma samples from six patients had mean levels of 71 ± 13 ng/ml R-FLU, 210 ± 50 ng/ml S-FLU, 157 ± 43 ng/ml R-NFLU and 226 ± 40 ng/ml S-NFLU.

The mean free urine levels of the enantiomers of both FLU and NFLU are shown in Table 4-1. Table 4-1 also indicates free levels of TFMP and NMHPPA. As mentioned in Chapter 3 of this thesis, it was hypothesized, based on the structure of TFMP, that this metabolite would be a good candidate for conjugation. This hypothesis was confirmed when rat urine was analysed (see section 3.3.1 for details), so it was also of interest to analyse human urine for conjugated levels of TFMP. The results were very similar to those found in the rat where 87 ± 5% (n=6) of TFMP was found to be conjugated. In human urine samples 86 ± 3% (n=6) of TFMP was conjugated.

Drug/Metabolite	Concentration (µg/24 h urine)
R-FLU	404 ± 159
S-FLU	964 ± 367
R-NFLU	746 ± 333
S-NFLU	638 ± 206
TFMP	229 ± 41
NMHPPA	7.6 ± 0.84

Table 4-1: Free 24 h urine levels of the enantiomers of FLU and NFLU and of free TFMP and NMHPPA. Patients had been taking FLU (Prozac®; 10-20 mg/day) for a period of 3 weeks. Values represent means ± S.E.M.

4.3.2 *Part 2 - Investigation of Metabolism of FLU and NFLU Using cDNA-Expressed Human CYP450 Isozymes and Human Liver Microsomes.*

Racemic FLU (100 μM) was incubated with human liver microsomes for 5, 10, 20, 30 or 60 min to determine the time course for the formation of the enantiomers of NFLU (Figure 4-1) and also of TFMP and NMHPPA. The results of these studies indicated that R- and S-NFLU, TFMP and NMHPPA production were all approximately linear for the first 30 min. Therefore, an incubation time of 20 min was chosen for subsequent experiments as it was within the linear range.

Next, racemic FLU (100 μM) was incubated with microsomes prepared from a panel of 16 human livers characterized for catalytic activity of some of the CYP isozymes. The rate of formation of S-NFLU showed significant correlation with both CYP2D6 (r^2= 0.31, F (1,14)=6.41, p=0.02) and CYP3A4 (r^2= 0.34, F=(1,14)=7.29, p=0.02) [see Figure 4-2]. The rate of formation of R-NFLU did not significantly correlate with any of the CYP isozymes. It is worth mentioning, however, that CYP3A4 activity did approach linearity (r^2=0.20), even though it was not found to be significant when analyzed. Similarly, the rate of formation of TFMP and NMHPPA from FLU did not significantly correlate with any of the CYP isozymes whose activity was characterized. The rate of formation of HPPA from NFLU did, however, correlate with the activity of CYP1A2 (r^2=0.27, F(1,14)=5.07, p=0.04) [Figure 4-3].

Experiments utilizing cDNA-expressed human CYP isozymes revealed additional information on the individual isozymes that could be involved in the formation of metabolites of FLU or NFLU. The results from these studies are sum-

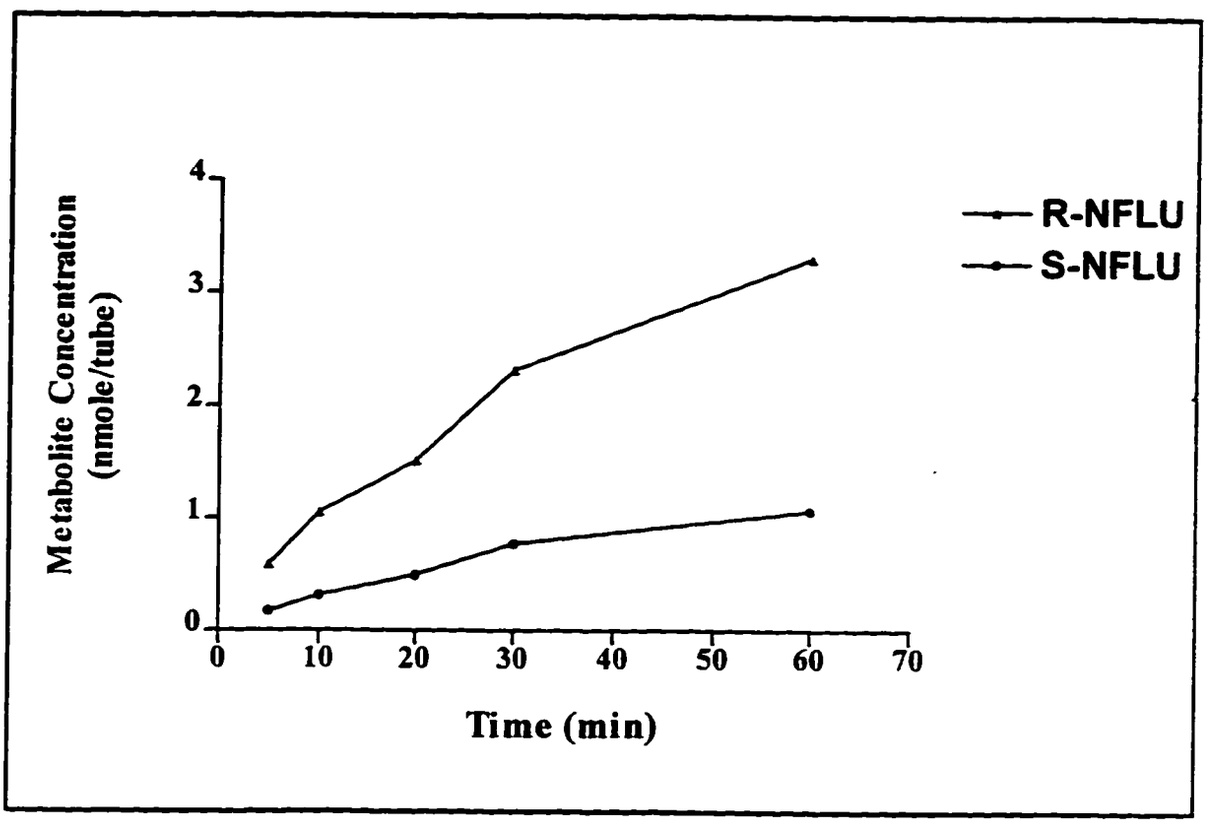

Figure 4-1: Time course for the formation of the enantiomers of norfluoxetine in human liver microsomal preparations.

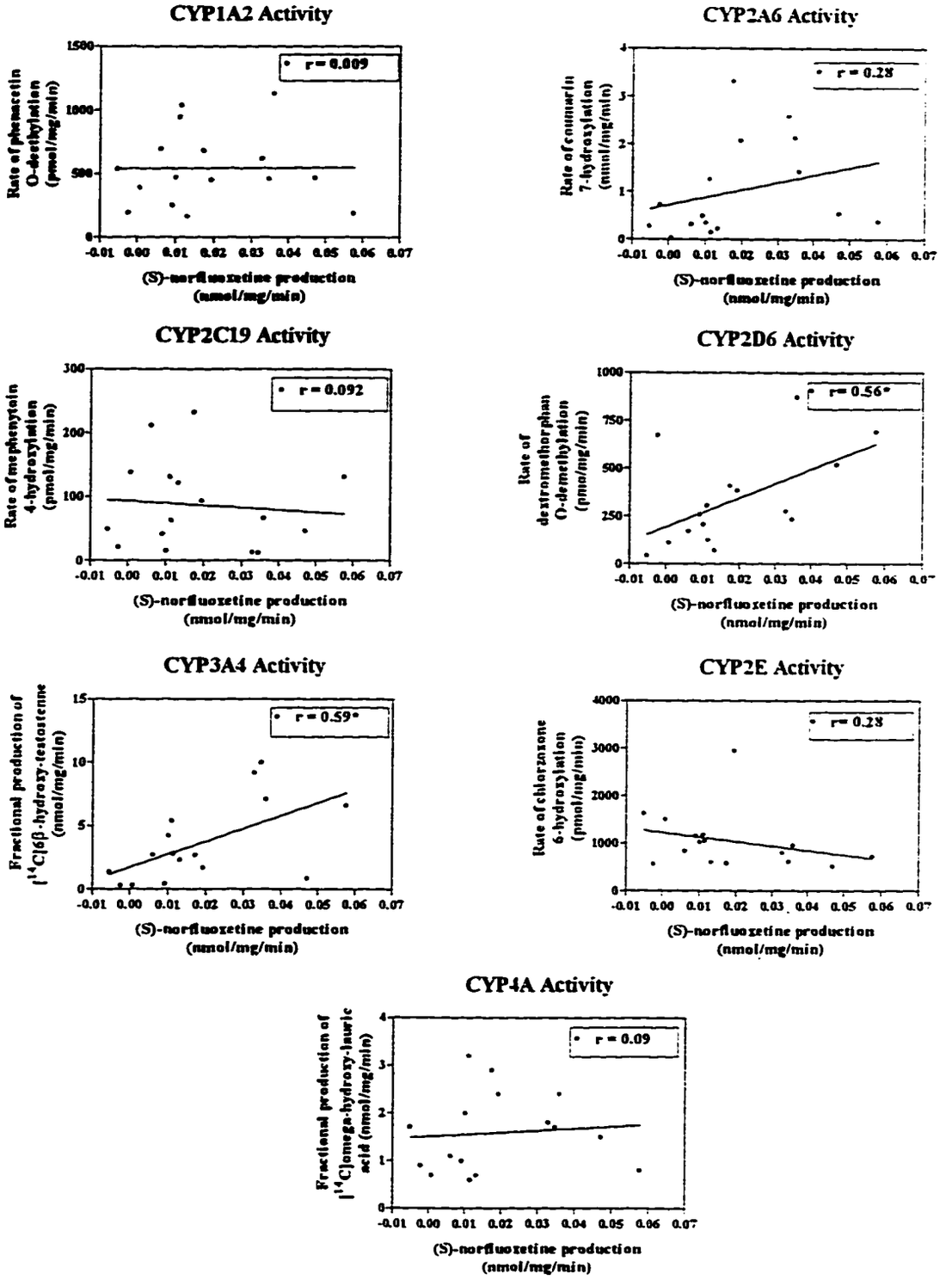

Figure 4-2: Correlations of S-norfluoxetine production from racemic fluoxetine and enzyme activity in microsomes from 16 human livers characterized for activity of CYP1A2, CYP2A6, CYP2C19, CYP2E, CYP3A4, CYP4A and CYP2D6.

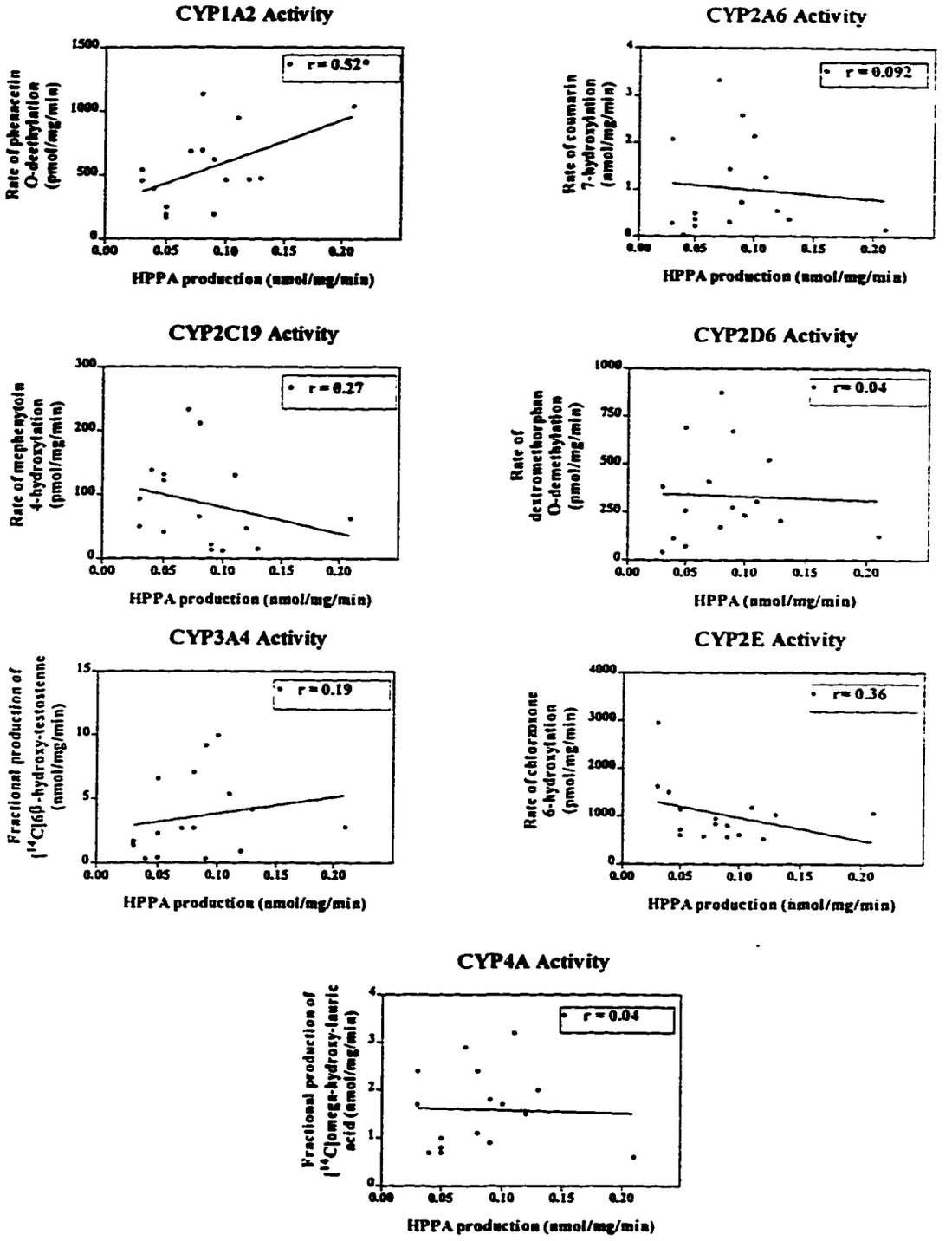

Figure 4-3: Correlations of HPPA production from racemic norfluoxetine and enzyme activity in microsomes from 16 human livers characterized for activity of CYP1A2, CYP2A6, CYP2C19, CYP2E, CYP3A4, CYP4A and CYP2D6.

marized in Table 4-2. When racemic FLU (100 μM) was incubated with 10 μl of a microsomal preparation expressing a single CYP isozyme, the data indicated that several isozymes were able to produce NFLU. The isozymes shown to be involved in the formation of S-NFLU included CYP1A1, CYP1A2, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, CYP2D6 and CYP3A4, with CYP2D6, CYP2C19 and the two variants of CYP2C9 producing the largest quantities of S-NFLU in the time studied. Those involved in the formation of R-NFLU included CYP1A2, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, CYP2D6 and CYP3A4, with CYP $2C9_{arg}$ $CYP2C9_{cys}$ and CYP2C19 forming the largest amount of R-NFLU from racemic FLU. The isozymes that were able to produce TFMP from racemic FLU included CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, CYP2D6 and CYP3A4, with CYP3A4 producing the largest concentration of TFMP. The isozymes shown to produce NMHPPA from racemic FLU were CYP2C8, CYP2C19 and CYP2D6, with CYP2D6 being predominantly involved according to the amount of metabolite produced.

Racemic NFLU (100 μM) was also studied using individual cDNA-expressed isozyme preparations (see Table 4-2). Results from these preliminary experiments suggest that CYP1A1, CYP1A2, CYP2C8 and CYP3A4 could be involved in the metabolism of NFLU to TFMP while CYP1A1, CYP1A2, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, and CYP3A4 could be involved in the formation of HPPA.

Preliminary studies using specific isoform inhibitors were conducted to further assess the role of CYP3A4 in the production of TFMP and CYP2D6 in the formation of NMHPPA from racemic FLU. Because no significant correlation with CYP3A4 for the formation of TFMP or CYP2D6 for the formation of NMHPPA had been found

	Isozymes implicated in the formation of :				
Substrate	**R-NFLU**	**S-NFLU**	**TFMP**	**NMHPPA**	**HPPA**
Racemic FLU (100 µM)	CYP1A2, 2C8, **2C9$_{arg}$, 2C9$_{cys}$, 2C19**, 2D6, 3A4	CYP1A1, 1A2, 2C8, **2C9$_{arg}$, 2C9$_{cys}$, 2C19, 2D6**, 3A4	CYP2C8, 2C9$_{arg}$, 2C9$_{cys}$, 2C19, 2D6, **3A4**	CYP2C8, 2C19, **2D6**	-
Racemic NFLU (100 µM)	-	-	CYP1A1, 1A2, 2C8,3A4	-	CYP1A1, 1A2, 2C9$_{arg}$, 2C9$_{cys}$, 2C19, 3A4

The isozymes indicated in **bold type** are those that produced the largest amount of the metabolite in question.

Table 4-2: Summary of experiments conducted with racemic FLU or NFLU and microsomal preparations expressing either CYP1A1, CYP1A2, CYP2C8, CYP2C9$_{arg}$, CYP2C9$_{cys}$, CYP2C19, CYP2D6, CYP3A4 or CYP2E1.

in human liver microsomes, production of each of these metabolites was examined using several concentrations of racemic FLU and a constant concentration of the isozyme in question to ensure that production of the the metabolite increased accordingly. When racemic FLU (100 μM) was then incubated with 100 μM final concentration of ketoconazole, a known potent inhibitor of CYP3A4, the production of TFMP was significantly inhibited (Figure 4-4). Likewise, when racemic FLU was incubated with quinidine, a known potent inhibitor of CYP2D6, the formation of NMHPPA was also inhibited significantly (Figure 4-5).

4.4 DISCUSSION

4.4.1 Part 1 - Drug and Metabolite Levels in Human Urine and/or Plasma Samples.

The finding of higher S-FLU than R-FLU levels in human plasma and urine extracts is consistent with the findings of several other groups (Torok-Both et al., 1992; Baumann and Bertschy, 1993; Stevens and Wrighton, 1993; Aspeslet et al., 1992). These data also support reports that R-FLU is metabolized about 50% faster than S-FLU (see Preskorn, 1996a for review).

The levels of free TFMP found in the human urine extracts was ~⅓ of the level of NFLU, which suggests that TFMP could be an important metabolite. TFMP was found to be extensively conjugated (86%) in the urine of humans, which is consistent with data from rat urine samples, where TFMP was 87% conjugated. This extensive conjugation of TFMP is not surprising in light of the fact that it is a

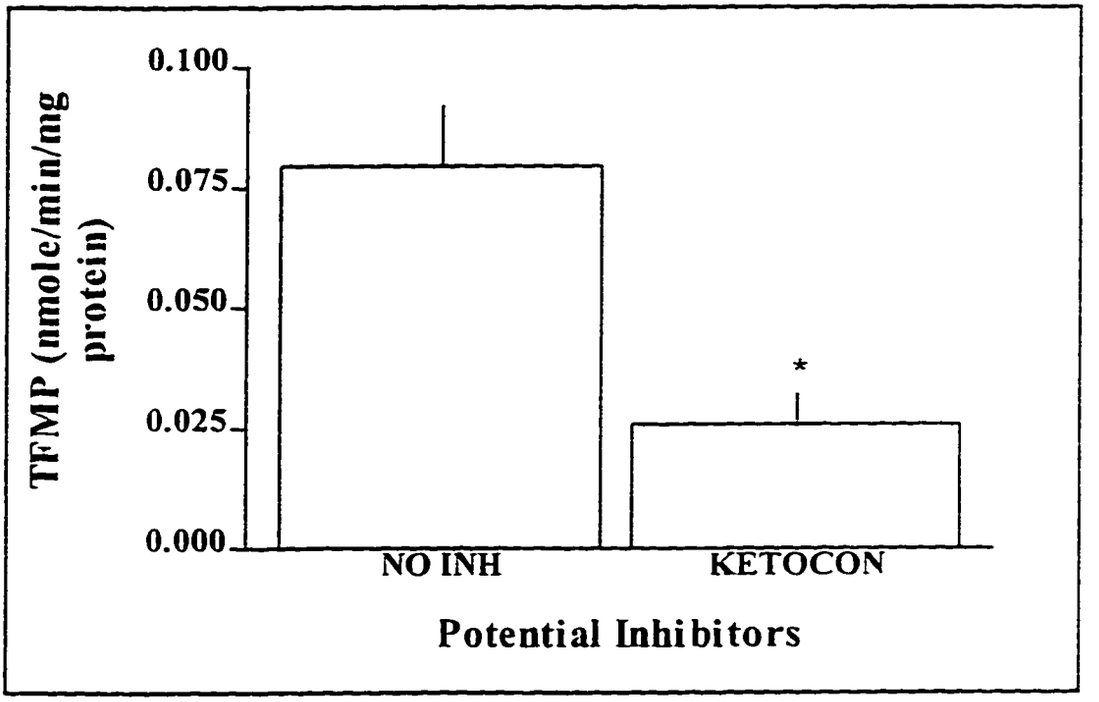

Figure 4-4: Metabolism of fluoxetine to TFMP by CYP3A4 with no inhibitor present (NO INH) or in the presence of ketoconazole (KETOCON), 100 μM. Data shown are means ± S.E.M. (*n*=4). *Signifies $p<0.05$.

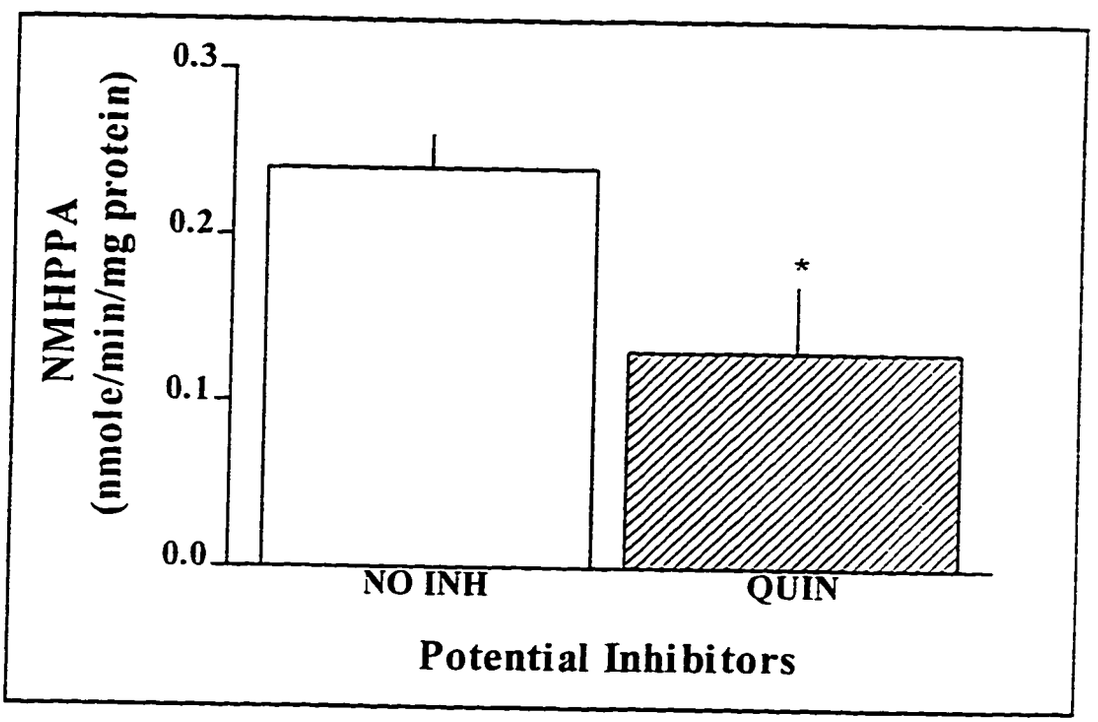

Figure 4-5: Metabolism of fluoxetine to NMHPPA by CYP3A4 with no inhibitor present (NO INH) or in the presence of quinidine (QUIN), 100 μM. Data shown are means ± S.E.M. (*n*=3). *Signifies $p<0.05$.

phenol, and compounds with hydroxy groups are often readily conjugated (Gibson and Skett, 1994).

The level of NMHPPA, a novel amine metabolite of FLU, was also substantial which means that if it is active, it could contribute to FLU's therapeutic or toxic effects. Thus, further examination of this metabolite is warranted. Unfortunately, as discussed in Chapter 3, the levels of HPPA could not be accurately quantitated in human urine due to interference in the GC traces. It may be necessary to resort to another analytical technique, such as combined HPLC-MS for the analysis of this compound in urine extracts.

4.4.2 Part 2 - Investigation of Metabolism of Fluoxetine and Norfluoxetine Using cDNA-Expressed Human CYP450 Isozymes and Human Liver Microsomes.

Valuable information about how a compound is metabolized can be obtained *in vitro* using currently available enzyme preparations. Using individual cDNA-expressed human CYPs, it was determined that multiple isozymes may play a role in the metabolism of racemic FLU to S- and R-NFLU and TFMP and in the catabolism of FLU and NFLU to the two phenylpropylamine metabolites, NMHPPA and HPPA, respectively. It is possible that more than one isoform may be involved in the metabolism of a drug (see Lane, 1996; Preskorn, 1996a for discussion). Drugs may also have major and minor pathways of metabolism that are mediated by different isozymes. In addition, studies revealed that ketoconazole, an inhibitor

of CYP3A4, significantly inhibited the production of TFMP from racemic FLU. Likewise, quinidine, an inhibitor of CYP2D6, was able to inhibit the production of NMHPPA from racemic FLU.

Interestingly, human liver microsome studies did not reveal significant correlations with the isozymes for the formation of any of the metabolites of interest, with the exception of S-NFLU whose formation significantly correlated with the activities of both CYP2D6 and CYP3A4. In addition, expressed isozyme studies indicated that CYP2D6, CYP2C19, $CYP2C9_{arg}$, and $CYP2C9_{cys}$ produced the largest amounts of S-NFLU, while CYP3A4 produced lower levels. These data are confusing, to say the least, but some possible explanations for the results are discussed below.

It is important to recognize the fact that in the human body many factors contribute to how a drug is biotransformed; these factors include the availability and affinity of enzymes, concomitant medications, age, diet, race and even personal habits such as smoking or drinking (see Coutts and Urichuk, 1997 for review). From these important observations, it follows that the metabolic situation in human liver microsomal preparations may certainly differ from cDNA-expressed cell lines and that both of these *in vitro* preparations may differ from the situation in the living human. With these points in mind, the findings from the present study should be interpreted cautiously. In cDNA-expressed cell lines expressing a single human CYP, CYP3A4 was shown to produce less S-NFLU than some of the other isozymes (see table 4-2). Yet, in human liver microsomes, the formation of S-NFLU correlated with the activities of both CYP2D6 and CYP3A4. An explanation for this

may be that when only individual P450s are present, with no direct competition from other isoforms for a substrate, they may be able to metabolize the substrate. When several P450s are present and are competing for the substrate (as in microsomal preparations), however, only those isoforms with higher affinity for the substrate may play a role in metabolism.

The observation that no CYP activities significantly correlated with the formation of R-NFLU, TFMP or NMHPPA from FLU may be possible if other isozymes whose activities were not characterized are the ones important for the production of these metabolites. At present, human liver microsomal preparations are a relatively new pharmacological tool and, thus, not only are they very difficult to aquire, they are also not fully characterized for all of the known human P450s. It is our understanding that work is progressing to more fully assess the activities of the isozymes present in the microsomal preparations, but, as can be envisioned, this is an enormous task and will take some time to accomplish. Therefore, since only a small number of isozymes were characterized, it is possible that the formation of R-NFLU, TFMP and NMHPPA is correlated with the activity of another, as yet uncharacterized, CYP.

The major isoform responsible for the formation of HPPA from racemic NFLU appears to be CYP1A2. The activity of CYP1A2 significantly correlated with the formation of HPPA in human liver microsomes and evidence of its involvement was further corroborated using cDNA-expressed human cell lines which also confirmed that CYP1A2 played a role.

Even though *in vitro* metabolism studies cannot be directly applied to the *in*

vivo situation, they can give us valuable information about the biotransformation of drugs and the CYPs that could potentially be involved. This information is helpful clinically to predict possible adverse effects and interaction. There is still considerable knowledge lacking about the biotransformation of FLU and the isozymes involved, but the present studies have opened the door for further experimentation in this area.

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5.0 CHAPTER 5

Drug-drug interactions involving fluoxetine and moclobemide.

(Parts of the work presented in this chapter were presented at the *British Association of Pharmacology/Canadian College of Neuropsycopharmacology, joint summer meeting* in Cambridge, England)

5.1 INTRODUCTION

Both SSRIs and MAOIs are extensively used in the treatment of major depression. Even in cases of refractory depression, however, it is not recommended that these treatments be combined (Ebert et al., 1995; Bakish et al., 1995) due to the emergence of adverse events such as the serotonin syndrome. In combination with MAOIs, other agents that are serotonergic, including certain TCAs (i.e. clomipramine), SSRIs and buspirone, may result in a mild (i.e. tachycardia, hypertension, fever) or severe (i.e. hyperthermia, coma, convulsions, death) serotonin syndrome (Baldessarini, 1989; Hyman et al., 1995). With the advent of specific, reversible inhibitors of MAO-A, such as MOC, the psychiatric literature is now documenting an increasing frequency of MOC/SSRI combination therapy for the treatment of refractory depression (Power et al, 1995). Clinicians are becoming less reluctant to try these combinations because of the knowledge that interactions are less likely to occur with drugs that act on only one receptor type or one primary mechanism of action (Amrein et al., 1992). In addition, with drugs that have one primary target, possible adverse interactions are more predictable. Both MOC and SSRIs appear to fit this criterion.

Recently, MOC has been found to be an inhibitor of CYP2D6, CYP2C19 and CYP1A2 (Gram and Brøsen, 1993; Gram et al., 1995). Because many commonly prescribed drugs are substrates for these isozymes, especially CYP2D6, the inhibition produced by MOC could be important if coadministered with these drugs. Likewise, FLU has been shown to be an inhibitor of not only CYP2D6 (see

Preskorn, 1996 for review), but also of CYP2C19 and CYP3A4 (Lemberger et al., 1988; Grimsley et al., 1991; Bergstrom et al., 1992; Greenblatt et al., 1992; Spina et al., 1993; Preskorn et al., 1994; Preskorn, 1996). In addition, the extensive hepatic biotransformation of MOC increases its potential for possible metabolic interactions when administered with other drugs (Härtter et al., 1996). Of interest to this thesis are the possible interactions that could occur when MOC and FLU are coadministered. A summary of MOC/FLU interaction data, although limited, is provided below.

The combination of FLU and MOC has demonstrated good efficacy in cases of refractory depression, but has created controversy as to whether or not toxic side effects such as the serotonin syndrome result from the combination (Bakish et al., 1995; Liebenberg et al., 1996; Dingemanse, 1993). Seven fatal cases of the serotonin syndrome have been reported after an overdose of combined therapy with MOC and another antidepressant, but in these cases the drugs used were citalopram and clomipramine (Neuvonen et al., 1993; Spigset et al., 1993; Kuisma, 1995; Hernandez et al., 1995; Power et al., 1995; Liebenberg et al., 1996). Out of thirteen post-marketing surveillance cases reported to Hoffmann La-Roche of concomitant MOC/FLU treatment, headache was the only adverse event reported (see Dingemanse et al., 1995). When this combination was given to healthy volunteers, the adverse events were similar to those seen when each compound was given separately (Dingemanse, 1993) and there was no indication of the development of a serotonergic syndrome. Recently, an adverse reaction was reported to occur after four weeks of concomitant use of FLU and MOC (see

Liedenberg et al., 1996). This latent response may have been due to the fact that, after a week of multiple dosing, the clearance of MOC decreased (Hyman et al., 1995). Thus, MOC accumulation may have inhibited the CYP isozyme(s) involved in FLU metabolism, resulting in increased levels of FLU and possible adverse effects (Liedenberg et al., 1996). Another recent report examining cases meeting criteria for the serotonin syndrome, found that out of 105 cases, only 3 involved MOC in multi-drug combinations and/or in mixed drug overdose (Hilton et al., 1997).

The aim of the present investigation was to further examine potential drug-drug interactions between MOC and FLU. Human liver microsomal preparations were utilized to help elucidate if concomitant treatment with these drugs affected each other's metabolism and to determine the potential CYPs involved.

5.2 MATERIALS AND METHODS

5.2.1 Analysis of drug-drug interactions between fluoxetine and moclobemide in vitro.

5.2.1.1 Isozymes and Microsome Preparations

The individual isozymes were prepared (as described in section 4.2.2.1 of this thesis) by and purchased from Gentest Co.

Human liver microsomal preparations were obtained from the International Institute for the Advancement of Medicine.

5.2.1.2 Analysis of Moclobemide and Three of its Metabolites

MOC and three of its metabolites (namely Ro 16-3177, Ro12-5637 and Ro 12-8095) were analysed using a variation of the HPLC method developed by Geschke et al. (1987). The specific HPLC apparatus and mobile phase composition are indicated in section 2.2.5 of this thesis. Figure 5-1 shows representative chromatograms of MOC and its three metabolites from incubations in human liver microsomal preparations.

Before interactions between MOC and FLU could be examined it was necessary to obtain information on the time course of formation of each of the three metabolites of interest. Thus, the first step was to incubate MOC with human liver microsomes for 5, 10, 20, 30 or 60 min to determine the time course of metabolite formation. A time was chosen where formation of each metabolite was approximately linear and this was the incubation time utilized for subsequent experiments. Experiments were then conducted where FLU was incubated with MOC to determine its effects on metabolism. FLU (200, 100, 50, 25, 12.5, or 0 μM) was incubated with MOC (100 μM final concentration), human liver microsomes and NADPH-generating system. The final volume in the microfuge tubes was 100 μl. In addition, MOC (200, 100, 50, 25, 12.5, or 0 μM) was incubated with FLU (100 μM) to determine its effects on FLU metabolism.

To obtain information on which specific isozymes might be implicated in the production of the metabolites of MOC, MOC was incubated with microsomes prepared from a panel of 16 livers that were characterized for their catalytic activity for CYP1A2, CYP2A6, CYP2C19, CYP2D6, CYP2E, CYP3A4 and CYP4A (see

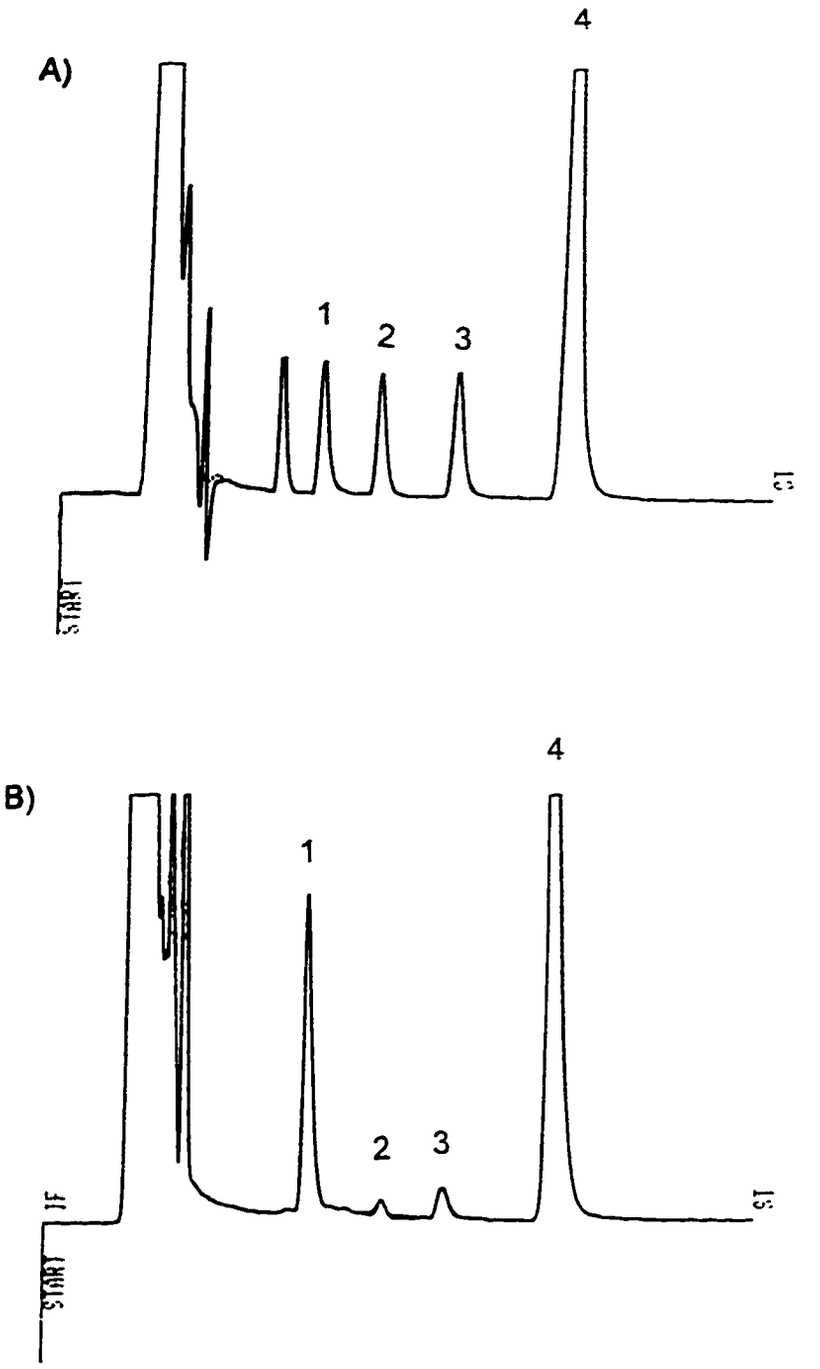

Figure 5-1: Representative chromatograms from incubations with human liver microsomes. (A) Control microsomal preparations and (B) Medium from microsomes incubated with 100 µM MOC (for 40 min). Peaks are as follows: 1= Ro 16-3177, 2= Ro12-8095, 3= Ro12-5637 and 4= MOC.

section 4.2.2.2). The rates of formation of Ro 16-3177, Ro 12-5637 and Ro 12-8095 were correlated with the activities of the specific isozymes for each of the 16 livers. Any isozyme producing a significant correlation was implicated in the formation of that specific metabolite. The correlations were analysed by simple linear regression and slopes were tested for linearity with an F test (Graph Pad Prism, version 2.0).

For further information on the individual isozymes that might be involved in the catabolism of MOC to each specific metabolite, MOC (100 μM) was incubated with a 10 mg/ml cDNA microsomal preparation expressing one of the following: CYP1A1, CYP1A2, CYP2C8, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, CYP2D6, CYP3A4 or CYP2E1 to determine which individual isoforms were able to produce the metabolites of interest.

Isoform-specific inhibitors were then utilized to further assess the role of those CYPs that were implicated in the metabolism of MOC. The inhibitors (100 μM final concentration) were added to a mixture containing MOC (100 μM), cells expressing the particular isozyme of interest and the NADPH generating system. The final volume in the microfuge tube was 100 μl.

The reaction mixture for all of the above experiments was the same as that described in section 4.2.2.2 of this thesis. Following incubation, however, the samples were placed on ice and 50 μl of ice-cold acetonitrile was added to terminate metabolism and precipitate the proteins. After 10 min the tubes were centrifuged for 5 min and the supernatant was removed for HPLC analysis.

5.3 RESULTS

When MOC (100 μM) was incubated with human liver microsomes at different time intervals, it was determined that the production of Ro 12-5637, Ro 12-8095 and Ro 16-3177 was approximately linear for the entire 60 min that were examined (Figure 5-2). Therefore, an incubation time of 40 min was chosen for subsequent experiments as it was within the linear range.

To determine if the coadministration of MOC and FLU affected the metabolism of either of these drugs, MOC (100 μM) was incubated with several concentrations of FLU (200, 100, 50, 25, 12.5, or 0 μM) to determine the effects of FLU on the metabolism of MOC. The results are expressed in Figure 5-3 and indicate that FLU inhibits the formation of Ro 16-3177, Ro 12-5637 and Ro 12-8095.

Next, FLU (100 μM) was incubated with several concentrations of MOC (200, 100, 50, 25, 12.5, or 0 μM) to determine if MOC interfered with the metabolism of FLU to its major, active metabolite, NFLU. The results of this experiment indicated that when FLU was incubated with MOC, the formation of both S- and R-NFLU was inhibited. This inhibition was concentration-dependent only below 25 μM (see Figure 5-4).

To assess which specific isozymes might be involved in the formation of the metabolites of MOC, and, thus, which ones may be inhibited, MOC (100 μM) was then incubated with microsomes prepared from a panel of 16 human livers characterized for their catalytic activity for some of the known CYPs. The rate of formation of Ro 16-3177 showed significant correlation (r^2= 0.47, F(1,14)=12.52,

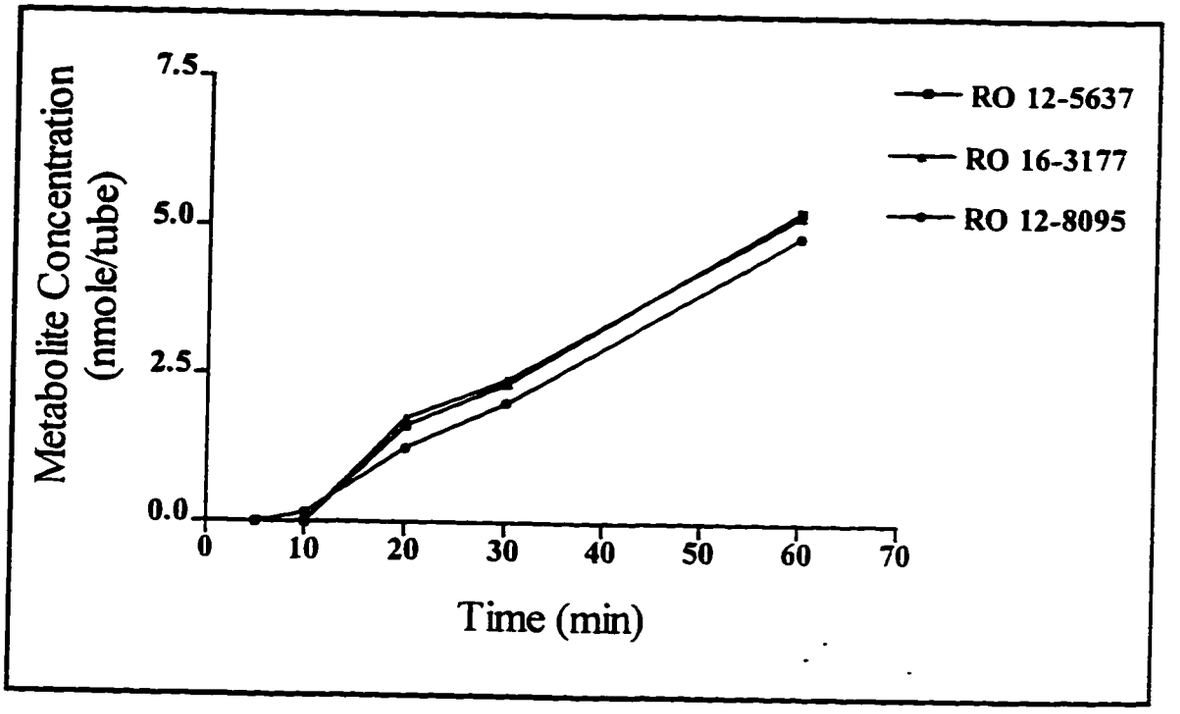

Figure 5-2: Time course of formation of MOC metabolites from human liver microsomal preparations.

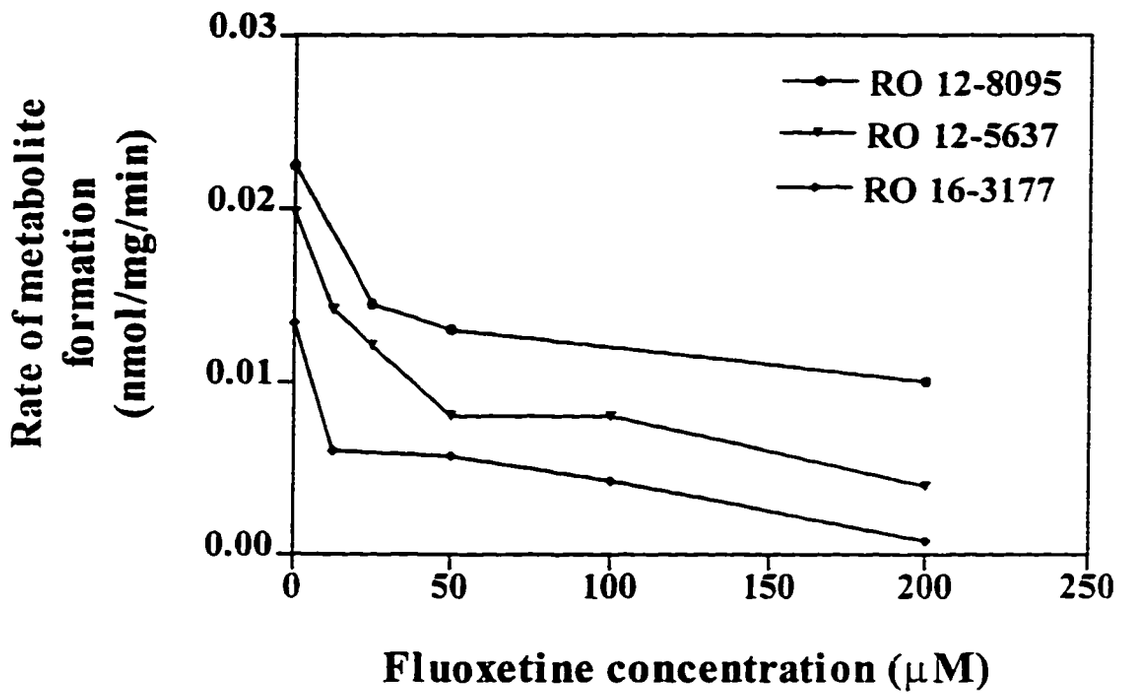

Figure 5-3: Production of three metabolites of moclobemide in the presence of fluoxetine (200, 100, 50, 25, 0 μM) and human liver microsomes (*n*=3).

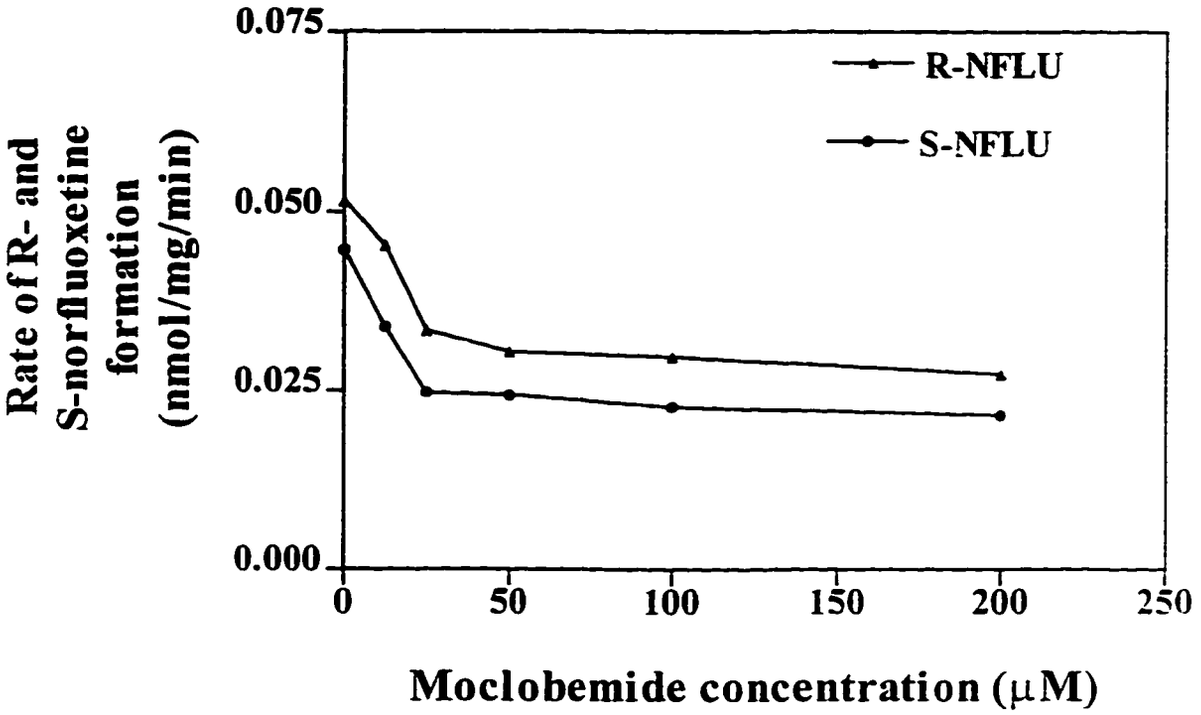

Figure 5-4: Production of S- and R-norfluoxetine in the presence of moclobemide (200, 100, 50, 25, 0 μM) and human liver microsomes (*n*=3).

p=0.003) [Figure 5-5] with CYP3A4 activity while the formation of both Ro12-5637 and Ro 12-8095 did not significantly correlate with the activity of any of the CYPs whose catalytic activity was characterized. So from these initial experiments, CYP3A4 was implicated as the isoform responsible for the formation of Ro 16-3177. It should be noted, however, that CYP2D6 activity did approach linearity (even though it was not significant) for the formation of Ro 16-3177 (see Figure 5-5).

To further assess which individual isozymes might be involved in the formation of these metabolites from MOC, MOC (100 μM) was incubated with a 10 mg/ml microsomal preparation expressing one of CYP1A1, 1A2, 2C8, $2C9_{arg}$, $2C9_{cys}$, 2C19, 2D6, 3A4, or 2E1. Incubation with CYP1A2 resulted in the formation of Ro 12-8095, whereas incubations with the other isozymes did not. Likewise, incubation with CYP2C19 resulted in the formation of Ro 16-3177, while incubation with the other isozymes did not.

Specific inhibitors of the CYPs that were implicated in the production of these individual metabolites were then utilized to further assess their role in the metabolism of MOC. Paroxetine (specific inhibitor of CYP2D6, weak inhibitor of CYP1A2), ketoconazole (specific inhibitor of CYP3A4) or tranylcypromine (specific inhibitor of CYP2C19) were incubated with MOC and human liver microsomes. An inhibitor of CYP2D6 was included in these experiments as CYP2D6 activity appeared to have a positive correlation with the formation of Ro 16-3177, even though this correlation was not significant when analysed. The results of the inhibition experiments are shown in Figure 5-6. Incubation with both ketoconazole and paroxetine inhibited the formation of Ro12-8095, whereas incubation with all

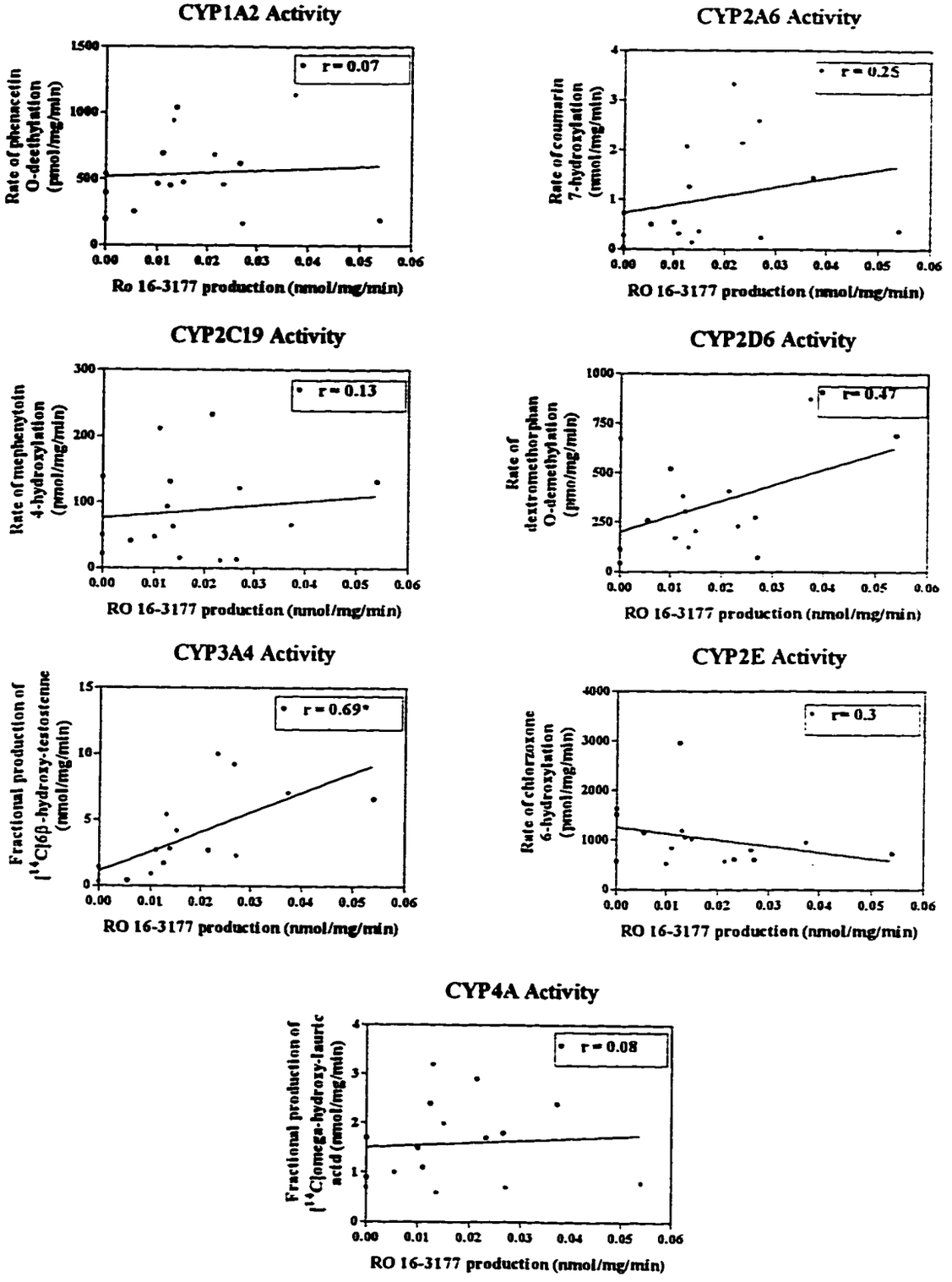

Figure 5-5: Correlations of Ro 16-3177 production from moclobemide and enzyme activity in microsomes from 16 human livers characterized for activity of CYP1A2, CYP2A6, CYP2C19, CYP2E, CYP3A4, CYP4A and CYP2D6.

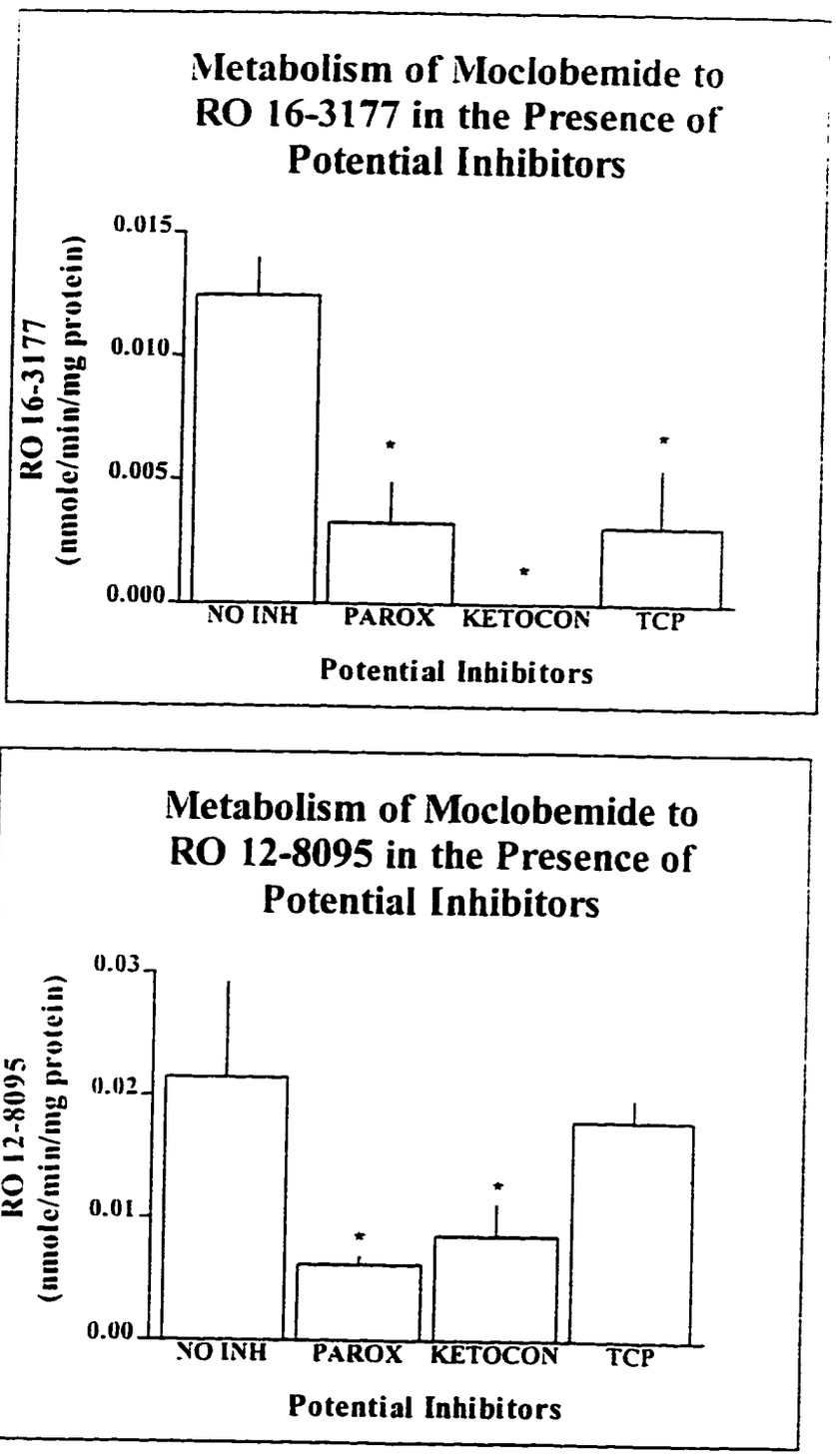

Figure 5-6: Metabolism of moclobemide to Ro 16-3177 (top) or Ro 12-8095 (bottom) by human liver microsomes with no inhibitor present (NO INH), or in the presence of paroxetine (PAROX; 100 μM), ketoconazole (KETOCON; 100 μM), or tranylcypromine (TCP; 100 μM). Data shown are mean ± S.E.M. (*n*=3).

of the inhibitors decreased the formation of Ro 16-3177.

5.4 DISCUSSION

It is important to realize that the purpose of the present investigation was to determine if there is a potential for drug-drug interactions between MOC and FLU and not to describe the metabolic pathways of MOC. Individual isozymes that appeared to play a role in the metabolism of MOC were investigated to enable comment on which isozymes might be inhibited by the coadministration of these two drugs. As described in Chapter 1 (section 1.2.2), MOC is extensively metabolized. In addition, the formation of the metabolites that were investigated in the present study would involve multiple CYPs (see Figure 1-3). So to reiterate, the primary purpose of this study was to investigate potential interactions between MOC and FLU, and, for this pupose, preliminary data on the individual CYPs that might be involved were collected.

The present experiments first employed human liver microsomal preparations to determine if combining FLU and MOC disrupted or changed the metabolism of either or both drugs. When MOC was incubated with FLU, the formation of Ro 12-5637, Ro 12-8095 and Ro 16-3177 was inhibited in a concentration-dependent manner. Similarly, when FLU was incubated with MOC, the formation of both S- and R-NFLU was inhibited. This inhibition was concentration-dependent only below 25 μM. The observation that higher concentrations of MOC did not further inhibit S- and R-NFLU activities may be attributed to the involvement of CYPs that are not

inhibited by MOC.

In order to comment on which particular isozymes may be inhibited, preliminary experiments were conducted *in vitro* using human liver microsomes. These data revealed that the activity of CYP3A4 significantly correlated with the formation of Ro 16-3177. Utilization of a cell line expressing human cDNA for individual isozymes, however, indicated that CYP2C19 was involved in the formation of Ro 16-3177. The discrepancy in these results is not surprising because, as previously mentioned, several steps are involved in the catabolism of MOC to Ro 16-3177. Thus, even though a cell line expressing only CYP2C19 produces this metabolite, CYP3A4 activity may predominate when all of the isozymes compete for the substrate. It is possible that CYP3A4 may be responsible for producing an intermediate metabolite that is necessary for the ultimate formation of Ro 16-3177 (perhaps *via* the activity of CYP2C19).

The finding that the production of Ro 12-8095 did not correlate with the activity of any of the CYPs, but was formed by CYP1A2 in a cell line expressing that specific isozyme, again may be because this metabolite requires multiple steps for its formation. The formation of Ro 12-5637 involves N-oxidation of the morpholine ring of MOC (Figure 1-3), and another enzyme implicated in N-oxidation is flavin-containing monooxygenase (F.M.O.; see section 1.4.1 of this thesis) [Gibson and Skett, 1994]. Because F.M.O. activity was not characterized for the human liver microsomes utilized for these experiments, the formation of Ro 12-5637 could not be correlated with its activity. This characterization is apparently underway at the International Institute for the Advancement of Medicine, and once these data are

available it will be interesting to re-analyse the data to determine if F.M.0. does play a role.

To further assess the roles of the particular isozymes that were implicated in the formation of these metabolites of MOC, studies using isoform-specific inhibitors were conducted. The observation that ketoconazole, a potent CYP3A4 inhibitor, inhibited the formation of Ro 16-3177 is consistent with the human liver microsome results where CYP3A4 activity correlated with the production of this metabolite. Both paroxetine and tranylcypromine also inhibited the formation of Ro 16-3177, although this inhibition was not as pronounced as that produced by ketoconazole. The reason for inhibition by these agents may be due to the fact that paroxetine is a weak CYP3A4 inhibitor (von Moltke et al., 1994; Rasmussen et al., 1995) and tranylcypromine is a relatively potent inhibitor of CYP2C19 (Parkinson, 1996). As discussed previously, CYP2C19 is also implicated in the formation of Ro 16-3177, although the particular pathway it mediates remains unknown. These results are interesting because FLU has been shown to have mild inhibitory effects on both CYP3A4 and CYP2C19 (Lane, 1996). Paroxetine has also been shown to be a weak inhibitor of CYP1A2 (Brøsen et al., 1993; Rasmussen et al., 1995; von Moltke et al., 1996), which may explain why it was also able to inhibit the formation of Ro 12-8095. These data corroborate the observation that CYP1A2 catalysed the formation of Ro 12-8095 in a human cDNA-expressed cell line.

In summary, although there is a modest amount of contradictory data on whether or not MOC can be safely co-administered with an SSRI, the present series of *in vitro* experiments suggest that there may be a potential for pharmacokinetic

MOC/FLU interactions if they are administered concomitantly. If these agents are coadministered, metabolism of the parent compounds may be inhibited, which may result in excessive 5-HT neurotransmission and, hence, contribute to a serotonergic syndrome. This syndrome has also been reported to occur after a latent period (Power et al., 1995), so both drug dosages and the duration of treatment are important factors to consider when using this combination drug therapy.

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6.0 CHAPTER 6

General Discussion

6.1 SUMMARY

In chapter 2 of this thesis is discussed a series of acute and chronic experiments that were conducted to investigate and compare the effects of a nonselective, irreversible MAOI (PLZ) with those of the selective, RIMAs (MOC and BROF). These data revealed that all 3 drugs produced a down-regulation of T receptors in brain, with PLZ and MOC producing a decrease in the density of receptors and BROF producing an apparent decrease in the affinity of the radioligand for the receptor. The finding that MOC and PLZ significantly increased the concentration of T in rat brain is in agreement with their ability to produce a reduction in B_{max}, as persistently high concentrations of a ligand at its receptor site is a favorable condition for this phenomenon (Hrdina, 1986). The effect of BROF on K_d, however, is not clear at this time. In contrast to the effects on T receptors, only PLZ produced a significant down-regulation of 5-HT_{2A} receptors despite the fact that all three drugs significantly increased brain levels of 5-HT. GABAergic investigations revealed that inhibition of GABA-T is a characteristic of all three drugs *in vitro*, although PLZ is more potent than the other two drugs, perhaps explaining why it was the only one of the three that elevated rat brain GABA *ex vivo*. Of the three drugs, only BROF inhibited GABA uptake *in vitro*. The studies on urinary levels of T, MHPG, and PEA and on levels of MAO-A and -B activity in rat brain were consistent with the selectivity of MOC and BROF for MAO-A and the lack of selectivity of PLZ. The data presented in Chapter 2 revealed marked differences between both MOC and BROF and PLZ on aminergic and GABAergic mechanisms.

Attention was then directed towards investigation of the formation of some novel metabolites of FLU. The studies described in Chapter 3 resulted in the identification and quantitation of several novel metabolites of FLU (namely TFMP, HPPA and NMHPPA) in rat and human biological samples. These data also revealed that both HPPA and NMHPPA can be deaminated by MAO, a finding which supports a metabolic pathway that we have proposed for FLU, which is different from the pathways previously proposed (Altamura et al., 1994; Benfield et al., 1986). These investigations also included analysis of conjugated levels of the enantiomers of both FLU and NFLU and of conjugated TFMP, which, to our knowledge, have not been reported previously. All three metabolites were extensively conjugated.

It was then of interest to conduct a preliminary investigation of the CYPs that might mediate the metabolism of FLU by utilizing cDNA-expressed human CYP isozymes and human liver microsomes. Analyses revealed that multiple CYP isozymes may play a role in the formation of metabolites of FLU *in vitro* including CYP1A2, $CYP2C9_{arg}$, $CYP2C9_{cys}$, CYP2C19, CYP2D6 and CYP3A4.

Recently, there has been an increasing frequency of combination therapy with MOC and an SSRI for the treatment of refractory depression (Power et al., 1995). There is considerable controversy, however, as to whether or not this combination results in toxic side effects due to drug-drug interactions (Bakish et al., 1995; Liedenberg et al., 1996; Dingemanse, 1993). In chapter 5 is described a series of experiments that were conducted to examine potential drug-drug interactions between MOC and FLU using human liver microsomal preparations.

The data from these experiments suggest that there may be a potential for pharmacokinetic MOC/FLU interactions if they are administered concomitantly and this may be due to the inhibition of CYP3A4, CYP2C19 and CYP1A2.

In summary, a number of studies pertaining to metabolic and neurochemical issues of some serotoninergic antidepressants have been addressed in this thesis.

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