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Effects of the Monoamine Oxidase Inhibitors, Tranylcypromine and Phenelzine, on Selected Cytochrome P450 Enzymes

by

Mahnaz Salsali 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Medical Sciences (Psychiatry)

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Abstract

The inhibitory effects of two non-selective irreversible inhibitors of monoamine oxidase (MAO), tranylcypromine (TCP) and phenelzine (PLZ), on three cytochrome P450 (CYP) enzymes, namely CYP2C9, CYP2C19, and CYP2D6, have been evaluated *in vitro*. Individual TCP enantiomers as well as ring-substituted analogues of TCP were also investigated for their effects on CYP2C19. In addition possible formation of amphetamine as a metabolite of TCP was investigated using human liver microsomes.

Sensitive and reproducible gas chromatographic and high-pressure liquid chromatographic assays were developed to quantify hydroxymephenytoin (OHMEP), hydroxytolbutamide (OHTOL), and dextrorphan (DXR), metabolites of S-mephenytoin (MEP), tolbutamide (TOL), and dextromethorphan (DM), probe substrates of CYP2C19, CYP2C9 and CYP2D6, respectively.

Using human liver microsomes, it was demonstrated that of the two enantiomers of TCP, (+)-TCP is a much stronger inhibitor of CYP2C19 than (-)-TCP. Of the TCP analogues tested, 4-fluorotranylcypromine (FTCP) had the weakest inhibitory effects on CYP2C19.

The type of inhibition and inhibition constant (K_i) values for PLZ and TCP against the various CYP enzymes were determined with studies conducted on cDNA-expressed CYP enzymes. The results demonstrated that TCP is a competitive inhibitor of CYP2C19 (K_i = 32μM) and CYP2D6

(K_i = 367μM), and a non-competitive inhibitor of CYP2C9 (K_i = 56μM), and that PLZ is a non-competitive inhibitor of CYP2D6 (K_i = 0.7 μM), CYP2C19 (K_i = 0.7 μM) and CYP2C9 (K_i = 30 μM).

Neither amphetamine nor 1-amino-3-phenylpropane was detected after the incubation of TCP with human liver microsomes, suggesting that opening of the cyclopropyl ring of TCP is not a significant metabolic pathway for TCP; such findings are in accord with the findings of other researchers using *in vivo* techniques.

The studies described in this thesis have resulted in the development of several novel analytical procedures for metabolites of CYP substrates and have provided important information about the interactions of two clinically valuable antidepressants, PLZ and TCP, with CYP enzymes.

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List of Symbols and Abbreviations

v	Initial reaction velocity at any given substrate concentration
AMP	Amphetamine
cDNA	Complementary deoxyribonucleic acid
CO	Carbon monoxide
CYP	Cytochrome P450
DA	Dopamine
DM	Dextromethorphan
DXR	Dextrorphan
EDTA	Disodium ethylenediamine-tetraacetate
FAD	Flavin adenine dinucleotide
Fe^{2+}	Ferrous (oxidized) iron
Fe^{3+}	Ferric (reduced) iron
FMN	Flavin mononucleotide
FTCP	4-Fluorotranylcypromine
GC	Gas chromatography
GC-ECD	Gas chromatography with an electron-capture detector
HPLC	High performance liquid chromatography
HPLC-UV	High performance liquid chromatography with a UV detector
5-HT	5-Hydroxytryptamine (serotonin)
[I]	Inhibitor concentration

IU	International Units
K_D	Partition coefficient
K_2HPO_4	Dibasic potassium phosphate
KH_2PO_4	Monobasic potassium phosphate
K_i	Dissociation constant for an inhibitor
K_m'	Apparent K_m
K_m	Michaelis-Menten constant
k_{on}	The rate constant for binding of the compound to the enzyme
LC	Liquid chromatography
MAO	Monoamine oxidase
MAOI	MAO inhibitor(s)
MEP	S-Mephenytoin
$MgCl_2$	Magnesium chloride
MTCP	4-Methoxytranylcypromine
$NADP^+$	β-Nicotine adenine dinucleotide phosphate
NE	Norepinephrine
OHMEP	Hydroxymephenytoin
OHTOL	Hydroxytolbutamide
4-OH-PAA	*para*-Hydroxyphenylacetic acid
4-OH-PLZ	4-Hydroxyphenelzine
PAA	Phenylacetic acid
PCP	*Para*-chlorophentermine

PEA	2-Phenylethylamine
PFBC	Pentafluorobenzoyl chloride
PFBSC	Pentafluorobenzenesulfonyl chloride
PHB	Phenobarbital
PLZ	Phenelzine (β-phenylethylhydrazine)
PPA	Phenylpropylamine (1-amino-3-phenylpropane)
RIMAs	Reversible inhibitors of MAO-A
[S]	Substrate concentration
SSRIs	Selective serotonin reuptake inhibitors
TCAs	Tricyclic antidepressants
TCP	(±)-Tranylcypromine
(+)-TCP	Pure optical isomer of TCP
(-)-TCP	Pure optical isomer of TCP
TOL	Tolbutamide
V_{max}	Maximum velocity of an enzyme-catalyzed reaction

CHAPTER 1

Review of the Literature

1.1 General Introduction

Drugs that could inhibit monoamine oxidase (MAO) were introduced as a treatment for depression in the 1950s (Crane, 1957; Kline 1958; Sandler 1990). Interest in the MAO inhibitors (MAOIs) later dropped off because of their interactions with dietary constituents and other drugs and also because of reports of toxicity, such as liver damage, particularly in the case of the hydrazide-based agents (Blackwell, 1963; Callingham, 1986; Blackwell, 1991). However, in recent years with more research on these drugs, there has been a resurgence in the use of MAOIs in psychiatry and neurology (Kennedy et al., 2000).

Most of the side effects following clinical use of MAOIs stem from: 1) the inhibition of gut, liver, and vasomotor nerve MAO-A; 2) the inhibition of MAO within the central nervous system; and 3) the inhibition of microsomal and other enzymes involved in metabolizing drugs and xenobiotics (Callingham, 1993). Addressing the last of these, the study reported in this thesis investigated the inhibitory effects of two nonselective, irreversible inhibitors of MAO, tranylcypromine (TCP) and phenelzine (PLZ), on three cytochrome P450 (CYP) enzymes involved in drug metabolism. Among numerous CYP enzymes, only six of them have been shown thus far to play a major role in the metabolism of drugs in common clinical use; these are CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP1A2 and CYP2E1. The present study focused on three of these enzymes: CYP2D6, CYP2C9, and CYP2C19.

An extensive experimental program was designed to determine the kinetics of metabolite formation from substrates of these CYP enzymes as well as the kinetics of inhibition of these enzymes by TCP and PLZ. The experiments were conducted *in vitro* using human liver microsomes and cDNA-expressed individual human CYP enzymes. To quantify compounds of interest in these microsomal experiments, several chromatographic assays were developed.

The present chapter reviews the literature and provides a background on the relevant subjects, including MAO enzymes, MAOIs, drug-drug interactions involving TCP or PLZ, drug biotransformation, cytochrome P450 enzymes, enzyme kinetics, enzyme inhibition and the kinetics of inhibition. In addition, the basic concepts of stereoisomers, gas chromatography (GC) and high performance liquid chromatography (HPLC) are discussed.

1.2 Monoamine Oxidase

Monoamine oxidase (MAO; EC 1.4.3.4) is a ubiquitous flavin-containing mitochondrial enzyme present in both vertebrate and invertebrate species (Blaschko, 1952). This enzyme is distributed in numerous organ systems, most notably the heart, lung, liver, kidney and brain where it catalyses the oxidative deamination of biogenic and xenobiotic amines. MAO oxidizes most primary amines very effectively, while it oxidizes secondary amines at a slower rate. Drugs containing amine groups with substituents

larger than methyl are not generally favorable substrates for MAO (Blaschko, 1952; Tipton, 1975; Youdim, 1975). The ability of MAO to oxidize tertiary amines has been more recently documented (Benedetti & Tipton, 1998).

Two MAO subtypes, MAO-A and MAO-B, have been distinguished by their respective substrate and inhibitor specificities (Cawthon et al., 1981). SDS-gel electrophoresis (Weyler & Salach, 1985), immunological research (Denny et al., 1982a,b), peptide mapping from enzyme digestion (Cawthon & Breakefield, 1979) and combined quantitative enzyme radioautography and *in situ* hybridization histochemistry (Saura et al., 1990) have added further support to the existence of two MAO subtypes.

Type A preferentially deaminates norepinephrine (NE) and 5-hydroxytryptamine (5-HT, serotonin) and is selectively inhibited by clorgyline (Johnston, 1968). 5-HT can be oxidized by MAO-B when MAO-A is inhibited (Wolf et al., 1985). Type B preferentially deaminates 2-phenylethylamine (PEA) and the synthetic substrate, benzylamine and is sensitive to inhibition by deprenyl or pargyline. At concentrations greater than its physiological concentrations, PEA can also be metabolized by MAO-A (Kinemuchi et al., 1982). Dopamine (DA) and tyramine are oxidized by both forms of the enzyme. Although DA is considered to be a mixed substrate for both MAO-A and MAO-B, the breakdown of DA in the striatal regions of the brain is preferentially by MAO-B. In other regions, MAO-A may be more important (Cesura & Pletscher, 1992).

Most tissues, including the brain (Murphy and Donnelly, 1974) and the blood-brain barrier (Yu, 1984) contain a mixture of both isozymes. However, only MAO-A is present in placental tissue (Salach and Detmer, 1979), while lymphocytes (Bond and Dundall, 1977) and platelets (Donnelly and Murphy, 1977) contain solely MAO-B. The regional brain distribution of MAO-A and MAO-B also varies. Both MAO-A and MAO-B are present in discrete cell populations within the CNS. MAO-A is present in both DA- and NE-containing neurons, whereas MAO-B is present to a greater extent in serotonin-containing neurons. They are also present in nonaminergic neurons in various subcortical regions of the brain. Glial cells also express MAO-A and MAO-B (Cesura & Pletscher, 1992).

Brain MAO-B activity has been shown to increase with age in both rats and humans (Robinson, 1975; Benedetti & Keane, 1980; Fowler et al., 1980; Leung et al., 1981; Gottfries et al., 1983; Cao Danh et al., 1984). The age-dependent increase in MAO-B may reflect glial cell proliferation accompanying neuronal loss (Peng & Lee, 1979; Knoll, 1982).

MAO-B activity in hypothalamus, caudate nucleus, hippocampus, and cortex gyrus cinguli has been found to be increased in postmortem tissue from patients with dementia of the Alzheimer type (Adolfsson et al., 1980; Oreland & Gottfries, 1986). Platelet MAO-B is also significantly higher in Alzheimer patients compared with matched control subjects (Donnelly & Murphy, 1977; Adolfsson et al., 1980).

MAO activity in the caudate nucleus and substantia nigra from patients with Parkinson's disease has been reported as either higher than (Riederer et al., 1983) or not different from that observed in matched control subjects (Lloyd et al., 1975; Jellinger & Riederer, 1984). Since anti-Parkinsonian drugs such as L-dopa and carbidopa could have influenced MAO activity, the results of such studies are recommended to be interpreted with caution (Callingham & Lyles, 1980; Naoi & Nagatsu, 1987).

Platelets, which contain only MAO-B, have been studied in many psychiatric conditions, mainly due to ease of access. Although significant alterations in platelet MAO have been reported in a variety of neuropsychiatric conditions, it is generally accepted that there is currently not a "disease marker function" for MAO activity, irrespective of the source of the enzyme (Mosnaim et al., 1996). Platelet MAO activity sometimes has been reported to be high in depressed patients (Mann, 1979; Davidson et al., 1980) and patients with panic disorder (Yu et al., 1983), but low in alcoholics and bipolar disorder patients (Sullivan et al., 1978). While some studies have reported low platelet MAO activities in schizophrenic patients (Murphy & Wyatt, 1972; Sandler et al., 1981), others have not observed a difference between MAO activity of schizophrenic and control subjects (Owen et al., 1977; Reveley et al., 1981). Also, Maj et al. (1985) have observed a reduction of platelet MAO activity following chronic treatment of patients with some neuroleptic drugs used in schizophrenia.

1.3 Monoamine Oxidase Inhibitors

MAOIs were first identified as effective antidepressants in the late 1950s. They have also been used in treatment of other psychiatric and neurologic conditions, including panic disorder and agoraphobia (Sheehan et al., 1980), social phobia (Versiani et al., 1988), bulimia nervosa (Kennedy et al., 1988), neurodermatitis (Friedman et al., 1978), Parkinson's disease (Birkmayer et al., 1985; Marsden, 1990), and Alzheimer's disease (Sunderland et al., 1987; Tariot et al., 1987a, 1987b).

The inhibitors of MAO can be classified into two main types, reversible and irreversible. The latter group is usually divided into two principal chemical categories, hydrazine and non-hydrazine. Further subdivision into selective and non-selective inhibitors of MAO-A or MAO-B is possible. Until recently, the only MAOIs available were irreversible and relatively non-selective inhibitors of MAO-A and MAO-B, with the exception of clorgyline, which is highly selective for MAO-A, and (-)-deprenyl, which is selective for MAO-B.

Over the years, there have been many reports of adverse reactions, both with dietary constituents and with other drugs, following treatment with irreversible MAOIs. Although much of this information is anecdotal comment or reports of single cases, they all contribute to the sense of unease or apprehension limiting the widespread use of MAOIs in psychiatry (Cooper, 1989). However, there are some well-documented and potentially serious

interactions, which cannot be ignored, and these are summarized in Table 1.1 (Callingham, 1993). In addition to side effects related to food or drug interactions, other side effects associated with MAOIs have been reported. Table 1.2 lists these side effects.

In patients treated with the older irreversible, nonselective MAOIs, such as PLZ and TCP, the potentially life-threatening hypertensive crisis that could follow ingestion of foods containing tyramine (cheese effect) is ascribed to the irreversible inhibition of MAO-A in the gastrointestinal tract, liver, and in postganglionic noradrenergic vasomotor neurons (Da Prada et al., 1988). The importance of the MAO in the gastrointestinal tract as a barrier against the absorption into the circulation of tyramine and other susceptible dietary amines was recognized by Blackwell and Marley (1966), and has been confirmed many times (Ilett et al., 1980; Davies et al., 1984; Hasan et al., 1988). Clearly, the gut is an important area, where the metabolism of MAO-susceptible amines could be inhibited by MAOIs, leading to increased absorption into the systemic circulation of potentially harmful concentrations.

The greatest drawback with classical MAOIs, the interaction with dietary tyramine and other drugs, has been minimized by the development of reversible, selective inhibitors of MAO-A (RIMAs), such as moclobemide and brofaromine. These newer agents have been used successfully as antidepressants, and have also produced encouraging results as a treatment

for social phobia and panic disorder (van Vliet et al., 1993, 1996; Dingemanse et al., 1995; Volz et al., 1996). Moclobemide has been available commercially in several countries for a few years, but brofaromine has been withdrawn because of expiry of its patent. There is a paucity of information comparing the efficacy of the newer reversible compounds with the classical irreversible MAOIs (Kennedy & Glue, 1994). However, there is some indirect information to suggest that irreversible compounds may be more potent than the newer reversible compounds (Kennedy & Glue, 1994). For example, the onset of efficacy in social phobic patients was more rapid in those treated with phenelzine (PLZ) than in those treated with moclobemide (Versiani et al., 1992).

There are relatively few studies investigating the mechanisms of action of MAOIs compared with the number of studies using other antidepressants such as tricyclic antidepressants (TCAs) or selective serotonin reuptake inhibitors (SSRIs). It was initially believed that the mechanism of action of MAOIs was due to a simple elevation of brain levels of catecholamines and 5-HT, subsequent to inhibition of MAO (Finberg & Youdim, 1984; Murphy et al., 1984, 1987). More recently, it has been suggested that secondary adaptive mechanisms may be important for their mechanism of action.

In general, the effects on receptor number and sensitivity after chronic MAOI therapy are similar to those reported with TCA use, particularly as

they relate to changes in central serotonergic and noradrenergic pathways (Kennedy & Glue, 1994). The overall effects of MAOIs on central NE pathways are increased functional activity, and adrenoceptor down-regulation. Similar effects are also observed on central 5-HT activity, with increased transmission and 5-HT_2 receptor down-regulation (Da Prada et al., 1984, 1989; Glue et al., 1994). Selective MAO-B inhibitors lack the above effects (Glue et al., 1994).

There is a paucity of data describing effects of MAOIs on DA receptor binding characteristics. Decrease in total number of DA receptor binding sites and no change in their affinity have been reported by Paetsch and Greenshaw (1992) after chronic treatment of rats with TCP (1mg/kg/day) or PLZ (5 or 10 mg/kg/day). The reversible MAO-A inhibitors, moclobemide and brofaromine have been assessed and found not to alter D_1-like DA receptors labeled by ^{3}H-SCH 23390 or D_2-like DA receptors labeled by ^{3}H-spiperone binding (Klimek et al., 1990). MAOIs appear to decrease peripheral indices of DA turnover (Linnoila et al., 1983). In addition to effects on other neurotransmitters, PLZ has been reported to increase brain gamma-aminobutyric acid (GABA) levels (Popov & Matthies, 1969; Baker et al., 1991, 1992; McManus et al., 1992; Paslawski et al., 1996; Parent et al., 2000), although this is due largely to inhibition of enzymes other than MAO.

Among the MAOIs, the two most widely used, PLZ and TCP, have been the focus of the studies described in this thesis.

Agent	*Effect*
Sympathomimetic amines	
• Directly acting, e.g., NE	Potentiation of pressor effects
• Indirectly acting	
MAO substrates, e.g., tyramine, PEA, phenylethanolamine	Hypertensive crisis
Not MAO substrates, e.g., amphetamine, ephedrine	Hypertensive crisis
L-DOPA	Hypertensive crisis
Fluoxetine	Central excitatory syndrome
Pethidine	Central excitatory syndrome
Dextromethorphan	Central excitatory syndrome
Tryptophan	Central excitatory syndrome
Reserpine	Central excitatory syndrome
Tricyclic antidepressants, especially clomipramine	Central excitatory syndrome
Hypoglycemic agents	Increased hypoglycemia

Table 1.1 Some well-established interactions with irreversible inhibitors of MAO (adapted from Callingham, 1993).

Atropine-like effects Dry mouth Blurred vision Drowsiness Urinary hesitancy Constipation
Cardiovascular effects Light-headedness Orthostatic hypotension Peripheral edema
Other side effects Restlessness/ insomnia Anorexia Nightmares Decreased sexual potency Ejaculatory disturbances Tiredness/ weakness Flushing/ sweating Skin rash/ purpura Hallucinations, hypomania Myoclonic twitches
Acute toxicity reaction (hypertensive crisis) Nausea Vomiting Sweating Severe occipital headache/pounding headache Stiff neck/ sore neck Palpitations Tachycardia/ bradycardia Acute blood pressure increase (20-30 mm Hg) Chest pain Seizure

Table 1.2 Side effects of MAOIs (adapted from Jenkins and Hansen, 1995).

1.3.1 Phenelzine

Phenelzine (β-phenylethylhydrazine; PLZ; Nardil®) is an irreversible, nonselective MAOI. It is considered the most effective MAOI and is the most sedative (Keltner & Folks, 1997). PLZ has been found to be most effective in patients with atypical depression (Nies & Robinson, 1982; Keltner & Folks, 1997), which refers to a type of depression in which many of the classical symptoms of endogenous depression are absent, or present in the reverse forms, such as feeling of well being only in the morning. Also, certain features such as phobic anxiety symptoms and panic episodes are present or are more prominent (Nies & Robinson, 1982).

Patients have shown better clinical response to treatment with PLZ when platelet MAO inhibition was above 80% (Robinson et al., 1978a, 1978b). PLZ dosage (mg/kg body weight) strongly associates with clinical response and percent MAO inhibition (Robinson et al., 1979). Controlled clinical trials suggest that 1 mg/kg body weight is the optimal dose for PLZ (Nies & Robinson, 1982), and lower doses are only marginally effective (Ravaris et al., 1976).

PLZ, like iproniazid, belongs to the hydrazine group of MAOIs. Hydrazine itself is known to be hepatotoxic in animals, contributing to the concern that drugs of this class might carry a special liability. An incidence of hepatotoxicity of 1% to 2% has been seen with iproniazid, with an estimated

15% mortality rate (Robinson & Kurtz, 1987). However, this side effect is considered rare with PLZ (Nies & Robinson, 1982).

PLZ can be oxidized by MAO (Tipton & Spires 1971). In the past it was assumed that PLZ was metabolized by acetylation because of its similarity in structure to other drugs, such as isoniazid, which were known to be acetylated (Narasimhachari et al., 1980; Robinson et al., 1985). However, Robinson and coworkers (1985) administered $^{13}C_6$-labeled PLZ and followed the excretion products, and their study indicated that N-acetylation of PLZ is not a significant metabolic pathway in humans. Sixty-six to 79 percent of the administered dose of PLZ was excreted in urine as either phenylacetic acid (PAA) or *para*-hydroxyphenylacetic acid (4-OH-PAA) within 96 hours.

PLZ has been shown to undergo biotransformation to the bioactive amine, 2-phenylethylamine (Baker et al., 1982; Dyck et al., 1985). There is also evidence for formation of 4-hydroxyphenelzine (4-OH-PLZ; McKenna et al., 1990, 1991) and for possible N-methylation of PLZ (Yu et al., 1991). In addition, ethylbenzene has been identified as an *in vitro* and *in vivo* metabolite of PLZ (Danielson et al., 1984; Ortiz de Montellano & Watanabe, 1986). Figure 1.1 summarizes PLZ biotransformation routes.

Available pharmacokinetic data indicate that PLZ is rapidly absorbed, with concentration maxima occurring between 2 and 4 hours post oral dose (Robinson 1985). In the same study, plasma elimination half-lives ranged from 1.5 to 4 hours. Steady state plasma PLZ concentrations have been noted

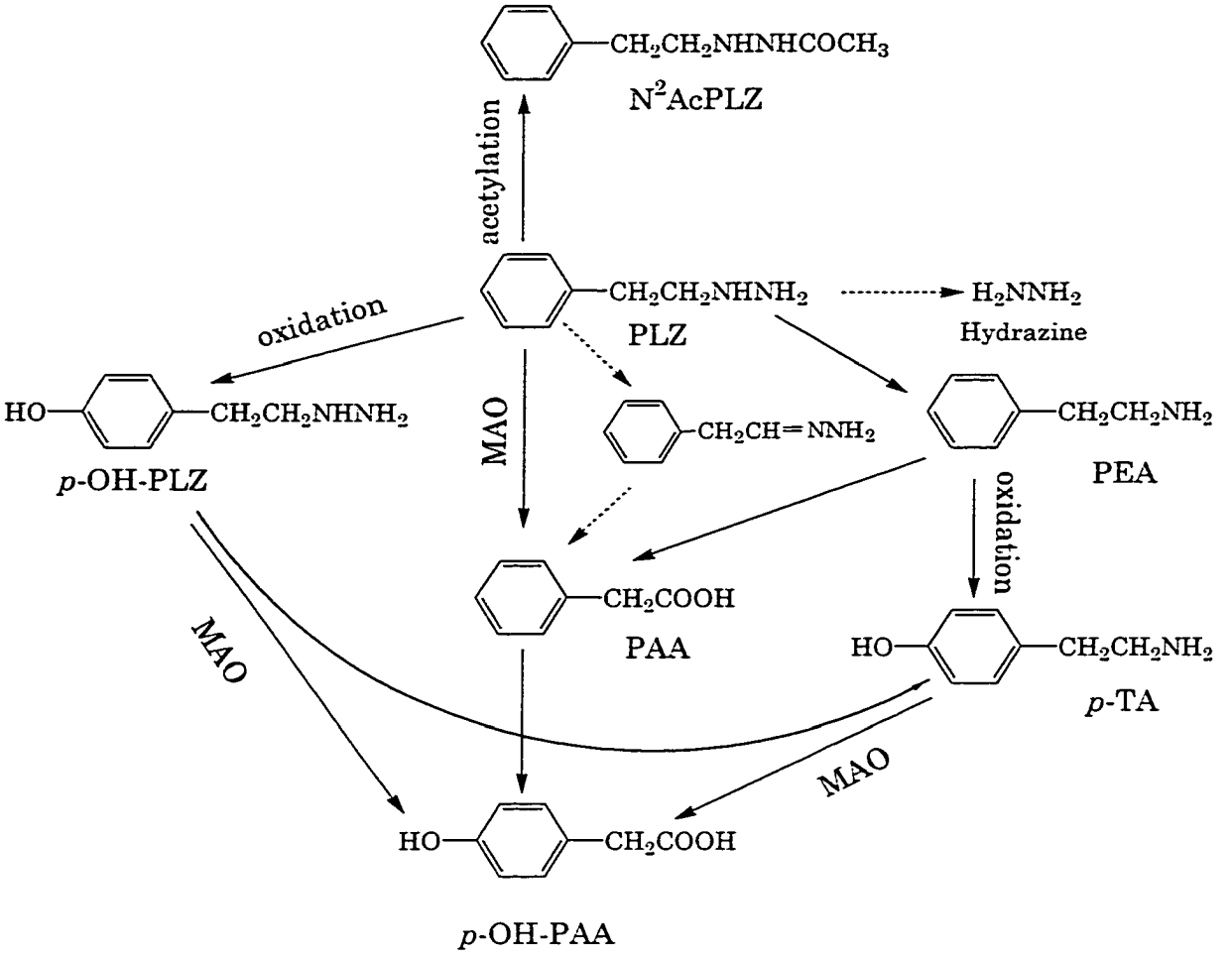


Figure 1.1 Identified and potential metabolites of phenelzine. Ethylbenzene has also been shown to be a metabolite. Phenylethylidene hydrazine ($PhCH_2CH{=}NNH_2$), 1-(2-phenylethyl)diazene ($PhCH_2CH_2N{=}NH$), and N-methylphenelzine have also been proposed as metabolites. MAO, monoamine oxidase; PLZ, phenelzine; *p*-OH-PLZ, *p*-hydroxyphenelzine; PEA, 2-phenylethylamine; *p*-TA, *p*-tyramine; PAA, phenylacetic acid; *p*-OH-PAA, p-hydroxyphenylacetic acid.

to increase gradually over the initial 6 to 8 weeks of chronic treatment. The drug or metabolites may thus be inhibiting PLZ's metabolism in liver and other tissues (Robinson et al., 1980; Bieck et al., 1989). Hence, increases in bioavailability could increase the magnitude of effects correlated with maximum plasma concentration such as hypotension and fatigue (Preskorn, 1993).

1.3.2 Tranylcypromine

Tranylcypromine (TCP; Parnate ®), the first non-hydrazine MAOI, was synthesized by converting the isopropyl side chain of amphetamine to a cyclopropyl side chain in an attempt to develop a nasal decongestant (Burger & Yost, 1948). The drug was not successful as a nasal decongestant, and it was not until the late 1950s that its MAO-inhibiting properties were discovered (Maass & Nimmo, 1959; Tedeschi et al., 1959).

TCP exists as a mixture of (+)- and (-)-*trans*-2-phenylcyclopropylamine (Coutts & Baker, 1989), and is used clinically as the racemate. The (+)-isomer has been shown to be more potent than the (-)-isomer at inhibiting MAO, whereas (-)-TCP has been demonstrated to be more effective than (+)-TCP as an inhibitor of catecholamine reuptake (Smith, 1980; Nickolson & Pinder, 1984). The two enantiomers also differ in their interaction with 5-HT_1 receptors in human post-mortem frontal cortex, with (-)-TCP displaying a higher affinity than (+)-TCP (Reynolds, 1985). TCP also has a mild beneficial

effect in some Parkinsonian patients, and in general (-)-TCP has a better effect on psychiatric symptoms, while (+)-TCP has a better effect on motor function (Reynolds & Riederer, 1981). In addition, the two enantiomers demonstrate different pharmacokinetics. Compared to the (+)-isomer, the (-)-TCP has been reported by some researchers to be more rapidly absorbed and more slowly eliminated (Weber-Grandke et al., 1993). In contrast, findings of another study suggest more rapid elimination of (-)-TCP than of (+)-TCP (Reynolds & Riederer, 1981).

TCP has been used for the treatment of a variety of psychiatric disorders, most notably atypical depressions (Himmelhoch et al., 1982, 1991; Thase et al., 1989). TCP seems to be the most effective MAOI for treatment of severe or endogenous depression, and its clinical effect may be experienced more rapidly compared with other MAOIs (Keltner & Folks, 1997). TCP also is the most stimulating MAOI (Nies & Robinson, 1982; Keltner & Folks, 1997). It has been reported to be an anticonvulsant, and has thus been suggested as a viable alternative antidepressant for patients predisposed to epilepsy (Fischer, 1991). It has been reported that (+)-TCP is more potent than the (-)-TCP as an anticonvulsant (Fischer, 1991). The combination of lithium and TCP appears to be an effective treatment for bipolar depression characterized by anergia (Himmelhoch et al., 1972). The combination of MAOIs, including TCP, with TCAs seems to reduce depression in some patients who have not responded to TCAs alone, but increases their anxiety

(Raskin, 1983). TCP in combination with amphetamine (Sovner, 1990) or with carbamazepine (Ketter et al., 1995) may be useful in treating some cases of refractory depression.

Despite several decades of clinical use, much is still unknown about the metabolism of TCP. Theoretically, one of the potential metabolic pathways of TCP is the opening of its cyclopropyl ring, which could yield three potential metabolites (Figure 1.2). They are 1-amino-3-phenylpropane, 1-amino-2-phenylpropane, and 2-amino-1-phenylpropane (amphetamine). Alleva (1965) reported hippuric acid as a metabolite of TCP, following subcutaneous or oral administration of ^{14}C-TCP, which was the first evidence supporting the cleavage of the cyclopropyl ring. However, he concluded that amphetamine was not involved as an intermediate in this metabolism. The metabolic formation of amphetamine from TCP continues to be debated. While one study reported the presence of amphetamine in the plasma of a patient with TCP overdose (Youdim et al., 1979), another study reported the absence of amphetamine in two cases of overdose with TCP (Iwersen & Schmoldt, 1996). Also, studies conducted by Reynolds et al. (1980) on humans and by Sherry et al. (2000) on humans and rats have not revealed amphetamine in human urine or rat brain, respectively after the administration of pharmacologically relevant doses of TCP. In addition, the two other potential metabolites of cyclopropyl ring cleavage have not been identified as metabolites of TCP (Sherry et al., 2000).

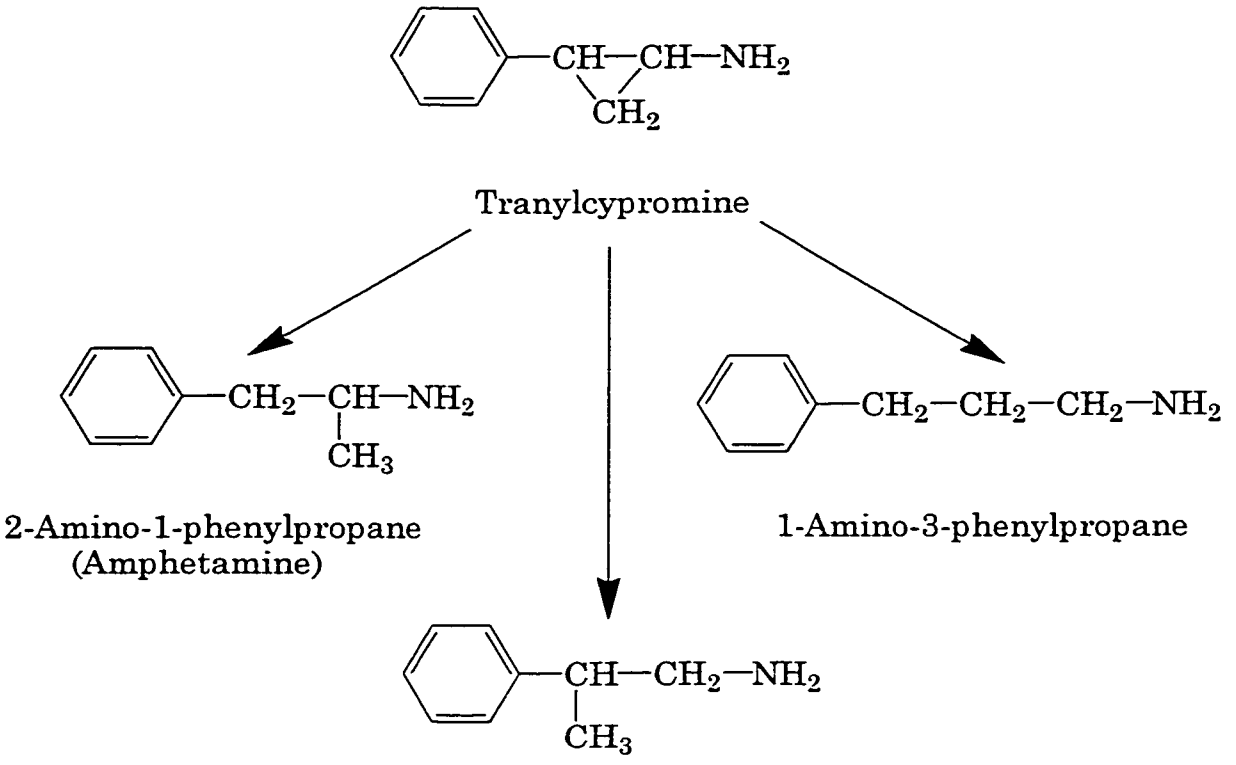


Figure 1.2 Potential metabolites of tranylcypromine formed by cleavage of the cyclopropyl ring.

Some other metabolic pathways of TCP have been reported. The presence of the N-acetyltranylcypromine (Calverley et al., 1981; Kang & Chung, 1984) and 4-hydroxytranylcypromine (OHTCP; Baker et al., 1986; Nazarali et al., 1987) has been demonstrated in rat brain and urine samples after TCP administration. Kang and Chung (1984) also identified N-acetyl-4-hydroxytranylcypromine as a TCP metabolite in rat urine. In a study on the biotransformation of TCP by the fungus *Cunninghamella echinulata*, Foster et al. (1991) found N-acetyl-TCP and N,O-diacetylated OHTCP as major metabolites.

It has been suggested that ring hydroxylated metabolites of TCAs contribute to cardiovascular side effects (Jandhyala et al., 1977; Kutcher et al., 1985; Young et al., 1991), but it is not presently known whether the 4-hydroxy metabolite of TCP contributes to the side effects associated with TCP.

In an attempt to improve pharmacokinetic and side effect profiles of TCP, researchers in the Neurochemical Research Unit at the University of Alberta synthesized several analogues of TCP substituted at the 4-position of the phenyl ring to protect these compounds from ring hydroxylation. Two of these drugs, namely 4-fluorotranylcypromine (FTCP) and 4-methoxy-tranylcypromine (MTCP), were found to be more potent than TCP at inhibiting rat brain MAO *in vitro* (Rao et al., 1986). FTCP compared to TCP attained higher levels in rat brain after intraperitoneal injection of equimolar

amounts of the drugs (Coutts et al., 1987). The levels of FTCP in brain, unlike TCP, were not altered by coadministration of drugs such as iprindole, chlorpromazine, or trifluperazine (Sherry et al, 1999). It was observed that FTCP causes a down-regulation of 5-HT_2, tryptamine, β-adrenergic, and α_2-adrenergic receptors in rat brain, characteristics shared by other MAOIs that are antidepressants (Greenshaw et al., 1988; Sherry et al., 1999).

There are a limited number of studies on pharmacokinetics of MAOIs. TCP is absorbed rapidly, with peak plasma concentration levels ranging from 64.5 to 190 ng/mL attained within 40 minutes to 3.5 hours following a 20 mg oral dose administration (Mallinger et al., 1986). A significant correlation has been found between the mean orthostatic hypotension, orthostatic pulse rate rise and the mean plasma TCP concentration; this finding suggests that at least some cardiovascular effects of TCP are strongly related to the free TCP concentration and unrelated to irreversible MAO inhibition (Mallinger et al., 1986). Elimination of TCP is rapid, and the calculated half-life in humans is 2.5 ± 0.77 hours (mean ± S.D.; Mallinger et al., 1990).

1.4 Drug-Drug Interactions Involving Tranylcypromine or Phenelzine

Drug-drug interactions can be grouped into two principal groups, pharmacokinetic and pharmacodynamic. Pharmacokinetic drug interactions happen at the level of those processes that involve transport to and from the

receptor site and consist of absorption, distribution in body tissue, plasma protein binding, metabolism, and excretion. Pharmacodynamic interactions occur at biologically active sites (Ciraulo et al., 1995; Preskorn, 1995). Understanding of the sites of interaction is essential for rational drug treatment.

Drug-drug interactions have been observed after coadministration of PLZ or TCP with other drugs, including TCAs, hypnotics, SSRIs and narcotic analgesics such as pethidine (Blackwell, 1981; Mitchell, 1997). These interactions can be termed pharmacodynamic interactions (such as interaction of MAOIs with SSRIs) or pharmacokinetic interactions (such as inhibitory actions of MAOIs on CYP enzymes).

It is well known that the administration of meperidine (pethidine) to patients being treated with a MAOI, including PLZ or TCP, can produce toxic interactions (London & Milne, 1962; Eade & Renton, 1969). Hence, pethidine must never be used in the presence of MAOIs. The clinical picture resulting from such an interaction has two distinct forms; an excitatory form, which is thought to be due to central serotonergic over-activity, and a depressive form due to inhibition of hepatic microsomal enzymes by MAOIs. The excitatory form is characterized by sudden agitation, unmanageable behavior, headache, hyper- or hypotension, rigidity, convulsions, hyperpyrexia, and coma. The depressive form is characterized by respiratory depression, hypotension and coma (Davidson et al., 1984; Browne & Linter, 1987).

Several studies investigated the inhibitory effects of TCP and PLZ on some oxidative reactions catalyzed by microsomal enzymes using rat liver microsomes. The type of inhibition caused by PLZ or TCP seems to vary depending on the utilized substrate and the metabolic reaction involved. The experiments conducted by Belanger & Atitse-Gbeassor (1982) have shown that TCP inhibits competitively the N-demethylation of aminopyrine (K_i=0.07 mM) and O-demethylation of *p*-nitroanisole (K_i=0.025 mM), but is a non-competitive inhibitor of the N-demethylation of N,N-dimethylaniline (K_i=0.15 mM) and aniline hydroxylation (K_i=0.10 mM). Preincubation of TCP for 30 min with the microsomal homogenate prior to substrate addition resulted in a decrease in the observed inhibitory effect of TCP, and the authors concluded that the metabolic products of TCP have weaker inhibitory effects than the parent drug (Belanger & Atitse-Gbeassor, 1982). Another study also reported that after a 2-hour preincubation of TCP with rat liver microsomes in the presence of NADPH, TCP did not inhibit hydroxylation of bufuralol, a substrate for CYP2D6 in humans (Dupont et al., 1987). A study using human liver microsomes has demonstrated that the inhibition of mephenytoin hydroxylase by TCP is competitive (Inaba et al., 1985; K_i=8 μM).

Eade and Renton (1969) have shown that PLZ and iproniazid are competitive inhibitors of meperidine N-demethylation, while pargyline and TCP are non-competitive inhibitors. However, another study has demonstrated that both PLZ (K_i=0.07 mM) and TCP (K_i=0.14 mM) are

competitive inhibitors of meperidine N-demethylation (Clark, 1967; Clark & Thompson, 1972). The inhibition of bufuralol hydroxylation by PLZ has been reported to be rapidly time-dependent and irreversible, and requires the presence of NADPH for complete inhibition (Dupont et al., 1986). The inhibition of 7-ethoxycoumarin O-deethylation by PLZ is also irreversible, time-dependent and NADPH-dependent (Dupont et al., 1986).

A study by Mukkassah and Yang (1981) has shown that at least part of the inhibition of oxidative metabolism of aminopyrine produced by PLZ is related to metabolism of PLZ to a reactive intermediate that can cause heme destruction in the CYP. The reactive intermediate has been reported to be a 2-phenylethyl radical, and the metal-free porphyrin derived from the prosthetic heme group has been isolated and identified as N-(2-phenylethyl) protoporphyrin IX (Ortiz de Montellano et al., 1983).

1.5 Drug Biotransformation

All organisms are exposed daily to a wide variety of foreign compounds called xenobiotics, including drugs. Many xenobiotics are absorbed through the skin, lungs, or gastrointestinal tract, due to their lipophilic characteristics. Because of the lipophilic nature of renal tubular membranes these compounds are reabsorbed. Consequently, the elimination of xenobiotics often depends on their conversion to water-soluble chemicals by a process known as biotransformation (Parkinson, 1996; Correia, 1998). An

exception to this general rule is the elimination of volatile compounds by exhalation (Parkinson, 1996).

In addition to a change in pharmacokinetic behavior, the consequence of xenobiotic biotransformation is termination or alteration of biological effects of xenobiotics, including drugs. In some cases, drugs must undergo biotransformation to exert their beneficial pharmacodynamic effect, and in other cases to exert their toxic or tumorogenic effects (Parkinson, 1996).

Biotransformation is usually catalyzed by enzymes, and xenobiotic-biotransforming enzymes are widely distributed throughout the body. In vertebrates, the liver is the richest source of these enzymes. At the subcellular level, enzymes involved in biotransformation reactions are located primarily in the endoplasmic reticulum or cytosol, with lesser amounts in mitochondria, nuclei, and lysosomes (Parkinson, 1996).

The reactions catalyzed by xenobiotic-biotransforming enzymes are generally divided into two groups, phase I and phase II (Williams, 1971). Phase I reactions involve oxidation, reduction, and hydrolysis. These reactions expose or introduce a functional group (-OH, -NH_2, -SH, or –COOH), and usually result in a small increase in hydrophilicity. Phase II biotransformation reactions include glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione (mercapturic acid synthesis) and conjugation with amino acids such as glycine, taurine, or glutamic acid. Most

phase II reactions result in a large increase in xenobiotic hydrophilicity, markedly promoting the excretion of chemicals (Parkinson, 1996).

Among phase I biotransforming enzymes, the CYP system ranks first in terms of catalytic versatility and the number of xenobiotics it detoxifies or activates to reactive intermediates (Guengerich, 1987; Waterman & Johnson, 1991).

1.6 Cytochrome P450 Enzymes

Cytochrome P450 monooxygenase is one of the major enzyme systems and plays an important role in determining the organism's capability of dealing with drugs and other chemicals. The enzyme system consists of two protein components; a hemoprotein called cytochrome P450 (CYP), which is the focus of this section, and a flavoprotein called NADPH-cytochrome P450 reductase, which is discussed briefly here.

CYP is the substrate- and oxygen-binding site of the enzyme system, while the reductase serves as an electron carrier, shuttling electrons from NADPH to the CYP-substrate complex (Meyer, 1996). The NADPH-cytochrome P450 reductase is sometimes named NADPH-cytochrome c reductase because of its ability to reduce cytochrome c in the presence of NADPH + H^+ (Gibson & Skett, 1994; Meyer, 1996; Parkinson, 1996). Liver microsomes contain numerous forms of CYP, but contain a single form of

NADPH-cytochrome P450 reductase and cytochrome b_5 (Parkinson, 1996). The precise role of cytochrome b_5 remains controversial.

1.6.1 Nomenclature and Classification

The name cytochrome P450 is derived from the fact that the cytochrome, initially identified as a red liver pigment (P), exhibits a spectral absorbance maximum at approximately 450 nm when reduced and complexed with carbon monoxide (CO). All other hemoproteins that bind CO absorb light maximally at 420 nm. The unusual absorbance maximum of CYP is due to an unusual fifth ligand to the heme (a cysteine-thiolate). The amino acid sequence around the cysteine residue that forms a thiolate bond with the heme moiety is highly conserved in all CYP enzymes ranging from those in bacteria through lower mammals to man (Gibson & Skett, 1994; Parkinson, 1996).

The broad and often overlapping substrate specificity of liver microsomal CYP enzymes precludes the possibility of naming these enzymes for the reactions they catalyze. The amino acid sequences of numerous CYP enzymes have been determined, and such sequences now form the basis for classifying and naming CYP enzymes (Gonzalez, 1989; Nelson et al., 1993). The prefix "CYP" is used to designate the cytochrome P450 system (Nebert et al., 1987). In general, CYP enzymes with less than 40% amino acid sequence identity are assigned to different gene families. An Arabic number after CYP

designates gene families (1, 2, 3, etc). CYP enzymes that are 40% to 55% identical are assigned to different subfamilies. A letter indicates the gene subfamily (e.g., 2**A**, 2**B**, 2**C**, etc.). In other words, the amino acid sequences within a gene family are usually > 40%, and within a gene subfamily >55% identical. The individual enzymes within a subfamily are designated by a terminal Arabic number on an arbitrary basis (e.g., 2A**1**, 2A**2**, 2A**3**, etc) (Nelson et al., 1996; Parkinson, 1996).

1.6.2 Catalytic Cycle of Cytochrome P450

The CYP system catalyzes the insertion of an active oxygen species into a substrate molecule. The CYP contains iron protoporphyrin IX as the prosthetic group, with the heme group non-covalently bound to the apoprotein. The central features of the CYP catalytic cycle are the ability of the heme iron to undergo cyclic oxidation/reduction reactions in conjunction with substrate binding and oxygen activation (Gibson & Skett, 1994). The precise molecular details of this catalytic cycle have not all been fully elucidated. As illustrated in Figure 1.3, different steps of this catalytic cycle can be summarized as follows.

Step one involves drug binding to the oxidized (Fe^{3+}, ferric) form of CYP. The second step involves the first electron reduction of substrate-bound ferric (Fe^{3+}) to the ferrous (Fe^{2+}) form of the hemoprotein. The reducing equivalent is derived from NADPH + H^+ and is transferred by the NADPH-

cytochrome P450 reductase. Step three involves the binding of molecular oxygen to the ferrous CYP-substrate adduct. The remaining steps involve electron rearrangement, introduction of the second electron and subsequent oxygen insertion, product release, and regeneration of ferric CYP. The precise oxidation states of iron and oxygen in these intermediates are unknown (Gibson & Skett, 1994).

The input of the second electron usually derives from NADPH-cytochrome P450 reductase, and possibly cytochrome b_5. Donation of the second of two electrons by cytochrome b_5 seems to increase the rate of catalysis by CYP. Cytochrome b_5 can also increase the apparent affinity with which certain CYP enzymes bind their substrates. Hence, cytochrome b_5 can increase the V_{max} and/or decrease the apparent K_m of CYP-catalyzed reactions (Parkinson, 1996).

Phospholipids also appear to play an important role in CYP reactions, although the precise mode of action of these lipids is still unknown. It has been suggested that lipid may be required for substrate binding, facilitation of electron transfer and/or providing a template for interaction of CYP and NADPH-cytochrome P450 reductase molecules (Gibson & Skett, 1994; Parkinson, 1996).

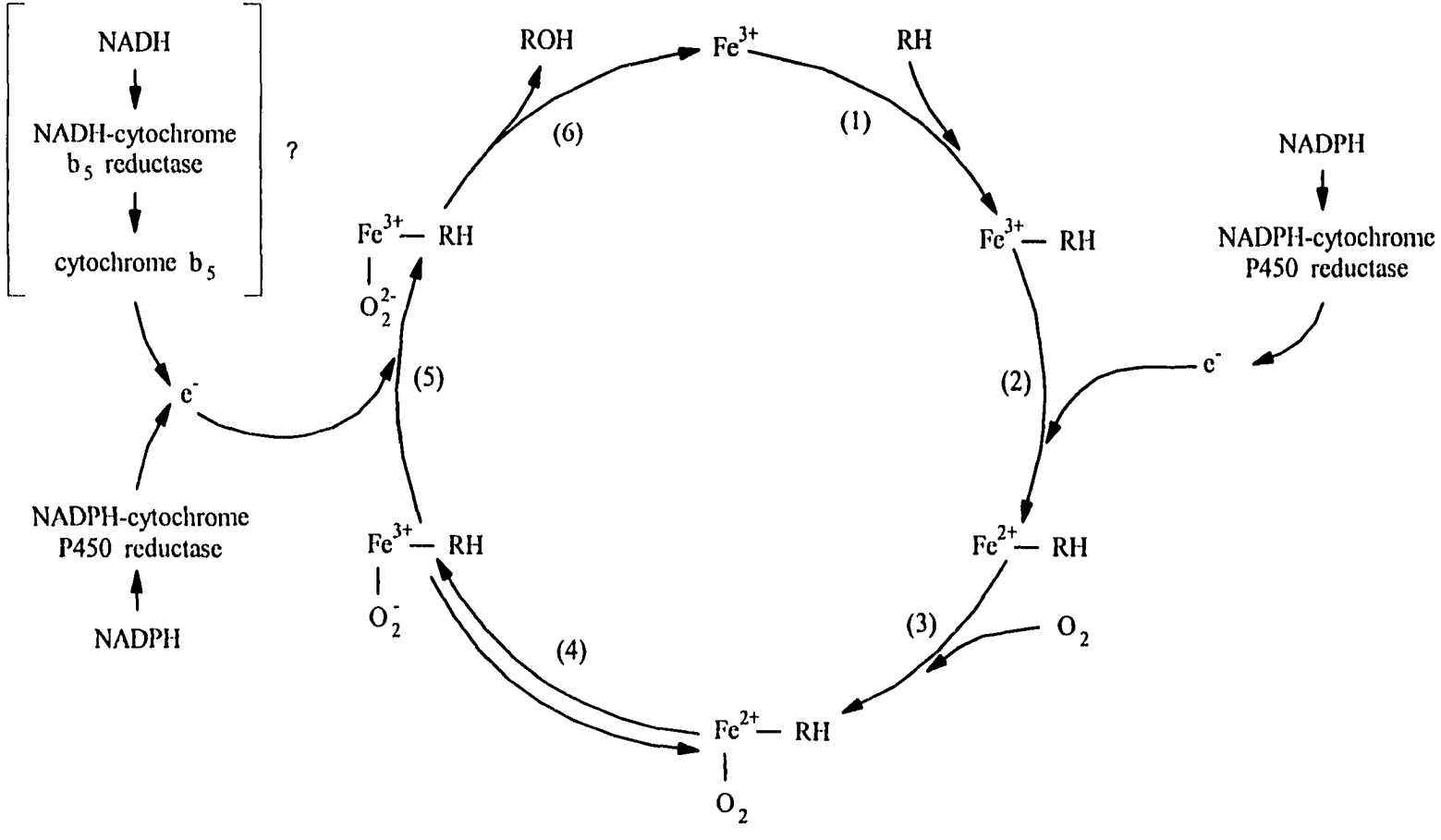


Figure1.3 Catalytic cycle of cytochrome P450. RH represents the drug substrate and ROH the corresponding hydroxylated metabolite. The numbers in brackets refer to the catalytic steps. Adapted from Gibson and Skett, 1994.

1.6.3 Variability in Enzyme Activity

Although total levels of liver microsomal CYP do not vary markedly among individual humans (Distlerath & Guengerich, 1987), there is considerably more variation in the levels of individual CYPs (Guengerich, 1994). Age, gender, genetic and environmental factors may produce considerable variability in enzyme expression between individuals, which may account for the high variability in plasma concentrations of some drugs.

1.6.3.1 Effects of Genetics on CYP Enzyme Activity

Heritable DNA changes that lead to a lack of production or lack of inducibility of a CYP enzyme, or to synthesis of a form of the CYP with altered catalytic activity, are referred to as genetic polymorphisms. A polymorphism is usually defined as a genetically determined difference affecting ≥2% of the population under consideration (Guengerich, 1994). Less frequent genetic changes associated with disorders are often referred to as "genetic deficiencies" or " inborn errors of metabolism" (Guengerich, 1994).

In studying the effects of genetic influences on enzyme activity, most research has been carried out on CYP2D6 and CYP2C19. In these studies, subjects are usually administered substrates for these enzymes, and the rate of metabolism of these compounds is determined from the ratio of urinary concentrations of parent to metabolite. This procedure is called phenotyping. The parent to metabolite ratios falls into two principal groups within

populations; the majority of subjects are classified as "extensive metabolizers" and a minority with reduced metabolism is called "poor metabolizers" (Glue & Banfield, 1996).

Almost all poor metabolizers have two mutant genes, while extensive metabolizers have one or two normal genes (Dahl et al., 1992). There is also an "ultra-rapid metabolizer" group that metabolizes drugs much more rapidly than most of the population due to the presence of multiple copies of the relevant gene (Coutts & Urichuk, 1999). Although CYP2D6 is the only CYP enzyme that has been shown to result in ultra-rapid metabolism, a recent report suggests that ultra-rapid metabolism of maprotiline was caused by enzymes other than CYP2D6 and suggested that CYP2C19 may be a candidate (Vormfelde et al., 1997).

Poor, extensive, and ultra-rapid metabolizers are also recognized by genotyping. In genotyping, molecular techniques such as polymerase chain reaction combined with restriction fragment length polymorphism provide complete structures of mutant alleles, and thus the mutant enzyme resulting from them can be ascertained (Coutts & Urichuk, 1999).

1.6.3.2 Environmental Factors and CYP Enzyme Activity

Another reason for variation in levels of CYP enzymes is enzyme induction. Several of the CYP enzymes are known to be induced by drugs, endogenous compounds, foods, social habits such as smoking and drinking

alcoholic beverages and disease status (Gibson & Skett, 1994; Guengerich, 1994; Glue & Banfield, 1996; Wrighton et al., 1996). For example, CYP1A2 activity may be increased by smoking, consumption of cruciferous vegetables such as cabbage and foods cooked over charcoal, and diets high in protein and low in carbohydrates (Kappas et al., 1976, 1978; Yang et al., 1992; Bailey et al, 1994). Activity of CYP2E1 may be induced by fasting (Hong et al., 1987) and chronic consumption of alcohol (Perrot et al., 1989). Also, an increase in CYP2D6 activity during pregnancy has been reported, and may be caused by induction of CYP2D6 (Wadelius et al., 1997).

CYP inducers can increase CYP enzyme expression by increasing synthesis, decreasing degradation, activating pre-existing components or a combination of these processes (Gibson & Skett, 1994). Induction of CYP enzymes lowers blood levels of the parent drug, which can compromise the therapeutic goal of drug therapy. For example, induction of ethinylestradiol metabolism by phenobarbital and rifampin can decrease the contraceptive effect of the former drug and lead to unplanned pregnancy (Parkinson, 1996). However, as an underlying cause of adverse effects, CYP induction is less important than CYP inhibition, because the latter can cause a rapid and profound increase in blood levels of a drug that can lead to symptoms of overdose and toxicity. One exception to this general rule is the potentiation of acetaminophen hepatotoxicity due to CYP induction by alcohol or isoniazid (Parkinson, 1996).

Another source of interindividual variations is enzyme inhibition or suppression of CYP expression by drugs, endogenous compounds, foods, social habits, and disease status (Glue & Banfield, 1996; Parkinson, 1996; Wrighton et al., 1996). For instance, CYP1A2 activity is reduced by grapefruit juice, psoralens (found in parsley, parsnip and celery), and diets low in protein and high in carbohydrates (Kappas et al., 1978, 1976; Yang et al., 1992; Bailey et al, 1994). Grapefruit juice also inhibits activity of CYP3A4 (Bailey et al., 1994), and cruciferous vegetables inhibit CYP2E1 activity (Koop, 1992; Yang et al., 1992).

The inhibition of CYP enzymes may be reversible (competitive, non-competitive, uncompetitive, or mixed type) or irreversible. The clinical relevance of the inhibition in terms of drug-drug interactions in humans depends on the relative affinities of the two compounds for the enzyme, the concentrations of drugs at the enzyme site, the role of the CYP enzyme in the overall elimination of two drugs and the therapeutic index (Halpert, 1995).

1.6.3.3 Age and CYP Enzyme Activity

Age is among the factors that may cause interindividual variations. Studies in animals and humans of different ages have shown marked alterations in expression of CYP enzymes in liver tissue. A negative correlation between age and total CYP content, NADPH-cytochrome P450 reductase activity and levels of CYP2E1 and CYP3A proteins has been

reported in samples of human liver microsomes (George et al., 1995). There is preliminary evidence that CYP3A4 activity is lowest in neonates and increases to maximal levels in adulthood (Ratanasavanh et al., 1991), and that CYP3A4 activity decreases between 20 and 80 years of age (May et al., 1994).

1.6.3.4 Gender and CYP Enzyme Activity

Effect of gender on CYP enzyme expression in humans is less clear. One study reported no gender influence on the expression of CYP proteins (George et al., 1995), while other studies indicated higher activity of CYP2C19 (May et al., 1994), greater or similar activity of CYP3A4 (Watkins et al., 1989; Horsmans et al., 1992; Hunt et al., 1992; May et al., 1994), and lower activity of CYP1A2 in females compared to males (Relling et al., 1992; Ford et al., 1993).

1.6.4 Diversity of Cytochrome P450 Enzymes

The CYP families by the last count comprise 481 genes and 22 pseudogenes that have been described in 85 eukaryotes and 20 bacteria. Of the 74 gene families that have been described, 14 exist in all mammals examined to date. These 14 families comprise 26 mammalian subfamilies (cluster of genes), of which 20 have been mapped in the human genome

(Nelson, 1996). The 14 gene families are 1, 2, 3, 4, 5, 7, 8, 11, 17, 19, 21, 24, 27, and 51.

Families 1, 2, and 3 are involved in xenobiotic biotransformation. Substrates of CYP4 are several fatty acids and eicosanoids and relatively few xenobiotics. CYP5, CYP7, CYP8, CYP24, CYP27 and CYP51 are involved in the metabolism and biosynthesis of eicosanoids, vitamins and bile acids. CYP11, CYP17, CYP19 and CYP21 are involved in biosynthesis of steroid hormones from cholestrol (Parkinson, 1996; Baker et al., 1998).

Among numerous CYP enzymes, only six of them play a major role in the metabolism of drugs in common clinical use. Prominent among them with respect to a number of substrate drugs are CYP3A4 and CYP2D6, with smaller numbers of drugs metabolized by CYP2C9, CYP2C19, CYP1A2, and CYP2E1 (Meyer, 1996). Major features of these six CYP enzymes will be described in the following section. CYP2A6, CYP2B6 and CYP2C8 are also implicated in the metabolism of xenobiotics (Parkinson, 1996), but are not addressed in this thesis.

1.6.5 CYP3A4

The most abundant CYP enzymes in human liver microsomes belong to the CYP3A gene subfamily. The CYP3A subfamily in humans includes at least four forms of CYP. These are CYP3A3, CYP3A4, CYP3A5 and CYP3A7 (Wrighton et al., 1996). CYP3A3 and CYP3A4 are so highly related, differing

only by 11 amino acid substitutions, that the majority of studies have not been able to distinguish their catalytic activity contributions (Gonzalez, 1992; Wrighton et al., 1996). Thus they are often referred to as CYP3A3/4 (Wrighton et al., 1996). All human livers appear to contain CYP3A4, although the levels vary enormously (>10 fold) among individuals (Wrighton & Stevens, 1992; Shimada et al., 1994). In addition to liver, CYP3A4 is also expressed in other tissues, including lung (Shimada et al., 1989) and especially small intestine (Kaminsky & Fasco, 1992). CYP3A5 is expressed in only 10-30% of adult human livers, and CYP3A7 is found primarily in the human fetal liver (Parkinson, 1996; Wrighton et al., 1996).

The CYP3A enzymes play a very prominent role in human metabolism of xenobiotics (Wrighton & Stevens, 1992), and have very broad substrate specificities (Guengerich, 1994). The CYP3A4 in the small intestine where extensive transformation may occur during absorption across the wall plays an important role in the metabolism of orally administered drugs (Kolars et al., 1991).

Table 1.3 lists some substrates, inducers and inhibitors of CYP3A4. Nifedipine oxidation, erythromycin N-demethylation, midazolam hydroxylation, testosterone 6β-hydroxylation, and cortisol 6β-hydroxylation are all *in vitro* probe substrates of CYP3A3/4 catalytic activity (Wrighton et al., 1996). In humans, CYP3A enzymes are inducible by numerous drugs, such as rifampin, dexamethasone, phenobarbital, and phenytoin (Pichard et

Substrates		Inhibitors	Inducers
Acetaminophen	Loratadine	Clotrimazole	Carbamazepine
Aldrin	Losartan	Erythromycin	Dexamethasone
Alfentanil	Lovastatin	Ethinylestradiol	Phenobarbital
Alpidem	Midazolam	Fluvoxamine	Phenytoin
Alprazolam	Nefazodone	Gestoden	Rifampin
Amiodarone	Nifedipine	Itraconazole	Sulfadimidine
Astemizole	Omeprazole	Ketoconazole	Sulfinpyrazone
Benzphetamine	Progesterone (6β,	Miconazole	
Budesonide	some 16α)	Naringenin	
Carbamazepine	Propafenone	Nefazodone	
Cisapride	Quinidine	Troleandomycin	
Clonazepam	Rapamycin		
Codeine	Retinoic acid		
Cortisol	Sertraline		
Cyclophosphamide	Steroids (e.g. cortisol)		
Cyclosporin	Sulfamethoxazole		
Dapsone	Sulfentanil		
Dexamethasone	Tacrolimus		
Dextromethorphan	Tamoxifen		
Digitoxin	Taxol		
Diltiazem	Teniposide		
Diazepam	Terfenadine		
Ebastine (alcohol)	Testosterone		
Erythromycin	Tetrahydrocannabinol		
17β-Estradiol	Theophylline		
17α-Ethinylestradiol	Toremifene		
	Triazolam		
Etoposide	Trimethadone		
Felodipine (dihydropyridine)	Troleandomycin		
	Venlafaxine		
Flutamine	Verapamil		
Gestodene	Warfarin		
Hydroxyarginine	Zatosetron		
Ifosphamide	Zolpidem		
Imipramine	Zonisamide		
Lansoprazole			
Lidocaine			

Table 1.3 Substrates, inhibitors and inducers of CYP3A4 (data compiled from Guengerich, 1995; Parkinson, 1996; Richelson, 1997).

al., 1990). Several inhibitors of CYP3A have been recognized, including troleandomycin, gestodene, ketoconazole, clotrimazole, and flavones such as naringenin and quercetin found in grapefruit juice (Parkinson, 1996; Wrighton et al., 1996). The roles of CYP3A5 and CYP3A7 in drug metabolism have not been studied as extensively as those of CYP3A3/4.

The function and regulation of the CYP3A enzymes are fairly well conserved among mammalian species, with some notable exceptions (Parkinson, 1996). For example, in rat CYP3A is a major enzyme for S-mephenytoin hydroxylation whereas in human CYP2C catalyzes this reaction (Smith, 1991). Rifampin is an inducer of the CYP3A enzymes in humans and rabbits but not in rats or mice, whereas the opposite appears to be true of pregnenolone-16α carbonitrile (Pichard et al., 1990). Sex differences in expression of CYP3A are seen in rats but not humans (Smith, 1991; Parkinson, 1996).

1.6.6 CYP2D6

CYP2D6 is the only CYP2D enzyme expressed in humans. CYP2D7 and CYP2D8 are pseudogenes (Nelson et al., 1993). CYP2D6 is expressed in liver, intestine, kidney and brain (Gonzalez, 1992). Although CYP2D6 accounts for 1-5% of total CYP hepatic protein mass, it is responsible for the metabolism of numerous drugs (Bertz & Granneman, 1997). The β-adrenoceptor blocking agent bufuralol is metabolized almost exclusively at

the 1′-position by CYP2D6 to 1′-hydroxybufuralol, and can be used as a very sensitive and specific substrate with which to assay CYP2D6 activity (Gentest, 1992).

Dextromethorphan is also used as a probe drug *in vivo* and *in vitro* for evaluation of CYP2D6 activity. Quinidine is a potent competitive inhibitor of CYP2D6, but it is not a CYP2D6 substrate (Guengerich, 1994; Wrighton et al., 1996). CYP2D6 does not seem to be inducible by drugs or other organic chemicals (Coutts, 1994). Table 1.4 shows some substrates and inhibitors of CYP2D6.

CYP2D6 exhibits genetic polymorphism, and the poor metabolizer phenotype is inherited as an autosomal recessive trait. Considerable racial differences are seen in CYP2D6. In Caucasians, CYP2D6 is defective in 5-10% of the population; however, it is defective in less than 2% of African Americans, Africans, Thai, Chinese, and Japanese subjects (Parkinson, 1996).

There are numerous reports in the literature that have associated the risk of onset of various disorders with CYP2D6 status. For example, low incidences of some chemically induced neoplastic diseases such as lung cancer, bladder cancer and hepatocellular carcinoma have been reported in poor metabolizers of CYP2D6 substrates (Idle, 1991). However, it remains to be determined whether there is a causal or coincidental relationship.

Substrates		Inhibitors	Inducers
Ajmaline	Maprotiline	Ajmalicine	None known
Amiflamine	Methoxyamphetamine	Chimidin	
Amitriptyline	Methoxyphenamine	Corynanthine	
Amphetamine	Metoprolol	Fluoxetine	
Aprindine	Mexiletine	Lobeline	
Brofaromine	Mianserine	Paroxetine	
Bufurolol	Minaprine	Propidin	
Captopril	Nefazodone	Quinidine	
Chlorpromazine	Nortriptyline	Trifluperazine	
Cinnarizine	Olanzapine	Yohimbine	
Citalopram	Ondansetron		
Clomipramine	Papaverine		
Clozapine	Paroxetine		
Codeine	Penbutolol		
Debrisoquine	Perhexiline		
Deprenyl	Perphenazine		
Desmethylcitalopram	Phenformin		
Desipramine	Propafenone		
Dextromethorphan	Propranolol		
Dihydrocodeine	N-Propylajmaline		
Diphenhydramine	Remoxipride		
Encainide	Risperidone		
Flecainide	Sparteine		
Fluoxetine	Thioridazine		
Flunarizine	Timolol		
Fluperlapine	Tomoxetine		
Fluphenazine	Trifluperidol		
Guanoxan	Tropisetron		
Haloperidol (reduced)	Venlafaxine		
Hydrocodone	Yohimbine		
Imipramine	Zuclopenthixol		
Indoramin			

Table 1.4 Substrates, inhibitors and inducers of CYP2D6 in humans (data compiled from Guengerich, 1995; Parkinson, 1996; Richelson, 1997; Coutts & Urichuk, 1999).

Although substrates of CYP2D6 represent a variety of chemical structures, most of them possess common structural features and physical properties. They are lipophilic compounds and have one or more basic nitrogen atoms that become protonated at physiological pH; they also possess a planar, usually aromatic, ring system, with a negative electronic potential above the planar part of the molecule (Koymans et al., 1992; Strobl et al., 1993; Coutts, 1994). The biotransformation of substrates for CYP2D6 occurs 5 to 7.5A° from the basic nitrogen, which interacts with an anionic residue (Glu^{301}) in the substrate-binding site of enzyme (Strobl et al., 1993). This model has some limitations and sometimes known substrates of CYP2D6 cannot be fitted into this model (Groot et al., 1997).

1.6.7 CYP2C Subfamily

The CYP2C subfamily proteins were among the first to be purified from human liver (Wang et al., 1980). This subfamily accounts for approximately 20% of CYP based on hepatic protein mass (Bertz & Granneman, 1997). The CYP2C gene family is composed of 4 closely related genes (Gray, 1995; Guengerich, 1995). However, the various CYP2C members seem to have distinct roles in human drug metabolism. Of the various CYP2C members, CYP2C9 and CYP2C19 will be discussed in more detail.

1.6.7.1 CYP2C9

Evidence suggests that CYP2C9 may be the most abundantly expressed of the human CYP2C subfamily proteins, at least in liver (Guengerich, 1995). A number of allelic variants of CYP2C9, which are generated by one or more amino acid substitutions at positions 144, 358, 359, and 417, have been described (Parkinson, 1996). These amino acid substitutions can influence catalytic activity in a substrate-dependent manner. For example, both wild type CYP2C9 (Arg^{144}), and its allelic variant ($Arg^{144} \rightarrow Cys^{144}$) catalyze the methylhydroxylation of tolbutamide. However, the allelic variant of CYP2C9 is virtually devoid of S-warfarin 6- and 7-hydroxylase activity (Rettie et al., 1994). Genetic polymorphisms in tolbutamide hydroxylation (Scott & Poffenbarger, 1978) and phenytoin 4'-hydroxylation (Vasko et al, 1980) have been suggested but not confirmed (Guengerich, 1995).

CYP2C9 is involved in the metabolism of some drugs in common clinical use, including tolbutamide (methyl) hydroxylation (Relling et al., 1990), phenytoin 4'-hydroxylation (Veronese et al., 1991), (S)-warfarin 7-hydroxylation (Rettie et al., 1992), 4'-hydroxylation of diclofenac and 5'-hydroxylation of piroxicam and tenoxicam (Parkinson, 1996). Sulfaphenazole is a potent inhibitor of CYP2C9, both *in vitro* and *in vivo* (Parkinson, 1996). There is *in vivo* evidence of induction of tolbutamide oxidation by treatment of an individual with barbiturates or rifampicin, which may be interpreted as

evidence of CYP2C9 inducibility (Zilly et al., 1977). Figure 1.5 lists some substrates, inhibitors and inducers of CYP2C9.

Substrates	Inhibitors	Inducers
Diclofenac	Fluoxetine	Rifampin
Ibuprofen	Sulfaphenazole	
Mefenamic acid	Sulfinpyrazone	
Naproxen		
Phenytoin		
Piroxicam		
S-warfarin		
Tenoxicam		
Tetrahydrocannabinol		
Tienilic acid		
Tolbutamide		
Torsemide		

Table 1.5 Some Substrates and Inhibitors and inducers of CYP2C9 (data adapted from Parkinson, 1996; Richelson, 1997).

1.6.7.2 CYP2C19

CYP2C19 accounts for approximately 1% of the total CYP2C subfamily based on hepatic protein mass (Coutts & Urichuk, 1999). Expression of CYP2C19 has not been detected in extrahepatic tissue (Gonzalez, 1992). The gene encoding for CYP2C19 is inducible and is polymorphically expressed, being inherited as an autosomal recessive trait (Nelson, 1996; Wrighton et al., 1996).

There is considerable interethnic variation in the incidence of the poor metabolizer phenotype. In Caucasians, CYP2C19 is defective in as few as 2 to 5% of populations, but it is defective in as many as 12 to 23% of Asian populations (Wrighton & Stevens, 1993; Parkinson, 1996). African Americans demonstrate a poor metabolizer rate of 18% and East Indians have a rate of 21% (Smith & Lin, 1996).

Substrates, inhibitors, and inducers of CYP2C19 are listed in Table 1.6. CYP2C19 is responsible for the 4′-hydroxylation of (S)-mephenytoin, and is highly stereoselective for the S-enantiomer of mephenytoin. The R-enantiomer is not converted to 4′-hydroxymephenytoin, but it is N-demethylated to R-nirvanol. Rifampicin administration to extensive metabolizers of (S)-mephenytoin has been shown to increase mephenytoin 4′-hydroxylation. Tranylcypromine is a relatively potent, but not specific inhibitor of CYP2C19 *in vitro* (Wrighton & Stevens, 1992; Parkinson, 1996).

Substrates	Inhibitors	Inducers
Chloroproguanil	Fluvoxamine	Rifampin
Citalopram	Fluoxetine	
Clomipramine	Tranylcypromine	
Diazepam		
Diphenylhydantoin		
Hexobarbital		
Imipramine		
Lansoprazole		
S-Mephenytoin		
Mephobarbital		
Moclobemide		
Omeprazole		
Pentamine		
Phenobarbital		
Proguanil		
Propranolol		

Table 1.6 Substrates, inhibitors and inducers of CYP2C19 in humans (data compiled from Guengerich, 1995; Parkinson, 1996; Richelson, 1997; Coutts & Urichuk, 1999).

1.6.8 CYP1A1/2

The human CYP1A subfamily contains two inducible members, CYP1A1 and CYP1A2, which are about 70% identical in their sequences (Guengerich, 1995). Human liver microsomes contain relatively high levels of CYP1A2, but not CYP1A1. The latter is essentially an extrahepatic enzyme (Shimada et al., 1992; Schweikl et al., 1993), while CYP1A2 is expressed essentially in the liver and not in extrahepatic tissues (Shimada et al., 1989).

A list of some substrates, inhibitors and inducers of CYP1A2 appears in Table 1.7. Among substrates of CYP1A2, Caffeine has been used as an *in vivo* metabolic probe for CYP1A2 (Guengerich, 1995). CYP1A2 also catalyzes the N-hydroxylation of carcinogenic aryl amines and heterocyclic amines (Butler et al., 1989; McManus et al., 1990), which in many cases represents the initial step in the conversion of aromatic amines to tumorogenic metabolites. The heterocyclic amines are found in charbroiled food and cigarette smoke (Sugimura, 1992). Because CYP1A1 and CYP1A2 are inducible by polycyclic aromatic hydrocarbons, their activity is higher in smokers than non-smokers. Attempts to correlate the inducibility of the CYP1A1 with the incidence of cigarette smoking-induced lung cancer have been non-conclusive (Kouri et al., 1984). Both CYP1A1 and CYP1A2 can be inhibited by α-naphthoflavone. Ellipticine preferentially inhibits CYP1A1, whereas furafylline is a specific inhibitor of CYP1A2 (Parkinson, 1996).

Substrates	Inhibitors	Inducers
Acetaminophen	Fluvoxamine	Charcoal-broiled beef
Acetanilide	Furafylline	Cigarette smoke
Antipyrine	α-Naphthoflavone	Cruciferous vegetables
Aromatic amines		Omeprazole
Bufuralol		
Caffeine		
Clozapine		
Estradiol		
Ethoxyresorufin		
Imipramine		
Maprotiline		
Methoxyresorufin		
Ondansetron		
Phenacetin		
Propranolol		
Tacrine		
Tamoxifen (N-demethylation)		
Theophylline		
R-Warfarin		

Table 1.7 Substrates, inhibitors and inducers of CYP1A2 in humans (data compiled from Guengerich, 1995; Parkinson, 1996; Richelson, 1997).

1.6.9 CYP2E1

CYP2E1 is the only gene in the CYP2E subfamily in most species, including humans (Nelson et al., 1993). This enzyme appears to be expressed in liver and a number of other tissues (Guengerich, 1995). CYP2E1 accounts for 7% of total CYP based on hepatic protein mass (Bertz & Granneman, 1997).

A list of substrates, inhibitors, and inducers of CYP2E1 is given in Table 1.8. Few drugs are oxidized by CYP2E1; these include acetaminophen (oxidation) and chlorzoxazone (6-hydroxylation) (Peter et al., 1990). However, the list of carcinogens oxidized by CYP2E1 is quite extensive (Guengerich et al., 1991; Guengerich & Shimada, 1991). The enzyme is also of interest because of its possible relevance to alcoholism, chemical carcinogenesis, diabetes, and other illnesses (Yang et al., 1990; Guengerich et al., 1991; Koop, 1992). CYP2E1 is induced by ethanol and also catalyzes oxidation of ethanol (Perrot et al., 1989). This induction by ethanol is likely to be the major reason for the increased hepatotoxicity of paracetamol among chronic alcoholics (George et al., 1991). CYP2E1 levels are also induced in the diabetic state and by starvation (Johansson et al., 1991) and are decreased by insulin treatment (Richardson et al., 1992).

Some polymorphisms have been identified in humans, including one in a 5′-upstream region where a transcription factor (HNF-1) putatively binds (Hayashi et al., 1991). However, none of these has been yet associated with

levels of the enzyme or catalytic activity (Guengerich, 1995). Cytochrome b_5 appears to be necessary for optimal activity of the enzyme, at least with regard to several catalytic activities (Wrighton et al., 1987; Gillam et al., 1994). The function and regulation of CYP2E1 are well conserved among mammalian species (Parkinson, 1996).

Substrates	Inhibitors	Inducers
Acetaminophen (ring)	3-Amino-1,2,4-triazole	Ethanol
Alcohols	Diethyldithiocarbamate	Isoniazid
Aniline	Dihydrocapsaicin	
Benzene	Dimethyl sulfoxide	
Caffeine	Disulfiram	
Chlozoxazone	4-Methylpyrazole	
Dapsone	Phenethyl isothiocyanate	
Enflurane		
Halogenated alkanes		
Isoflurane		
Methylformamide		
p-Nitrophenol		
Nitrosamines		
Styrene		
Theophylline		

Table 1.8 Substrates, inhibitors and inducers of CYP2E1 in humans (adapted from Parkinson, 1996).

1.6.10 Probe Substrates of CYP Enzymes Used in This Study

In the present study, tolbutamide (TOL), S-mephenytoin (MEP), and dextromethorphan (DM) have been used as substrates of CYP2C9, CYP2C19, and CYP2D6, respectively.

TOL is an oral hypoglycaemic agent, and belongs to the sulfonylurea class of drugs. Tolbutamide's action is terminated *via* hydroxylation, primarily by CYP2C9. Although most studies have implicated CYP2C9 as the exclusive catalyst of hepatic TOL hydroxylation in humans, there is evidence that other CYP2C enzymes such as CYP2C19 may also participate (Wester et al., 2000). TOL has been used as a probe substrate of CYP2C9 in previous *in vitro* and *in vivo* studies (Eagling et al., 1998; Venkatakrishnan et al., 1998; Yamazaki et al., 1998).

MEP, an antiepileptic drug, is a racemic mixture of optically active isomers and is metabolised stereoselectively by two major pathways, namely aromatic hydroxylation of the S-enantiomer to 4′-hydroxymephenytoin (OHMEP) by CYP2C19 and N-demethylation of the R-enantiomer to nirvanol (Kupfer et al., 1981; Jurima et al., 1984). S-(+)-Mephenytoin has been used as a probe substrate for CYP2C19 in a number of studies (Inaba et al., 1984; Wedlund et al., 1984; Jurima et al., 1985; Baumann et al., 1988; Dahlof et al., 1992; Kiivet et al., 1993; Caslavska et al., 1994; Brockmoller et al., 1995; Endres et al., 1996).

DM, a constituent of many over-the-counter cough syrups, acts centrally to elevate the threshold for coughing (Reisine and Pasternak, 1996). The metabolism of DM is primarily by O-demethylation to dextrorphan (DXR), a reaction that is mediated primarily by CYP2D6 (Schmid et al., 1985; Jacqz-Aigran et al., 1993; Von Moltke et al., 1998). DM is also metabolized to 3-methoxymorphinan, mediated primarily by CYP3A4, and a secondary didemethylated metabolite, 3-hydroxymorphinan (Jacqz-Aigran et al., 1993). DM has been used in *in vitro* studies, including the present study as a probe substrate for CYP2D6 (Kronbach et al., 1987; Kronbach, 1991; Rodrigues, 1996; Vielnascher et al., 1996).

1.7 Principles of Enzyme Kinetics

1.7.1 General Overview

Enzyme kinetics is the study of enzyme reaction rates and the factors that affect them. To explain kinetic properties, the simplest model assumes that a single substrate (S) is metabolized to a single product (P) *via* an intermediate complex (ES) formed between the enzyme (E) and substrate, as represented in the following equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_p} E + P \qquad \text{(Equation 1.1)}$$

The rates of forward and backward reactions for the interaction between E and S are given by the rate constants k_1 and k_{-1}, respectively. The rate of product formation from ES is given by the rate constant k_p. In steady-

state equilibrium, it is assumed that during a short initial period of equilibration, the concentration of ES builds up to a constant steady-state level. For most enzymes, ES decomposes to E + P extremely rapidly, and so the rate of product formation is directly proportional to [ES] (Holt, 1999).

The Michaelis-Menten equation describes the kinetic behavior of enzymes in a steady-state system and can be derived from equation 1.1, when a number of basic assumptions are made (Hohnadel, 1984). The Michaelis-Menten equation predicts that the relationship between [S] and v is hyperbolic in nature. Figure 1.4 represents the graphical relationship between [S] and v which is referred to as the Michaelis-Menten plot.

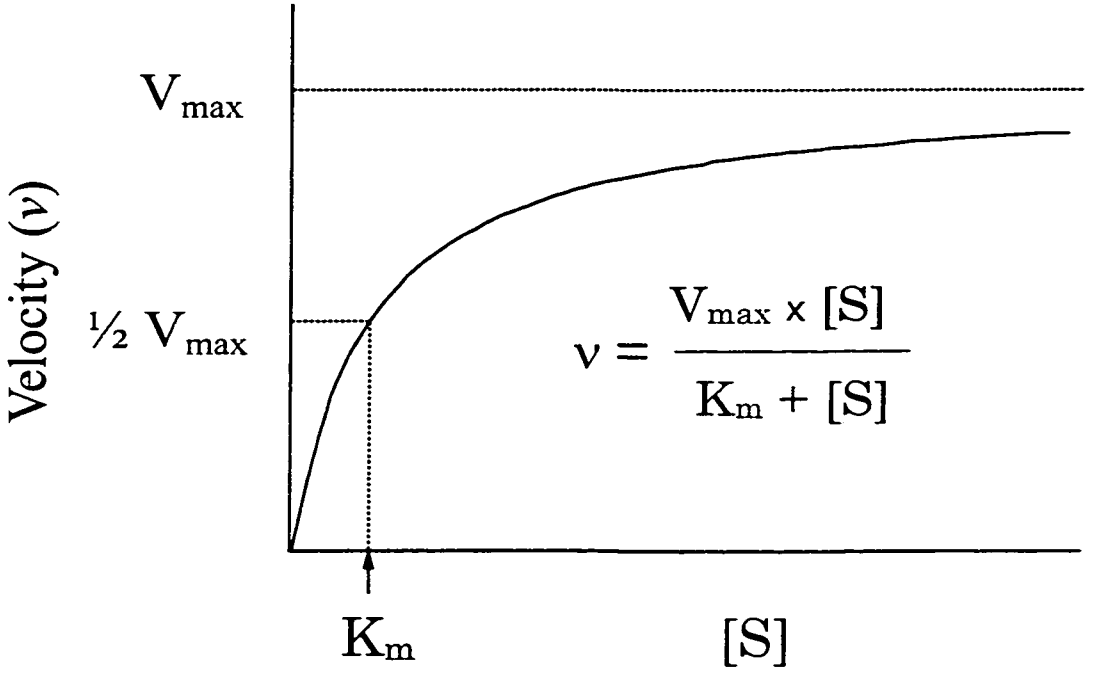


Figure 1.4 A Michaelis-Menten plot showing the hyperbolic relation between substrate concentration, [S], and velocity (v). K_m is the Michaelis-

Menten constant and V_{max} is the maximum rate of reaction (adapted from Hohnadel, 1984).

The Michaelis-Menten equation is mathematically represented as follows:

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \qquad \text{(Equation 1.2)}$$

Where:

v = Initial reaction velocity at any given substrate concentration

V_{max} = The maximum possible reaction velocity that occurs when all of the enzyme active sites are saturated with substrate.

[S] = Substrate concentration.

K_m = The Michaelis constant, which is the concentration of substrate at which the enzyme is operating at half of the maximum possible velocity.

Characterization of an enzyme usually includes determination of K_m and V_{max} for each substrate. Although K_m is independent of enzyme concentration, V_{max} will vary with total amount of enzyme (Palmer, 1995). Knowledge of K_m and V_{max} is useful for a number of biochemical purposes (Henderson, 1998; Holt, 1999). K_m provides an indication of the affinity between substrate and enzyme; a low K_m indicates a high affinity for the substrate, and vice versa. Generally, a compound with a low K_m and a high V_{max} is considered a better substrate than one with a high K_m and low V_{max}.

In many cases, the K_m value provides an approximate estimation for the physiological concentration of substrate (Holt, 1999).

K_m and V_{max} values are also useful in comparative situations such as comparisons of the catalytic activity of enzymes obtained from different tissues or species for a particular substrate or studies of characteristics that make a substrate more suitable for a specific enzyme. Finally, calculating these valuable parameters allows researchers to establish optimal conditions in experiments designed to investigate a specific question (Henderson, 1998; Holt, 1999).

1.7.2 Determination of K_m and V_{max}

K_m and V_{max} can be determined easily from basic experimental data by several methods. The advantages and disadvantages of these methods are discussed by Henderson (1998). One reliable method to calculate the values of K_m and V_{max} is the least-squares fit of data points to a v against [S] hyperbola with the use of a computer program. This method calculates best-fit values of K_m, V_{max}, K_m/V_{max}, $1/V_{max}$ and their standard deviations (Henderson, 1998).

Another statistically sound method for the determination of K_m and V_{max} values is the direct linear plot. In a direct linear plot, values of [S] are plotted on a negative horizontal axis and experimentally determined values of v are plotted on the vertical axis (Figure 1.5). Straight lines drawn through corresponding [S] and v points for two or more pairs of ([S] and v) values

intersect at [S]= K_m and $v = V_{max}$. In practice, because of measurement errors, a number of different intersections may be obtained. The coordinates of each intersection provide estimates of K_m and V_{max}. In this case, the median values of these estimates are the best-fit values of K_m and V_{max} (Cornish-Bowden & Eisenthal, 1974).

Alternatively, the Michaelis-Menten equation can be transformed to give equations yielding a straight line. K_m and V_{max} can then be obtained from slopes and intercepts of the best-fit straight line. Examples of these linear plots are the Hanes-Woolf plot ([S]/ v *versus* [S]; Figure 1.6), the Hofstee plot (v *versus* v/[S]; Figure 1.7), and the Lineweaver-Burk plot (1/v *versus* 1/[S]; Figure 1.8). Compared with Hofstee and Lineweaver-Burk plots, plotting of data on a Hanes-Woolf plot causes less distortion of the experimental error on each point and thus yields the most reproducible values for kinetic constants. The calculated values are also closest to values obtained from computerized analysis of the hyperbola or a direct linear plot (Holt, 1999).

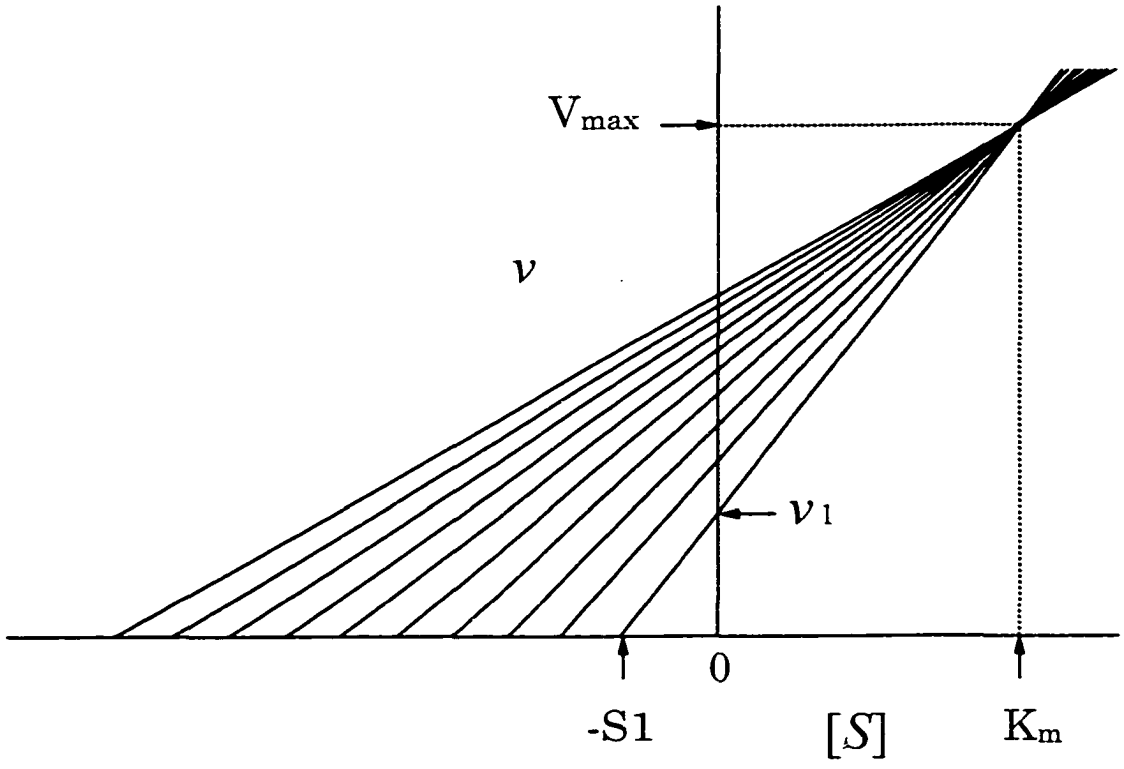


Figure 1.5 A direct linear plot of data that fits the Michaelis-Menten equation. Deviation from Michaelis-Menten kinetics is not apparent in this plot (adapted from Henderson, 1998).

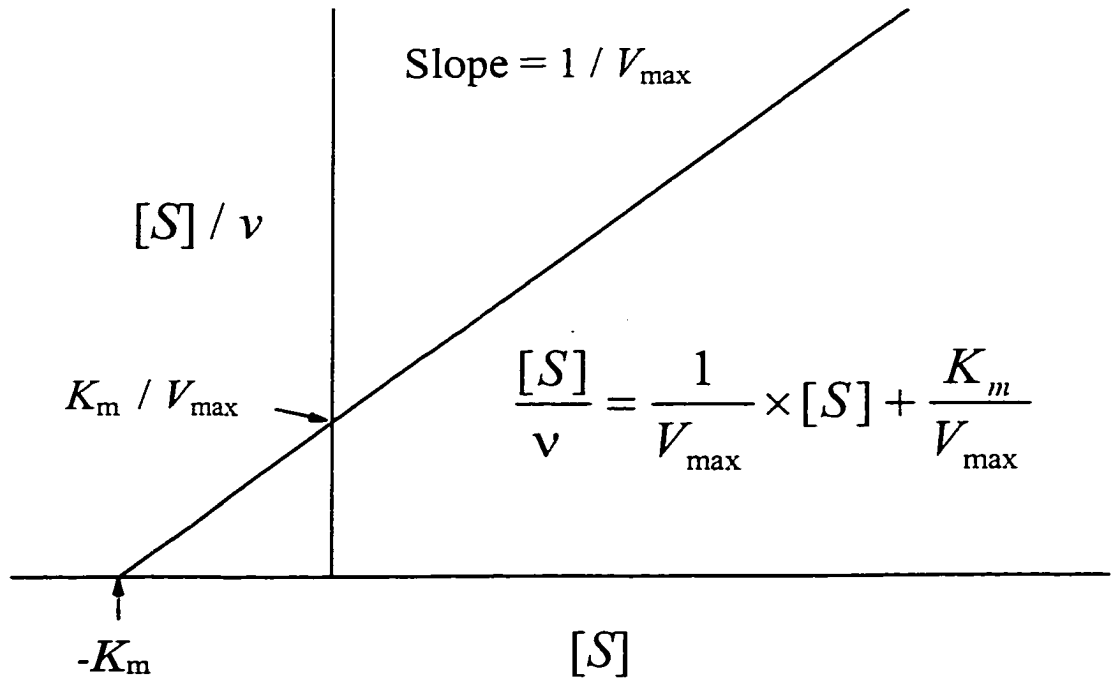


Figure 1.6 A Hanes-Woolf plot. Plotting of data in this transformation causes the least distortion of the experimental error. Thus, it gives the best-fit values for K_m and V_{max} (adapted from Hohnadel, 1984).

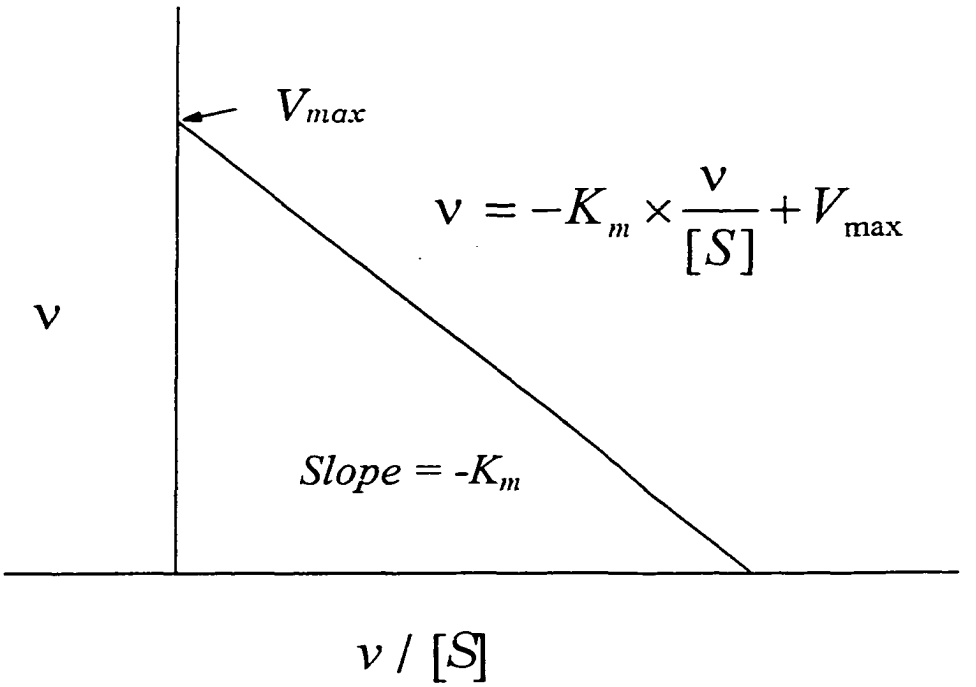


Figure 1.7 An Eadie-Hofstee plot. This type of plot provides excellent confirmation that data obtained can be fitted to a straight line and hence that the enzyme-substrate interaction follows Michaelis-Menten kinetics (adapted from Hohnadel, 1984).

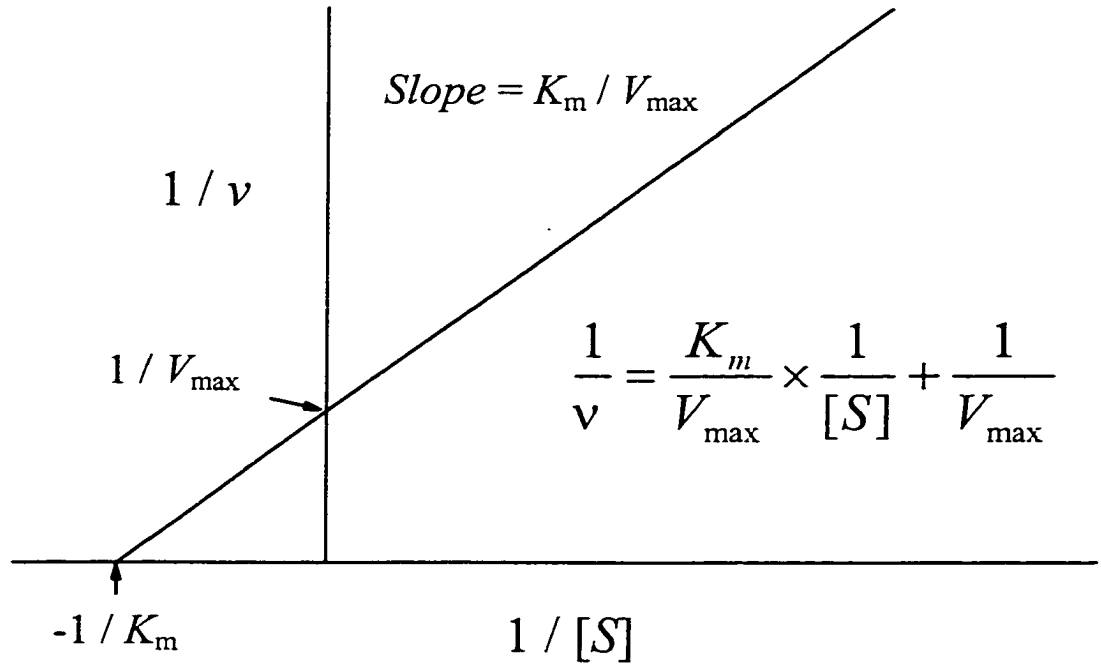


Figure 1.8 A Lineweaver-Burk plot. The largest errors occur in reciprocals of the lowest values of v. Thus, in order to obtain reliable kinetic constants, careful choices of substrate concentrations must be coupled with use of an appropriate method to determine the best fit line that gives more weighting to those data with smallest errors. For illustrative purposes the effects of inhibitors can be most clearly displayed in this plot (adapted from Hohnadel, 1984).

1.8 Enzyme Inhibitors and Principles of Inhibitor Kinetics

Inhibitors are substances that decrease the rate of an enzyme-catalyzed reaction. Inhibitors may act on the substrate, cofactor or enzyme (Palmer, 1995). This section concentrates on inhibitors that combine directly with an enzyme. All enzyme inhibitors are classified either as reversible or irreversible, and most are reversible. Reversible inhibitors bind to an enzyme in a reversible fashion and can be removed by dialysis, dilution, or gel filtration to restore enzyme activity. An irreversible inhibitor is usually bound covalently or very tightly and cannot be removed from an enzyme by dialysis or other processes. Reversible inhibitors usually rapidly form an equilibrium system with an enzyme to show a definite degree of inhibition that is independent of time. The potency of a reversible inhibitor can be quantified by determining an equilibrium constant or dissociation constant (K_i). In contrast, the degree of inhibition by irreversible inhibitors may increase over a period of time (Palmer, 1995; Holt, 1999).

1.8.1 Reversible Inhibition

1.8.1.1 Fully Competitive Inhibition and Calculation of K_i Value

A fully competitive inhibitor binds either at the enzyme active site or at a separate allosteric site such that binding of the inhibitor causes a conformational change at the active site, thereby preventing substrate binding (Holt, 1999). The effect of a competitive inhibitor depends on the

inhibitor concentration, the substrate concentration and the relative affinities of the substrate and the inhibitor for the enzyme (Palmer, 1995). Inhibition by a fully competitive inhibitor can be overcome by increasing the concentration of substrate. At very high substrate concentrations, molecules of substrate will greatly outnumber molecules of inhibitor and the effect of inhibitor will be negligible. Hence the V_{max} for the reaction is unchanged. However, the apparent K_m (K_m') is increased since a higher substrate concentration is required in the presence of inhibitor to achieve half the maximum velocity (Palmer, 1995; Holt, 1999). Figure 1.9 represents a Lineweaver-Burk plot in the presence and absence of a competitive inhibitor.

Assuming steady state conditions, the velocity equation in the presence of a competitive inhibitor is an equation of the same form as the Michaelis-Menten equation, with the only difference being that the K_m has been increased by a factor $[1 + ([I]/K_i)]$. Therefore, for simple competitive inhibition, V_{max} is unchanged but K_m is altered so that $K_m' = K_m [1 + ([I]/K_i)]$. It can be deduced that K_i is equal to the concentration of competitive inhibitor that apparently doubles the value of K_m (Palmer, 1995).

To calculate the inhibitor constant (K_i), a graphical method is preferred to a direct substitution of numbers to allow errors in individual determinations to be averaged out. K_i can be calculated by several graphical methods. Since $K_m' = K_m / K_i [I] + K_m$, plotting K_m' values against [I] gives a line with the x-intercept equal to $-K_i$ (Palmer, 1995). A graph of the slopes of

the Lineweaver-Burk plot (K_m'/ V_{max}) against [I] is also linear and the x-intercept gives $-K_i$ (Figure 1.10). An alternative graphical means of calculating K_i was suggested by Dixon (1953); plotting 1/v against [I] at a fixed [S] is linear, and the intersection of plots for different values of [S] is equal to $-K_i$ (Figure 1.11).

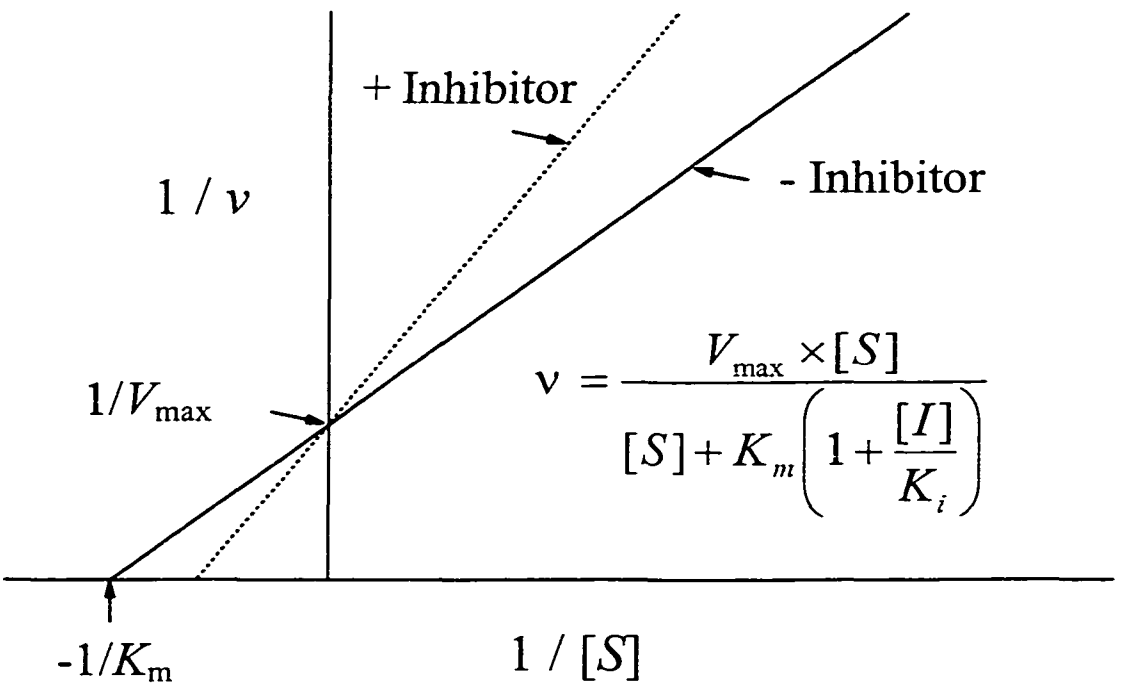
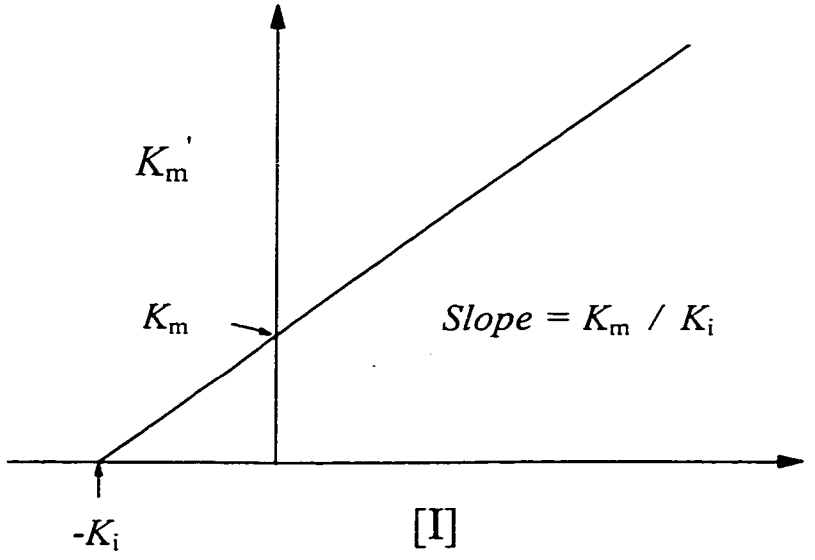


Figure 1.9 The effects of competitive inhibition on K_m and V_{max} are represented by use of a Lineweaver-Burk plot (adapted from Hohnadel, 1984; Palmer, 1995).



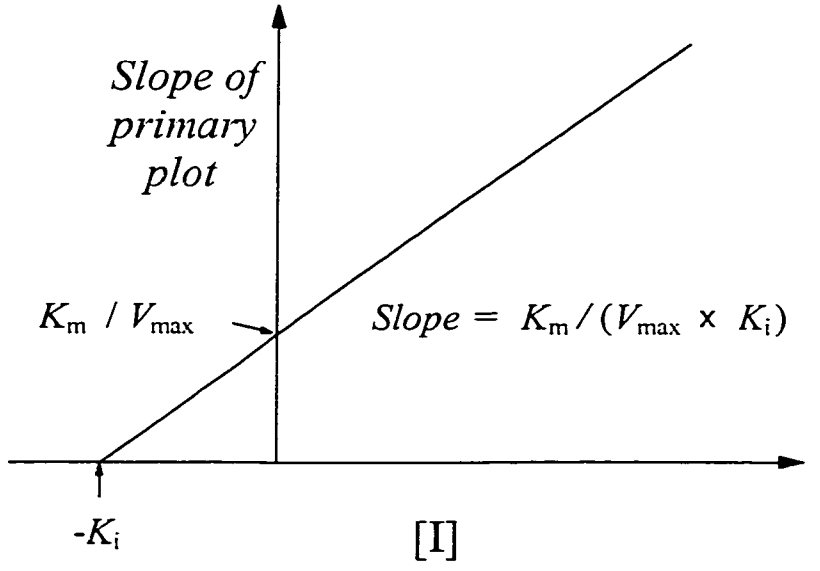


Figure 1.10 Calculation of K_i for competitive inhibition using secondary plots (adapted from Palmer, 1995).

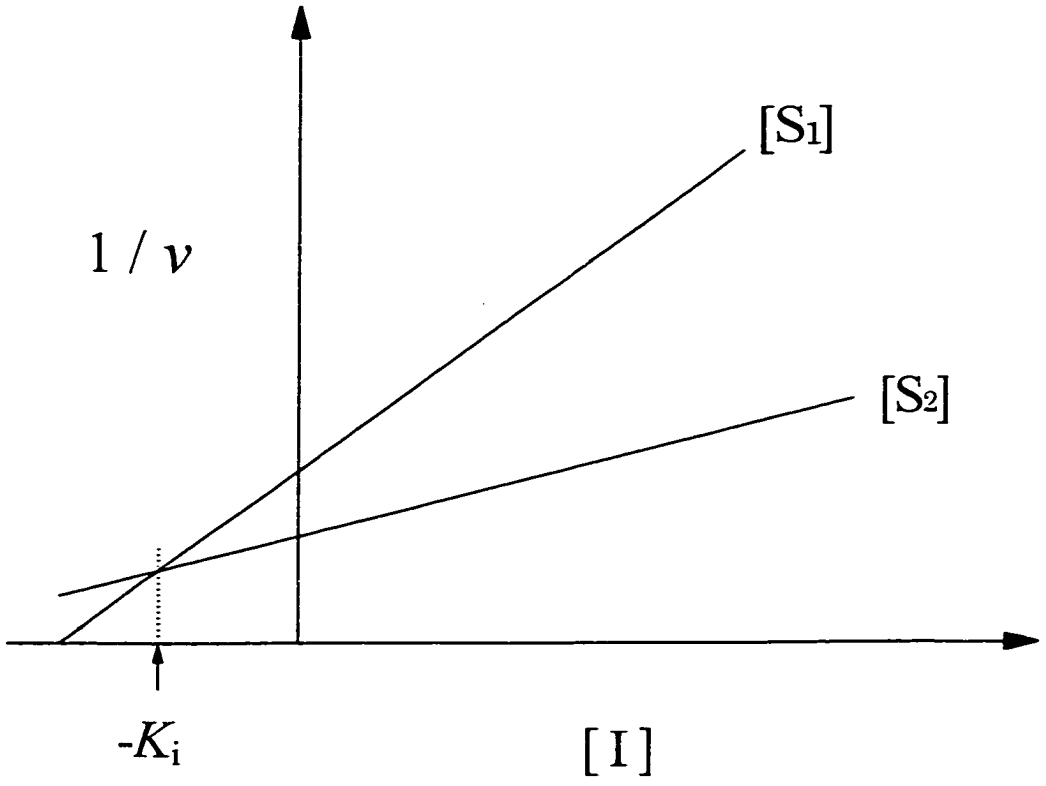


Figure 1.11 Calculation of K_i for competitive inhibition by a Dixon plot (adapted from Palmer, 1995).

1.8.1.2 Fully Non-Competitive Inhibition and Calculation of K_i Value

A non-competitive inhibitor can combine with an enzyme molecule to produce a dead-end complex, regardless of whether a substrate molecule is bound or not. Hence the inhibitor must bind at a different site from the substrate. Since neither inhibitor nor substrate affects the binding of the other, K_m remains unaffected. However, the net effect of a non-competitive inhibitor is to give the impression that less enzyme is present, thus decreasing the value of V_{max} (Palmer, 1995; Holt, 1999). The dissociation constant (K_i) for the ESI complex yielding ES + I or for the EI complex yielding E + I has the same value. The K_i for such a system is the inhibitor concentration that halves the value of V_{max} (Palmer, 1995). Figure 1.12 represents the Lineweaver-Burk plot in the presence and absence of a non-competitive inhibitor.

Assuming steady state conditions, the velocity equation in the presence of a non-competitive inhibitor is an equation of the same form as the Michaelis-Menten equation, with the only difference being that V_{max} has been divided by a factor $[1 + ([I]/ K_i)]$. Therefore, for simple non-competitive inhibition K_m is unchanged but V_{max} is altered so that $V_{max}' = V_{max}/[1 + ([I]/K_i)]$ or $1/ V_{max}' = 1/ V_{max} [1 + ([I]/ K_i)]$ (Palmer, 1995).

K_i can be calculated by several graphical methods. Since $1/ V_{max}' = 1/ V_{max} [1 + ([I]/ K_i)]$, plotting $1/ V_{max}'$ values against [I] or slopes of the

Lineweaver-Burk plot against [I] gives a line with an x-intercept equal to $-K_i$ (Palmer, 1995; Figure 1.13).

A Dixon plot may also be used to determine K_i. In this case, the Dixon plots for different values of [S] intersect on the x-axis, where $[I]=-K_i$ (Figure 1.14).

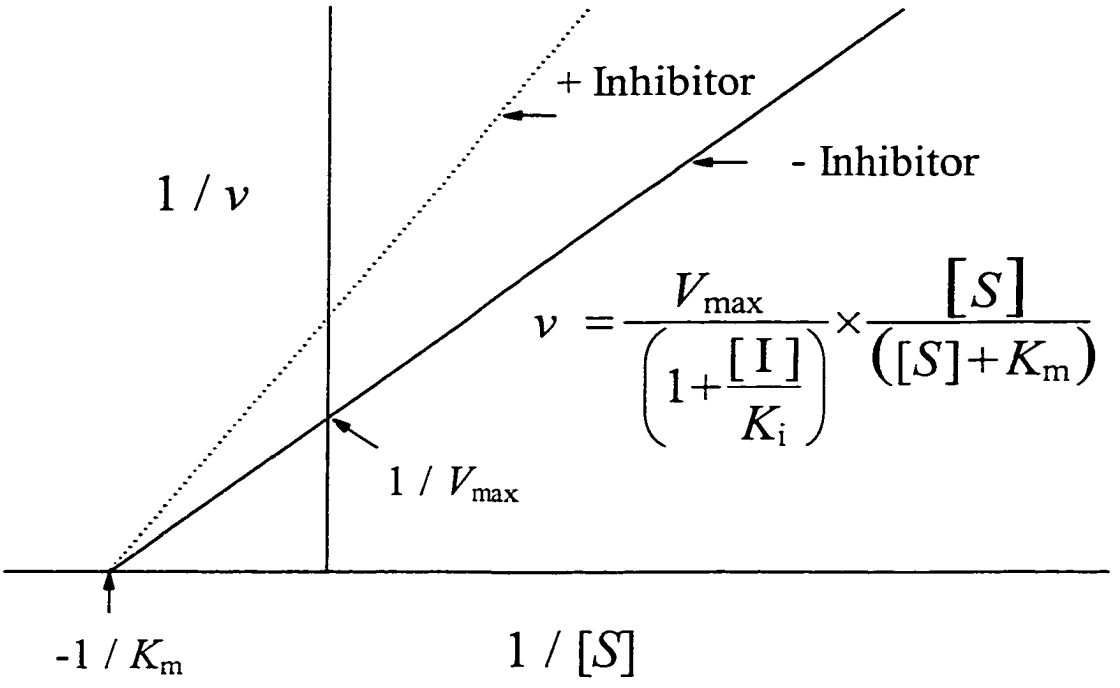
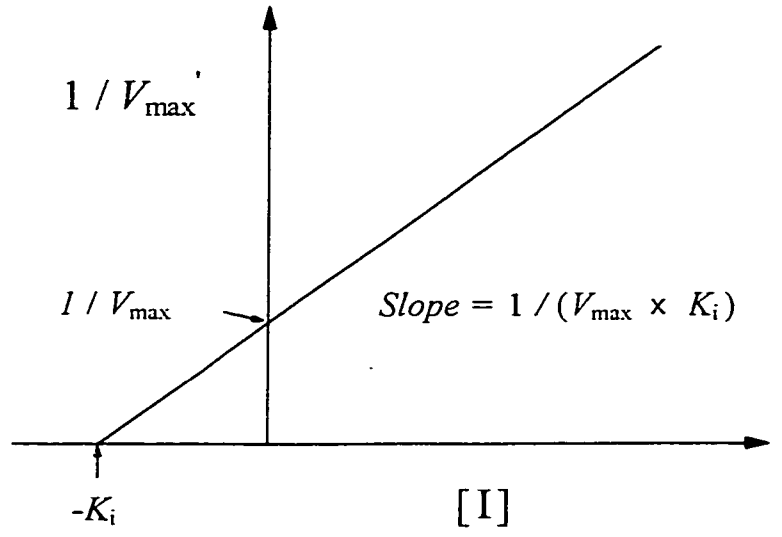


Figure 1.12 The effects of non-competitive inhibition on K_m and V_{max} are represented by use of a Lineweaver-Burk plot (adapted from Hohnadel, 1984; Palmer, 1995).



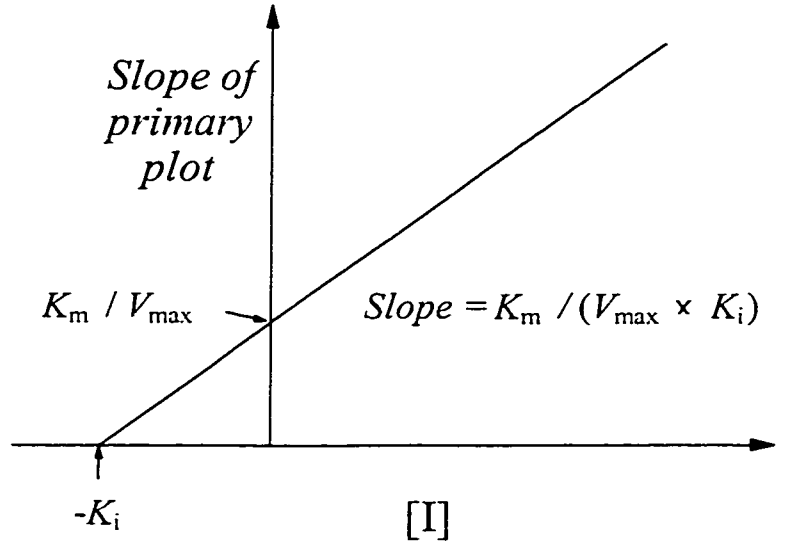


Figure 1.13 Calculation of K_i for non-competitive inhibition using secondary plots (adapted from Palmer, 1995).

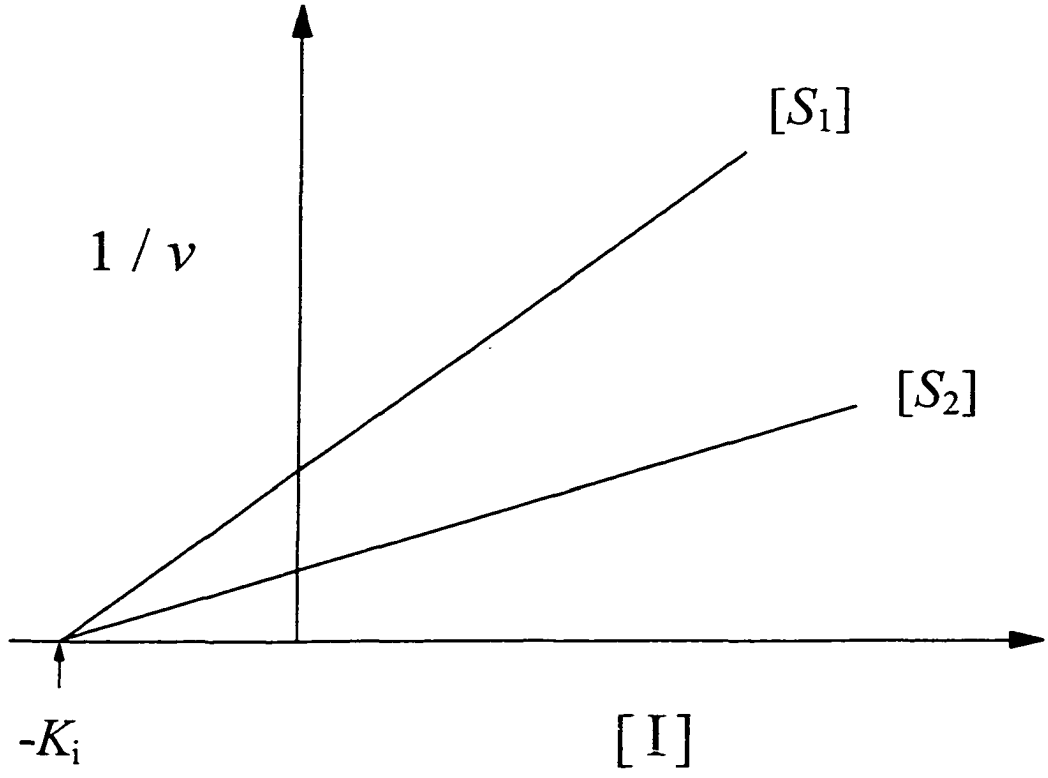


Figure 1.14 Calculation of K_i for non-competitive inhibition by a Dixon plot (adapted from Palmer, 1995).

1.8.1.3 Fully Uncompetitive Inhibition and Calculation of K_i Value

Uncompetitive inhibitors bind only to the enzyme-substrate complex and not to the free enzyme. The ESI complex is a dead-end complex, and cannot break down to yield product. Since the inhibitor does not compete with the substrate for the same binding site, the inhibition cannot be overcome by increasing substrate concentration. A fully uncompetitive inhibitor decreases K_m and V_{max} values to the same extent (Palmer, 1995; Holt, 1999; Figure 1.15).

Uncompetitive inhibition of a single-substrate enzyme-catalyzed reaction is a rare phenomenon, one of the few examples known being the inhibition of arylsulphatase by hydrazine. However, uncompetitive inhibition patterns are seen with two-substrate reactions (Palmer, 1995).

Under steady state conditions, the velocity equation in the presence of an uncompetitive inhibitor is an equation of the same form as the Michaelis-Menten equation, with the constants K_m and V_{max} both being divided by the factor $[1 + ([I]/ K_i)]$. Thus, for uncompetitive inhibition, $K_m' = K_m/[1 + ([I]/K_i)]$ and $V_{max}' = V_{max}/[1+([I]/K_i)]$. The inhibitor concentration equal to K_i will halve the values of both K_m and V_{max} (Palmer, 1995).

The inhibitor constant K_i can be determined using secondary plots. For uncompetitive inhibition, plots of $1/ V_{max}'$ or $1/ K_m'$ against [I] are linear, the intercepts on [I] axes giving $-K_i$ (Palmer, 1995; Figure 1.16). The K_i value for

a fully uncompetitive inhibitor corresponds to the dissociation constant for the ESI complex yielding ES + I.

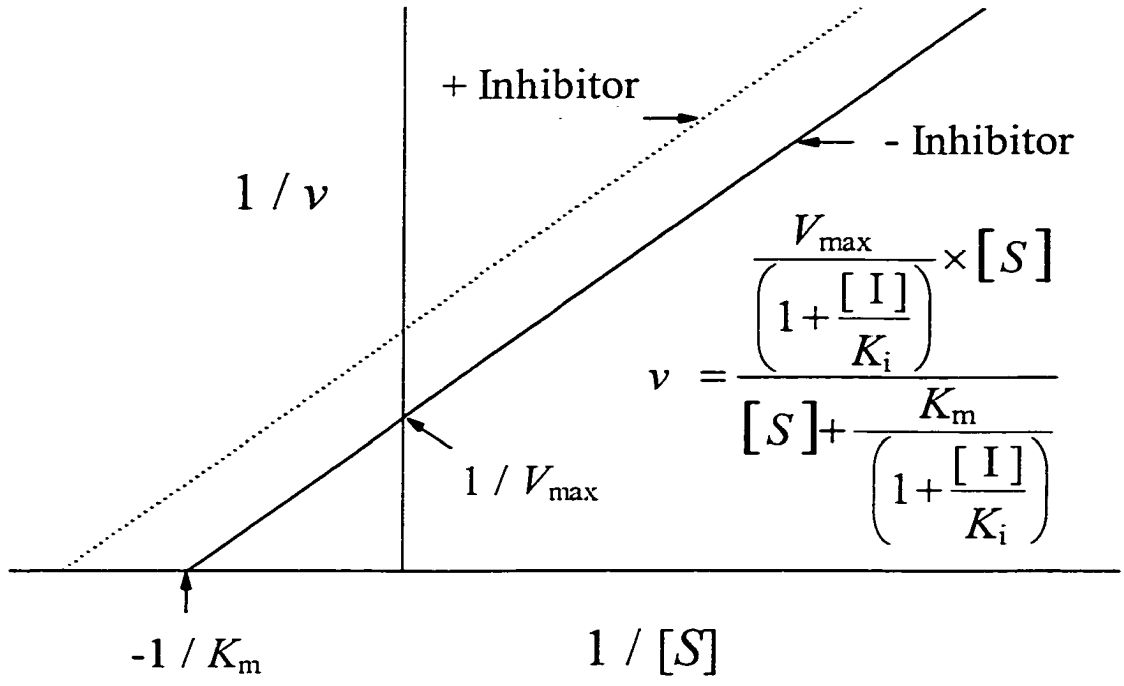


Figure 1.15 The effects of uncompetitive inhibition on K_m and V_{max} are represented by use of a Lineweaver-Burk plot (adapted from Hohnadel, 1984; Palmer, 1995).

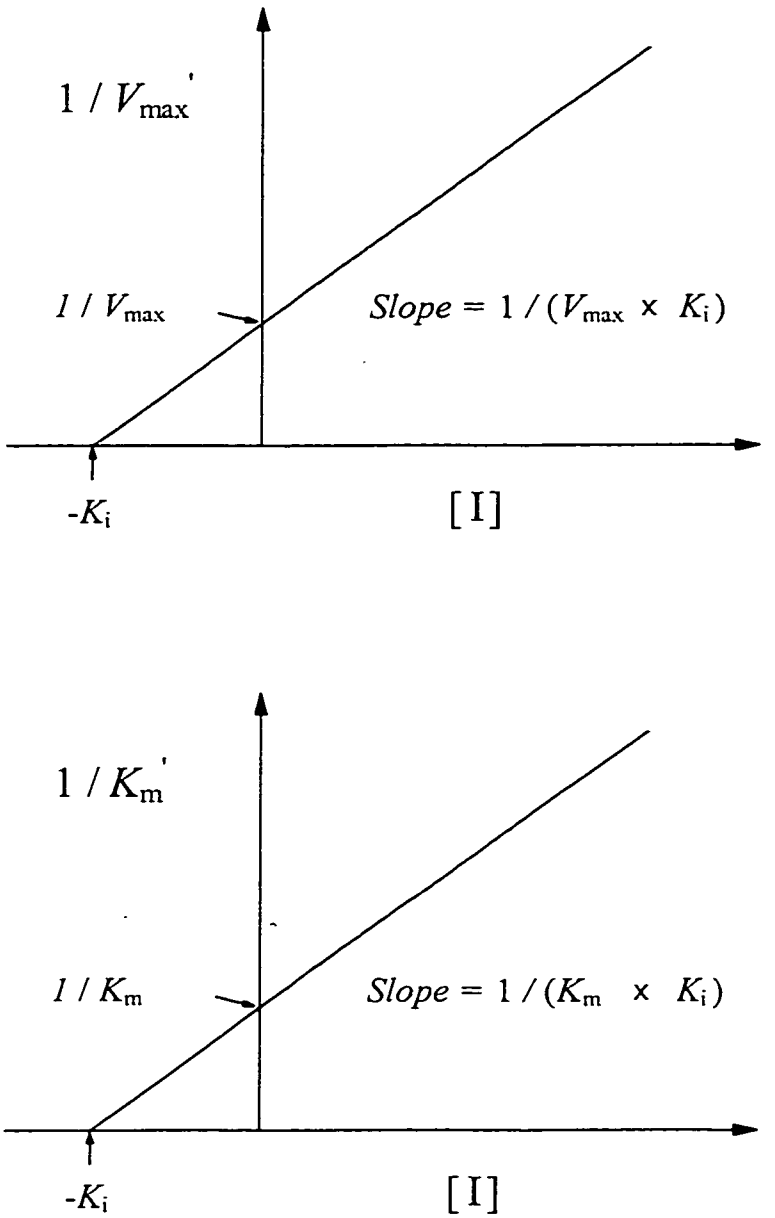


Figure 1.16 Calculation of K_i for uncompetitive inhibition using secondary plots (adapted from Palmer, 1995).

1.8.1.4 Fully Mixed Inhibition and Calculation of K_i Value

Fully mixed inhibition, the simplest form of mixed inhibition system, is one in which EI has a lower affinity than E for S. Also, binding of S affects affinity of E for I. Thus, the dissociation constants for the equilibrium between EI yielding E + I, and for ESI yielding ES + I, are not the same. The inhibitor constant for the former is termed K_i and for the latter K_I (Holt, 1999).

In equilibrium conditions, the velocity equation in the presence of a fully mixed type inhibitor is an equation of the same form as the Michaelis-Menten equation, with the constants K_m and V_{max} both being changed as follows:

$$V_{max}' = V_{max} / [1+([I]/K_i)]$$

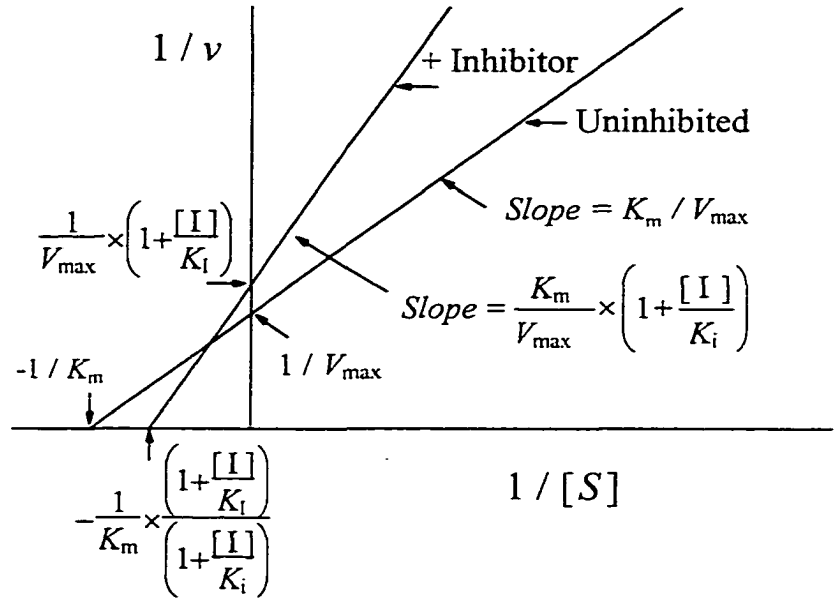
$$K_m' = K_m \times \frac{1+[I]/K_i}{1+[I]/K_I}$$

While V_{max}' is always decreased by a fully mixed inhibitor, K_m' can be higher or lower than K_m, measured in the absence of inhibitor. Fully mixed type inhibition always causes Lineweaver-Burk plots at different inhibitor concentrations to intersect to the left of the y-axis, and above or below, but not on, the x axis.

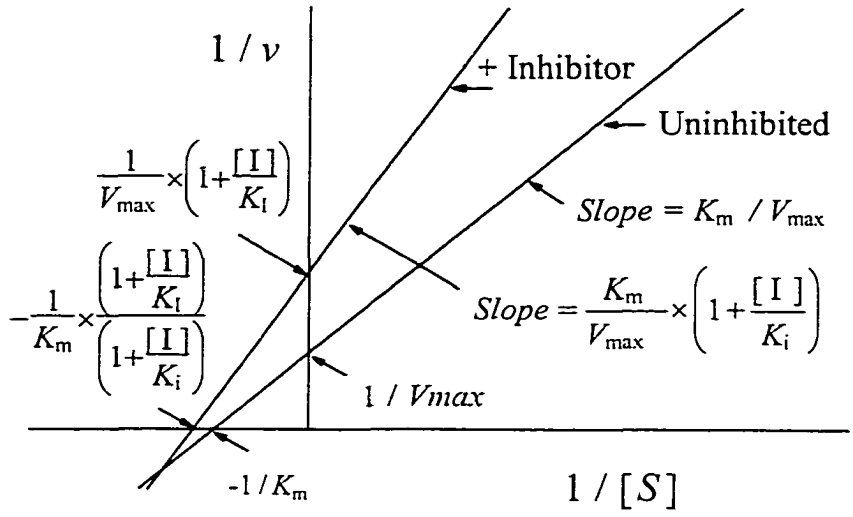
In the situations where $K_I > K_i$, the plots cross to the left of the y-axis but above the x-axis (Figure 1.17.A). This situation has been termed competitive-non-competitive inhibition, because the pattern observed lies between those for competitive and non-competitive inhibition.

In situations where $K_I < K_i$, the plots cross to the left of the y-axis and below the x-axis (Figure 1.17.B). This form has been named non-competitive-uncompetitive inhibition because the pattern is intermediate between those for non-competitive and uncompetitive inhibition (Palmer, 1995; Holt, 1999).

Analogous to the procedure used with fully competitive inhibitors, a graph of the slope of the Lineweaver-Burk plot against [I] gives $-K_i$. Analogous to the procedure used with fully non-competitive inhibitors a secondary plot of $1/ V_{max}'$ against [I] gives $-K_I$, (Palmer, 1995; Holt, 1999; Figure 1.18).

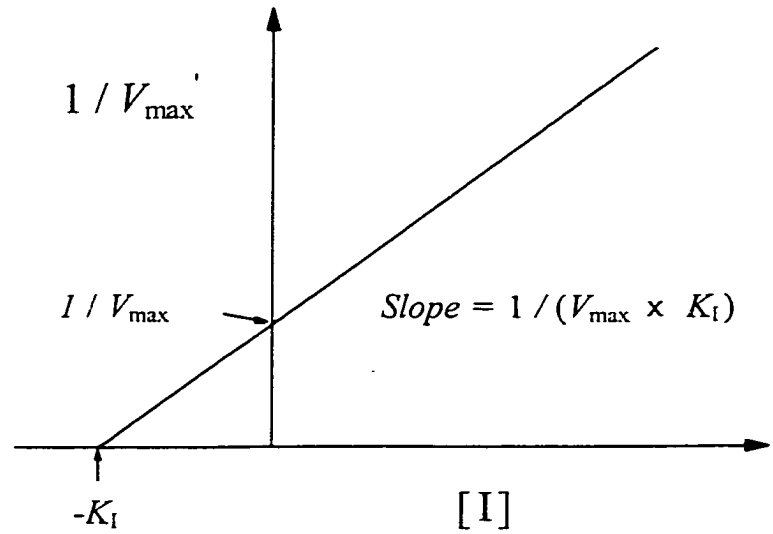


(A)



(B)

Figure 1.17 Lineweaver-Burk plots showing the effects of mixed inhibition: (A) $K_I > K_i$; (B) $K_I < K_i$ (adapted from Palmer, 1995).



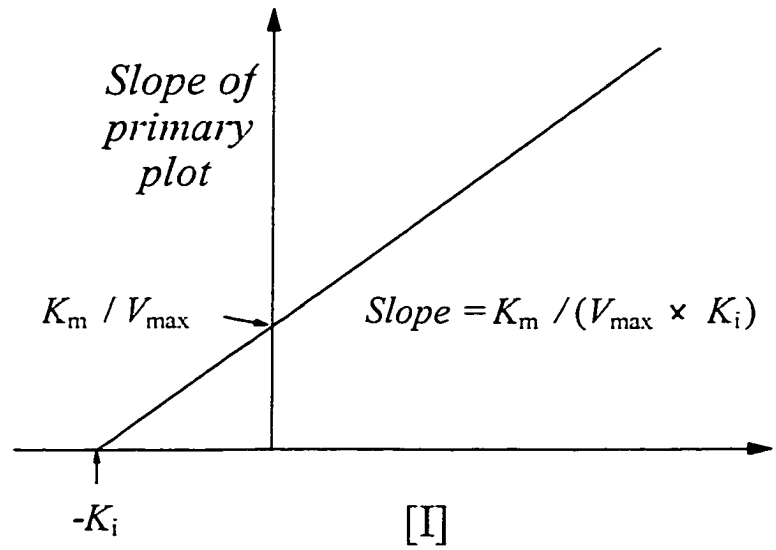


Figure 1.18 Calculation of K_I and K_i for mixed type inhibition using secondary plots (adapted from Palmer, 1995).

1.8.2 Irreversible Inhibitors

An irreversible inhibitor binds to an enzyme extremely tightly, so that it does not dissociate significantly from the enzyme by dialysis during the time period of the kinetic studies. Since there is no appreciable reversal of inhibition, no dissociation constant between enzyme and inhibitor exists. Unlike reversible inhibition, irreversible inhibition is progressive and increases with time until either all the inhibitor or all the enzyme present has been used up in forming enzyme-inhibitor complex.

Although irreversible inhibitors often yield a kinetic plot resembling those for reversible non-competitive inhibition, with unchanged K_m and reduced V_{max}, calculation of K_i values is meaningless. Hence, if a pattern of non-competitive inhibition is obtained in the investigation of a system, it is important to establish reversibility *versus* irreversibility of inhibition before the results can be interpreted (Palmer, 1995; Holt, 1999).

Non-specific irreversible inhibitors act in a manner described by Equation 1.3.

$$E + I \xrightarrow{k_{on}} EI \qquad \text{(Equation 1.3)}$$

These inhibitors usually bind to a specific residue, or group of residues, in the enzyme, but will bind to any enzyme or other cellular constituent containing that residue. The non-specific inhibitors are useful in studies of enzyme structure and function, but their lack of enzyme specificity has

restricted their pharmacological and medical uses (Tipton, 1980; Holt, 1999). The potency of these inhibitors can be described by a pseudo-first-order rate constant (Tipton, 1980), although it is possibly sufficient to quote an IC_{50} value (Holt, 1999).

1.8.3 IC_{50} value

The IC_{50} is the concentration of inhibitor that reduces enzyme activity to 50% of activity of a control sample; its value may depend on substrate concentration, and depends on type of inhibition. For simple, reversible inhibition systems, IC_{50} can be related to K_i by the Cheng-Prusoff equation as follows (Cheng & Prusoff, 1973).

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}} \qquad \text{(Equation 1.4)}$$

In the case of irreversible inhibitors, tight binding, and slow tight-binding inhibitors, the degree of inhibition depends on the concentration of inhibitor and also on the concentration of enzyme. For these classes of inhibitors, reporting the IC_{50} value is acceptable if the concentration of enzyme is also indicated. If the enzyme concentration is unknown, then the activity of the enzyme in International Units (*IU*) or, if possible, its specific activity (in *IU* mg^{-1}) should be provided (Holt, 1999).

1.9 Stereochemical Terms

In general, stereoisomers are those isomers whose atoms, or group of atoms, differ with regard to the spatial arrangement of the ligands. Stereoisomers can be either geometric or optical isomers.

Geometric isomers are stereoisomers without optically active centers: for those compounds terminology such as *cis* or Z isomer (meaning together or same side), and *trans* or E isomer (meaning opposite side) are used to describe the spatial arrangement of the atoms around a functional group such as a double bond (Hyneck et al., 1990).

Optical isomers are a subset of stereoisomers, of which at least two isomers are optically active; these compounds are said to possess chiral or asymmetrical centers. Many drugs in clinical use, including TCP and MEP, have a chiral center. The most common chiral center is carbon, but phosphorus, sulfur and nitrogen can also form chiral centers. The importance of chirality comes to attention when a chiral molecule interacts with chiral environments such as enzymes or receptors.

If the isomer and its mirror image are not superimposable, the molecules are referred to as enantiomers or optical antipodes. A mixture of equal portions of each is called a racemate. Enantiomers have physically identical characteristics such as lipid solubility, melting and boiling points (Hyneck et al., 1990). The method of differentiating one enantiomer from its antipode is by assigning the d or (+) designation to stereoisomers that cause a

clockwise rotation of a beam of polarized light, and l or (-) to stereoisomers that cause a counterclockwise rotation of the polarized light. It is important to realize that the optical rotation caused by the enantiomer is not an independent variable and can be changed by a number of factors such as the solvent that the enantiomer is dissolved in, the temperature at which the measurement was taken, the sample concentration, the light wavelength, and pH of aqueous solutions (Hyneck et al., 1990).

Enantiomers may also be named according to their actual spatial arrangements about a chiral atom. In the Fisher convention (Freudenberg, 1966), the molecule under investigation had to be converted to a compound of known configuration such as (D)-(+)-glyceraldehyde and then named accordingly (Hyneck et al., 1990). The two enantiomers of a pair are labeled D- (from the Latin *dexter*, "right") and L- (from the Latin *laevus*, "left") to distinguish them. Currently, the Cahn-Ingold-Prelog convention is recommended for specifying the absolute configuration of the isomers (Cahn et al., 1966). In this method, the ligands around the chiral center are sized according to their atomic number. The molecule is then positioned with the smallest ligand(s) away from the viewer (into the page). If the sequence of the remaining three ligands is arranged so that the largest to the smallest size is in a clockwise manner, the molecule is assigned "R" or *rectus*. A counterclockwise direction is assigned "S" or *sinister* configuration.

1.10 Analytical Procedures

A significant amount of time was devoted to the development of sensitive and efficient methods for detection and quantification of the compounds of interest. In this regard, several methods were developed using gas chromatography with electron-capture detection (GC-ECD) and high performance/pressure liquid chromatography with UV detection (HPLC-UV). To understand better the approach to method development, the basic concepts of gas chromatography and high performance liquid chromatography are discussed in this section.

Chromatography is a powerful analytical technique that is widely used to separate or purify a mixture of compounds into its individual components by partitioning the sample between a moving phase which can be gas or liquid, and a stationary phase that can be either liquid or solid (Johnson & Stevenson, 1978).

The objective is to select those conditions that optimize the resolution of separated components in the fastest and most reproducible manner. This requires knowledge of the physical properties of a compound such as volatility, solubility, and ionic state as well as the chemical properties that make its detection possible, such as UV or visible light absorption, fluorescence, electrochemical activity, and electronic capture capability. The two broad classes of chromatographic methods are gas chromatography and liquid chromatography (Burtis et al., 1987).

1.10.1 High Performance Liquid Chromatography (HPLC) with a UV Detector

HPLC is an advanced type of liquid chromatography (LC) that separates components of a mixture more efficiently than LC. An HPLC instrument usually has several components: (1) a solvent reservoir, (2) a pump, (3) an injector, (4) a chromatographic column, (5) a detector, (6) a data recorder, and (7) a microprocessor (Burtis et al., 1987).

In an HPLC system, mobile phase is taken from the solvent reservoir by the use of the pump, and is driven through the injector, the column, and one or more detectors. An aliquot of sample is introduced into a liquid chromatograph *via* some type of sample injector (Burtis et al., 1987).

The pump in a liquid chromatograph operates in two different modes: isocratic or gradient. In the isocratic mode the mobile phase composition remains constant throughout the chromatographic run. In the gradient mode, the mobile phase composition is changed either in a stepwise or in a continuous fashion throughout the run (Burtis et al., 1987).

The chromatographic column consists of a stainless steel tube that contains the column packing (stationary phase). Numerous materials are used as column packing, including, alumina, charcoal, organic polymers, and the most widely used material, silica. When the stationary phase is more polar than the mobile phase, HPLC is called normal-phase, and when the mobile phase is more polar than the stationary phase, HPLC is classified as

reversed phase (Johnson & Stevenson, 1978). Reversed-phase HPLC, in which polar analytes would elute first, is believed to be the most widely practiced form of liquid chromatography (Burtis et al., 1987).

The function of the detector is to detect compounds as they elute from the chromatographic column. Ultraviolet and visible wavelength photometers are reported to be the most popular HPLC detectors, although fluorometers and electrochemical detectors are becoming more widely used for various applications (Burtis et al., 1987).

Ultraviolet and visible wavelength photometers measure the absorption of radiant energy by the compounds as they elute from the chromatographic column. The wavelengths and the magnitude of the absorption depend on the molecular structure and concentration of the compounds. Most organic compounds will absorb in the ultraviolet and a few in the visible region of the electromagnetic spectrum (Burtis et al., 1987).

A liquid chromatograph produces data in a response-*versus*-time format. The time required for the apex of a compound to pass through a liquid chromatograph is called retention time. The detector response is used for quantitative purposes, since the magnitude of the response is proportional to the quantity of the compound passing through it (Burtis et al., 1987).

Improvement of resolution in HPLC can be achieved by adjustment of several factors, including the strength, composition and flow rate of the mobile phase, the type of the column, its packing and its length, the sample

chemistry through derivatization and the separation temperature (Burtis et al., 1987).

1.10.2 Gas Chromatography with an Electron Capture Detector

Gas chromatography (GC) is a process by which a mixture of compounds in volatized form is separated into its constituent components by moving a mobile phase (gas) over a stationary phase. A gas chromatograph consists of six basic parts: (1) a carrier gas supply with flow control; (2) an injector; (3) a chromatographic column located in an oven with temperature control; (4) a detector; (5) an electrometer (amplifier); and (6) a data recorder (Burtis et al., 1987).

After introduction of the sample into the gas chromatograph and vaporization of its components, the carrier gas sweeps the sample vapor to the column. The components separate from each other based on their partition coefficients between the carrier gas and the stationary phase. As the components elute, they enter a detector and each eluate produces a signal. The response signal of the detector, after amplification in an electrometer, is fed into a recorder for display. Retention time, which is the elapsed time between injection of sample and the appearance of a peak apex, is a distinctive property of a particular component under the special conditions of an assay (Burtis et al., 1987).

The carrier gas that is the mobile phase in GC system is an inert gas such as nitrogen, helium, or argon. The type of detector utilized in the system determines the type of carrier gas. Precise control of the flow rate of carrier gas is important, since the retention time of individual sample components is in part determined by the gas flow rate (Burtis et al., 1987).

The sample injection system is kept at a temperature that will cause quick vaporization of sample components. Excessive dead volume in the injection port results in diffusion of the sample and hence in increased peak width and tailing. Efficiency of separation is also affected by the sample volume, which should be kept as small as possible (Burtis et al., 1987).

The separation of sample components takes place in the gas chromatographic column, which is located in the column oven. Gas chromatographic columns are commercially available in materials such as borosilicate glass, fused silica, stainless steel, copper, aluminum, or Teflon® and nylon. Two types of GC columns are packed and capillary columns (Coutts & Baker, 1982). In general, capillary columns produce better resolution of components of a mixture compared to packed columns. Capillary columns are usually made of glass and coated with a thin layer of the stationary phase (Coutts & Baker, 1982).

The column oven maintains the constancy and uniformity of the column temperature, which are necessary for reproducibility of retention times. The use of temperature programming allows the analysis of complex

mixtures containing analytes with a wide range of boiling points. It also shortens the total analysis time (Burtis et al., 1987).

Many types of detectors have been invented, including the thermal conductivity detector, the flame ionization detector, the nitrogen-phosphorus detector, the electron capture detector and the mass-spectrometer detector. In the present study an electron capture detector was used, and hence the principles of its mechanism of action are explained briefly here (Burtis et al., 1987).

Electron capture detectors are very sensitive and also very selective, since they detect only components with affinity for electrons. Thus, the use of an electron capture detector (ECD) is suitable for detection of compounds that contain halogen atoms, ketone or nitro groups or other electrophoric groups (Baker et al., 1982).

In an ECD, a radioactive isotope releases beta particles that collide with the carrier gas molecules, producing a stream of secondary electrons, which result in a small measurable standing current (Baker et al., 1982; Polkis, 1984). As compounds with electrophoric groups pass the detector, the standing current decreases. The changes in the current can be amplified and polarity can be reversed so that a peak is depicted on the recorder chart. The size of peak is proportional to the concentration of the sample component that produces the peak.

Resolution in GC depends mostly on the characteristics of the stationary phase and components of the sample. The flowing gas (mobile phase) has little interaction with the solutes and separation is achieved with the stationary phase (Johnson & Stevenson, 1978). Since retention of a solute depends on its affinity for the stationary phase, polar solutes have greater partition coefficient values (K_D; the ratio of the time a compound is in stationary phase to the time it is in mobile phase) and longer retention times on polar phases (hydrophilic) than non-polar (hydrophobic) phases and *vice versa*. Temperature is also an important factor in GC. The higher the column temperature, the lower the K_D, and the shorter the retention time (Poklis, 1984).

1.11 Objectives and Hypotheses

It has been suggested that part of the drug-drug interactions observed when TCP or PLZ co-administered with other drugs stems from the inhibitory effects of these drugs on CYP enzymes. The main goal of the studies described in this thesis was to evaluate in considerable detail the inhibitory effects of TCP and PLZ on three CYP enzymes; CYP2D6, CYP2C9, and CYP2C19. These CYP enzymes are major enzymes involved in the metabolism of a number of drugs. As mentioned earlier in the introduction, previous studies by other investigators had suggested that metabolism of substrates for these enzymes was inhibited by TCP and PLZ, but those

investigations were conducted before the various CYP enzymes had been characterized and named definitively and before the availability of the characterized human liver microsomes for their content of the various CYP enzymes, as well as individual c-DNA expressed CYP enzymes.

TCP had been reported to be a relatively potent inhibitor of CYP2C19 (Inaba et al., 1985), but detailed studies on the nature of this inhibition had not been conducted. Similarly, indirect *in vivo* studies in the Neurochemical Research Unit at the University of Alberta utilizing drug-drug interaction studies had suggested that TCP and PLZ both interacted with drugs that were inhibitors or substrates of CYP2D6. Such indirect studies had led members of the Unit to synthesize analogues of TCP that had potential as antidepressants (Sherry et al., 1999), and it was now important to see if they interacted with CYP enzymes in a similar fashion to TCP. The initial studies in the literature on the effects of TCP on CYP2C19 had not investigated the action of the individual enantiomers; given their marked differences on inhibition of MAO and on uptake of catecholamines, it was of interest to determine if they differed in their effects on the activity of CYP2C19. The final experiment was conducted to provide further data with respect to the ongoing controversy about whether or not TCP is metabolized to amphetamine. To reach the main goal, the following objectives formed the basis of the studies described here.

1. To develop sensitive assays for quantification of dextrorphan (DXR), hydroxytolbutamide (OHTOL) and hydroxymephenytoin (OHMEP), metabolites of dextromethorphan (DM), tolbutamide (TOL) and S-mephenytoin (MEP), respectively. DM, TOL, and MEP are now known to be appropriate probe substrate for CYP2D6, CYP2C9, and CYP2C19, respectively.
2. To conduct kinetic studies on the inhibitory effects of TCP and PLZ on CYP2C19, CYP2C9 and CYP2D6. It was hypothesized that both drugs would inhibit CYP2C9, CYP2C19 and CYP2D6 competitively.
3. To compare the inhibitory effects of TCP enantiomers on CYP2C19. It was hypothesized that the two enantiomers differ considerably from one another in their potency of inhibition of CYP2C19.
4. To evaluate the inhibitory effects of ring-substituted analogues of TCP, including FTCP, MTCP, and OHTCP, on CYP2C19. It was hypothesized that the analogues of TCP would be similar in potency to TCP with regard to ability to inhibit CYP2C19.
5. To evaluate *in vitro* whether TCP can be metabolized *via* cyclopropyl ring cleavage to amphetamine and other potential metabolites by CYP enzymes. It was hypothesized that TCP would not form amphetamine when incubated with the CYP enzymes.

CHAPTER 2

Materials and Methods

2.1 Materials

Table 2.1 illustrates a list of chemicals and reagents used in this study along with their suppliers. The human liver microsomes and cDNA expressed purified human microsomal enzymes were purchased from the International Institute for the Advancement of Medicine and Science (IIAMS; Exton, PA, USA), and Gentest Corporation (Woburn, MA), respectively.

The human liver microsomes used in all experiments were from lot number HHM-0205. The manufacturer characterized these microsomes for their protein content and CYP enzyme activity. The protein content was determined by the Pierce protein assay and the enzyme activities were evaluated based on the rate of metabolism of specific substrates (Table 2.2). The protein content of the HHM-0205 microsomes is 23 mg/mL.

Characterization of the CYP enzymes from cDNA-expressed cells is summarized in Table 2.3. The catalog and lot number for the enzymes used in this study, namely, $CYP2C9_{Arg}$, CYP2C19, and CYP2D6 were P258-5, P219-11, and P217-15, respectively. The cDNAs for the human enzymes were expressed in either metabolically competent derivatives of the AHH-1 TK+/- human lymphoblastoid cell line or baculovirus (*Autographa californica*)-infected insect cells (BTI-TN-5B1-4). The insect host cell line does not seem to have detectable CYP activity. However, the parent AHH-1 TK+/- cell line contains a low level of native human CYP activity, which is inducible by

polycyclic aromatic hydrocarbons. All enzymes were co-expressed with P450 reductase. $CYP2C9_{Arg}$ was also co-expressed with cytochrome b_5.

Table 2.1 List of chemicals and reagents along with their suppliers

Chemical	Suppliers
Acetonitrile (HPLC grade)	Fisher Scientific (Nepean, ON)
Ammonium hydroxide	Fisher Scientific (Nepean, ON)
L-Ascorbic acid	Fisher Scientific (Nepean, ON)
p-Chlorophentermine	Dr. R.T. Coutts, University of Alberta, Edmonton
Dextromethorphan	Research Biochemicals International (Natick, MA)
Dextrorphan	Research Biochemicals International (Natick, MA)
EDTA (Disodium ethylenediamine-tetraacetate)	Fisher Scientific (Nepean, ON)
Ethyl acetate	Fisher Scientific (Nepean, ON)
Glacial acetic acid	Fisher Scientific (Nepean, ON)
Glucose-6-phosphate dehydrogenase	Sigma Chemical Company (St. Louis, MO)
Glucose-6-phosphate (monosodium salt)	Sigma Chemical Company (St. Louis, MO)
Hydrochloric acid	Fisher Scientific (Nepean, ON)
p-Hydroxymephenytoin	Research Biochemicals International (Natick, MA)
4-Hydroxytolbutamide	Research Biochemicals International (Natick, MA)
Magnesium chloride	Fisher Scientific (Nepean, ON)

S (+)-Mephenytoin	Salford Ultrafine Chemicals and Research (Manchester, England)
Methanol (HPLC grade)	Fisher Scientific (Nepean, ON)
β-Nicotinamide adenine dinucleotide phosphate (oxidized form; $NADP^+$)	Sigma Chemical Company (St. Louis, MO)
Pentafluorobenzoyl chloride	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Pentafluorobenzenesulfonyl chloride	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Perchloric acid (60%)	Fisher Scientific (Nepean, ON)
Potassium phosphate dibasic	JT Baker (Philipsburg, NJ)
Potassium phosphate monobasic	Fisher Scientific (Nepean, ON)
Phenelzine sulfate	Sigma Aldrich Canada Ltd. (Oakville, ON)
Sodium bicarbonate	Fisher Scientific (Nepean, ON)
Sodium carbonate	Fisher Scientific (Nepean, ON)
Toluene (HPLC grade)	Fisher Scientific (Nepean, ON)
Tolbutamide	Research Biochemicals International (Natick, MA)
(±)-Tranylcypromine hydrochloride	Sigma Chemical Company (St. Louis, MO)
Tranylcypromine analogues 4-Fluorotranylcypromine 4-Hydroxytranylcypromine 4-Methoxytranylcypromine	Synthesized by Dr. R.T. Coutts, University of Alberta, Faculty of Pharmacy and Pharmaceutical Sciences
(+)-Tranylcypromine and (-)-tranylcypromine	SmithKline & French (Philadelphia, PA)
Triethylamine (HPLC grade)	Fisher Scientific (Nepean, ON)
Tris	Fisher Scientific (Nepean, ON)

Table 2.2 Characterization of protein content and specific CYP enzyme activities in human liver microsomal samples (information supplied by IIAM Company for lot number HHM-0205).

Components	Determination Process	Determined Values
Protein content	Microsomal protein was determined by the Pierce protein assay.	23 mg/mL
P450	P450 was determined by the carbon monoxide spectrum.	0.47 nmol/mg protein
P450 reductase	CYP P450 reductase activity was determined by the rate of reduction at room temperature.	50 nmol/mg/min
ECOD Mix	7-ECOD activity was determined by the rate of 7-ethoxycoumarin O-deethylation	177 pmol/mg/min
CYP1A2	CYP1A2 activity was determined by the rate of phenacetin O-deethylation.	347 pmol/mg/min
CYP2A6	CYP2A6 activity was determined by the rate of coumarin 7-hydroxylation.	0.67 nmol/mg/min
CYP2C	CYP2C activity was determined by the rate of mephenytoin 4-hydroxylation.	152 pmol/mg/min
CYP2D	CYP2D activity was determined by the rate of dextromethorphan O-demethylation.	180 pmol/mg/min
CYP2E	CYP2E activity was determined by the rate of chlorzoxazone 6-hydroxylation .	811 pmol/mg/min
CYP3A	CYP3A activity was determined by the rate of fractional production of [^{14}C] 6β-hydroxy-testosterone	3.6 nmol/mg/min
CYP4A	CYP4A activity was determined by the rate of fractional production of [^{14}C]ω-hydroxylauric acid	1.4 nmol/mg/min

Table 2.3 Characterization of cDNA-expressed CYP enzymes used in this study

CYP Enzyme Expressed	Assay	Activity (pmol/mg/min)	Protein Content (mg/mL)	Expression System
$CYP2C9_{Arg}$	Diclofenac 4'-hydroxylase	13810	2.1	Baculovirus infected insect cells
CYP2C19	S-(+)-mephenytoin 4'-hydroxylase	1545	2.2	Baculovirus infected insect cells
CYP2D6	(±)-Bufuralol 1'-hydroxylase	6129	6.2	Baculovirus infected insect cells

2.2 Equipment

2.2.1 Block Heater

A Canlab Temp-Block Module Heater (Lab Line Instruments, Melrose Park, II, USA) was used to concentrate solvents or carry out derivatizations for GC analysis.

2.2.2 Centrifuges

A Sorvall GLC-2B General Laboratory Centrifuge (Dupont Instruments, Wilmington, DE) was used for samples that required low speed centrifugation (up to 1500 rpm). The maximum allowable volume of samples for this centrifuge is 10 mL. Centrifugation of samples requiring higher speed (i.e., 10,000-13,000 g) was carried out using a Beckman Microfuge B (Palo Alto, CA) or a Micro-Centaur Centrifuge (MSE Scientific Instruments,

Sussex, England). The maximum sample volume for these micro-centrifuges is 1.5 mL.

2.2.3 Filter Apparatus for Mobile Phase Preparation

Mobile phase was filtered and degassed using a Millipore filtering system (Millipore Corporation, Bedford, MA) in which the mobile phase was forced under vacuum through a Nylon Filter Membrane with pore size of 0.2 μm, and diameter of 47 mm (Phenomenex, Torrance, CA).

2.2.4 pH Meter

An Accumet ® 915 pH meter (Fisher Scientific, Nepean, ON) was used to measure the pH value of solutions, and was routinely standardized with certified buffer solutions (Fisher Scientific, Nepean, ON).

2.2.5 Vacuum Evaporator

A Savant Speed Vac SSI (Savant Instruments, Inc., Farmington, NY) was used to remove solvent and concentrate and dry samples using a combination of vacuum and centrifugal force. This system consisted of a concentrator (a rotor chamber with heater), a chemical trap with disposable cartridge, a refrigerated condensation trap and a vacuum pump.

2.2.6 Vortex Mixer

A thermolyne Maxi Mix® vortex mixer (Sybron/Thermolyne Instruments, Dubuque, IO) was used for mixing samples.

2.2.7 Vortex Shaker

An IKA-Vibrax-VCVR (R) shaker (Janke and Kunkle Instruments, Staufen, Germany) was used for processing samples.

2.2.8 Water Bath

Microsomal incubations were carried out in a Fisher Isotemp® Water bath (Fisher Scientific, Nepean, ON) that was set at 37°C.

2.2.9 Water Still

Double-distilled water was prepared using a Mega-Pure® Three Liter Automatic Water Still (Corning Waterware, Corning, NY) that utilized a central deionized water source. This double-distilled water was further purified for HPLC purposes using a Mixed Bed Organic Removal Cartridge (Fisher Scientific, Palo Alto, CA).

2.2.10 Weighing Balance

All compounds were weighed on a Mettler AE160 electronic balance (Mettler Instrument Corporation, Hightstown, NJ).

2.2.11 Glassware Cleaning

All test tubes were manually scrubbed using Sparkleen® Manual Washing Soap (Fisher Scientific, Nepean, ON) followed by sonication in an Ultra-Sonic Cleaner (Mettler Electronics, Hightstown, NJ) and rinsed in a G7704 Lavador dishwasher (Miele Laboratory Technology, Unionville, ON). All other glassware was rinsed with tap water and then washed with Sparkleen® Dishwasher Soap in the dishwasher. The water utilized by the dishwasher was from a central deionized water source. A mechanical convection oven (Model 28, Precision Scientific Group, Chicago, IL) was used to dry all glassware upon removal from the dishwasher.

2.3 Instrumentation

2.3.1 High Performance Liquid Chromatograph (HPLC)

The HPLC system consisted of a Waters Model 510 solvent delivery system, a Waters WISP 710B autosampler (Waters Associates, Milford, MA), a 5μm Spherisorb 5 ODS2 (Phenomenex) column (4.6 × 250 mm), and a Waters Model 481 LC spectrophotometer. A Waters 740 data Module was used to record, store and analyze chromatograms.

2.3.2 Gas Chromatograph

A Hewlett-Packard (HP) 5880A gas chromatograph equipped with a 15mCi ^{63}Ni linear electron-capture detector and a narrow-bore fused HP5

silica capillary column (25 m × 0.32 mm) coated with 1.05 μm film of 5% phenylmethylsilicone as stationary phase (Hewlett Packard, Mississauga, Ontario, Canada) was employed. The gas chromatograph was linked to a HP 5880A integrator. The carrier gas was helium and the make-up gas was methane-argon (5:95) (Praxair Canada Inc, Edmonton, AB).

2.3.3 Gas Chromatograph Equipped with Mass Spectrometer Detector

The structures of the derivatives of OH-MEP and DXR were confirmed using combined GC-mass spectrometry (GC-MS) (electron-impact mode). A VG 7070E mass spectrometer, linked to a Varian Vista gas chromatograph was utilized.

2.4 Solution Preparation

2.4.1 NADPH-Generating System

The NADPH-generating system was prepared fresh with each experimental run. It contained 4 mg/mL of β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), 4 mg/mL of glucose-6-phosphate, 3.2 units/mL of glucose-6-phosphate dehydrogenase, and 2.64 mg/mL $MgCl_2.6H_2O$, all dissolved in either a 0.1 M potassium phosphate buffer or a 0.1 M Tris buffer.

2.4.2 1.0M Perchloric Acid

The perchloric acid solution was prepared by addition of 10.88 mL 60% perchloric acid, 8.8 mg of ascorbic acid, and 100 mg of EDTA to 60 mL of double-distilled water. The solution was mixed until all components were dissolved and then the volume was brought to 100 mL with double-distilled water in a volumetric flask.

2.4.3 0.1M Potassium Phosphate Buffer (pH=7.4)

The 0.1M potassium phosphate buffer was prepared by acidifying a solution of dibasic potassium phosphate (K_2HPO_4) with a 0.1M solution of monobasic potassium phosphate (KH_2PO_4). The solution of monobasic potassium phosphate was prepared by dissolving 1.361 g of monobasic potassium phosphate (molecular weight = 136.09) in 100mL of double-distilled water. To prepare solution of potassium phosphate buffer, 1.7418 g of dibasic potassium phosphate (molecular weight = 174.18) was dissolved in about 60 mL of double-distilled water. Then it was acidified to a pH value of 7.4 with monobasic solution, and the volume was brought to 100 mL with double-distilled water in a volumetric flask.

2.4.4 0.1M Tris Buffer (pH=7.5)

The Tris buffer was prepared by dissolving 1.211 g of Tris (molecular weight= 121.14) in 50 mL of double-distilled water and acidifying to a pH

value of 7.5 with 1.0 M hydrochloric acid. The solution was transferred to a 100mL volumetric flask and brought to volume with double-distilled water.

2.5 GC and HPLC Analyses

2.5.1 GC analysis of TCP, AMP, PPA, and PCP

GC analysis of TCP, amphetamine (AMP), 1-Amino-3-phenylpropane (PPA), and PCP (*p*-chlorophentermine) involved essentially a modification of methods developed previously in the Neurochemical Research Unit laboratories at the University of Alberta (Paetsch et al., 1992; Sherry et al., 1999). The procedure consisted of extractive derivatization of compounds using Pentafluorobenzoyl chloride (PFBC) or Pentafluorobenzenesulfonyl chloride (PFBSC) followed by analysis on a GC equipped with a fused silica capillary column and an electron capture detector.

2.5.2 Assay Development for Detection and Quantification of OHMEP and DXR in Human Liver Microsome Incubations Using GC-ECD

A simple, sensitive and economical electron-capture gas chromatographic procedure was developed for determination and quantification of OHMEP and DXR, metabolites of MEP and DM respectively. After incubation of DM or MEP with human liver microsomes or microsomes from cDNA-expressed cells for a predetermined period of time,

the reaction was stopped by addition of perchloric acid (1M). The resulting metabolite (DXR or OHMEP) was then assayed by the following procedure.

When MEP was used as substrate, DXR was added as internal standard, and when DM was used as substrate, OHMEP was added as internal standard. The incubation mixture was then diluted to 1.0 mL with distilled H_2O and basified by the addition of 2 mL of saturated sodium carbonate solution. Following addition of a solution (3 ml) of ethyl acetate: acetonitrile: PFBC / PFBSC (9:1: 0.02) to each tube, the tubes were shaken vigorously for 10 min and centrifuged (1000 *g*) for 5 min. The organic layers were retained and transferred to another set of tubes and taken to dryness under a stream of nitrogen at 60°C or by using a SAVANT evaporator. Each residue was reconstituted in 300 μL of toluene. After adding 450 μL of ammonium hydroxide (1M) to each sample, they were vortexed for a few seconds and centrifuged briefly. Each top layer was transferred to a microfuge tube and 1 μL of sample was used for GC analysis.

A GC equipped with an electron-capture detector and capillary column was employed. The carrier gas was helium at a flow-rate of 2 mL/min, and the make-up gas at the detector was methane-argon (5:95) at a flow rate of 30 mL/min. Injection port and detector temperatures were set at 250°C and 325°C, respectively. An initial oven temperature of 105°C was maintained for 0.5 min, and was programmed to increase at a rate of 15°C/min to a final

temperature of 295°C, which was maintained for 10 min. A splitless mode of injection with a purge off time of 0.5 min was used.

A standard curve based on a series of tubes containing a fixed amount of internal standard (1000 ng) and varying amounts of metabolite (from 18 ng to 600 ng) was run in parallel with each assay. Quantitation was carried out by determining peak-height ratio of compound of interest to internal standard for each sample and by comparing the ratios to the values on the calibration curve obtained from the standard runs for that particular assay.

The structure of the derivatives of OHMEP and DXR were confirmed using combined GC-mass spectrometry (GC-MS) (electron-impact mode). A VG 7070E mass-spectrometer, linked to a Varian Vista gas chromatograph was used. Operating conditions were as follows: ion source temperature of 200°C; interface temperature of 295°C; column pressure of 34.5 kPa; accelerating voltage of 2200 eV; ionization voltage of 70 eV; scan speed of 200 amu/sec; and dwell time of 200 msec.

2.5.3 A Simple HPLC Assay to Measure CYP2C19 Activity, in the Presence of MAOIs, TCP and PLZ

An assay method to measure the activity of CYP2C19 in the presence of its potential inhibitors, TCP and PLZ was developed. This method was a modification of the method of Chiba et al. (1993), and was used to detect the applied substrate of CYP2C19 (S-mephenytoin; MEP), the produced

metabolite (*para*-hydroxymephenytoin; OHMEP), the MAOIs (TCP or PLZ), and the internal standard (phenobarbital; PHB). The HPLC system consisted of a Waters Model 510 solvent delivery system, a WISP 710B, a 5 μm Spherisorb 5 ODS2 (Phenomenex) column (4.6 × 250 mm), and a Waters Model 481 LC spectrophotometer. A Waters 740 Data Module was used to record, store and analyze chromatograms.

2.5.3.1 Mobile Phase Combination in the Presence of TCP

When TCP was used as a potential inhibitor, the mobile phase consisted of acetonitrile/ methanol/ dibasic potassium phosphate (5 mM) in the proportion 20/5/75 by volume, and was delivered at 0.8 mL/min. A final pH value of 3.4 was attained using phosphoric acid. Each analyte was monitored at 204 nm. Retention times for OHMEP, PHB, TCP, OHTCP, and MEP were respectively 10.59, 19.15, 11.91, 5.81, and 30.36 min.

2.5.3.2 Mobile Phase Combination in the Presence of PLZ

To improve resolution when evaluating the inhibitory effects of PLZ on CYP2C19, the mobile phase was changed as follows. The mobile phase consisted of acetonitrile/ methanol/ dibasic potassium phosphate (3.5 mM, pH=3.4) in the proportion 20/10/70 by volume. Retention times for OHMEP, PLZ, PHB, and MEP were 8.18, 10.67, 13.84, and 20.83 min, respectively.

2.5.4 HPLC Assay to Measure CYP2C9 Activity in the Presence of TCP

An assay method to measure the activity of CYP2C9 in the presence of its potential inhibitor, TCP, was developed. Tolbutamide was used as a substrate for CYP2C9 and *para*-chlorophentermine (PCP) was used as an internal standard. The same HPLC system as described in section 2.3.1 was used. The mobile phase consisted of acetonitrile/ methanol/ dibasic potassium phosphate (2.5 mM, pH=3.4) in the proportion 25/10/65 by volume and 50 μL of triethylamine per 1L of mobile phase. Flow rate was 0.8 mL/min. Each analyte was monitored at 204 nm. Retention times for OHTOL, TCP, PCP, and TOL were 8.87, 9.78, 22.68 and 31.80 min, respectively.

2.5.5 HPLC Assay to Measure CYP2C9 Activity in the Presence of PLZ

In this assay, TOL was used as a substrate for CYP2C9 and PCP as the internal standard. The HPLC system described in section 2.3.1 was used. The mobile phase consisted of acetonitrile/methanol/dibasic potassium phosphate (1.5 mM, pH=3.4) in the proportion 25/10/65 by volume and 90 μL of triethylamine per 1L of mobile phase. Flow rate was 0.8 mL/min. Each analyte was monitored at 204 nm. Retention times for OHMEP, PLZ, PHB, and MEP were 8.79, 9.60, 27.22, and 32.70 min, respectively.

2.5.6 HPLC Assay to Measure CYP2D6 Activity, in the Presence of TCP or PLZ

In this assay, dextromethorphan was used as a substrate for CYP2D6, and PCP was used as the internal standard. The HPLC system described in section 2.3.1 was used. The mobile phase consisted of acetonitrile/ methanol/ potassium phosphate dibasic (2.5 mM, pH=3.4) in the proportion 35/15/50 by volume, and 120 μL of triethylamine per 1L of mobile phase. Flow rate was 0.8 mL/min. Each analyte was monitored at 204 nm. Retention times for DXR, TCP, PLZ, PCP, and DM were respectively 10.61, 7.09, 9.98, 12.81, and 33.26 min.

2.5.7 HPLC Assay for Detection of 4-OHTCP in Human Liver Microsomes

An assay method was developed to measure OHTCP, a metabolite of TCP, in the presence of human liver microsomes. Benzylamine (BZA) was used as the internal standard. The mobile phase consisted of acetonitrile/ dibasic potassium phosphate (2.5 mM, pH=3.4) in the proportion 10/90, v/v, and was delivered at 0.8 mL/min. Each analyte was monitored at 204 nm. Retention times for OHTCP, BZA, and TCP were 10.09, 14.21, and 45.12 min, respectively.

2.5.8 Validation of GC and HPLC Assays

The developed assays were validated by determining the limit of detection, inter-assay and intra-assay coefficients of variation and recoveries. The coefficients of variation were calculated according to the following equation:

Coefficient of variation = (Standard deviation / Mean) x 100 (Equation 2.1)

The intra-assay coefficients of variation were calculated by analysing the data obtained from several injections of standards (containing a known amount of the drug of interest and an internal standard) in the same analytical run. The inter-assay coefficients of variation were calculated by analysing the data obtained from the injection of standards from several analytical runs.

The recoveries were calculated by dividing the peak-height ratio of authentic standards determined in the presence of control microsomes to that determined in the absence of any microsomes. The reported mean recovery is the average of the calculated recoveries for 4 to 6 samples.

2.6 Evaluation of Amphetamine as a Metabolite of TCP

The study was conducted by incubation of TCP with human liver microsomes at 37°C for 1 hour. The incubations were carried out in 1.5mL polypropylene microcentrifuge tubes. Each incubation mixture contained 200μM TCP, 10μL of human liver microsomes, 25μL of an NADPH

generating system as cofactor, and 0.1M potassium phosphate buffer (pH=7.4) to bring the volume up to 100μL. Human liver microsomal protein was the last component added. The reaction was started by placing the microcentrifuge tubes in a water bath. At the end of the incubation period, 50μL $HClO_4$ were added to the incubation mixtures, which were centrifuged for 5 minutes. The upper layer from each tube was transferred to test tubes, and the internal standard, PCP, was added. The presence of amphetamine was evaluated using a GC-ECD analysis procedure.

2.7 Inhibition Studies

2.7.1 Calculation of Initial Reaction Velocity (v), in the Presence and Absence of Inhibitor

To minimize experimental errors in determination of v, several points are taken into consideration. First, the effect of experimental errors can be reduced by making several measurements of v at each concentration of substrate and taking the average. Thus, when setting up the experiment, running triplicate samples at each substrate concentration helps to reduce experimental errors. Secondly, because of the discontinuous nature of the microsomal assays used in this study, the assumption of linear production of metabolite during the incubation period must be satisfied. If metabolite production is not linear due, for example, to substrate depletion with time, the value of v would be underestimated. The assumption of linear production

of metabolite for the entire incubation period at the predetermined substrate concentration can reasonably be satisfied in a time course study. In addition to incubation time, several factors may affect substrate depletion, and consequently linear production of metabolite, such as enzyme concentration and substrate concentration. Higher concentrations of enzyme cause higher reaction rates and faster depletion of substrate. The lowest concentration of enzyme necessary to catalyze formation of a measurable amount of product during the incubation period could be determined from a preliminary study. Therefore, before setting up the main experiment, several preliminary experiments should be conducted. A calibration curve was always run with each set of experiments to quantify the resultant metabolite by comparison with the standard curves using the peak-height ratio method.

2.7.1.1 Time Course Study

Preliminary experiments were designed to determine optimum incubation times for microsomal studies involving 2C19, 2C9, 2D6, and human liver microsomes. With each enzyme, a set of samples (with predetermined substrate concentration, NADPH-generating system and microsomal solution) was placed in a water bath at 37°C for varying periods of time (5, 10, 15, 20, 30, 45, and 60 min). The total incubation volume was 100 μL in all samples. Table 2.4 provides additional information about the samples.

After the elapsed time, the samples were placed on ice, and 50 μL of perchloric acid (1M) or acetonitrile was added to precipitate the protein and terminate the reaction. The samples were placed on ice for about 10 min, and then were centrifuged for 2 minutes. Supernatants were transferred to another set of tubes for quantitation of metabolite produced during the incubation period, using the appropriate assay method. A plot of the peak-height ratio of metabolite to internal standard against incubation time demonstrates how long production of metabolite is linear.

Table 2.4 Samples in Time Course Study

Microsomal Enzyme		Substrate		NADPH generating system (μL)	Total incubation volume (μL) & its Protein Content (mg/mL)	Incubation times (min)
Name	Volume (μL)	Name	Concentration (μM)			
CYP2C19	5	MEP	25	25	100 & 0.11	0, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 75, 90
			100	25	100 & 0.11	0, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 75, 90
CYP2C9	10	TOL	50	25	100 & 0.21	0, 10, 15, 20, 25, 30, 35, 40, 50, 60, 75, 90
			200	25	100 & 0.21	0, 10, 15, 20, 25, 30, 35, 40, 50, 60, 75, 90
CYP2D6	5	DM	200	25	100 & 0.31	0, 10, 15, 20, 25, 30, 35, 40, 50, 60, 75, 90
			10	25	100 & 0.31	0, 4, 7, 11, 21, 31, 41
HLM	10	MEP	200	25	100& 2.3	0, 10, 15, 20, 25, 30, 35, 40, 50, 60, 75, 90

2.7.1.2 The Enzyme Concentration Study

To determine the optimum concentration of enzyme to be used in the microsomal experiments, a set of samples containing a predetermined substrate concentration, 25 μL NADPH-generating system, and decreasing concentrations of microsomal protein, was incubated in a water bath at 37°C for a predetermined incubation time.

At the end of the incubation time, the reaction was stopped and the production of metabolite was measured as described in section 2.5. To evaluate linearity of metabolite production with the increasing enzyme concentration, the peak-height ratio of metabolite to internal standard was plotted against the corresponding concentration of enzyme. Table 2.5 provides further information about the samples.

Table 2.5 Samples in Enzyme Concentration Study

Enzyme	Volume of microsomal solution (μL)	Substrate		NADPH generating system (μL)	Total incubation volume (μL)	Incubation time (min)
		Name	Concentration (μM)			
CYP2C19	1, 2, 3, 5, 10	MEP	200	25	100	30
CYP2C9	0, 2, 4, 6, 8, 10	TOL	200	25	100	30
CYP2D6	0, 2, 4, 6, 8, 10, 20	DM	200	25	100	30
HLM	0, 2, 4, 6, 8, 10	MEP	200	25	100	30

2.7.1.3 Concentration Curve Study and Determination of Kinetic Constants (K_m and V_{max})

A range of substrate concentrations (a few concentrations below and a few above the estimated K_m concentration) was incubated with an NADPH-generating system and microsomal solution for a constant period of time in a water bath at 37°C. The total volume of the incubation mixture was 100μL. Table 2.6 provides further information regarding the samples.

Table 2.6 Samples in Concentration Curve Study

Enzyme	Volume of microsomal solution (μL)	Substrate		NADPH generating system (μL)	Total incubation volume (μL)	Incubation times (min)
		Name	Concentrations (μM)			
CYP2C19	5	MEP	12.5, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 400, 500	25	100	40
CYP2C9	10	TOL	25, 50, 100, 125 150, 200, 300, 400, 500, 750, 1000	25	100	40
CYP2D6	5	DM	25, 50, 100, 150 200, 300, 400, 600, 800, 1000	25	100	30
HLM	10	MEP	25, 50, 100, 150 200, 400	25	100	30

At the end of the incubation period, the reaction was stopped by addition of 1M perchloric acid or acetonitrile and placing the samples on ice. After centrifugation, the supernatant was retained for quantitation of product formation using the developed assay for that particular metabolite.

The kinetic constants were obtained by iterative nonlinear regression analysis of data according to the Michaelis-Menten equation using GraphPad Prism (GraphPad Software Inc., Menlo Park, CA). Linear transformation of data and construction of linear plots are alternative ways to calculate the kinetic constants.

2.7.2 Comparison of the Inhibitory Effects of TCP, TCP Enantiomers, and Ring Substituted Analogues of TCP on CYP2C19

After preliminary experiments, a constant concentration of substrate (200 μM) was incubated with varying concentration of the drug of interest as inhibitor (25, 50, 100, 200, and 400 μM) in the presence of human liver microsomes (10 μL) and an NADPH-generating system (25 μL) in a water bath at 37°C. The total incubation volume was brought to 100 μL with 0.1 M potassium phosphate buffer (pH=7.4). The control sample contained buffer in place of inhibitor (drug of interest). At the end of the incubation period, the reaction was stopped by placing the samples on ice and adding 50μL perchloric acid. The metabolite was extracted, derivatized and analyzed on a GC-ECD as described in section 2.3.2. This set of experiments was performed in triplicate over three consecutive days.

The effect of the drug of interest (potential inhibitor) on CYP2C19 activity was evaluated by comparing the rate of substrate turnover (v) in samples containing different inhibitor concentrations to the rate of substrate

turnover (v') in the control sample. A non-linear best-fit analysis was performed to relate the enzyme activity to the concentration of inhibitor and to calculate IC_{50}.

2.8 Kinetic Studies of Inhibition

2.8.1 Preliminary Studies

Prior to any kinetic studies of inhibition, it is necessary to determine whether inhibition is time-dependent or not, and thereafter if it is reversible or irreversible.

2.8.2 Time-Dependence of Inhibition

To evaluate whether preincubation of inhibitor (TCP or PLZ) with enzyme (CYP2C19, CYP2C9, or 2D6) affects the degree of inhibition, a series of experiments was conducted. For each series, a set of microcentrifuge tubes (1.5 mL) containing microsomes was placed in a water bath at 37°C. At predetermined time intervals (preincubation times of 0, 2, 4, 7, 10, 15, 20, 25, and 30 min), one of the samples was removed from the water bath. A constant submaximal concentration of inhibitor was added to the sample. The solution was then returned to the water bath (each sample corresponding to a specific preincubation time was done in triplicate). When the inhibitor was added to all samples at different time intervals, samples were placed on ice for a few minutes. Then constant concentrations of substrate and NADPH-generating system were added to each sample. The final mixture

was incubated further in the water bath at 37°C for a fixed period of time. At the end of the incubation period the reaction was stopped and the presence of metabolite was assayed as described previously. Table 2.7 provides details of samples in the time dependence study of inhibition.

Table 2.7 Samples in time dependence study of inhibition

Enzyme	Volume of microsomal solution (μL)	Substrate		Inhibitor concentration (μM)	Preincubation time (min)	Incubation time (min)
		Name	Concentration (μM)			
TCP as Inhibitor						
CYP2C19	5	MEP	300	50	0, 2, 4, 7, 10, 15, 20, 25, 30	40
CYP2C9	10	TOL	75	50	0, 2, 4, 7, 10, 15, 20, 25, 30	60
CYP2D6	5	DM	100	100	0, 2, 4, 7, 10, 15, 20, 25, 30	30
PLZ as Inhibitor						
CYP2C19	5	MEP	300	10	0, 2, 4, 7, 10, 15, 20, 25, 30	40
CYP2C9	10	TOL	80	10	0, 2, 4, 7, 10, 15, 20, 25, 30	60
CYP2D6	5	DM	100	7.5	0, 2, 4, 7, 10, 15, 20, 25, 30	30

The peak-height ratio of metabolite to internal standard, an indicator of the remaining enzyme activity in each sample, was plotted against the corresponding preincubation time. If enzyme activity does not decrease with time, inhibition is not time-dependent and preincubation of enzyme with inhibitor would not be necessary in subsequent experiments. In addition, a

lack of time-dependence suggests that inhibition is likely to be reversible, although this should still be confirmed.

2.8.3 Reversibility versus Irreversibility of Inhibition

Different methods, including dialysis, dilution, gel filtration and size-exclusion chromatography can be used to examine reversibility of inhibition. Dilution has been the principle of our approach for determination of reversibility of inhibition. In this experiment four sets of microcentrifuge tubes were specified as control, group A, group B, and group C. Control contained microsomal protein and buffer. The remaining groups contained microsomal protein, buffer and inhibitor. The variables are the concentration of inhibitor (TCP or PLZ) and total volume of mixture.

As shown in Table 2.8, the concentration of inhibitor in group A was equal to that in group C and approximately 10 times larger than that in group B. The total volume of mixture in group A was equal to that in the control group, and less than that in groups B or C.

All tubes were incubated at 37°C for 20 min. After 20 min, buffer was added to groups A and control to equalize the total volume of incubation medium in all 4 sets of tubes, and in the case of reversibility of inhibition equalize the concentration of inhibitor in groups A and B. All tubes were returned to the water bath and incubated for another 10 min. The tubes were then removed and placed on ice. Constant amounts of NADPH and substrate

were added to all tubes. The tubes were then incubated for a predetermined period of time.

Table 2.8 Samples in reversibility study of inhibition

Microsomal enzyme & its volume (μL)	Substrate		Inhibitor concentration (μM)		Preincubation volume (μL)		Total volume (μL)
	Name	Concentration (μM)	Groups A and C	Group B	Control & Group A	Groups B and C	
TCP as Inhibitor							
CYP2C19 (3)	MEP	300	100	11.1	10	90	100
CYP2C9 (10)	TOL	80	100	14.8	20	135	150
CYP2D6 (3)	DM	100	1000	111.1	10	90	100
PLZ as Inhibitor							
CYP2C19 (5)	MEP	300	50	5	10	90	100
CYP2C9 (10)	TOL	90	50	5	20	200	220
CYP2D6 (5)	DM	100	50	5	10	90	100

At the end of the incubation time, the reaction was stopped and the production of metabolite was quantified by the appropriate developed HPLC method. In the case of reversible inhibition, the enzyme activities in groups A and B should be almost equal, and higher than that in group C. In the case of irreversible inhibition, the enzyme activities in groups A and C should be almost equal, and higher than that in group B.

2.8.4 Evaluation of Inhibitory Effects of TCP or PLZ on CYP2C9, CYP2C19, and CYP2D6

Following preliminary studies, a range of substrate concentrations (TOL, MEP, and DM as substrates of CYP2C9, CYP2C19, and CYP2D6, respectively) was incubated at 37°C with microsomes from c-DNA expressed cell lines in the presence of an NADPH-generating system and varying concentrations of TCP or PLZ as inhibitor, for an appropriate period of time. At the end of the incubation period, the enzyme was inactivated with ice-cold acetonitrile. Table 2.9 provides further information regarding samples in this study.

After adding internal standard and centrifuging briefly, a portion of the supernatant was analyzed for levels of the metabolite of that specific substrate, on an HPLC equipped with a UV detector. Calibration curves were generated at concentrations ranging from 0.781 to 25 μM by processing the authentic standard substances throughout the entire procedure. The produced metabolite was quantified by comparison with the standard curves using the peak-height ratio method.

Table 2.9 Samples in Main Study

Microsomal enzyme & its volume (μL)	Substrate		Inhibitor concentrations (μM)	Incubation time (min)	Total incubation volume (μL)
	Name	Concentrations (μM)			
TCP as Inhibitor					
CYP2C19 (5)	MEP	50, 100, 150, 200, 300, 450	0, 10, 30, 100, 300	40	100
CYP2C9 (10)	TOL	10, 25, 50, 100, 150	0, 10, 30, 100, 300	60	100
CYP2D6 (3)	DM	5, 10, 50, 100, 200	0, 100,400, 800, 1200	30	100
PLZ as Inhibitor					
CYP2C19 (10)	MEP	50, 100, 150, 200, 300, 450, 1000	0, 2, 5, 10, 15	35	100
CYP2C9 (10)	TOL	10, 25, 50, 100, 150	0, 5, 10, 20, 50	70	100
CYP2D6 (5)	DM	5, 10, 20, 50, 100	0, 2, 5, 10, 15	15	100

2.9 Data Analysis

The data were plotted on Michaelis-Menten, Hanes-Woolf or Lineweaver-Burk graphs. The pattern of variations in K_m and V_{max} values, in the presence of TCP or PLZ, yielded information regarding the type of inhibition. In each experiment, K_i was determined by graphical methods as explained in sections 1.8.1.1 through 1.8.1.4.

CHAPTER 3

Results

3. Results

To achieve the objectives of the study described in section 1.11, a series of tests and experiments was conducted starting with the development of appropriate GC-ECD as well as HPLC-UV assays for detection and quantification of compounds of interest from microsomal studies.

Human liver microsomal studies were conducted *in vitro* to investigate the inhibitory effects of TCP, its enantiomers and its ring-substituted analogues on CYP2C19.

The next series of experiments addressed the kinetics of inhibitory effects of TCP and PLZ on CYP2C9, CYP2C19 and CYP2D6 using human cDNA-expressed cell lines. These experiments included preliminary studies on appropriate enzyme concentration, optimum incubation time, appropriate substrate concentration by estimation of K_m and V_{max}, and evaluation of time dependency and reversibility of inhibition.

3.1 Detection and Quantitation of DXR and OHMEP Using GC-ECD

The GC-ECD assay described in section 2.5.2.1 was used to detect and quantify DXR and OHMEP in microsomal studies. The method was rapid and sensitive and the derivatives produced were stable. As illustrated in Figure 3.1, the derivatives had excellent chromatographic properties. The retention times of the PFB derivatives of DXR and OHMEP were 17.82 and 16.78 min,

respectively. The standard curve of the PFB derivatives of DXR and OHMEP were linear from 10 to 2000 ng (r^2>0.99 was obtained routinely).

The limit of detection was less than 5 ng per 1mL sample for DXR or OHMEP (<17 pg "on column"). Recoveries, determined using 100 ng of DXR and 140 ng of OHMEP, were virtually quantitative. To determine the coefficients of variation, 6 data points (n=6) were used in each case. Intra- and inter-assay coefficients of variation for DXR, determined at 100 ng, were 5.5% and 7.3%, respectively. Intra-assay coefficients of variation for OHMEP, determined at drug concentrations of 140, 70 and 17 ng (n=6), were 5.5, 6.7 and 9.5%, respectively. Inter-assay coefficients of variation, determined at the same drug concentrations (n=6), were 7.3, 5.0 and 4.5%, respectively. The derivatized samples were stable for at least 48 hours when stored at -80°C.

In another experiment PFBSC was used instead of PFBC as the derivatizing agent. Figure 3.2 shows that the GC traces of the PFBS derivative are cleaner than those of the PFB derivatives. The retention times of the PFBS derivatives of OHMEP and DXR were 17.66 and 18.93 min, respectively. However, the PFBS derivatives of compounds are not as stable or reproducible as the PFB derivatives, so the PFB derivatives were used in subsequent studies.

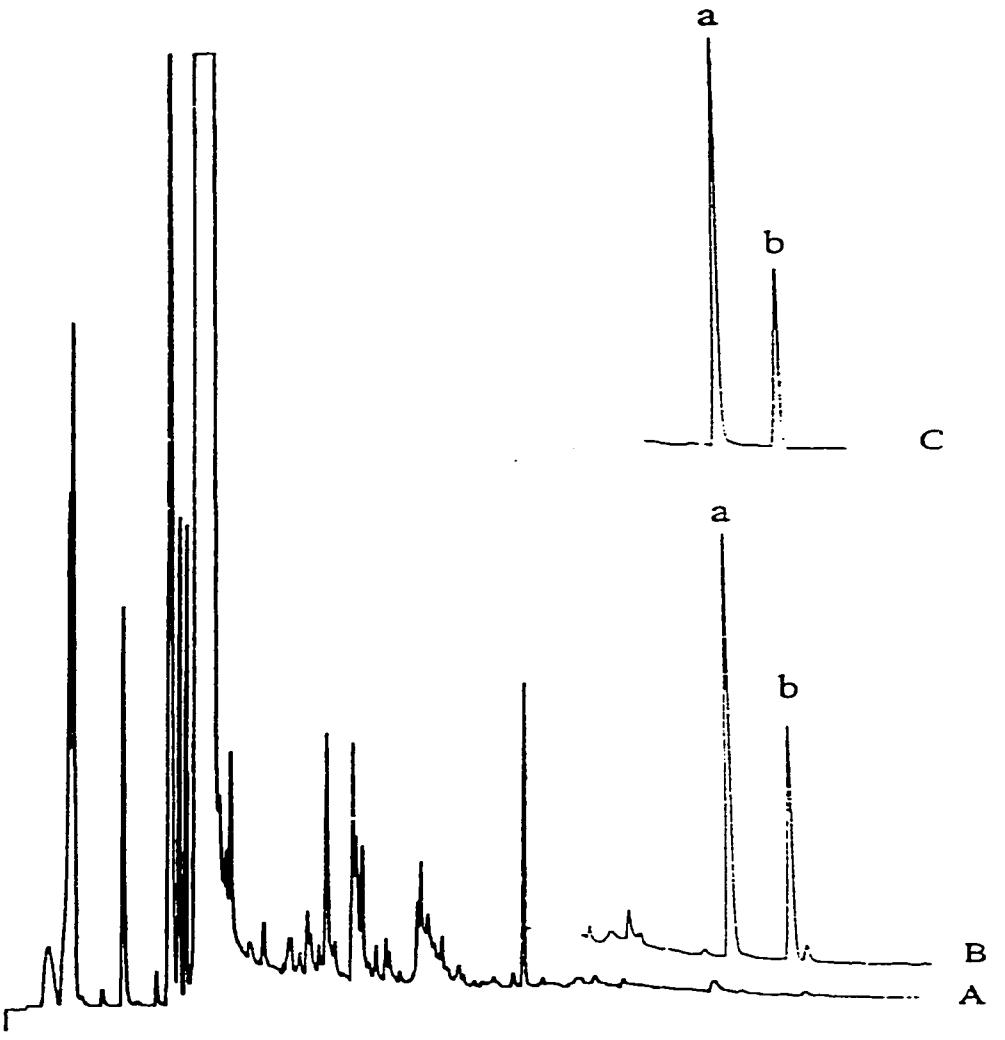


Figure 3.1 Typical GC traces of PFB-derivatized extracts of; A) an incubation medium containing human liver microsomes and no substrate (DM); B) an incubation medium containing human liver microsomes, DM as substrate, NADPH-generating system, and 0.1 mM potassium phosphate buffer (the mixture was incubated for 30 min and OHMEP was added as internal standard before extraction); and C) authentic standards of OHMEP and DXR. Peak "a" with retention time of 16.78 min, and peak "b" with retention time of 17.82 min correspond to OHMEP and DXR, respectively.

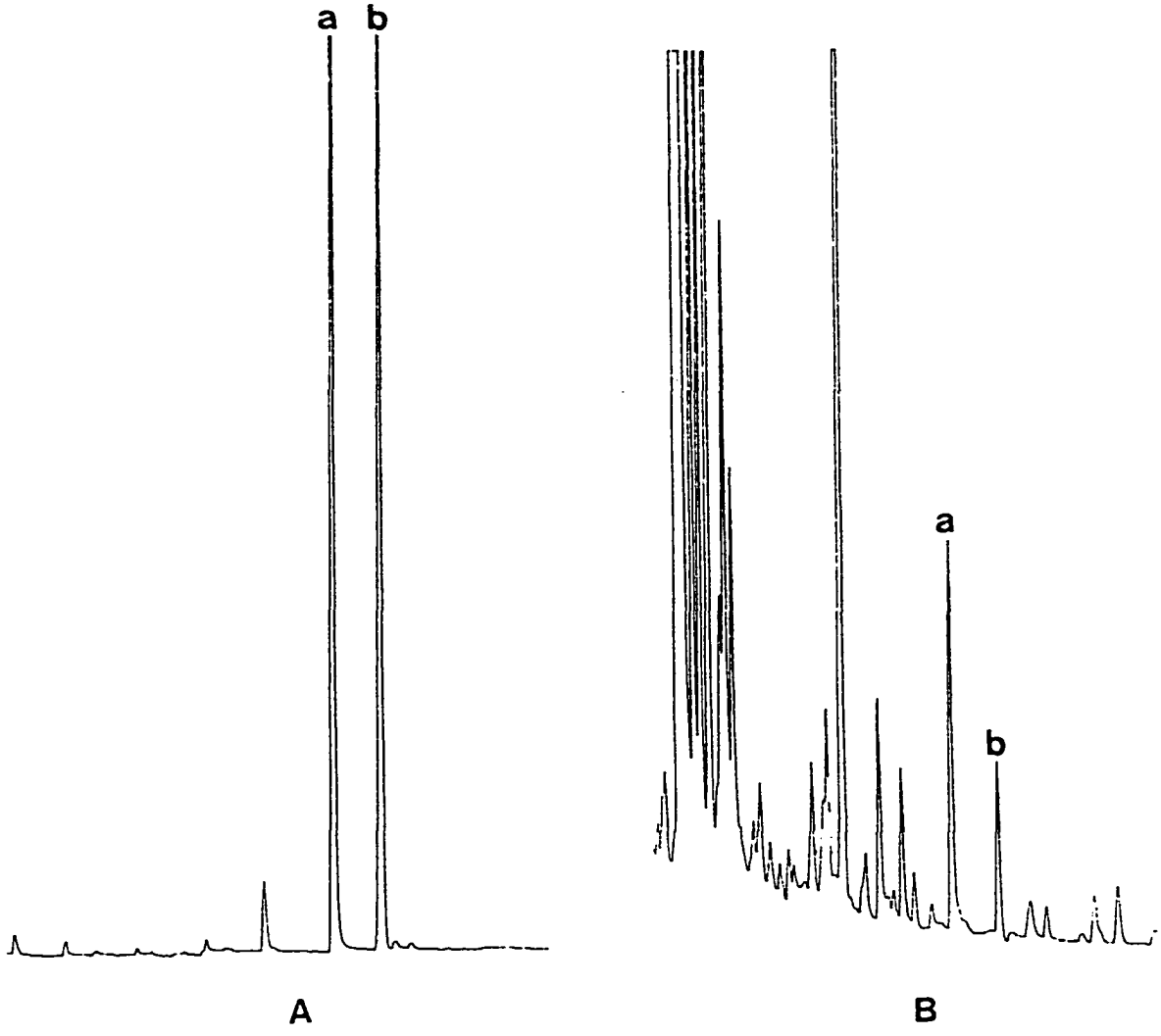


Figure 3.2. Typical GC traces of PFBS derivatives of OHMEP and DXR; A) an extract from authentic standards of OHMEP and DXR; and B) an extract from an incubation of MEP with human liver microsomes and an NADPH generating system at 37°C for 30 minutes (DXR was added as internal standard before extraction). Peak "a" with retention time of 17.66 min, and peak "b" with retention time of 18.93 min correspond to OHMEP and DXR, respectively.

Figures 3.3 and 3.4 show the proposed electron-impact mass fragmentation of the PFB derivatives of DXR and OHMEP, respectively. The proposed electron-impact mass fragmentation of the PFBS derivatives of DXR and OHMEP were consistent with the structures shown in Figures 3.5 and 3.6, respectively.

The GC-ECD method was first developed in the absence of microsomes. Then it was applied to the incubations from human liver microsomes or microsomes from cDNA-expressed cell lines. To study the effect of the protein content of microsomes, calibration curves were constructed in the presence as well as in the absence of control microsomes. The results showed that the developed method can be applied to microsomal experiments, and the small amount of microsomal protein remaining after precipitation of protein by perchloric acid at the end of the incubation period does not interfere with analyses.

Figures 3.7 and 3.8 represent respectively the application of this method to quantify DXR formation from DM and OHMEP from MEP in a time-course study using human liver microsomes. Although the method has been applied to quantify DXR and OHMEP in microsomal experiments, it should be readily adaptable to other studies such as investigation of levels of these metabolites in tissues or body fluids.

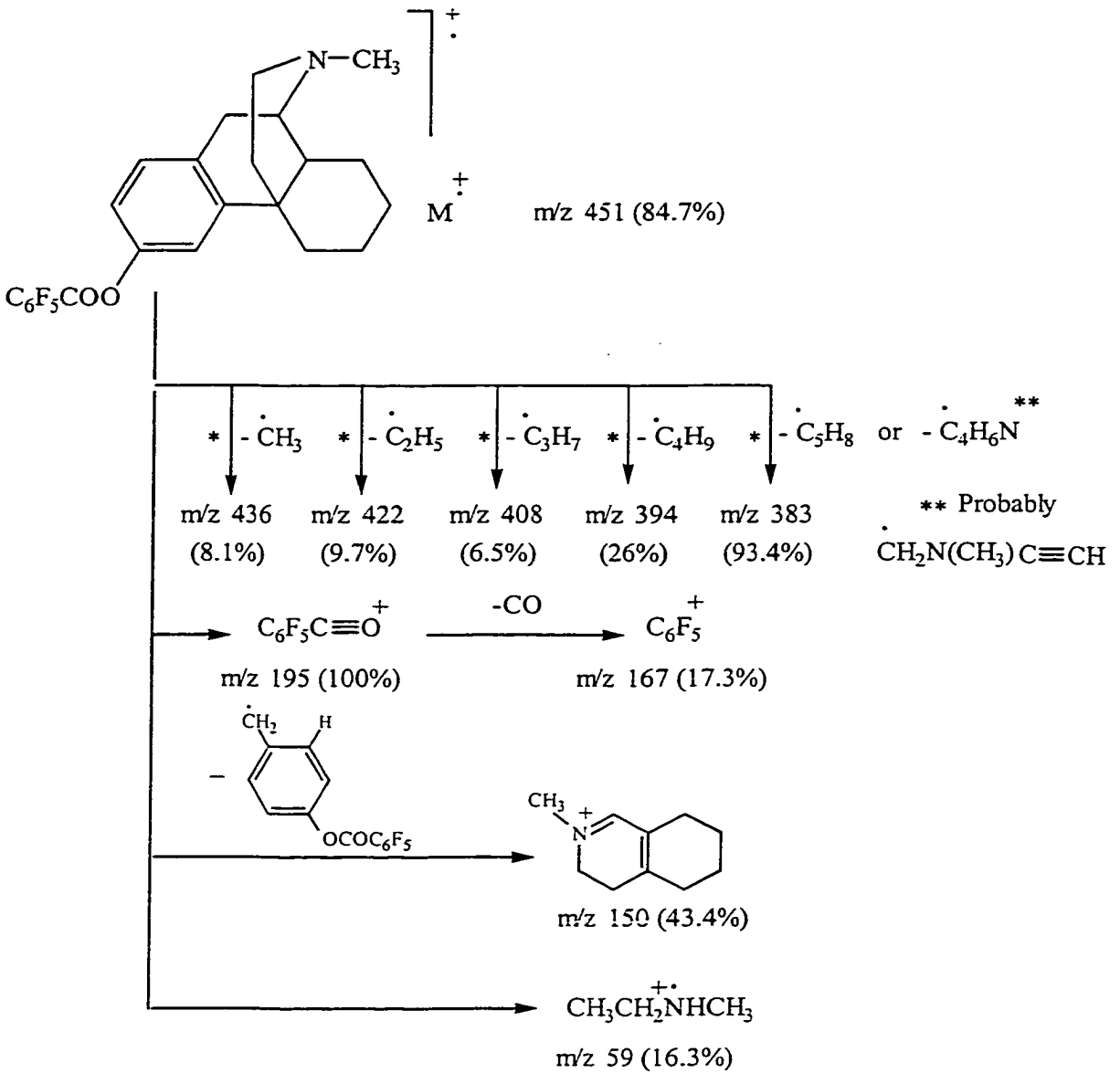


Figure 3.3 Proposed electron-impact mass fragmentation of the PFB derivative of DXR. Values in parentheses are relative abundances.

* Expulsion of hydrocarbon radicals from the molecular ion with structural rearrangements gives these fragments for which it is not possible to provide exact molecular structures.

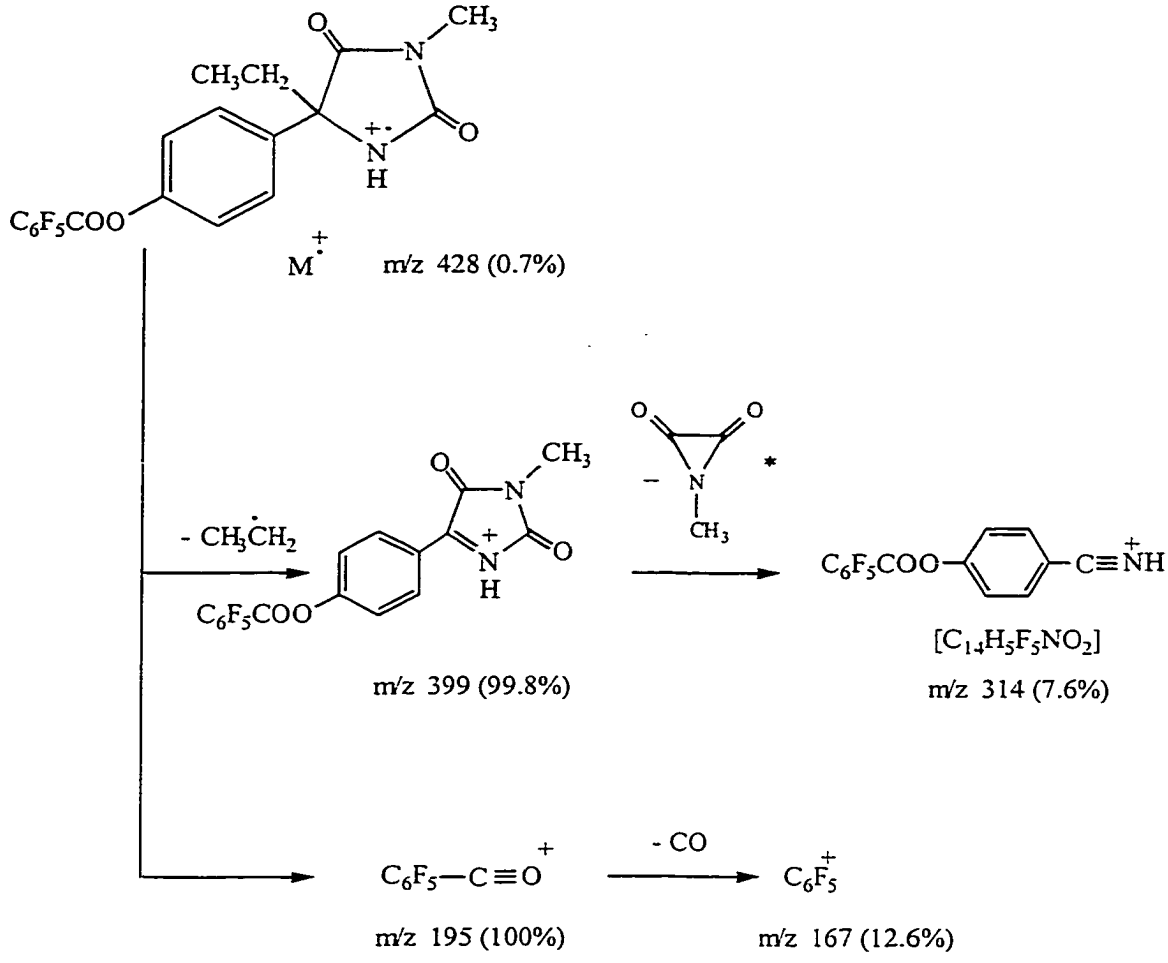


Figure 3.4 Proposed electron-impact mass fragmentation of the PFB derivative of OH-MEP. Values in parentheses are relative abundance.

* Locock and Coutts (1970) have previously demonstrated the loss of this group from structurally similar compounds.

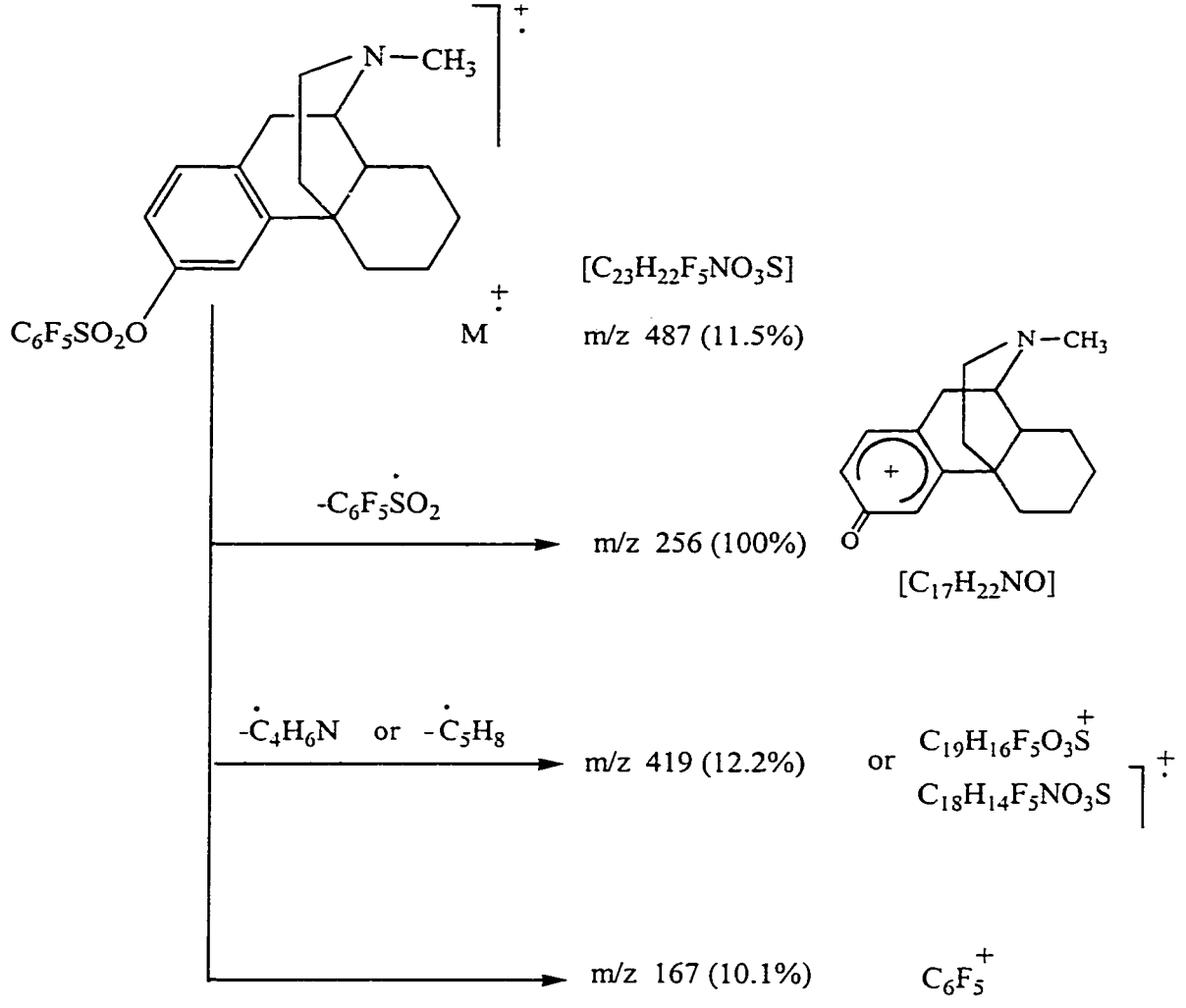


Figure 3.5 Proposed electron-impact mass fragmentation of the PFBS derivative of DXR. Values in parentheses are relative abundances of the individual fragments.

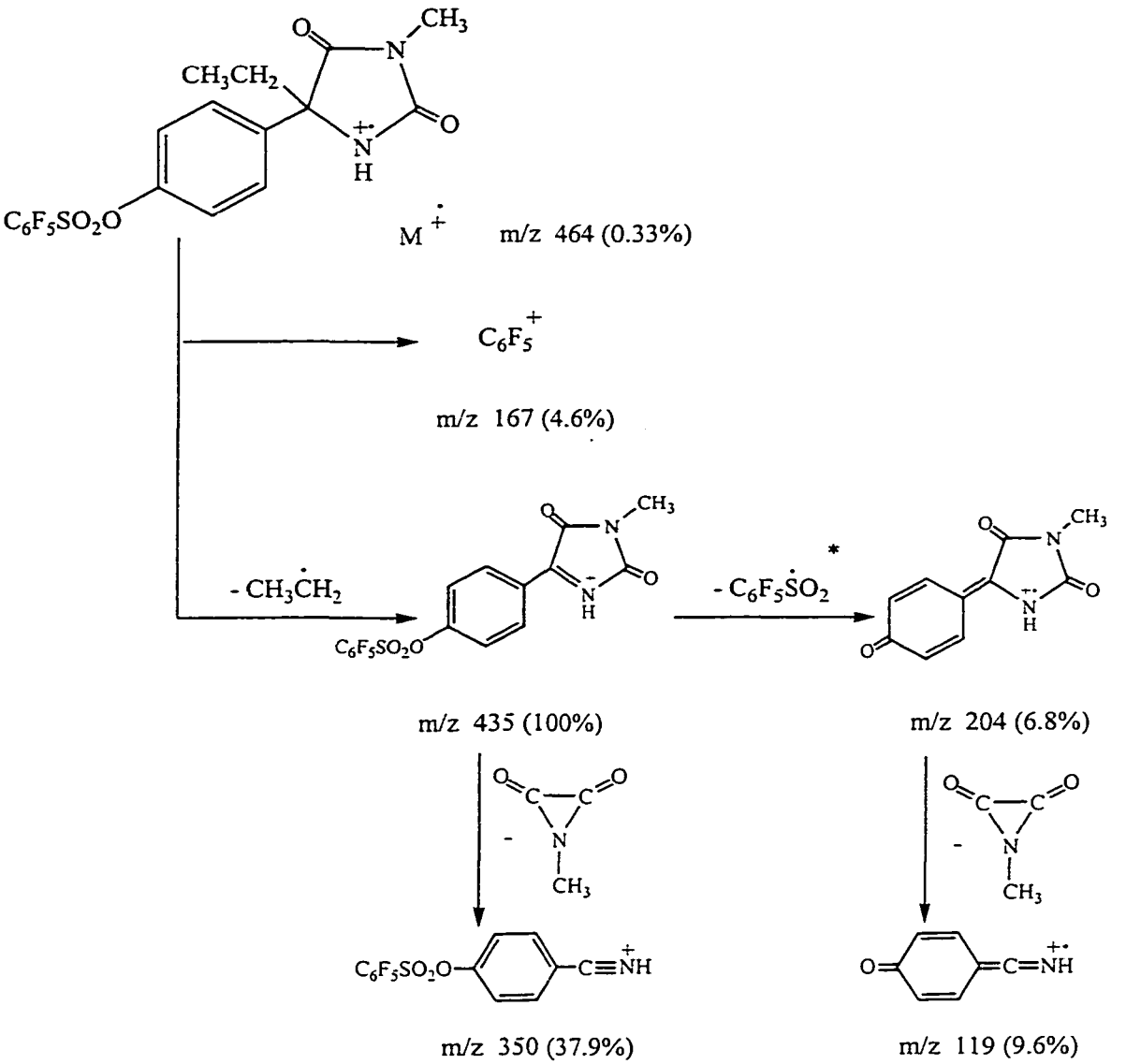


Figure 3.6 Proposed electron-impact mass fragmentation of the PFBS derivative of OHMEP. Values in parentheses are relative abundance of the molecular and fragment ions.

* This is an unusual fragmentation. Even electron ions are generally more stable than odd-electron ions and almost always fragment by expelling a molecule to give another even-electron ion of lower mass.

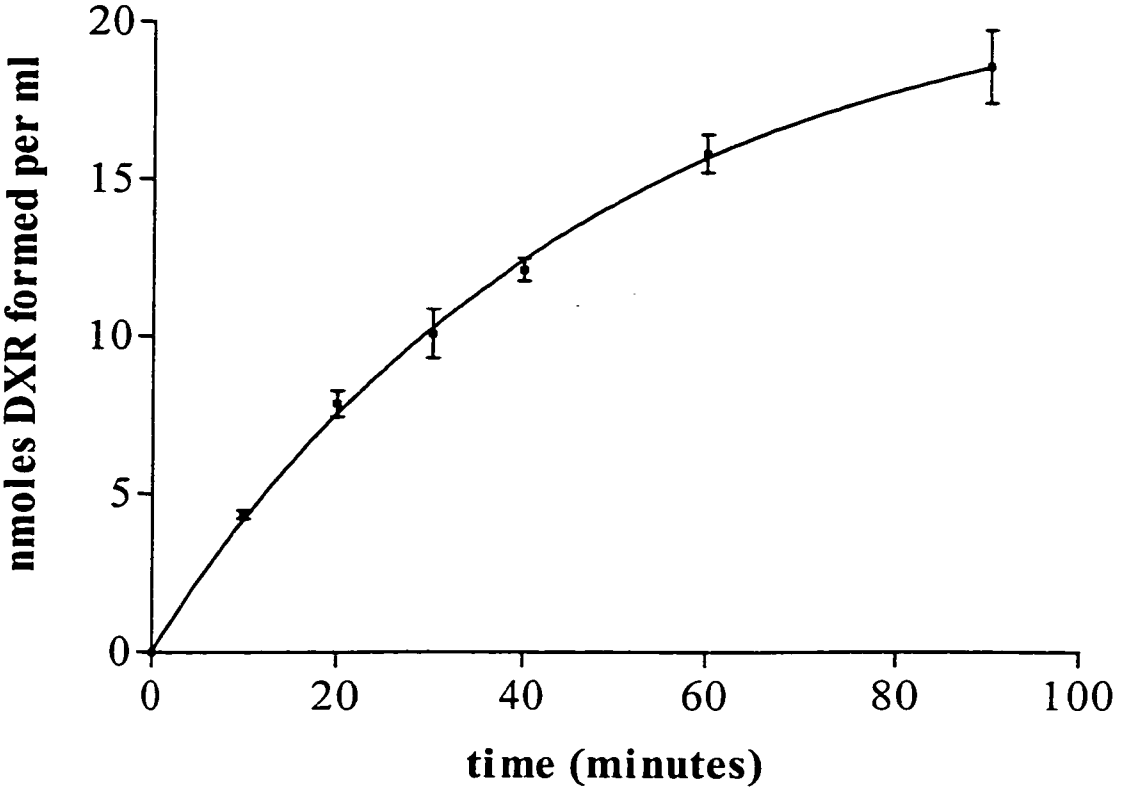


Figure 3.7 Time-dependent production of DXR from DM (200 μM), in an incubation medium containing human liver microsomes and an NADPH-generating system. Extracts were derivatized with PFBC. Results are expressed as means ± SEM (n=3).

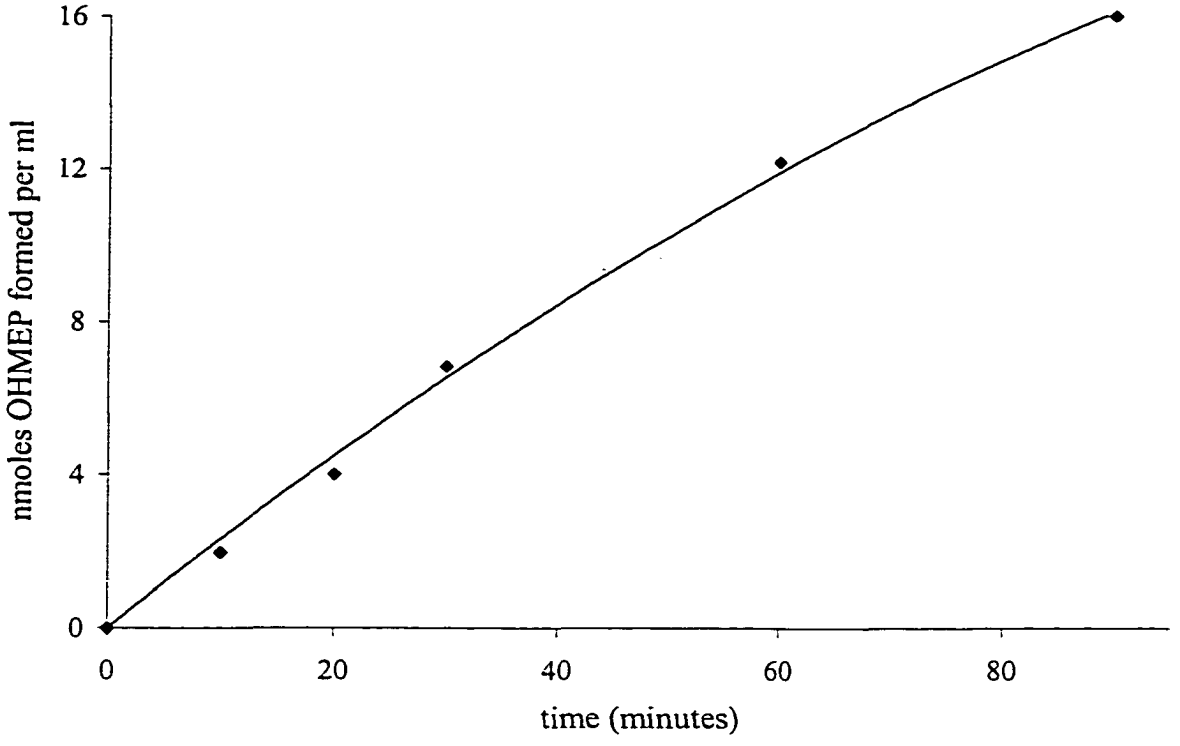


Figure 3.8 Time-dependent production of OHMEP from MEP (150 μM), in an incubation medium containing human liver microsomes and an NADPH-generating system. Extracts were derivatized with PFBC.

3.2 HPLC-UV Assay for Quantification of OHMEP in Microsomal Experiments in the Presence of TCP

Figure 3.9 shows an HPLC trace of peaks related to OHMEP, MEP, internal standard (PHB), and TCP. The developed assay can also detect OHTCP, a metabolite of TCP, in case of its production from incubated microsomes. Retention times for OHMEP, TCP, PHB, OHTCP, and MEP were approximately 10.59, 11.91, 19.15, 5.81, and 30.36 min, respectively. As presented in Figure 3.10, the calibration curve of OHMEP was linear for concentrations of 0.781 μM to 25 μM ($r^2 > 0.99$ was obtained routinely). The limit of detection was less than 0.5μM (or less than 11.7 ng/100μL of incubation volume). Recoveries, determined using 2.5 μM OHMEP, were virtually quantitative. The intra-assay coefficients of variation (n=6) for concentrations of 12, 6, 3, and 1.5 μM were between 0.5 and 2%. The inter-assay coefficients of variation (n=3) for the range of concentrations from 0.781μM to 25 μM were between 4% and 7%.

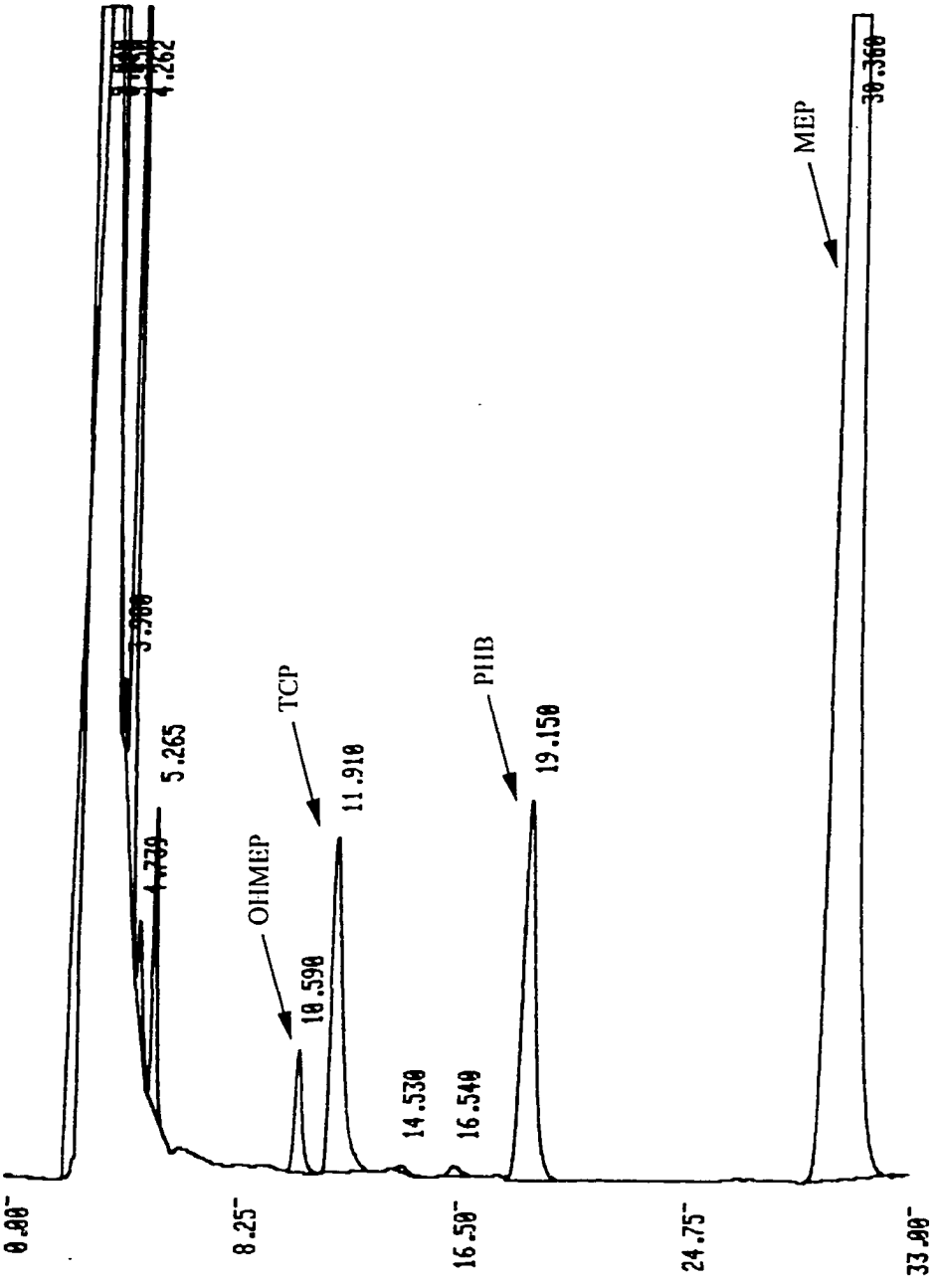


Figure 3.9 A typical HPLC trace from the study on the production of OHMEP from MEP in an incubation mixture of CYP2C19 in the presence of TCP. OHMEP, *para*-hydroxymephenytoin; TCP, (±)-tranylcypromine; PHB, phenobarbital; MEP, S-(+)-mephenytoin.

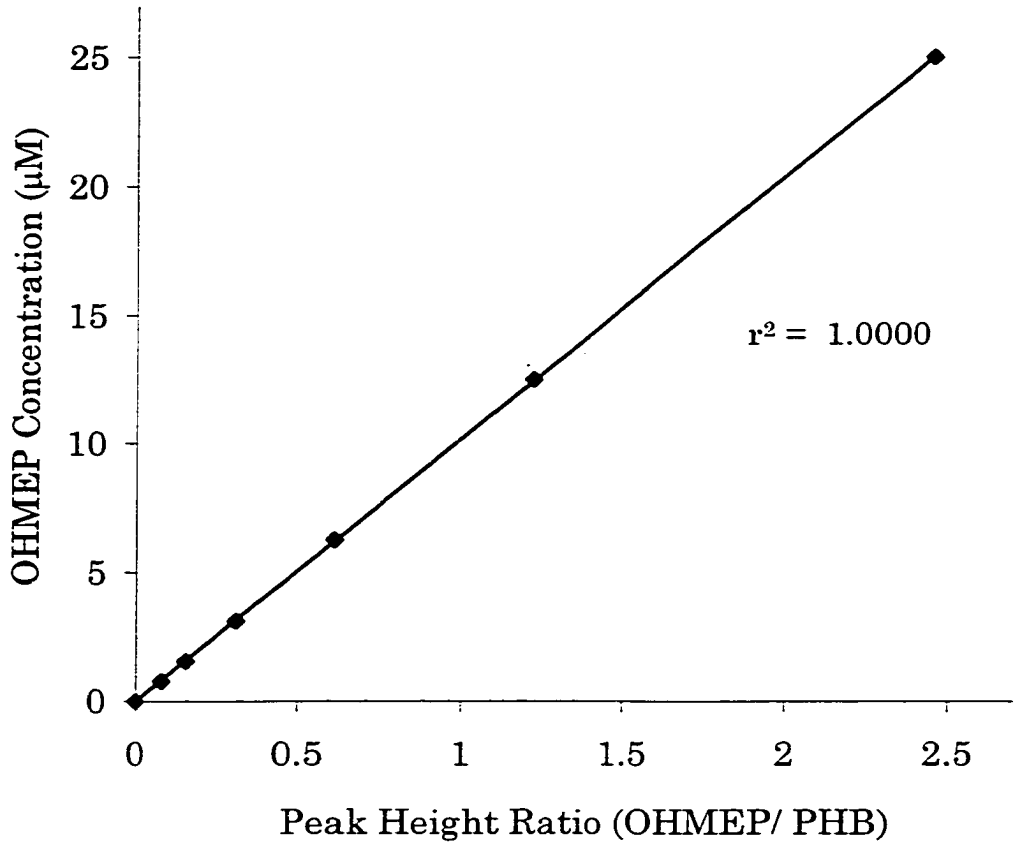


Figure 3.10 Typical calibration curve for the quantification of OHMEP in microsomal experiments

3.3 HPLC-UV Assay for Quantification of OHMEP in Microsomal Experiments in the Presence of PLZ

Figure 3.11 shows an HPLC trace of peaks related to OHMEP produced from MEP, PLZ, internal standard (PHB), and MEP. Retention times for OHMEP,

PLZ, PHB, and MEP were 8.18, 10.67, 13.84, and 20.83 min, respectively. The calibration curve of OHMEP was linear for concentrations of 0.781 μM to 25 μM ($r^2 > 0.99$ was obtained routinely). The limit of detection was less than 0.5μM (or less than 11.7ng/100μL of incubation volume). The intra-assay coefficients of variation (n=6) for concentrations of 12, 6, 3, and 1.5 μM were between 1% and 2%. The inter-assay coefficients of variation (n=3) for a range of concentrations from 1.5 μM to 25 μM were between 5% and 7%.

3.4 HPLC-UV Assay for Quantification of OHTOL in Microsomal Experiments in the Presence of TCP

Retention times for OHTOL, TCP, PCP, and TOL were 8.87, 9.78, 22.68 and 31.80 min, respectively (Figure 3.12). The calibration curve of OHTOL was linear for concentrations of 0.781 μM to 25 μM ($r^2 > 0.99$ obtained routinely). The limit of detection was less than 0.5 μM (or less than 14.3 ng/100μL of incubation volume). Recoveries, determined using 2.5 μM OHTOL, were virtually quantitative. The intra-assay coefficients of variation (n=6) for concentrations of 12.5, 6, 3, and 1.5 μM were between 1% and 2%. The inter-assay coefficients of variation (n=3) for a range of concentrations from 1.5 μM to 25 μM were between 1.5% and 4%.

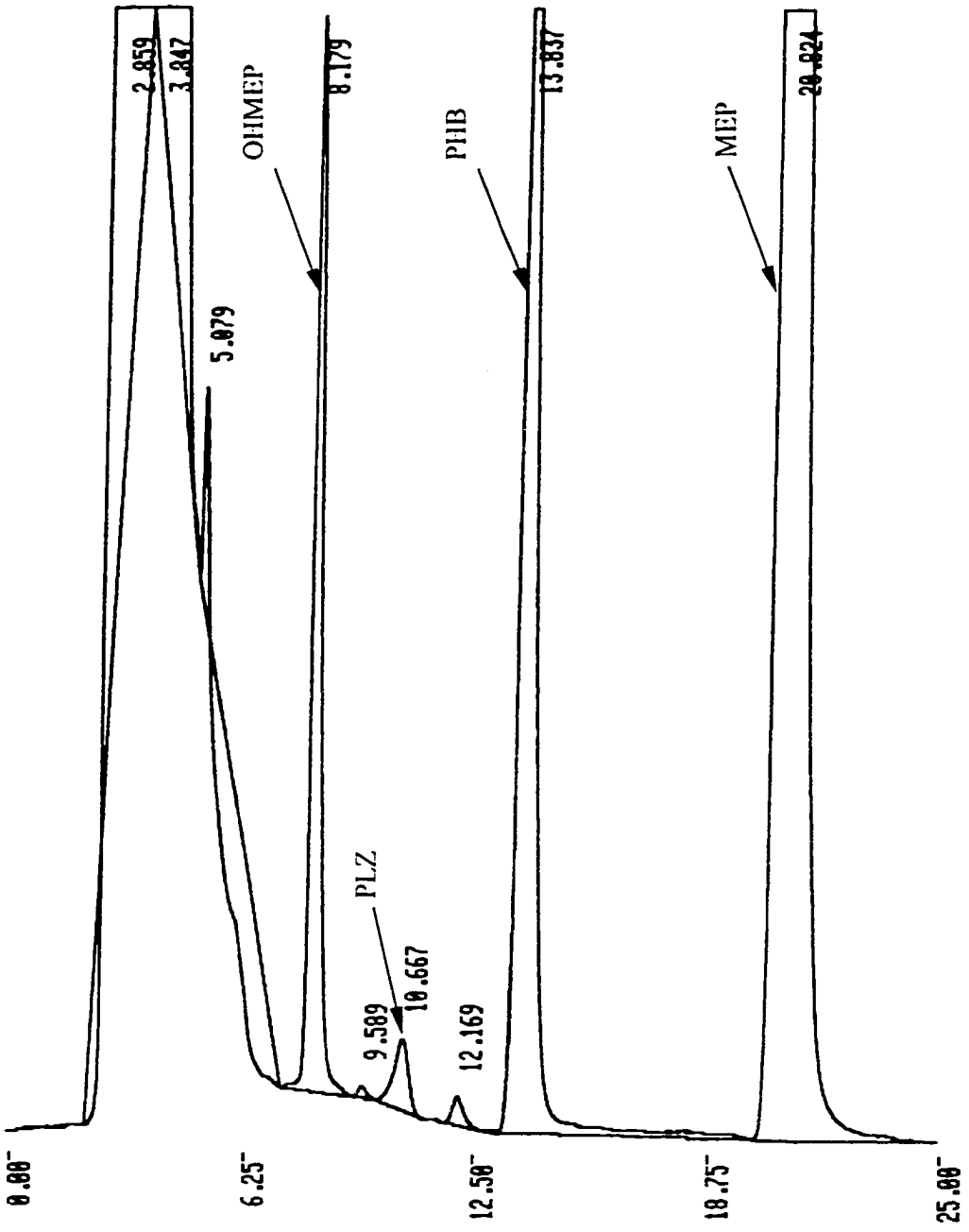


Figure 3.11 A typical HPLC trace from the study on the production of OHMEP from MEP in an incubation mixture of CYP2C19 in the presence of PLZ. OHMEP, *p*-hydroxymephenytoin; PLZ, phenelzine; PHB, phenobarbital; MEP, S-mephenytoin.

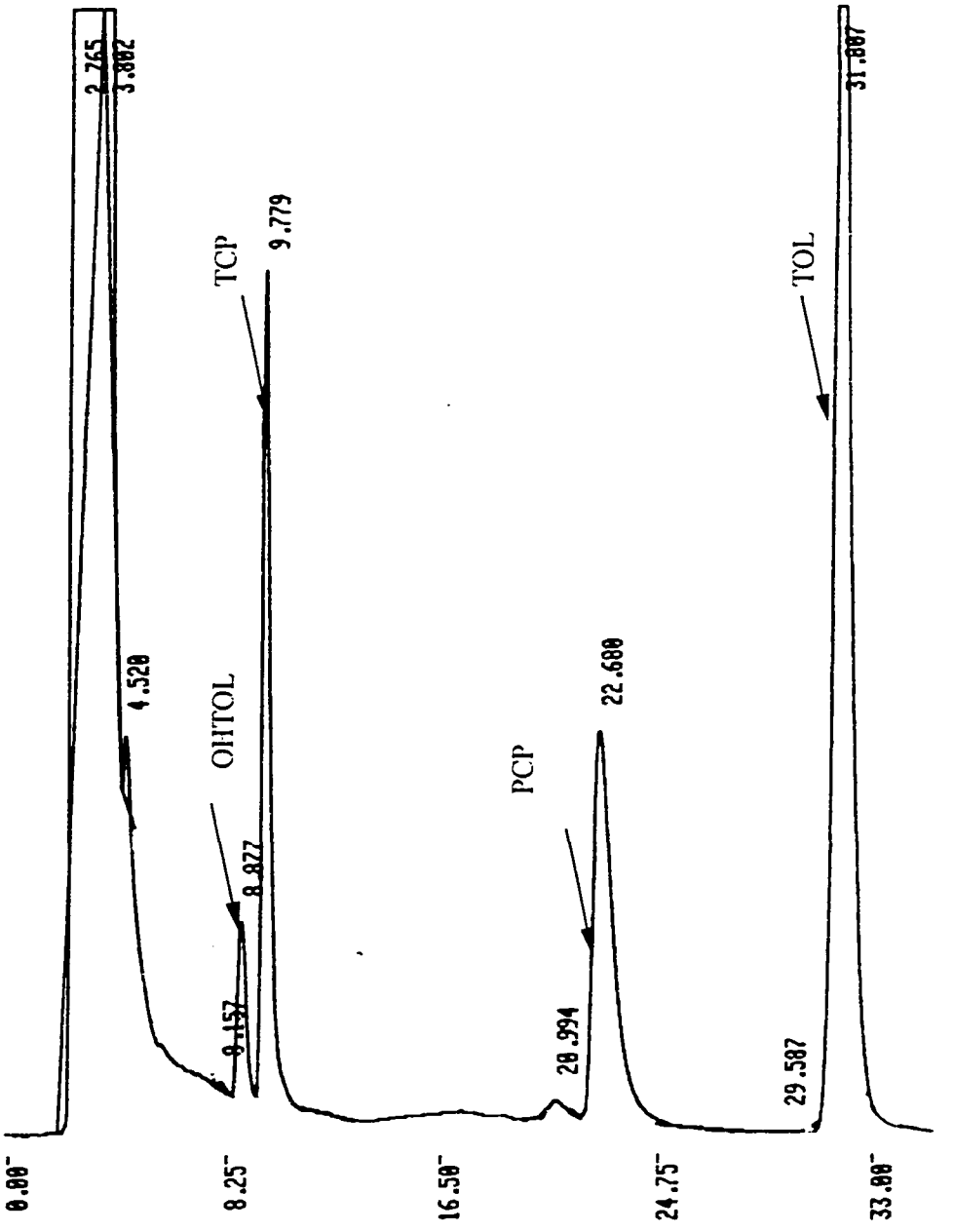


Figure 3.12 A typical HPLC trace from the study on the production of OHTOL from TOL in an incubation mixture of CYP2C9 in the presence of TCP. OHTOL, hydroxytolbutamide; TCP, tranylcypromine; PCP, *p*-chlorophentermine; TOL, tolbutamide.

3.5 HPLC-UV Assay for Quantification of OHTOL in Microsomal Experiments in the Presence of PLZ

Retention times for OHTOL, PLZ, PCP, and TOL were respectively 8.79, 9.60, 27.22, and 32.70 min (Figure 3.13). The calibration curve of OHTOL was linear for concentrations of 0.781 μM to 25 μM ($r^2 > 0.99$ obtained routinely). The limit of detection was less than 0.5 μM (or less than 14.3 ng/100μL of incubation volume). Recoveries, determined using 2.5 μM OHTOL, were virtually quantitative. The intra-assay coefficients of variation (n=6) for concentrations of 12.5, 6, 3, and 1.5 μM were between 1% and 2.5%. The inter-assay coefficients of variation (n=3) for a range of concentrations from 1.5 μM to 25 μM were between 1% and 7%.

3.6 HPLC-UV Assay for Quantitation of DXR in Microsomal Experiments in the Presence of TCP or PLZ

Retention times for DXR, TCP, PLZ, PCP, and DM were 10.61, 7.09, 9.98, 12.81, and 33.26 min, respectively (Figures 3.14 and 3.15). The calibration curve of DXR was linear for concentrations of 0.781 μM to 25 μM ($r^2 > 0.99$ was obtained routinely). The limit of detection was less than 0.5 μM (or less than 12.9 ng/100μL of incubation volume). Recoveries, determined using 2.5 μM DXR, were virtually quantitative. The intra-assay coefficients of variation (n=6) for concentrations of 12.5, 6, 3, and 1.5 μM were between 2%

and 3%. The inter-assay coefficients of variation (n=3) for a range of concentrations from 1.5 μM to 25 μM were between 6% and 8%.

3.7 Absence of Amphetamine as a Metabolite of Tranylcypromine

Neither 1-amino-3-phenylpropane nor amphetamine was detected following incubation of TCP with human liver microsomes.

3.8 The Inhibitory Effects of TCP, its Enantiomers and Ring Substituted Analogues on CYP2C19

The inhibitory effects of (+)-TCP, (-)-TCP, and (±)-TCP on CYP2C19 activity in human liver microsomes are presented in Figure 3.16. As illustrated in this figure, (+)-TCP (IC_{50}≈25μM) is a much stronger inhibitor of CYP2C19 than is (-)-TCP (IC_{50} >400μM).

From Figures 3.17-3.20, it can be seen that OHTCP (a metabolite of TCP; IC_{50}≈20μM), and the ring-substituted analogues of TCP namely, MTCP (IC_{50}≈41μM) and FTCP (IC_{50}≈78μM) also have inhibitory effects on CYP2C19. Among them, OHTCP has the strongest, and FTCP has the weakest inhibitory action (Figure 3.20).

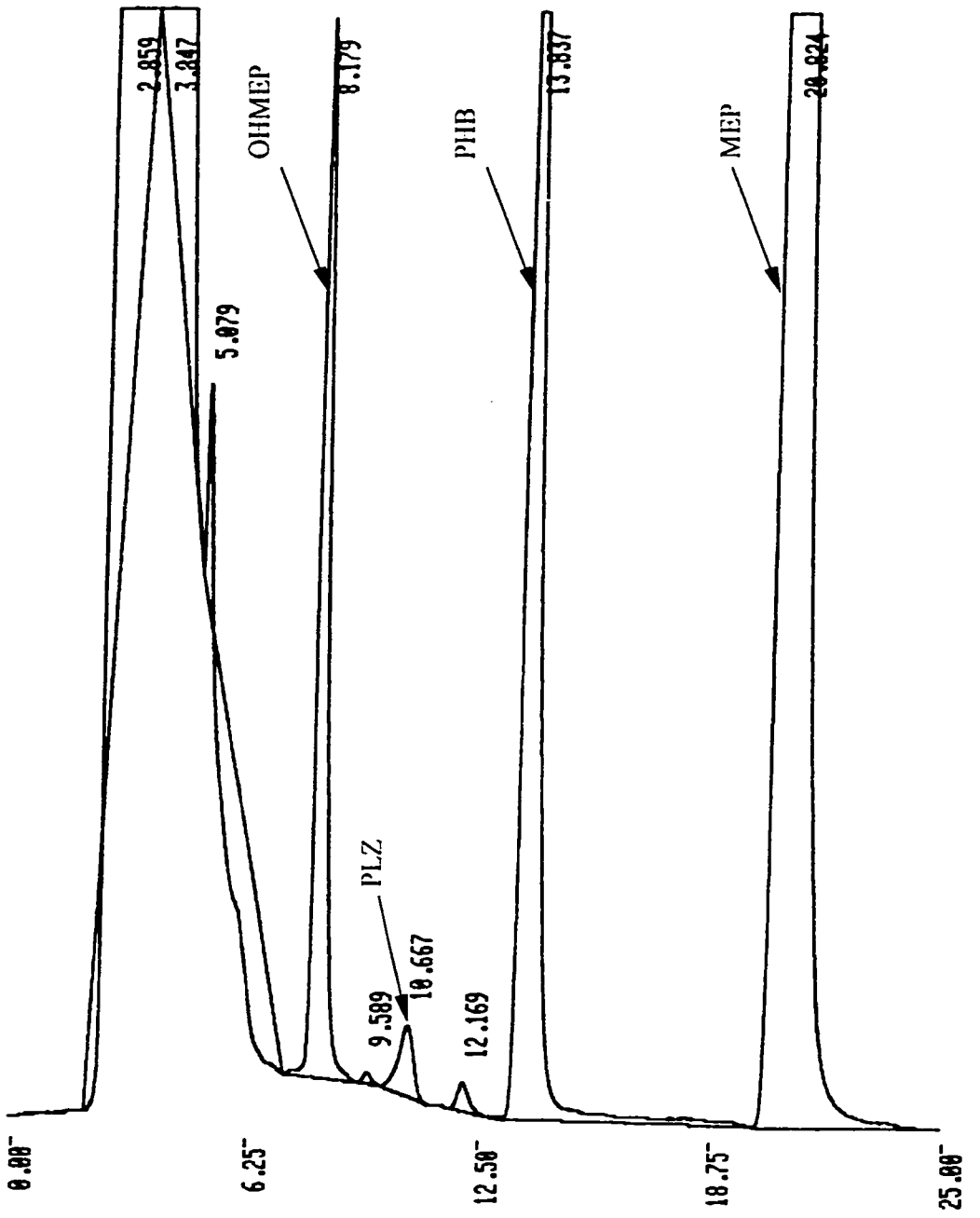


Figure 3.11 A typical HPLC trace from the study on the production of OHMEP from MEP in an incubation mixture of CYP2C19 in the presence of PLZ. OHMEP, *p*-hydroxymephenytoin; PLZ, phenelzine; PHB, phenobarbital; MEP, S-mephenytoin.

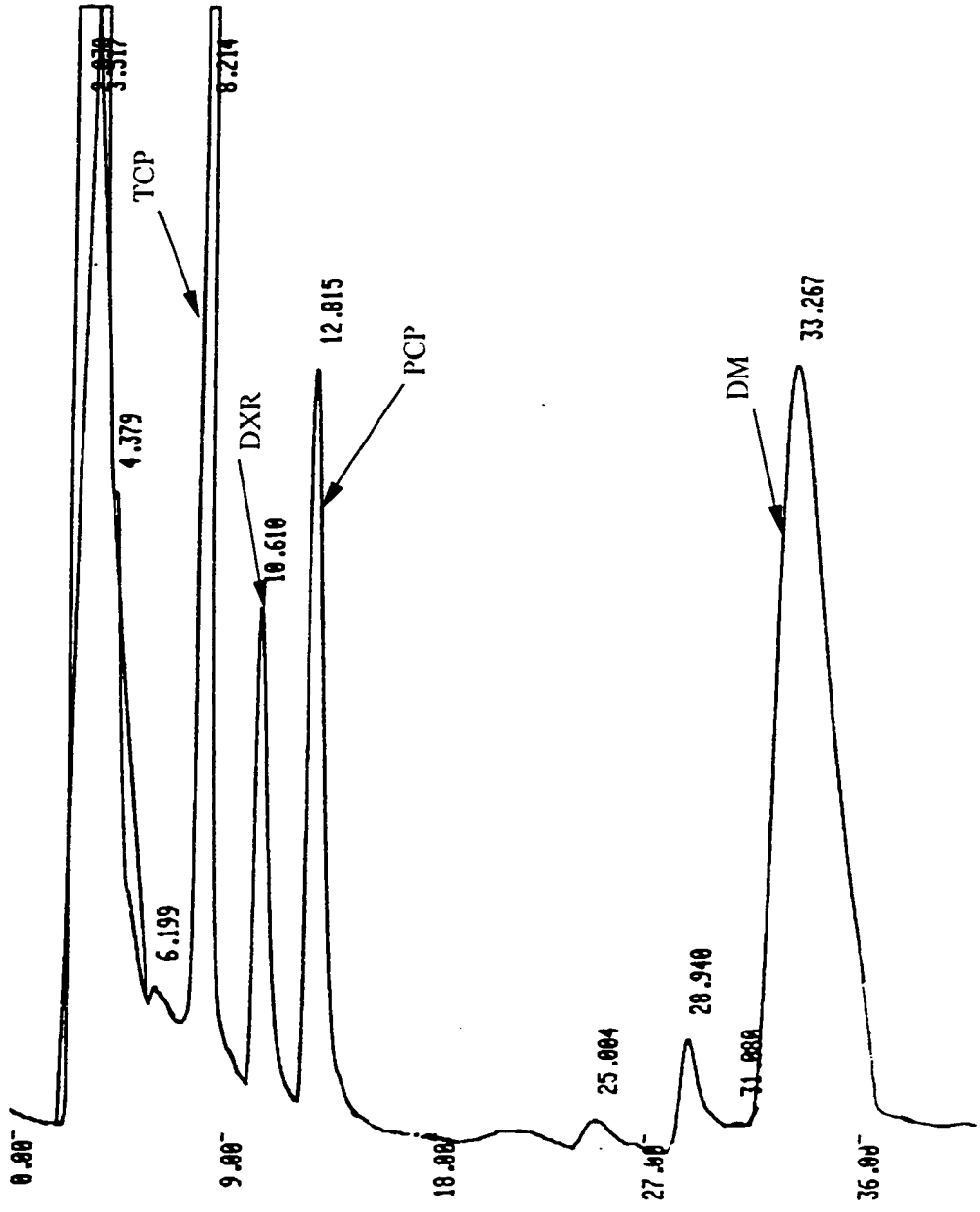


Figure 3.14 A typical HPLC trace from the study on the production of DXR from DM in an incubation mixture of CYP2D6 in the presence of TCP. DXR, dextrorphan; TCP, tranylcypromine; PCP, *p*-chlorophentermine; DM, dextromethorphan.

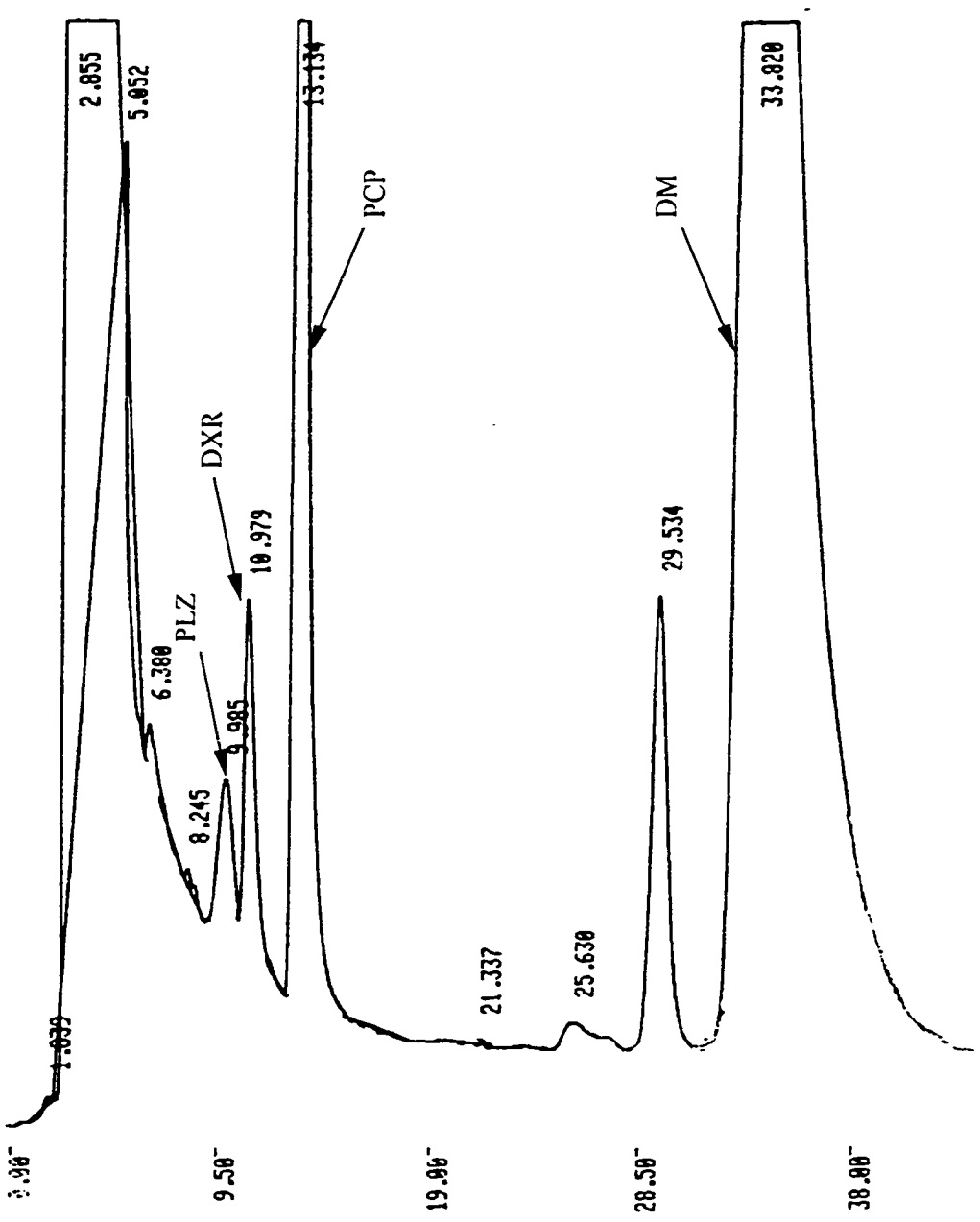


Figure 3.15 A typical HPLC trace from the study on the production of DXR from DM in an incubation mixture of CYP2D6 in the presence of PLZ. DXR, dextrorphan; PLZ, phenelzine; PCP, *para*-chlorophentermine; DM, dextromethorphan.

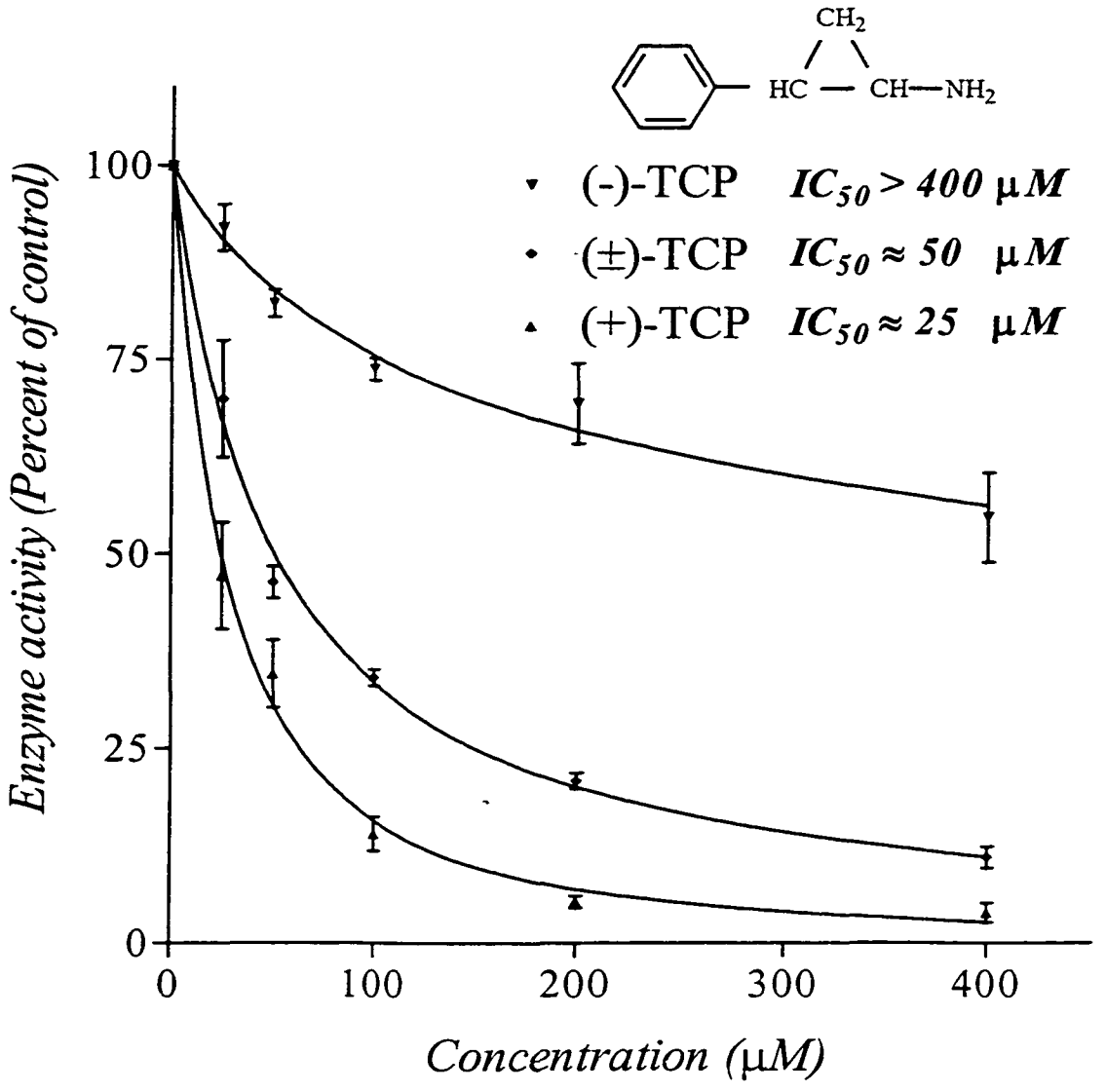


Figure 3.16 Comparison of the inhibitory effects of (+)-TCP and (-)-TCP on CYP2C19 activity in human liver microsomes. The results represent means ± SEM (n=3).

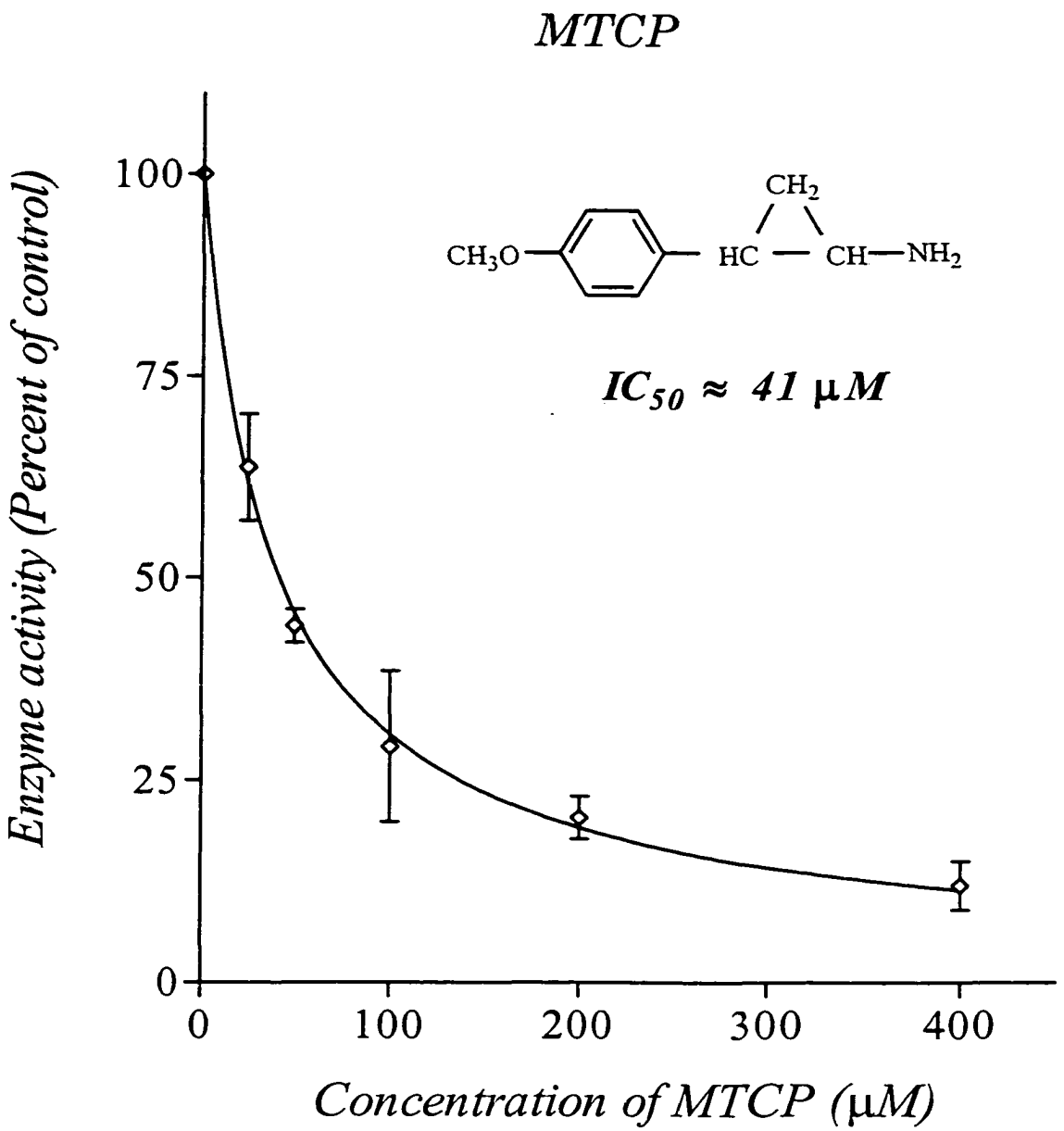


Figure 3.17 The inhibitory effect of MTCP on CYP2C19 activity in human liver microsomes. The results represent means ± SEM (n=3).

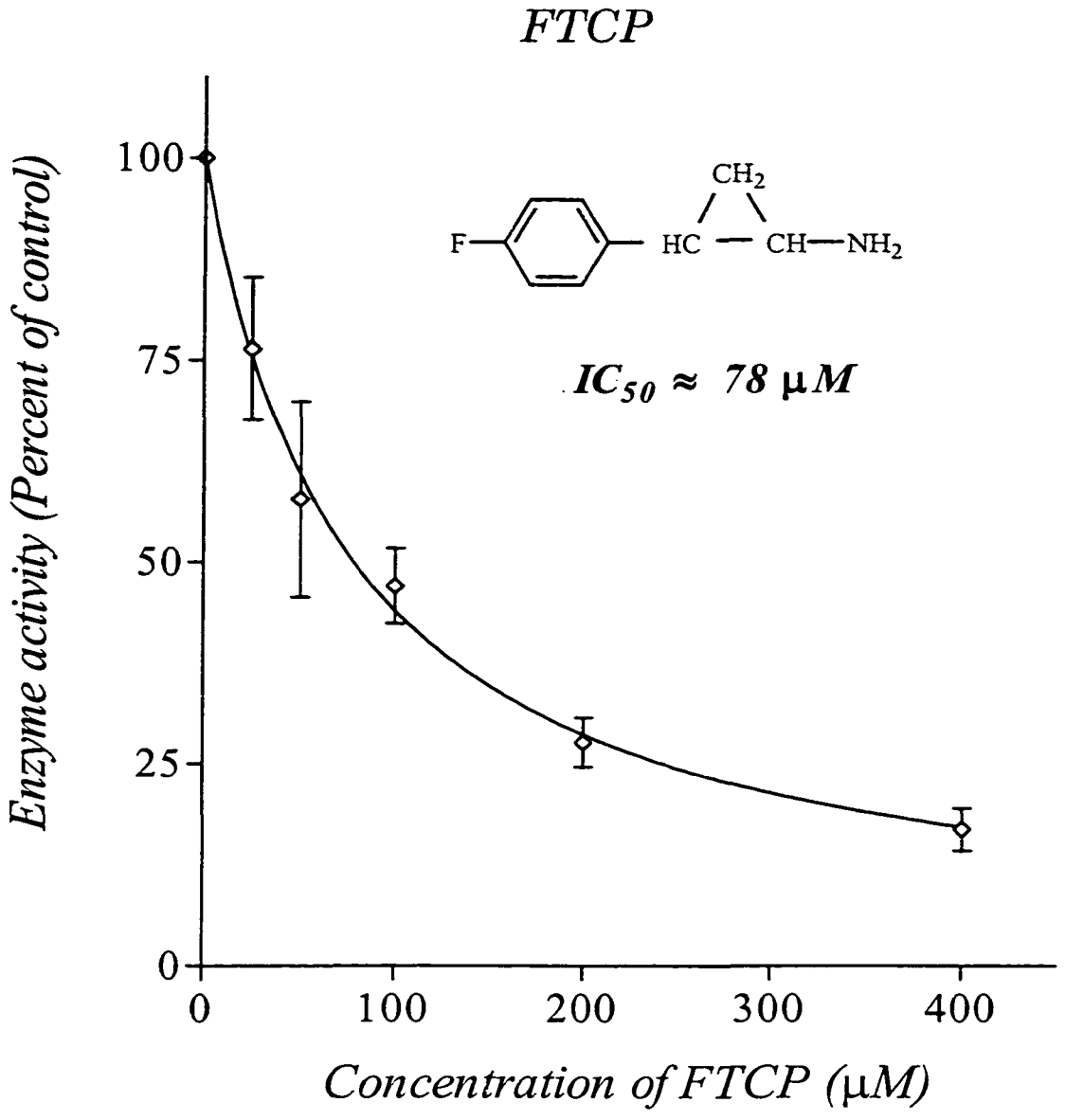


Figure 3.18 The inhibitory effect of FTCP on CYP2C19 activity in human liver microsomes. The results represent means ± SEM (n=3).

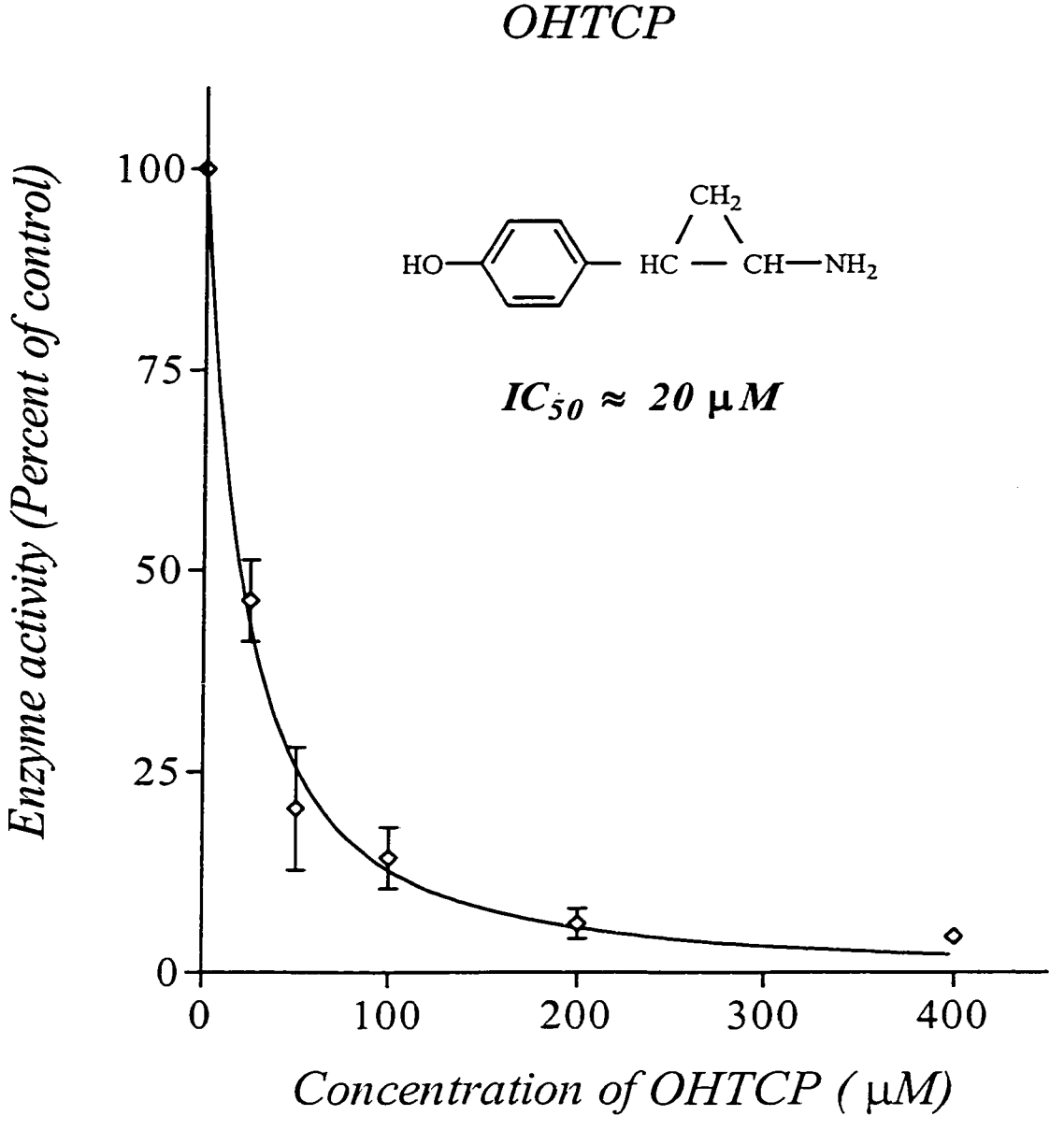


Figure 3.19 The inhibitory effect of OHTCP on CYP2C19 activity in human liver microsomes. The results represent means ± SEM (n=3).

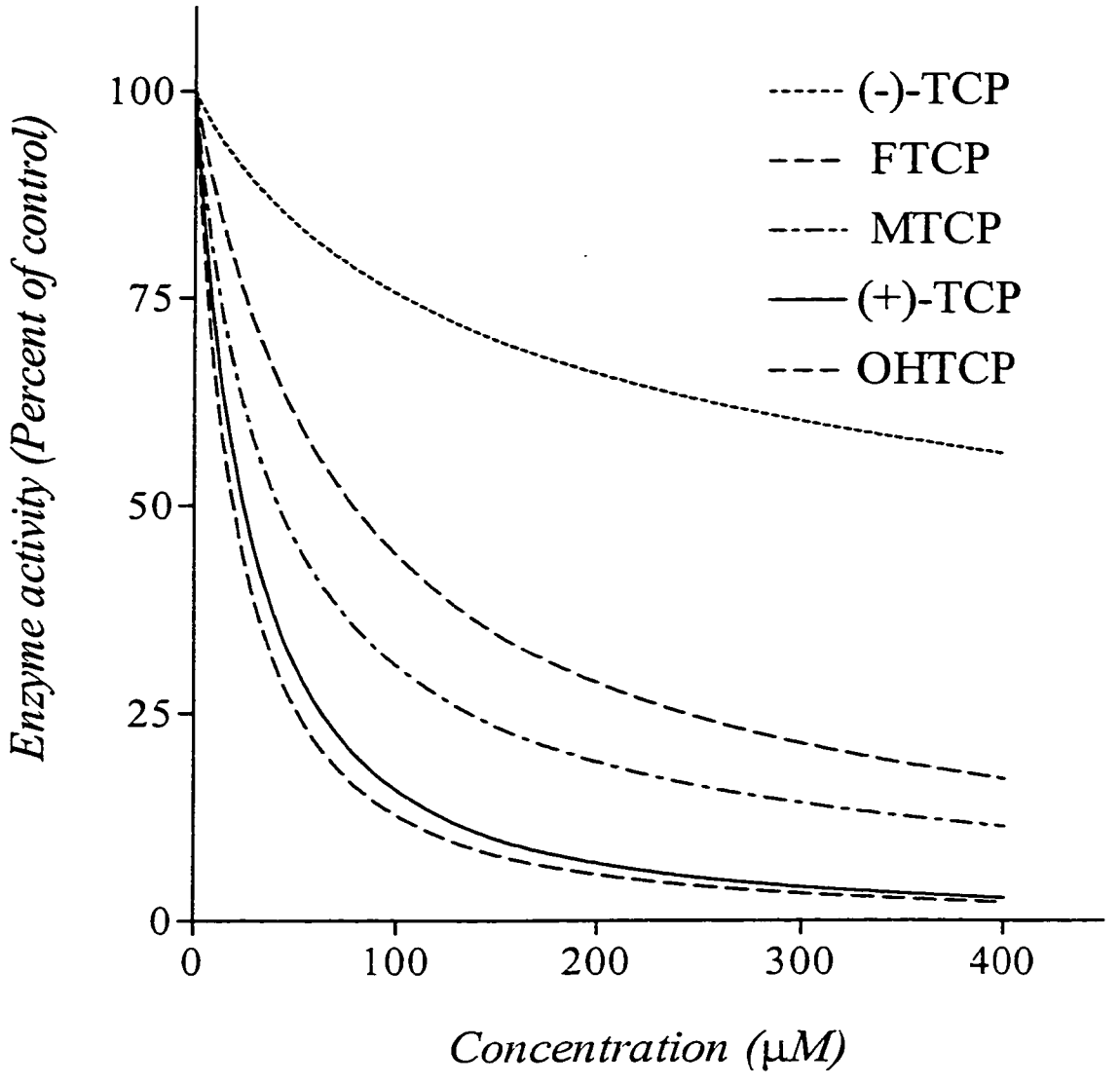


Figure 3.20 Comparison of the inhibitory effects of enantiomers of TCP and its ring substituted analogues.

3.9 Kinetics of Inhibitory Effects of TCP on CYP2C19

3.9.1 Preliminary Studies

3.9.1.1 Enzyme Concentration Study

The increase in substrate (MEP) turnover was approximately linear with increasing microsomal protein, as shown in Figure 3.21. When 5 μL of CYP2C19 (protein content of 11μg in 100 μL of incubation mixture) from a cDNA expressed cell line were added to the incubation mixture, the amount of metabolite produced was sufficient to be detectable by the developed HPLC method. Therefore, 5 μL of CYP2C19 were added to the incubation mixtures in subsequent microsomal studies.

3.9.1.2 Optimum Incubation Time

As shown in Figure 3.22, the production of OHMEP from MEP was linear in the first 40 minutes of the test for both lower (25μM) and higher (100μM) concentrations of MEP. Thus, for the rest of the studies the incubation time was set at 40 min.

3.9.1.3 Estimation of K_m and V_{max} Values

As illustrated in Figure 3.23, the plot of velocity of OHMEP production against MEP concentrations is a hyperbola that fits the one-enzyme Michaelis-Menten equation. The K_m and V_{max} values obtained from the Michaelis-Menten plot were 168 μM and 1.69 nmol/mg/min, respectively.

Similar K_m and V_{max} Values were obtained by plotting the data on a Hanes-Woolf plot (Figure 3.24).

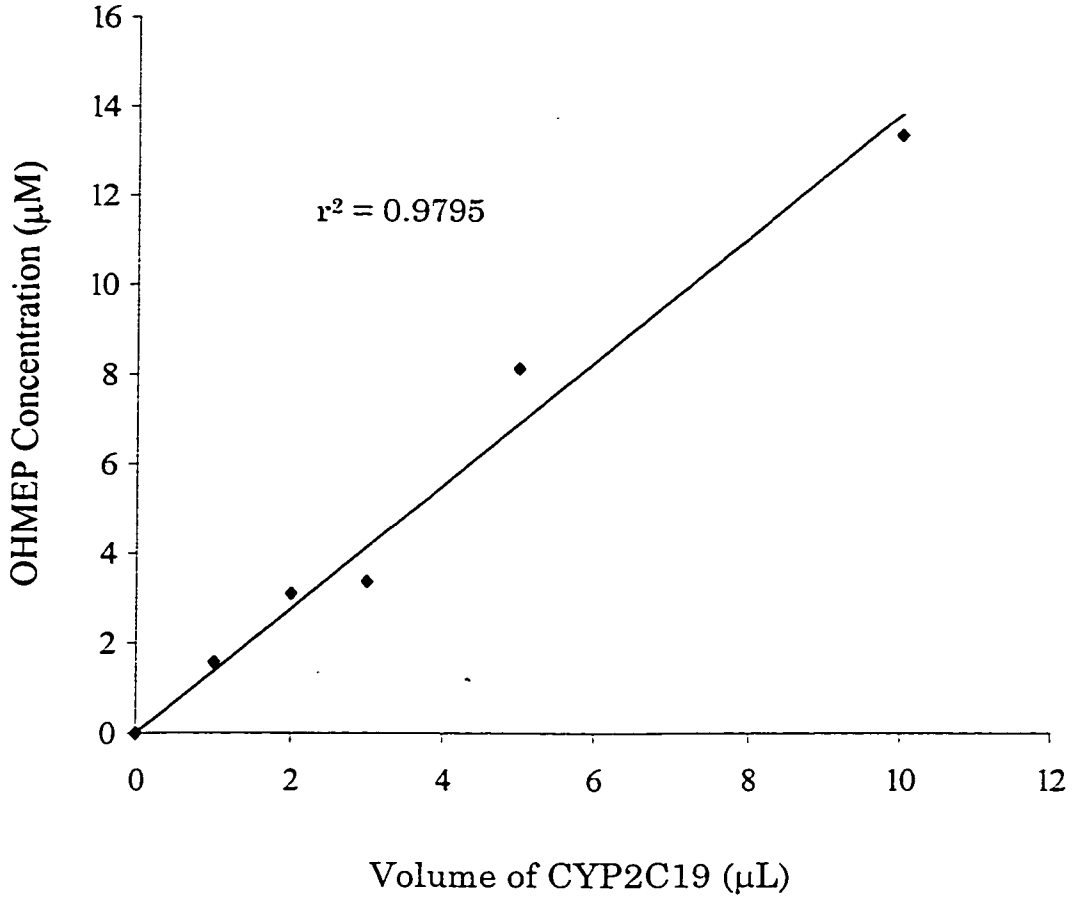


Figure 3.21 Production of OHMEP versus volume of CYP2C19 preparation (enzyme concentration).

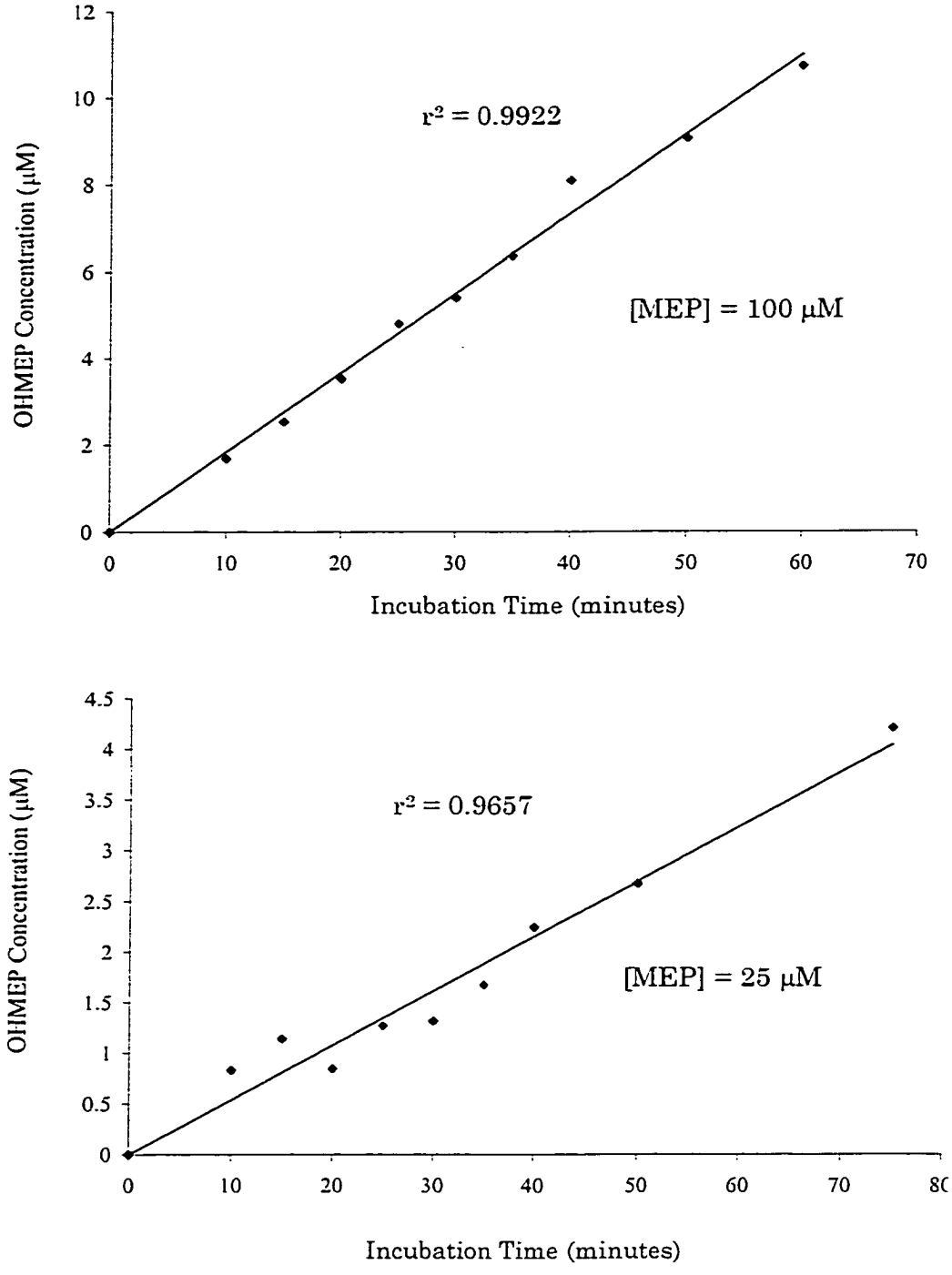


Figure 3.22 Effect of incubation time on the formation of OHMEP from MEP by CYP2C19.

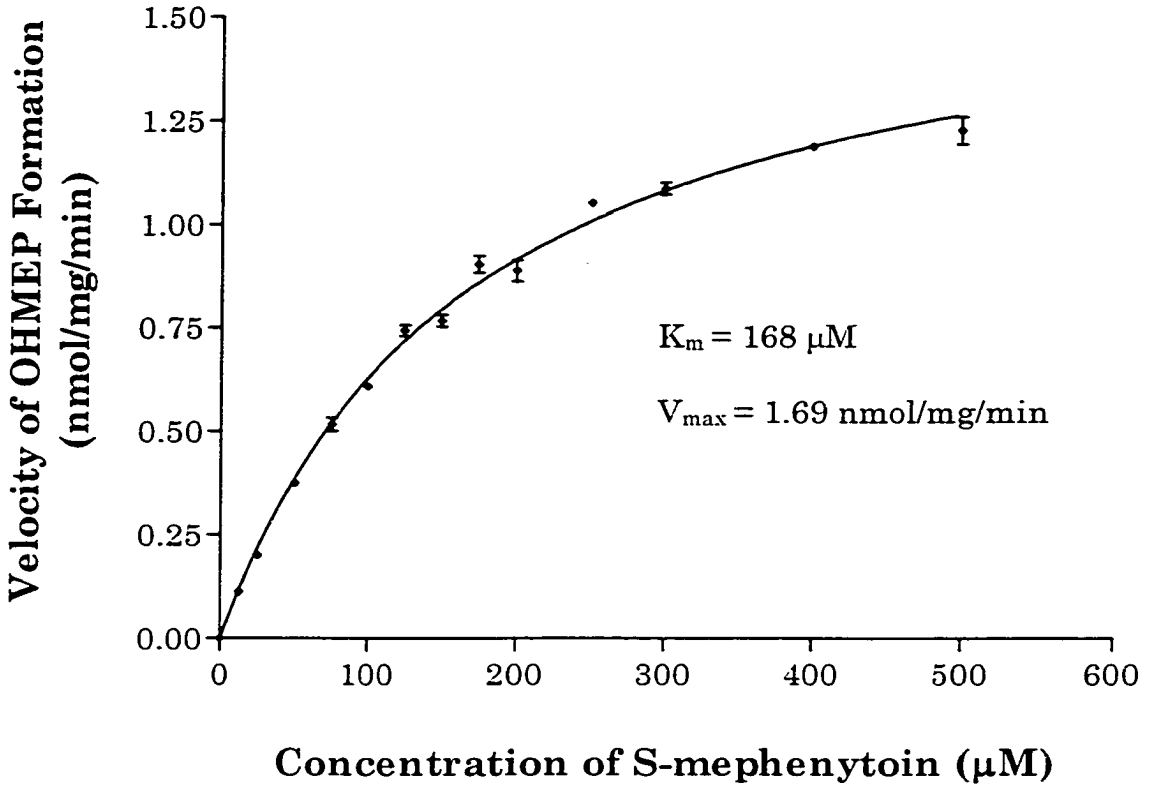


Figure 3.23 Michaelis-Menten plot of the velocity of OHMEP formation versus MEP concentration. Results represent means ± SEM (N=3).

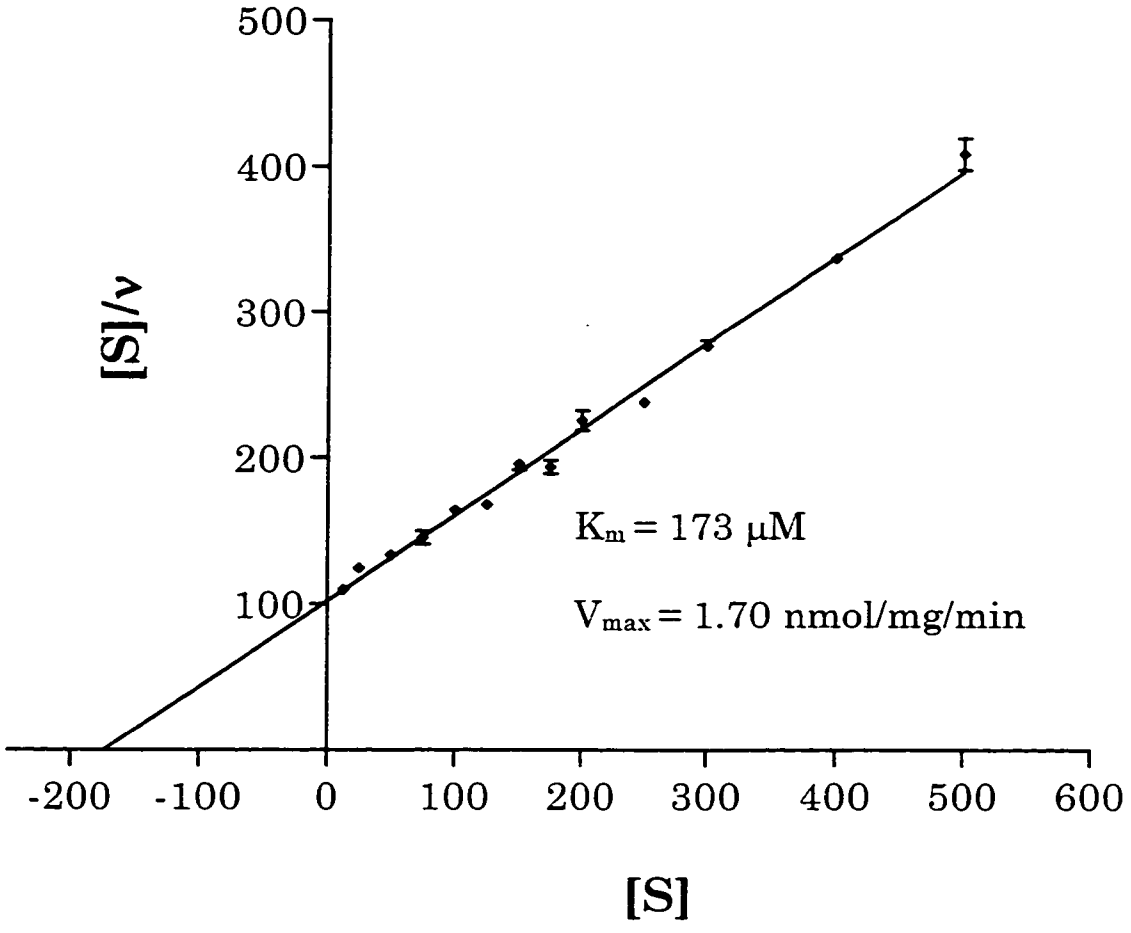


Figure 3.24 Graphical determination of kinetic constants for MEP metabolism by CYP2C19 using the Hanes-Wolf plot. Results represent means ± SEM (N=3).

3.9.1.4 Time-Dependence of Inhibition

Figure 3.25 shows the percent of CYP2C19 activity versus preincubation time of the drug with the enzyme. It can be seen that the inhibitory effect of TCP on CYP2C19 does not depend on the preincubation time.

3.9.1.5 Reversibility of Inhibition

As described in section 2.2, a dilution method has been used to evaluate the reversibility of inhibition. The result of this experiment showed that remaining enzyme activity (as a percent of control) was 89%, 99% and 57% for Groups A, B and C, respectively. It was observed that addition of buffer to Group A (the set of tubes containing the higher concentrations of TCP) after preincubation of TCP with CYP2C19 for a period of time resulted in a large recovery of enzyme activity. This suggested that TCP is not bound tightly to the enzyme, and it is concluded that the inhibitory effect of TCP on CYP2C19 is reversible.

3.9.2 Main Study

Figure 3.26 shows the effects of increasing concentrations of TCP on production of OHMEP from MEP in a Michaelis-Menten plot. The fully competitive inhibitory effects of TCP on CYP2C19 can be seen clearly in a Lineweaver-Burk plot (Figure 3.27) and in a Hanes-Woolf plot (Figure 3.28).

The values of K_m and V_{max} obtained from Hanes-Woolf plot, and the inhibitor constant (K_i) for TCP has been determined from a replot of K_m/V_{max} against TCP concentration (Figure 3.29). The value of x-intercept in this plot is equal to $-K_i$, resulting in $K_i = 32 \pm 6$ μM.

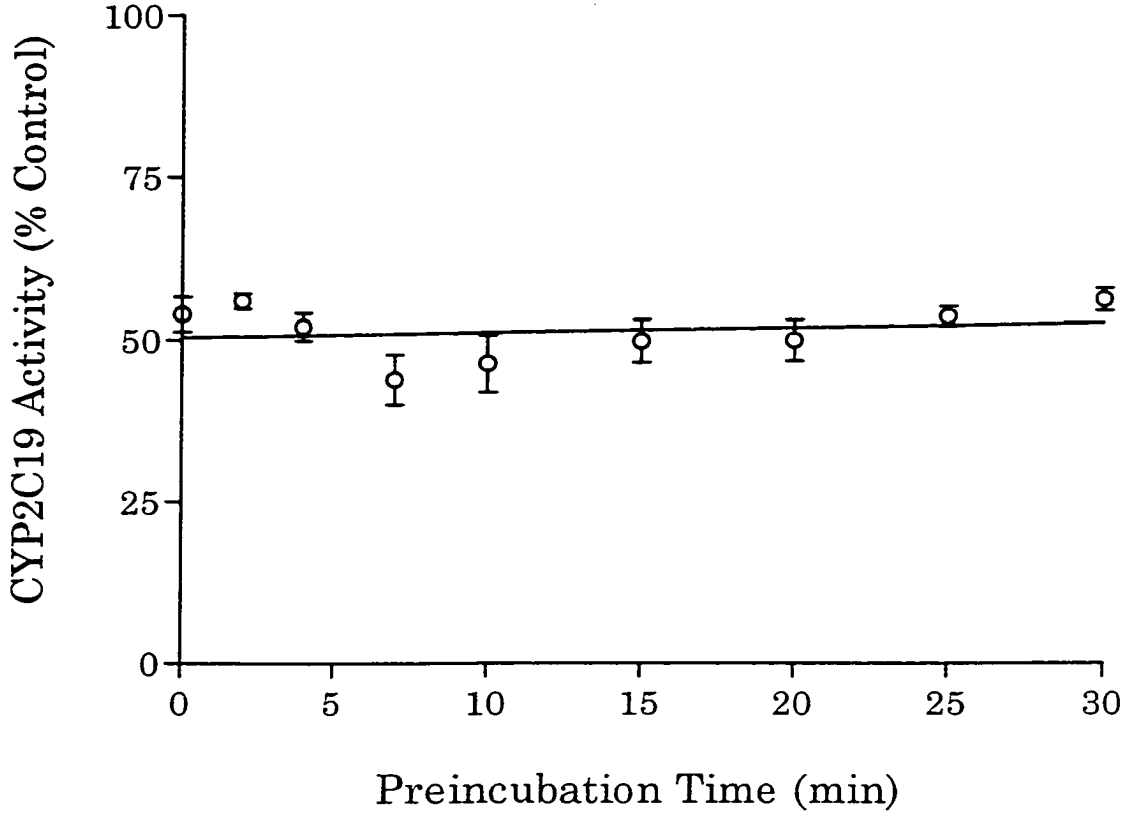


Figure 3.25 The effect of preincubation time on the inhibition of CYP2C19 activity by TCP. Results represent means ± SEM (N=3).

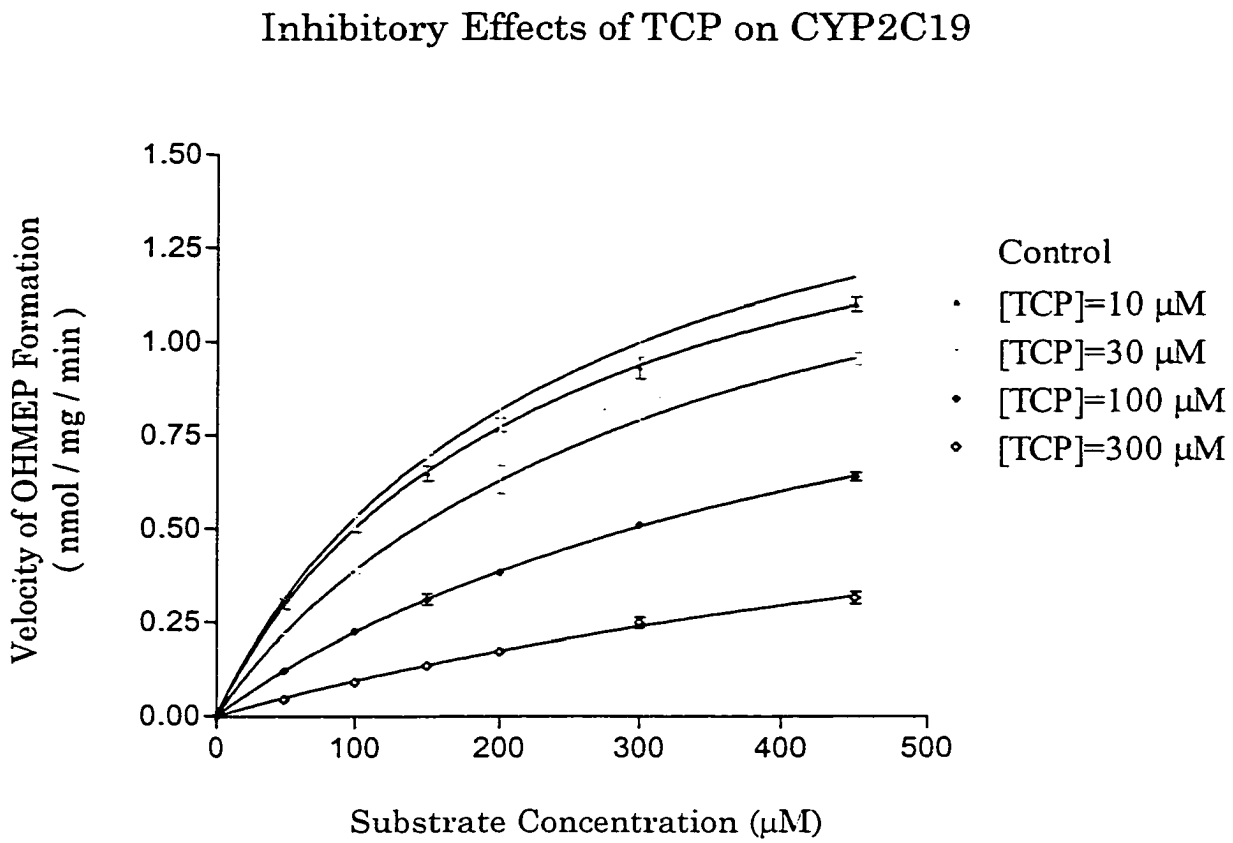


Figure 3.26 Michaelis-Menten plot showing the effect of increasing concentration of TCP on production of OHMEP from MEP. Results represent means ± SEM (N=3).

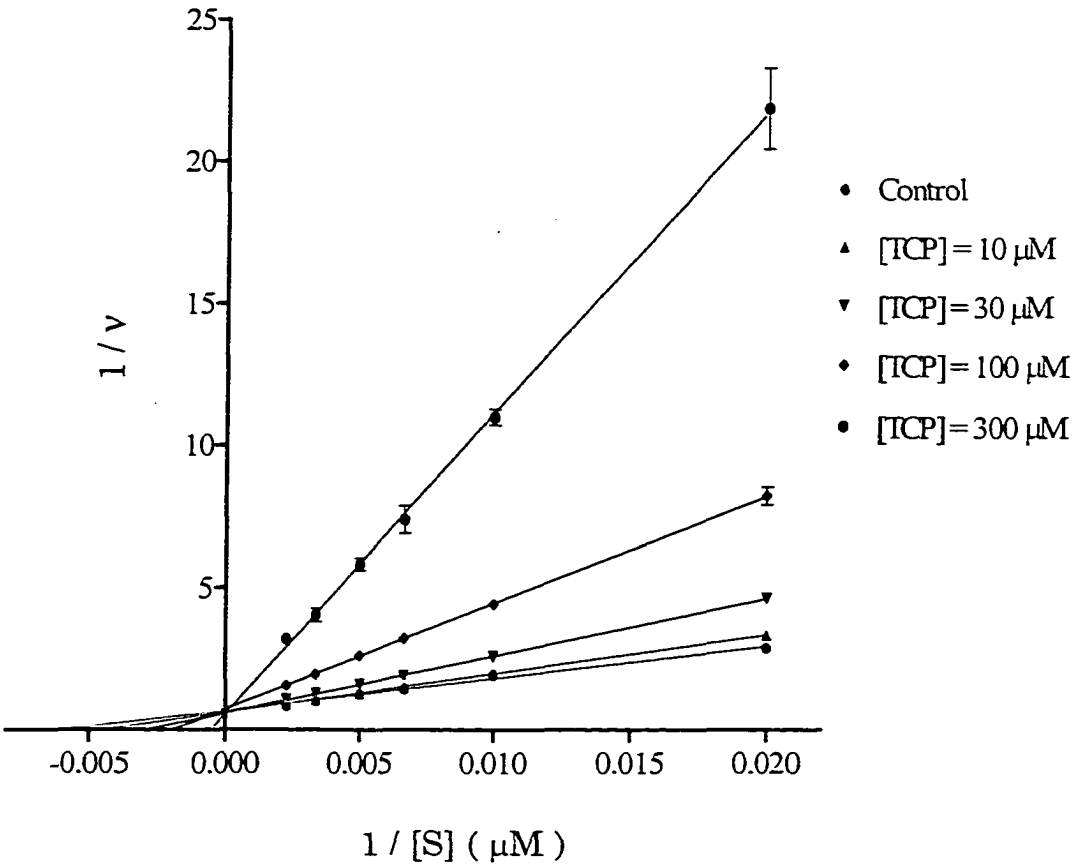


Figure 3.27 Lineweaver-Burk plot showing the competitive inhibitory effects of TCP on CYP2C19. Results represent means ± SEM (N=3).

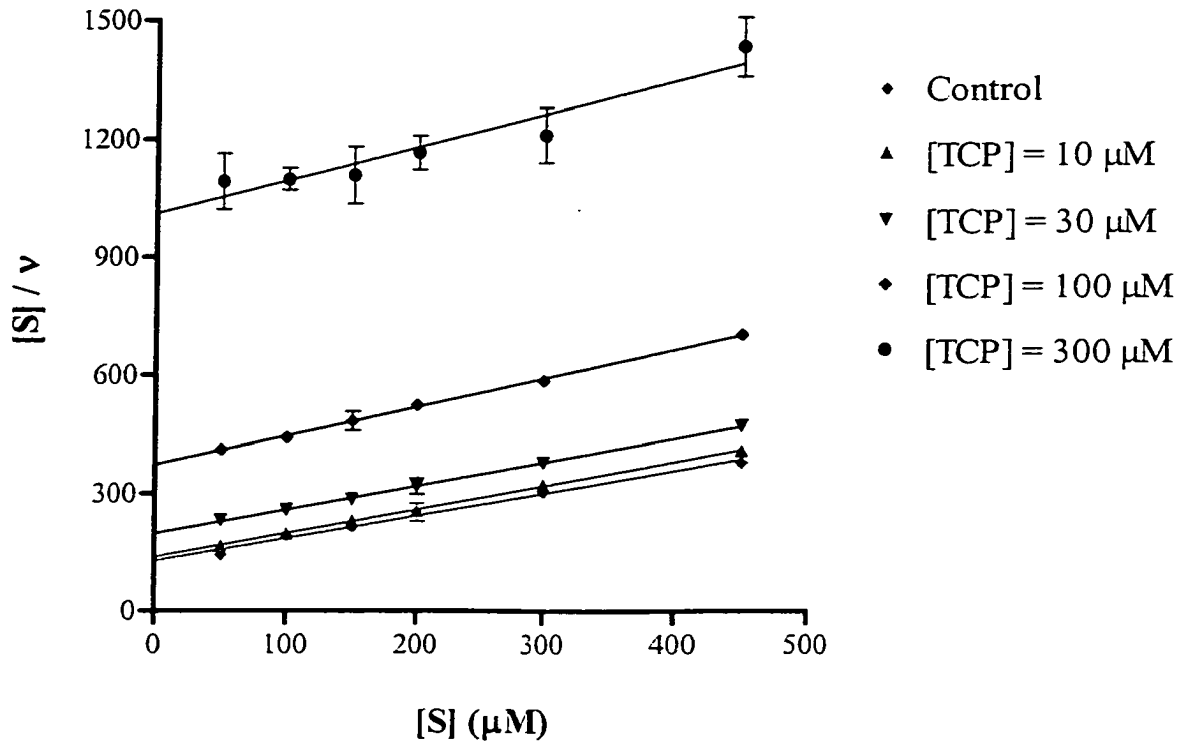


Figure 3.28 Hanes-Woolf plot showing the competitive inhibitory effects of TCP on CYP2C19. Results represent means ± SEM (N=3).

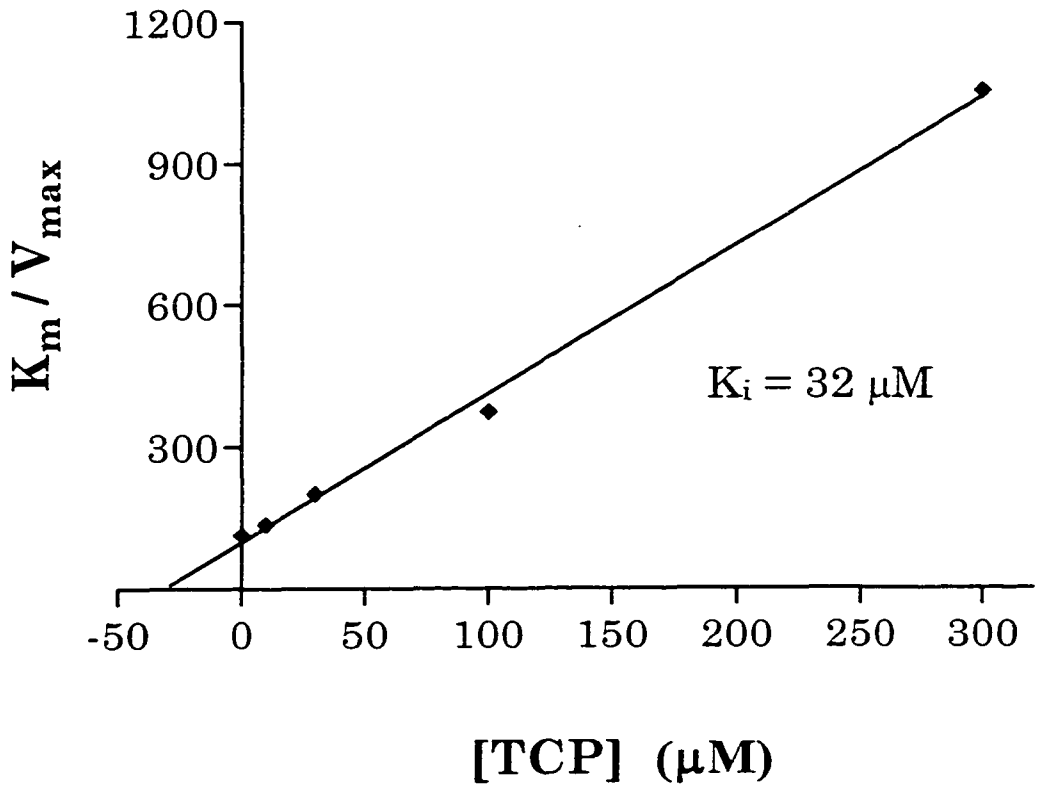


Figure 3.29 Graphical determination of inhibitory constant (K_i) of TCP *versus* CYP2C19.

3.10 Kinetics of Inhibitory Effects of PLZ on CYP2C19

3.10.1 Preliminary Studies

3.10.1.1 Time-Dependency of Inhibition

As demonstrated in Figure 3.30, the percent of CYP2C19 activity does not change with respect to the time PLZ is preincubated with the enzyme. Therefore, the inhibitory effect of PLZ on CYP2C19 is not time-dependent.

3.10.1.2 Reversibility of Inhibition

The results of dilution study for evaluation of reversibility of inhibition showed that the remaining enzyme activities (as a percent of control) were 42%, 46%, and 8% for Groups A, B and C, respectively. It was observed that addition of buffer to Group A (the set of tubes containing the higher concentrations of PLZ) after preincubation of PLZ with CYP2C19 for a period of time resulted in large recovery of enzyme activity. This indicates that PLZ is not bound tightly to the enzyme. Hence, the inhibitory effect of PLZ on CYP2C19 is reversible.

3.10.2 Main Study

Figure 3.31 shows the effects of increasing concentrations of PLZ on production of OHMEP from MEP in a Michaelis-Menten plot. The fully non-competitive inhibitory effects of PLZ on CYP2C19 can be seen clearly in a Lineweaver-Burk plot (Figure 3.32) and in a Hanes-Woolf plot (Figure 3.33).

The inhibitor constant for PLZ has been determined from a replot of K_m/V_{max} against the PLZ concentration, and its value is 0.7 ± 0.3 μM (Figure 3.34).

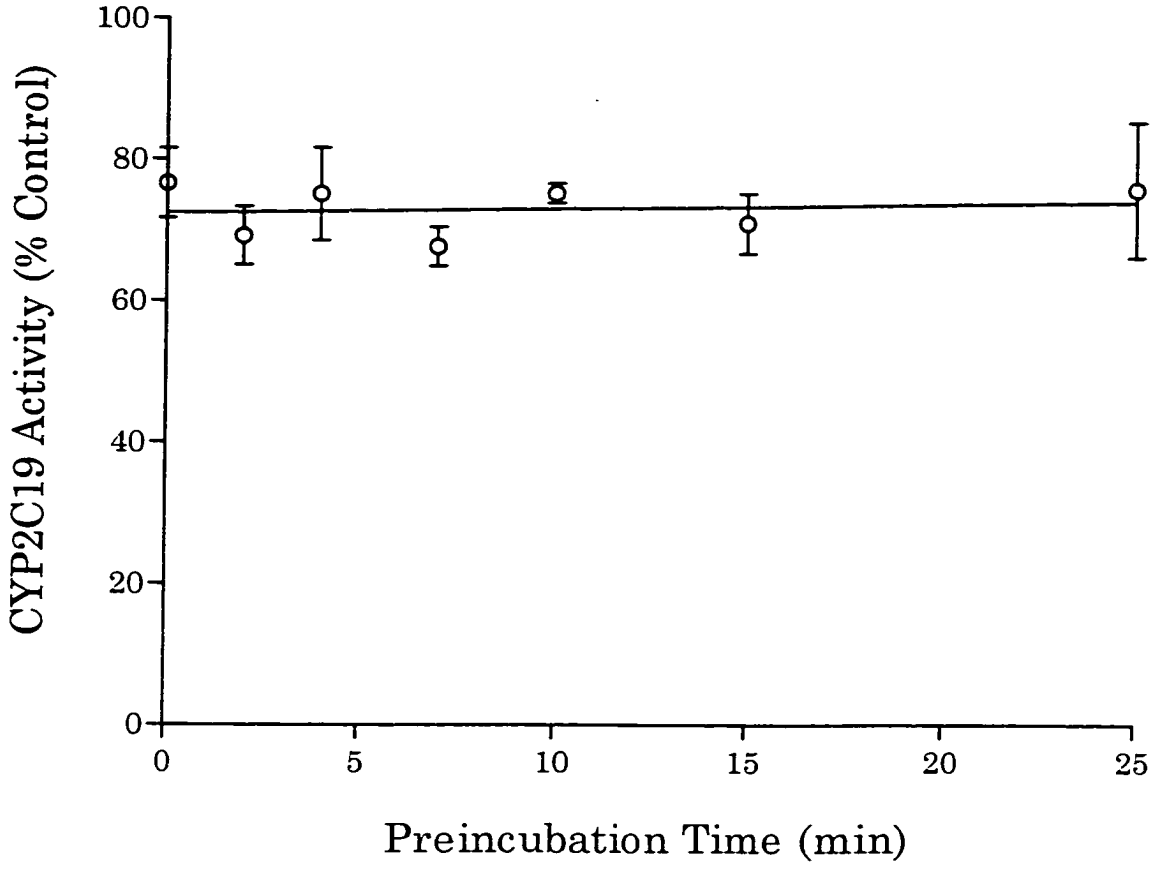


Figure 3.30 The effects of pre-incubation time on inhibition of CYP2C19 activity by PLZ. Results represent means ± SEM (N=3).

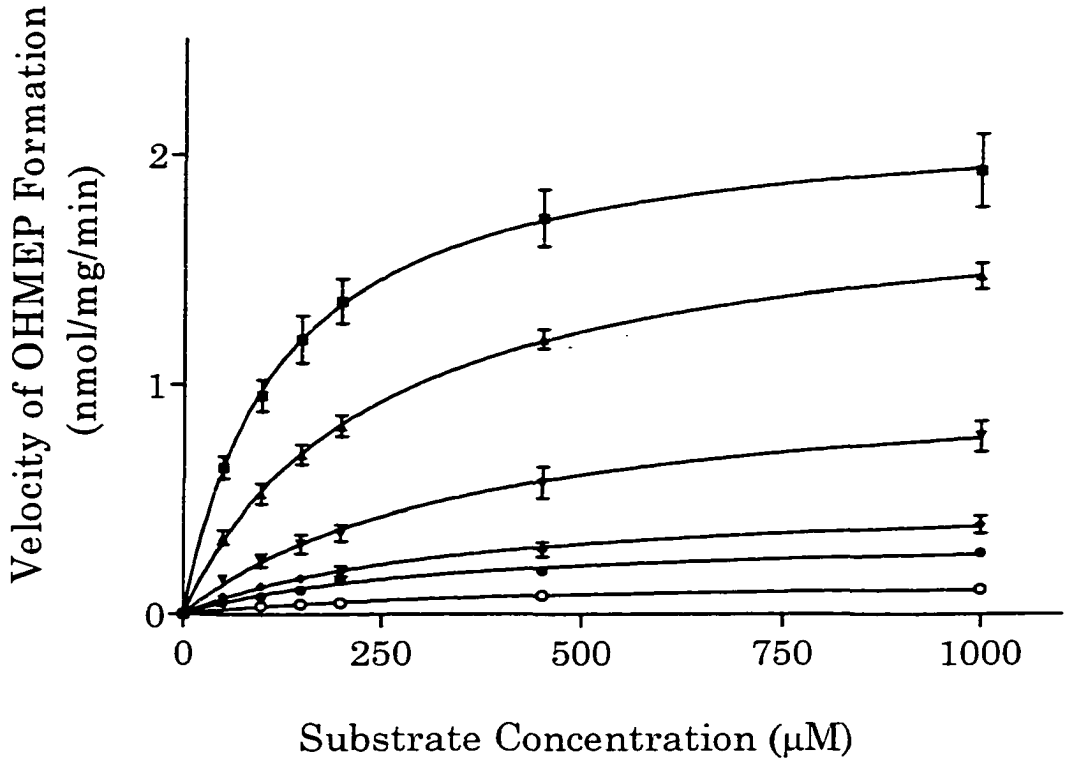


Figure 3.31 Michaelis-Menten plot showing the effect of increasing concentration of PLZ on production of OHMEP from MEP by CYP2C19. Results represent means ± SEM (N=3).

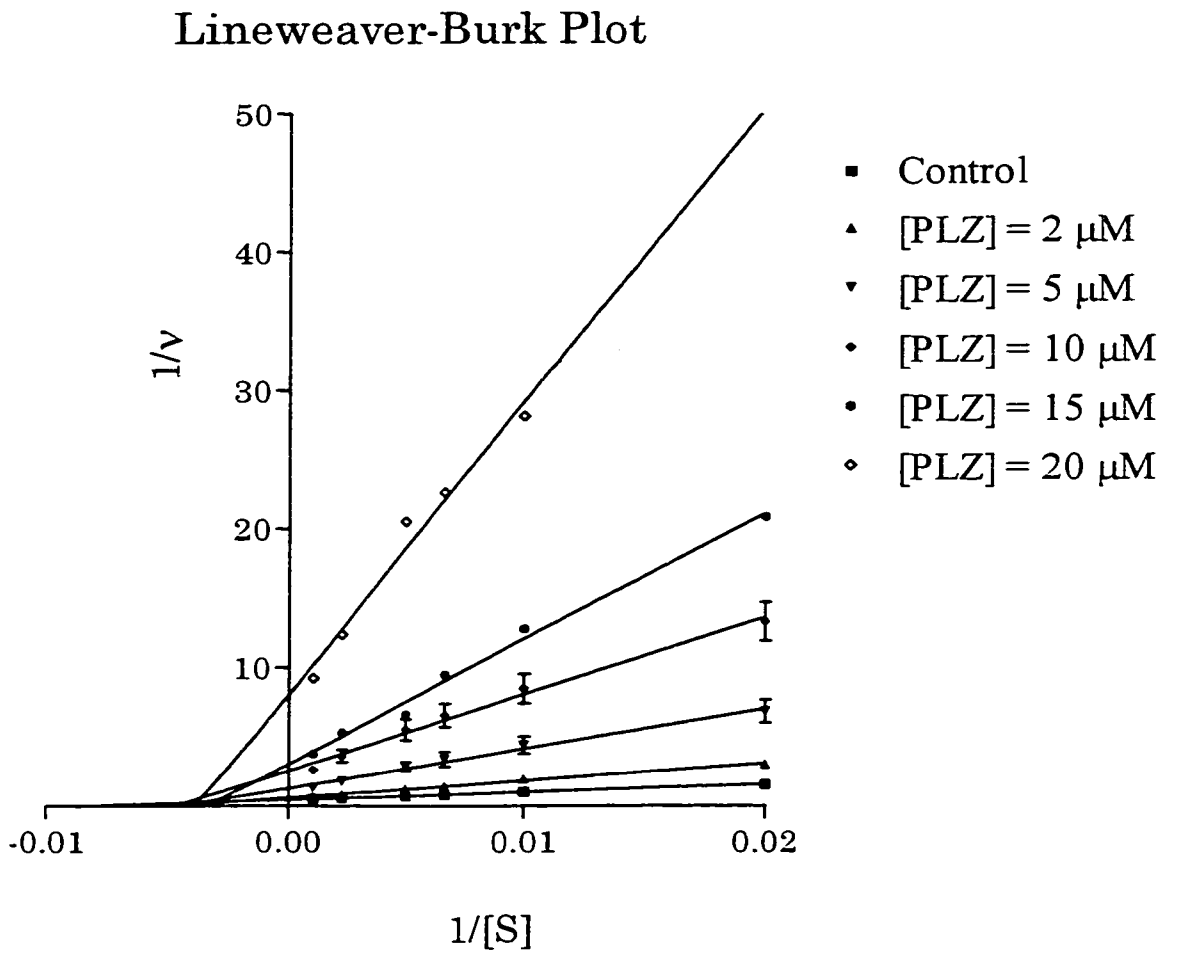


Figure 3.32 Lineweaver-Burk plot showing the non-competitive inhibitory effects of PLZ on CYP2C19. Results represent means ± SEM (N=3).

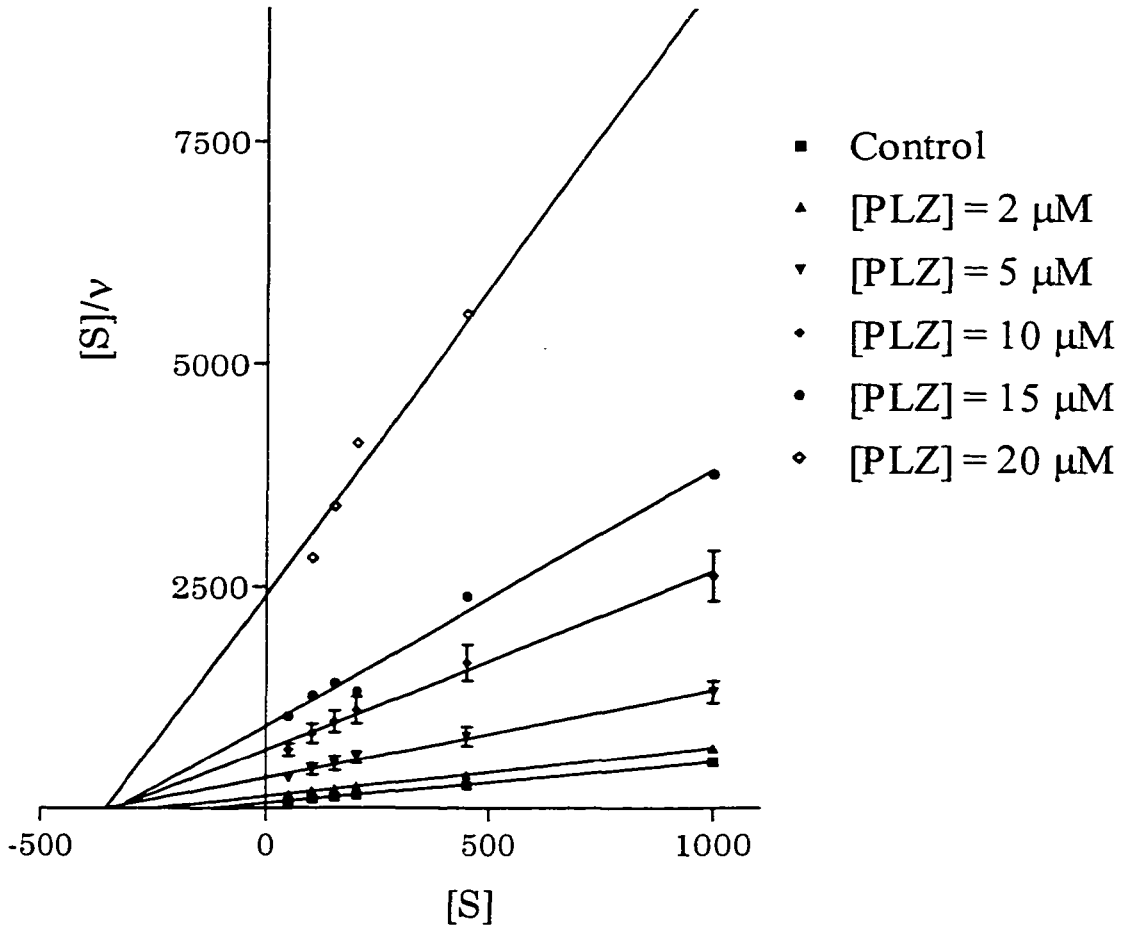


Figure 3.33 Hanes-Woolf plot showing the non-competitive inhibitory effects of PLZ on CYP2C19. Results represent means ± SEM (N=3).

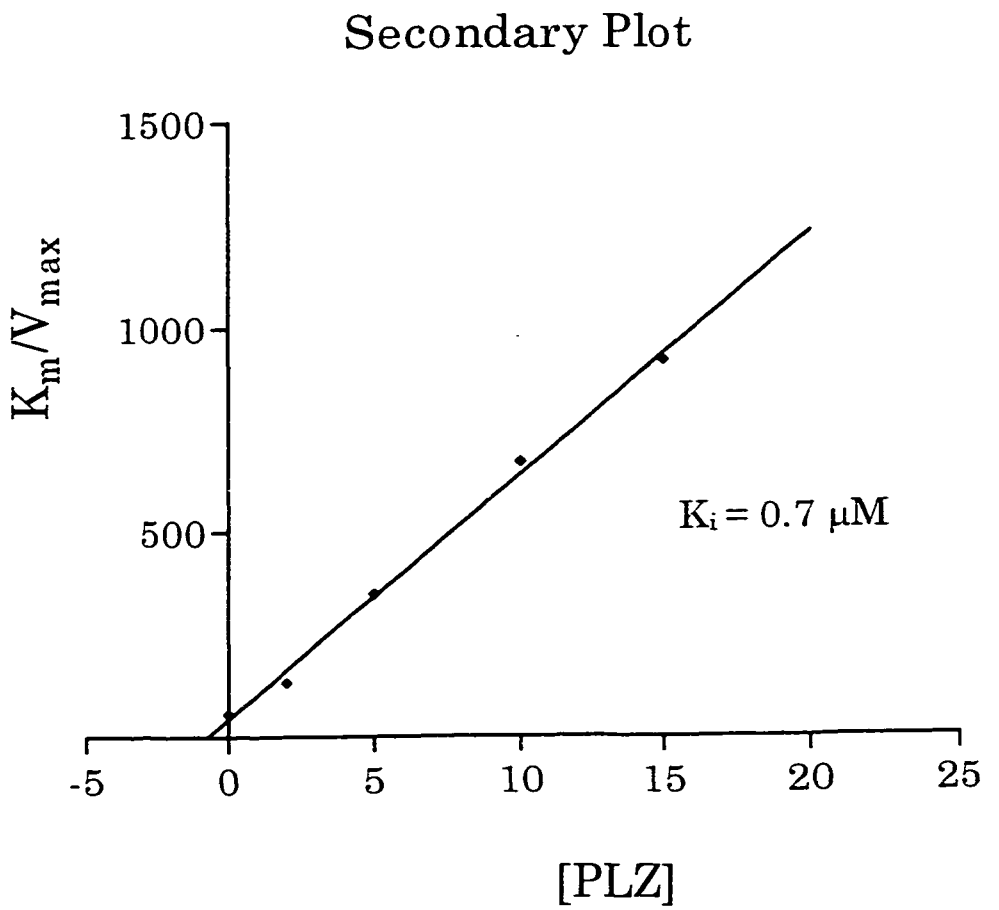


Figure 3.34 Graphical determination of inhibitory constant (K_i) of PLZ *versus* CYP2C19.

3.11 Kinetics of Inhibitory Effects of TCP on CYP2C9

3.11.1 Preliminary Studies

3.11.1.1 Enzyme Concentration Study

The increase in TOL metabolism to OHTOL was approximately linear with increasing microsomal protein, as shown in Figure 3.35. With 10 μL of CYP2C9 preparation (protein content of 21 μg in 100 μL of incubation mixture) from a cDNA expressed cell line added to incubation mixture, the produced metabolite in the presence of inhibitor was detectable by the developed HPLC method. Consequently, in subsequent microsomal studies, 10μL of CYP2C9 were added to the incubation mixtures.

3.11.1.2 Optimum Incubation Time

As shown in Figure 3.36, the production of OHTOL from TOL was approximately linear for the first 60 minutes of the test for both lower (50μM) and higher (200μM) concentrations of TOL. Thus, for the rest of studies the incubation time was set at 60 min.

3.11.1.3 Estimation of K_m and V_{max} Values

The plot of velocity of OHTOL production versus TOL concentrations is a hyperbola that fits the one-enzyme Michaelis-Menten equation (Figure 3.37). The K_m and V_{max} values obtained from the Michaelis-Menten plot were 65μM and 1.32 nmol/mg/min, respectively. It was observed that at higher

concentrations of TOL, the initial velocity is less than the maximum value. It seems that the substrate, in very high concentrations, can inhibit its own conversion to metabolite (Figure 3.38).

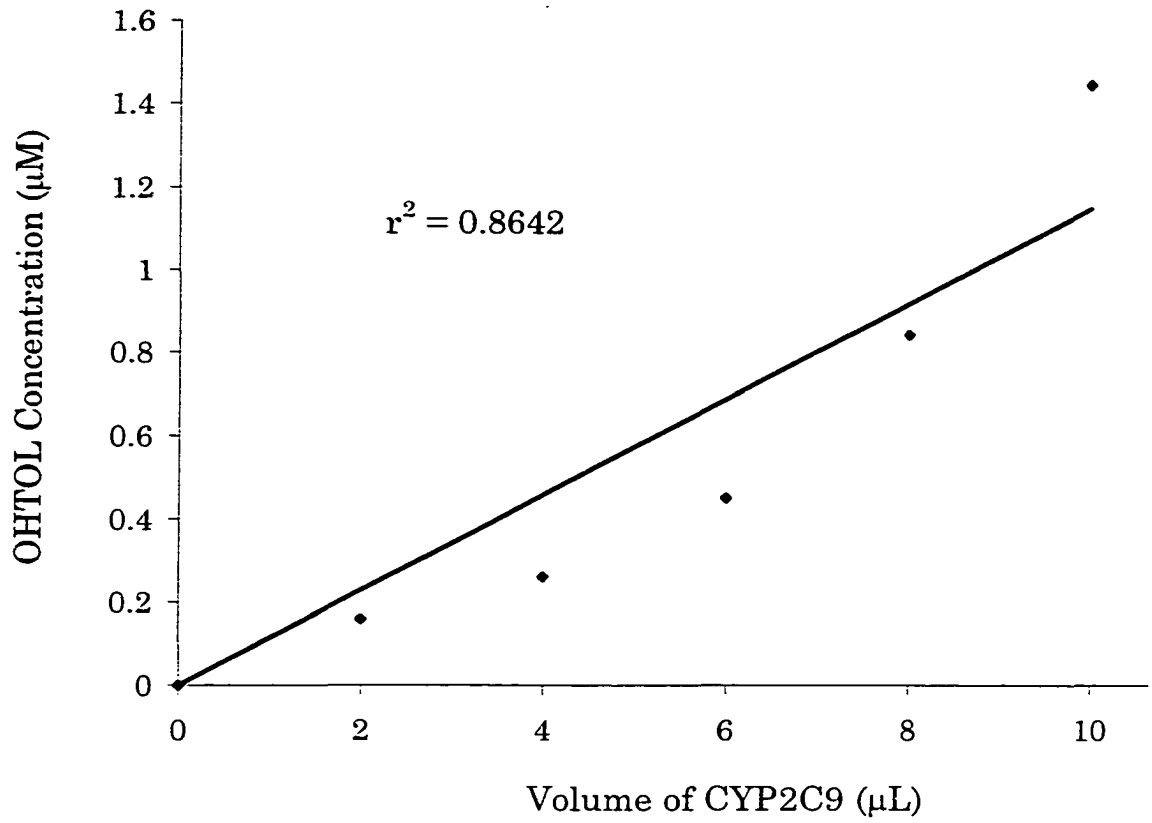


Figure 3.35 Production of OHTOL *versus* volume of CYP2C9 preparation (enzyme concentration).

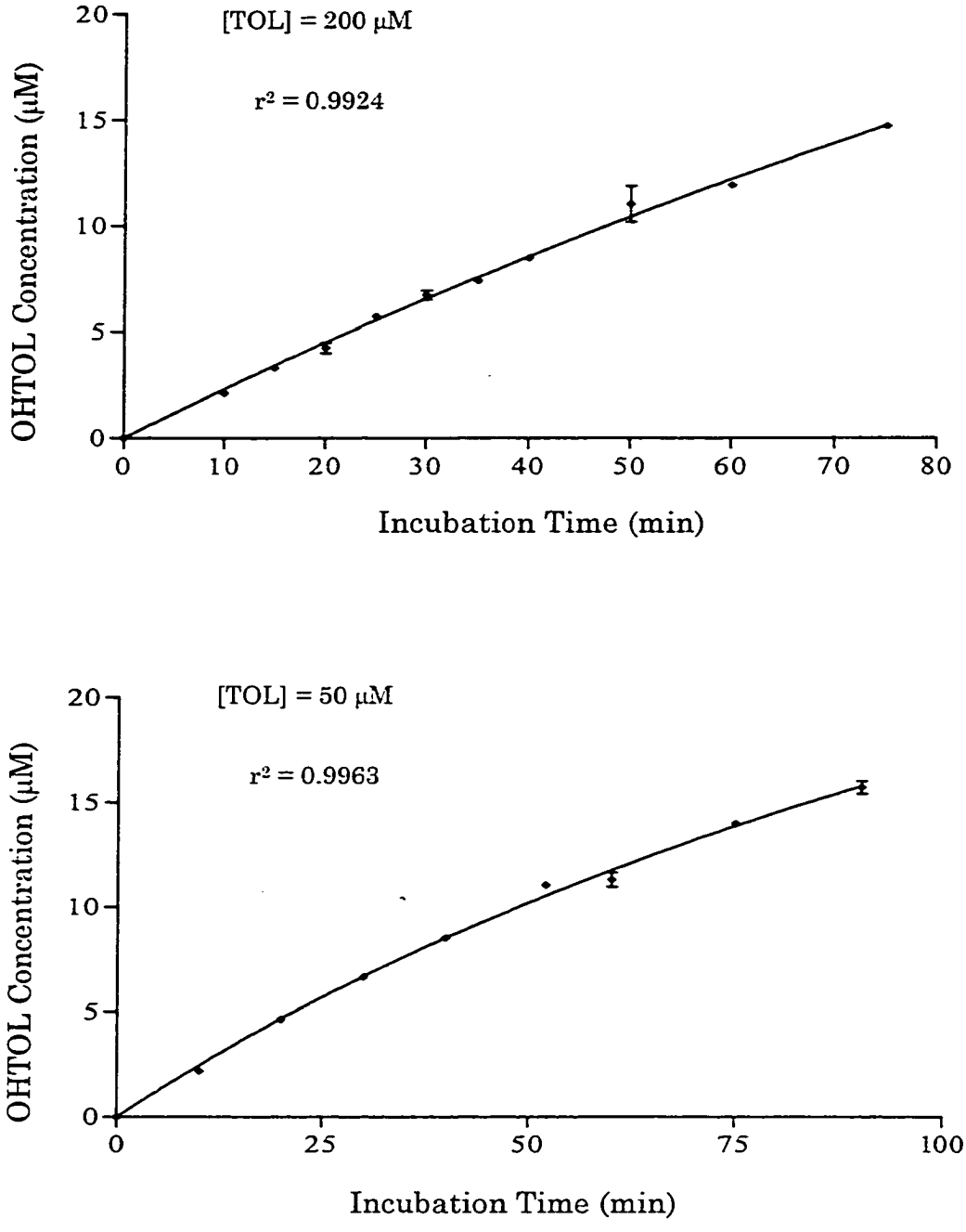


Figure 3.36 Effect of incubation time on the formation of OHTOL from TOL.

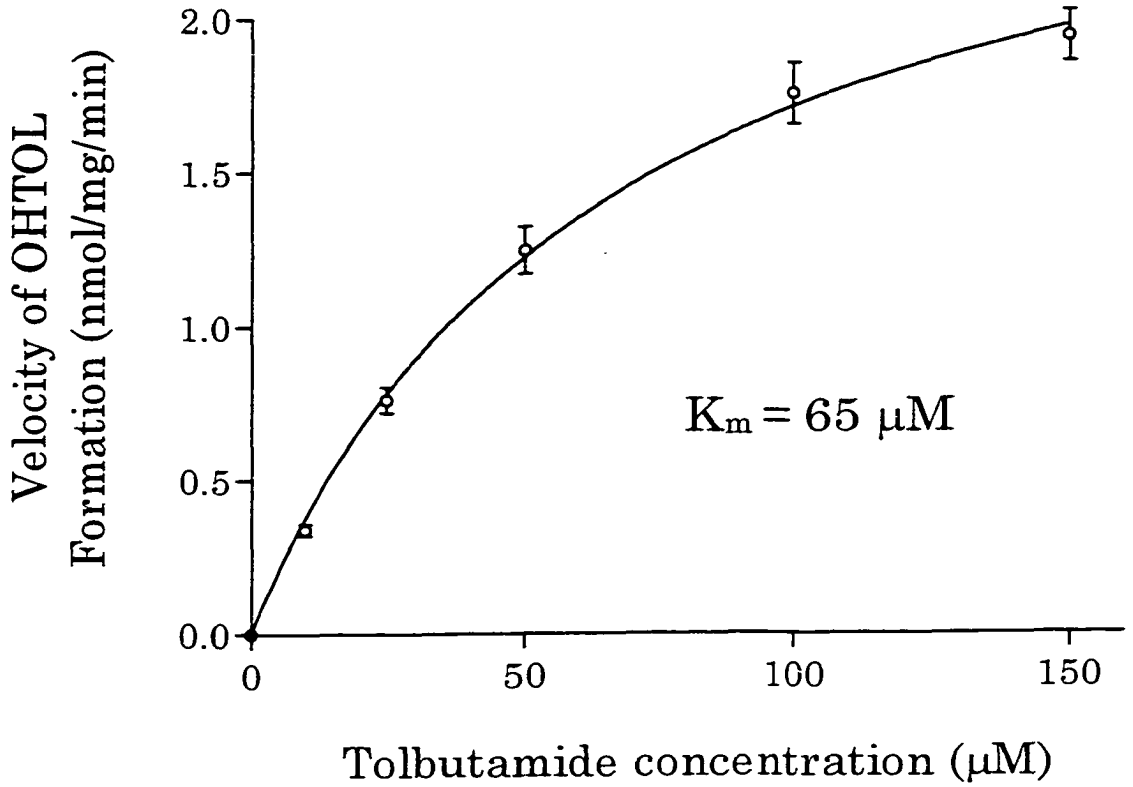


Figure 3.37 Michaelis-Menten plot of the velocity of OHTOL formation versus TOL concentration. Results represent means ± SEM (N=3).

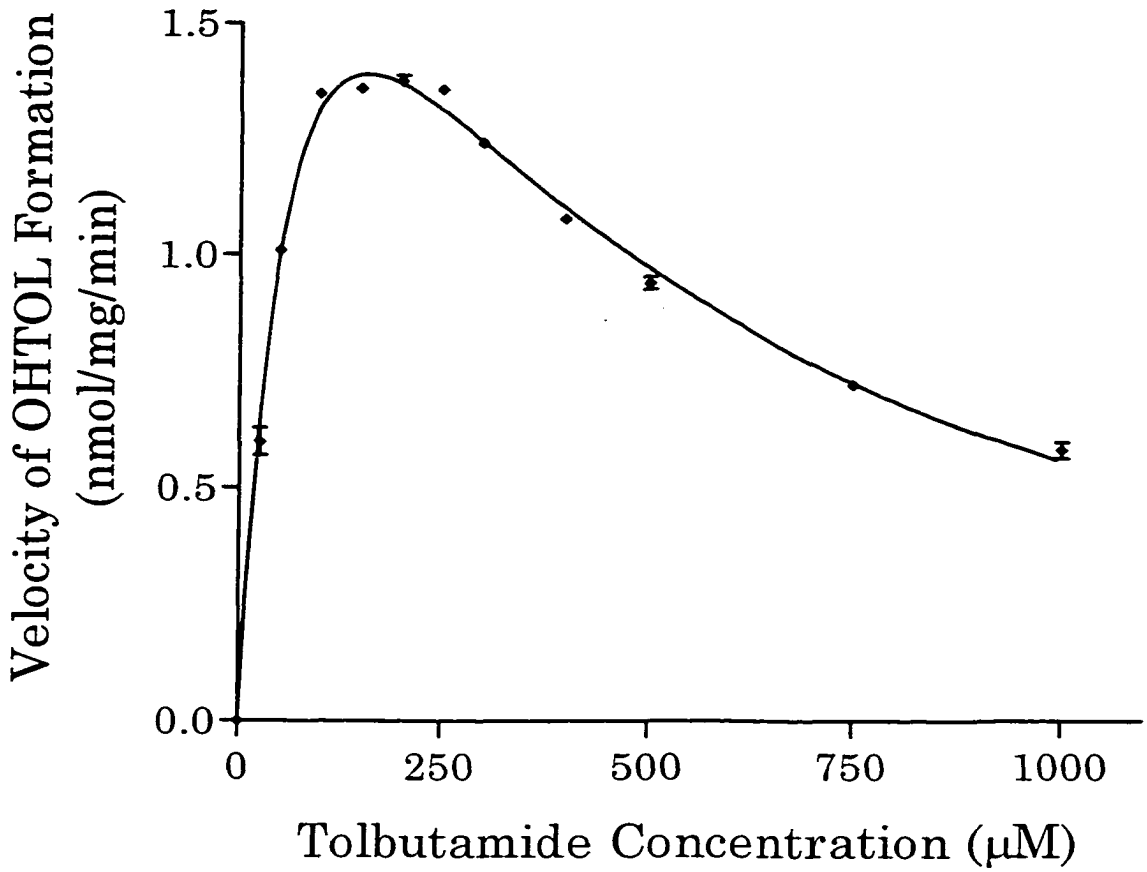


Figure 3.38 The velocity of OHTOL formation versus TOL concentration showing the inhibitory effects of substrate (TOL) at high concentrations. Results represent means ± SEM (N=3).

3.11.1.4 Time-Dependence of Inhibition

Figure 3.39 shows the percent of CYP2C9 activity in relation to preincubation time with TCP; the loss of CYP2C9 activity in the presence of TCP does not change with the time of preincubation, and thus the inhibitory effect of TCP on CYP2C9 is not time-dependent.

3.11.1.5 Reversibility versus Irreversibility of Inhibition

The remaining enzyme activities (as a percent of control) for Groups A, B and C were respectively 65%, 72%, and 26%, showing that the inhibitory effect of TCP on CYP2C9 is reversible. As discussed earlier, after a preincubation period, the percent of enzyme inhibition produced as a result of incubation of enzyme with TCP was decreased by dilution, implying the reversible nature of TCP binding to CYP2C9 enzymes.

3.11.2 Main Study

Figure 3.40 shows the effects of increasing concentrations of TCP on production of OHTOL from TOL in a Michaelis-Menten plot. The fully non-competitive inhibitory effects of TCP on CYP2C9 can be seen clearly in a Lineweaver-Burk plot (Figure 3.41) and in a Hanes-Woolf plot (Figure 3.42). The inhibitor constant for TCP has been determined from a replot of K_m/V_{max} against the TCP concentration, and is 56 ± 2 μM (Figure 3.43).

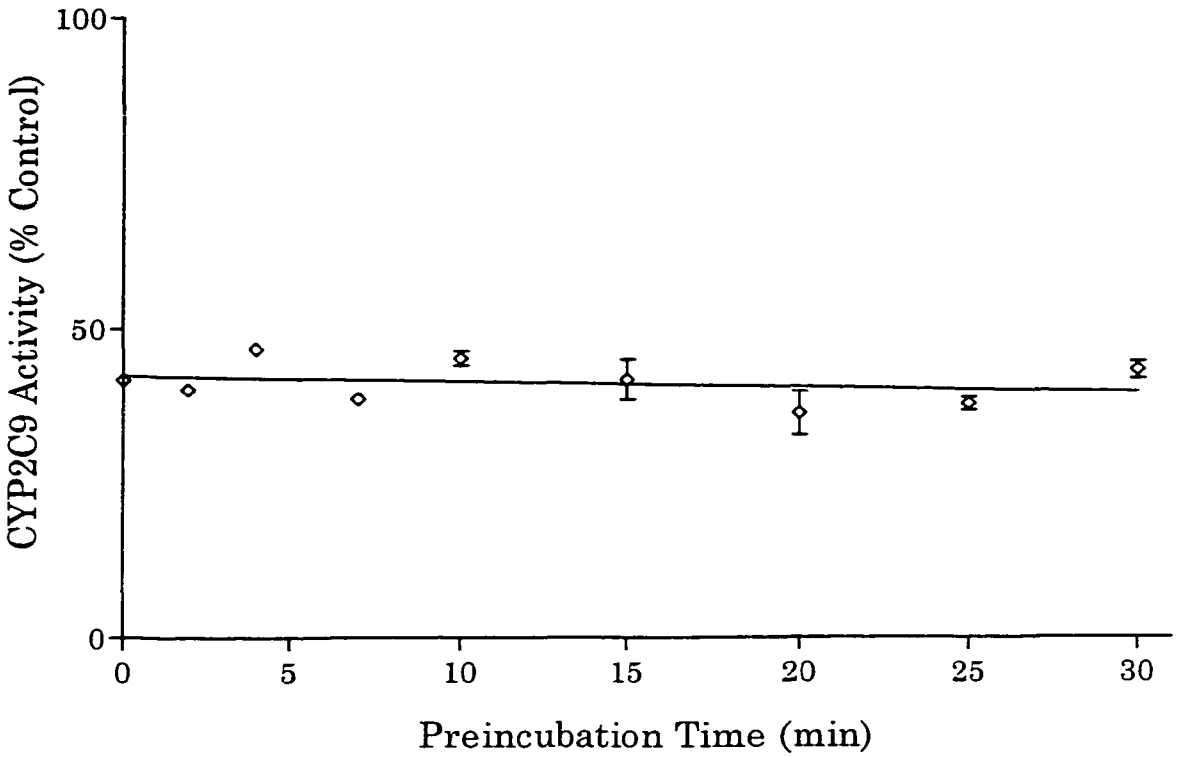
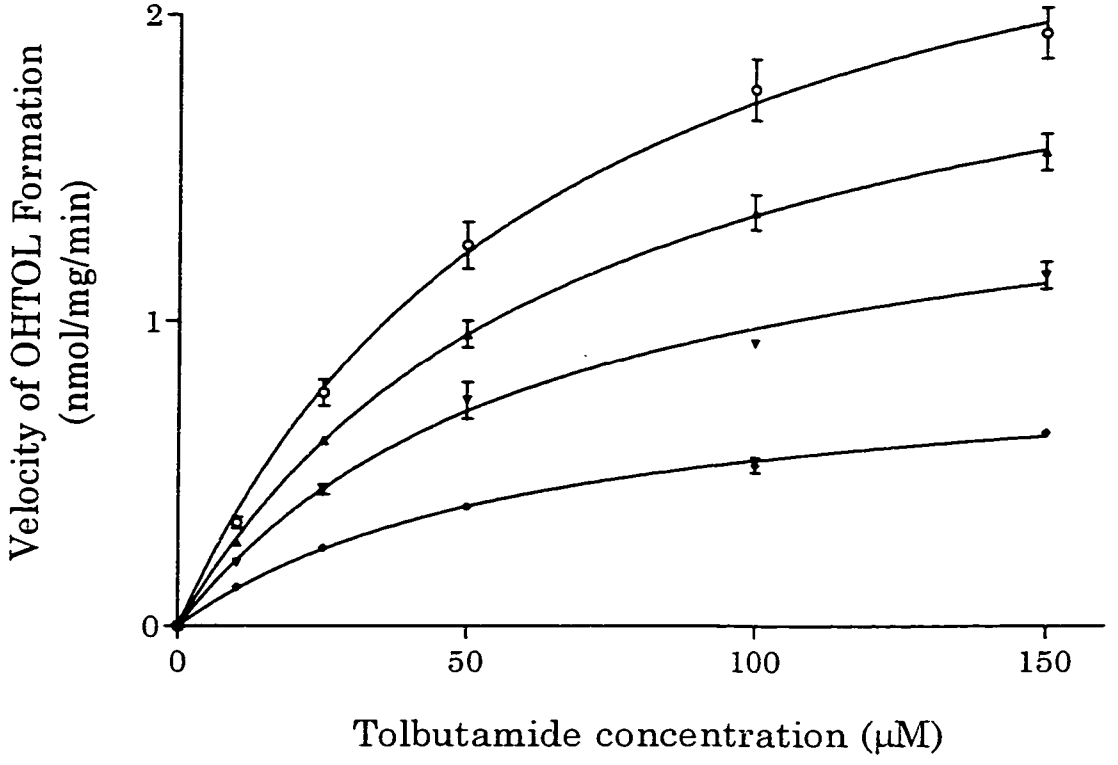


Figure 3.39 The effects of preincubation time on inhibition of CYP2C9 activity by TCP. Results represent means ± SEM (N=3).



- ○ Control
- ▲ [TCP] = 10 μM
- ▼ [TCP] = 30 μM
- ◆ [TCP] = 100 μM

Figure 3.40 Michaelis-Menten plot showing the effect of increasing concentration of TCP on production of OHTOL from TOL. Results represent means ± SEM (N=3).

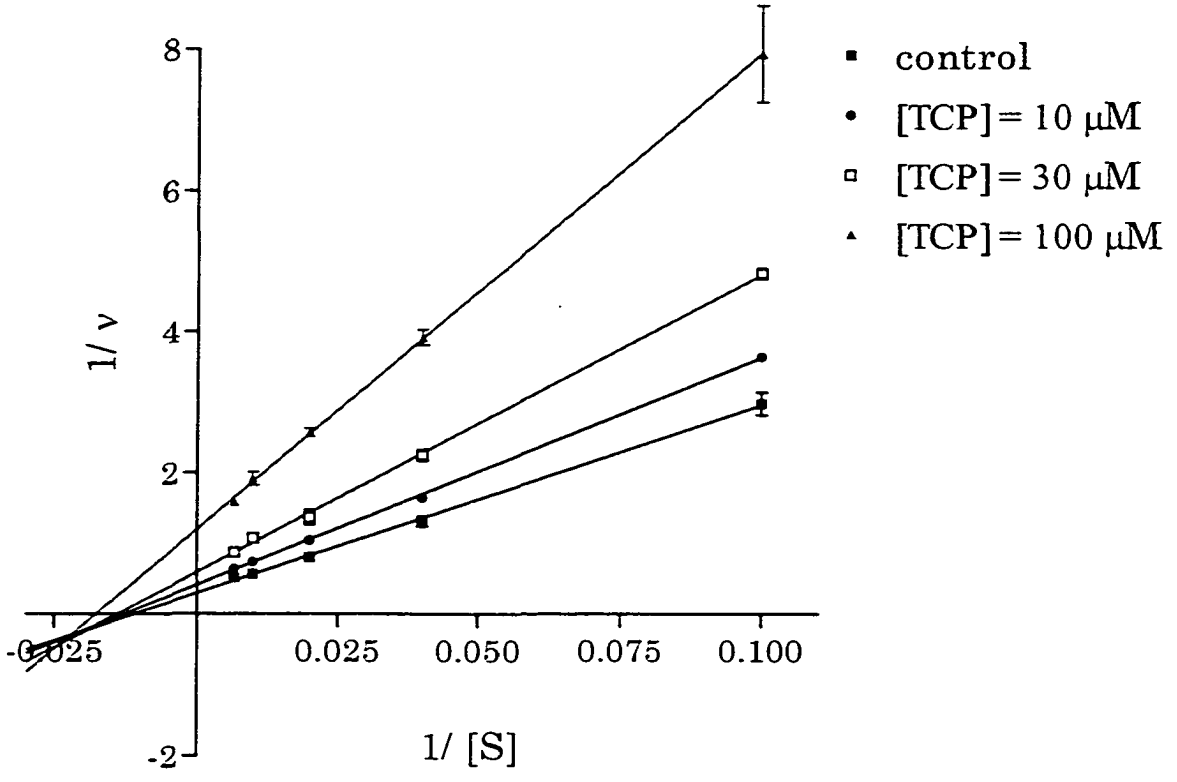


Figure 3.41 Lineweaver-Burk plot showing the non-competitive inhibitory effects of TCP on CYP2C9. Results represent means ± SEM (N=3).

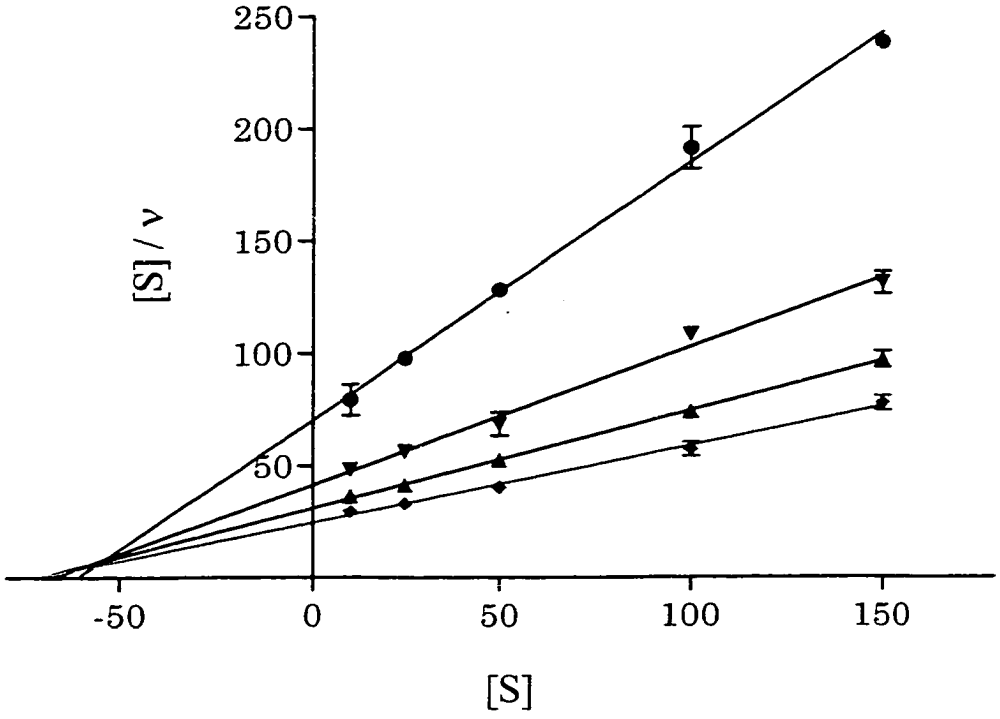


Figure 3.42 Hanes-Woolf plot showing the non-competitive inhibitory effects of TCP on CYP2C9. Results represent means ± SEM (N=3).

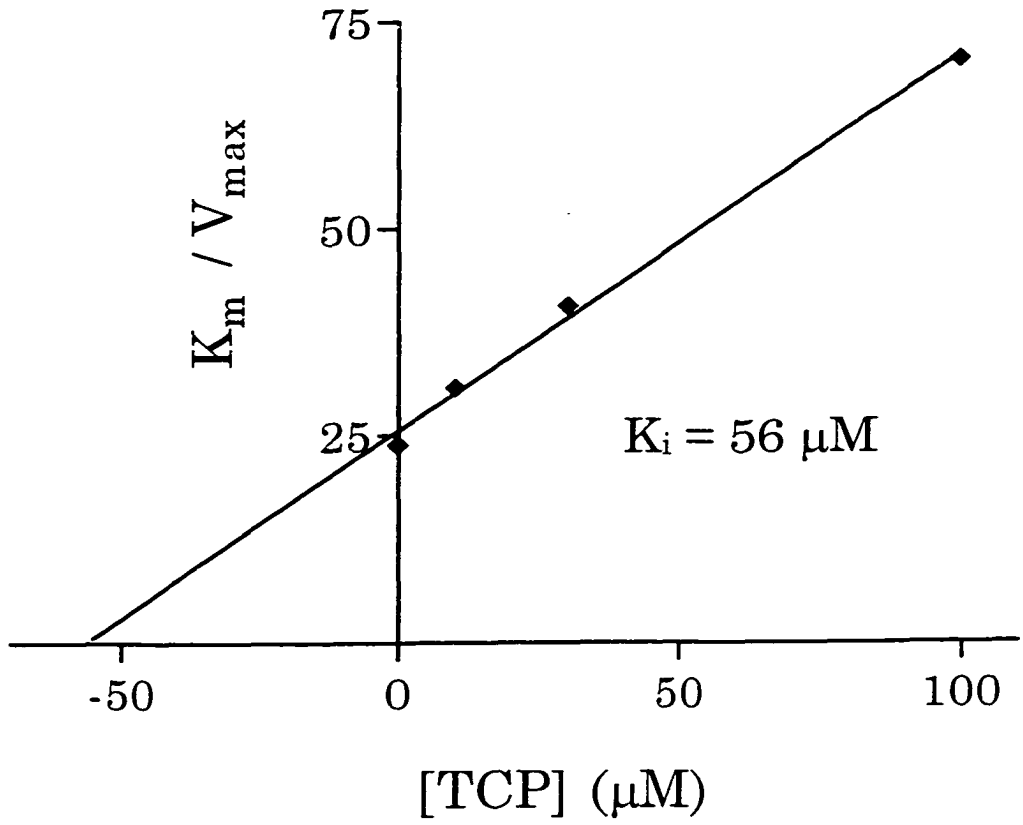


Figure 3.43 Graphical determination of inhibitory constant (K_i) of TCP *versus* CYP2C9.

3.12 Kinetics of Inhibitory Effects of PLZ on CYP2C9

3.12.1 Preliminary Studies

3.12.1.1 Time-Dependence of Inhibition

As demonstrated in Figure 3.44 the degree of CYP2C9 inhibition by PLZ does not change with preincubation time. Therefore, the inhibitory effect of PLZ on CYP2C9 is not time-dependent.

3.12.1.2 Reversibility versus Irreversibility of Inhibition

The remaining enzyme activities (as a percent of control) for Groups A, B and C were respectively 87%, 96%, and 62%. After preincubation of PLZ with CYP2C9, addition of buffer to Group A (containing higher concentrations of PLZ) resulted in some recovery of enzyme activity. This indicates that PLZ is not bound tightly to the enzyme. Hence, the inhibitory effect of PLZ on CYP2C9 is reversible.

3.12.2 Main Study

Figure 3.45 shows the effects of increasing concentrations of PLZ on production of OHTOL from TOL in a Michaelis-Menten plot. The fully non-competitive inhibitory effects of PLZ on CYP2C9 can be seen clearly in a Lineweaver-Burk plot (Figure 3.46) and in a Hanes-Woolf plot (Figure 3.47). The inhibitor constant (K_i) for PLZ has been determined from a replot of K_m/V_{max} against the PLZ concentration, and is 30 ± 3 μM (Figure 3.48).

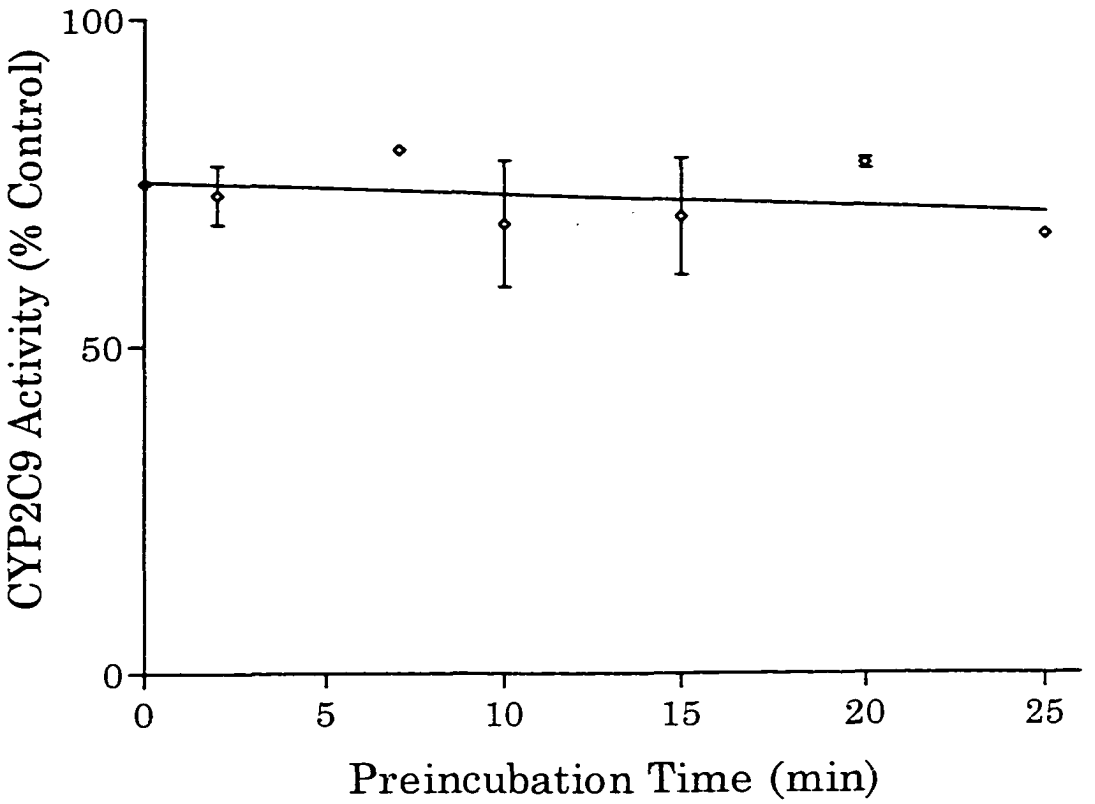
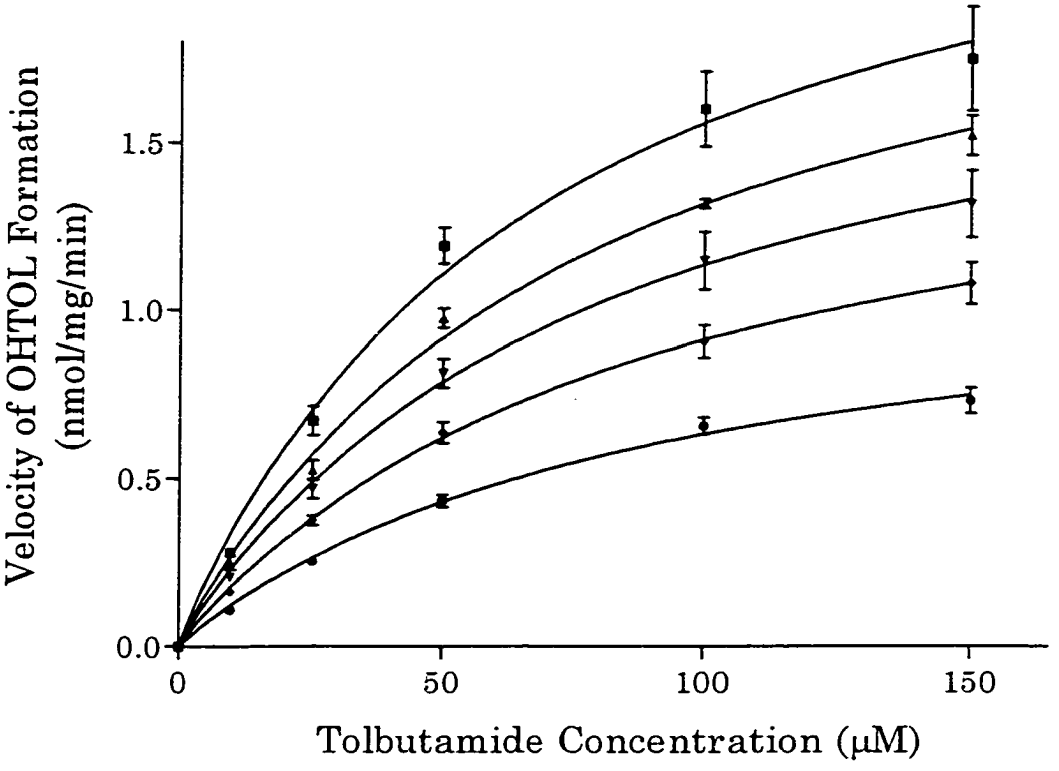


Figure 3.44 The effect of preincubation time on the inhibition of CYP2C9 activity by PLZ. Results represent means ± SEM (N=3).



- Control
- [PLZ] = 5 μM
- [PLZ] = 10 μM
- [PLZ] = 20 μM
- [PLZ] = 50 μM

Figure 3.45 Michaelis-Menten plot showing the effect of increasing concentration of PLZ on production of OHTOL from TOL. Results represent means ± SEM (N=3).

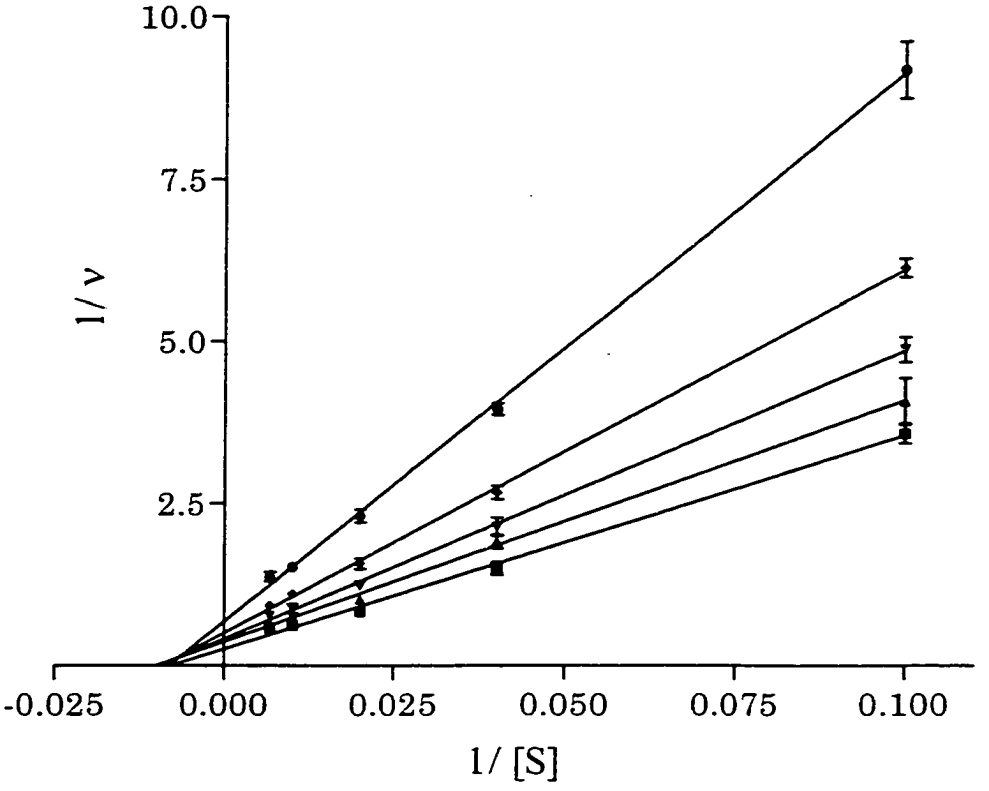
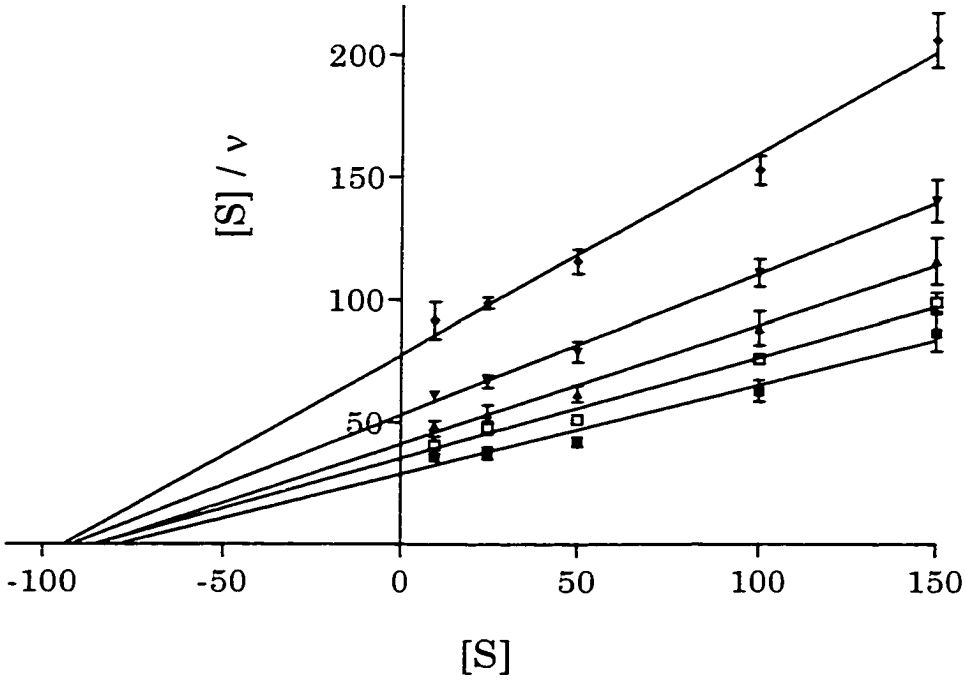


Figure 3.46 Lineweaver-Burk plot showing the non-competitive inhibitory effects of PLZ on CYP2C9. Results represent means ± SEM (N=3).



- Control
- [PLZ] = 5 μM
- [PLZ] = 10 μM
- [PLZ] = 20 μM
- [PLZ] = 50 μM

Figure 3.47 Hanes-Woolf plot showing the non-competitive inhibitory effects of PLZ on CYP2C9. Results represent means ± SEM (N=3).

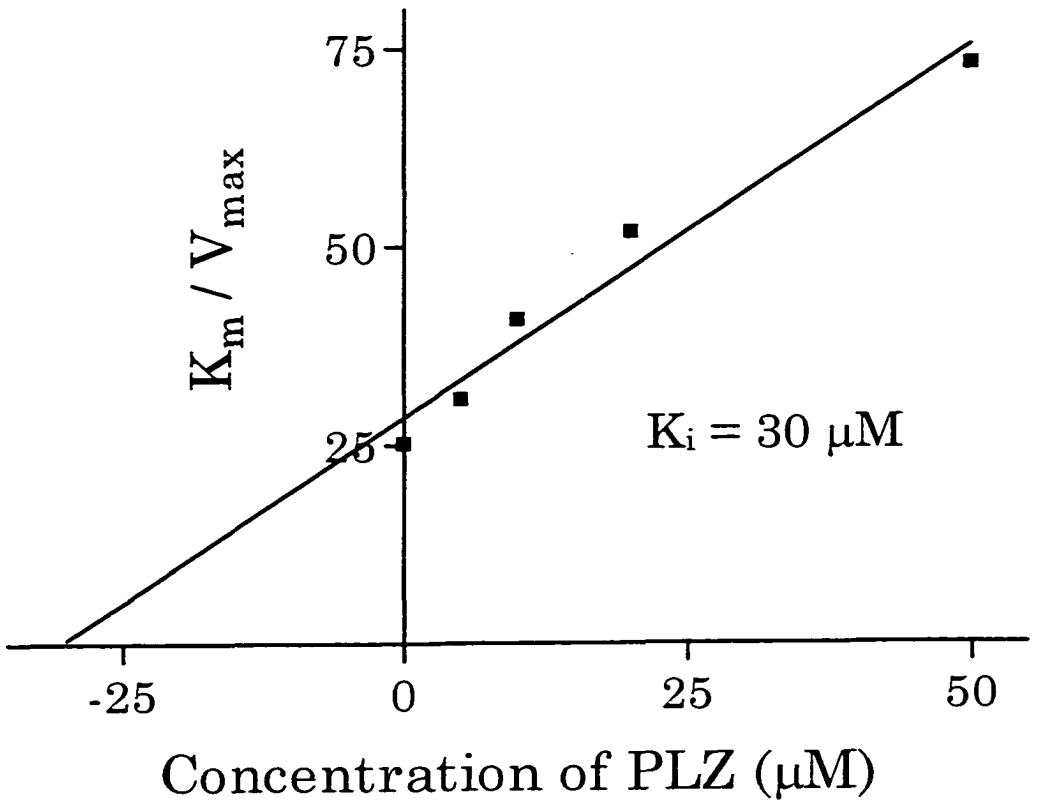


Figure 3.48 Graphical determination of inhibitory constant (K_i) of PLZ *versus* CYP2C9

3.13 Kinetics of Inhibitory Effects of TCP on CYP2D6

3.13.1 Preliminary Studies

3.13.1.1 Enzyme Concentration Study

The increase in substrate (DM) turnover was approximately linear with increasing microsomal protein (Figure 3.49). When 5 μL of CYP2D6 (protein content of 31 μg in 100 μL of incubation mixture) from a cDNA-expressed cell line were added to the incubation mixture, the production of metabolite over the chosen incubation time was detectable by the developed HPLC method. Therefore, 5 μL of CYP2D6 were added to the incubation mixtures in subsequent microsomal studies.

3.13.1.2 Optimum Incubation Time

As shown in Figure 3.50, the production of DXR from DM was linear in the first 35 minutes of the test at the higher concentration of DM (200 μM). Thus, for the rest of studies the incubation time was set at 35 min.

3.13.1.3 Estimation of K_m and V_{max} Values

As illustrated in Figure 3.51, the plot of velocity of DXR production against DM concentrations is a hyperbola that fits the one-enzyme Michaelis-Menten equation. The K_m and V_{max} Values from Michaelis-Menten plot were 12 μM and 1.83 nmol/mg/min, respectively.

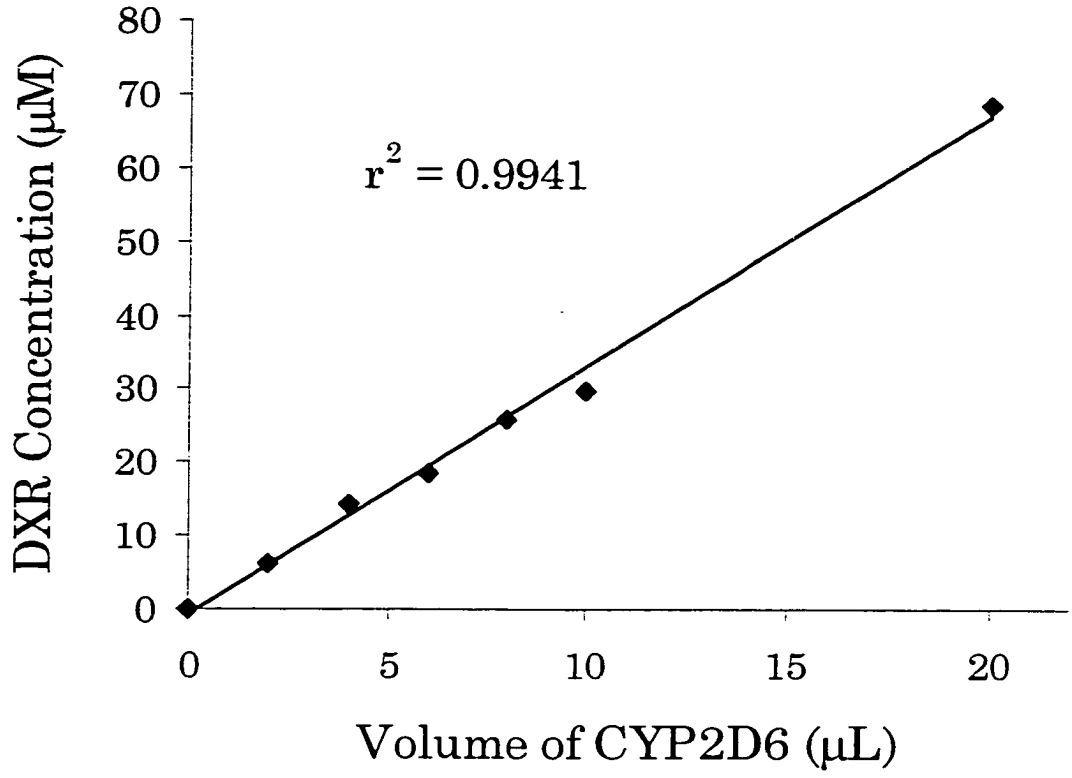


Figure 3.49 Production of DXR *versus* volume of CYP2D6 preparation (enzyme concentration).

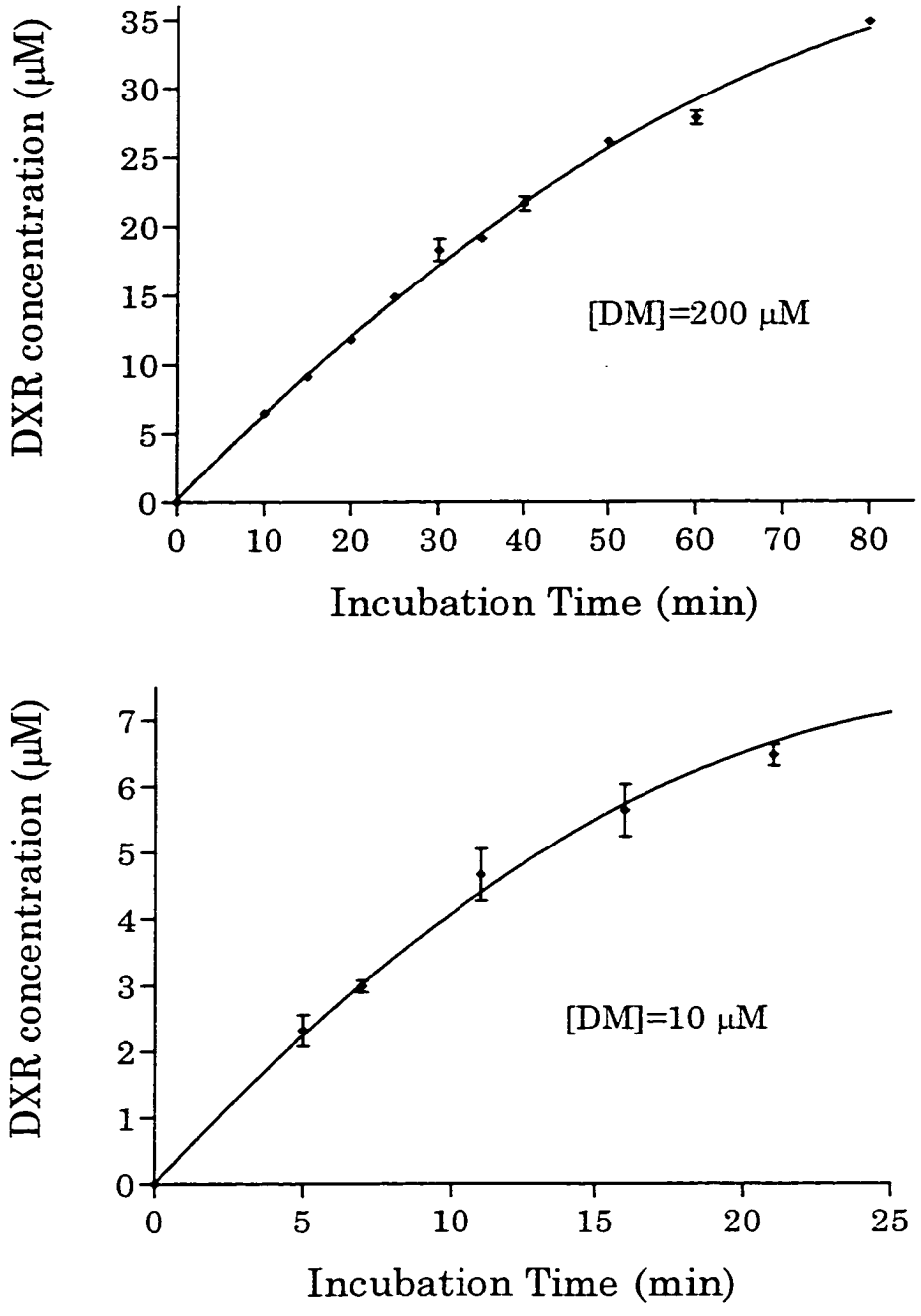


Figure 3.50 Effect of incubation time on the formation of DXR from DM. Results represent means ± SEM (N=2).

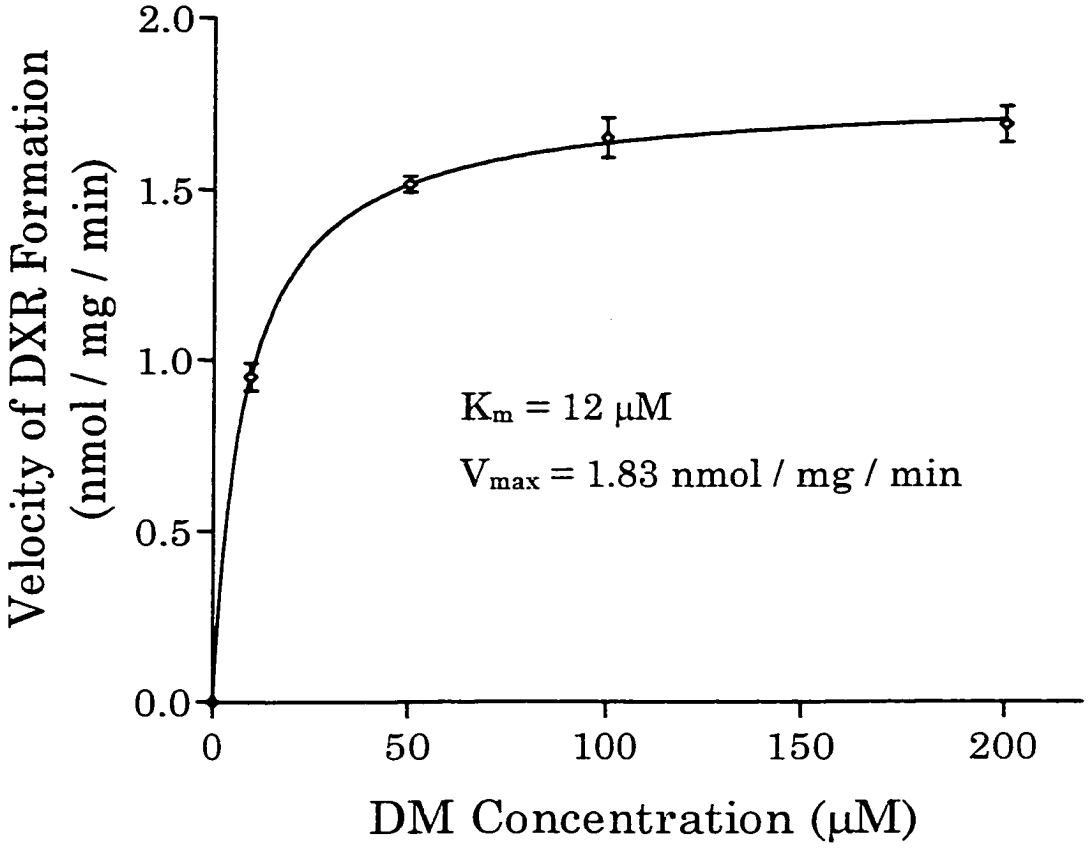


Figure 3.51 Michaelis-Menten plot of the velocity of DXR formation versus DM concentration. Results represent means ± SEM (N=3).

3.13.1.4 Time-Dependence of Inhibition

Figure 3.52 shows remaining CYP2D6 activity versus preincubation time with TCP; the inhibition of CYP2D6 activity by TCP does not increase with preincubation, and thus the inhibitory effect of TCP on CYP2D6 is not time-dependent.

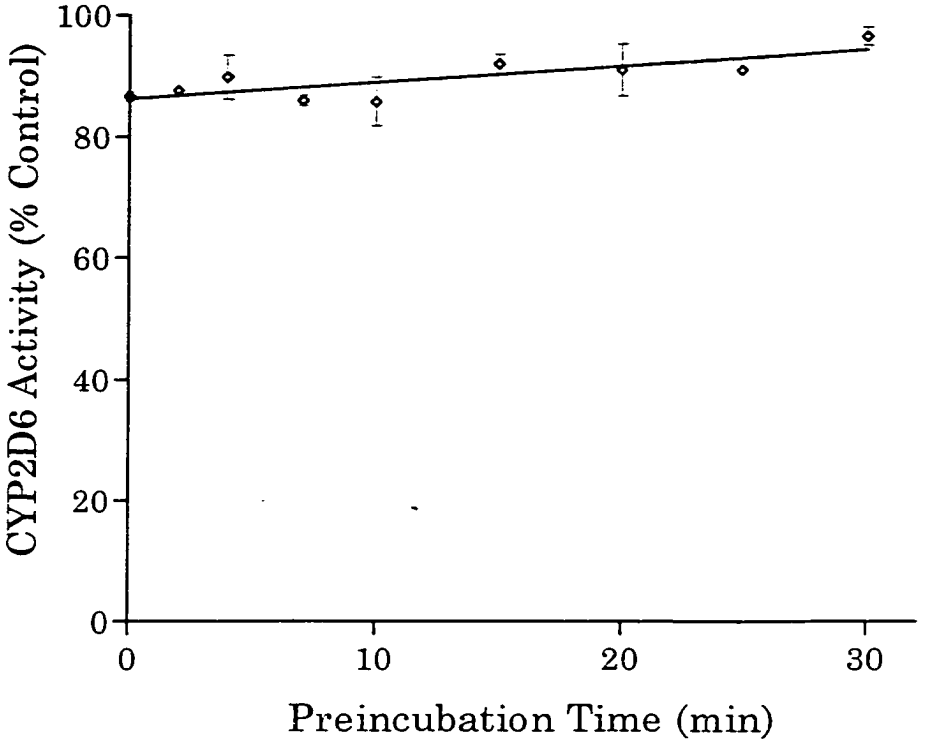


Figure 3.52 The effects of pre-incubation time on the inhibition of TCP on CYP2D6 activity. Results represent means ± SEM (N=3).

3.13.1.5 Reversibility of Inhibition

The remaining enzyme activities (as a percent of control) for Groups A, B and C were respectively 86%, 87%, and 45%. After preincubation of TCP with enzyme, addition of buffer to the set of tubes containing higher concentrations of TCP resulted in some recovery of enzyme activity. This implies that TCP is not bound tightly to the enzyme. Thus, the inhibitory effect of TCP on CYP2D6 is reversible.

3.13.2 Main Study

Figure 3.53 shows the effects of increasing concentrations of TCP on production of DXR from DM in a Michaelis-Menten plot. The competitive inhibitory effects of TCP on CYP2D6 can be seen clearly in a Lineweaver-Burk plot (Figure 3.54) and a Hanes-Woolf plot (Figure 3.55). However, as illustrated in Figure 3.56, a replot of K_m/V_{max} against TCP concentration is parabolic, suggesting a two-site pure competitive system. When not one but two molecules of inhibitor can bind to the substrate binding site, two-site competitive inhibition is said to occur (Segel, 1993; Palmer, 1995). Two-site, pure competitive inhibition is not distinguishable from one-site pure competitive inhibition by the usual Michaelis-Menten plot, Lineweaver-Burk plot or Hanes-Woolf plot. However, the secondary plot is linear for one-site pure competitive inhibition, while it is parabolic for two-site pure competitive inhibition (Segel, 1993).

In the case of two-site competitive inhibition, K_i cannot be determined directly from the replot of K_m/V_{max} *versus* inhibitor concentration. The x-intercept calculated based on the K_m/V_{max} of the control and the K_m/V_{max} of a certain [I] is referred to as K_i' for that inhibitor concentration. Replot of the reciprocal of the K_i' *versus* [I], as presented in Figure 3.57, is a straight line that intercepts the vertical axis at 2 / K_i. The calculated K_i for the inhibition of CYP2D6 by TCP is 367 μM.

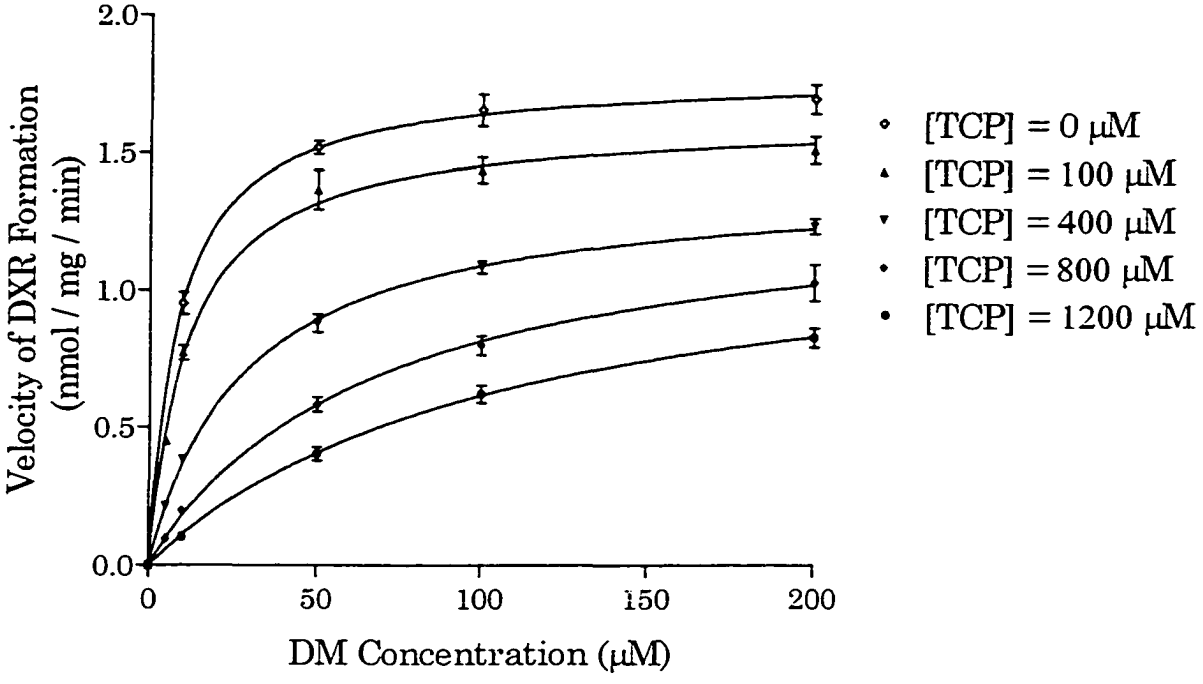


Figure 3.53 Michaelis-Menten plot showing the effect of increasing concentration of TCP on production of DXR from DM. Results represent means ± SEM (N=3).

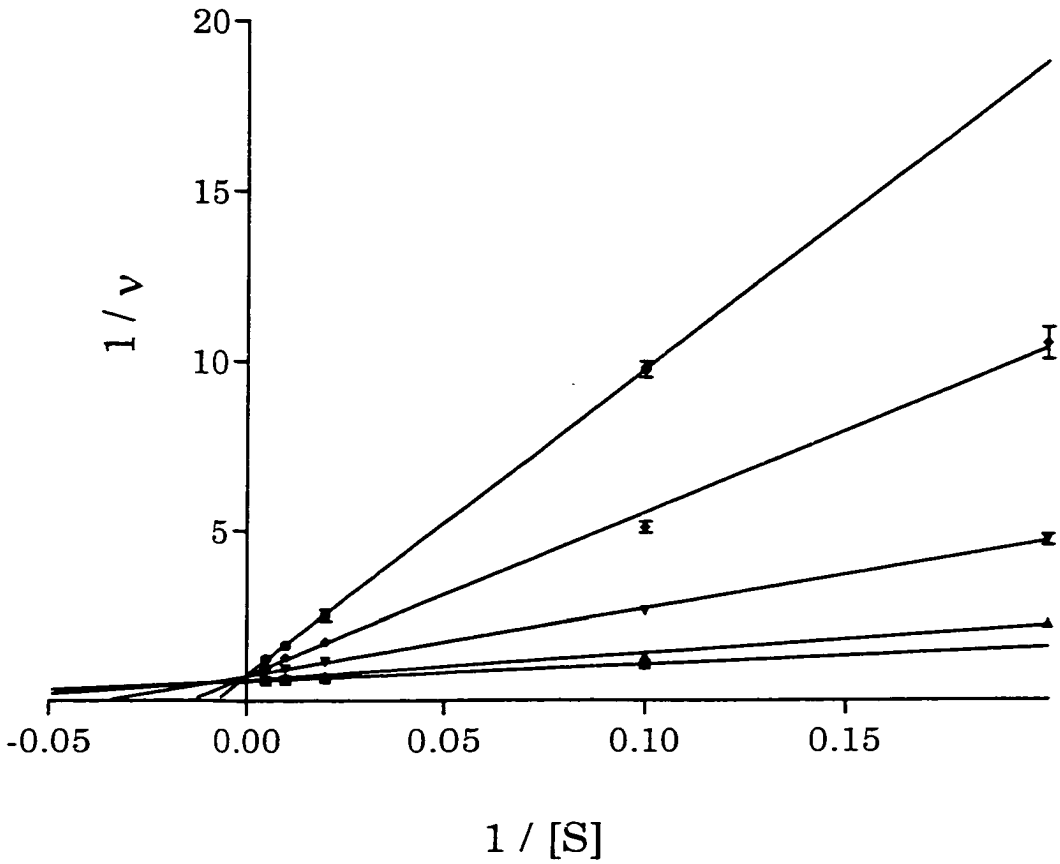


Figure 3.54 Lineweaver-Burk plot showing the competitive inhibitory effects of TCP on CYP2D6. Results represent means ± SEM (N=3).

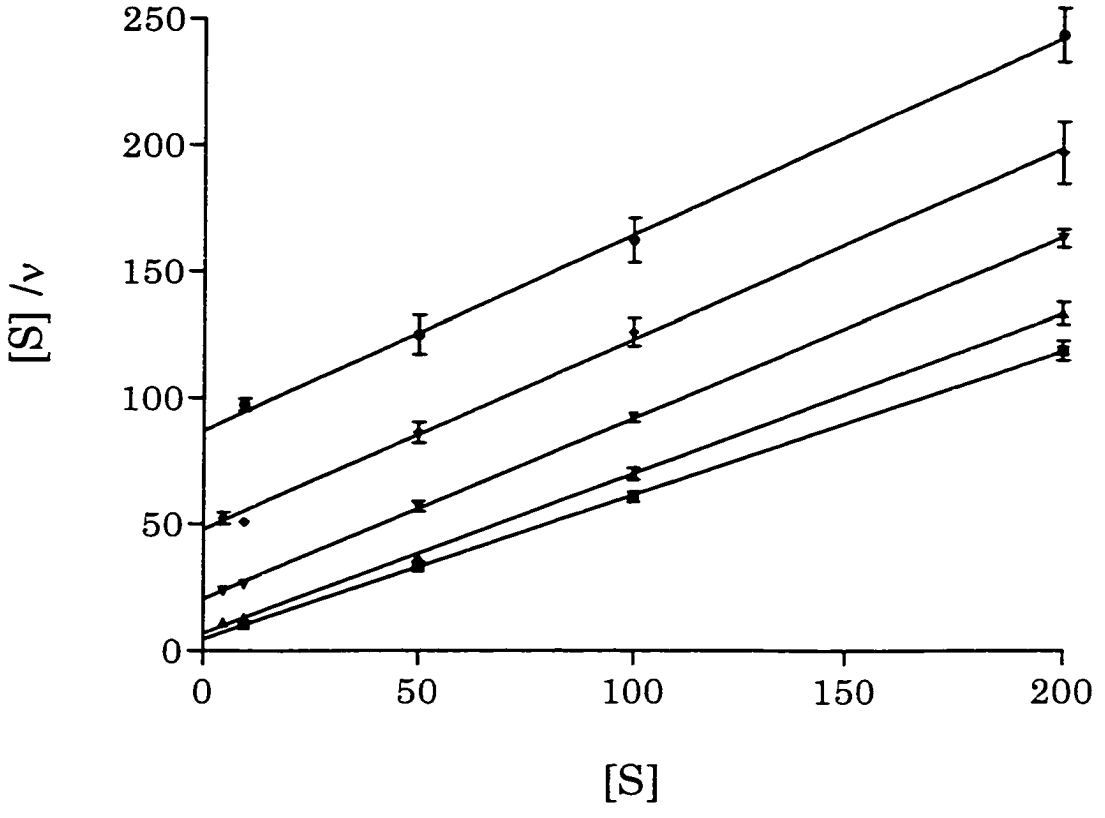


Figure 3.55 Hanes-Woolf plot showing the competitive inhibitory effects of TCP on CYP2D6. Results represent means ± SEM (N=3)

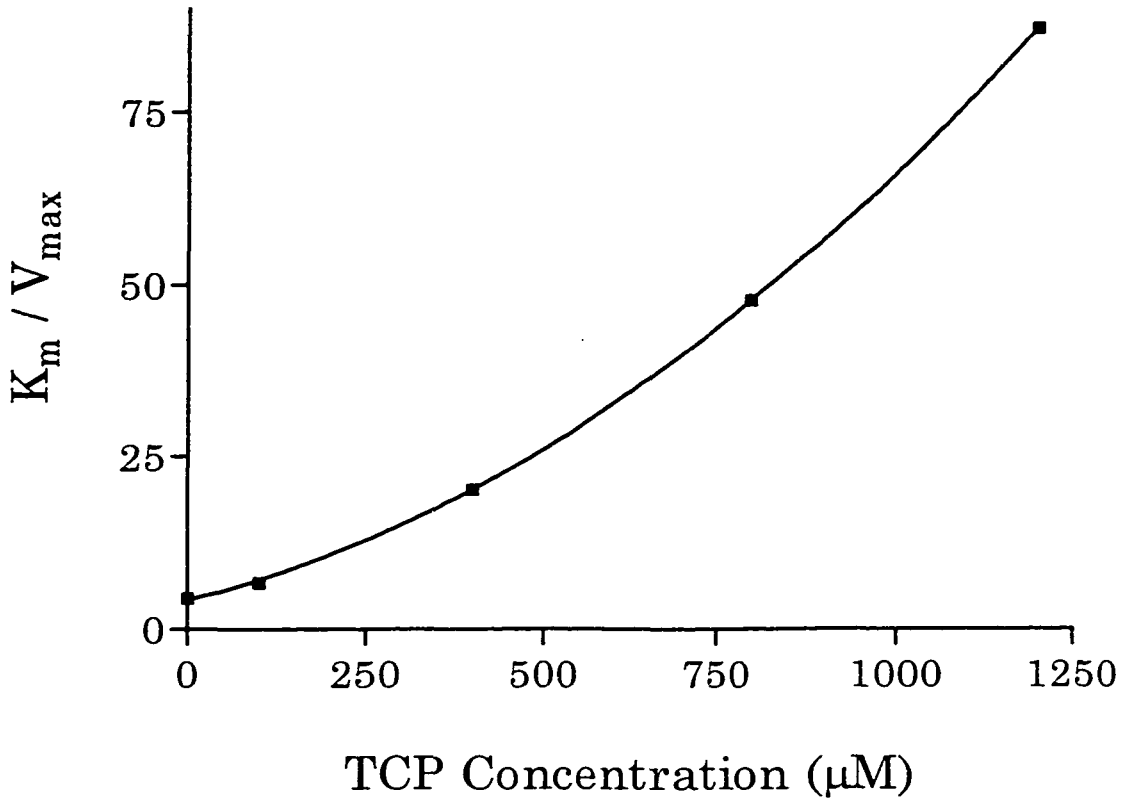


Figure 3.56 K_m/V_{max} *versus* TCP concentration plot, showing two-site competitive inhibition of TCP on CYP2D6

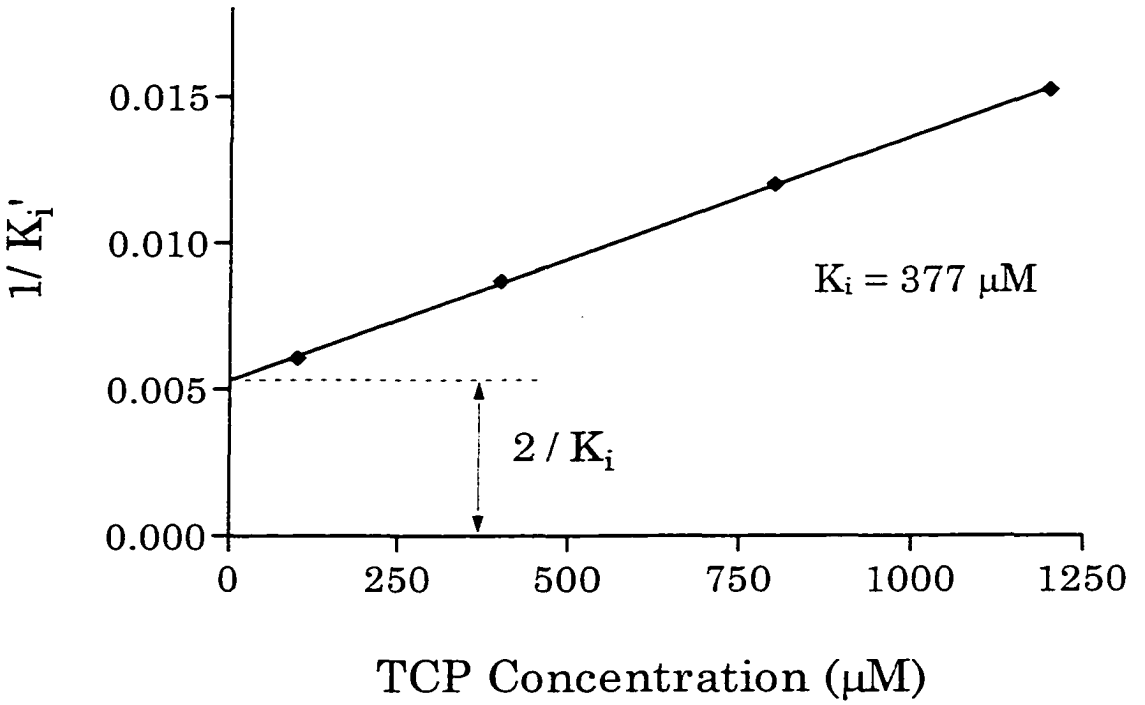


Figure 3.57 Graphical determination of inhibitory constant (K_i) of TCP *versus* CYP2D6.

3.14 Kinetics of Inhibitory Effects of PLZ on CYP2D6

3.14.1 Preliminary Studies

3.14.1.1 Time-Dependence of Inhibition

Figure 3.58 shows remaining CYP2D6 activity (% control) versus preincubation time with PLZ in the absence and presence of an NADPH generating system; the inhibition of CYP2D6 activity by PLZ does not increase with preincubation time, and thus the inhibitory effects of PLZ on CYP2D6 is not time-dependent.

3.14.1.2 Reversibility of Inhibition

The experiment was run in the presence and absence of an NADPH generating system. In the absence of the NADPH system, the remaining enzyme activities (as a percent of control) for Groups A, B and C were respectively 55%, 56%, and 11%. In the presence of NADPH system, the remaining enzyme activities (as a percent of control) for Groups A, B and C were respectively 46%, 48%, and 10%. After preincubation of PLZ with enzyme, addition of buffer to the set of tubes containing higher concentrations of PLZ resulted in large recovery of enzyme activity. This implies that PLZ is not bound tightly to the enzyme. Thus, the inhibitory effect of PLZ on CYP2D6 is reversible.

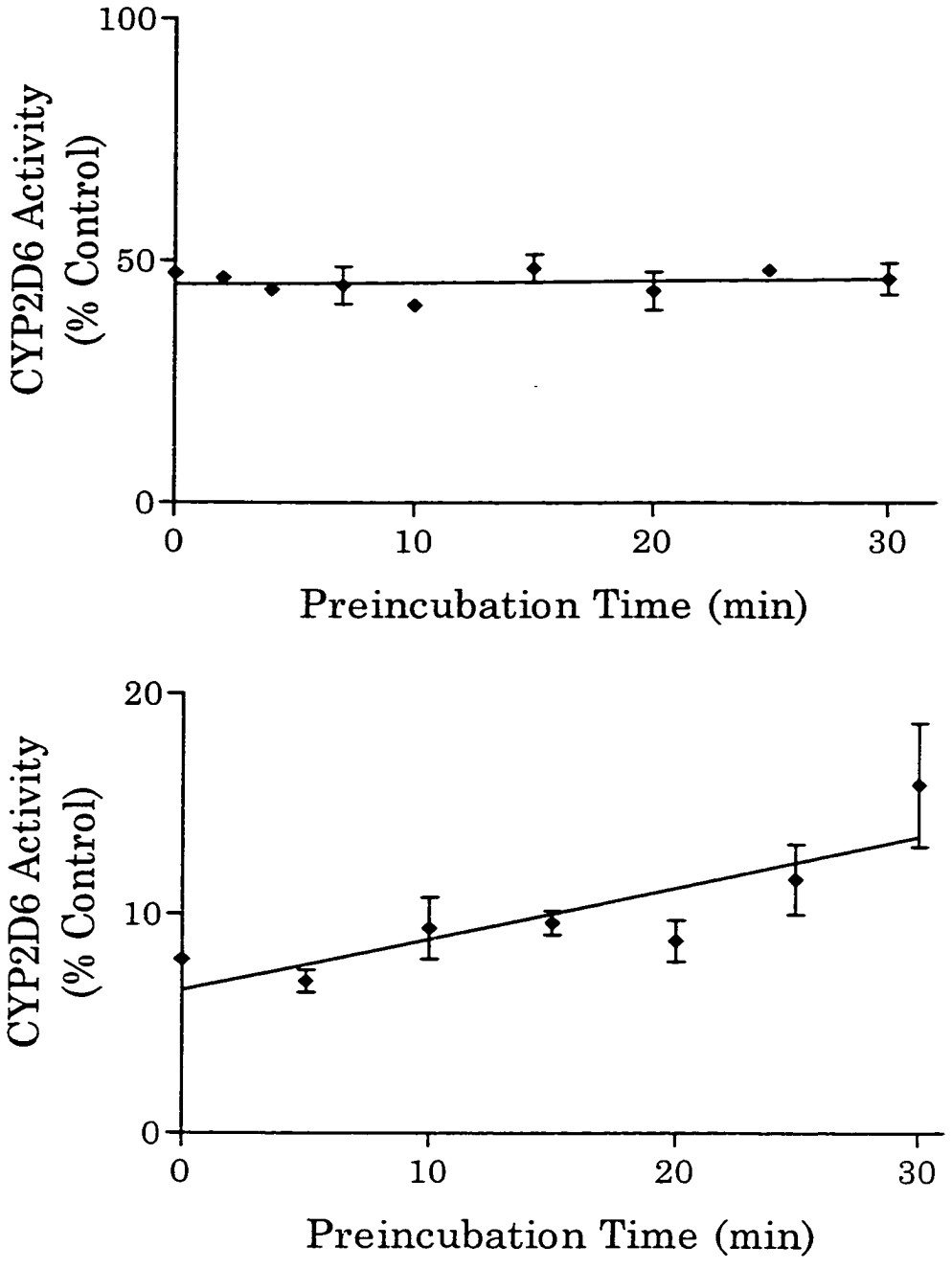
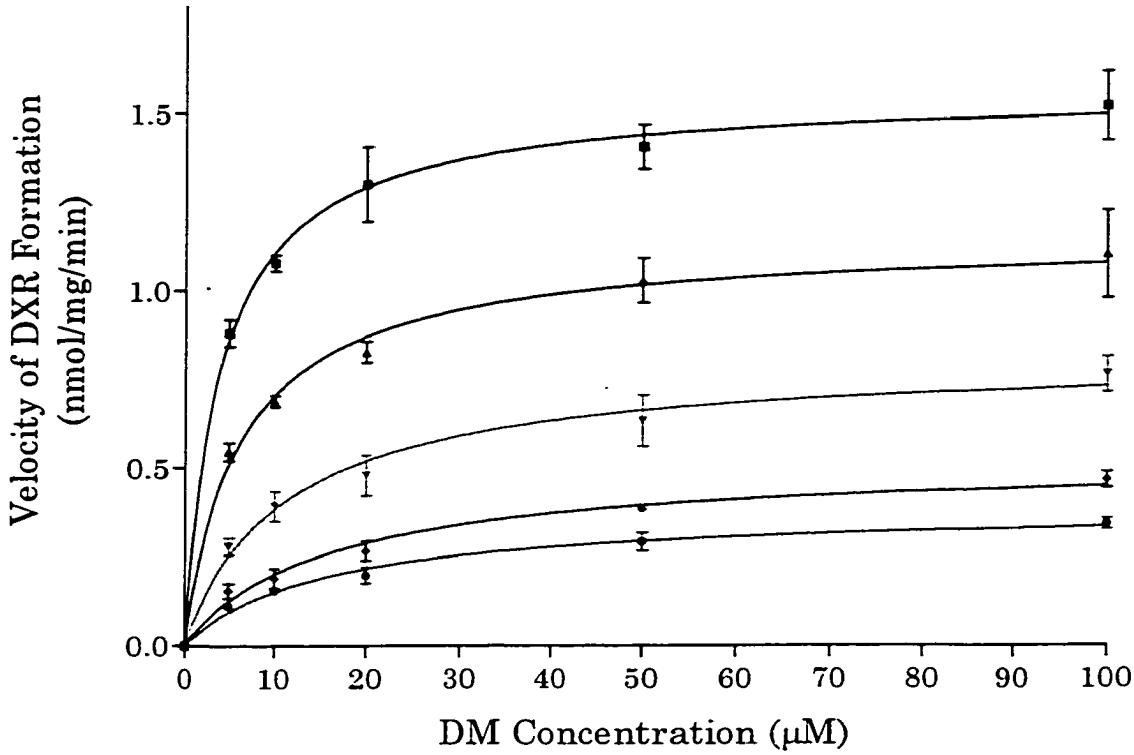


Figure 3.58 The effect of preincubation time on the inhibition of CYP2D6 activity by PLZ in the absence (top) and presence (bottom) of NADPH. Results represent means ± SEM (N=2).

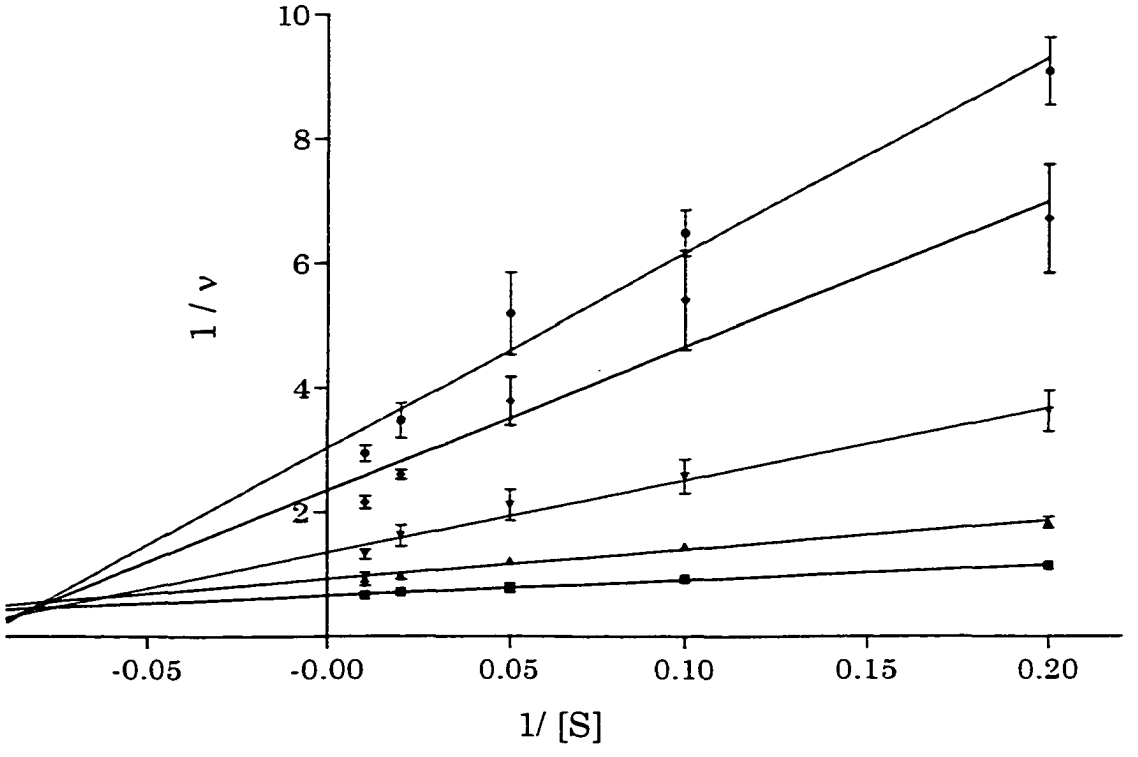
3.14.2 Main Study

Figure 3.59 shows the effects of increasing concentrations of PLZ on production of DXR from DM in a Michaelis-Menten plot. The non-competitive inhibitory effects of PLZ on CYP2D6 can be seen in a Lineweaver-Burk plot (Figure 3.60) and Hanes-Woolf plot (Figure 3.61). The inhibitor constant (K_i) for PLZ has been determined from a replot of K_m/V_{max} against the PLZ concentration, and is 0.7 ± 0.5 μM (Figure 3.62).



- Control
- [PLZ] = 2 μM
- [PLZ] = 5 μM
- [PLZ] = 10 μM
- [PLZ] = 15 μM

Figure 3.59 Michaelis-Menten plot showing the effect of increasing concentration of PLZ on production of DXR from DM. Results represent means ± SEM (N=3).



- Control
- [PLZ] = 2 μM
- [PLZ] = 5 μM
- [PLZ] = 10 μM
- [PLZ] = 15 μM

Figure 3.60 Lineweaver-Burk plot showing the non-competitive inhibitory effects of PLZ on CYP2D6. Results represent means ± SEM (N=3).

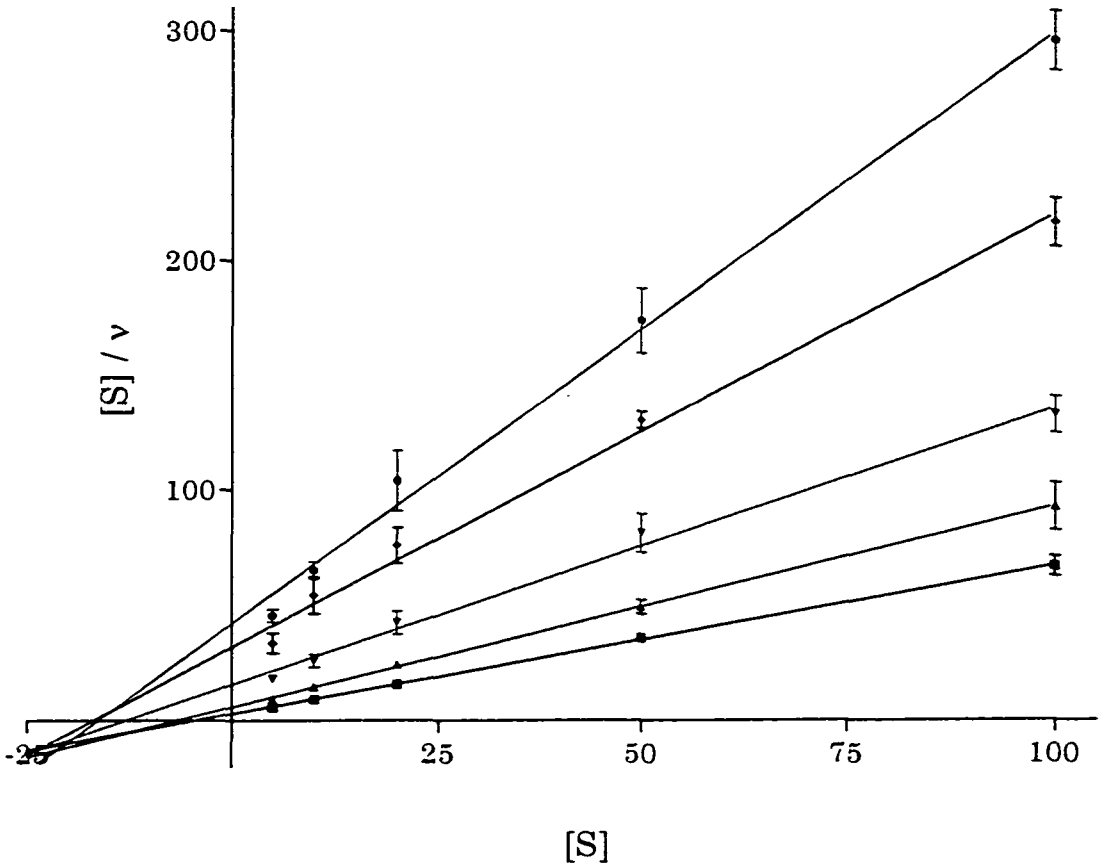


Figure 3.61 Hanes-Woolf plot showing the non-competitive inhibitory effects of PLZ on CYP2D6. Results represent means ± SEM (N=3)

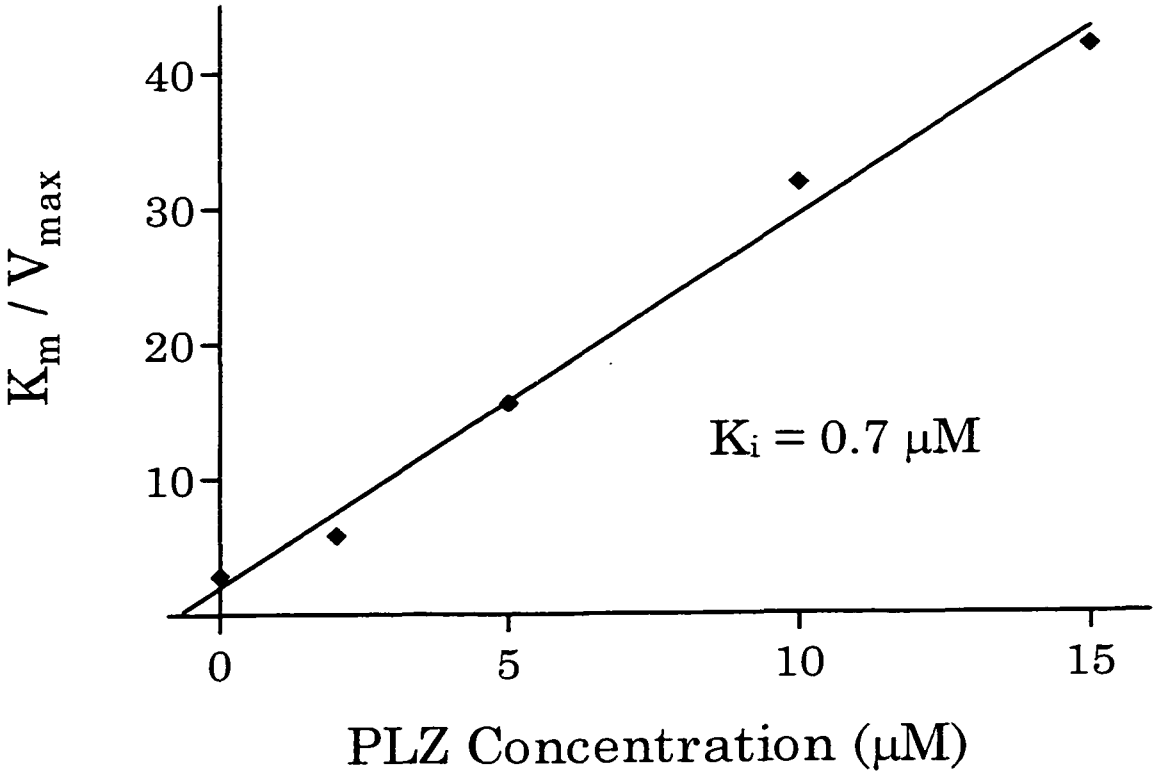


Figure 3.62 Graphical determination of inhibitory constant (K_i) of PLZ *versus* CYP2D6.

CHAPTER 4

Discussion

4.1 General Overview

The CYP enzymes are among the major enzymes involved in drug biotransformation. Information regarding the metabolic pathways, inducers, and inhibitors of CYP enzymes offers a basis for the explanation and prediction of certain drug interactions, establishes guidelines for dose selection, and provides insight into some cases of unexpected toxicity or non-response to treatments.

Many of the drugs used to treat psychiatric disorders are substrates and/or inhibitors of CYP enzymes, and the possibility of pharmacokinetic drug-drug interactions must be considered during clinical use of these drugs. This possibility must be considered seriously in psychiatric patients, since these patients are frequently taking two or more drugs.

If two or more drugs that are significantly metabolised by the same CYP enzyme are administered concomitantly to a patient, the pharmacokinetic properties of each drug may change markedly, because of the competition between drugs for that particular enzyme.

Another source of pharmacokinetic interaction originates from the inhibitory effects of a drug and/or its metabolites on CYP enzymes involved in drug metabolism. When such drugs [e.g. the antidepressant fluoxetine (Prozac®)] that have strong inhibitory effects on particular CYP enzymes are coadministrated with substrates of the enzymes, a dramatic increase in blood levels of substrate drugs may occur, with possible toxicological consequences.

There is very little information available on the inhibitory effects of MAOI antidepressants on CYP enzymes. In the present study, the inhibitory effects of two non-selective irreversible inhibitors of MAO, TCP and PLZ, on three CYP enzymes, CYP2C9, CYP2C19, and CYP2D6, have been evaluated, *in vitro*. In addition, the inhibitory effects of individual enantiomers of TCP, and some ring-substituted analogues of TCP on CYP2C19 have been investigated, using *in vitro* models of human liver microsomes.

In recent years, pharmacokinetic studies have been facilitated by the availability of human liver microsomes and individual cDNA-expressed CYP enzymes from human sources (Glue and Banfield, 1996; Lane, 1996; Parikh et al., 1997). Microsomal preparations of human liver *in vitro* contain the various human CYP enzymes in proportion to their quantitative representation in human liver *in vivo*. cDNA-transfected human lymphoblastoid cells or other expression systems contain individual human CYP enzymes (Parikh et al., 1997; Crespi, 1995).

Using the *in vitro* models of human liver microsomes and cDNA-expressed human CYP enzymes, quantitative inhibitory potency of a series of drugs and/or their metabolites against specific CYP enzymes can be screened relatively quickly with no clinical drug exposure. Also, when a drug being studied undergoes *in vivo* biotransformation, any observed metabolic inhibition could be due to parent drug, its metabolites, or both; there is no direct method to determine *in vivo* the relative contributions or the

quantitative inhibitory potency of multiple potential inhibitors that are present simultaneously (Greenblatt et al., 1998).

In this chapter, first the validity of the applied methodologies and the developed assay procedures are briefly discussed. This is followed by a discussion of the new supportive evidence regarding the lack of formation of amphetamine as a metabolite of TCP. The inhibitory effects of the individual enantiomers of TCP, and TCP analogues on CYP2C19 are discussed. Finally, the kinetics of the inhibitory effects of TCP and PLZ on CYP2C9, CYP2C19 and CYP2D6, as well as the possible clinical significance of these inhibitions are discussed.

4.2 Discussion of Methodology

In laboratory experiments, specimens are usually run on a repeated basis. Thus, it is important to devote significant time to develop an efficient analytical method for quantification of the compounds of interest. In the present study, several sensitive and reproducible GC-ECD and HPLC-UV methods were developed for detection and quantification of the compounds of interest in microsomal studies.

4.2.1 Detection and Quantification of OHMEP and DXR by GC-ECD

A GC method was developed to quantify DXR and OHMEP, metabolites of DM and MEP, respectively. The method involves extractive

derivatization of DXR and OHMEP with PFBC, followed by quantification by GC-ECD. PFBS was also used for extractive derivatization instead of PFBC, but the derivatives are not as stable as PFBC derivatives.

Extractive derivatization with PFBC or PFBS under aqueous conditions has been shown previously to be particularly useful for analysis of drugs and neurochemicals containing phenolic groups and/or primary or secondary amines (Cristofoli et al., 1982; Nazarali et al., 1986; Nazarali et al., 1987a, b; Rao et al., 1987).

The assay developed was sensitive, with a limit of detection less than 17 pg on column. The low inter- and intra-assay coefficients of variation indicate that the assay is reproducible. As shown in Figure 3.1, the derivatives have excellent chromatographic properties. The sensitive and reproducible method developed has been applied successfully in quantifying DXR and OHMEP in the microsomal experiments described in this thesis. It is anticipated that the procedure would be readily adaptable to other studies such as investigations of levels of these metabolites in tissues or body fluids.

The derivatives prepared for the GC method are stable for at least 48 hours when stored at $-80^{\circ}C$. However, they are not stable at room temperature for the time required to inject a relatively large number of samples by autosampler. Therefore, in kinetic studies, where a relatively large number of samples should be analyzed comparatively at room temperature, alternative methods are preferred.

4.2.2 Detection and Quantification of OHMEP, DXR, and OHTOL by HPLC-UV in the Presence of TCP or PLZ

Different combinations of mobile phase were developed and applied in separate experiments to detect and quantify OHMEP, OHTOL, DXR, and their related parent drugs as well as TCP, PLZ, and the internal standards. In all of the assays, after precipitation of microsomal protein with acetonitrile, the samples were injected onto the HPLC directly. The procedure was simple and convenient.

As presented in Sections 3.2 through 3.6, the low inter- and intra-assay coefficients of variation indicate that the developed assays are reproducible. The methods are sensitive, and there is no need for derivatization. The metabolites from microsomal experiments are stable at room temperature. Being sensitive, reproducible and stable, the HPLC-UV assays were used for the kinetic studies involving OHMEP, DXR, and OHTOL.

4.3 Absence of Amphetamine as a Metabolite of Tranylcypromine

There has been considerable discussion in the literature about whether or not TCP is metabolized to amphetamine. Youdim et al. (1979) detected high concentrations of amphetamine and methamphetamine in a case of tranylcypromine overdose and postulated the metabolic formation of amphetamine from tranylcypromine by cleavage of the cyclopropyl ring. However, other researchers have been unable to detect amphetamine as a

metabolite of TCP, even in overdose cases (Reynolds et al., 1980; Calverley et al., 1981; Mallinger et al., 1986; Jefferson, 1992; Iwerson & Schmoldt, 1996; Crifasi & Long, 1997; Sherry et al., 2000).

In the present study, neither 1-amino-3-phenylpropane (another potential metabolite arising from cleavage of the cyclopropyl ring of TCP, Fig.1.2) nor amphetamine was detected as a result of incubation of TCP with human liver microsomes. Thus, the results of this study do not confirm the proposal of Youdim and co-workers. It seems that cleavage of the cyclopropyl ring is not a significant pathway for metabolism of TCP.

In fact, two other routes of biotransformation of TCP, namely ring hydroxylation (Baker et al., 1986; Nazarali et al., 1987) and N-acetylation (Calverly et al., 1981; Kang & Choi, 1986) have been reported. *Para*-hydroxytranylcypromine (OHTCP) has been identified as a metabolite of TCP in the heart and brain of rats treated with intraperitoneal administration of TCP (Baker et al., 1986; Nazarali et al., 1987), and N-acetyltranylcypromine has been detected in brain and urine of rats treated with TCP via the subcutaneous route (Calverly et al., 1981; Kang & Chung, 1984).

4.4 Comparison of the Inhibitory Effects of TCP, TCP Enantiomers, and Ring Substituted Analogues of TCP on CYP2C19

The inhibitory potency of compounds against activity of specific CYP enzymes can be screened readily using *in vitro* models of human liver

microsomes or individual cDNA expressed CYP enzymes. In the present study, a fixed concentration of index substrate (MEP) for CYP2C19 was co-incubated with various concentrations of the potential inhibitor (TCP, its individual enantiomers and some of its analogues). The relation of decrement in metabolite formation rate *versus* inhibitor concentration yields an estimate of a concentration which produces 50% inhibition (IC_{50}). IC_{50} values are suitable for comparing the relative potency of a series of inhibitors studied under identical conditions.

Although (±)-TCP has been reported to be a relatively strong inhibitor of CYP2C19 (Inaba et al., 1985), to the knowledge of the author the inhibitory effects of individual enantiomers of TCP or the hydroxylated metabolite of TCP on CYP2C19 have not been studied previously. In addition, MTCP and FTCP, two analogues of TCP synthesized in the Neurochemical Research Unit and shown to possess potent MAO-inhibiting properties (Sherry et al., 1999), were also included in the current study.

The results demonstrate that the two enantiomers of TCP differ in their inhibitory potency on CYP2C19. (+)-TCP is a much stronger inhibitor of CYP2C19 ($IC_{50}\approx25\mu M$) compared to (-)-TCP ($IC_{50}>400\mu M$). This finding in addition to other pharmacodynamic differences (Smith, 1980; Reynolds & Riederer, 1981; Nickolson & Pinder, 1984; Reynolds, 1985) between the two enantiomers emphasizes the importance of testing individual stereoisomers for pharmacological activity.

Of the TCP analogues tested, OHTCP had the strongest ($IC_{50}\approx20\mu M$) and FTCP had the weakest ($IC_{50}\approx78\mu M$) inhibitory effects on CYP2C19. Previous studies have shown that FTCP is more potent than TCP at inhibiting MAO (Rao et al., 1986) and has a longer elimination half-life in rat brain (Coutts et al., 1987). Therefore, lower doses of FTCP than that of TCP are required to give the same degree of MAO inhibition. It now appears that FTCP is less likely than the parent compound to inhibit CYP2C19, hence reducing the probability of drug-drug interactions involving this CYP enzyme. It thus seems that FTCP is worth pursuing further as an antidepressant.

In this regard, it is of interest that FTCP also causes a down-regulation of 5-HT_2, tryptamine, α-adrenergic, and β-adrenergic receptors in rat brain, characteristics shared by TCP and PLZ (Greenshaw et al., 1988; Sherry et al., 1999), and has recently been shown to act as an antidepressant in an animal screening model (forced swimming test) for such drugs (Bourin et al., 2000).

4.5 Kinetic Studies of Inhibition

In addition to the calculation of IC_{50} values, another approach to determine quantitative inhibitory potency of a drug against a specific CYP enzyme is to calculate the inhibition constant (K_i). Determination of K_i involves more labor, time and cost, since it requires studies of multiple

substrate concentrations and multiple inhibitor concentrations. IC_{50} values depend on substrate concentration, while K_i is independent of substrate concentration and can be used under some defined conditions for quantitative *in vitro / in vivo* scaling of drug interactions (von Moltke et al., 1998).

The microsomal preparations of human liver used *in vitro* contain various human CYP enzymes in proportion to their quantitative representation in human liver *in vivo*. In the experiments conducted to determine K_i values, pure CYP enzymes expressed in cDNA-transfected human lymphoblastoid cells or other expression systems have been used. However, when interpreting results from cDNA-expressed human CYP enzymes, intrinsic limitations caused by study of a single CYP enzyme removed from its usual mixture of enzymes must be considered.

4.5.1 Kinetics of Inhibitory Effects of TCP on CYP2C19 and Its Clinical Significance

Preliminary studies showed that the inhibitory effect of TCP on CYP2C19 is not time-dependent (Figure 3.26) and is reversible. As described in section 2.8.4, to determine the type of inhibition, a range of MEP concentrations was incubated with cDNA-expressed CYP2C19 and various concentrations of TCP. As presented in Figure 3.28, K_m values increased with increasing TCP concentrations, while V_{max} remained almost unchanged. It is concluded that TCP is a competitive inhibitor of CYP2C19 (K_i = 32μM).

The preliminary studies of time dependency and reversibility were conducted in the absence of NADPH, in order to prevent TCP turnover during the preincubation time. A previous study conducted on rat liver microsomes has shown that the inhibitory effect of TCP is NADPH-independent (Dupont et al., 1987). Hence, it has been assumed that the absence of NADPH during the preincubation period would not affect test results. This was proven to be a valid assumption, as the results of the main study clearly indicated a competitive pattern, and hence an inhibition of reversible nature.

In general, the clinical relevance of a pharmacokinetic drug-drug interaction is determined by a number of factors. The major factors are the frequency with which the drug combination is administered, patient age and physical condition, the presence of medical illness, the pharmacokinetic profile (half-life and clearance) and the therapeutic index of the drugs, and whether the interaction may be prevented by clinical dose titration (Lane, 1996; Devane, 1998). The focus of the studies described in this thesis is on those types of pharmacokinetic drug interactions that are caused by the inhibitory effects of drugs on enzymes responsible for drug biotransformation. *In vitro* studies on the type of inhibitory effect and the percent of inhibition can establish a base to determine if the interaction may be clinically significant.

In competitive inhibition, the percent of inhibition depends on substrate and inhibitor concentrations as well as K_i and K_m. Equation 4.1

represents the percent inhibition in the presence of a competitive inhibitor (Segel, 1993).

$$i_{100} = \frac{[I]}{[I] + K_i \times \left(1 + \frac{[S]}{K_m}\right)} \times 100 \qquad \text{(Equation 4.1)}$$

In acute studies, at clinical dosages (20-50 mg/day), peak TCP plasma concentrations have been reported to be about 0.48-1.4 μM (Mallinger et al., 1986). In chronic studies, plasma concentrations levels have been reported to be about 1 μM (Keck et al., 1991). There is no information regarding the liver levels of TCP. Since the drug is lipophilic and lipophilic drugs partition extensively into the liver, we would expect liver levels to be greater than plasma concentrations. For SSRIs, liver to plasma concentration ratios of 12 to 27 have been reported by Preskorn (1996). Since TCP is very lipophilic, we would expect liver to plasma concentrations of TCP to be at least as high as those for the SSRIs. Therefore, the liver concentration of TCP would be expected to be at about 30μM, which is similar to the calculated K_i for the inhibition of CYP2C19. This means that in the absence of substrate, TCP occupies approximately 50% or less of enzyme active sites.

In the presence of substrate, the value of enzyme occupancy with TCP would be even less due to the competition between substrate and inhibitor, suggesting that the inhibitory effect of TCP on CYP2C19 is not clinically significant. However, in certain situations such as high dose TCP therapy (4-5 times the common clinical dose) used previously for some refractory

patients (Robinson, 1983; Guze & Baxter, 1987; Pearlman, 1987; Amsterdam & Berwish, 1989), the percent of enzyme inhibition would be expected to reach high levels. Such inhibition could cause relevant metabolic changes and clinically significant interactions, when co-administrated with another CYP2C19 inhibitor or substrate.

4.5.2 Kinetics of Inhibitory Effects of PLZ on CYP2C19 and its Clinical Significance

The preliminary studies conducted in the absence of NADPH have shown that the inhibitory effect of PLZ on CYP2C19 is not time-dependent and is reversible. As described in the Section 2.8.4, a range of MEP concentrations were incubated with cDNA-expressed CYP2C19 and various concentrations of PLZ. As presented in Figure 3.33, the K_m remained unaffected, whereas V_{max} decreased with increasing PLZ concentrations. Therefore, it is concluded that PLZ is a non-competitive inhibitor of CYP2C19 ($K_i = 0.7$ μM).

In noncompetitive inhibition, the percent of inhibition depends only upon inhibitor concentrations and K_i. Equation 4.2 represents the percent inhibition in the presence of a noncompetitive inhibitor (Segel, 1993).

$$i_{100} = \frac{[I]}{[I] + K_i} \times 100 \qquad \text{(Equation 4.2)}$$

There is limited information about PLZ plasma concentrations. In one study, after single dosages of 30 mg PLZ, peak plasma PLZ concentration was

reported to be about 0.11μM (Robinson et al., 1985). In another study, steady state plasma concentrations of PLZ in outpatients treated with 60 mg/day PLZ was reported to increase gradually over the 2 to 6 weeks of administration from 5.2±0.1 to 7.4±0.1 nM, respectively (Robinson et al., 1980). It was concluded that PLZ or its metabolites might inhibit the metabolism of PLZ. Therefore, PLZ concentrations and hence its inhibitory effects on CYP enzymes depends on the length of therapy.

There is no information about liver concentrations of PLZ in humans. In rats, 45 minutes after intraperitoneal administration of PLZ (dose of 0.38 mmol/kg) the plasma and liver levels of PLZ have been reported to be 1144 and 958 ng/g, respectively (Coutts et al., 1991). Also, 1 hour after intraperitoneal administration of PLZ (dose of 0.2 mmol/kg) the brain levels of PLZ have been reported to be 183 ng/g (McKenna, 1995). Based on the data from rat studies, it seems that PLZ is not as lipophilic as TCP, and its liver concentration seems to be of the same order of magnitude as its plasma concentration.

For PLZ concentrations as high as 0.1 μM corresponding to the reported peak plasma concentration at a clinical dose of 30 mg, and with the determined K_i of 0.7 μM, Equation 4.2 results in a percent of inhibition equal to 12.5%. With the increased PLZ dose of 90 mg/day, which is more in line with usual clinical doses, the peak concentration might be expected to reach as high as 0.3 μM. Consequently, the percent of inhibition might be as high

as 30%. Such levels of inhibition indicate that the inhibitory effect of PLZ on CYP2C19 is probably not clinically significant. However, other factors such as patient age, the presence of medical illness, poor metabolizer status for CYP2C19, the pharmacokinetic and the therapeutic index of the co-administered drugs, affect the incidence of drug interactions and their clinical significance. Hence, PLZ should be co-administered cautiously with substrates of CYP2C19. In addition, PLZ is extensively metabolized (Robinson et al., 1985), and no studies appear to have been conducted on the effects of those metabolites on CYP enzymes.

4.5.3 Kinetics of Inhibitory Effects of TCP on CYP2C9 and its Clinical Significance

Preliminary studies demonstrated that the inhibitory effect of TCP on CYP2C9 is not time-dependent (Figure 3.39) and is reversible. Further studies demonstrated that TCP is a noncompetitive inhibitor of CYP2C9 (K_i = 56 μM). As illustrated in Figure 3.41, by increasing TCP concentrations, V_{max} values are reduced, while K_m values remained almost unchanged. The net effect of a fully noncompetitive inhibitor is to give the impression that less enzyme is present.

As described in Section 4.5.1, at usual clinical doses, the TCP plasma concentration is about 1μM. This leads to an estimated TCP liver concentration with the same order of magnitude as (and usually not

exceeding) the calculated K_i for CYP2C9. In non-competitive inhibition, the percent of inhibition depends only upon inhibitor concentrations and K_i, and with $[I]=K_i$, we observe 50% inhibition at all substrate concentrations. Therefore, at clinical doses of TCP, the percent inhibition would be less than 50%, indicating that the inhibitory effect of TCP on CYP2C9 is not usually clinically significant. In some situations, such as high dose TCP therapy (4-5 times the common clinical dose), the percent of enzyme inhibition could reach about 65%, regardless of the substrate concentration. This can cause relevant metabolic changes and clinically significant interactions, particularly when co-administrated with drugs with narrow therapeutic indices.

4.5.4 Kinetics of Inhibitory Effects of PLZ on CYP2C9 and its Clinical Significance

Preliminary studies showed that the inhibitory effect of PLZ on CYP2C9 is not time-dependent and is reversible. Further studies demonstrated that PLZ is a noncompetitive inhibitor of CYP2C9 (K_i =30 μM). As illustrated in Figure 3.46, by increasing PLZ concentrations, V_{max} values are reduced, while K_m values remained practically unchanged. Equation 4.2 can be used to determine the percent inhibition in the presence of PLZ as a noncompetitive inhibitor of CYP2C9.

As stated in Section 4.5.2, PLZ plasma peak concentrations might be as high as 0.3 μM. For the determined K_i of 30 μM, according to Equation 4.2,

the percent of inhibition at this concentration would be only 1%. Therefore, it appears that at clinical doses the inhibitory effect of PLZ on CYP2C9 is not significant.

4.5.5 Kinetics of Inhibitory Effects of TCP on CYP2D6 and its Clinical Significance

Preliminary studies, which were conducted in the absence of an NADPH generating system, showed that the inhibitory effect of TCP on CYP2D6 is not time-dependent (Figure 3.52) and is reversible. Hence, for the remaining studies, preincubation of TCP with enzyme was not required. Further studies demonstrated that TCP is a reversible two-site pure competitive inhibitor of CYP2D6 (K_i = 367 μM). Previous studies also suggest that CYP enzymes can have kinetic characteristics consistent with a two-site model (Korzekwa et al., 1998). This model suggests that the CYP enzyme can bind two substrate molecules simultaneously, probably on either side of the active oxygen species. Thus either bound substrate can be metabolized, but not at the same time. This could be extrapolated to a situation where one inhibitor and one substrate, or two inhibitor molecules, are bound on either side of the active oxygen, giving rise to two-site kinetic behavior. Many CYP enzymes can metabolize substrates of varying sizes. If an active site can accommodate a very large substrate molecule, it might be expected that more than one small molecule can bind to the active site (Korzekwa et al., 1998).

As discussed earlier, considering expected plasma concentrations of TCP and a calculated K_i of 367 μM, it seems likely that the inhibitory effect of TCP on CYP2D6 is not clinically significant. A previous study using rat liver microsomes has also shown that after a 2-hour preincubation of TCP with rat liver microsomes in the presence of NADPH, TCP did not inhibit hydroxylation of bufuralol, a substrate for CYP2D6 in humans (Dupont et al., 1987).

4.5.6 Kinetics of Inhibitory Effects of PLZ on CYP2D6 and its Clinical Significance

The preliminary studies conducted in either the presence or absence of the NADPH-generating system showed that the inhibitory effect of PLZ on CYP2D6 is not time-dependent and is reversible. Further studies have demonstrated that PLZ is a non-competitive inhibitor of CYP2D6 (K_i=0.7μM). In non-competitive inhibition, the percent of inhibition depends only on the inhibitor concentration and K_i.

As stated in Section 4.5.2, PLZ peak plasma concentrations might reach as high as 0.3 μM. For the determined K_i of 0.7 μM, according to Equation 4.2, the percent of inhibition might be as high as 30%. Such a degree of inhibition suggests that the inhibitory effect of PLZ on CYP2D6 is not clinically significant at usual therapeutic doses. However, other factors such as patient age, the presence of medical illness, poor metabolizer status

for CYP2D6, and the pharmacokinetics and the therapeutic index of the co-administered drugs, affect the incidence of drug interactions and their clinical significance. In addition, PLZ is extensively metabolized (Robinson et al., 1985), and no studies appear to have been conducted on the effects of those metabolites on CYP2D6. Hence, PLZ should be co-administered cautiously with substrates of CYP2D6.

4.5.7 Possible Interactions of TCP with CYP Enzymes in the Rat

Although the enzymes CYP2C19, CYP2C9 and CYP2D6 are not present in the rat, very similar CYP enzymes, which metabolize similar substrates, are present. Metabolic drug-drug interactions involving CYP enzymes are frequently not taken into account in animal studies in which combinations of drugs are administered. As indicated in recent studies from members of the Neurochemical Research Unit, such interactions can indeed be important and may be contributing significantly to observed behavioral effects (Sills et al., 1999a,b). At the doses of TCP that have been used in animal studies cited in the literature, levels of TCP in brain may reach high micromolar concentrations (Fuentes et al., 1976; Calverley et al., 1981) at which pharmacokinetic interactions with co-administered drugs which are substrates for CYP enzymes would be expected.

Hampson et al (1986) demonstrated that coadministration of TCP with iprindole, chlorpromazine and trifluperazine, drugs which are inhibitors or

substrates of CYP2D6 in humans, results in significantly increased brain levels of TCP. Similarly, coadministration of iprindole and PLZ in rats produces significant increases in brain levels of PLZ (McKenna et al., 1991).

It is of interest here that TCP is sometimes given in combination with antipsychotics, including chlorpromazine and trifluperazine and in Great Britain there is a commercially available preparation which is a combination of TCP and trifluperazine. There appears to have been no work done on the effects of TCP or PLZ on the coadministered drugs in such combinations.

CHAPTER 5

Summary and Conclusions

5. Summary and Conclusions

In the present project, the inhibitory effects of two non-selective irreversible inhibitors of MAO, TCP and PLZ, on three CYP enzymes, CYP2C9, CYP2C19, and CYP2D6, have been evaluated using *in vitro* models of human liver microsomes and cDNA-expressed human CYP enzymes. The project also included experiments on the inhibitory effects of individual TCP enantiomers as well as the ring-substituted analogues of TCP on CYP 2C19, and investigated the possible importance of opening of the cyclopropyl ring in the metabolism of TCP.

In order to conduct the studies described, it was necessary to develop sensitive and reproducible GC-ECD and HPLC-UV assays to measure levels of OHMEP, OHTOL, and DXR, metabolites of the probe substrates of CYP2C19, CYP2C9 and CYP2D6, respectively. Although the GC assays utilizing GC-ECD after derivatization with PFBC and PFBS were useful and sensitive procedures, the HPLC-UV assays proved to be more convenient and were used for most of the subsequent analysis in the kinetic studies. With the HPLC-UV assays, there was no need for any derivatization, and the resultant metabolites were stable at room temperature, making the procedure simple and convenient. The HPLC-UV assays are recommended for future kinetic studies involving OHMEP, DXR, or OHTOL.

Prior to any kinetic studies, a series of preliminary experiments was conducted. These studies comprised an enzyme concentration study, a time course study, a concentration curve study, and studies of time-dependence and reversibility of inhibition. These experiments were used to optimise and improve the design of experiments in the main kinetic studies.

In the principal experiments, the inhibitory potencies of PLZ, TCP, TCP enantiomers and analogues against specific CYP enzymes were screened using *in vitro* models of human liver microsomes as well as individual cDNA-expressed CYP enzymes.

In studies conducted on human liver microsomes, a fixed concentration of probe substrate for the specific CYP enzyme was co-incubated with various concentrations of the compounds of interest as potential inhibitors. The relationship of decrement in metabolite formation rate *versus* inhibitor concentration yielded an estimate for the 50% inhibitory concentration (IC_{50}). IC_{50} values are suitable for comparing the relative potencies of a series of inhibitors studied under identical conditions, and were determined in an investigation of TCP enantiomers and analogues

The results demonstrated that the two enantiomers of TCP differ in their inhibitory potency on CYP2C19. (+)-TCP is a much stronger inhibitor of CYP2C19 ($IC_{50}\approx25\mu M$) than (-)-TCP ($IC_{50}>400\mu M$). These findings on the differences of the two enantiomers of TCP are interesting and an important addition to the overall profile of the drug. Since previous studies on other

pharmacological (inhibition of MAO, effects on reuptake of biogenic amines) and pharmacokinetic properties of TCP had also shown marked differences between the two enantiomers.

It was observed that the TCP analogues OHTCP (IC_{50}≈20μM), MTCP (IC_{50}≈41 μM), and FTCP (IC_{50}≈78 μM), like TCP (IC_{50}≈50 μM) had inhibitory effects on CYP2C19. Of the TCP analogues tested, OHTCP (a metabolite of TCP) had the strongest and FTCP had the weakest inhibitory effects on CYP2C19. This finding in combination with the findings of previous studies on various neurochemical and behavioral effects of FTCP suggest that FTCP would be worth pursuing further as an antidepressant.

The type of inhibition produced by TCP and PLZ and inhibition constants were determined with studies conducted on individual cDNA-expressed CYP enzymes. In these studies, multiple substrate concentrations were co-incubated with multiple concentrations of inhibitor. Since the K_i value, unlike the IC_{50} value, is independent of substrate concentration, it is a better index of the inhibitory potency of a drug, and may be useful for predicting the potential for possible *in vivo* metabolic drug-drug interactions.

The kinetic studies demonstrated that TCP is a competitive inhibitor of CYP2C19 (K_i = 32μM) and CYP2D6 (K_i = 367μM) and a non-competitive inhibitor of CYP2C9 (K_i = 56μM). Currently, a major obstacle in determining the clinical significance of these inhibitory effects of TCP is the lack of knowledge about its liver to plasma ratio in humans. Based on the estimated

range of TCP concentrations, neither of these inhibitory effects is considered clinically significant at usual therapeutic doses. However, in certain situations such as high dose TCP therapy, or in poor metabolizers of CYP2C19 substrates, clinically significant interactions might occur, particularly when TCP is co-administrated with drugs with a narrow therapeutic index.

The kinetic studies showed that PLZ is a non-competitive inhibitor of CYP2D6 (K_i = 0.7 μM), CYP2C19 (K_i = 0.7 μM) and CYP2C9 (K_i = 30 μM). Based on limited information available on PLZ plasma concentrations, and assuming that PLZ liver concentration are of the same order of magnitude or higher than its plasma concentration, none of these inhibitory effects seem likely to be clinically relevant at usual therapeutic doses.

An important observation regarding biotransformation of TCP was that neither GC-ECD nor HPLC detected amphetamine and/or 1-amino-3-phenylpropane as a result of the incubation of TCP with human liver microsomes. These compounds are potential metabolites that could arise from cleavage of the cyclopropyl ring of TCP, and their absence suggests that the cleavage of the cyclopropyl ring is not a significant pathway for metabolism of TCP.

The studies described in this thesis have filled a gap in our knowledge of PLZ and TCP, two MAO inhibitors that have been used clinically for over 35 years. The recent availability of well characterized human liver

microsomes and individual cDNA-expressed CYP enzymes has facilitated more comprehensive studies on interactions of these two drugs with CYP than was previously possible. The results suggest that interactions of PLZ with CYP enzymes are probably not clinically relevant but that such interactions could be possible in the case of high doses of TCP. The possibility of metabolic drug-drug interactions in animal studies is often ignored, but recent findings by other researchers in the Neurochemical Research Unit and the results of this thesis indicate that such interactions with CYP enzymes could certainly be occurring at the brain and liver TCP concentrations attained in such studies.

The additional studies on TCP and its enantiomers and analogues have emphasized the importance of studying individual enantiomers of drugs and have provided further evidence that FTCP is worth pursuing as a potential antidepressant drug with some advantages over TCP.

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