UNIVERSITY OF ALBERTA

SEPARATION AND QUANTITATION OF THE ENANTIOMERS OF TRIMIPRAMINE

by

KUEN-SHAN STEVEN LEE

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ABSTRACT

A novel high-performance liquid chromatography (HPLC) assay (with ultraviolet detection) was developed for quantification of the individual enantiomers of trimipramine (TMP), a frequently prescribed antidepressant. The assay also permitted separation of the individual enantiomers of TMP's major metabolites from the parent drug and each other. The effects of pH and solvents on extraction efficiencies for TMP and its demethylated and hydroxylated metabolites from biological matrices were investigated. The HPLC assay was applied to the determination of (+)- and (-)-TMP in tissues and body fluids of rats that had been administered (±)-TMP, alone and in combination with fluoxetine (FLU). In rats given (±)-TMP, the ratio of (+)- to (-)-TMP 5 hours after was similar in liver and plasma (< 0.5), but much different in brain (1.4). Coadministration of FLU resulted in increases in liver, brain and plasma levels of both enantiomers to about the same extent, although the ratio did decrease significantly in brain.

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ABBREVIATIONS

(-)	levorotatory
(+)	dextrorotatory
AMI	amitriptyline
ANOVA	analysis of variance
APA	American Psychiatric Association
aq	aqueous
cAMP	cyclic adenosine monophosphate
CI	chemical ionization
C_{max}	maximum concentration
CMI	clomipramine
CNS	central nervous system
CSF	cerebrospinal fluid
CV	coefficient of variance
CYP	cytochrome P450
CYP2D6	cytochrome P450 2D6
CYP450	cytochrome P450
CYP_{MP}	cytochrome P450 mephenytoin hydroxylase
d-	dextrorotatory
DA	dopamine
DDMI	N,N-didesmethylimipramine
DMI	N-desmethylimipramine, desipramine
DMSO	dimethylsulfoxide
dopa	3,4-dihydroxyphenylalanine
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th Ed.

ξ	molar absorptivity
ECD	electron capture detector
ECT	electroconvulsive therapy
EDTA	ethylenediaminetetraacetate, disodium salt
EEG	electroencephalography
EI	electron impact
EM(s)	extensive metabolizer(s)
FID	flame ionization detector
FLU	fluoxetine
FLUV	fluvoxamine
GABA	γ-aminobutyric acid
GC	gas chromatography
h	hour
HP	Hewlett-Packard
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine, serotonin
IC_{50}	concentration producing 50% inhibition
ID	inside diameter
IDB	iminodibenzyl
IMI	imipramine
ip	intraperitoneally
IPR	iprindole
IS	internal standard
K'	capacity factor
kg	kilogram

l-	levorotatory
M	molar
MAO	monoamine oxidase
MAOI(s)	monoamine oxidase inhibitor(s)
(-)-MC	(-)-menthyl chloroformate
mg	milligram
min	minute
ml	millilitre
MP	mephenytoin
MS	mass spectrometer
MSD	male Sprague-Dawley
N	normality
NA	noradrenaline, norepinephrine
NADPH	nicotinamide adenosine monophosphate, reduced
NFLU	norfluoxetine
ng	nanogram
nm	nanometer
nM	nanomolar
NPD	nitrogen phosphorus detector
NTMP	nortrimipramine, N-desmethyltrimipramine
NNTMP	bis-nortrimipramine, N,N-didesmethyltrimipramine
NTR	nortriptyline
OH	hydroxy
p	probability
p-	*para-*

pg	picogram
PFPA	pentafluoropropionic anhydride
PKA	protein kinase A
PKC	protein kinase C
PLZ	phenelzine
PM(s)	poor metabolizer(s)
po	*per os*, orally
QND	quinidine
QNN	quinine
rac-	racemic
REM	rapid eye movement
R_s	resolution
SCOT	support-coated open tubular
SEM	standard error of the mean
$t_{1/2}$	half-life
TCA(s)	tricyclic antidepressant(s)
TCD	thermal conductivity detector
TCP	tranylcypromine
TFAA	trifluoroacetic anhydride
(-)-TFPC	S-(-)-trifluoroacetylprolyl chloride
TMP	trimipramine
t_R	retention time
µg	microgram
µl	microlitre
µM	micromolar

µmole	micromole
v	volume
WCOT	wall-coated open tubular

1 *INTRODUCTION*

1.1 Antidepressant Drugs

Depression, one of the most common psychiatric disorders, will affect one out of seven North Americans during a lifetime (Lapierre, 1991). Its major complication, suicide, is among the leading causes of death in most western societies. In a recent survey (Stinson, 1990), it was found that depression afflicts 11 million Americans, 40% of the 27 million who suffer from mental disorders. Depressed mood is an occasional component of daily life, but it may also be a symptom of primary affective disorder. When depression is left untreated, the mortality rate due to suicide has been reported to be a staggering 15%, a rate 30 times that in the general population (Robins and Guze, 1970). The recurrence rate of depression is as high as 75-80% (Zis and Goodwin, 1979), leading to chronic disruption of the patient's life.

Nosology is the science of classification of disorders. Of the classification schemes that have been proposed in psychiatry over the years, currently the most popular in North America is the Diagnostic and Statistical Manual of the American Psychiatric Association. This manual is now in its fourth edition (1994), which is referred to by the abbreviation DSM-IV. This classification system allows multiple diagnoses and is theoretical with regard to the etiology or origin of mental disorders. The affective and anxiety disorders are among the most common conditions diagnosed in clinical psychiatry. These disorders often coexist and overlap with other serious psychiatric illnesses such as schizophrenia, anorexia

nervosa, bulimia and alcohol or drug abuse. A mixture of several disorders is often seen in the same patient (Torgersen, 1990; Thompson et al. 1989) and treatments are often effective for more than one disorder, suggesting fundamental connectivity rather than discontinuity. It can be difficult to study "pure disorders" because of this pervasive overlap.

1.1.1 Affective Disorders

The affective or mood disorders in DSM-IV include bipolar disorder, depressive disorder, cyclothymia, and dysthymia. In bipolar disorder and cyclothymia, the mood will periodically swing from euthymia (normal mood) to depression or mania. Cyclothymia refers to a milder variant where the mood swings are attenuated, causing less dysfunction and disruption of the patient's life. The essence of these disorders is the manic episode, in which the patient experiences a distinct period of an expansive, elevated mood, accompanied by increased self-esteem (grandiosity) and energy, a decreased need to sleep, the subjective sense that thoughts are racing, excess talkativeness, distractibility and an increase in the time spent in goal-directed, pleasurable and often dangerous activities. The cornerstones in the pharmacologic management of bipolar disorders are mood-stabilizing drugs which include lithium salts and the anticonvulsants valproic acid, carbamazepine and clonazepam (Ballenger and Post, 1980; Prien and Gelenberg, 1989; Jefferson, 1990; Post, 1990). Exposure to an antidepressant in the absence of a mood stabilizer may precipitate mania, an event that confirms the diagnosis of bipolar disorder.

Major depressive and dysthymic disorders are approximately ten times more common than bipolar disorder (Myers et al., 1984; Weissman and Boyd, 1982), with reported incidences in the general population of approximately 5% or greater (Kaplan et al., 1994). Patients with major depressive disorder experience a sustained sadness that is not solely in reaction to adverse circumstances, an inability to experience pleasure (anhedonia), a sense of hopelessness that may lead to suicidal ideation or intent, diminished energy, impaired concentration and changes in appetite and sleep patterns. Dysthymia is a mild form of depressive illness which never meets the full criteria for a major depression and also tends to be chronic in nature. Dysthymic patients often describe themselves as having been unhappy for as long as they can remember in contrast to the more episodic and discrete nature of major depressive episodes.

In medicine, the word "depression" is taken to mean a serious pervasive disorder that is distinctly different from normal behaviour and is not solely a reaction to unpleasant circumstances. Grief is an experience similar in phenomenology to depression but considered a normal reaction to loss. If grief becomes severe and protracted and causes significant dysfunction it would eventually be considered a depression (Zisook and Shuchter, 1991). Depression is a syndrome, a group of illnesses which share common symptoms and signs. In all likelihood, patients arrive at the common syndrome through varying paths. For some, a genetic predisposition is paramount, whereas in others emotional trauma, loss of a loved one, medical disorders or the use (or abuse) of drugs may be

causative factors. In most cases a number of factors may be necessary to arrive at the endstate, and their relative importance remains a matter of conjecture. Regardless of the etiology, it appears that 70% of patients with depression will respond following several weeks of continual antidepressant therapy (Morris and Beck, 1974; Baldessarini, 1985).

Major depression has been said to be a heterogenous disorder both in terms of origin and presenting symptoms. That is, the sequence of events contributing to the clinical state of depression will be influenced by the biological predisposition of the individual and, in turn, environmental factors may have a marked impact on the individual (Jesberger and Richardson, 1985). This heterogeneity is also indicated in the observation that any one antidepressant drug does not work in all types of depression or in all individuals.

1.1.2 Classes of Antidepressants

It was only in the 1950s that a large number of drugs became readily available for the effective treatment of major mental disorders. These advances were initiated by a number of fortuitous discoveries (see below), rather than the planned and rational development of drugs that would have specific biologic effects. Subsequently, attempts were made to alter compounds structurally in specific ways in order to increase the selectivity of their effects. Drugs found effective in the treatment of depression have been classified in a simple fashion into the (1) monoamine oxidase inhibitors (MAOIs), (2) tricyclic antidepressants (TCAs), and (3) novel (or

atypical) antidepressants.

1.1.2.1 Monoamine Oxidase Inhibitors (MAOIs)

The discoveries that isoniazid and iproniazid were inhibitors of the enzyme monoamine oxidase (MAO) (Zeller et al., 1952a,b) and reversed the depressant effects of reserpine (Chessin et al., 1956) suggested that inhibition of MAO might be important in producing the antidepressant response. This suggestion has been supported by the development of several clinically useful MAOIs, including the hydrazine compound phenelzine (PLZ) and the nonhydrazine tranylcypromine (TCP). Chemical structures of common MAOIs are shown in Figure 1. Both PLZ and TCP are classified as irreversible and nonselective MAOIs, that is, they inhibit both the A and B forms of MAO non-selectively, and the dysfunction of the enzyme activity is only slowly reversible after discontinuation of the drug. A second generation of MAOIs is currently being studied in order to develop safer, and more specific compounds. Selective irreversible [(-)-deprenyl (MAO-B), clorgyline (MAO-A)] and reversible inhibitors [moclobemide and brofaromine (MAO-A)] are now available commercially or undergoing clinical trials (Möller et al., 1991; Elsworth and Roth, 1993; Tiller, 1993; Chouinard et al., 1994). However, (-)-deprenyl, an irreversible but selective MAO-B inhibitor, has not proven to be a particularly effective antidepressant (Elsworth and Roth, 1993), but is used in idiopathic Parkinson's disease, both alone and in combination of L-DOPA (Birkmayer et al., 1985; Tetrud and Langstrom, 1989; Parkinson Study Group, 1989, 1993).

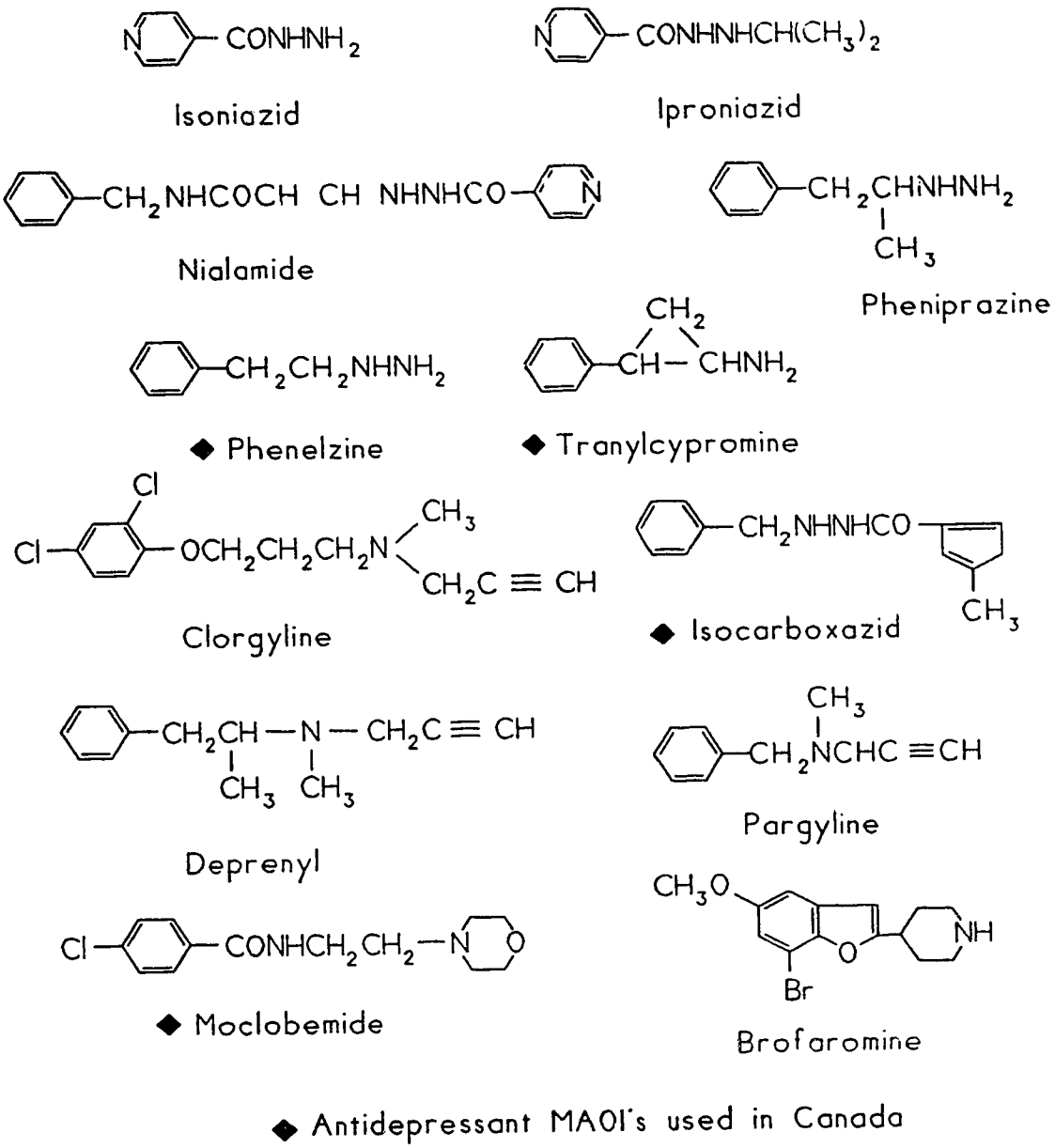


Figure 1: **Chemical structures of common monoamine oxidase inhibitors (MAOIs).**

Clorgyline is an effective antidepressant but there is the danger of the "cheese effect", so named because ingestion of foods such as aged cheeses, which contain high concentrations of the sympathomimetic amine tyramine, in combination with irreversible MAO-A inhibitors can result in a number of adverse effects ranging from headaches to a hypertensive crisis. This is the result of inhibition of metabolism of tyramine (which is extensively metabolized by MAO-A in the gut under ordinary circumstances). Moclobemide and brofaromine, both reversible and selective type A MAOIs, retain the antidepressant effects of clorgyline, but the chances of the cheese effect are much reduced.

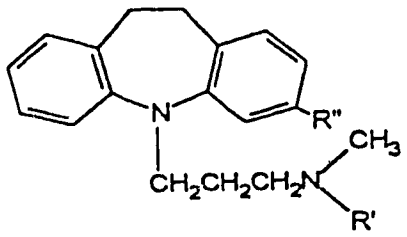
1.1.2.2 Tricyclic Antidepressants (TCAs)

In early 1950s, chemists at J. R. Geigy in Basel, Switzerland, synthesized a group of iminodibenzyl derivatives as potential antihistaminic agents (Schindler and Hafliger, 1954). Pharmacologic testing of these compounds revealed that some compounds possessed hypnotic and analgesic properties in addition to their antihistaminic and atropine-like actions (Grünthal, 1958). Meanwhile, the demonstration of the antipsychotic effects of the phenothiazines had also stimulated interest in the iminodibenzyl derivatives because of their structural similarity to the phenothiazines. For these reasons, one compound of the group, imipramine (IMI), was evaluated in chronic schizophrenic and other psychotic patients. Following his careful observations, Kuhn (1957) reported that IMI, a tricyclic compound, was efficacious in the treatment of

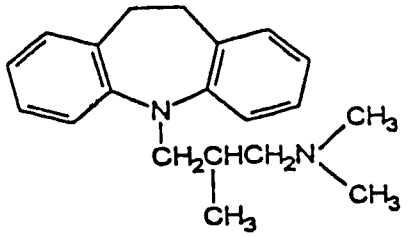
serious depression. Most TCAs share a common structure consisting of two benzene rings joined by a central seven-member ring (Baldessarini, 1985). Numerous examples of this class are available, including desmethylimipramine (DMI), amitriptyline (AMI), nortriptyline (NTR), TMP, doxepin, protriptyline, and clomipramine (CMI) [Figure 2].

The TCAs possess a wide spectrum of biological activity but in almost all cases share the property of blocking the presynaptic uptake pump for the neurotransmitter amines noradrenaline (NA) and/or serotonin (5-hydroxytryptamine, 5-HT), but with a much weaker effect on dopamine (DA) [Baker and Dewhurst, 1985]. In addition, most TCAs are antagonists of several neurotransmitter receptors, including presynaptic and postsynaptic muscarinic (acetylcholine), histaminic, adrenergic and serotoninergic receptors (Baldessarini, 1985; Rudorfer and Potter, 1989). A knowledge of a TCA's antagonist potency for each type of receptor permits some insight into its propensity to cause side-effects related to receptor blockade. Common side-effects of TCAs related to blockade of receptors include dry mouth, blurred vision, urinary retention and constipation (muscarinic), sedation and weight gain (histamine) and postural hypotension (α_1-adrenergic) [Pollack and Rosenbaum, 1987].

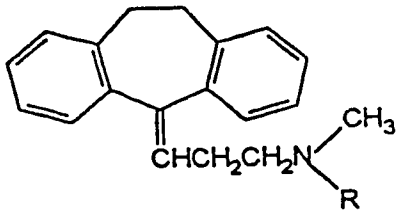
The N-demethylated metabolites of IMI and AMI, DMI and NTR respectively, are more potent NA uptake inhibitors and possess fewer anticholinergic properties than the parent compounds and are marketed as antidepressants in their own right. Because of the greater selectivity and decreased toxicity, DMI and NTR are popular in elderly and medically



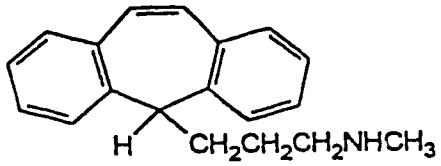
Imipramine R' = CH_3 R" = H
Desipramine R' = R" = H
Clomipramine R' = CH_3 R" = Cl



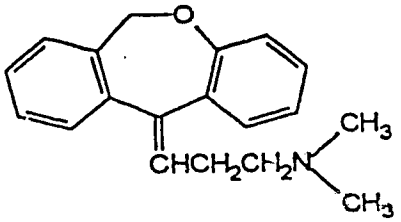
Trimipramine



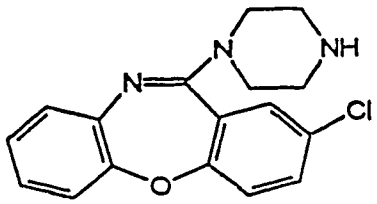
Amitriptyline R = CH_3
Nortriptyline R = H



Protriptyline



Doxepin



Amoxapine

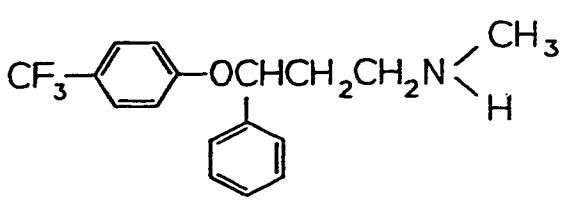
Figure 2: Chemical structures of common tricyclic antidepressants (TCAs).

unstable patients, who might be intolerant of side-effects. In addition, the TCAs are efficacious in the treatment of anxiety disorders such as panic disorder, obsessive compulsive disorder and generalized anxiety disorder (Liebowitz, 1989; Dubovsky, 1990).

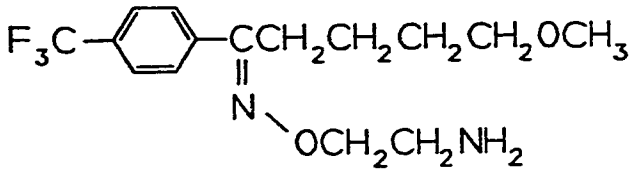
1.1.2.3 Novel Antidepressants

Novel antidepressants represent drugs that do not fall readily into one of the other two classes mentioned above. Some have no effects on MAO or amine uptake (mianserin, iprindole, viloxazine) [Damlouji et al., 1985; Rosenbaum et al., 1990) and some selectively inhibit the uptake of NA, DA or 5-HT, but are not TCAs.

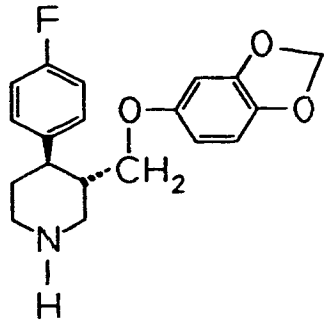
Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine (FLU), fluvoxamine (FLUV), sertraline and paroxetine have become popular and well tolerated treatments for depression (Fuller and Wong, 1989; Rudorfer and Potter, 1989; Boyer and Feighner, 1991; Baumann, 1992; Richelson, 1994; Hyttel, 1994; Preskorn, 1994). Chemical structures of common SSRIs are shown in Figure 3. Citalopram (Miline and Goa, 1991; Luo and Richardson, 1993), fluoxetine (Altamuru et al., 1994), fluvoxamine (Wilde et al., 1993; Perucca et al., 1994; Palmer and Benfield, 1994), paroxetine (Dechant and Clissold, 1991; Bloomer et al., 1992; Hiemke, 1994) and sertraline (Murdoch and McTavish, 1992) differ widely in structure, which helps to explain why they show differences in their metabolic and pharmacokinetic profiles (Van Harten, 1993). The SSRIs have no anticholinergic or sedative properties but rather tend to be



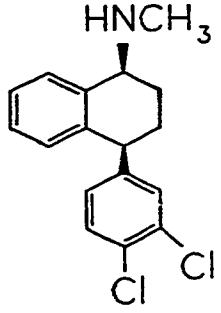
Fluoxetine



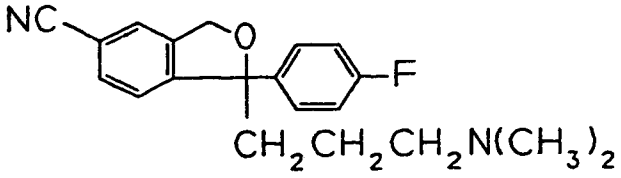
Fluvoxamine



Paroxetine



Sertraline



Citalopram

Figure 3: **Chemical structures of common selective serotonin reuptake inhibitors (SSRIs).**

arousing and cause appetite suppression (Wernicke, 1985; Fuller and Wong, 1989; Leonard, 1993). Such agents exhibit a significantly lower discontinuation rate due to adverse events than do other conventional antidepressants (review: Tollefson, 1993). Adverse events clearly detract from patient compliance and, thus, ultimately contribute to the risk of depressive recurrence. Overall adverse events do not increase with chronic adminstration of SSRIs. When side effects occur, they typically emerge early in the course of acute treatment and wane in the face of continued treatment. Upon drug discontinuation, these adverse events usually reverse at a much faster pace than the drug's half-life

Trazodone, a triazolopyridine derivative, in addition to inhibiting 5-HT uptake, is a partial 5-HT agonist, and its metabolite, m-chlorophenylpiperazine (m-CPP), is a potent 5-HT agonist (Coccaro and Siever, 1985; Zohar et al., 1987). Nonselective DA uptake inhibitors such as nomifensine and bupropion have been synthesized, but the former, because of an association with fatal haemolytic anemia, has been withdrawn from clinical use and the latter is not available in Canada (van Scheylen et al., 1977; Ferris et al., 1982; Rudorfer and Potter, 1989). Bupropion only weakly inhibits the uptake of DA and its mechanism of action is controversial (Bryant et al., 1983; Golden et al., 1988; Nemeroff, 1994). A relatively selective NA uptake inhibitor, the tricyclic maprotiline, has antidepressant effects but is also associated with an increased risk of seizures (Rudorfer et al., 1984). Two new antidepressants, venlafaxine (a combined 5-HT/NA reuptake inhibitor) and nefazodone (a 5-HT reuptake inhibitor and a 5-HT_2 receptor antagonist) have recently been introducted on the market (Cusak

et al., 1994; Holliday and Benfield, 1995; Morton et al., 1995; Rickels et al., 1995; Taylor et al., 1995).

The division of antidepressants into MAOIs, TCAs and novel (atypical) drugs was based on the history of their development and is now a rather confusing and outdated system of classification. The antidepressants are now known to be effective in the treatment of a wide array of mental disorders and several could also be called antipanic or antianxiety drugs. Elucidation of the complete pharmacodynamic profile of each drug and its metabolites would facilitate our understanding of how these drugs work. The development of drugs with very selective effects might then greatly improve the specificity and safety of antidepressant therapy.

1.1.3 Antidepressant Mechanisms of Action

There is now a significant body of evidence indicating that biochemical abnormalities in the brain are directly involved in the pathology of depression and other psychiatric disorders. Several hypotheses have been put forward to explain how antidepressants exert beneficial effects. Understanding antidepressant mechanisms of action may suggest which neurotransmitter systems are involved in the origin of mental illness.

1.1.3.1 Biogenic Amine Theory

The biogenic amine theory hypothesized that depression resulted from a functional deficiency of NA and/or 5-HT at certain central synapses (Bunney and Davis, 1965; Schildkraut, 1965; Lapin and Oxenkrug, 1969).

Antidepressants were thought to correct this deficit by elevating levels of the amine neurotransmitters through inhibition of their catabolism (MAOIs) or uptake mechanisms (TCAs). This theory was also based on the observation that treatments which deplete (reserpine) or inhibit the synthesis (e.g. α-methyl-p-tyrosine) of the biogenic amines NA and 5-HT resulted in depression (Chessin et al., 1956; Bunney and Davis, 1965; Schildkraut, 1965). The biogenic amine hypothesis of depression was a useful concept, but several observations mitigated against such a simple mechanism. In particular, the requirement for several weeks of drug administration prior to symptom remission suggests that inhibition of MAO and blockade of the uptake mechanism, which occur shortly after drug intake, are only partial explanations.

1.1.3.2 Receptor and Second-Messenger Theories

Recent theories of antidepressant mechanisms of action have emphasized the delayed effects of elevated amine levels on presynaptic and postsynaptic receptor density and sensitivity and on second-messenger systems (reviews: Baker and Greenshaw, 1989; Hrdina, 1993; Nestler and Duman, 1995). Several subtypes of receptor respond to each neurotransmitter, with each subtype differing in form and effect from the others. Ionotropic (affecting ion channels directly) and metabotropic (affecting enzymes and second messenger systems) receptors represent the two major families of receptors. Depression is hypothesized to be linked with a super-sensitivity of these receptors and the antidepressant drugs work to reduce the sensitivity of these receptors.

In contrast, mania is associated with sub-sensitized (desensitized) receptors, and anti-manic drugs, e.g. lithium, presumably increase the sensitivity of these receptors (Richelson, 1994).

Changes in adrenergic receptor sensitivity are observed following administration of many antidepressants, and the time-course of these changes approximates the onset of clinical recovery. The most commonly reported change is a decreased sensitivity of the β-adrenoreceptor-coupled adenylate cyclase system (Vetulani et al., 1976a,b; Sugrue, 1982; Baker and Greenshaw, 1989). This observation has been relatively consistent among many antidepressants studied (including ECT), regardless of their primary biologic effect. It is likely that several mechanisms are involved in this heterologous desensitization, including shared second messenger systems and receptor cross-talk. Utilizing radioligand binding assays, a reduction in the density of β-adrenoreceptors has been reported in rat cortical tissue following chronic, but not acute, administration of MAOIs, TCAs, some novel antidepressants and ECT (Banerjee et al., 1977; Sellinger-Barnette et al., 1980; Kellar et al., 1981; Sugrue, 1983; Baker and Greenshaw, 1989). There is, however, a great deal of controversy about the effects of SSRIs on β-adrenoceptor down-regulation (Hyttel, 1994; Goodnough and Baker, 1994; Wong et al., 1995). Effects of chronic antidepressant treatment on α-adrenergic receptors (α_1 and α_2) have also been reported, with evidence supporting enhanced sensitivity of α_1 receptors and diminished sensitivity of inhibitory α_2 autoreceptors (Svensson and Usdin, 1978; Cohen et al., 1982; Sugrue, 1983; Maj et al., 1984; Baker and Greenshaw, 1989). It has been suggested that changes in

α_2-receptor density precede and may be responsible for the decrease in β-adrenoreceptors (Reisine et al., 1982).

Serotoninergic receptors are also affected by antidepressants. Radioligand binding studies reveal a reduction in 5-HT_2 receptor density following chronic treatment with many antidepressants (Peroutka and Snyder, 1980; Snyder and Peroutka, 1984; Scott and Crews, 1986; Eison et al., 1991) but not ECT (Kellar and Bergstrom, 1983). It is of interest that, as with the β-adrenergic receptors, there is considerable controversy with regard to the effects of SSRIs on down-regulation of 5-HT_{2A} (5-hydroxytryptamine-2A) receptors (Eison et al., 1991; Hrdina and Vu, 1993; Baker and Goodnough, 1994; Hyttel, 1994). Blier and de Montigny (1994) have proposed that chronic administration of the major classes of antidepressants results in a net increase in 5-HT neurotransmission, although the different types of antidepressants act through different mechanisms or combinations of mechanisms on 5-HT receptors, e.g. on responsiveness of somatodendritic 5-HT_{1A} autoreceptors, on function of terminal 5-HT autoreceptors, or on responsiveness of postsynaptic 5-HT receptors. Interactions between NA and 5-HT systems may be important in antidepressant action (Manier et al., 1987; Sulser and Sanders-Bush, 1987), but this remains a controversial area of research, particularly with regard to possible mechanisms involved (Asakura et al., 1987; Hensler et al., 1991; Eiring et al., 1992).

1.1.3.3 Other Theories

Several other neurotransmitters, including DA, acetylcholine, GABA

and neuromodulating peptides and steroids, are apparently important in the etiology and treatment of depression (review: Heninger and Charney, 1987; Baker and Greenshaw, 1989; Lloyd et al., 1989; Pfeiffer et al., 1991; Brown and Gershon, 1993; Papp et al., 1994; Tanda et al., 1994). The brain is a dynamic organ with multiple interacting neurotransmitter systems implicated in the etiology and treatment of depression. A unitary mechanism of antidepressant action may not exist and antidepressants may potentially act through several neurotransmitter systems.

Although radioligand binding methodologies have done much to enhance our knowledge of the effects of antidepressants on noradrenergic and 5-HT receptors, they deal with only an early stage in the action of neurotransmitters and may, thus, be of limited value for interpreting functional changes in neural transmission (Hrdina, 1993). There is also considerable controversy with regard to the effects of some antidepressants on receptor regulation, depending on the techniques used to study those receptors. For example, radioligand binding, behavioral studies or measurement of activities of second messenger components directly related to receptors such as adenylyl cyclase or phospholipid metabolism sometimes give apparently conflicting results with regard to effects of antidepressants (Heninger and Charney, 1987; Sanders-Bush, 1989; Johnson, 1991; Cadogan et al., 1993; Sulser, 1993). Because of such discrepancies, several researchers are now examining more closely transduction mechanisms occurring downstream of the interaction at the receptor; such research includes studies on the effects of such drugs on G proteins and associated protein kinases involved in the mediation of second-messenger systems.

Actions of G protein-linked receptors are often mediated through the adenylyl cyclase (AC) or phosphoinositide (PI) systems. The resultant second messengers, cAMP and diacylglycerol (DAG), activate cAMP-dependent protein kinase (PKA) or calcium/phospholipid-dependent protein kinase C (PKC), respectively, and also the calcium/calmodulin dependent kinase (CaM-kinase II). These protein kinases then phosphorylate a range of cellular proteins. β-Adrenergic receptors and some subtypes of 5-HT receptors are linked to either the AC-PKA or PI-PKC cascades [(review: Hrdina, 1993). In recent years, there has been a great deal of interest in the effects of chronic administration of antidepressant drugs on the levels and expression of these G proteins and protein kinases (Lesch et al., 1991; Li et al., 1993; Hudson et al., 1993; Duman and Nestler, 1995; Manji et al., 1995; Mann et al., 1995).

Two other aspects of antidepressants which are often neglected when considering the actions of these drugs are the influences of metabolism and chirality.

1.2 Chirality

1.2.1 Introduction to Stereoisomerism

Stereoisomerism arises from the occurrence within compounds of chiral centres, also known as stereogenic or asymmetric centres. By far the most common type of chiral centre arises when a tetravalent atom, whose valencies are directed to the four corners of a regular tetrahedron, carries four different substituents. The number of possible stereoisomers is 2^n, where n is the number of chiral centres. If two stereoisomers are mirror

images of each other, the relationship between the two is enantiomeric and the two stereoisomers are called a pair of enantiomers. Enantiomers differ in their optical activity, with one rotating plane polarized light to the right [(+) or dextrorotatory] and the other to the left [(-) or levorotatory]. The enantiomers are thus designated as (+) and (-) or prefixed by dextro- and levo- or more simply d- and l-. Nevertheless, optical activity can be influenced by the solvent, temperature or light wavelength used. The optically inactive racemic mixture of 50% of each of the individual enantiomers will be prefixed by rac-. Where no prefix is indicated in the drugs mentioned in this thesis, it will be assumed the racemate is inferred.

However, enantiomers may also be described according to their absolute configuration, referring to the order of the arrangement of the constituents about the chiral centre. Unlike optical activity, which can be influenced by the solvent, temperature or light wavelength used, absolute configuration can only be modified by breaking and reforming chemical bonds. Absolute configuration is expressed using the Cahn-Ingold-Prelog method (Cahn et al., 1966), prefixing compounds with R (rectus) or S (sinister) to indicate the location of the constituents about the chiral centre. With this method, the substituents about the chiral centre are first ranked according to their atomic number, from largest to smallest. The substituents are then oriented such that the smallest substituent is directed away from the viewer. The absolute configuration is then determined by whether the ordered substituents from highest rank to lowest rank follows a clockwise path, designated as R, or a counterclockwise path, designated as S. For historical reasons the absolute configuration of amino acids and

carbohydrates are still referred to as D- and L-, using gylceraldehyde as the standard (Horn, 1984). The absolute configuration is not related to the optical activity in any way, and thus experimental procedures are required in addition to a polarimeter to determine absolute configuration of (+)- and (-)-isomers.

1.2.2 Implications of Chirality in Pharmaceuticals

Enantiomers, if in an achiral environment, have similar chemical properties and almost identical physical properties (except optical activity), and are difficult to distinguish by conventional chemical techniques. However, differences can arise when enantiomeric compounds are introduced into a chiral environment, such as exists in biological systems. Differences in pharmacological and behavioral effects, pharmacokinetic properties (absorption, distribution, metabolism and excretion), pharmacological efficacy and toxicity can occur (Coutts and Baker, 1989; Jamali and Pasutto, 1989). Often only one of the enantiomers, the eutomer, is largely responsible for the therapeutic action, while the inactive or less active enantiomer, the distomer, can often contribute to or is even the main cause of the undesired actions of racemate (Ariens, 1984). The ratio of pharmacological activity, the eudismic ratio, is a good indication of the degree of stereoselectivity (Lehmann, 1976).

Differences in the physiological and behavioral effects induced by the two enantiomers of TMP have been demonstrated in animals (Julou et al., 1961). In radioligand binding studies in male Wistar rats, (-)-TMP shows higher affinity than (+)-TMP does for D_1 and D_2 (DA), alpha 1A/B,

2A, 2B (NA), and the 5-HT receptor subtypes (Gross et al., 1991). In humans, the antidepressant property of TMP appears to reside in the (+)-enantiomer. Surprisingly, the (-)-isomer is said to have a depressant action (Bowman and Rand, 1980; Reynolds, 1982).

Beauchamp et al. (1992), in a study on inhibition of net K^+-induced uptake of ^{45}calcium in synaptosomes prepared from the cortex of the rat brain, observed IC_{50} values for *rac*-TMP, NTMP, (+)-TMP and (-)-TMP of 31, 39, 17 and 95 μM, respectively. The order of potency of the stereoisomers of TMP for inhibition of calcium channels was the same as their reported order of potency as antidepressants: (-)-TMP was less effective than (+)-TMP (Lambert and Guyotat, 1961), and is currently considered a clinically much weaker antidepressant (Beauchamp et al., 1992, personal communication with Rhône-Poulenc Rorer). It is noteworthy that NTMP has an IC_{50} value of 39 μM which is not significantly different from the value of 31 μM for rac-TMP.

While most chiral drugs from natural sources exist as only one of the possible enantiomers, synthetically produced drugs that contain a chiral centre are usually produced as a racemic mixture (Drayer, 1986; Caldwell, 1992). The stereoisomeric composition of therapeutic drug substances is rapidly becoming a key issue in the development, approval and clinical use of pharmaceuticals (Campbell, 1990a,b; Testa and Trager, 1990). Potentially clinically useful racemic mixtures are no longer likely to be approved for preclinical trials without supportive research data on the individual enantiomers and on any possible interactions between the two (Cayen, 1993).

1.3 Drug Metabolism

1.3.1 General Principles of Metabolism

The metabolism of exogenous compounds or xenobiotics is generally a process of detoxification. There are two basic purposes for the metabolism of drugs: (1) to convert drugs to products (metabolites) that are less pharmacologically active, or in other words, to terminate a pharmacological action; (2) to convert drugs to products that are much more water-soluble (i.e. more polar or ionized) than the parent drug and therefore more readily and rapidly excreted.

However, there are also many examples of metabolism resulting in the production of active metabolites (reviews: Rudorfer and Potter, 1985; Young, 1991; Baker et al., 1994). In other words, metabolites that possess a pharmacological activity equal to, greater than, or different from the parent drug may be formed. In fact, such metabolism may be the basis of the actions of certain prodrugs, i.e. compounds that are inactive in the administered form become active compounds by being metabolized by the biological system (Albert, 1958; Harper, 1959; Gardner and Alaxander, 1985).

The chemical modifications are generally catalyzed by metabolic enzymes. Because of its relative richness in enzymes and large mass, drug metabolism occurs mainly in the liver, but has also been shown to occur to a lesser extent in the kidney, blood, brain, lungs, gastrointestinal tract, skin and other tissues (Gibson and Skett, 1986). Regardless of where metabolism takes place, a single drug may undergo several biotransformation steps, any of which may profoundly influence its

biological activity. For the majority of xenobiotics, metabolism involves 2-step processes, designated phase 1 and phase 2 metabolism (review: Gibson and Skett, 1986). A phase 1 metabolic reaction is one in which a new chemical group is introduced into a drug molecule, especially by oxidative, reductive or hydrolytic methods. A phase 2 metabolic reaction is one in which a drug or phase 1 metabolite is conjugated by an enzymatic process with a small endogenous molecule such as glucuronic acid, sulphates, glycine or acetate. Conjugates are water- soluble products which can be excreted in bile or urine. While many enzymes are involved in drug metabolism reactions, for example, esterases, amidases, and amine oxidases, the cytochrome P-450 (CYP450) enzymes are of particular importance in the oxidative metabolism of exogenous compounds or xenobiotics, including drugs.

As indicated earlier in section 1.2, chirality can play a significant role in the metabolism of drugs. Because the configuration of molecules interacting with the active sites of metabolic enzymes is important for charge attraction and three-dimensional fit, there is often a difference in fit between (+)- and (-)- isomers. This can result in enantioselective metabolism favouring the isomer whose fit allows for maximal interaction. Studies of structure-activity relationships involving enantiomers and structural analogues have allowed researchers to gain valuable insights and make predictions about the active sites on many protein structures.

1.3.2 The Cytochrome P450 Enzyme System

Oxidation, a prominent route of phase 1 biotransformation, involves

insertion of an oxygen atom into the substrate in its transition state. The process is catalyzed by an enzyme system called a monooxygenase or mixed function oxidase (MFO) system (Kaufman, 1977) and it occurs mainly in the liver. The most extensively studied MFO system is the CYP450 system made up of NADPH CYP reductase, the CYP450 heme protein and a lipid component, phosphatidylcholine (Lu and West, 1980). The oxidase enzymes are located in the smooth endoplasmic reticulum. The exact number of CYP450 isozymes is unknown. The CYP450 superfamily is classified into families, subfamilies, and individual enzymes. Human CYP450 isozymes have been allocated to ten different gene families (1, 2, 3, 4, 7, 11,17, 19, 21 and 27) based on the similarity in their amino acid sequences. Each gene family displays less than 40% amino acid similarity with the other gene families and all members of a particular family display 40% or greater homology. Families 1 to 4 are involved in the metabolism of numerous drugs (review: Coutts, 1994; Wrighton and Stevens, 1992; Gonzalez, 1992). Within these different gene families, there is only one (family 2) that contains a large number of subfamilies, each of which is designated by a different capital alphabet letter. Individual CYP450s within a subfamily have greater than 59% homology and are distinguished from each other by the terminal arabic number (Nebert et al., 1989 and 1991; Nelson et al., 1993).

The five human CYP450 isozymes that have received most attention are CYP1A1, CYP1A2, CYP2D6, CYP3A4 and CYP_{MP} (mephenytoin hydroxylase). Normally, CYP1A1 is virtually absent in humans, but can be induced by cigarette smoking and polycyclic aromatic hydrocarbons.

CYP1A1 is expressed in placenta and lung tissue (Pelkonen et al., 1986; Sesardic et al., 1988; Sarkar et al., 1992; Gentest, 1992), and when its production is induced, it becomes widely distributed in human tissue. CYP1A2 is expressed mainly in liver and is identical to phenacetin O-deethylase, and also named 7-ethoxyresorufin O-deethylase. Typical substrates are acetanilide (4-hydroxylation), 7-ethoxy- and 7-methoxy-resorufin (O-dealkylation), estradiol (2-hydroxylation), and phenacetin (O-deethylation). CYP1A2 is predominantly responsible for the major metabolic pathway of the methylxanthines (N-demethylation) and of caffeine and theophylline (8-hydroxylation and N-demethylation). CYP_{MP} is a member of the CYP2C subfamily that 4′-hydroxylates S-mephenytoin. Its substrates include tolbutamide, hexobarbital, diazepam, flurazepam, methylphenobarbital, N-desmethyldiazepam and alprenolol. CYP3A4 is also named nifedipine oxidase. Its substrates are diverse in structure and include lidocane, quinidine (QND), nifedipine, alprazolam, midazolam, triazolam, erythromycin, cyclosporin A, cortisol and testosterone. CYP2D6 and CYP_{MP} are the two isozymes that exhibit polymorphism. Approximately 5-10% of Caucasians, 2% of Orientals and 1% of Arabics are poor metabolizers (PMs) of debrisoquine to its 4-hydroxy metabolite, because their body cannot synthesize CYP2D6 isozyme (Gaedigk et al., 1991). About 3-5% of Caucasians and over 20% of Japanese are PMs of mephenytoin to the 4′-hydroxy metabolite, because their body cannot produce CYP_{MP} isozyme (Gonzalez, 1990).

CYP2D6 is the most extensively studied cytochrome enzyme by far (Brøsen and Gram, 1989a; Eichelbaum and Gross, 1990: Gaedigk et al.,

1991). CYP2D6 is also referred to as debrisoquine 4-hydroxylase, sparteine dehydrogenase and debrisoquine-sparteine oxidase. Numerous structurally diverse basic drugs, including antidepressants (IMI, DMI, AMI, NTR, CMI, FLU and paroxetine) and neuroleptics (haloperidol, clozapine, risperidone) have been identified or suggested as substrates of CYP2D6. TMP was only recently identified as a substrate of CYP2D6 (Eap et al., 1992a; Bolaji et al., 1993). In most cases, particularly with TCAs, CYP2D6 is involved in aromatic hydroxylation.

Appropriate substrates for CYP2D6 have been characterized as lipophilic aromatic compounds that possess a basic centre which is protonated at physiological pH and then attaches itself to an anionic location (COO^- group) at the active site of the CYP2D6 molecule. The substrate orientates itself at the protein's active site, such that the distance between the site of metabolic oxidation and the protonated N atom is 0.5-0.7 nm (Guengerich et al., 1986).

Some SSRIs (those in Figure 3) are eliminated in human *via* oxidation catalyzed by CYP450 in the liver. Some investigations have shown that elimination of FLU and NFLU (Altamura et al., 1994; Brøsen and Skjelbo, 1991; Stevens and Wrighton, 1993), paroxetine (Sindrup et al., 1992; Bloomer et al., 1992) and desmethylcitalopram (Sindrup et al., 1993) is partly controlled, directly or indirectly, by CYP2D6. However, specific isozyme(s) other than CYP2D6 implicated in the biotransformations of SSRIs are still under investigation (review: Brøsen, 1993; Baumann and Rochat, 1995; Rasmussen et al., 1995).

1.3.2.1 Involvement of CYP450s in N-Dealkylation

Metabolic studies on various basic drugs have led to the general conclusion that aromatic hydroxylation and N-dealkylation are two commonly observed phase 1 metabolic pathways. Many N-dealkylations of drugs are catalyzed in humans by CYP2D6 (partly), CYP3A (often CYP3A4), CYP1A2 and CYP_{MP}. However, the results from *in vitro* single isozyme studies and *in vivo* inhibition studies are confusing and sometimes contradictory regarding the issue of which specific isozyme(s) is (are) involved in the N-dealkylation of a certain drug (review: Coutts et al., 1994).

Identified substrates for CYP3A4-mediated N-dealkylation are alfentanil, amiodarone, benzphetamine, CQA206-291 (an alkaloid), codeine, dextromethorphan, diltiazem, erythromycin, lidocaine, tamoxifen, terfenadine, troleandomycin, verapamil, propafenone and IMI (to some extent). Examples of drugs in which CYP1A2 contributes to N-dealkylation in humans are methylxanthines, caffeine, theophylline, propafenone and IMI (to some extent). CYP_{MP} has been identified in N-dealkylation of citalopram, diazepam, TMP (Seifritz et al., 1994), and AMI (Baumann et al., 1986; Breyer-Pfaff et al., 1992).

CYP2D6 is generally not considered to be capable of catalyzing the N-dealkylation of basic drugs, and controversy exists in the literature. Following the "CYP2D6 substrate model" theory of Guengerich et al. (1986), the distance between the protonated N atom and adjacent C atom in the N-dealkylation reaction is only about 0.15 nm, not within the optimal 0.5-0.7 nm range, and, therefore, N-dealkylation should not be considered to be mediated by CYP2D6. Nevertheless, N-dealkylations of a limited

number of basic drugs, e.g. AMI, IMI, FLU and NFLU (Stevens and Wrighton, 1993), amiflamine and desmethylcitalopram, are identified as partially under the control of the CYP2D6 isozyme (Coutts et al., 1994). Stevens and Wrighton (1993) found QND inhibited the N-demethylation of each enantiomer of FLU by only 20% at a concentration 300 times greater than the K_i determined for the QND inhibition of bufuralol 1'-hydroxylase. However, Skjelbo and Brøsen (1992) screened 20 compounds for their abilities to inhibit the N-demethylation of IMI, including the SSRIs (paroxetine, NFLU, FLU, FLUV, citalopram and desmethylcitalopram) and QND. Only FLUV, a weak inhibitor of CYP2D6, was found to be a potent inhibitor of IMI N-demethylation. Bolaji et al. (1993) and Eap et al. (1992a) reported that N-demethylation of TMP was not mediated by the human CYP2D6 isozyme.

Noteworthy is the observation that N-dealkylation of some drugs, especially TCAs, is catalyzed by more than one CYP450 isozyme. For example, the N-dealkylation of IMI is partly controlled by CYP2D6, and is also mediated by CYP1A2 and CYP3A4 (Lemoine et al., 1993), but may or may not involve CYP_{MP} (Skjelbo et al., 1991; Skjelbo and Brøsen, 1992). Besides CYP2D6 (Coutts et al., 1994), the N-dealkylation of AMI may also involve the CYP_{MP} isozyme (Breyer Pfaff et al., 1992).

1.3.3 Enzyme Inhibition and Induction

It has been shown that administration of certain drugs or other organic chemicals can cause an induction or inhibition of the synthesis of some CYP isozymes (Gibson and Skett, 1986; Coon et al., 1992). This

alteration in levels may occur at any of several modes of regulation such as transcription activation or mRNA or protein stabilization (Coon et al., 1992; Guengerich, 1992). Most inducers stimulate the production of more than one species of CYP450 and stimulate their own metabolism as well as the metabolism of many other chemicals. An example is the induction of CYP1A1 caused by the polycyclic aromatic hydrocarbons found in cigarette smoke. However, not all CYP450 species are inducible. CYP2C7, CYP2C13 and CYP2D6 are relatively refractory to induction (Eichelbaum et al., 1986; Okey, 1990). No substrates of CYP2D6 are known to stimulate their own metabolism, and common CYP450 inducers such as rifampicin, phenazone (antipyrine), ethanol, polyaromatic hydrocarbons, carbamazepine and phenobarbital have no effect on the activity of CYP2D6 (Coutts, 1994: Okey, 1990).

Inhibition of the CYP450-mediated MFO system has been the subject of many reviews (Guengerich, 1992; Boobis et al., 1990; Murray, 1987; Testa and Jenner, 1981). This inhibition of drug metabolism by drugs or xenobiotics can take place in several ways, including reversible inhibition, destruction of pre-existing enzymes, inhibition of enzyme synthesis or by complexing and thus inactivating the drug-metabolizing enzyme (Gibson and Skett, 1986). The onset of inhibition is usually rapid following a single dose of the inhibitory compound and from this point of view interactions involving inhibitors of drug metabolism are probably of greater significance than those involving enzyme induction (Testa and Jenner, 1976).

The co-administration of drugs sometimes results in active site

inhibitory interactions and may result in impaired drug elimination. This can be reversible or irreversible, competitive or non-competitive inhibition. Competitive inhibition occurs when two drugs, administered at the same time, compete for the same particular isozyme. This competition will cause differences in the pharmacokinetic properties of each drug when compared to those properties observed when the drugs are administered alone. Competitive inhibition is also observed when one of two drugs administered is a non-substrate (is not metabolized by the enzyme) inhibitor but has good binding affinity to the enzyme. For example, QND is a potent non-substrate inhibitor of CYP2D6 (Otton et al., 1984; Brøsen et al., 1989a,b), since it is predominantly metabolized *via* CYP3A4. A compound may also be oxidized by a particular CYP isozyme to form a product which then can inhibit that enzyme's activity by binding tightly to it (Guengerich, 1992).

Perhaps the most potent reversible inhibitors of MFO are the nitrogen heterocycles (Murray, 1987). This class of compounds includes the imidazoles and quinolines. These ring systems are present in a large number of therapeutic agents. An imidazole ring system is present in the antifungal agents ketoconazole and miconazole and in the H2-receptor antagonist cimetidine; a quinoline ring is present in antimalarial drugs like chloroquine, primaquine and quinine (QNN) and in antiarrhythmic agents such as QND.

1.3.3.1 Inhibition of CYP450s by SSRIs

As discussed in the previous section 1.3.2, several SSRIs can inhibit

the oxidative metabolism of other drugs. Using human liver microsomes, paroxetine, NFLU, FLU, sertraline, citalopram and FLUV, have been shown, in decreasing order of potency, to inhibit CYP2D6 activity. On a molar basis, the apparent inhibition constant K_i with respect to oxidation of sparteine is 0.15, 0.43, 0.60, 0.70, 5.1 and 8.2 μM, respectively; those of the TCAs are as follows: CMI (2.2 μM), DMI (2.3 μM) and AMI (4.0 μM) (Skjelbo and Brøsen, 1992; Crewe et al., 1992; Otton et al., 1993; von Moltke et al., 1994; Altamura et al., 1994). *In vitro* studies on O-demethylation of oxycodone further indicated that FLU and NFLU are non-selective inhibitors of CYP2D6 (Otton et al., 1993).

Besides CYP2D6, *in vitro* and clinical studies suggest that SSRIs are also capable of inhibiting the activities of CYP1A1, CYP1A2, CYP3A4 and CYP_{MP}, but not of CYP2A6 or CYP2E1 (Rasmussen et al., 1995; Baumann and Rochat, 1995). Fuller and Perry (1989) found FLU and NFLU could inhibit N-demethylation and ring hydroxylation of DMI. FLUV is a weak inhibitor of CYP2D6, but is a potent inhibitor of CYP1A2 with respect to (1) all metabolic pathways of theophylline (Rasmussen et al., 1995), (2) O-deethylation of phenacetin (Jensen et al., 1995; Brøsen et al., 1993a), (3) metabolism of clozapine (Hiemke et al., 1994; Jerling et al., 1994), and (4) N-dealkylation of IMI (Skjelbo and Brøsen, 1992). Other SSRIs, e.g. paroxetine, litoxetin, sertraline, NFLU, FLU and desmethylcitalopram, in decreasing order, have weak or no CYP1A2 inhibition potency (Rasmussen et al., 1995).

NFLU is a potent inhibitor of CYP3A4 with respect to hydroxylation of alprazolam (von Moltke et al., 1995) and 6β-hydroxylation of cortisol and

testosterone (Rasmussen et al., 1995). FLUV was as potent as NFLU at inhibiting hydroxylation of alprazolam (von Moltke et al., 1995), but in contrast, similar to FLU, was a weak CYP3A4 inhibitor in 6β-hydroxylation of cortisol and testosterone (Rasmussen et al., 1995). Paroxetine, sertraline, litoxetine, citalopram and desmethylcitalopram caused little or no inhibition of CYP3A4. FLUV has also been identified as a potent inhibitor of the N-demethylation of the tertiary amine TCAs IMI, AMI, CMI (Bertschy et al., 1991, 1992; Spina et al., 1993) and TMP (Seifritz et al., 1994). The possible involvement of CYP2D6, CYP1A2 and CYP3A4 in N-dealkylation of IMI (Brosen and Gram, 1989b, 1991a,b; Coutts et al., 1993; Lemoine et al., 1993), and of CYP_{MP} in N-dealkylation of TMP were speculated upon in comedication studies with fluvoxamine (Seifritz et al., 1994).

Rasmussen et al. (1995) also showed that NFLU and sertraline were potent inhibitors of CYP1A1 in O-deethylation of 7-ethoxyresorufin, while FLUV was a weak inhibitor and other SSRIs showed no inhibition in human microsomes from placenta.

1.3.4 Drug-Drug Interactions

In the treatment of mental illness, there is a widespread occurrence of polypharmacy (Grahame-Smith, 1977). Drug-drug interactions may occur when the predominant metabolic enzyme of one drug is induced or inhibited by another drug, particularly at the level of the CYP isozymes. Examples of drug interactions in the comedication with SSRIs are; FLU and TCAs (Bergstrom et al., 1992), FLU and alprazolam (Greenblatt et al., 1992), paroxetine and DMI (Brøsen et al., 1993b), FLUV and TCAs

(Bertschy et al., 1991; Spina et al., 1993), FLUV and clozapine (Hiemke et al., 1994: Jerling et al., 1994), and FLUV and TMP (Seifritz et al., 1994).

Drug interactions can either be of clinical benefit or be the cause of adverse effects. In extreme cases, the potentiation of the pharmacological effects can lead to toxic effects that are life-threatening (Shen and Lin, 1991). In the opposite situation, the decrease in activity of one drug caused by another drug may lead to a relapse or worsening of the psychiatric problems of the patients (Shen and Lin, 1991). Drug interactions can be pharmacodynamic or pharmacokinetic (Grahame-Smith, 1977). A pharmacodynamic drug interaction is an interaction that involves either a quantitative or a qualitative change in the action of one of the drugs (Grahame-Smith, 1977). This interaction is very difficult to assess in a clinical setting. A pharmacokinetic interaction is one that results in an alteration in the expected plasma level of a drug through some effect on absorption, distribution, metabolism and/or elimination.

It is also possible that the individual enantiomers of a racemic mixture have the potential to be a source of drug interactions, or the individual enantiomers may be involved to different extents in interactions with other drugs (Jamali and Pasutto, 1989). Such an interaction has been observed when cimetidine is co-administered with verapamil. The increase in bioavailability of the more active (-)-enantiomer of verapamil is almost twice that of the (+)-enantiomer (Mikus et al., 1988). In humans, cimetidine interacts only with R-warfarin, leaving the more active S-warfarin unaffected (Choonara et al., 1986). (S)-Enantiomers of FLU and NFLU are more potent CYP2D6 inhibitors than their (R)-enantiomers in bufuralol 1'-

hydroxylation (Stevens and Wrighton, 1993).

1.4 Metabolism of Tricyclic Antidepressants (TCAs)

1.4.1 General Metabolism of TCAs

The major factor responsible for observed inter-individual and inter-species pharmacokinetic variations is considered to be the drug metabolism process, whereas absorption, distribution and excretion show less pronounced variation. The steady-state plasma levels of TCAs show large individual variation (Preskorn, 1989; Preskorn and Fast, 1991; Furlanut et al., 1993). Of the two factors determining steady-state plasma levels, i. e., distribution and elimination, the latter is more important (Wagner et al., 1965). Thus, it has been demonstrated that plasma half-lives (Hammer and Sjöqvist, 1967) and urinary excretion of metabolites (Christiansen et al., 1967) show individual variations in the same order of magnitude as those of steady-state plasma levels. Variations in elimination capacity must, therefore, be considered as important factors determining the effect of TCAs. However, the scientific literature on the dose-effect relationships of TCAs is very limited (Gram, 1993). Insufficient dosing will not produce a clinical effect while overdosing may worsen the illness.

The TCAs are highly lipophilic and therefore subject to multiple biotransformation steps yielding polar metabolites that can be readily excreted in the urine or bile (Potter and Calil, 1981; Rudorfer and Potter, 1987). The major site of drug metabolism is the liver, where TCAs undergo mainly demethylation and hydroxylation followed by glucuronide conjugation (Gram, 1974; DeVane, 1986; Potter and Manji, 1990). The

oxidative reactions are usually the rate-limiting ones and are subject to stimulation (induction) or inhibition by exogenous agents (Rudorfer and Potter, 1987). Overall, renal clearance accounts for elimination of less than 5% of administered doses as unchanged drug (Sutfin et al., 1984; Sjoqvist et al., 1969). TCAs that have received the most attention in drug metabolism and pharmacokinetic research studies are IMI, DMI, AMI and NTR. In contrast, TMP's metabolic and pharmacokinetic properties have been virtually ignored. Typical metabolic pathways of TCAs are discussed below using IMI as an example.

1.4.2 Metabolism of Imipramine

IMI is eliminated by N-demethylation to the active metabolite DMI and by aromatic hydroxylation to 2-OH-IMI. DMI is further metabolized by aromatic hydroxylation to 2-OH-DMI (Sallee and Pollock, 1990). Quantitatively, hydroxylation is the most important intermediate metabolic pathway, facilitating the clearance of IMI (Rubinstein et al., 1983). The hydroxylated metabolites are excreted either in the urine (60-80%) or *via* the biliary system (22%) mainly as glucuronide conjugated forms (Judd and Ursillo, 1975). Minor metabolic pathways for IMI are N-dealkylation of the entire side chain to form iminodibenzyl (IDB) or further N-demethylation of DMI to N,N-didesmethyl-IMI (DDMI) and subsequent 10-hydroxylation (Sallee and Pollock, 1990).

The aromatic hydroxylations of IMI and its metabolite DMI are catalyzed by CYP2D6 isozyme, and as such, inter-individual differences exist in their metabolism (Sallee and Pollock, 1990). N-Dealkylation of IMI

may involve CYP2D6 isozyme to some extent, (Brøsen and Gram, 1989b; Brøsen et al., 1991a,b; Coutts et al., 1993), CYP1A2 and CYP3A4 (Lemoine et al., 1993), but may or may not involve CYP_{MP} (Skjelbo et al., 1991; Skjelbo and Brøsen, 1992). Less than 5% of an oral dose of IMI is excreted unchanged in the urine (Sjöqvist et al., 1969). The most important IMI metabolites (free and conjugated) are 2-OH-DMI (40-50%), 2-OH-IMI (15-25%) and 2-OH-IDB (15%) (Crammer et al., 1969; Rudorfer and Potter, 1985; Potter et al., 1982; Potter and Manji, 1990). Other metabolites, IMI-N-oxide and IDB, are also formed in humans in significant amounts. IMI-N-oxide is metabolically reconverted into IMI. IDB and 2-OH-IDB are of little clinical significance (Potter and Calil, 1981).

Poor hydroxylators of IMI and DMI demonstrated appreciable plasma levels of DDMI, exceeding those of 2-OH-IMI (Gram et al., 1983). There is no evidence of enzyme saturation in the hydroxylation of IMI at therapeutic dose levels. However, the aromatic hydroxylation of IMI and DMI is under the influence of genetic polymorphism (Sallee and Pollock, 1990).

1.4.3 Metabolism of Trimipramine (TMP)

1.4.3.1 Dose-Plasma Concentration Relationship in Humans

Metabolic and pharmacokinetic studies of TMP were largely ignored before 1980. Part of the reason was probably the relatively low concentration of TMP and its metabolites in human plasma at therapeutic dosage, which requires a highly sensitive and specific analytic method. In single dose studies in humans, with a 50-100 mg TMP tablet, p.o., maximum plasma concentrations (C_{max}) of TMP reached 16-39 ng/ml, with

a high inter-individual variability (Caille et al., 1980; Abernethy, 1984; Cowan et al., 1988; Bougerolle et al., 1988, 1989; Gulaid et al., 1991). Only trace amounts of major metabolites were detected: nortrimipramine (or N-desmethyltrimipramine, NTMP), <10 ng/ml; 2-OH-TMP, <3 ng/ml; and bis-nortrimipramine (or N,N-didesmethyltrimipramine, NNTMP), <1 ng/ml.

Following daily oral doses of 75 or 150 mg to 29 depressed patients, plasma concentrations at steady-state were: TMP, 86 ng/ml (range 11-241 ng/ml); NTMP, 65 ng/ml (range 3-382 ng/ml); 2-OH-TMP (total), 379 ng/ml (range 58-1126 ng/ml); 2-OH-NTMP (total), 317 ng/ml (range 63-800 ng/ml); and trace amounts of NNTMP (<10 ng/ml). Four to five percent of the total plasma hydroxy metabolites is in free form and the rest is glucuronide-conjugated (Suckow and Cooper, 1984; Eap and Baumann, 1994).

In another steady-state study of TMP with a daily oral dose of 75 or 150 mg to each group of 10 patients for 6 weeks, Simpson et al., (1988) reported that the plasma concentration of the N-desmethylated metabolite increased by over five-fold when the dose of TMP was doubled. Musa (1989) suggested this non-linear dose-concentration relationship is most likely due to the saturation within the therapeutic dosage range of the subspecies of CYP450 responsible for hydroxylation of NTMP. This situation is similar to the dose-dependent kinetics of DMI, but not of NTR.

At higher daily doses of 200-350 mg TMP given to 4 patients for 5-44 days, the results further confirmed lower plasma concentrations of TMP (range 114-305 ng/ml) and NTMP (range 0-310 ng/ml) than of 2-OH-TMP (range 264-755 ng/ml) and 2-OH-NTMP (range 966-1754 ng/ml), and a

significant inter-individual variability in pharmacokinetics was also observed (Eap et al., 1992b).

1.4.3.2 TMP Metabolic Pathways in Humans

Suckow and Cooper (1984) showed that the metabolism of TMP in humans paralleled that of IMI. It was demethylated and hydroxylated at the C-2 position, and the hydroxylated metabolites were excreted mainly as glucuronide conjugates. It is noteworthy that N-demethylation did not appear to occur with TMP as readily as with IMI or AMI. At therapeutic steady-state, the ratio of desmethyl metabolite to parent compound ranged from 1.0 to 1.6 for AMI and was 1.0 for IMI and 0.61 for TMP. Suckow and Cooper (1984) suggested the difference in the side chain of TMP probably accounted for the retardation in demethylation.

Two GC/MS studies on the urinary metabolites of TMP in humans were reported in recent years (Köppel and Tenczer, 1988; Maurer, 1989). Both were qualitative studies and the results varied somewhat. In addition to the unchanged drug TMP, Köppel and Tenczer (1988) reported the formation of 8 metabolites: NTMP, NNTMP, 2-OH-TMP, 2-OH-NTMP, TMP N-oxide, 10-oxo-NTMP, IDB and 2-OH-IDB. They concluded that the metabolic degradation of TMP was similar to that of IMI and included N-demethylation, C-2 aromatic hydroxylation, C-10 (or C-11) oxidation, N-1-dealkylation and N-oxidation.

In analysis of urine samples from 5 human subjects who received 100 mg TMP and from 2 overdose victims, Maurer (1989) identified 15 metabolites besides unchanged TMP. The metabolites included

2-OH-TMP, NTMP, 2-OH-NTMP, IDB, 2-OH-IDB, 2,3-di-OH-IDB, 2-OH-3-methoxy-IDB (or 3-OH-2-methoxy-IDB), 2,3-di-OH-TMP, 2,3-di-OH-NTMP, NNTMP, 2-OH-NNTMP, 2,3-di-OH-NNTMP, 2-OH-3-methoxy-TMP (or 3-OH-2-methoxy-TMP), 2-OH-3-methoxy-NTMP (or 3-OH-2-methoxy-NTMP) and 2-OH-methoxy-NNTMP (or 3-OH-2-methoxy-NNTMP). The hydroxylated metabolites were excreted mainly as glucuronide conjugates. It is interesting that no C-10 (or C-11) oxidation was observed, which is in agreement with the findings of Populaire et al. (1970). Maurer postulated three overlapping phase-1 metabolic pathways of TMP. In one pathway, the iminodibenzyl (IDB) ring is dealkylated at the N-1 position; in another, the tertiary amine on the side chain is successively N-demethylated; and in the third, the IDB ring undergoes mono- or bi-fold aromatic hydroxylation followed by some methylation of one of the hydroxy groups. Because the amounts of the hydroxy metabolites in urine after enzymatic cleavage of the conjugates were several times greater than without cleavage, it can be concluded that these compounds also undergo phase-2 metabolism (Maurer, 1989).

1.4.3.3 Drug Interactions Involving TMP in Humans

In vitro metabolic studies of TMP using human CYP2D6 isozyme expressed in the human AHH-1 TK+/- cell line revealed two metabolites, 2-OH-TMP and a previously unreported metabolite, 2,10 (or 2,11)-di-OH-TMP (Bolaji et al., 1993). Unlike the case with IMI (Coutts et al., 1993), no N-dealkylation of TMP was observed. Prior adminstration of QND before TMP produced a large reduction (80%) in 2-OH-TMP (Bolaji et

al., 1993). This finding suggests that 2-hydroxyation, but not N-demethylation, of TMP in humans is mediated by CYP2D6 isozyme.

Coadministration of QND and TMP in 2 healthy human subjects markedly altered the pharmacokinetics of TMP, doubling its plasma half-life and reducing its apparent clearance to 35% and volume of distribution to 60% of the values when TMP was given alone. Higher concentrations of TMP and NTMP, and lower concentrations of 2-OH-TMP and 2-OH-NTMP, were generally observed than when TMP was given alone. These results indicate that TMP is a substrate of CYP2D6 and that N-demethylation of TMP is mediated by isozymes other than CYP2D6 (Eap et al., 1992a). The addition of FLUV to TMP treatment at steady-state in one depressed subject resulted in a two-fold increase of TMP plasma concentrations, and only a slight increase of levels of NTMP, 2-OH-TMP and 2-OH-NTMP (Seifritz et al., 1994). The patient was phenotyped as an extensive metabolizer (EM) of both dextromethorphan and mephenytoin. The reduction of the ratios of NTMP/TMP and [(2-OH-TMP + 2-OH-NTMP)/TMP] by 42% and 41%, respectively, indicated a significant FLUV-induced inhibition of N-demethylation of TMP and that of 2-hydroxylation of TMP and NTMP (Seifritz et al., 1994). Earlier clinical studies suggest that the N-demethylation of AMI (Baumann et al., 1986; Breyer-Pfaff et al., 1992), IMI (Skjelbo et al., 1991; Skjelbo and Brøsen, 1992) and of other psychotropic drugs (Brøsen, 1990) is at least partly co-regulated by CYP_{MP}. FLUV has been shown to be an inhibitor of the N-demethylation of tertiary amines and, therefore, possibly CYP_{MP} (Bertschy et al., 1991, 1992; Skjelbo and Brøsen, 1992). *In vitro* studies (Crewe et al., 1992; Skjelbo

and Brøsen, 1992) and clinical data show that FLUV inhibits the metabolism of both dextromethorphan and mephenytoin (Bertschy et al., 1992). Based on these rationale, Seifritz et al. (1994) suggest that the interaction between FLUV and TMP occurred at the level of CYP2D6 and CYP_{MP}.

1.4.3.4 TMP Metabolic Pathways in Rats

MSD rats have five CYP2D genes (CYP2D1, 2, 3, 4 and 5), although one (CYP2D1) appears to be predominantly expressed in rat liver. The substrate specificity of CYP2D1 enzyme is similar, but not identical to, that of human CYP2D6 isozyme (Gonzalez, 1990; Souček and Gut, 1992). Analogous metabolic profiles for TMP are expected in rats and humans. Therefore, the rat is a suitable species in which drug metabolism and drug-drug interactions can be investigated. In a study of the metabolism of TMP in the rat, Coutts et al. (1990) and Hussain et al. (1991), found unchanged TMP and 20 metabolites in the urine. The four major urinary metabolites were identified as 10-oxo-TMP, 2-OH-TMP, 2-OH-NTMP and 2-OH-10-oxo-TMP. A total of 12 out of 20 metabolites had undergone alicyclic C-10 (or C-11) oxidation, involving either 10-oxidation or 10-hydroxylation in the alicyclic ring.

In drug-drug interaction studies, when iprindole (IPR), QND or QNN was administered to rats prior to TMP treatment, the effect on TMP metabolism was profound. In all cases, the production of 2-OH-TMP was significantly reduced while the formation of 2-OH-NTMP was unexpectedly increased. However, the alicyclic ring oxidation at C-10 (or C-11) position

was inhibited markedly by IPR, while QND had no effect and QNN inhibited this pathway to a certain extent (Coutts et al., 1991; Hussain and Coutts, 1991; Hussain, 1992).

Theoretically, the formation of 2-OH-NTMP could be either from 2-OH-TMP by N-demethylation or from NTMP by C-2 aromatic hydroxylation. To delineate the probable *in vivo* metabolic pathway of formation of 2-OH-NTMP in the rat, two separate studies were conducted by Hussain (1992). In one study the rats were dosed with NTMP and its urinary metabolites were compared to those of another study with rats dosed with 2-OH-TMP. The major metabolites of NTMP were 2-OH-NTMP (22% of the dose) and 10-OH-NTMP (22% of the dose) together with a small amount of unchanged NTMP (0.9% of the dose). The major identified metabolites of 2-OH-TMP were 2-OH-10-oxo-TMP (% unreported) and a low amount of 2-OH-NTMP (4% of the dose) together with large amounts of unchanged 2-OH-TMP (31% of the dose). Furthermore, administration of QND prior to NTMP treatment doubled the level of 2-OH-NTMP (44% of the dose) with a slight increase of unchanged NTMP (1.5% of the dose). The results from this study clearly showed that *in vivo* metabolic formation of 2-OH-NTMP in MSD rats is mainly from NTMP by C-2 hydroxylation. It also suggests that CYP2D6 is involved in metabolic C-2 hydroxylation but not C-10 (or C-11) hydroxylation of TMP. Unlike the case with DMI, the C-2 aromatic hydroxylation of NTMP is apparently under the influence of an isozyme other than CYP2D6 (Hussain, 1992).

In an *in vitro* metabolic study of TMP using MSD rat liver microsomes, Hussain (1992) characterized 5 metabolites besides

unchanged TMP: NTMP, 2-OH-TMP, 2-OH-NTMP, 10-OH-TMP, and 10-OH-NTMP. However, N-TMP was the major *in vitro* metabolite and, in contrast to the *in vivo* study, no 10-oxo-TMP metabolite was found. In the time course study of formation of these metabolites, 2-OH-NTMP was once again confirmed to be formed from NTMP but not from 2-OH-TMP.

Comparing the urinary metabolites of TMP in humans to MSD rats, only one of 8 metabolites, 10-oxo-NTMP, involved the C-10 (or C-11) alicyclic ring oxidation, has been reported by Köppel and Tenczer (1988), but none of the C-10 (or C-11) hydroxylation metabolites have been found by Köppel and Tenczer (1988) or Maurer (1989). Apparently significant species differences in the metabolism of TMP occur. It is possible that the CYP450 isozyme responsible for alicyclic hydroxylation of TMP is absent in humans.

1.4.3.5 Stereoselective Aspects in TMP Metabolism

As indicated earlier in sections 1.2 and 1.3, chirality plays a significant role in the metabolism of drugs. TMP has a chiral centre and is used as a racemic drug. Therefore, all metabolites that retain the chiral centre will also be racemates, each possessing a pair of enantiomers at possibly different concentrations. Since the initial 50:50 enantiomeric ratio of racemic TMP used may rapidly change to an unknown ratio in the chiral biological environment, the value of "total drug" plasma concentrations and pharmacokinetic parameters is highly questionable. If pharmacokinetic and pharmacological investigations are to be meaningful, it is imperative to know blood or tissue concentrations of individual stereoisomers.

Unfortunately, the detection of these enantiomeric compositions in the biological samples and identification of the active enantiomers of TMP and its metabolites in humans or animals have been ignored. To my knowledge, the only method reported for analysis of the enantiomers of TMP is a relatively time-consuming two-step HPLC method (Eap et al., 1992b). In the first step, separation and quantification of TMP and the metabolites (NTMP, NNTMP, 2-OH-TMP and 2-OH-NTMP) were performed with a Nucleosil CN column using reverse phase chromatography. In the second step, for the quantification of enantiomers, fractions containing peaks corresponding to TMP and its metabolites were collected manually and taken to dryness. The residue was taken up in an appropriate volume of mobile phase solution, and an aliquot of 100 µl was injected onto a chiral α_1-acid glycoprotein (AGP) column. The (+)- and (-)-isomers were then analyzed on the basis of their relative peak areas. However, TMP was slightly contaminated by 2-OH-TMP and NTMP by 2-OH-NTMP, after separation with the CN column. Furthermore, because of the losses during the peak collection steps, and peak broadening on the AGP column, the detection limit for the quantification of the enantiomers was estimated to be 300 ng/ml of original plasma. Therefore, only 2-OH-TMP and 2-OH-NTMP enantiomers were measured in patients' plasma. Thus a more sensitive analytical method is warranted in order to study the enantiomeric metabolism of TMP and its drug-drug interactions.

1.5 Development of Analytical Methods

1.5.1 Isolation and Purification of Drugs

The study of xenobiotic metabolism has developed over the last few years into a specialized science with its own technical skills, three of which are the isolation, identification and quantification of metabolites. Drugs can be classified as acidic, basic, amphoteric, or neutral on the basis of their pKa values. The TCAs are mostly lipophilic bases with pKa values of 8.5-10 or higher. Most extraction procedures take advantage of these properties to separate the drug and its metabolites from biological matrices. A three step liquid-liquid extraction procedure is the most commonly used method for the initial purification (Reed, 1988). This involves first adjusting the pH of the sample to >10 with a base or buffer solution and extracting with a suitable organic solvent. The organic layer, which now contains basic and neutral analytes, is dried under vacuum or under a stream of dry nitrogen. The residue obtained is dissolved in dilute hydrochloric acid (or pH 3.0 buffer for HPLC) and then washed with a suitable organic solvent. The acidic aqueous phase which now contains the basic drug and basic metabolites is made alkaline by the addition of a base or buffer solution and the analytes are extracted into a suitable organic solvent. The organic layer is evaporated as described above and finally the residue obtained is reconstituted or derivatized with a suitable derivatizing agent before being analyzed by gas chromatography (GC). When high performance liquid chromatography (HPLC) is used for analysis, the third step can be omitted and the pH 3.0 aqueous extract is used directly in HPLC.

Solid phase extraction techniques have recently been proposed for

extracting antidepressants from their biological matrix (Gupta, 1992; Kaplan and Pesce, 1989). These techniques involve the use of small columns containing a packing material such as silica, C18 bonded silica, resin, etc. These small columns are first washed with methanol, then with an acidic buffer containing approximately 5% methanol; this is followed by application of the sample. Analytes are then collected by elution with a small amount of organic solvent such as methanol. The sample can now be injected or taken to dryness, derivatized and reconstituted for analysis.

1.5.2 Derivatization Techniques

The scope of GC would be quite limited without sample derivatization. For example, benzoic acid, because of its polarity, is not suited for gas chromatographic analysis, but derivatization with methanol in the presence of boron trifluoride readily converts it to methyl benzoate, which is more stable and volatile and easier to chromatograph. Therefore, analytes are derivatized for several reasons: (a) to increase volatility; (b) to increase stability; (c) to reduce polarity; (d) to introduce specific groups which enhance detectability; (e) to increase extractability from aqueous solution; (f) and to improve chromatographic separation of different components present in the sample mixture (Coutts and Baker, 1982; Reidman, 1973).

Derivatization of amines, alcohols and carboxylic acids reduces the adsorption of these compounds by the GC column (Vandenheuvel and Zacchei, 1976). Most derivatization reactions involve the replacement of active hydrogen in polar groups (e.g. NH, OH, COOH), and the main types

of derivatizations are alkylation, acylation, silylation and condensation (Knapp, 1979; Blau and King, 1978; Coutts and Baker, 1982). The principal types of derivatization used in GC analysis are illustrated in Figure 4 (Baker et al., 1982).

1.5.3 Gas Chromatography (GC)

Adsorption chromatography was first applied by Tswett in 1906, and in 1952 James and Martin introduced gas chromatography (Ettre and Horvath, 1975) (GC), also known as gas-liquid chromatography (GLC). This technique is an analytical procedure used to separate mixtures of organic compounds prior to their identification and/or quantification. The volatile components of an analyte mixture are carried through a column by an inert carrier gas (called the mobile phase) and the components are separated from one another according to their partition coefficients between the carrier gas and the liquid stationary phase. GC can be used for the analysis of gas, liquid, and solid samples, providing that the latter two can be thermally vaporized without significant decomposition (Kline and Soine, 1984).

The choice of the GC columns depends on the polarity of the compound under investigation. In general, separations are best achieved through matching the polarities of the solute and the liquid phase. Much better resolution is attained on capillary columns than on packed columns, so capillary columns are now used frequently. Two types of capillary columns, wall-coated open tubular (WCOT) and support-coated open

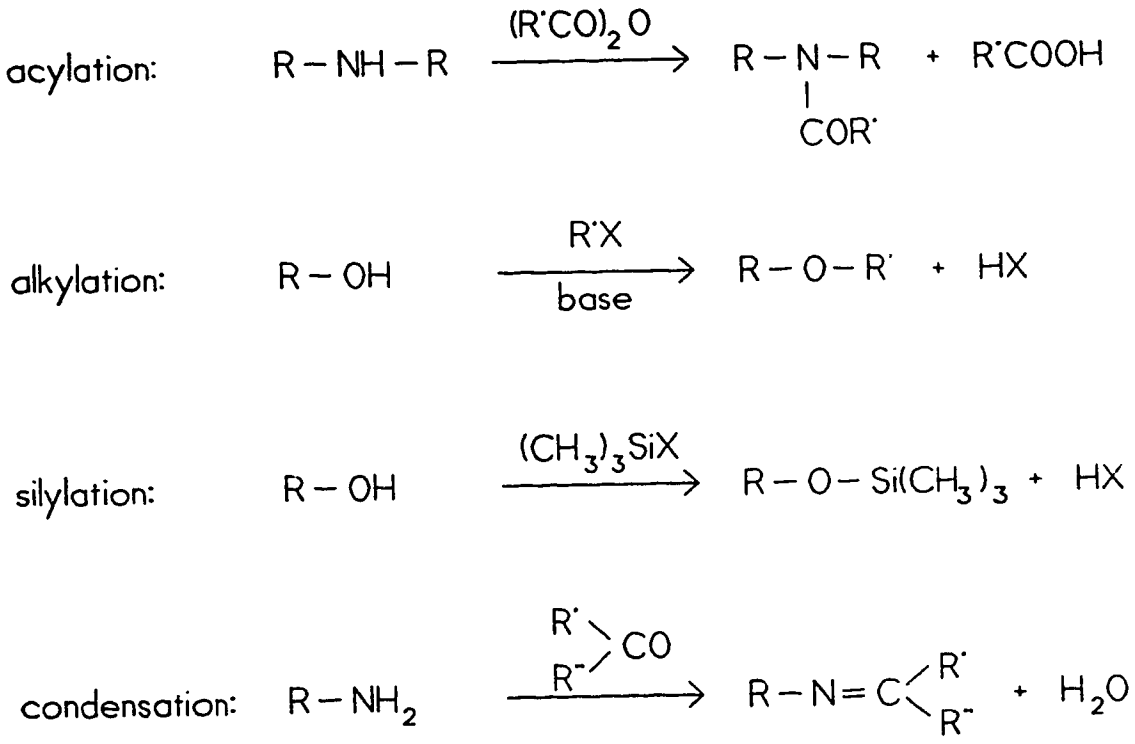


Figure 4. **Principal types of derivatization used in GC analysis. Adapted from Baker et al. (1982).**

tubular (SCOT), are available, differing in the absence or presence of a support between the liquid phase and the inside surface of the capillary tubing. Compounds eluting from a GC are detected by a variety of techniques. The choice of GC detectors depends on the properties of the analyte itself and of its derivatives. The flame ionization detector (FID) is an universal detector, while the electron capture detector (ECD) and nitrogen-phosphorous detector (NPD) are more selective and sensitive. The minimum detection limit for ECD and NPD is in the picogram range (Kline and Soine, 1984). The NPD has been extensively used for the analysis of TCAs (Cooper, 1988).

1.5.4 High Performance Liquid Chromatography (HPLC)

HPLC is a form of partition chromatography in which a liquid mobile phase is forced at high pressure through a column coated with a thin layer of stationary phase (review: Kaplan and Pesce, 1989). The differential equilibrium of the analytes between the mobile and stationary phases results in their separation. Interactions involve competition in terms of solubility. The components of a HPLC system include: (a) a solvent reservoir for the mobile phase; (b) a pump, most commonly a reciprocating piston pump, to provide the driving force for the mobile phase; (c) a sample delivery system, commonly a nitrogen-driven automated sample system; (d) a guard column between the injector and the analytical column protecting the analytical column from particulate matter or any strongly retained components of the sample; (e) an analytical column, usually of

stainless steel with a stationary phase on a support matrix; (f) some type of detector (ultraviolet, fluorescence or electrochemical); and (g) an integrator and recording device.

The most popular stationary phase support matrix for reversed phase HPLC is octadecylsilane (ODS, C-18) because of the ease with which polar biological molecules are separated. The stationary phase consists of organic moieties chemically bonded onto the support matrix, accomplished by reacting silanol groups with the ODS matrix, resulting in a surface containing octadecyl groups bound to the surface by siloxane bonds (Si-O-Si). Chemical bonding is advantageous because polar and ionic compounds are readily separated and the phase is not easily stripped with repeated use. This type of column operates most effectively at pH values between 2 and 8.

The characteristics of the highly polar water-based mobile phase include: (a) being buffered to a fixed pH value and maintained there to limit ionization of analytes; (b) the use of organic modifiers such as acetonitrile to decrease the surface tension between the stationary and mobile phases, thus decreasing the retention times of the analytes; and (c) ion-pairing reagents to decrease the analytes' polarity.

1.5.5 Analysis of Racemic Mixtures

When racemic mixtures are studied, it is often of interest to analyze the two enantiomers separately. Conventional analytical methods do not distinguish between enantiomers, but rather measure the sum of the

impurity. However, the chiral columns are more costly than conventional columns and often are not as rugged or efficient (Pasutto, 1992). Furthermore, there is often a great deal of variability from column to column (Matuszewski et al., 1990; Takahashi et al., 1990). In addition to chromatographic analysis, racemic mixtures can be analyzed using competitive binding assays (Cook, 1993). This type of analysis is performed with the use of receptors and antibodies that are enantioselective.

1.6 *In Vitro* and *In Vivo* Models

1.6.1 In Vitro Model

Drug metabolism can be studied either *in vivo*, i.e., whole body experiments, or *in vitro* where specific enzyme(s) system are isolated from human or animal tissues or organs. *In vitro* metabolism studies reveal the liver's or other organ's contribution to the metabolism of a drug substrate. The major purposes of *in vitro* drug metabolism studies are: (1) to determine in which tissues drug metabolism takes place; (2) to determine the steps involved in the formation of metabolites; and (3) to discover the components of the enzyme systems that catalyze these various steps. To achieve these objectives, investigators may choose a variety of tissue preparations and techniques, each of which has its advantages and disadvantages (Gillette, 1971).

1.6.2 *In Vivo Model*

While *in vitro* studies are required to understand individual organs' contributions to metabolism of any xenobiotic, ultimately *in vivo* metabolism studies are necessary to delineate different biotransformation pathways and to determine various factors which affect the levels of drugs and their metabolites in the body at the same time. While the use of enzyme inducers and inhibitors can aid in the identification of specific enzymes, information from such studies can be equivocal, due to the complexity of competing metabolic pathways and the dynamics of transport and elimination. One of the problems of *in vivo* studies is to identify a suitable animal model. The difficulties of extrapolation of animal data to humans has stimulated the search for suitable animal models. MSD rats were chosen in this study for the following reasons:

1. MSD rats have CYP2D1 genes predominantly expressed in the liver. The substrate specificity of CYP2D1 is similar to that of human CYP2D6 isozyme. Thus analogous metabolic profiles for TMP are expected in rats and humans.
2. Urinary and *in vitro* microsomal metabolic studies of TMP in MSD rats have shown similar metabolic pathways as in humans except for excessive C-10 (or C-11) alicyclic oxidation and hydroxylation in the rats.
3. It is an animal that is easy to handle, readily available, economical and commonly used for metabolic studies.
4. The drug-drug interactions in MSD rats are expected to be of clinical

relevance to humans.

1.7 The Drug of Interest - Trimipramine (TMP)

1.7.1 Physicochemical Properties

TMP, a tricyclic antidepressant, was approved for use in the USA in 1979, but it has been used in Europe since 1960, and in Canada prior to 1979. TMP maleate is currently marketed under the proprietary name "Surmontil". It is a white crystalline powder, melting point 140-144°C, with a molecular weight (MW) of 410.5 (TMP base, MW=294.42) [Al-Badr, 1983]. TMP maleate is moderately soluble in water and ethanol, freely soluble in chloroform, and practically insoluble in ether (Moffat et al., 1986). The common therapeutic dose of TMP is 50-300 mg/day (Moffat et al., 1986). The structure of TMP is closely related to that of IMI, with replacement of a hydrogen atom by a methyl group on the side chain carbon beta to the dimethylamino group, resulting in a chiral centre in the molecule. The chemical structure of TMP, 5-(3-dimethylamino-2-methylpropyl)-10,11-dihydro-5H-dibenz[b,f]azepine, is depicted in Figure 2. Due to the presence of an asymmetric centre, TMP exists as enantiomers and is marketed as the racemic mixture.

1.7.2 Pharmacology

TMP is as effective as other antidepressants, and its anxiolytic and sedative effects enhance its clinical utility (review: Settle and Ayd, 1980; Gastpar, 1989; Lapierre, 1989). Onset of its antidepressant action is

generally delayed for 2-4 weeks. It is still a frequently prescribed antidepressant drug, but its mechanism of action remains unknown (Hauser et al., 1985; Delini-Stula, 1986) and the relationship of plasma concentration to therapeutic response is not yet well established.

The properties of TMP differ appreciably in many respects from those of IMI, AMI and other TCAs. TMP does not inhibit presynaptic reuptake of noradrenaline (NA) and 5-HT, which makes TMP compatible with MAO inhibitors in combined treatment (Gastpar, 1989). Long-term administration of TMP to rats does not down-regulate β-adrenoceptors nor does it impair the NA-induced increase in accumulation of cAMP in cerebral tissue (Kopanski et al., 1983; Hauser et al., 1985). However, TMP does produce an increase in the brain regional levels of some monoamines and metabolites, indicating a greater synthesis rate for DA and 5-HT coinciding with an adaptive down-regulation of DA D_2 and 5-HT_2 receptors upon chronic administration in rats (Juorio et al., 1990). Unlike typical TCA drugs, TMP has no effect on suppression of REM sleep (Wiegand and Berger, 1989; Feuillade et al., 1992). The potency of TMP in inhibition of the net K^+-induced uptake of ^{45}calcium suggested the possible involvement of blockade of calcium channels in the antidepressant effect (Beauchamp et al., 1992).

In addition to being an effective antidepressant, large doses of TMP have been reported to have antipsychotic properties and have been used for the treatment of schizophrenia without inducing extrapyramidal side effects (Gastpar, 1989; Eichmeier et al., 1991). In radioligand binding

studies on rat brain, TMP showed fairly high affinity for 5-HT_2, α_1, and D_2 receptors and intermediate affinity for D_1 receptors, similar to the antipsychotic drug clozapine. TMP is also a potent histamine H_2 receptor antagonist (Alvarez et al., 1986a,b) and effective in the treatment of duodenal and gastric ulcer (MacKay et al., 1984). It also reduces joint pain and tenderness in rheumatoid arthritic patients (Macfarlane et al., 1986). These diverse pharmacological properties of TMP may be associated with some of its many active metabolites. Knowledge of the pharmacology of TMP is still fairly limited and largely ignored.

1.7.3 Pharmacokinetics

TMP is more highly bound to protein (95%) than other TCAs (80-90%). Its absolute bioavailability is low (41.4%) and very variable (17.8-62.7%) (Abernethy et al., 1984) and this variability has been attributed to a high, but individual-dependent, first-pass hepatic clearance. TMP, like other TCAs, is rapidly absorbed from the gastrointestinal tract and peak plasma levels follow a single oral dose by 2 to 4 hours (Lapierre, 1989; Köppel and Tenczer, 1988). The elimination half-life ($t_{1/2}$) of 24h for TMP is similar to that of most TCAs (Abernethy et al., 1984). Metabolism of TMP takes place mainly in liver and the drug is excreted in urine mainly as metabolites (Köppel and Tenczer, 1988; Maurer, 1989; Coutts et al., 1990 and 1991; Hussain, 1992).

Reported absolute volumes of distribution (30 l/kg), and total metabolic clearance (16 ml/min/kg) were higher than values found for other

TCAs. Unbound (intrinsic) clearance was 317 ml/min/kg (Abernethy et al., 1984), greater than would be expected on the basis of hepatic clearance of unbound drug. Assuming normal hepatic blood flow to be 1.5 l/min, the extraction ratio of TMP is 0.65 to 0.70; therefore, it is a non-restrictively cleared drug. Detailed metabolic pathways of TMP were discussed in section 1.4.3.

1.7.4 Side-Effects and Toxicity

Minor adverse effects associated with tertiary amine TCAs generally reflect the drugs' anticholinergic and CNS activities. The anticholinergic effects include dry mouth, blurred vision, constipation and urinary retention (Robinson, 1984; Horwell, 1985). Adverse CNS and neuromuscular effects occur frequently. Drowsiness, weakness, lethargy, and fatigue are also common reactions. The side effects of TMP are similar to the profiles of other tertiary amine TCAs, with some variations more characteristic of TMP. The marked sedative properties have been attributed to its high affinity for histamine H_1 receptors (Richelson and Nelson, 1984). TMP is considered to be less cardiotoxic, has minimal effect on orthostatic hypotension, and has less epileptogenic potential than other tertiary amine TCAs (Pecknold et al., 1985; Assalian et al., 1985; Cournoyer et al., 1987; Gastpar, 1989).

Toxicity effects have been associated with plasma concentrations greater than 1 µg/ml. Fatalities due to TMP overdose or due to comedication-elevated tissue and plasma concentrations of TMP have

been reported (Meatherall et al., 1983; Fraser et al., 1987), although not as frequently as with amitriptyline and some other TCAs. It has been suggested that in patients with genetically slow rates of metabolism of TMP, the parent drug and its active metabolites may accumulate gradually in the brain and heart tissue until they exceed a toxic threshold, resulting in convulsions, cardiac arrhythmias, and death (Meatherall et al., 1983). Therefore, a closely monitored therapeutic dosage is essential for optimal clinical response (Preskorn, 1989; Preskorn and Fast, 1991; Furlanut et al., 1993). Determination of the plasma concentrations of unchanged TMP and NTMP may help to prevent the acute toxicity. Measurement of hydroxylated metabolites may be important in the elderly due to possible cardiotoxicity of these metabolites and possible renal dysfunction in such patients; such measurements are particularly important in the situations where the total concentrations of parent drug (TMP) and desmethyl metabolite (NTMP) are near the toxic-fatal range (Fraser et al., 1987).

2 *RATIONALE AND OBJECTIVES*

2.1 Rationale

TMP has been marketed as an antidepressant since the 1960s in Europe and is still frequently prescribed in Europe and Canada. Unlike other TCAs, the mechanism of action remains unknown and the knowledge of the pharmacological, pharmacodynamic and pharmacokinetic properties of TMP is still fairly limited and largely ignored.

TMP's structure is similar to IMI but it has a chiral centre in the molecule and is used as a racemic drug. The difference in the side chain of TMP probably accounts for the metabolic retardation in N-demethylation, uniqueness in side-effects, receptor binding profile and its diverse pharmacological functions. As indicated earlier in sections 1.2 and 1.3, chirality can play a significant role in pharmacologic, pharmacodynamic and pharmacokinetic properties of drugs. Differences between the two enantiomers of TMP have been demonstrated in: physiological and behavioral effects in animals; affinity for DA, NA, and 5-HT receptors; inhibition of calcium channels; and antidepressant properties in humans (see section 1.2.2).

Since the initial 50:50 enantiomeric ratio of racemic TMP used may rapidly change to an unknown ratio in the chiral biological environment, pharmacokinetic and pharmacologic investigations will be only meaningful if blood and tissue concentrations of the individual enantiomers of TMP are known. Unfortunately, the determination of the ratios of the two enantiomers of TMP in human or animal biological samples has been

ignored.

In the treatment of mental illness, there is a widespread occurrence of polypharmacy. This use of multiple drugs leads to potential drug interactions. For example, FLU, a popular SSRI antidepressant, is increasingly being administered in combination with other psychoactive drugs, such as TMP. Research has shown that both FLU and its active metabolite NFLU are potent inhibitors of CYP2D6 and NFLU is also a strong inhibitor of CYP3A4 and CYP1A1 (Baumann and Rochet, 1995; Rasmussen et al., 1995; von Moltke et al, 1995). Therefore, both would affect the metabolism of other drugs metabolized by these isozymes. In fact, coadministration of FLU has now been shown to affect the metabolism and plasma levels of numerous other drugs, including TCAs, antipsychotics and benzodiazepines (Messiha, 1993; Preskorn et al., 1995).

Studies in man and MSD rats have shown that TMP is a substrate for CYP2D6, and C-2 hydroxylation, but not N-demethylation, of TMP is mediated by CYP2D6 isozyme. Research in the MSD rat also suggests that C-10 (or C-11) alicyclic hydroxylation of TMP, and C-2 aromatic hydroxylation of NTMP are under the influence of an isozyme other than CYP2D6. However, neither tissue (brain or liver) drug levels nor enantiomeric identification and quantification has been investigated in any of these studies.

In order to understand more fully the metabolic pathways of TMP and to investigate possible drug-drug interactions, a technique to measure the concentrations of each enantiomer of TMP in biological samples is

necessary. The research in this thesis should do much to improve our knowledge of metabolic and stereochemical profiles of TMP in rats, and the techniques developed could be of great utility in investigations of this and other drugs in humans.

2.2 Objectives

The primary objectives of the thesis can be summarized as follows:

1. To investigate direct and indirect GC and HPLC analytical methods for enantiomeric separation and quantification of TMP enantiomers.
2. To develop a suitable assay for analysis of the individual enantiomers and separation of them from TMP and major metabolites of TMP in biological matrices of the MSD rat.
3. Using the MSD rat as an animal model, to study metabolism of TMP and drug-drug interactions between TMP and FLU at the tissue level.
4. To study the steroselectivity of individual enantiomers of TMP in rat tissues (brain and liver) and biological fluids (plasma and urine).

3 *MATERIALS AND METHODS*

3.1 Chemicals

The chemicals used in the thesis research are summarized as follow:

Chemicals	Abbrev.	Suppliers
acetonitrile, distilled		BDH (Toronto, ON)
clomipramine HCl	CMI	Sigma (St. Louis, MO)
N-desmethyltrimipramine maleate	NTMP	Rhône-Poulenc (Montréal, PQ)
dichloromethane		BDH
N,N-didesmethyltrimipramine maleate	NNTMP	Rhône-Poulenc
diethyl ether		BDH
ethyl acetate		BDH
fluoxetine HCl	FLU	Lilly Research Lab. (Indianapolis, IN)
2-hydroxyclomipramine	2-OH-CMI	Sigma
2-hydroxydesmethyltrimipramine fumarate	2-OH-NTMP	Rhône-Poulenc
2-hydroxyimipramine HCl	2-OH-IMI	Ciba-Geigy (Mississauga, ON)
2-hydroxytrimipramine fumarate	2-OH-TMP	Rhône-Poulenc
isopropanol, HPLC grade		Fisher Scientific (Edmonton, AB)
n-butanol		Fisher Scientific
n-hexane, HPLC grade		BDH
perchloric acid, 60%		Fisher Scientific

potassium carbonate, anhydrous		Fisher Scientific
potassium bicarbonate, anhydrous		Fisher Scientific
S-(-)-trifluoroacetylprolyl chloride	(-)-TFPC	Aldrich (Milwaukee, WI)
sodium phosphate, monobasic		Fisher Scientific
sodium bicarbonate, anhydrous		Fisher Scientific
sodium phosphate, tribasic		J.T.Baker Chemicals (Toronto, ON)
sodium perchlorate, HPLC grade		Fisher Scientific
sodium carbonate, anhydrous		Fisher Scientific
sodium phosphate, dibasic		Fisher Scientific
toluene, glass-distilled		BDH
(+)-trimipramine maleate	(+)-TMP	Rhône-Poulenc
(-)-trimipramine maleate	(-)-TMP	Rhône-Poulenc
trimipramine maleate	TMP	Sigma

3.2 Instrumentation

3.2.1 GC

Gas chromatographic analysis with nitrogen-phosporus detection (NPD) was performed on a Hewlett Packard (HP) 5730A gas chromatography (Palo Alto, CA, USA) equipped with HP 3390A integrator using the splitless injection mode. Helium at a flow rate of 1 ml/min was employed as a carrier gas and the make-up gas was also helium at a flow rate of 35 ml/min. The injection port and detector temperatures were maintained at 250°C and 300°C respectively and the oven temperature was

programmed differently in each assay.

A DB-5 GC column was used in the indirect analytical method for the enantiomeric separation of TMP metabolites. It had a 5% diphenyl-95% dimethylpolysiloxane crossed-linked and bonded stationary phase, and the following dimensions: 25m x 0.25mm ID with a film thickness of 0.25 µm. The DB-5 capillary column was purchased from J&W Scientific (Folsom, CA, USA).

The direct GC analytical method for enantiomeric separation of TMP and its metabolites was attempted on a Chiralsil-Val capillary column, 25m x 0.25mm ID, purchased from Mandel Scientific (Guelph, ON, Canada). Chirasil-Val is a silicone-based polymer with a chiral functionality incorporated into the "backbone" to provide the separation of optical enantiomers.

3.2.2 HPLC

Liquid chromatography was performed with a Waters solvent delivery system (Model 510) equipped with a Waters variable wavelength ultraviolet detector (Model 481), a Hewlet Packard integrator (Model 3392A) and a Waters automatic sample injector (Model WISP-710B).

Enantiomeric analysis was performed by the reversed phase, direct analytical method at ambient temperature using a 25cm x 4.6mm ID Chiralcel OD-R column with 10 µm particle size (Daicel Chemical, Japan) and fitted with a C-18 guard column. The Chiralcel column was packed with tris(3,5-dimethylphenyl carbamate)cellulose on a silica gel base to provide

chiral separation.

3.2.3 *Tissue Homogenizer*

A combination of a TRI-R S63C (TRI-R Instruments, Rockville Centre, NY, USA) variable speed laboratory motor with a Teflon® glass pestle and a glass grinding tube was used for homogenizing tissue samples at a temperature of 0-4 °C.

3.2.4 *Centrifuges*

A Sorvall GLC-2b or Sorvall GLC-1 General Laboratory Centrifuge (Dupont Instruments, Wilmington, DE, USA) was used for low-speed, small volume centrifugations.

3.2.5 *Shaker-Mixer*

Two types of vortex-shakers were used: an Ika-Vibrax VXR2 Shaker (Janke and Kundel GMBH and Co., Staufen, Germany) and a Thermolyne Maxi Mix vortex mixer (Thermolyne Corp., Dubuque, LO, USA).

3.2.6 *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 involved the combination of centrifugal force

and vacuum.

3.3 Glassware Cleaning

All glassware was initially rinsed with tap water. Further cleaning was completed using a Miele Electronic 6715 dishwasher and biodegradable Sparkleen (Fisher Scientific). For test tubes, an additional cleaning step was added. The tubes were sonicated (Ultra-sonic cleaner, Mettler Electronics) in a solution of Decon 75 concentrate (BDH Chemicals). Tubes were then cleaned in the dishwasher and finally rinsed with distilled water. All glassware was then air-dried in a mechanical convection oven (Model 28, Precision Scientific Group, Chicago, IL, USA).

3.4 Animal Handling

The MSD rats (200-300 g) used for all experiments in this thesis were obtained from Bio-Science Animal Services, Ellerslie, Alberta, Canada. The animals were housed in pairs in plastic cages on cedar chip bedding in a temperature controlled room (21 °C). They were subjected to a 12h light/dark cycle (lights on at 07:30 am) throughout all experiments. Water and lab chow were provided *ad libitum*. The lab chow (Lab-Blox feed, Wayne Feed Division, Continental Grain Company, Chicago, IL, USA) was 4.0% (min) crude fat, 4.5% (max) crude fibre and 24% (min) crude protein.

During urine collection time periods, the rats were housed individually in plastic metabolic cages (Nalgene, model E1000, Maryland Plastic Inc., New York, NY, USA), designed for separate collection of urine and feces.

Procedures involving the use of animals were approved by the University of Alberta Health Sciences Animal Welfare Committee and were conducted according to the guidelines put forth by the Canadian Council on Animal Care.

3.4.1 Administration of Drugs

Animals were randomly allocated to drug or vehicle treatment groups. All drugs were administered intraperitoneally (i.p.) and in a solvent volume of 2 ml/kg body weight. The drugs, rac-TMP and rac-FLU, were dissolved in N,N-dimethylsulfoxide (DMSO)/water (20:80 v/v). DMSO was necessary to dissolve TMP and FLU in water at the required concentration. In each study the control animals were injected with the corresponding vehicle solution.

3.4.2 Sample Collection and Storage

Urines were separately collected for 24h in the metabolic cages after drug administration and stored at -20°C until analyzed. For tissues and plasma sample collections, animals were sacrificed by guillotine decapitation at specific times following drug administration. The blood samples were collected from the neck region into Vacutainer tubes containing EDTA and centrifuged later at 1000x g for 10 min to obtain plasma. The brains were removed and immediately frozen in dry ice-cooled isopentane. Livers were removed and immediately placed on dry ice. Until the time of analysis, brain samples were stored at -80°C and liver and

plasma samples were stored at -20°C.

3.5 Metabolic Studies of TMP in Rats -- Experimental

3.5.1 Drug Treatment

For the metabolic study of TMF, 15 rats were injected with rac-TMP (10 mg/kg) and 9 rats received vehicle. At times of 1h, 2h, and 4h post-TMP injection, five rats from the drug treatment group and 3 rats from the control group were sacrificed.

3.5.2 Development of a GC Method

3.5.2.1 Indirect (Derivatization) GC Method

An aliquot (100 µl) of aqueous stock solutions (0.01 mg/ml) of the racemic standards NTMP, 2-OH-TMP, 2-OH-NTMP and FLU was placed in 1 ml water in a screw-cap culture tube (16x125 mm). To each sample was added a fixed amount of the internal standard IPR (500 ng). Each solution was basified to a pH value of 11 by adding 40 µl of 25% potassium carbonate solution. The basic solution was vortex-mixed with 6 ml of ethyl ether: dichloromethane (13/11 v/v) for 10 min and centrifuged for 5 min. The top (organic) layer was transferred to a smaller screw-cap culture tube (13x100 mm) and evaporated under a stream of nitrogen at 50°C in a water bath. To the dry residue was added 100 µl of 0.004M (-)-TFPC dichloromethane: toluene [40 µl of 0.1M (-)-TFPC in dichloromethane made up to 1 ml with toluene]. Each reaction was allowed to proceed at room temperature or 60°C for 30 min. Higher reaction temperature at 60°C for

30 min was also tried for 2-OH-TMP and 2-OH-NTMP. After evaporation of the organic solvent, the residue was redissolved in 200 µl of toluene. An aliquot (1 µl) of this solution was analyzed using a GC capillary column (DB-5, 25m x 0.25mm ID, and film thickness 0.25 µm). The column temperature was initially set at 100°C for 2 min and then programmed to increase to 280°C at a rate of 8°C/min and held at that temperature for 8 min.

3.5.2.2 Direct GC Method

Following a similar extraction procedure as in section 3.5.2.1 except for the derivatization step with (-)-TFPC, the free base forms (ca. 3 nmoles) of racemic TMP, NTMP, 2-OH-TMP, 2-OH-NTMP, FLU and NFLU were prepared in 200 µl of toluene. An aliquot (1 µl) of this solution was analyzed on a GC chiral column (Chiralsil-Val, 25m x 0.25mm ID). The column temperature was initially set at 100°C for 2 min and programmed to increase to 170°C at a rate of 35°C/min; the temperature of 170°C was held for 2 min and increased again to 210°C at 8°C/min and held at that level for 32 min.

3.5.3 Development of a Direct HPLC Method

3.5.3.1 Retention Time Study

Stock solutions of each authentic racemic drug (TMP, NTMP, NNTMP, 2-OH-TMP, and 2-OH-NTMP) and of the internal standard CMI were prepared in water at a concentration of 0.1 mg/ml (as free base).

These solutions were stored at -4°C and protected from light. Appropriate aliquots of the stock standards were diluted 10x or 100x and used as working solutions. Proportions of working solutions of each drug were mixed for HPLC analysis. In initial experiments, these solutions were prepared by someone else in the laboratory so that I was blind to which enantiomer was in each tube and had to correctly identify each on the basis of retention times on the HPLC. The HPLC conditions included a chiral reversed phase column (Chiralcel OD-R, 25cm x 4.6mm ID) and a UV detector at 254 nm. The mobile phase was 0.5 M aqueous sodium perchlorate/ acetonitrile, 60/40 (v/v), adjusted to pH 5.0, pumped at a flow rate of 0.3 ml/min. The injection range of each racemic drug was 5-20 ng on column with an injection volume of 10-30 µl.

3.5.3.2 Concentration-Absorption Relationship Study

UV spectra of each racemic standard (TMP, NTMP, 2-OH-TMP, 2-OH-NTMP and CMI) were recorded over the 200-400 nm range at a concentration of 0.01 mg/ml (as free base) in water in an LKB Biochrim Ultraspec II spectrophotometer. Calibration curves were prepared by using known, varying amounts of racemic standards (equivalent to 50-1,200 ng of the free base) in water. A fixed amount (250 ng) of CMI was added each sample tube. An aliquot (30 µl) of the resulting mixture was analyzed by HPLC at 254 nm. Peak area ratios (TMP or metabolite relative to CMI) were plotted against concentrations of TMP or metabolite to produce calibration curves.

3.5.4 *Enantiomeric Assay for Biological Samples*

3.5.4.1 Extraction and Isolation Method

Rat brain and liver tissue were partially thawed, weighed and homogenized in five volumes of ice-cold water. A 2 ml portion of this homogenate was placed in a screw-cap culture tube (16x125 mm). To this homogenate was added CMI (250 ng). The samples were then basified by adding 400 µl carbonate buffer solution (1.0M, pH 9.8). The basic solution was vortex-mixed with 6 ml of 2% butanol in hexane for 10 min in an Ika Vibrex VXR vortex-mixer and centrifuged for 5 min at 1000 g in a benchtop centrifuge. The top (organic) layer was transferred to a smaller screw-cap culture tube (13x100 mm) and reduced to 0.5-1 ml in a water bath at 50°C under a gentle stream of nitrogen. A 200 µl volume of 0.05 M phosphate (pH 3.0) buffer was added and the mixture was shaken for 10 min in an Ika Vibrex VXR vortex-mixer before being centrifuged for 5 min at 1000x g in a benchtop centrifuge. The contents were then transferred to a small plastic conical vial (10x30 mm, 1.5 ml) and centrifuged for 2 min at a Microcentaur mini-benchtop centrifuge. From the bottom (aqueous) solution two 75 µl portions were pipetted into automatic sample vials for direct chiral HPLC analysis. All plasma and urine samples were thawed completely and 2 ml volumes were used in the assay procedures described above for tissue samples.

3.5.4.2 Recovery Study

Known amounts (100 and 300 ng as free base) of racemic standards of TMP, NTMP, NNTMP, 2-OH-TMP and 2-OH-NTMP were mixed with 2 ml of drug-naive rat biological matrices and worked up as described in section 3.5.4.1. using 6 ml of 2% n-butanol in n-hexane as extraction solvent. To the final acidic extract (200 µl), a fixed amount of external standard CMI (250 ng in 25 µl water) was added and the components of the solution were analyzed by the HPLC method.

In the studies of pH effect on extraction efficiency, a fixed amount (500 ng) of each standard TMP, NTMP, NNTMP, 2-OH-TMP, 2-OH-NTMP and CMI was added to 2 ml of water. The resulting solution was adjusted to a specific pH value (6.0, 8.0, 9.0, 9.8, 11.8 or 12.6). Each solution was worked up as described in section 3.5.4.1. To the final 200 µl acidic extract, an external standard (2-OH-CMI, 200 ng) was added and the components of the solution were analyzed by HPLC. The buffer solutions or bases used for adjusting pH values were: 1.0M phosphate buffer (pH 6.0 and 8.0); 1.0M carbonate buffer (pH 9.0 and 9.8); 1.0M sodium carbonate (pH 11.8); and 10% trisodium phosphate (pH 12.6).

In the studies of solvent effects on extraction efficiency, various solvents were used to replace 2% butanol in hexane. The same procedure as just described in this section was followed except the pH value was maintained at 9.8. The solvents used were: ethyl acetate; toluene; 25% and 50% ethyl acetate in toluene; hexane; and 2%, 5% and 10% butanol in hexane.

3.5.4.3 Evaluation of the Enantiomeric Assay

The calibration curves were constructed by preparing a series of concentrations of known, varying amounts (equivalent to 50-1,200 ng of the free base) of racemic TMP and its major metabolites NTMP, NNTMP, 2-OH-TMP and 2-OH-NTMP in 2 ml of drug-naive rat biological matrices (tissue homogenates, urine, and plasma). A fixed amount (250 ng) of CMI was added to each sample tube. The resulting mixture was worked up as described in section 3.5.4.1. Peak area ratios (TMP or metabolite relative to CMI) were plotted against concentrations of TMP or metabolite to produce calibration curves that were linear over the desired concentration range. The quantification of each enantiomer of TMP and its metabolites in the biological matrices extract from TMP-treated rats was determined from the [illegible]tion of the straight line derived from each calibration curve.

3.6 TMP-Fluoxetine (FLU) Drug Interactions in Rats

3.6.1 Drug Treatment

Twelve rats were first injected with rac-TMP (20 mg/kg). One hour later, 6 of these rats were injected with either vehicle or rac-FLU (10 mg/kg). In parallel, a control group of 6 rats were first injected with vehicle and 1h later 3 of these rats received either vehicle or FLU (10 mg/kg). The rats were housed individually for 3 days in metabolic cages for urine collection. The animals were then housed in pairs in plastic cages for a washout period of 14 days. The same animals were then injected randomly

with the same treatment regimen as just described. All animals were then sacrificed at 5h after the initial TMP injection.

3.6.2 *Enantiomeric Assay*

The same assay procedures as described in section 3.5.4 were used for the analysis of enantiomers of TMP and NTMP using the direct chiral HPLC method.

3.7 Statistical Analysis

Statistical analysis consisted of analysis of variance (ANOVA) followed by independent t-tests in the case of single pair comparisons. A two-tailed probability distribution was used and the general convention of a probability value of $p<0.05$ was used to establish statistical significance.

4 *RESULTS AND DISCUSSION*

4.1 Attempts to Develop a GC Method

Using a technique similar to the one used in the separation and quantification of the enantiomers of both FLU and NFLU by Torok-Both et al. (1992), the free base forms of racemic standards of NTMP, 2-OH-TMP and 2-OH-NTMP were isolated and reacted with (-)-TFPC as described in section 3.5.2.1. Racemic NTMP gave two diastereomeric peaks of (-)-TFPC derivatives on a DB-5 column with retention times of 26.13 and 26.29 min. Unlike NTMP, racemic 2-OH-TMP and 2-OH-NTMP failed to show pairs of (-)-TFPC derivatives under similar conditions and degradation was observed in the GC traces. This result is possibly due to the instability of these (-)-TFPC ester derivatives under GC conditions. Eap et al. (1994) have reported that good derivatization with trifluoroacetic acid anhydride (TFAA) was obtained with demethylated TMP metabolites (NTMP and NNTMP) but not with the hydroxylated metabolites (2-OH-TMP and 2-OH-NTMP). They also reported derivatization of all TMP metabolites with pentafluoropropionic acid anhydride (PFPA), but the esters of the hydroxy metabolites were unstable under GC-MS conditions.

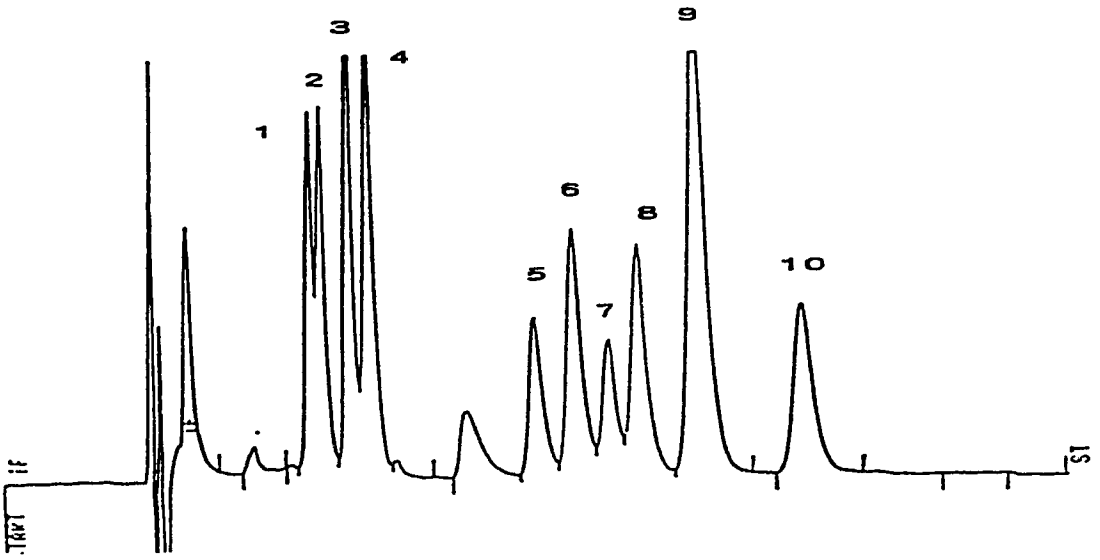
TMP is a tertiary amine and possesses no active N-H (as in demethylated metabolites) or phenolic OH groups (as in hydroxy metabolites) that would react directly with (-)-TFPC to form amide or ester diastereomers to be separated on the achiral GC column. On the other hand, the direct non-derivatization GC method using a Chiralsil-Val chiral column as described in section 3.5.2.2 failed to provide separation of the

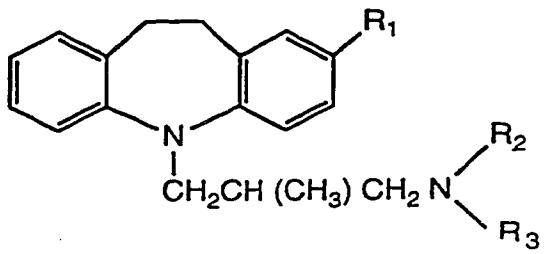
enantiomers of TMP and its metabolites (NTMP, 2-OH-TMP and 2-OH-NTMP). Another derivatizing agent, (-)-menthyl chloroformate [(-)-MC)], has been used successfully for the resolution of the enantiomers of warfarin (Jeyaraj and Porter, 1984) by derivatizing the enolic OH group in warfarin and analyzing the derivative on an achiral GC column. This derivatizing agent may be worthwhile trying with TMP, but regardless of what the outcome might be, separate derivatizing procedures and/or separate GC analyses will be inevitable for the analysis of TMP and its metabolites. Therefore, it was decided that a direct chiral HPLC method should be developed.

4.2 Development of a Direct Chiral HPLC Method

TMP and its major metabolites, NTMP, NNTMP, 2-OH-TMP and 2-OH-NTMP, all exist as pairs of enantiomers. It would be time-saving if all isomers could be analyzed in one single analysis. A novel direct non-derivatization reversed phase HPLC method has been developed in this thesis project to separate simultaneously the enantiomers of TMP and its major metabolites, NTMP, NNTMP, 2-OH-TMP and 2-OH-NTMP.

TMP, NTMP, NNTMP, 2-OH-TMP and 2-OH-NTMP were separated in a single chromatogram within 61 min using the conditions described in section 3.5.3.1. Each racemic standard gave a pair of enantiomeric peaks as illustrated in Figure 5. Only the (+)- and (-)- optical isomers of TMP





Peaks	Compound	Isomer	R_1	R_2	R_3
1, 2	2-OH-NTMP	1, 2	OH	CH_3	H
3, 4	2-OH-TMP	1, 2	OH	CH_3	CH_3
5, 7	NNTMP	1, 2	H	H	H
6, 8	NTMP	1, 2	H	CH_3	H
9, 10	TMP	(+), (-)	H	CH_3	CH_3

Figure 5: HPLC separation of the enantiomers of TMP and its metabolites using a Chiracel OD-R column. Only the (+)- and (-)-enantiomers of TMP were identified by comparison with authentic samples. Other virtually pure isomers were not available. Peak #9 was increased by the addition of (+)-TMP standard.

could be identified unequivocally because authentic samples were available. Enantiomer standards of the metabolites were not available and the peaks could only be identified as isomer-1 and isomer-2 according to their retention time order in the chromatogram. The relative peak area ratios at an equimolar concentration of 10 μM was; 2-OH-NTMP:2-OH-TMP:NNTMP:NTMP:TMP = 0.91:1.35: 0.66:1.12:1.0, respectively. The chromatographic parameters, retention time (t), capacity factor (K′), relative resolution (R_s) and selectivity (α) between each pair of enantiomers and the equations for their determination are shown in Table 1.

4.2.1 Chromatographic Parameters Study

The separation between NNTMP-2 and NTMP-1 isomers (peak 6 and 7 in Figure 5) was acceptable, with a resolution value of 1.2 using the chromatographic conditions described in section 3.5.3.1. Increasing the flow rate of the mobile phase from 0.3 to 0.5 ml/min decreased the retention time of the last peak, (-)-TMP, 30% from 61.0 to 42.3 min. However, the 2-OH-NTMP enantiomers (peaks 1 and 2) and NNTMP-2 and NTMP-1 isomers (peaks 6 and 7) overlapped significantly.

Various organic modifiers were used in combination with 0.5M sodium perchlorate for the preliminary retention time study. Different proportions [10, 20, 30, 40, and 50% v/v] of methanol, ethanol and acetonitrile were added to the mobile phase. It was found that 40/60 (v/v) of acetonitrile/0.5 M aqueous sodium perchlorate gave the best resolution and a reasonable

Table 1: HPLC chromatographic parameters of TMP and its metabolites. Parameters: retention time (t); resolution (R_s); capacity factor (K′); and selectivity (α). The values shown represent the means of 4 experiments.

$R_s=(t_2-t_1)/[1/2(W_1+W_2)]$; $K'=(t-t_o)/t_o$; $\alpha=K'_2/K'_1$, where the symbols are as follows: peak width of the enantiomer isomer-1 (W_1) and isomer-2 (W_2), elution time of non-retained compound (t_o), and capacity factor of the enantiomer isomer-1 (K'_1) and isomer-2 (K'_2).

Compound	Isomer	t (min)	R_s	K′	α
2-OH-NTMP	1	22.14	0.63	1.2	1.1
	2	23.08		1.3	
2-OH-NTMP	1	25.26	0.91	1.5	1.1
	2	26.85		1.7	
NNTMP	1	39.95	2.90	3.0	1.2
	2	45.70		3.6	
NTMP	1	43.07	2.10	3.3	1.2
	2	48.20		3.8	
TMP	(+)-	52.83	3.00	4.3	1.2
	(-)-	61.06		5.1	

retention time span. Isopropanol, 2% in 40/60 CH_3CN/0.5M $NaClO_4$ helps to narrow the peak shapes of NNTMP, NTMP and TMP, particularly if the column has been used over a period of time.

The addition of the ion-pairing agent triethylamine 0.4%(vol) to the mobile phase at pH 5.0 extended the retention time of (-)-TMP from 61.0 to 73.3 min, and gave background interferences in the baseline. Decreasing the concentration of $NaClO_4$ from 0.5 M to 0.25 M markedly decreased the resolution of all enantiomers, while increasing the concentration from 0.5 M to 1.0 M created a problem in column pressure, which increased from 300 psi to 500 psi at a flow rate of 0.3 ml/min (the manufacturer recommends less than 430 psi for maximum column life). Adjusting the pH value of the mobile phase from pH 5.0 to 7.0 increased the retention time of (-)-TMP 32% to 82 min but did not markedly improve the resolution of all peaks.

4.2.2 Linear Regression Study

Reported UV wavelength values used in HPLC analyses of TMP and its metabolites vary widely; typical examples are 254 nm (Pok Phak et al., 1986; Haupt et al., 1993), 205 nm (Fraser, 1987), 210 nm (Eap et al. 1992b), and 285 nm (Haupt et al. 1993). In order to select the best single wavelength for simultaneous quantification of TMP and its metabolites in a sample matrix, UV absorption spectra over the range 200-400 nm were recorded for TMP, NTMP, 2-OH-TMP, 2-OH-NTMP and CMI (Figure 6). The maximum absorption wavelength (λ_{max}), absorbance (A) and measured molar absorptivities (ξ) are listed in Table 2.

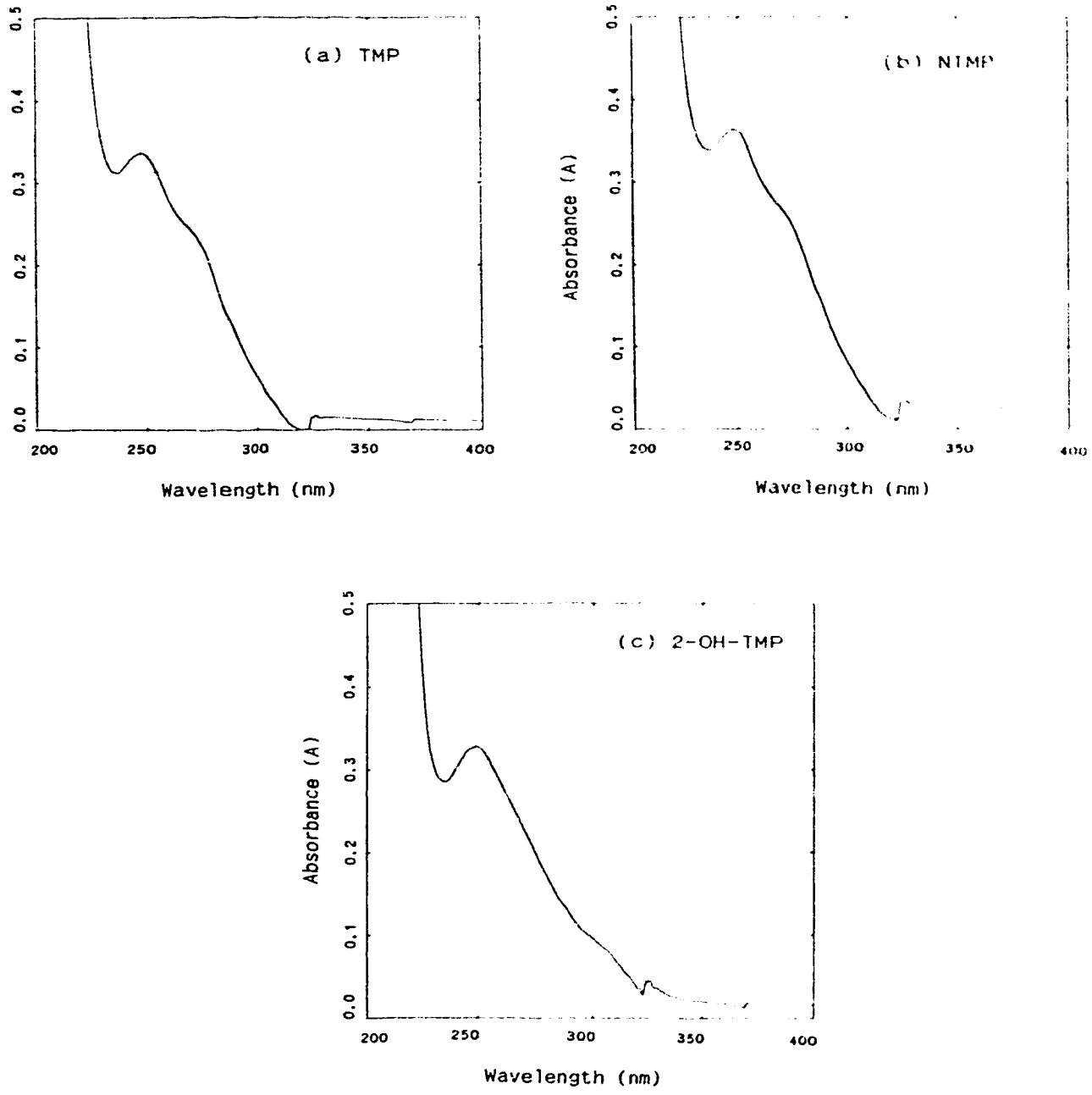
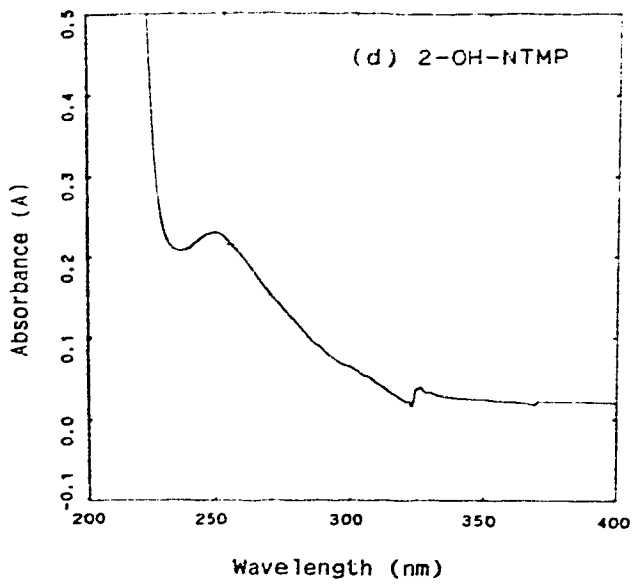


Figure 6: UV absorption spectra of rac-TMP, NTMP, 2-OH-TMP, 2-OH-NTMP and CMI (0.01 mg/ml in water).



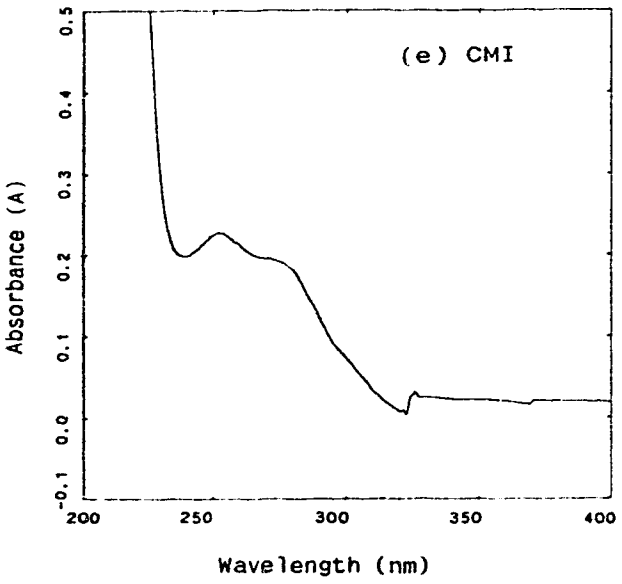


Figure 6: (continued) UV absorption spectra of rac-TMP, NTMP, 2-OH-TMP, 2-OH-NTMP and CMI (0.01 mg/ml in water).

Table 2: UV absorption parameters of TMP and its metabolites.

Parameters: maximum absorption wavelength (λ_{max}); absorbance (A); and molar absorptivity (ξ).

$A=\xi cd$, where: c=molar concentration (mole/l) and d=path length (1 cm).

Parameter	TMP	NTMP	2-OH-TMP	2-OH-NTMP	CMI
Conc. (mg/ml)	0.010	0.010	0.0083	0.0072	0.010
λ_{max} (nm)	248.0	247.5	248.8	248.1	252.3
A	0.336	0.364	0.328	0.229	0.227
ξ (l/mole/cm)	9,892	10,206	12,266	9,427	7,146

As a rule of thumb, maximum linearity in a UV concentration-absorption relationship (Lambert-Beer Law) can be achieved by selecting a peak wavelength where sample absorptivity changes little over a short wavelength range. It was found that 254 nm was the best single wavelength which gave both high sensitivity and maximum linearity for TMP and all its metabolites. Even though much higher (>3-fold) sensitivity (absorbance) is produced at shorter wavelengths (200-220 nm region) for all compounds, the linearity is not as good as at 254 nm. The calibration curves of TMP and its metabolites were thus constructed using 254 nm as the detection wavelength; all calibration curves gave a linear regression r^2 value greater than 0.99 over the concentration range of 50-1,200 ng.

4.3 The HPLC Assay for Enantiomers of TMP in Biological Samples

The HPLC method developed in section 4.2 was used to quantify the enantiomers of TMP and NTMP-2 in extracts of rat biological samples (brain, liver, plasma and urine). The extraction and isolation protocol described in section 3.5.4.1 was validated by the linearity of calibration curves, absolute recoveries (%), and day-to-day and within-day variation (Table 3). The parameter precision, expressed as coefficient of variation (CV%), is a measure of the distribution of a number of repeated measurements of the same sample around the mean value. The parameter accuracy expressed in %, is a measure of how close a measured value of a known sample is to its actual value. Except for the hydroxylated metabolites (2-OH-TMP and 2-OH-NTMP), the assay provides good linear

Table 3: Validation of the enantiomeric assay (n=5). Racemic standards of 50-1,200 ng were used in the linear regression study. The recovery study was conducted at 300 ng, and variation studies were measured at 100 ng and 300 ng in 2 ml biological matrices. The limit of detection is based on a signal/noise peak height ratio greater than 3:1.

(for each enantiomer)	TMP	NTMP	NNTMP	2-OH-TMP	2-OH-NTMP
Linear Regression, r^2 (25-600 ng)	>0.98	>0.99	>0.98	>0.85	>0.77
Recovery % : (150 ng)	86	84	70	57	15
<u>Within-day Variation</u>					
Precision: 50 ng	7	9	10	9	13
(CV %)150 ng	5	7	6	6	10
Accuracy (%): 50 ng	[illegible]	91	88	87	58
150 ng	94	96	93	94	84
<u>Day-to-day variation</u>					
Precision: 50 ng	13	15	15	11	42
(CV%) 150 ng	8	3	6	6	33
Accuracy (%): 50 ng	87	85	83	79	57
150 ng	89	93	94	93	81
Limit of detection: on-column (ng)	1.5	1.0	2.0	0.5	0.7

regressions, with r^2 >0.98 over a concentration range of 25-600 ng for each enantiomer of TMP and its demethylated metabolites (NTMP and NNTMP). The low recoveries of 2-OH-TMP and 2-OH-NTMP (57% and 15%, respectively) and poor linearity (r^2 values of 0.85 and 0.77, respectively) are probably due to their amphoteric properties, which result in incomplete and inconsistent extraction from the aqueous solution at pH 9.8. For TMP, NTMP and NNTMP at concentrations of 50 and 150 ng, the within-day precisions (n=5) were within 10% and 7%, respectively of the mean values and the accuracy measurements were within 15% and 7% of the actual values. The day-to-day precisions (n=5) were within 15% and 8% of the mean values, and the accuracy measurements were within 17% and 11% of the actual values. The absolute recoveries (%) of 150 ng of TMP and its N-demethylated metabolites, NTMP and NNTMP, were 86%, 84% and 70%, respectively.

Calibration curves generated on different days (n=5) were reproducible, with mean slopes (± standard deviations) for (+)-TMP and (-)-TMP of 200.2 ± 18.3 and 198.1 ± 18.7, respectively. The mean y-intercepts (± standard deviations) were 1.7 ± 11.4 and -2.0 ± 4.3, respectively. The mean slopes (± standard deviations) for (+)-NTMP and (-)-NTMP were 223.2 ± 13.1 and 212.0 ± 2.4, respectively, and the mean y-intercepts (± standard deviations) were -1.0 ± 2.6 and 0.5 ± 5.4, respectively.

4.3.1 Solvent and pH Effects on Extraction Efficiency

The extraction solvents and pH values used in the TMP literature vary individually. Examples are: ether, pH 10 (Pok Phak et al., 1986); ethyl acetate:heptane (20:80), pH 9.8 (Suckow and Cooper, 1984); dichloromethane:isopropanol (9:1), pH 10 (Köppel and Tenczer, 1988); dichloromethane:isopropanol:ethyl acetate (1:1:3), pH 8-9 (Maurer, 1989); dichloromethane:ether (11:14), pH 8.5-9.0 (Coutts et al. 1990); hexane:amyl alcohol (98:2), pH 14 (Abernethy et al. 1984); and isoamyl alcohol:hexane (2:98), pH 11 (Gulaid et al. 1991).

Before the extraction and isolation protocol for TMP and its metabolites as described in section 3.5.4.1 was finalized, different solvents and extraction pH values were tested. Toluene gave the worst gel formation problem and hence incomplete extraction upon shaking with biological matrices; the addition of ethyl acetate (25-50%) to the toluene helped suppress gel formation while higher concentrations created interference peaks in the chromatogram baseline. Hexane by itself also gave gel formation, and 2% n-butanol in hexane was the best combination for preventing gel formation and giving clean baselines and good recoveries (Table 3).

In the study of pH effects on extraction efficiency, it was found that pH 9.8 was the best for extraction of TMP, its metabolites and CMI in a sample mixture (Table 4). TMP and CMI, relatively nonpoplar compounds, showed no difference in extraction efficiencies in pH range 6.0-12.6. The more polar the metabolite is, the narrower the pH window for good extraction

Table 4: The effect of pH value on extraction efficiencies for TMP and its metabolites. Samples of 500 ng of each racemic standard and CMI in 2 ml water were extracted with 6 ml butanol/hexane (2/98 v/v) at different pH values. A fixed amount of external standard, 2-OH-CMI 200 ng, was then added in the final acid extract and the solution was analyzed by HPLC. Data shown are peak area ratios of TMP and its metabolites relative to external standard, 2-OH-CMI (n=2).

pH	2-OH-NTMP	2-OH-TMP	NNTMP	NTMP	TMP	CMI
6.0	0.00	0.21	0.34	0.51	1.05	0.95
8.0	0.00	0.76	0.71	0.76	1.04	0.95
9.0	0.65	0.80	0.74	0.79	1.00	0.92
9.8	0.84	0.72	0.73	0.78	1.07	0.94
11.8	0.66	0.75	0.67	0.82	1.03	0.88
12.6	0.28	0.71	0.63	0.74	1.05	0.93

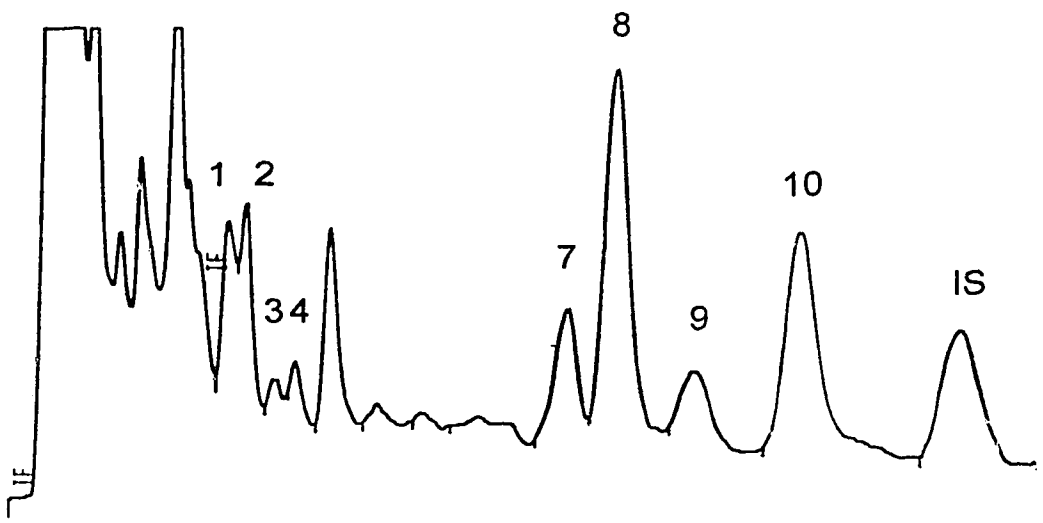
efficiency. The optimal pH value for extraction of 2-OH-NTMP was 9.8, while higher or lower pH values caused dramatic reductions in extraction efficiency. The less polar metabolite 2-OH-TMP showed a broader extraction pH window (8.0-11.8), with a peak efficiency at pH 9.0.

4.4 Metabolic Studies of TMP in Rats

In rats treated only with rac-TMP (10 mg/kg, ip), the major compounds that can be identified 1-5 h post-injection in liver, brain, plasma or in 0-24 h urine were TMP, NTMP, NNTMP, 2-OH-NTMP and 2-OH-TMP. However, the concentrations of (+)- and (-)-enantiomers of each compound were quite different among biological matrices. Typical HPLC chromatograms of extracts of brain (1 h), liver (1 h), plasma (5 h), and urine (0-24 h) after TMP injection are illustrated in Figure 7.

The quantities of (+)-TMP and (-)-TMP and NTMP-2 enantiomers in the biological matrices were determined from calibration curves and are listed in Table 5. A graphic presentation of the data from Table 5 is shown in Figure 8. It is noteworthy that the peak concentrations (C_{max}) of (+)-TMP, (-)-TMP and NTMP-2 were all attained by 1 h in both brain and liver. At each time interval (1, 2, and 4 h), the levels (ng/g tissue) of parent drug [(+)-and (-)-TMP] were much higher in brain than in liver, 8-9 fold and 2-3 fold, respectively. [Similar patterns have been reported for imipramine after administration to rats -- Bickel et al. (1983); Daniel et al. (1982).] In contrast, the concentrations of the desmethylated metabolite, NTMP-2, were found to be 2-3-fold greater in liver than in brain at 1, 2, and 4 h post-

(a) Drug-treated liver at 1 h



Drug-naive liver

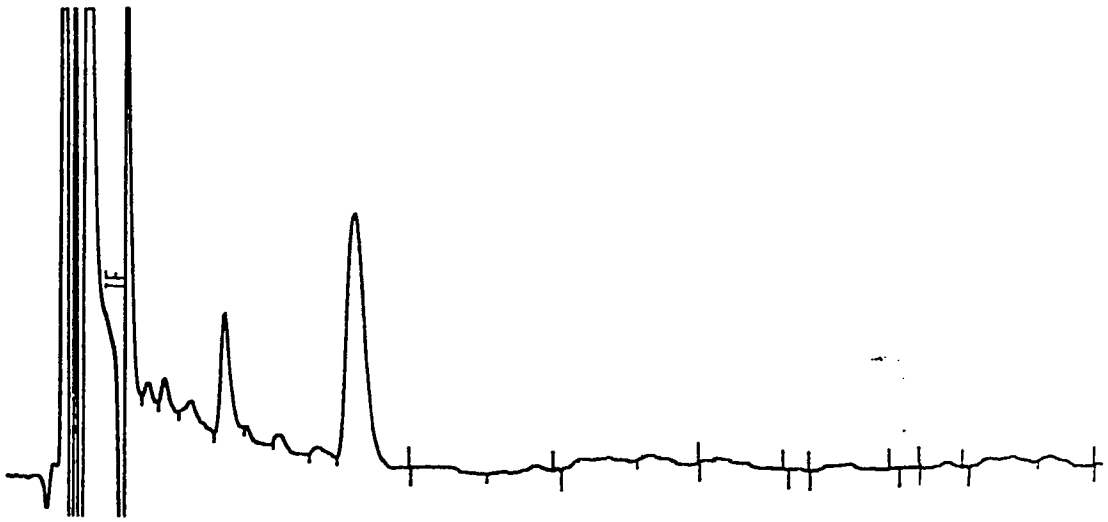
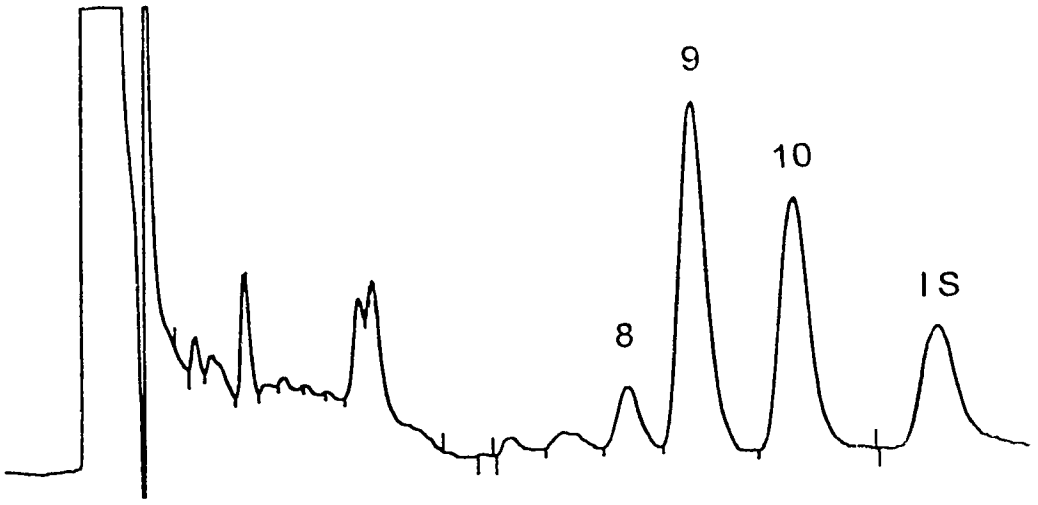


Figure 7: Typical HPLC chromatograms of extracts from biological matrices after administration of rac-TMP (10 mg/kg, ip) in rats: (a) liver at 1 h; (b) brain at 1 h; (c) plasma at 5 h; (d) urine at 0-24 h. Peaks: 1&2 (2-OH-NTMP), 3&4 (2-OH-TMP), 5&7 (NNTMP), 6&8 (NTMP), 9 [(+)-TMP], and 10 [(-)-TMP]. Corresponding traces from extracts of biological matrices from drug-naive rats are also shown.

(b) Drug-treated brain at 1 h



Drug-naive brain

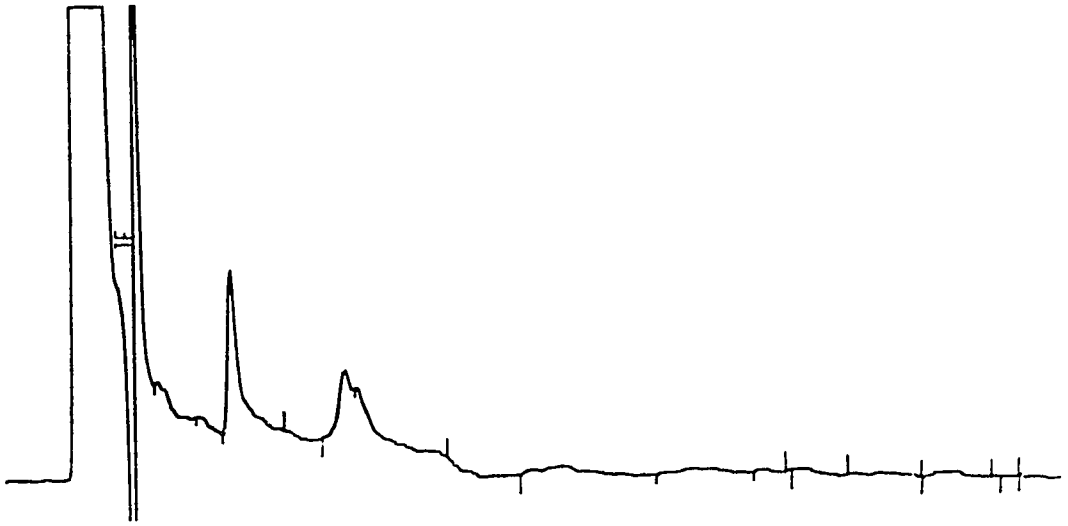
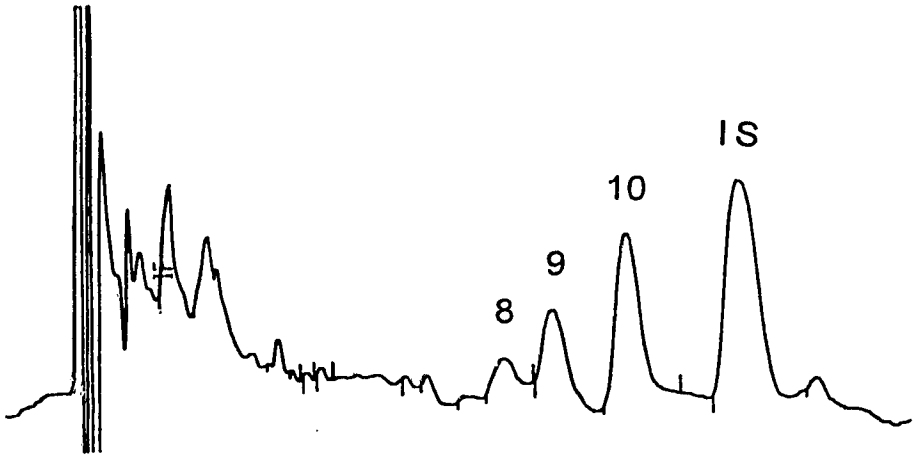


Figure 7: continued. Typical HPLC chromatograms of extracts from biological matrices after administration of rac-TMP (10 mg/kg, ip) in rats: (a) liver at 1 h; (b) brain at 1 h; (c) plasma at 5 h; (d) urine at 0-24 h. Peaks: 1&2 (2-OH-NTMP), 3&4 (2-OH-TMP), 5&7 (NNTMP), 6&8 (NTMP), 9 [(+)-TMP], and 10 [(-)-TMP]. Corresponding traces from extracts of biological matrices from drug-naive rats are also shown.

(c) Drug-treated plasma at 5 h



Drug-naive plasma

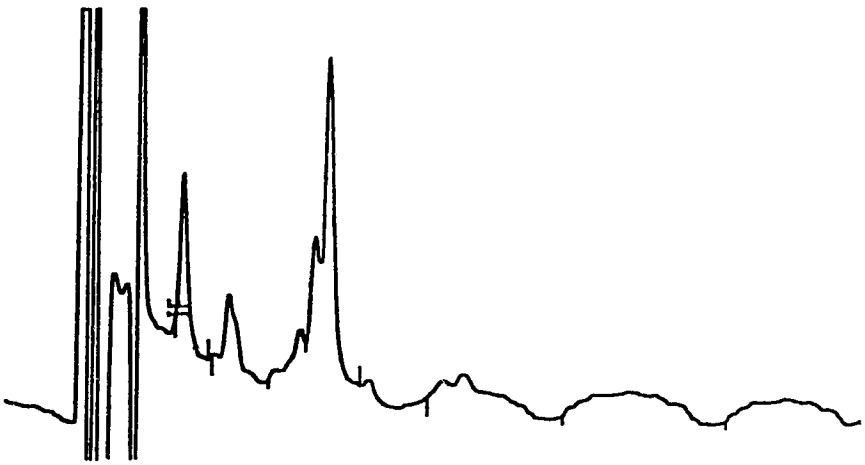
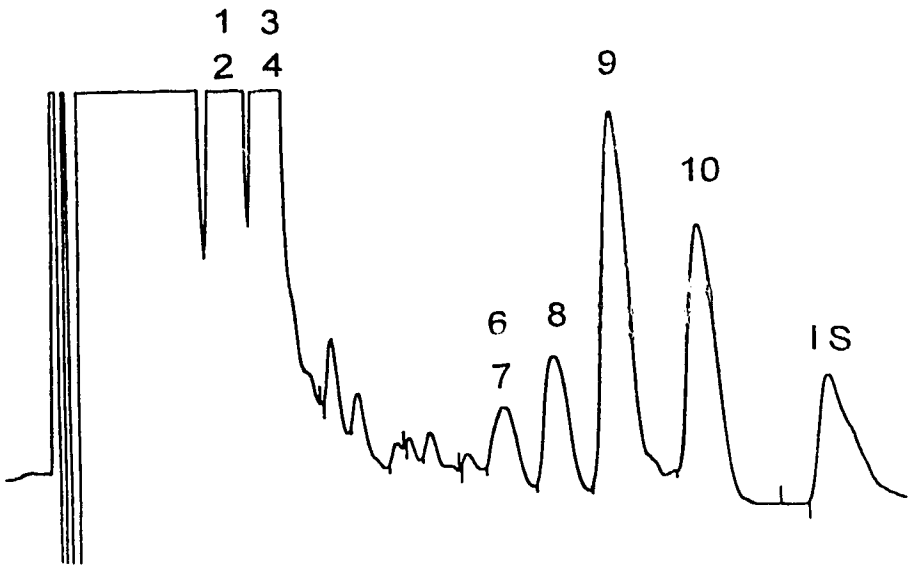


Figure 7: continued. Typical HPLC chromatograms of extracts from biological matrices after administration of rac-TMP (10 mg/kg, ip) in rats: (a) liver at 1 h; (b) brain at 1 h; (c) plasma at 5 h; (d) urine at 0-24 h. Peaks: 1&2 (2-OH-NTMP), 3&4 (2-OH-TMP), 5&7 (NNTMP), 6&8 (NTMP), 9 [(+)-TMP], and 10 [(-)-TMP]. Corresponding traces from extracts of biological matrices from drug-naive rats are aiso shown.

(d) Drug-treated 24 h urine



Drug-naive 24 h urine

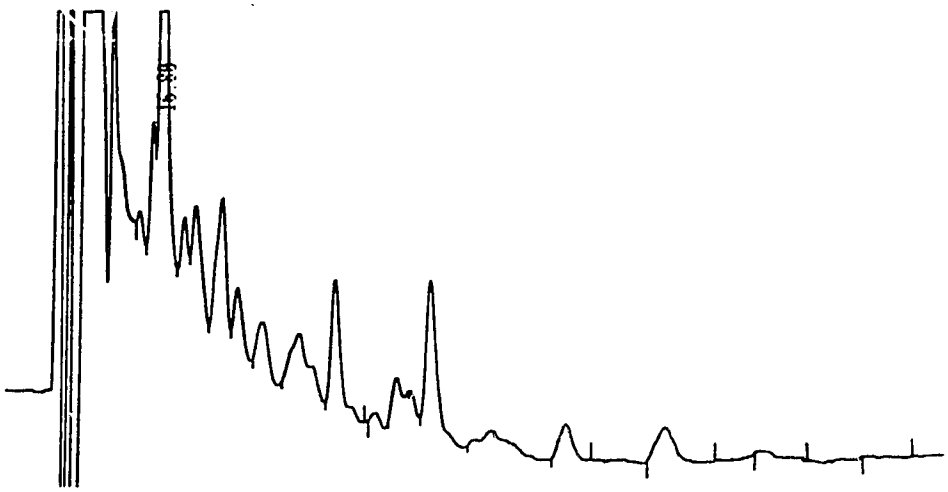


Figure 7: continued. Typical HPLC chromatograms of extracts from biological matrices after administration of rac-TMP (10 mg/kg, ip) in rats: (a) liver at 1 h; (b) brain at 1 h; (c) plasma at 5 h; (d) urine at 0-24 h. Peaks: 1&2 (2-OH-NTMP), 3&4 (2-OH-TMP), 5&7 (NNTMP), 6&8 (NTMP), 9 [(+)-TMP], and 10 [(-)-TMP]. Corresponding traces from extracts of biological matrices from drug-naive rats are also shown.

Table 5: Levels of enantiomers of (+)- and (-)-TMP and NTMP (isomer-2) in brain and liver after administration of rac-TMP (10 mg/kg, ip) in rats (n=5). Results are given as ng/g and represent means (±SEM). *p<0.05, (+)-TMP compared to (-)-TMP.

Sample	Conc.	h	NTMP-2	(+)-TMP	(-)-TMP	(+)/(-)-TMP
Brain	ng/g	1	207 (30)	1565 (276)	1237 (208)	1.26 (0.06)
		2	124 (19)	1068 (28)*	851 (38)	1.26 (0.04)
		4	117 (17)	546 (145)*	384 (97)	1.40 (0.08)
Liver	ng/g	1	461 (64)	171 (19)*	660 (77)	0.27 (0.04)
		2	378 (84)	124 (21)*	345 (18)	0.36 (0.07)
		4	136 (18)	67 (20)*	205 (34)	0.32 (0.07)

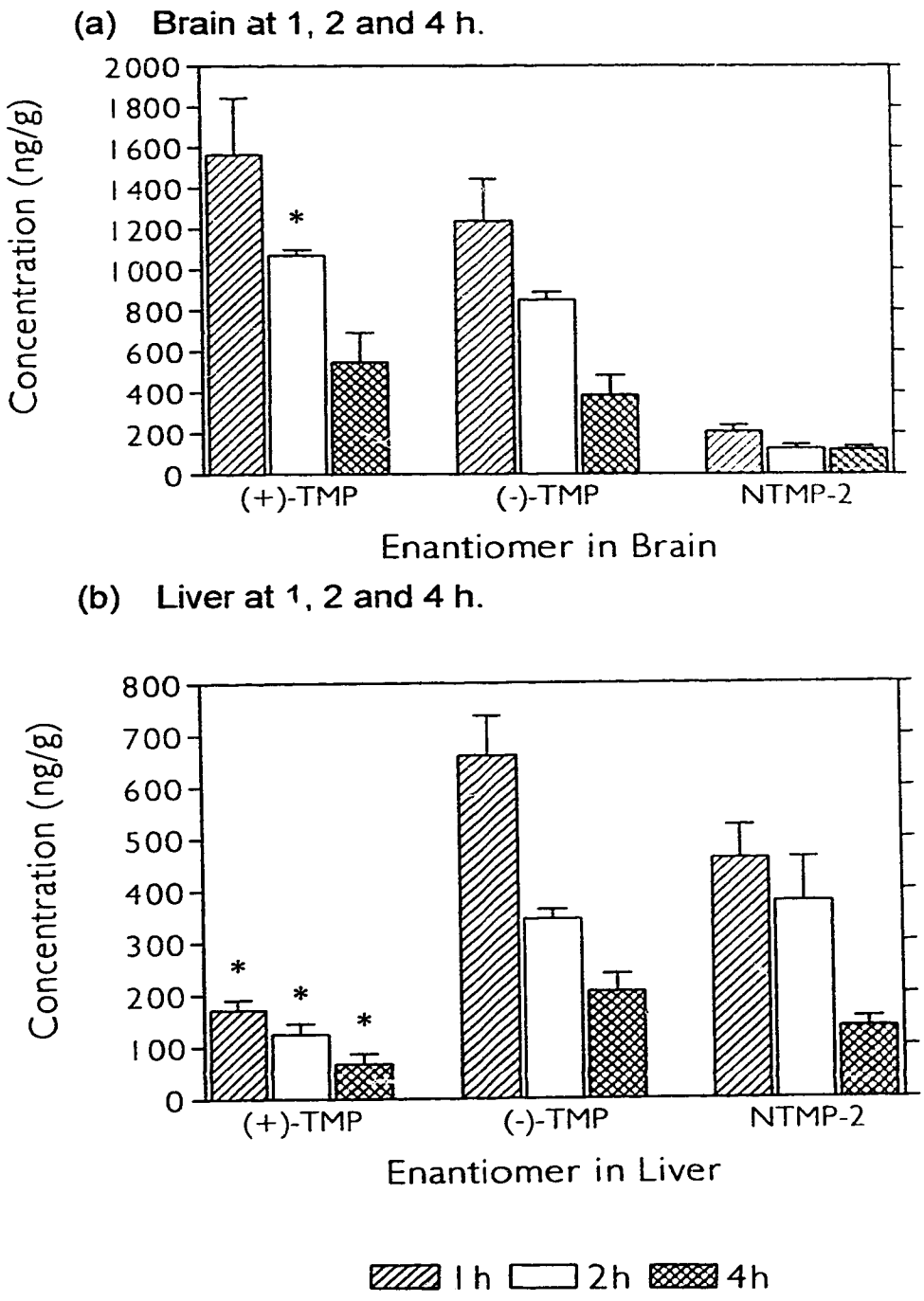


Figure 8: Levels of enantiomers of (+)- and (-)-TMP and NTMP-2 in brain and liver after administration of rac-TMP (10 mg/kg, ip) to rats (n=5). Results are expressed as ng/g and represent means ± SEM. * $p<0.05$, (+)-TMP compared to (-)-TMP.

injection of TMP. These results suggest that the metabolism and N-dealkylation of TMP take place mainly in the liver.

The ratio of (+)/(-)-TMP concentrations ranged from 1.26-1.40 in brain at 1-4 h and 0.27-0.36 in liver at 1-4 h. This ratio was 0.35 in 5 h plasma and 1.61 in 24-hour urine from rats treated with 20 mg/kg of TMP (see Table 6 in Section 4.5). Statistical analyses support the significant difference ($p<0.05$) between levels of (+)- and (-)-TMP in liver at 1, 2, and 4 h. In brain, the difference was only significant at 2 h, and even then it was a very small effect. The marked difference in the ratio of (+)-TMP to (-)-TMP shown here and in Table 6 (where a dose of TMP of 20 mg/kg and a time interval of 5 h were used for direct comparison of plasma and brain levels) are very interesting. In human subjects, plasma levels of antidepressants are sometimes monitored (review: Preskorn and Fast, 1991), although there continues to be an ongoing debate over whether a useful correlation exists between plasma levels of these drugs (Potter et al., 1995). There is no information available on levels of such drugs and their metabolites in brains of human subjects taking therapeutic doses, and there is a paucity of rat studies in which atempts have been made to correlate plasma and brain levels. Glotzbach and Preskorn (1982) found a high correlation between plasma and brain levels of amitriptyline in rats treated with this tricyclic, but did not measure levels of the individual geometric isomers of the parent drug or determine levels of the N-desmethylated metabolite, nortriptyline.

In 1 h liver samples, there were significant amounts of (-)-TMP (660

ng/g) and NTMP-2 (461 ng/g) and smaller amounts of (+)-TMP (171 ng). Measurable amounts of NNTMP-2 (260 ng/g, based on area ratio) and trace amounts of free (unconjugated) hydroxylated metabolites (2-OH-NTMP and 2-OH-TMP) were also found, as illustrated in Figure 7a. The ratio of total amount of N-desmethylated metabolites (NTMP-2 and NNTMP-2) to the total amount of TMP [(+)- and (-)-TMP] was 0.88. This result indicates that N-demethylation of TMP takes place extensively in the liver. The presence of unconjugated 2-hydroxylated metabolites in the HPLC trace suggests that 2-hydroxylation of TMP and NTMP also occurs in the liver. The findings are in agreement with the results of Hussain (1992) who reported that NTMP was the major metabolite of TMP (2-OH-TMP, 2-OH-NTMP, 10-OH-TMP and 10-OH-NTMP were also measured) in an *in vitro* metabolic study of TMP using rat liver microsomes. The 10-hydroxy metabolites were not identified in the present research due to the lack of availability of authentic standards.

In 1 h brain samples, there were large quantities of (+)-TMP (1,565 ng/g) and (-)-TMP (1,237 ng/g), and a much smaller amount of NTMP-2 (207 ng/g). No NNTMP-2 or 2-hydroxylated metabolites were observed, as illustrated in Figure 7b. The ratio of NTMP-2 to total TMP was 0.07. This finding suggests that N-demethylation of TMP does not takes place in brain to any significant extent, and the 2-hydroxylated metabolites formed in the liver do not readily pass the blood-brain barrier.

In 5 h plasma samples from rats treated with TMP at 20 mg/kg (Table 6), the concentrations of parent drug (+)-TMP (23 ng/ml) and (-)-

TMP (57 ng/ml), and of the N-desmethylated metabolite NTMP-2 (19 ng/ml) were relatively low compared to the values in 5 h brain or liver, in agreement with the findings of others in similar experiments with other TCAs (Bickel et al., 1983; Kurata et al., 1986; Miyake et al., 1990; Daniel et al., 1992). The ratio of NTMP-2 to total TMP was only 0.23, much smaller than that in the liver. These results suggest that when NTMP is formed in the liver, it is bound to the liver tissue and then undergoes further biotransformation before it is released to the general circulation. Much higher concentrations of the parent drug, (+)-TMP and (-)TMP, in the brain than in the plasma further support the concept that TMP is lipophilic, highly bound to the tissue protein, and slowly released to the general circulation. No detectable amounts of free (unconjugated) 2-hydroxylated metabolites were found in the plasma as illustrated in Figure 7c. This is in agreement with the results of Eap et al. (1994) who reported that more than 95% of hydroxylated metabolites of TMP were glucuronide-conjugated.

In 0-24 h urine samples from rats treated with TMP at a dose of 20 mg/kg, the unchanged parent drug accounted for only a trivial percentage of the dose administered [(+)-TMP (1,444 ng, <0.1% dose) and (-)-TMP (1,040 ng, <0.1% dose)] (Table 6). Significant amounts of free (unconjugated) 2-OH-TMP and 2-OH-NTMP metabolites were observed in the urine samples based on their peak areas. Also found in the urine was an enormous amount of unknown, as yet unidentified metabolites, as illustrated Figure 7d. Judging from the shorter retention times, these unknown metabolites probably are 10-oxo-TMP and 10-oxo-2-OH-TMP.

Both metabolites have been identified (using GC-MS analysis) together with 2-OH-TMP and 2-OH-NTMP as the four major urinary metabolites in rats (Coutts et al., 1990 and 1991). Because of the lack of availability of authentic standards, the quantification of 10-oxo-TMP and 10-oxo-2-OH-TMP was not attempted. The quantities of 2-OH-TMP and 2-OH-NTMP were not measured due to poor resolution between each pair of enantiomers (R_s= 0.91 and 0.63, respectively, as shown in Table 1).

Small amounts of NTMP-1 (or NNTMP-2) were also observed in the urine samples (peak 6 or 7 in Figure 7d), but quantification was not conducted because of the loss of resolution between them in a Chiralcel OD-R column after it was used over a very short period of time. This separation was not attainable in two other columns purchased from the same source, emphasizing the problem of variability among columns used for chirality studies.

4.5 TMP-FLU Drug Interactions in Rats

For TMP, there are only two clinical studies indicating interactions of TMP with other drugs which cause inhibition of CYP isozymes. One of these was with QND (Eap et al., 1992a) and the other was with FLUV (Seifritz et al., 1994). *In vivo* and *in vitro* studies of drug interactions between TMP and QND, QNN, and IPR were reported by Coutts et al. (1991) and Hussain (1992) in MSD rats. Only one *in vitro* study of a TMP-QND interaction using human CYP2D6 isozyme has been reported (Bolaji et al., 1993). No other drug interactions of TMP and SSRIs have been reported.

The results described in this thesis represent the first report of a metabolic interaction between FLU and TMP.

The addition of rac-FLU (10 mg/kg, ip) one hour after the administration of rac-TMP treatment (20 mg/kg, ip) in 6 MSD rats significantly increased the levels of both (+)-TMP and (-)-TMP in tissues (brain and liver) and plasma 5 h after administration of rac-TMP, compared to values in TMP-VEH control group (Table 6). A graphic representation of the data from Table 6 is shown in Figure 9. The time interval (5 h) was chosen because the metabolic studies of TMP in rats in this thesis (section 4.4) suggested that the concentrations of TMP in brain tissue had reached the terminal stage of the elimination phase, but were still measurable. This time interval had also been shown in previous studies in the Neurochemical Research Unit to be one at which drug interactions between FLU and other TCAs were readily discernible. The dose of 2:1 for TMP:FLU was chosen to be similar to that used for these drugs clinically.

The increases in (+)-TMP and (-)-TMP were 3.1-fold and 3.9-fold in liver, 1.5-fold and 2.0-fold in brain, and, 1.7-fold and 1.9-fold in plasm 5 h post-injection of TMP. It is noteworthy that the magnitude of the effect of FLU is consistently greater on the levels of (-)-TMP than on those of (+)-TMP. However, only in the brain did the coadministration of the drugs cause a significant change in this ratio: the overall (+)/(-)-TMP ratio is 1.05 (±0.03) compared to 1.41 (±0.05) when TMP was given alone.

Both FLU and its active metabolite NFLU are potent inhibitors of CYP2D6 (sections 1.3.2 and 1.3.3). TMP is a substrate of CYP2D6 and its

Table 6: The effects of rac-FLU (10 mg/kg, ip) on levels of (+)- and (-)-TMP and NTMP-2 after administration of rac-TMP (20 mg/kg, ip) in rats (n=6): brain at 5 h; liver at 5 h; plasma at 5 h and urine at 0-24 h. Results are expressed as means ± SEM. *$p<0.05$, values compared to control (TMP-VEH) treatments.

Sample	Treatment	NTMP-2	(+)-TMP	(-)-TMP	(+)/(-)-TMP
Liver (ng/g) 5 h	a.TMP-VEH	280 (17)	116 (16)	238 (22)	0.48 (0.04)
	b.TMP-FLU	788 (99)*	367 (25)*	936 (175)*	0.43 (0.05)
	b/a	2.81	3.16	3.93	0.90
Brain (ng/g) 5 h	a.TMP-VEH	97 (7)	472 (36)	335 (22)	1.41 (0.05)
	b.TMP-FLU	310 (74)*	708 (117)*	674 (107)*	1.05 (0.03)*
	b/a	3.19	1.50	2.01	0.74
Plasma (ng/ml) 5 h	a.TMP-VEH	19 (3)	23 (6)	57 (10)	0.35 (0.09)
	b.TMP-FLU	79 (20)*	39 (8)*	106 (19)*	0.36 (0.03)
	b/a	4.19	1.68	1.86	1.0
Urine ng/24 h 24 h	a.TMP-VEH	305 (107)	1444 (318)	1040 (310)	1.61 (0.20)
	b.TMP-FLU	853 (334)*	1517 (372)	1133 (313)	1.41 (0.13)
	b/a	2.80	1.05	1.09	0.88

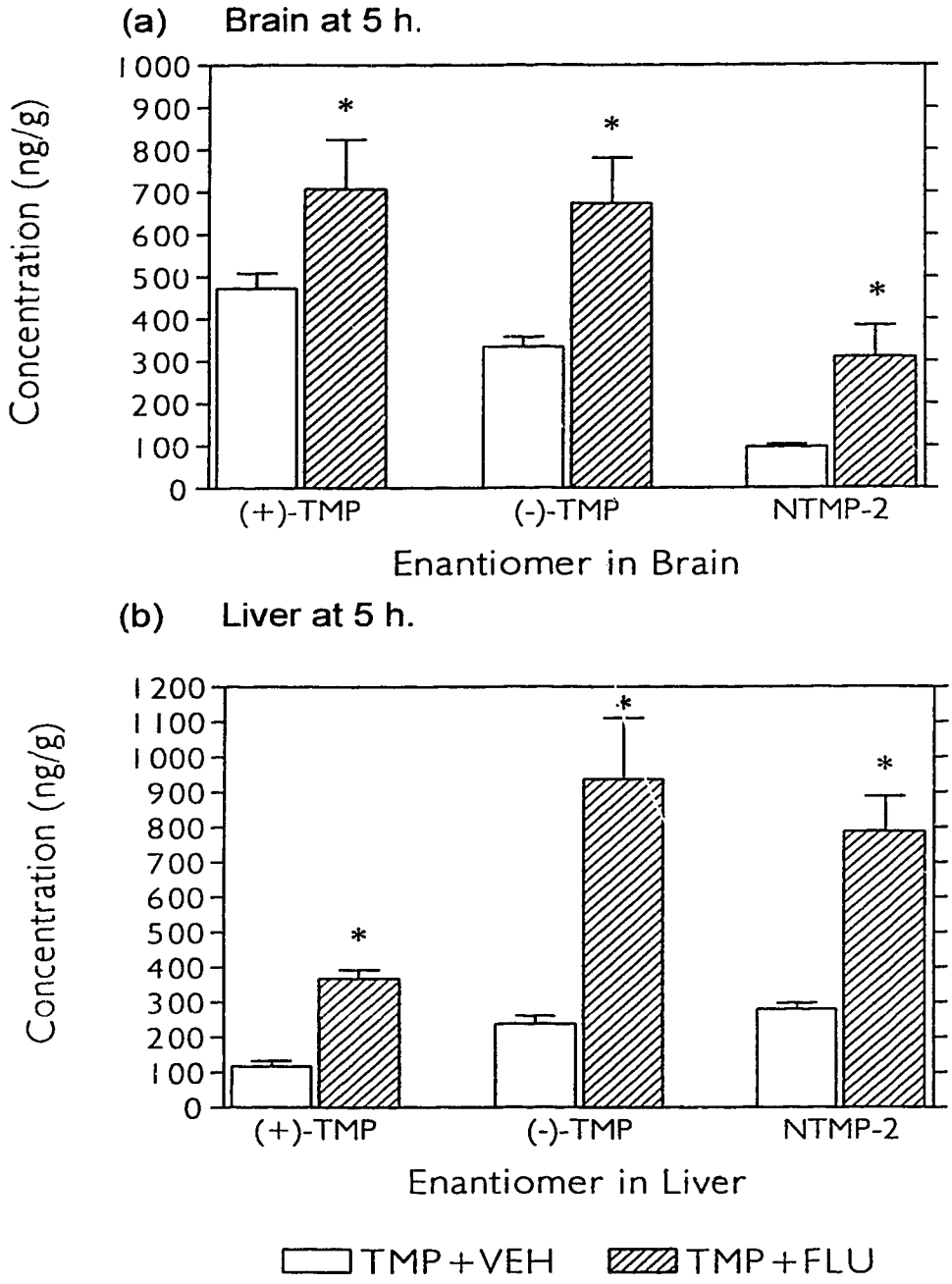
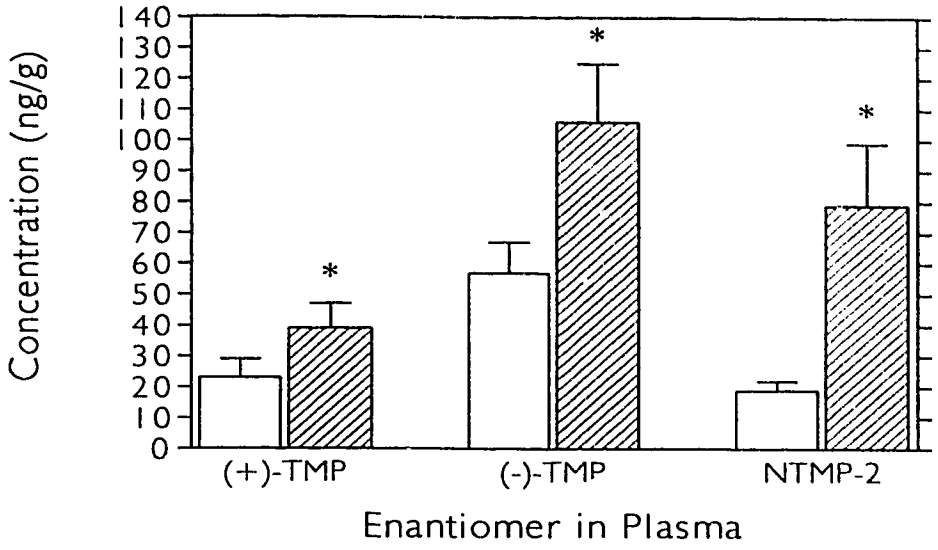


Figure 9: The effects of rac-FLU (10 mg/kg, ip) on levels of (+)- and (-)-TMP and NTMP-2 after administration of rac-TMP (20 mg/kg, ip) in rats (n=6): (a) brain at 5 h; (b) liver at 5 h; (c) plasma at 5 h and (d) urine at 0-24 h. Results are expressed as means ± SEM. *$p<0.05$, values compared to control (TMP-VEH) treatments.

(c) Plasma at 5 h.



(d) Urine at 0-24 h.

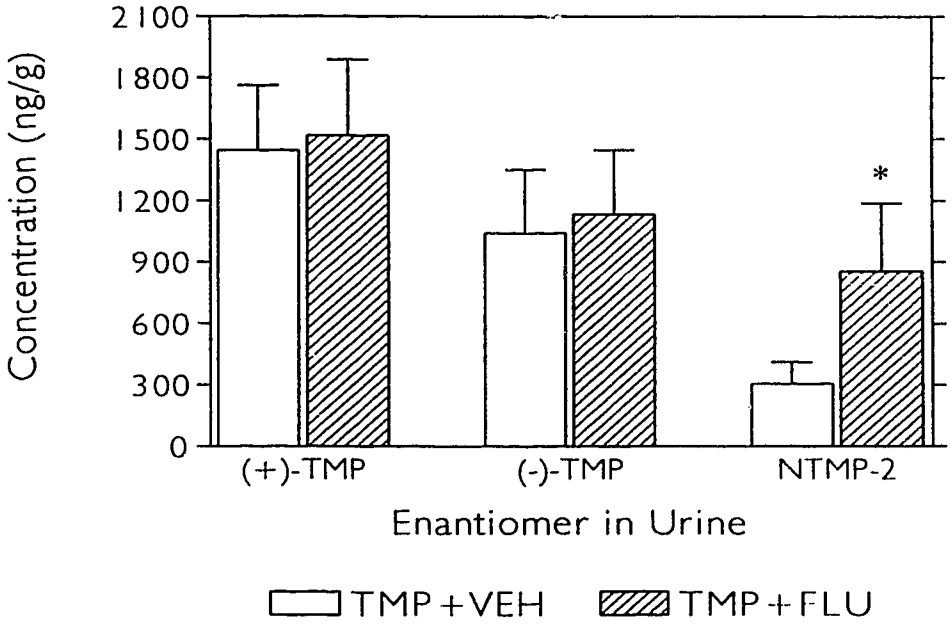


Figure 9: (continued). The effects of rac-FLU (10 mg/kg, ip) on levels of (+)- and (-)-TMP and NTMP-2 after administration of rac-TMP (20 mg/kg, ip) in rats (n=6). Results are expressed as means ± SEM. *$p<0.05$, values compared to control (TMP-VEH) treatments.

major metabolic pathway of 2-hydroxylation is controlled by CYP2D6 (section 1.3.2 and 1.4.3). The increases of TMP levels in tissues (liver and brain) and plasma upon comedication of TMP with FLU is thus probably the result of inhibition of the CYP2D6-mediated 2-hydroxylation of TMP in the liver where such metabolism predominantly takes place. The increased parent drug released from the liver to the general circulation could cause the higher concentration of TMP in plasma and brain.

The addition of FLU to TMP treatment also significantly increased the N-demethylated metabolite NTMP-2 observed 5 h after administration of rac-TMP, compared to values in TMP-VEH control group (Table 6 and Figure 9). The increases were nearly 3-fold in liver, 3-fold in brain, 4-fold in plasma and 3-fold in urine. However, the ratio of NTMP-2 to total TMP concentration in the liver samples decreased from a value of 0.80 in the TMP-VEH group to 0.60 in the TMP-FLU group. This implies that the N-demethylation of TMP, which takes place predominantly in the liver as indicated in section 4.4, was reduced 25% by the influence of FLU.

It will be of interest to conduct further time-response studies, including chronic investigations. As Juorio et al. (1990) observed, chronic (14 day) administration of TMP resulted in decreased density of 5-HT_2 receptors in cortex, an effect that has been suggested to be important in the antidepressant effects of the tricyclics (Peroutka and Snyder, 1980; Baker and Greenshaw, 1989; Eison et al., 1991). The increased levels of TMP may mean that when combined with FLU, lower doses of TMP may be required in humans to reach the same degree of 5-HT_2 down-regulation

as obtained with the drug given alone. Goodnough and Baker (1994) found, however, that when the tricyclic desipramine was given in combination with FLU [a combination that has been used successfully for treating refractory depression -- Nelson et al. (1991)], the brain levels of desipramine were increased relative to those seen in rats administered desipramine alone, but the 5-HT_2 down-regulation produced by desipramine alone was, surprisingly and inexplicably, reversed. These authors suggested that this effect was the result of direct competition of FLU with desipramine for the 5-HT_2 receptor site, since both FLU and desipramine have similar affinities for that receptor (Baldessarini, 1983). It would be of interest to see if a similar interaction between FLU and TMP also occurs at the molecular level.

This drug-drug interaction between TMP and FLU may also have important consequences with regard to the side effects and therapeutic actions. As mentioned previously in this thesis, the antidepressant properties of TMP appear to reside in the (+)-enantiomer, and the (-)-isomer may, in fact, have depressant actions (Bowman and Rand, 1980; Reynolds, 1982). Coadministration of FLU results in an elevation of both enantiomers to about the same extent in brain, liver and plasma, but in the brain, the ratio of active antidepressant to inactive enantiomers [i.e. (+)-TMP/(-)-TMP] decreases significantly. TMP has strong sedative effects and moderately strong anticholinergic effects (Hyman et al., 1995), and when given in combination with FLU, it may be necessary to lower the dose of TMP to get the same sedative effect and reduced anticholinergic

side effects such as dry mouth, urinary retention and constipation. TMP has also been reported to have antipsychotic effects (Gastpar, 1989; Eichmeier et al., 1991) and to produce galactorrhea in females (CPS, 1995), presumably because of its affinity for dopamine receptors (Gross et al., 1991), and these actions may be augmented when the drug is given in combination with FLU. Thus, the preliminary results on FLU and TMP coadministration indicate that extended time- and dose-response studies are warranted. Further investigations should also be extended to include discrete brain areas since several other authors have reported regional differences in the concentrations of other TCAs in rat brain (Kurata et al., 1986, 1988; Miyake et al., 1990).

5 *CONCLUSION*

1. A novel direct non-derivatization reversed phase HPLC method has been developed to separate the enantiomers of TMP, (+)-TMP and (-)-TMP, and its metabolite NTMP-2 from other metabolites [NTMP-1, NNTMP (1&2), 2-OH-TMP (1&2) and 2-OH-NTMP (1&2)]. For each enantiomer of TMP and NTMP-2, the assay developed in this thesis provides good linear regressions, with r^2 >0.98 over a concentration of 25-600 ng in 2 ml biological matrices. The absolute recoveries were 86%, 86% and 84%, respectively, at a concentration of 150 ng. The day-to-day precision for all three enantiomers were within 15% of the mean values and the accuracies were within 17% of the actual values at the concentration of 50 ng.
2. The wavelength of 254 nm was the best single UV wavelength for HPLC analysis of TMP and its N-dealkylated metabolites (NTMP and NNTMP) and hydroxylated metabolites (2-OH-TMP and 2-OH-NTMP) using CMI as the internal standard. The addition of 2% n-butanol to hexane was the best extraction solvent for preventing gel formation and giving clean baselines and good recoveries. The optimal pH value for extraction of TMP and its metabolites in rat biological matrices was 9.8. The more polar the compound, the narrower the extraction pH window for good recovery. The best HPLC analysis conditions used were 2/40/60 (v/v) of isopropanol/acetonitrile/0.5 M aqueous sodium perchlorate adjusted to pH 5.0 as mobile phase, 0.3 ml/min for flow rate, 254 nm for UV

detection; a Chiralcel OD-R column (250 x 4.6 mm ID) was employed in the analysis.

3. This is the first report of an enantiomeric study of TMP in tissues (brain and liver) and biological fluids (plasma and urine) in rats. The levels of parent drug, (+)-TMP and (-)-TMP, were much higher in brain than in liver, 8-9 fold and 2-3 fold, respectively, at each time interval (1, 2, and 4h) after administration of racemic TMP in rats. In contrast, the concentrations of desmethylated metabolite, NTMP-2, were 2-3-fold greater in liver than in brain. The ratio of concentrations of N-desmethylated metabolites (NTMP-2 and NNTMP-2) to TMP [(+)- and (-)-isomer] was 0.88 in liver (1 h), 0.23 in plasma (5 h) and 0.07 in brain (1 h) after administration of TMP. These results suggest that the metabolism and N-dealkylation of TMP take place mainly in the liver.

4. The major metabolite of TMP in brain and plasma was NTMP-2. The major metabolites found in liver were NTMP-2, NNTMP-2, 2-OH-TMP (1&2), and 2-OH-NTMP (1&2). The major urinary metabolites were 2-OH-TMP (1&2), 2-OH-NTMP (1&2) and unidentified metabolites presumed to be 10-oxo-TMP (1&2) and 2-OH-10-oxo-TMP (1&2).

5. Significant amounts of free (unconjugated) hydroxylated metabolites (2-OH-NTMP and 2-OH-TMP) were observed in 0-24 h rat urine, but only trace amounts were found in liver and no detectable amounts were measured in brain and plasma at 1-5 h post-injection of rac-

TMP. These findings suggest that polar compounds as 2-OH-TMP and 2-OH-NTMP do not pass brain-blood-barrier and the majority of hydroxylated metabolites of TMP were glucuronide-conjugated in liver before excretion into the urine. Furthermore, the unchanged parent drug found in 0-24 h urine accounted for only a trivial percentage (<0.1%) of the dose used. This suggests extensive biotransformation is responsible for the elimination of TMP.

6. It was found that the metabolism of TMP in rats was stereoselective. The ratio of (+)/(-)-TMP concentrations ranged from 1.26-1.40 in brain (1-4 h), 0.27-0.36 in liver (1-4 h), 0.35 in plasma (5 h) and 1.61 in urine (0-24 h) after administration of rac-TMP. Statistical analyses showed significant differences between (+)- and (-)-TMP in liver at 1, 2, and 4 h, in brain at 2 h and in plasma at 5 h.

7. Included in this thesis is the first report of drug-drug interaction of TMP and FLU. The addition of rac-FLU to rac-TMP significantly increased the levels of both (+)-TMP and (-)-TMP in tissues (brain and liver) and plasma. The increases in (+)-TMP and (-)-TMP were 3-fold and 4-fold in liver, 1.5-fold and 2.0-fold in brain, and 1.7-fold and 1.9-fold in plasma, respectively, at 5 h post-injection of TMP. It is noteworthy that the magnitude of the effect of FLU is slightly greater on the levels of (-)-TMP than on the (+)-TMP, but this difference was only significant in brain tissue.

8. The addition of FLU to TMP treatment also significantly increased the concentrations of N-demethylated metabolite, NTMP-2. The

increases were nearly 3-fold in liver, 3-fold in brain, 4-fold in plasma and 3-fold in urine. However, the ratio of concentrations of NTMP-2 to TMP in liver decreased from 0.80 in the TMP-VEH group to 0.60 in TMP-FLU group. This suggests that the N-demethylation of TMP, which takes place predominantly in liver, was reduced 25% by the influence of FLU.

9. Both FLU and its active metabolite NFLU are potent inhibitors of CYP2D6. Others have shown that FLU is also a weak inhibitor of CYP3A4 and NFLU is a potent inhibitor of both CYP1A1 and CYP3A4 and a weak inhibitor of CYP1A2. The increases of TMP levels in liver, brain and plasma upon comedication of TMP with FLU is probably the result of inhibition of the CYP2D6-mediated 2-hydroxylation of TMP. The effect of FLU on inhibition of N-demethylation of TMP is probably a result of inhibition of CYP3A4 by FLU and NFLU, but other isozymes (CYP2D6, CYP1A1, CYP1A2 and CYP_{MP}) may also play some part in this inhibition. Further research on the TMP-FLU drug interaction using single isozymes *in vitro* is thus warranted based on the findings reported in this thesis.

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