INFORMATION TO USERS

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

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

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

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

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

UMI®

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UNIVERSITY OF ALBERTA

THE DEVELOPMENT AND APPLICATION OF AN ELECTRON-CAPTURE GAS CHROMATOGRAPHIC ASSAY PROCEDURE FOR THE SIMULTANEOUS DETERMINATION OF SERTRALINE AND N-DESMETHYLSERTRALINE IN BIOLOGICAL SAMPLES

by

CHERIE LEANNE KLASSEN 

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

SPRING 1999

UNIVERSITY OF ALBERTA

LIBRARY RELEASE FORM

NAME OF AUTHOR:	Cherie Leanne Klassen
TITLE OF THESIS:	The development and application of an electron-capture gas chromatographic assay procedure for the simultaneous determination of sertraline and N-desmethylsertraline in biological samples
DEGREE:	Master of Science
YEAR THIS DEGREE GRANTED:	1999

Cherie Klassen

Box 746
Stettler, Alberta
T0C 2L0

DATED: January 29, 1999

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: THE DEVELOPMENT AND APPLICATION OF AN ELECTRON-CAPTURE GAS CHROMATOGRAPHIC ASSAY PROCEDURE FOR THE SIMULTANEOUS DETERMINATION OF SERTRALINE AND N-DESMETHYLSERTRALINE IN BIOLOGICAL SAMPLES hereby submitted by CHERIE LEANNE KLASSEN in partial fulfilment of the requirements for the degree of Master of Science.

G.B. Baker (Supervisor)

A.N. Bateson (Co-Supervisor)

R.T. Coutts (Committee Member)

W.F. Dryden (Examiner and Chair)

DATED: November 20, 1998

DEDICATION

This thesis is dedicated to my parents, Walter and Rita Klassen, and to my grandparents for the love, support and encouragement they have provided unconditionally throughout my life.

ABSTRACT

This thesis presents a rapid, sensitive gas chromatographic assay with electron-capture detection (GC-ECD) for the simultaneous analysis of sertraline (SER) and N-desmethylsertraline (DMS). This assay was applied to studies using rat brain tissue, human liver microsomes, microsomes expressing individual cytochrome P450 (CYP) enzymes and human plasma samples.

As cases of serious metabolic drug-drug interactions have been reported involving the SSRIs, it is important to elucidate the relative involvement of specific CYP enzymes and the relevance of their inhibition on the metabolism of SER. The experiments described in this thesis examined which CYP enzymes may be involved in SER metabolism. This work demonstrates that this novel GC-ECD assay will be a very valuable tool for future basic science and clinical studies on SER.

ACKNOWLEDGEMENTS

To my supervisor, Dr. Glen Baker, I extend a heartfelt thank you for providing me with exceptional guidance and tutelage during my time at the Neurochemical Research Unit; his commitment to excellence and personal integrity is something that I aspire to. I also extend my sincere gratitude to my co-supervisor, Dr. Alan Bateson, for his support and insights, to Dr. Ron Coutts for serving as a member of my supervisory and examining committees, and to Dr. William Dryden for serving as my thesis defense examiner and chair.

I also wish to thank Gail Rauw, for her endless patience, knowledge and guidance, and Jordyce Van Muyden and Pips Wolfaardt for their superb technical and administrative support. I will forever cherish the friendships I have formed while at the NRU, and especially appreciate the time spent in conversation and laughter with my officemates and friends; Teresa Paslawski, Penny Colburne and Susan Rotzinger. I am grateful for the constant support and encouragement I received from my family, and for the joy my nephews, Alejandro and Nicolas Luy have given me.

Finally, I would like to thank the Department of Pharmacology, the Faculty of Medicine, the Faculty of Graduate Studies and Research, and the Alberta Heritage Fund for Medical Research for financial assistance throughout my program.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ANOVA	analysis of variance
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CIT	citalopram
CPS	Compendium of Pharmaceuticals and Specialties: 15th Edition
CNS	central nervous system
CV	coefficient of variation
CYP	cytochrome P450
°C	degrees Celsius
DM	dextromethorphan
DMS	N-desmethylsertraline
DMSO	dimethylsulfoxide
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders: 4th Edition
ECD	electron capture detector
EI	electron impact
EMs	extensive metabolizers
eV	electron volt(s)
5-HT	5-hydroxytryptamine; serotonin
FID	flame ionization detector
FLU	fluoxetine
FLV	fluvoxamine
g	gravity

GC	gas chromatography
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
HHM	human hepatic microsomes
HP	Hewlett Packard
HPLC	high performance liquid chromatography
i.p.	intraperitoneal
I.S.	internal standard
kg	kilogram(s)
M	molar
MAOIs	monoamine oxidase inhibitors
max	maximum
min	minute(s)
mg	milligram(s)
ml	millilitre(s)
mM	millimolar
mol	mole(s)
mRNA	messenger ribonucleic acid
MS	mass spectrometer
ms	millisecond(s)
m/z	mass/charge
NA	noradrenaline (norepinephrine)
$NADP^+$	nicotinamide adenine dinucleotide phosphate

NADPH	nicotinamide adenine dinucleotide phosphate, reduced
ng	nanogram(s)
nmol	nanomole(s)
NPD	nitrogen-phosphorus detector
OCD	obsessive compulsive disorder
OR	oxido-reductase
p	probability
PAR	paroxetine
%	percentage
% RA	percent relative abundance
PFP	pentafluoropropionyl
PFPA	pentafluoropropionic anhydride
pg	picogram(s)
PMs	poor metabolizers
SCOT	support-coated open tubular
SEM	standard error of the mean
SER	sertraline
SIAHD	syndrome of inappropriate secretion of antidiuretic hormone
SSRIs	selective serotonin reuptake inhibitors
TCAs	tricyclic antidepressants
TCD	thermal conductivity detector
µg	microgram(s)
µl	microlitre(s)

μM	micromolar
μm	micrometre(s)
UM	ultra-rapid metabolizer
WCOT	wall-coated open tubular

1.0 INTRODUCTION

1.1 Depression and its treatment

With estimates between 4.4 and 19.5% for lifetime prevalence rates (Van Moffaert et al., 1995; Eap and Baumann, 1996), depressive disorders are common in the general population. Patients diagnosed with major depressive disorder are also likely to experience relapses (Finley, 1994). The symptoms of depression, including depressed mood and/or significantly diminished interest or pleasure in daily activities, cause individuals distress and impair functioning in social, occupational and other important roles. This is a potentially life-threatening illness, as the suicide rate among afflicted individuals is thirty times higher than among the general population (DSM-IV, 1994).

The biochemical basis of depression is complex and, at present, poorly understood. However, observations over the years have lead to the development of the hypothesis that absolute or relative deficiencies of the monoamines serotonin (5-hydroxytryptamine, 5-HT) and noradrenaline (NA) in the central nervous system (CNS) are responsible for endogenous depression (Baker and Greenshaw, 1989; Coppen and Doogan, 1988; Heym and Koe, 1988). Although different classes of antidepressant drugs have different mechanisms of action, preclinical and clinical evidence indicates that it is likely the therapeutic effects of most of these drugs are due to an enhancement of monoamine, particularly 5-HT, mediated neurotransmission (Blier et al., 1987; Blier and de Montigny, 1998). Although increased functional availability of

monoamines generally occurs within hours of the first administration of an antidepressant, there is an observed latency of clinical response of three weeks or more from the start of treatment (Doogan and Caillard, 1988). This delay has led to the suggestion that the increase in monoamine neurotransmission is only an initiating event, and the therapeutic actions of these drugs result from slower adaptive changes in response to this effect (Finley, 1994). Chronic administration of antidepressants has been shown to also affect monoamine receptor binding and lead to alterations in transduction pathways downstream of the interaction at the receptor (Montgomery, 1995; Bourin and Baker, 1996), for example, changes in adenylate cyclase activity (Baker and Greenshaw, 1989; Shelton et al., 1996). Changes in the genetic expression of these receptors or their associated second messenger systems (Toth and Shenk, 1994; Tilakaratne et al., 1995; Schwaninger et al., 1995) may be another mechanism of antidepressant action. These observations have resulted in the broadening of the hypotheses on the pathophysiology and pharmacotherapy of depression and an increased appreciation for the complexities of this illness (Duman et al., 1997; Nemeroff, 1998).

1.1.1 **Traditional treatment**

Traditional pharmacotherapy for the treatment of depression consisted of the "first generation" antidepressants, which include the monoamine oxidase inhibitors (MAOIs) and the tricyclic antidepressants (TCAs) (Preskorn, 1993).

The MAOIs enhance the actions of biogenic amines by blocking their intracellular metabolism (Heym and Koe, 1988). The TCAs act by blocking neuronal reuptake of the monoamines, thereby increasing the concentration of the amines in the synapse, making more available to act post-synaptically (Hyman et al., 1995). Unfortunately, treatment with the traditional antidepressant classes generally also meant a long list of undesirable side effects including central nervous system (CNS) and cardiovascular toxicity, orthostatic hypotension, sedation, urinary retention and memory impairment (Preskorn, 1993). Additionally, treatment with the older irreversible MAOIs is plagued with the potential for the life-threatening adverse effect known as the "cheese effect". This involves symptoms ranging from headaches to hypertensive crises secondary to pharmacodynamic interactions between irreversible MAOIs and tyramine-rich foods such as red wine and cheese or sympathomimetic drugs including pseudoephedrine (which is frequently found in over-the-counter medications). Consequently, patients taking irreversible MAOIs must avoid these products. However, the introduction of reversible, selective inhibitors of MAO-A has largely eliminated this concern (Preskorn, 1993; Bieck and Antonin, 1989).

1.1.2. **The Selective Serotonin Reuptake Inhibitor (SSRI) class of antidepressants**

The SSRI family represents the first group of rationally developed psychopharmaceuticals (Preskorn, 1997) and includes fluoxetine (FLU),

fluvoxamine (FLV), paroxetine (PAR), sertraline (SER) and citalopram (CIT) (Stahl, 1993). These drugs are part of the group of "third generation" antidepressants and, as their name implies, they selectively block the 5-HT transporter (Hyman et al. 1995). They followed the "second generation" antidepressants, introduced in the mid-1970s. These "second generation" antidepressants, which included trazodone, mianserin and viloxazine, differed from the traditional antidepressants in that they did not have potent effects on reuptake of 5-HT or NA or inhibit MAO, but their basic mechanisms of action were not entirely clear (Damlouji et al., 1985).

The SSRI class of drugs has become one of the most widely prescribed for the treatment of depressive disorders (DeVane, 1995). Their popularity is due in part to efficacies that are comparable to or better than seen with TCA treatment (DeVane, 1995; Tollefson, 1995) and a larger group of potential indications, including obsessive-compulsive disorder (OCD), panic disorder, eating disorders, substance abuse and chronic pain syndrome (Tollefson, 1995; Stahl, 1993). Although not free of adverse effects, the SSRIs possess a superior side effect and safety profile when compared to the TCAs or MAOIs, which translates into greater patient compliance (DeVane, 1995; Tollefson, 1995; Preskorn, 1993). This reduction of side effects is due to the lack of activity of the SSRIs at histaminergic, α_1-adrenergic and muscarinic receptors (Tollefson, 1995; Finley, 1994). The most common adverse effects seen with SSRIs are nausea, vomiting and diarrhea, insomnia, agitation and headache, sexual dysfunction and tremor (Finley, 1994, DeVane 1995) due to excessive blockade of peripheral and

central 5-HT uptake (Preskorn, 1993). While undesirable, the effects of SSRI overdose are generally well tolerated and are not fatal, indicating a wide therapeutic index and a much greater safety in overdose compared to the traditional antidepressants (Finley, 1994; Preskorn, 1993).

Although the SSRIs each possess a similar mechanism of action, they exhibit pharmacokinetic variability with respect to extent of absorption, protein binding, time to peak concentration, elimination half-life, and apparent volume of distribution (Finley, 1994; Baumann, 1996).

1.2 **Sertraline (SER)**

SER is marketed by Pfizer under the trade name Zoloft® and, as mentioned above, is a member of the SSRI class of antidepressants. (Koe et al., 1983).

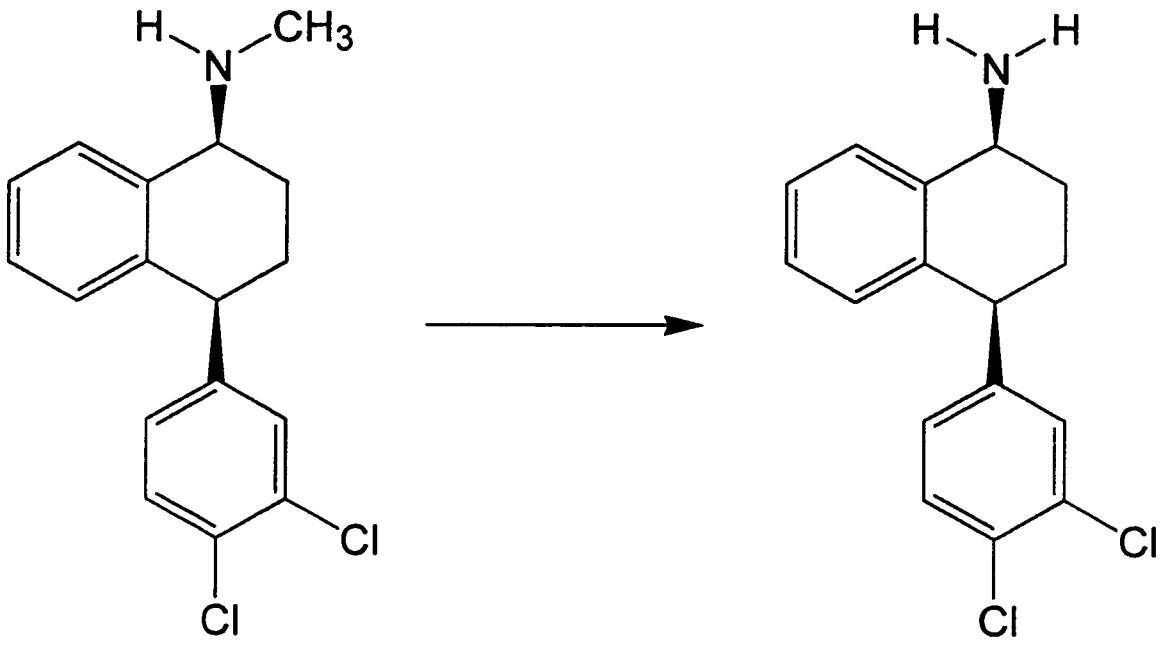
1.2.1. **Chemistry**

SER possesses a naphthylamine structure (Montgomery, 1995) and is marketed as a single enantiomer (Preskorn, 1997). Its chemical name is (+)-(1S,4S)-N-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthylamine (Koe et al., 1983; Heym and Koe, 1988), and its molecular weight is 306.23 (Merck Index, 1989). The structures of SER and its N-demethylated metabolite N-desmethylsertraline (DMS) are shown in Figure 1.

1.2.2. **General pharmacology**

1.2.2.1 **Pharmacodynamics**

Like the other SSRIs, SER has negligible affinity for cholinergic, histaminergic, dopaminergic, α_1-, α_2- or ß-adrenoreceptors *in vitro* (Doogan and Caillard, 1988; Koe et al., 1990; Montogomery, 1995). SER has the highest selectivity of the SSRIs for inhibiting 5-HT versus NE reuptake (Preskorn, 1993). Chronically administered, SER appears to possess an ability common to the SSRIs to desensitize the 5-HT autoreceptors involved in serotonergic neuronal feedback mechanisms (Blier et al., 1987; Briley and Moret, 1993; Tollefson, 1995). Consequently, SER's effect on 5-HT feedback systems would allow for the re-establishment of a normal neuronal firing rate as well as enhanced 5-HT release while reuptake inhibition continues, ultimately leading to increased synaptic 5-HT (Blier et al., 1987; Briley and Moret, 1993; Tollefson, 1995). SER is also noted, albeit at high doses, to produce down regulation of ß-adrenoreceptors and a decreased production of the second messenger cAMP stimulated by these receptors (Doogan and Caillard, 1988; Finley, 1994); however, the importance of this mechanism with regard to SER's antidepressant effect remains a subject of controversy. Another hypothesis to explain SER's mechanism of action is its ability to alter transduction pathways downstream of the interaction at the receptor. This was demonstrated in a radioligand study



SERTRALINE **N-DESMETHYLSERTRALINE**

Figure 1: The chemical structures of the SSRI SER and its major metabolite, DMS.

where SER was reported to induce a decrease in 5-HT-stimulated inositol phosphate formation (Sanders-Bush et. al., 1989). Additionally, Morinobu *et al.* (1995) suggest that chronic SER treatment has an effect on gene expression of certain effector systems, including *c-fos* mRNA. These theories attempt to reconcile SER's mechanism of action with the observed latency in therapeutic efficacy. They also demonstrate the true complexities of the antidepressant mechanism of action and the disorder itself. Although much headway has been made in this area recently, much work remains. Fortunately, there is considerable interest in pursuing this research.

1.2.2.2 **Pharmacokinetics**

SER's absorption from the gastrointestinal tract is >44%, and SER is >97% protein bound. SER exhibits an elimination half-life of approximately 26 hours; the half-life of DMS is approximately 66 hours. The peak plasma concentration of SER is reached in patients within 6 to 10 hours (Finley, 1994); plasma concentrations of DMS are maximal in 8 to 12 hours (Doogan and Caillard, 1988). SER's apparent volume of distribution in human subjects is approximately 20kg/L (Doogan and Caillard, 1988).

Unlike FLU and PAR, SER exhibits linear pharmacokinetics (proportional changes in plasma concentration levels with changes in dosages) in humans

(Preskorn, 1993; Preskorn, 1997). However, age and gender affect the pharmacokinetics of SER (Preskorn, 1996a). The half-life is shorter and the plasma concentrations of SER are generally 35 to 40% lower in young males than in elderly males and younger or elderly females (Ronfeld *et al.*, 1997).

1.2.2.2.1 **Metabolism**

SER undergoes extensive hepatic metabolism to the dominant metabolite N-DMS (Murdoch and McTavish, 1992). This metabolite, although only one-tenth as active as SER (Tollefson, 1995), is also selective for inhibition of reuptake of 5-HT (Koe et al., 1983; Heym and Koe, 1988). The metabolism of SER appears to be linear (Finley, 1994), and unlike FLU, FLV, and PAR, SER does not inhibit its own metabolism (Preskorn, 1997). Both SER and DMS are further metabolized to a ketone, with subsequent hydroxylation to major excretory metabolites; the α-hydroxy ketone appears in the urine and feces. Minor metabolites include a ketone, an alcohol and an oxime (Doogan and Caillard, 1988).

1.2.3. **Clinical efficacy**

The clinically effective dose range of SER is 50 to 200 mg/day (Preskorn and Lane, 1995), although increases in therapeutic benefit with increased dose tend to be small (Finley, 1994). If an increase in dosage is required, SER should

be increased in 50mg/day increments at intervals of at least one week to the 200mg/day maximum (Preskorn and Lane, 1995). SER (50mg/day) is as efficacious as FLU at 20mg/day (Montgomery, 1995; Van Moffaert et al., 1995), and patients suffering from depression that have discontinued FLU due to intolerable side effects have been successfully treated with SER (Rogowsky et al., 1994; Haider et al., 1995; Brown and Harrison, 1995).

SER has also been found to be useful in the pharmacotherapy of anxiety disorders (Nutt, 1995; Moon et al., 1993) and has recently been approved for the treatment of panic disorder and OCD in Canada (CPS, 1997). Considerable indirect evidence suggests 5-HT plays a role in the aetiology of OCD (Chouinard, 1992), a disorder characterized by disturbing, unwanted, anxiety-provoking thoughts or ideas and impulses to perform repetitive acts which may be considered abnormal, undesirable, or distasteful to the patient (DSM IV, 1994). OCD is estimated to have a lifetime prevalence of 2 to 3% of the general population (Chouinard, 1992).

SER has been reported to be a particularly suitable agent for the management of major depression in the elderly (Arranz and Ros, 1997) and has also demonstrated efficacy in treating social phobia (Katzelnich et al., 1996). It has been indicated for the prevention of relapse of depressive illness due to its effectiveness in long-term therapy (Montgomery, 1995). Presently, SER is licensed and marketed in the United Kingdom for such long-term treatment (Murdoch and McTavish, 1992).

1.2.4 **Adverse reactions**

Unlike the TCAs, SER has been shown to be effective as an antidepressant without sedative, cardiovascular, anticholinergic, or antidopaminergic side effects (Doogan and Caillard, 1988). Therefore SER is considered to be more tolerable (Ronfeld et al., 1997) and advantageous in the treatment of depression of compromised patients, particularly the elderly (Preskorn, 1993). Similar to all the SSRIs, side effects reported with SER include gastrointestinal effects (cramps, diarrhea), nausea and male sexual dysfunction (DeVane, 1995), with an incidence of 10 to 20% (Zoloft® Product Monograph, 1994). There is some indication that the incidence of initial anxiety as a side effect is decreased when treatment for depression is with SER rather than the other SSRIs (Nutt, 1995). SER is relatively safe in overdose (Finley, 1994).

One adverse effect that SER may share with the TCAs, however, is the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) with associated convulsions secondary to hyponatremia. Goldstein et al. (1996) report that a dysthymic patient treated with SER experienced a new-onset grand mal seizure during a transient episode of SIADH, which corroborated previous reports of SER-induced SIADH and convulsions. This side effect occurs rarely (DeVane, 1995).

1.3 **Drug metabolism**

1.3.1 **Human cytochrome P450 (CYP) enzymes**

The cytochrome P450 (CYP) enzyme system is responsible for the metabolism of endogenous and exogenous chemicals, including numerous drugs, prostaglandins, fatty acids and steroids (DeVane, 1994; Nemeroff et al., 1996). Oxidative metabolism renders these compounds more water-soluble and thus more readily excretable (Harvey and Preskorn, 1996a). Currently, at least 14 families of CYP enzymes have been identified as being common to all mammals (Nelson et al., 1996). CYP is particularly abundant in the liver, but is also expressed in gut mucosa and lung, brain and kidney tissue (Brøsen, 1996). This enzyme system is located in the smooth endoplasmic reticulum (DeVane, 1994; Harvey and Preskorn, 1996a).

At approximately 25% of the total CYP present in human adult liver, CYP3A is the most abundant of the isoform subfamilies (Ereshefsky et al., 1996). CYP2D6 makes up under 2% of the identifiable cytochromes in the human liver (von Moltke et al., 1995), but plays an important role in the metabolism of many basic drugs.

The term P450 was based on the initial identification of CYP as a red liver pigment (P) which produced a characteristic spectrophotometric absorption peak near 450nm when reduced and bound to carbon monoxide (Garfinkel, 1958; Omura and Sato, 1962; Glue and Banfield, 1996). Each CYP isozyme has an approximate molecular weight of 50kD (Spatzenegger and Jaeger, 1995).

1.3.1.1 **Genetic diversity of CYP enzymes**

The ancestral gene for CYP is thought to have been in existence before the divergence of prokaryotes and eukaryotes more than 3.5 billion years ago (Harvey and Preskorn, 1996a). During the past 800 million years there has been a vast expansion of CYP isoforms within eukaryotes. The reason for this has been postulated as "plant-animal warfare". As animals and plants diverged, the animals began to ingest the plants, and in retaliation the plants developed toxins to render them less palatable. Thus, new forms of CYP evolved to break down these plant toxins (Soucek and Gut, 1992; Harvey and Preskorn, 1996a). Today, each CYP enzyme is the product of a separate gene, and a number of these genes have multiple alleles, resulting in genetic polymorphism in the population (DeVane, 1994). In individuals, some of these polymorphisms may result in dysfunctional enzymes, and these patients will phenotypically present as 'poor metabolizers' (PMs), as opposed to 'extensive' or 'normal metabolizers' (EMs), due to their inability to efficiently biotransform drugs requiring those specific enzymes (Riesenman, 1995; DeVane, 1994). This phenomenon is termed genetic polymorphism if each allele exists in at least 1% of the population (DeVane, 1994), although the frequency with which polymorphism occurs differs between ethnic groups (Bertz and Granneman, 1997). In humans, polymorphism has been demonstrated for CYP2A6, CYP2D6, CYP2C19 (Ereshefsky et al., 1996) and CYP2E1 (DeVane, 1994), although not for CYP3A (Bertz and Granneman, 1997; von Moltke et al., 1995). Ultra-rapid metabolizers (UMs), with

expression of multiple genes for CYP2D6, have also been reported (Agúndez et al., 1995).

Known substrates for specific enzymes are used as 'probe' drugs in order to determine individual phenotypes, as well as to determine the effect of other drugs on the specific enzymes (Riesenman, 1995). Assessing an individual's phenotype can be helpful when selecting an appropriate drug dosage to initiate therapy (Coutts et al., 1994; Brøsen, 1996). At a dose generally accepted as effective, EMs (at the high range of normal) and UMs may not see a therapeutic response due to low plasma concentrations of a drug. Conversely, PMs may display side effects at a higher frequency than the normal population due to an accumulation of the parent drug (DeVane, 1994; Ereshefsky et al., 1996; Bertz and Granneman, 1997). It should be noted that interindividual differences in CYP enzyme activity are influenced by environmental and constitutional factors such as diet, lifestyle, age and gender, in addition to genetic disposition (Brøsen, 1996; Glue and Banfield, 1996).

1.3.1.2 **Classification and nomenclature**

More than 200 CYP genes (DeVane, 1994) and more than 30 human CYP enzymes (Ereshefsky et al., 1996) have been identified. CYP enzyme cDNAs have been cloned and sequenced over the past few years (Brøsen, 1996), permitting a unifying classification system to be developed (Harvey and Preskorn, 1996a). The enzymes of the human CYP system are grouped on the

basis of amino acid homology (DeVane, 1994) using a three-tiered classification system (Ereshefsky et al., 1996): families (demonstrating >40% homology in their amino acid sequences); subfamilies (>55% sequence homology); and individual genes (Riesenman, 1995; Harvey and Preskorn, 1996b). Nomenclature is as follows: the root 'CYP', the family number, the subfamily letter, and the number of the individual isoform (arbitrarily assigned); for example, CYP2D6 (Devane, 1994; Ereshefsky et al., 1996).

1.3.1.3 **Structure of CYP enzymes**

The CYP enzymes are a family of apoproteins (Glue and Banfield, 1996). The structure of the CYPs permits two binding modes: substrate binding (a productive binding to the hydrophobic substrate binding site) and ligand binding (an inhibitory binding to the thiolate-bound heme iron) (Testa, 1990; Soucek and Gut, 1992). CYPs require both oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) for catalytic activity (Harvey and Preskorn, 1996), and during catalysis oxygen is activated while the substrate is not (Soucek and Gut, 1992). Variant primary sequences and the resultant secondary structures are responsible for the different substrate specificities (Guengerich and MacDonald, 1990; Soucek and Gut, 1992).

1.3.1.4 **Mechanism of action of CYP enzymes**

Most drugs undergo biotransformation to more readily excretable metabolites *via* oxidative phase I and conjugative phase II metabolic reactions (DeVane, 1994; Riesenman, 1995). The hepatic CYP enzymes are involved in a variety of the phase I reactions, including hydroxylation, demethylation and dealkylation (Riesenman, 1995).

CYPs catalyze sequential electron transfer oxidations (Guengerich and MacDonald, 1990). The general reaction catalyzed by the CYPs is as follows: $NADPH + H^+ + O_2 + substrate\text{-}H \rightarrow NADP^+ + H_2O + substrate\text{-}OH$ (Spatzenegger and Jaeger, 1995). This reaction consists of at least seven steps: 1) Substrate binding to the ferric form of the enzyme; 2) Reduction to the ferrous form by NADPH-CYP reductase; 3) Binding of O_2; 4) Introduction of a second electron from CYP reductase or cytochrome b_5; 5) Dioxygen bond cleavage, releasing H_2O and forming the active oxidizing species; 6) Substrate oxygenation; and 7) Product release (Halpert, 1995). Any variation in this mode of operation is probably a function of substrate structural features (Guengerich and MacDonald, 1990).

1.3.2 **Drug metabolism and the CYP enzymes**

1.3.2.1 **The CYP enzymes**

Enzymes in the first three families, CYP1 – 3, are considered to be important in the metabolism of drugs (Riesenman, 1995), although the exact enzymes involved in the metabolism of most drugs still remain undefined (DeVane, 1994). The CYP system permits some substrate nonspecificity (Harvey and Preskorn, 1996a). Subsequently, more than one enzyme may be involved in the metabolism of a particular drug (Hansten and Horn, 1996). It has been estimated that CYP3A has some involvement in the metabolism of more than 50% of drugs in humans due to a binding site that accommodates molecules of varying size, charge and lipophilicity (Bertz and Granneman, 1997). In contrast, most substrates of CYP2D6 possess an extended hydrophobic region, a positively charged basic nitrogen, groups with a negative potential, and the ability to accept hydrogen bonds (Bertz and Granneman, 1997). The CYP2A and CYP2B subfamilies have not been studied in detail (Harvey and Preskorn, 1996a).

1.3.2.2 **Drug metabolism and CYP inhibition**

A drug may be a substrate of a specific CYP enzyme and/or alter the activity of the enzyme by inhibition or induction (Brøsen, 1996; Riesenman, 1995). Consequently, if a combination of drugs is administered, and each drug

acts upon a specific enzyme in one or more ways, the possibility of drug-drug interactions arises (Harvey and Preskorn, 1996a; Riesenman, 1995). Therefore, knowledge of prospective drug-drug interactions is imperative. Theoretically, this may be assessed using information about the inhibitory potency (K*i*) for a particular CYP isoform (obtained from *in vitro* data) and the nature of the metabolism of the potentially co-administered drugs and their active metabolites (Bertz and Granneman, 1997). Although the focus of drug interactions is upon increased toxicity, it should be noted that loss of efficacy may occur *via* enzyme inhibition if the medication administered is a pro-drug requiring metabolic activation (Ereshefsky et al., 1996) or *via* enzyme induction if a medication's concentration is reduced below therapeutic levels (Harvey and Preskorn, 1996a).

Inhibitors of the CYP enzymes act by a variety of mechanisms. Reversible mechanisms include competitive, noncompetitive, uncompetitive or mixed inhibition (Halpert, 1995). Irreversible inhibition occurs with mechanism-based inhibitors (substrates that when converted by CYP to a reactive species bind covalently to the enzyme) (Murray, 1987; Testa, 1990), and with drugs that possess the ability to degrade CYP450 to the inactive CYP420 [for example, coumarin derivatives (Murray, 1987)]. Most antidepressants and antipsychotics are either metabolized by, or inhibit to varying degrees, one or more CYP enzyme(s) (Nemeroff et al., 1996). Table 1 provides a selected list of substrates and inhibitors of the various CYP enzymes.

Beyond drug-drug interactions, certain dietary constituents also significantly inhibit the CYP enzymes. The flavonoid, naringin, and its aglycone,

naringenin, are compounds found in grapefruit juice and inhibit the enzymes CYP1A1/2, CYP2A6 and CYP3A3/4 (Bertz and Granneman, 1997; Merkel et al., 1994).

1.3.2.2.1 **Metabolism of SER**

In a study of EMs and PMs of debrisoquin (a probe drug for CYP2D6), no significant differences in the pharmacokinetics of SER were demonstrated. This finding suggests that CYP2D6 does not play an important role in SER demethylation (Hamelin et al., 1996). To date, the specific CYP enzymes involved in the metabolism of SER have not been identified, although data from unpublished *in vitro* experiments indicate that CYP3A involvement is more likely than that of CYP2D6, with correlation coefficients of r=0.93 and r=0.53, respectively (Harvey and Preskorn, 1996b).

CYP enzyme	Substrates	Inhibitors	References
CYP1A2	Phenacetin Caffeine Theophylline Imipramine Clozapine	FLV Erythromycin	DeVane (1994) Nemeroff et. al. (1996) Hansten and Horn (1996)
CYP2A6	Nicotine Coumarin	Imidazoles Methoxsalen Pilocarpine Grapefruit juice	Merkel et al. (1994) Bertz and Granneman (1997)
CYP2C **CYP2C19** **CYP2C9**	Tolbutamide (S)-Warfarin Phenytoin Diazepam Mephenytoin	FLU Tranylcypromine FLV	DeVane (1994) Parkinson (1996) Hansten and Horn (1996) Nemeroff (1996)
CYP2D6	Antiarrhythmic agents β-Adrenergic antagonists Morphine derivatives Neuroleptics SSRIs TCAs MAOIs	FLU Norfluoxetine PAR Quinidine	DeVane (1994) Harvey (1996a) Nemeroff et al. (1996) Hansten and Horn (1996) Preskorn (1996a)
CYP3A4	TCAs Nifedipine and other dihydropyridines Erythromycin Cyclosporin Quinidine Carbamazepine Benzodiazepines	FLV FLU Norfluoxetine Ketoconazole Quinidine Erythromycin Grapefruit juice	DeVane (1994) von Molke et al. (1995) Riesenman (1995) Nemeroff et al. (1996) Hansten and Horn (1996)

Table 1: Some substrates and inhibitors of various CYP enzymes.

1.3.2.3 **Pharmacokinetic drug – drug interactions involving the CYP enzyme system**

1.3.2.3.1 **Clinical implications of pharmacokinetic drug-drug interactions involving psychiatric drugs**

There are various reasons why psychiatric patients may be prescribed a multiple drug regimen. A proportion of depressed patients do not respond to traditional antidepressant treatment and may require therapy with a combination of antidepressants (Bakish et al., 1995; Sokolov and Joffe, 1995) before experiencing any therapeutic benefit. Alternately, patients may be switched from one antidepressant to another (DeVane, 1994). Further, combination therapy (polypharmacy) with other psychotropic drugs is commonly used in the treatment of co-morbid psychiatric conditions, recurring or resistant symptoms, or to manage adverse effects of the primary drug (Rosenbaum, 1995; Preskorn, 1996a). Psychiatric patients are at greatest risk for metabolic interactions when drug therapy is initiated or terminated, or the dosage or drug treatment is changed, particularly if the washout period for the original drug is insufficient (DeVane, 1994). Another consideration is the treatment of the elderly, a population in which depression is common (Rosenbaum, 1995). Many elderly patients are simultaneously undergoing pharmacotherapy for other medical conditions (Richelson, 1997). Finally, as many of the patients diagnosed with major depressive disorder require long term therapy with psychotropic drugs, the likelihood of co-therapy for other medical conditions increases (Rosenbaum,

1995). Along with the potential of serious adverse effects due to pharmacodynamic interactions between drugs (Bakish et al., 1995), pharmacokinetic drug-drug interactions must be considered (Rosenbaum, 1995). It should be noted that, in certain instances, polypharmacy may be used intentionally to produce controlled, beneficial interactions, for example, coadministration of a TCA and a SSRI (Harvey and Preskorn, 1996b).

As mentioned above, polypharmacy resulting in enzyme inhibition may lead to adverse effects due to toxic increases in blood levels of one or more of the drugs being co-prescribed (Ereshefsky et al., 1996). In this case, careful monitoring of drug plasma levels is strongly advised, especially when an agent has a narrow therapeutic window (Tollefson, 1995, Harvey and Preskorn, 1996b). In order not to dismiss a patient as unusually sensitive to the adverse effects of an affected drug, it is desirable that clinicians be aware of the presentation of pharmacokinetic drug-drug interactions, that is, changes in the nature of the patient's response are more likely to be quantitative than qualitative (Preskorn, 1996).

1.3.2.4.2 **Pharmacokinetic drug-drug interactions involving the SSRIs**

In the last few years it has become apparent that the SSRIs interfere with the metabolism of other drugs by inhibiting the CYP class of enzymes responsible for their oxidation (Brøsen, 1996).

As the coadministration of the TCAs with the SSRIs is fairly common in the treatment of depression (Perry, 1996), it is of importance to know that both FLU and PAR potently inhibit CYP2D6, which is the enzyme responsible, in part, for the metabolism of the TCAs (Preskorn, 1996a; von Moltke et al., 1995). FLV is a moderate inhibitor of this enzyme (Riesenman, 1995), and produces a clinically meaningful inhibition of CYP1A2, CYP2C19 and CYP3A3/4 (Preskorn, 1996a; Ereshefsky et al., 1996). FLU also produces clinically significant inhibition of CYP3A3/4 (Ereshefsky et al., 1996), CYP2C19, and CYP2C9 (Preskorn, 1996a). FLU's inhibition of CYP2C9 has been shown to affect the metabolism of phenytoin, an anticonvulsant. Warfarin is also a substrate of CYP2C9 and its plasma levels should be monitored when coadministered with FLU (Riesenman, 1995). FLU's active metabolite norfluoxetine is also a potent inhibitor of CYP2D6 in liver microsomes (Rasmussen et al., 1995) and also affects the hydroxylation of alprazolam, which is mediated by CYP3A4 (von Moltke et al., 1995).

Several clinical cases of serious adverse reactions involving co-administration of these SSRIs with TCAs and neuroleptics (antipsychotics) have been reported (DeVane, 1994; Otton et al., 1993; Harvey and Preskorn, 1996a). Vigilance in drug monitoring is suggested when certain SSRIs are concomitantly given with flecainide, quinidine, carbamazepine, beta-blockers (Riesenman, 1995), some of the benzodiazepines (Harvey and Preskorn, 1996a; Ereshefsky et al., 1996), and several antipsychotics (Tollefson, 1995). It is advantageous for the clinician to recall the differing patterns of CYP enzyme inhibition among

SSRIs to assist in choosing an appropriate SSRI when a patient requires treatment with multiple drugs (Glue and Banfield, 1996). For example, an individual on multiple medications including PAR was reported to have developed symptoms consistent with the serotonin syndrome (diaphoresis, tremor, confusion, restlessness) after using an over-the-counter cold medicine containing dextromethorphan (DM). This was diagnosed as a pharmacodynamic interaction between PAR and DM, as both are known to potentiate 5-HT in the CNS (Skop et al., 1994). Harvey and Burke (1995) suggested that, in actuality, an interaction at CYP2D6 had occurred due to PAR's potent inhibition of this isozyme. Further, they proposed that FLV and SER are less likely than PAR and FLU to lead to a serotonin syndrome if taken in combination with DM, due to less significant inhibition of CYP2D6 (Harvey and Burke, 1995).

1.3.2.4.3 **Pharmacokinetic drug-drug interactions involving SER**

Controversy exists over the clinical significance of the inhibition of the CYP enzyme system by SER. A case was reported where a patient co-administered SER and the TCA desipramine experienced tremor and anxiety to such an extent that the treatment was discontinued and the individual suffered a relapse into a depressive episode (DeVane,1994). Crewe *et al.* (1992) suggest SER has a consequential effect on CYP2D6, and Nemeroff *et al.* (1996) and Glue and Banfield (1996) mention SER's inhibition of CYP2C. Other studies propose that SER interacts with CYP3A isoforms with the potential of causing

drug interactions (Schmider et al., 1995; von Moltke *et al.*, 1995). Alternatively, Rapeport *et al.* (1996b,c) found an absence of any SER-mediated pharmacokinetic or pharmacodynamic effects on carbamazepine or phenytoin. Preskorn (1996a) noted that this finding suggests a lack of clinically meaningful effect of SER on either CYP3A3/4 or CYP2C9/10. *In vitro*, SER was not found to affect CYP1A2, CYP2A6 or CYP2E1 (Rasmussen et al., 1995). Generally, SER is considered safer than other SSRIs with respect to the inhibition of CYP1A2, CYP2C9/10, CYP2C19, CYP2D6 and CYP3A3/4 (Baumann,1996; Harvey and Preskorn, 1996b; Richelson, 1997). This may make SER advantageous in the treatment of compromised patients, for example, the elderly (Preskorn, 1993; 1994). Similarly, SER's metabolite DMS was predicted to not contribute significantly to the clinical inhibition of CYP3A-mediated metabolism, based on the *in vitro* studies of Ring and Binkley (1995).

Conversely, few publications address the issue of the effect on SER metabolism of other drugs which inhibit the CYP enzyme system (Baumann, 1996).

1.4 **Gas Chromatography**

Since gas chromatography was the technique used to conduct analyses of SER and its N-demethylated metabolite in this thesis, a brief summary of the principles of this technique follows.

Gas chromatography (GC) is a physical process that separates two or more compounds such that the compounds can be identified and/or quantitated. The components are separated based on their differential distribution between a stationary and a mobile phase (Tabor, 1989). Theoretically, any compound that can be vaporized or converted to a volatile derivative can be analyzed by this procedure (Poklis, 1989). The mobile phase (an inert gas) carries the vaporized mixture and flows through a heated column containing the stationary phase. Mobile carrier gases include nitrogen, helium, hydrogen, or argon-methane, and stationary phases are most commonly high-boiling nonvolatile liquids coating or bonded to inert supports (Coutts and Baker, 1982; Baker et al., 1982; Coutts et al., 1985).

Compounds in a mixture are separated based on their partition coefficients, detected as they leave the column, and displayed as peaks on an integrator/recorder connected to the gas chromatograph. Each component's retention time is the time between the injection of the sample onto the gas chromatograph and the apex of the peak corresponding to that specific component on the chromatographic trace. Retention times vary with changes in GC temperature, carrier-gas flow rate, and column length and diameter. However, under a controlled set of conditions they are characteristic of a given compound and thus used for component identification. The area and the height of the peak are proportional to the size of the signal arriving at the detector, and either may be used as means of quantitation. The basic parts of a gas chromatograph are an inlet to permit entry of a sample mixture and the inert

carrier gas, an oven with a variable temperature in which the column is contained, a detector that detects each component as it elutes from the column, and a recorder to record component peaks (Coutts and Baker, 1982; Baker et al., 1982; Coutts et al., 1985).

1.4.1 **Injection systems**

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

1.4.2 **Columns**

GC columns are commercially available in materials such as borosilicate glass, fused silica, stainless steel, nickel, copper, aluminum and nylon (Coutts et al., 1985). The columns used for the experiments described in this thesis were fused silica capillary columns, so only this type will be discussed. Two types of capillary columns are principally used. The wall-coated open tubular (WCOT)

has its liquid phase deposited directly on the inner glass of the column, whereas the liquid phase of the support-coated open tubular (SCOT) is deposited onto a thin layer of solid support material (Poklis, 1989). WCOT columns were employed in the present experiments as they permit analysis of larger sample volumes, and thus the relatively low quantities of drug investigated were more easily detected.

1.4.3 **Detectors**

The five detector types generally used for gas chromatography are the thermal conductivity detector (TCD), the flame ionization detector (FID), the nitrogen-phosphorus detector (NPD), the electron capture detector (ECD) and the mass spectrometer (MS) as a detector (Coutts et al., 1985). For this thesis, only the ECD and the MS were utilized.

In the ECD, a radioactive isotope (^{63}Ni or ^{3}H) releases β-particles (high energy electrons) that collide with the carrier gas molecules, producing many low-energy electrons which are collected on electrodes to produce a small standing current. When sample compounds containing chemical groups with high electron affinity flow through, they capture the low-energy electrons generated by the isotope to form negatively charged ions. The detector measures the loss of standing current and the integrator amplifies and records it as a peak on the graphic output. The ECD is particularly selective for compounds containing electronegative groups such as halogen atoms, ketone,

nitro or nitrile groups, and organometals (Coutts and Baker, 1982; Baker et al., 1982; Coutts et al., 1985). This detector is extremely sensitive and can quantitate as little as 1 picogram of analyte (Coutts et al., 1985).

The MS may be coupled to a gas chromatograph for structural determination of chemical compounds. The MS provides highly specific and reproducible spectra of ionic fragments formed by bombarding a volatilized compound with high energy electrons (Baker et al., 1982; Coutts et al., 1985). The production of the mass spectrum involves first ionizing the sample, and in this thesis the electron impact (EI) mode of ionization was utilized. In the EI mode, the sample molecules are bombarded with a beam of high energy electrons, which fragments them into positive, negative, and neutral fragments. The charged fragments are then separated according to mass by accelerating them out of the ion source and toward an ion collector. Usually the positively charged fragments are passed into an electron multiplier that generates an electric current, which is amplified and recorded. A plot of the relative abundance of the ions *versus* their mass-to-charge (m/z) ratios is produced (Poklis, 1989) and interpreted.

1.4.4 **Derivatization**

Derivatization of the substance of interest may be required in order to make it more suitable for GC analysis. This process typically involves the replacement of the active hydrogen in polar groups (-NH, -OH, -SH) *via* acylation,

alkylation, silylation or condensation (Baker et al., 1982). Derivatization is performed for one or more of the following reasons: to increase the volatility and/or the stability of a substance, to reduce a substance's polarity to improve chromatographic properties, to increase extraction efficiency from aqueous solutions, and to introduce a functional group that is sensitive to the detector being employed (Baker et al., 1982; Coutts et al., 1985).

1.5 **Human hepatic liver microsomes**

Currently, the *in vitro* model of human liver microsomes is used to determine the specific CYP enzymes involved in the metabolism of a particular drug and provide clinically relevant data used to predict potential drug-drug interactions (von Molke et al., 1995). As previously mentioned, the CYPs are located mainly in the endoplasmic reticulum of the liver (Spatzenegger and Jaeger, 1995; Harvey and Preskorn, 1996a). Human hepatic microsomes are formed by homogenization of these lamellar membranes, and can be precipitated as a pellet by centrifugation (Spatzenegger and Jaeger, 1995).

To determine the extent of inhibition of a specific enzyme by a particular drug, the drug is co-administered with a probe drug known to be metabolized by that enzyme, and changes in the metabolism of the probe drug are measured (DeVane, 1994). As known substrates for CYP2D6, sparteine and dextromethorphan are examples of commonly used probe drugs (Riesenman, 1995). There does not appear to be a "gold standard" probe to measure the

effects of drugs on CYP3A3/4 (Riesenman, 1995), but 1'-hydroxymidazolam formation from midazolam was used by Ring and Binkley (1995) as a probe for this enzyme.

2.0 **RATIONALE FOR THIS RESEARCH**

SER analysis methods using high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) have been developed by Logan *et al.* (1994) and Rogowsky *et al.* (1994). Tremaine and Joerg (1989) have developed a GC-ECD assay for SER in plasma. However, to the best of my knowledge, no GC-ECD assay method existed prior to this work to simultaneously determine SER and DMS levels in tissue samples. I proposed to develop a GC-ECD assay, which would permit simultaneous analysis of SER and DMS in tissues such as rat brain. Such an assay would be very beneficial since recently there have been numerous studies conducted in rats in order to elucidate possible mechanisms of action of SER, but very few of these studies have measured the brain levels of SER and DMS attained. As well, GC-ECD equipment is readily accessible to many laboratories.

It was hoped that such an assay would be applicable to two other areas of research which are very important in the study of antidepressants: 1) the use of *in vitro* studies with human liver microsomes and with microsomes from cells expressing individual CYP enzymes (important for studying the metabolism of the drug of interest and possible metabolic drug-drug interactions it may be involved in); and 2) measurement of levels of the antidepressant and its metabolite in plasma samples from patients undergoing treatment with SER. These three approaches are necessary for a comprehensive understanding of the actions of antidepressants, but apparently there was no assay procedure for SER and DMS

that could be used in all three approaches. It was thus the objective of the research described in this thesis to develop such an assay for SER and DMS.

3. MATERIALS AND METHODS

3.1 Chemicals and Reagents

The following chemicals (sources shown in square brackets) were used: concentrated hydrochloric acid (HCl), isopropanol, n-hexane and concentrated sulfuric acid (H_2SO_4) [Fisher Scientific, Nepean, Ont.]; magnesium chloride (crystalline, $MgCl_2 \cdot 6H_2O$), methanol, potassium carbonate (K_2CO_3), sodium borate, sodium chloride (NaCl), and Tris (crystalline free base) [Fisher Scientific, Fair Lawn, NJ]; dimethylsulfoxide (DMSO), D-glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroids* (G6PD) and NADP sodium salt (NADP+) from yeast [Sigma Chemical Company, St. Louis, MO]; potassium phosphate dibasic (K_2HPO_4), potassium phosphate monobasic (KH_2PO_4) and trisodium phosphate (Na_3PO_4) [J.T. Baker Chemical Company, Phillipsburg, NJ]; pentafluoropropionic anhydride (PFPA) [Aldrich Chemical Company, Milwaukee, WI]; isopentane [BDH, Toronto, Ont.]; solid carbon dioxide (CO_2) [Liquid Carbonic, Edmonton, Alta.]; silicone solution [SERVA, Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany]; glass-distilled ethyl acetate and glass-distilled toluene [from both Fisher Scientific, Fair Lawn, NJ; and BDH Inc., Toronto, Ont.]. Double-distilled water was prepared in-house.

3.1.1 **Preparation of SER and DMS**

3.1.1.1 **Rat brain tissue homogenate assay**

SER HCl and DMS maleate were generously provided by Pfizer Inc. (Groton, Conn.). Stock solutions (1mg/ml, based on base weight) in methanol were prepared and aliquots were stored at -20°C until required. The internal standard, FLU HCl (Eli Lilly Pharmaceuticals, Indianapolis, IN, U.S.A.) was similarly prepared and stored. Dilutions in double-distilled water were used when calibration curves were prepared.

3.1.1.2 **Human plasma assay**

SER HCl, DMS maleate and FLU HCl stocks were prepared and utilized as above.

3.1.1.3 ***In vitro* microsomal assay**

SER HCl, DMS maleate and FLU HCl were prepared as 10 mM stock solutions in methanol and stored as above. The SER HCl was prepared as the substrate for the microsomal experiments and DMS maleate was used for calibration curves. FLU HCl was used as the internal standard.

3.2 Instrumentation and apparatus

3.2.1 Gas chromatography (GC)

3.2.1.1 Electron capture detection

All samples were analyzed using a Hewlett Packard (HP) Model 5880 gas chromatograph equipped with a ^{63}Ni electron-capture detector and linked to a HP 5880A integrator/printer. A fused HP5 silica capillary column (25m x 0.32mm) coated with 1.05μm film thickness of 5% phenylmethylsilicone (Hewlett Packard) was employed for the animal time course experiment. An interfering peak co-eluting with the DMS peak which appeared after the animal time course experiment necessitated changing the column from an HP5 to a SP2100. Therefore, for all other experiments a fused SP2100 silica capillary column (15m x 0.25 mm) coated with 0.25 μm film thickness of poly(dimethylsiloxane) (SPB-1, Supelco) was used. The carrier gas was pure helium (Praxair Canada Inc.) at a flow rate of 3 mL/min. The make-up gas at the detector was argon-methane [95:5] (Praxair Canada Inc.) at a flow rate of 30 mL/min. The oven temperature was set at an initial temperature of 105°C for 0.5 min. The temperature was then increased by 5°C/min to 290°C, which was maintained for 15 min. Injection port and detector temperatures were 275°C and 325°C, respectively. All samples were hand-injected and injections were carried out using the splitless mode of injection with a purge-off time of 0.5 min.

3.2.1.2 **Mass spectrometeric confirmation of structures of derivatives**

Structures of the derivatives of SER and N-DMS were confirmed by combined GC-MS analysis performed in the electron-impact mode. The GC-MS system used was a HP 5840A gas chromatograph inlet coupled to a HP 5895A mass spectrometer and a HP 7920 data system. The GC-MS system also consisted of an HP 2648A graphics terminal, HP 9876A printer, HP 7920 disc drive (software) and HP 21 MX series E computer (hardware). Operating conditions were as follows: ion source temperature, 200°C; interface temperature, 275°C; column pressure, 34.5 kPa; accelerating voltage, 2200eV; ionization voltage, 70 eV; scan speed, 200 amu/sec [100 amu/sec]; and dwell time, 200 msec. The same column and oven conditions were used as for the GC.

3.2.2 **Tissue homogenizer**

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

3.2.3 **Centrifuges**

Centrifugations were carried out in a Sorvall GLC-2B General Laboratory Centrifuge (Dupont Instruments, Wilmington, DE, USA). A Micro Centaur MSE (John's Scientific, Toronto, Canada) was used occasionally to remove by centrifugation any solid material if the final sample taken up in toluene was cloudy.

3.2.4 **Savant evaporator**

For experiments involving rat brain tissue a Savant Speed Vac SC 110 (Fisher Scientific) was used for evaporating samples. The instrument consists of a concentrator (a rotor chamber with a heater), a chemical trap that accepts disposable cartridges, a refrigerated condensation trap, and a vacuum pump. The process of drying involves combining centrifugal force, vacuum and applied heat.

3.2.5 **Shaker - mixer**

Two types of vortex-shaker were used: an Ika Vibrex VXR vortex mixer (Janke and Kunkel, Staufen, Germany) and a thermolyne Maxi Mix vortex mixer (Thermolyne Corp., Dubuque, IO, USA).

3.2.6 **Weighing balances**

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

3.2.7 **Dry bath**

Samples were heated using a Multi-Blok Heater (Lab-Line Instruments Inc., Melrose park, Ill.).

3.2.8 **Water bath**

The water bath used for microsomal incubations was an Isotemp® water bath (Fisher Scientific, USA).

3.2.9 **Stirring hot plate**

A Thermix stirring hot plate (model 210T, Fisher, USA) was used for stirring and heating solutions.

3.2.10 **Glassware and glassware cleaning**

The test tubes used for the experiments in this thesis were 16x120mm and 13x100mm screw cap culture tubes (Fisher Scientific, USA). All glassware was hand washed with biodegradable Sparkleen (Fisher Scientific Co.). Additionally, test tubes were sonicated (Ultra-sonic cleaner, Mettler Electronics) in a 2 to 5% solution of Decon 75 concentrate (BDH Chemicals). All glassware was hand rinsed 6 times with in-house distilled water and then air-dried in a mechanical convection oven (Model 28, Precision Scientific Group, Chicago, IL, USA).

3.2.11 **Silanization of glassware**

Clean glassware was filled briefly with silicone solution (SERVA Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany), emptied and heated for one hour in a mechanical convection oven (Model 28, Precision Scientific Group, Chicago, IL, USA).

3.2.12 **Polyethylene and polypropylene tubes**

All toluene layers retained for GC analysis were stored in polyethylene (0.4ml) microcentrifuge tubes (Fisher Scientific, Pittsburg, PA). The initial

incubation of microsomes for the *in vitro* metabolism studies took place in polypropylene (1.5ml) microcentrifuge tubes (Fisher Scientific, Ottawa, Ont.).

3.3 **Animals**

The male Sprague-Dawley rats (100-150g) used for the experiments described in this thesis were obtained from Bio-Science Animal Services, Ellerslie, Alberta, Canada. The animals were housed in pairs in plastic cages on cedar chip bedding in a temperature controlled room (21±1°C). They were subjected to a 12 hour light/dark cycle (lights on at 7:30 a.m.). Water and lab chow were provided *ad libitum*. The lab chow (Lab-Blox feed, Wayne Feed Division, Continental Grain Company, Chicago, Ill., USA) was a 4.0% (min) crude fat, 4.5% (max) crude fibre and a 24% crude protein. Procedures involving animals were conducted according to the Canadian Council on Animal Care guidelines and were approved by the University of Alberta Health Sciences Animal Welfare Committee.

3.3.1 **Administration of Drugs**

Two separate experiments were conducted. In the time course study, the rats were randomly allocated into groups receiving either SER (20mg/kg) or vehicle (25% DMSO and 75% distilled water) injected intraperitoneally (i.p.). The DMSO was required in order to dissolve the SER completely at this

concentration. The animals were sacrificed either 1, 3 or 6 hours later, resulting in 6 treatment groups. In the dose-concentration experiment, the rats were also injected i.p. with either SER (5mg/kg or 10mg/kg) or the vehicle and sacrificed 6 hours later, resulting in 4 treatment groups. Results from the rats injected with 20mg/kg SER and sacrificed at 6 hours post-injection were also included in the analysis of the second experiment.

3.3.2 **Sample collection and storage**

At the appropriate time, animals were sacrificed by guillotine decapitation. Whole brains were removed, submerged in isopentane on solid carbon dioxide and then stored at -80°C until analysis.

3.4 **Human plasma samples**

Plasma samples were prepared from venous blood samples taken from patients under the care of Dr. Jacques Bradwejn at the Clarke Institute of Psychiatry, Toronto, Ontario, or Dr. Jean-Michel Le Mellédo, Department of Psychiatry, University of Alberta, Edmonton, Alberta. The plasma samples from Toronto were shipped on solid carbon dioxide to Edmonton. All samples were stored frozen at -20°C until analysis.

3.5 *In vitro* microsomal enzyme studies

3.5.1 Microsomal protein

Human liver microsomal preparations were obtained from the International Institute for the Advancement of Medicine (Exton, PA). For the preliminary experiments, human hepatic microsome (HHM) lot 201 was used. For the correlation experiments, a panel made up of HHM lots 53, 55, 59, 65, 67, 71, 74, 108, 110, 123, 124, 126, 151, 186, 198 and 211 was used. These 16 HHM preparations were all characterized for their various CYP enzyme activities as follows: CYP1A2 (characterized using phenacetin O-deethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2C19 (mephenytoin 4-hydroxylation), CYP2D6 (dextromethorphan O-demethylation), CYP2E (chlorzoxazone 6-hydroxylation), CYP3A4 ([^{14}C]-testosterone β-hydroxylation) and CYP4A ([^{14}C]-lauric acid omega-hydroxylation).

Individual CYP enzyme and control microsomal preparations were prepared by and purchased from Gentest Co. (Woburn, MA). cDNA-expressed human CYP450 enzymes were prepared by Gentest by individually transfecting metabolically competent derivatives of the human AHH-1TK +/- cell line with human CYP450 cDNA for CYP2A6 and CYP3A4. Control preparations (lot 101b) were from cells transfected with the vector alone. The microsomes contained adequate NADPH-reductase and cytochrome b_5 for the CYP450 form-specific metabolic assays.

3.5.2 **NADPH generating system**

The NADPH generating system was prepared by mixing stock solutions of NADP+ (1.3 mM), G6P (3.3 mM), G6DP (0.4 units/ml), and $MgCl_2 \cdot 6H_2O$ (3.3 mM). The final volume was made up with potassium phosphate buffer (pH = 7.4) or Tris buffer (pH = 7.5).

3.5.3 **Buffer systems**

The 0.1M potassium phosphate buffer system was prepared by mixing solutions of potassium phosphate dibasic (K_2HPO_4) and potassium phosphate monobasic (KH_2PO_4) until a pH of 7.4 was obtained. The 0.1M (pH = 7.5) Tris buffer system was prepared by dissolving Tris (crystalline free base) in double-distilled H_20.

3.5.4 **Preparation of inhibitors of CYP enzymes**

10mM stock solutions of quinidine sulfate (Shawinigan McArthur Chemical Company Ltd., Montréal, QC), ketoconazole (ICN Pharmaceuticals Inc., Costa Mesa, CA), erythromycin (Sigma Chemical Company, St. Louis, MO), (-)-nicotine hydrogen tartrate (Sigma Chemical Company, St. Louis, MO), coumarin (Sigma Chemical Company, St. Louis, MO) and fluvoxamine maleate (Solvay

Kingswood, Scarborough, Ont.) were prepared in methanol and stored at -20°C until use.

3.6 GC assay procedures for the simultaneous quantification of SER and N-DMS

3.6.1 Rat brain tissue homogenate samples

A novel GC procedure was developed for the simultaneous analysis of SER and DMS and is depicted in Figure 2. Rat brain was homogenized in five volumes of ice-cold double-distilled water. Aliquots (500 µl) were added to screw cap culture tubes containing 1.5mL of deionized water. To each tube 3 µg of the internal standard (I.S.) FLU was added. The samples were basified by the addition of 500 µl 25% K_2CO_3 and vortexed briefly. Sample pH values were checked randomly to ensure basicity. Glass-distilled ethyl acetate (5ml) was added to each tube. The tubes were then capped and shaken vigorously for 5 minutes and centrifuged for 5 min at 1000 g. The organic layers were transferred to a second set of screw cap culture tubes, and back extracted with 4 ml 1.0 N hydrochloric acid. This was done by shaking to mix the two immiscible phases for 5 min., centrifuging 5 min. at 1000 g, then aspirating the ethyl acetate layer. The HCl layers were basified by the addition of both 500 µl 25% K_2CO_3 and solid K_2CO_3, and vortexed. To these basified layers, 5 ml ethyl acetate was added. The tubes were capped, shaken vigorously for 5 min and centrifuged at 1000g for 5 min. The ethyl acetate layers were transferred to another set of smaller screw

cap tubes, and taken to dryness in a Savant evaporator. To the residue 25 µl ethyl acetate followed by 75 µl PFPA (derivatizing reagent) was added. Each tube was vortexed thoroughly, and the derivatization procedure was allowed to proceed for 30 min at 60°C. After cooling the tubes to room temperature, 300 µl of toluene was added to each. This solution was vortex-mixed, washed with 3 ml saturated sodium borate, and briefly vortex-mixed again. The samples were centrifuged at 1000g for 5 min and the toluene layer was pipetted off and retained for gas chromatographic analysis (1 µl was injected). This protocol was used for both the time course and dose-concentration experiments.

In this and the other GC procedures described in this thesis, calibration (standard) curves for SER and N-DMS were prepared using drug-naïve rat brain tissue homogenate, human plasma, or control microsomes (lacking significant CYP activity) to which were added a known range of dilutions of SER and/or its metabolite. Each standard sample also had a fixed amount of the I.S. (FLU) added. Calibration standards were run in parallel with each assay. The standard curve was calculated by plotting the peak height ratios of the derivatized compound of interest to the derivatized I.S. against the known concentrations of the compound of interest. The peak height ratios of SER to I.S. and N-DMS to I.S. of the experimental samples were compared to the values from the calibration curves to quantify levels of SER and/or DMS.

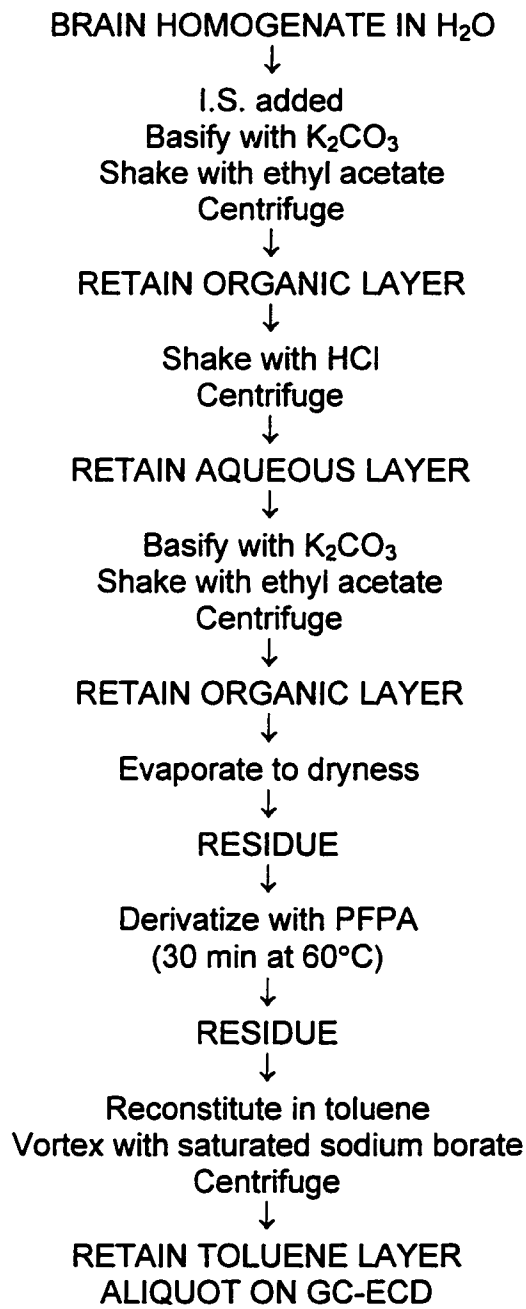


Figure 2: The GC procedure developed for the simultaneous analysis of SER and DMS in rat brain homogenate.

In attempts to improve recovery and eliminate a late-developing, interfering peak co-eluting with the DMS peak after the dose-concentration study had been completed, the assay was modified as follows for subsequent experiments involving rat brain tissue homogenate. All glassware was silanized before use and all tubes were kept on ice. The samples were basified at both basification steps by the addition of 10% Na_3PO_4 instead of 25% K_2CO_3. An ice-cold solution of 2% isopropanol in n-hexane (5ml) replaced ethyl acetate as the organic solvent. Frequently, emulsions formed when the aqueous and organic layers were shaken together. Thus, each sample was vortexed after the addition of solid NaCl and then centrifuged for 5 min at 1000 g. 4 ml 0.5M H_2SO_4 replaced 1.0N HCl for the back extraction step. The organic layers that were retained after the back extraction were transferred to a set of smaller, silonized tubes, and taken to dryness under nitrogen (N_2) gas instead of in a Savant evaporator. At this point, if necessary, the tubes were capped and stored at -80°C in order to inject the samples on the GC the same day they were derivatized. The derivatization and final wash steps of the procedure remained the same. All samples were stored at -20°C until immediately before hand injection on the GC. As mentioned previously, the column was also changed from an HP5 to an SP2100 column.

3.6.2 **Human plasma samples**

The assay protocol (Figure 3) was adapted as follows: 1 ml aliquots of plasma were added to clean glass screw cap culture tubes. All tubes were kept on ice. 2000ng of the I.S. (FLU) was added to each tube. The samples were basified by the addition of 500 µl 10% Na_3PO_4 and vortexed briefly. An ice-cold solution of 2% isopropanol in n-hexane (5ml) was added to each tube. The tubes were then capped and shaken vigorously for 5 min. Frequently, an emulsion formed at this stage. Thus, each sample was vortexed after the addition of solid NaCl and centrifuged for 5 min at 1000 g. The organic layers were transferred to a smaller set of screw cap culture tubes, and taken to dryness under nitrogen (N_2) gas. At this point, if necessary, the tubes were stored at -80°C in order to inject the samples on the GC the same day they were derivatized. To derivatize the residue in each tube, 25 µl ethyl acetate and 75 µl PFPA was added. Each tube was vortexed thoroughly, and the derivatization procedure was allowed to proceed for 30 min at 60°C. After cooling the tubes to room temperature, 300 µl of toluene was added to each. This solution was vortex-mixed, washed with 3 ml saturated sodium borate, and briefly vortex-mixed again. The samples were centrifuged at 1000g for 5 min and the toluene layer was pipetted off and retained for gas chromatographic analysis (1 µl was injected). All samples were stored at -20°C until immediately before hand injection on the GC.

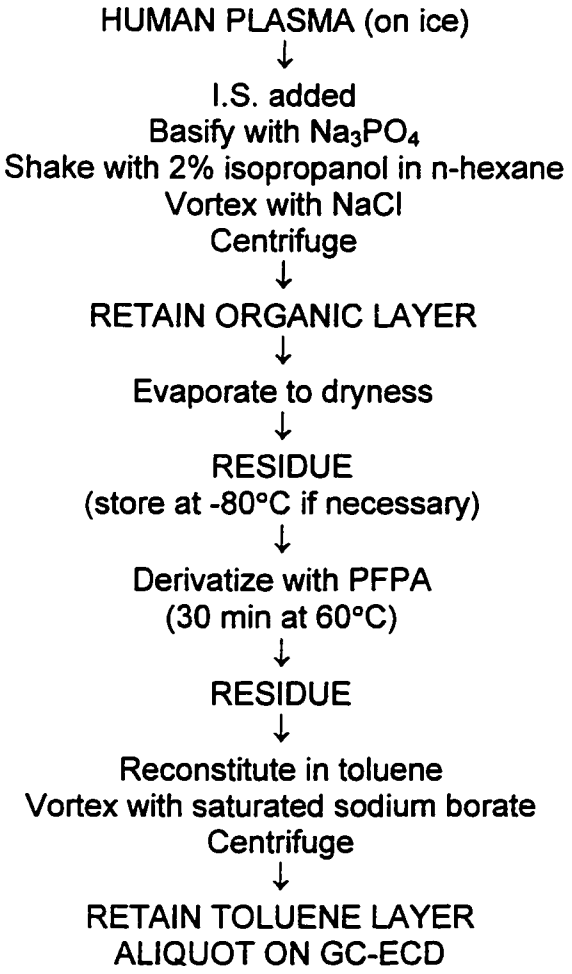


Figure 3: The GC procedure developed for the simultaneous analysis of SER and DMS in human plasma samples.

3.6.3 *In vitro* microsomal enzyme studies

The incubation and assay protocol is shown in Figure 4. The initial incubation of microsomes for the *in vitro* metabolism studies was performed in polypropylene microcentrifuge tubes. Microsomal protein (10µl) in potassium phosphate buffer (0.1M, pH 7.4) was added to an each tube (on ice) containing 25µl of NADPH generating system. The reaction was initiated by adding a known amount of the substrate SER in buffer (final volume of 100µl/tube), mixing gently and incubating in the water bath at 37°C for 20 min (unless otherwise indicated). The microsomal reaction was terminated by removing the tubes to ice, basifying the samples with 50µl 10% Na_3PO_4 and vortexing. The I.S. (FLU) was added to each tube to give a final concentration of 50µM. The samples were diluted to a volume of 500µl with double-distilled H_2O and vortexed. These mixtures were transferred to clean screw cap culture tubes and kept on ice. The tubes were shaken vigorously with 2ml of ice-cold 2% isopropanol in n-hexane. Frequently, an emulsion formed at this stage. Thus, each sample was vortexed after the addition of solid NaCl and centrifuged for 5 min. at 1000 g. The organic layers were transferred to a set of smaller screw cap culture tubes, and taken to dryness under nitrogen (N_2) gas. At this point, if necessary, the tubes were stored at -80°C in order to inject the samples on the GC the same day they were derivatized. To derivatize the residue in each tube, 25 µl ethyl acetate and 75 µl PFPA was added. Each tube was vortexed thoroughly, and the derivatization procedure was allowed to proceed for 30 min at 60°C. After cooling the tubes to

room temperature, 300 µl of toluene was added to each. This solution was vortex-mixed, washed with 3 ml saturated sodium borate, and briefly vortex-mixed again. The samples were centrifuged at 1000g for 5 min and the toluene layer was pipetted off and retained for gas chromatographic analysis (1 µl was injected). All samples were stored at -20°C until immediately before hand injection on the GC.

3.6.3.1 **Time course experiment**

In this preliminary experiment using HHM lot 201, the assay procedure was performed as described in section 3.6.3 "*In vitro* microsomal enzyme studies" with the following exception: samples were incubated with SER (100µM) for varying amounts of time. Individual samples were incubated for 5, 10, 20, 30, 45 or 60 min. Subsequent experiments used 20 min incubation periods based upon the observation that this was within the incubation time period where the metabolite formation rate was determined to be linear.

3.6.3.2 **Dose-concentration experiment**

In the second preliminary experiment using HHM lot 201, the "*in vitro* microsomal enzyme studies" protocol described above in section 3.6.3 was repeated except that individual samples had the following varying concentrations of substrate (SER): 25, 50, 100, 150, 200 or 400 µM.

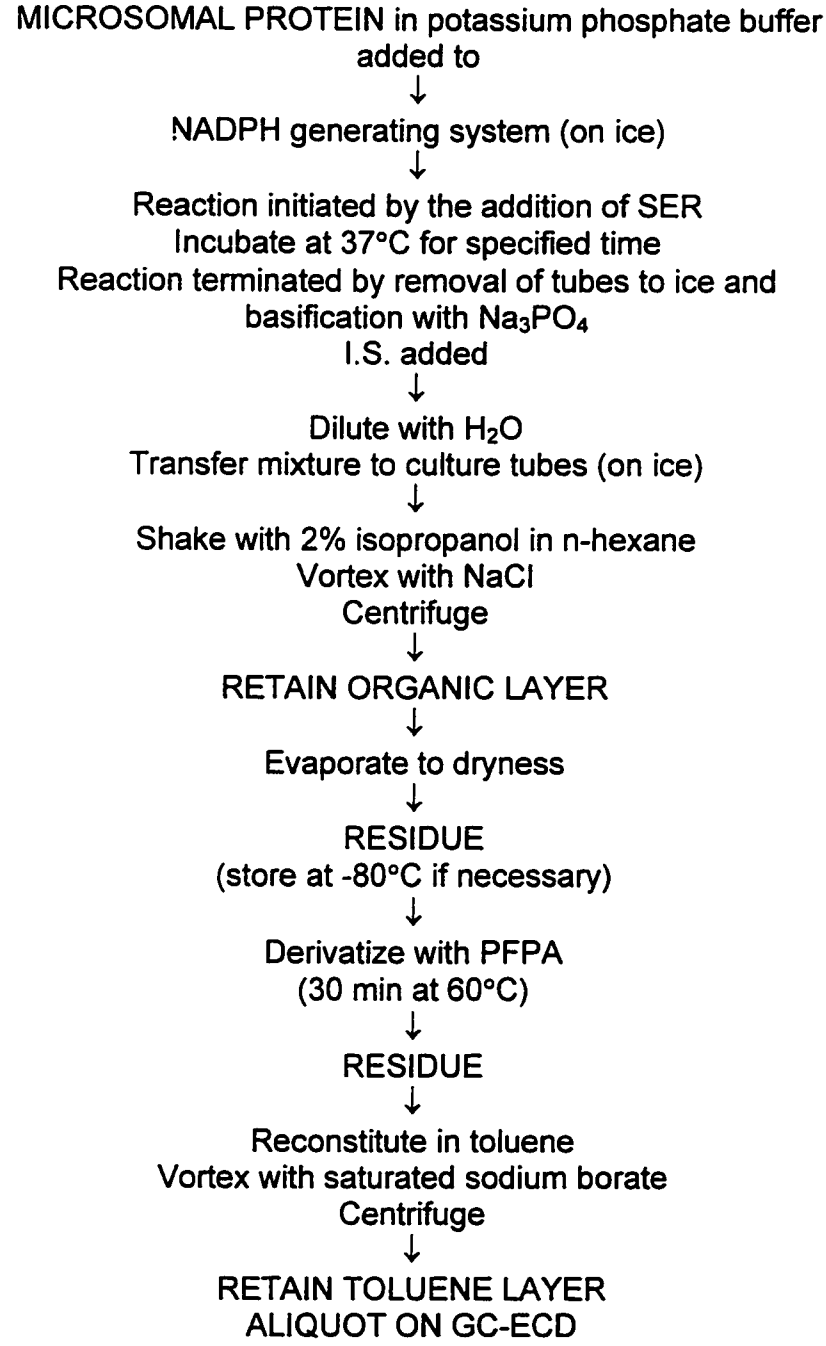


Figure 4: The incubation and GC assay procedure developed for the analysis of DMS formed from SER (*in vitro* microsomal CYP enzyme studies).

3.6.3.3 **Correlation experiment**

Using the panel of HHMs, the assay procedure was performed as described in section 3.6.3 "*In vitro* microsomal enzyme studies". The experiment was performed twice. Initially, the substrate (SER) concentration of 400μM was used based on the observation that it was a concentration at which the microsomal enzymes were saturated. However, to represent more pharmacologically relevant concentrations, the experiment was repeated using a SER concentration of 100μM.

3.6.3.4 **Inhibition experiment**

Using HHM lot 201 the "*in vitro* microsomal enzyme studies" protocol described above in section 3.6.3 was repeated except that individual samples were preincubated for 20min with known, varying amount of inhibitors. Fluvoxamine and ketoconazole were added to the reaction mixtures containing microsomal protein and NADPH generating system in buffer at the following concentrations: 0μM, 1μM, 10μM, 100μM and 200μM. Nicotine, quinidine, erythromycin and coumarin were used at 0μM, 10μM, 100μM, 500μM, and 1000μM concentrations. At the end of the preincubation time period, 100μM of SER was added to each tube and the incubation was continued for a further 20 minutes (final volume of 100μl/tube). This concentration of substrate was used based on the observation that it was a concentration at which the metabolite formation curve was linear. Control tubes containing 0μM SER and the high

standard concentration for each inhibitor were also included in the study as blanks.

3.6.3.5 **Experiment with individual CYP enzymes**

SER (100μM) was incubated with 10μl aliquots of the enzymes CYP2A6 + OR (oxido-reductase) and CYP3A4 + OR. Lot 101b (control; no significant CYP activity) was also included. The assay procedure was performed as described in section 3.6.3 "*In vitro* microsomal enzyme studies" with the following exception: for CYP2A6 + OR, Tris (0.1M, pH 7.5) and not potassium phosphate buffer was used in the reaction mixture, as this enzyme's activity is inhibited by phosphate (Gentest product inserts).

3.7 **Statistical Analysis**

Data for all calibration curves were analyzed using linear regression analysis (Lotus SmartSuite 97 1-2-3). GraphPad™ Prism was used for all other analyses. Calculations for the rat brain homogenate studies included mean ± standard error of the mean (SEM) and coefficients of variation (CV) using column statistics analyses. For the microsomal experiments, nonlinear regression analysis (line of best fit) was used for the time course and dose-concentration data and linear regression analysis was used to correlate the rate of production of DMS (nmol/min/mg) with the catalytic activities of the human livers. For the

inhibition experiments, inhibition was expressed as percent of control activity (DMS production). Values on the graphs represent the means ± SEM, and data were compared to control values using one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparisions post-test. The general convention of a probability value of $p \leq 0.05$ was used to establish statistical significance.

4.0 RESULTS

4.1 Simultaneous quantification of SER and N-DMS

This novel assay produces derivatives with good chromatographic properties, and no interfering peaks were noted. Figure 5 shows typical chromatographs of derivatized rat brain extracts from drug- and vehicle-treated animals, patient plasma samples and microsomal experiments. Peak heights, as provided by the integrator, were used in all calculations. The maximum sensitivity of the assay, based on a signal to noise ratio of 3:1, was less than 170 pg "on column".

4.1.1 Precision and Recovery

The reproducibility of the assay was determined using 200ng samples of SER and DMS in rat brain homogenate (n=4 – 6). Mean coefficients of variation are as follows: within-day, SER (6.03%), DMS (13.00%); and between-day, SER (16.27%), DMS (11.14%). Mean recoveries of SER and DMS were determined by comparing samples of spiked naïve brain homogenate which were carried through the assay procedure to tubes containing only the equivalent amounts of SER and DMS and reacted directly with PFPA. The mean recoveries of SER and DMS were determined to be 59% and 33% respectively (n=6).

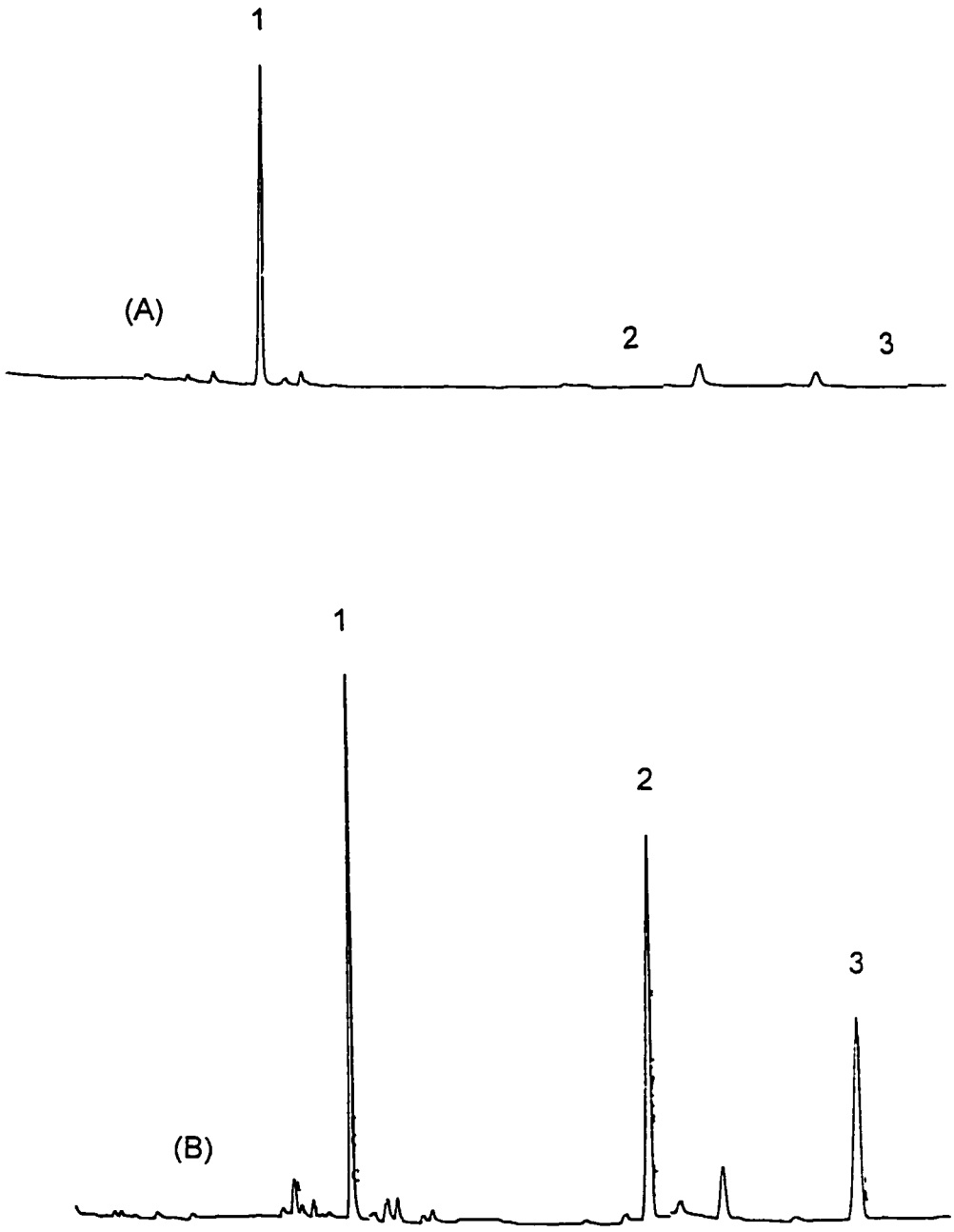


Figure 5a: Typical GC traces obtained using the procedures described for the quantification of SER and DMS. Shown are derivatives of: (A) extract of whole brain from a rat treated with vehicle; (B) extract of whole brain from a rat treated with SER. The peaks represent derivatives of I.S. FLU (1); DMS (2); and SER (3).

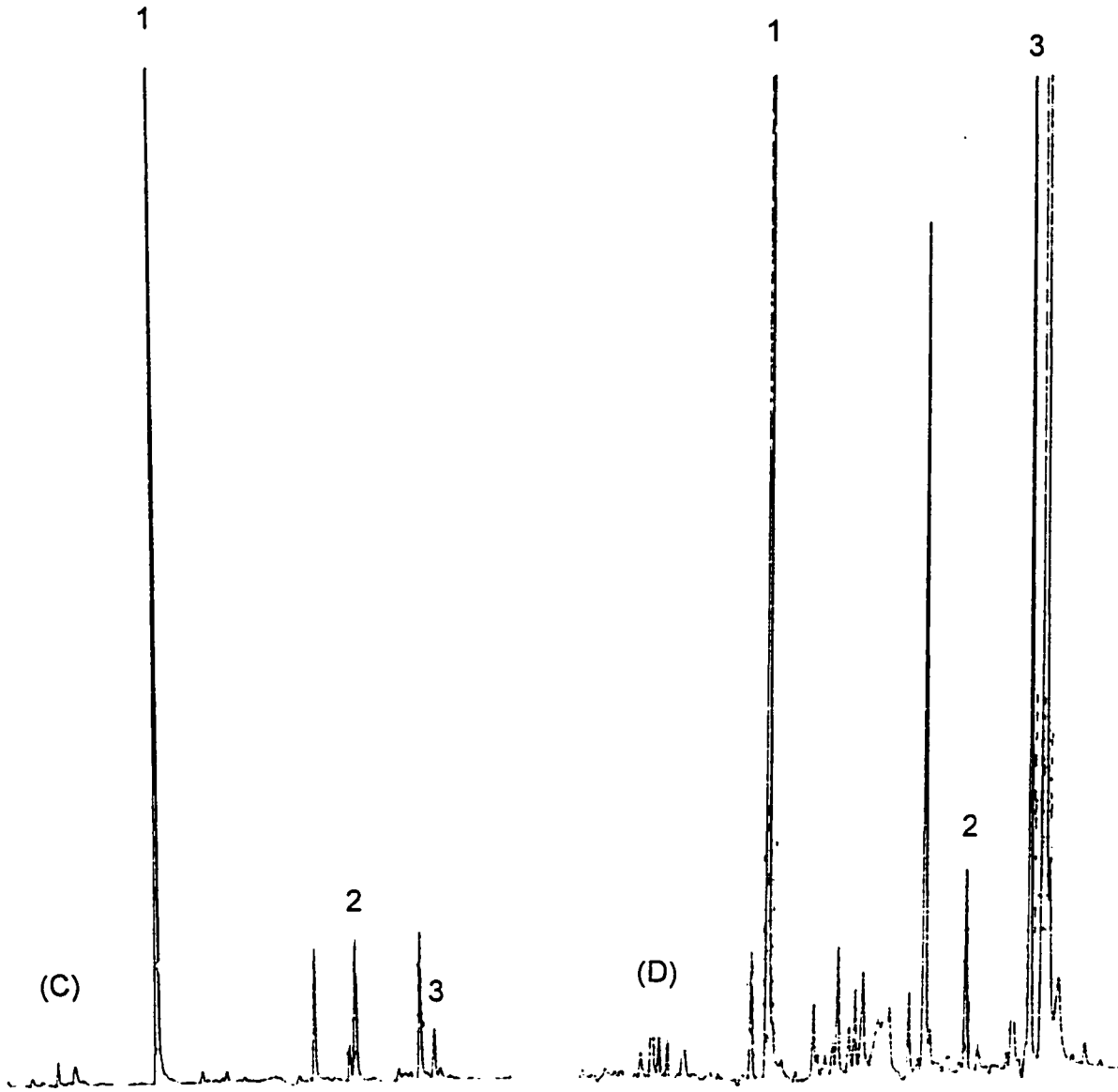


Figure 5b: Typical GC traces obtained using the procedures described for the simultaneous quantification of SER and DMS. Shown are derivatives of: (C) extract of plasma from a human patient treated with SER; (D) extract of human liver microsomal preparation treated with SER. The peaks represent derivatives of I.S. FLU (1); DMS (2); and SER (3).

4.1.2 **Mass spectrometeric confirmation**

Electron-impact (EI) MS was very helpful in confirming the structures of N-pentafluoropropionyl (PFP) derivatives of SER and N-DMS. The spectra of both derivatives contained molecular ions which were clearly of compounds that contained two chlorine atoms. The theoretical M^+:$[M+2]^+$:$[M+4]^+$ ratios of compounds containing 2 chlorine atoms are 100%:68.4%:10.5%. The m/z values and (% relative abundances) of these three molecular ions in the EIMS of PFP-SER ($C_{20}H_{16}Cl_2F_5NO$) were 451 (28.3%), 453 (18.2%) and 455 (3.30%). The same values in the EIMS of PFP-DMS ($C_{19}H_{14}Cl_2F_5NO$) were 437 (8.71%), 439 (5.80%) and 441 (1.09%).

There were several fragmentation pathways common to PFP-SER and PFP-DMS. 1) Chlorine-containing fragments were readily identified because of the isotope contributions of the ^{35}Cl and ^{37}Cl atoms to each chlorine-containing ion: a) m/z 274/276/278 ion; in the spectra of both derivatives this is the base peak, and the % relative abundances (% RA) of the three isotope components of this ion were consistent with the presence of 2 chlorine atoms ($C_{16}H_{12}Cl_2$). The PFP-SER % RA values were 100%:63.5%:11.5%; those of PFP-DMS were 100%:65.3%:14.5%. The mechanism of formation of this fragment ion is common to both derivatives and easily deduced (Figure 6a). b) m/z 239/241 ion; these masses are consistent with this ion being $C_{16}H_{12}Cl^+$. There is little doubt that it is formed by the expulsion of a Cl radical from the m/z 274/276/278 ion (Figure 6a). c) m/z 238/240 ion; this ion overlaps the m/z 239/241 peak. It is

probably formed by the expulsion of an HCl molecule from the m/z 274/276/278 ion by a mechanism that involves a complex rearrangement. d) m/z 159/161/163 ion: this fragment ion clearly contains 2 chlorine atoms. Its mass is consistent with a molecular formula of $C_7H_5Cl^+$. Such an ion could be directly formed by fragmentation of the molecular ions of PFP-SER and PFP-DMS or it could be the product of further fragmentation of the m/z 274/276/278 ion (Figure 6a).

2) Fluorine-containing fragments: a) the spectra of PFP-SER and PFP-DMS contain equivalent fragments of m/z 160 and 146, respectively, of low abundances, but of diagnostic value. In the spectrum of PFP-SER, the m/z 160 ion is masked by the presence of the m/z 159/161/163 isotopic cluster. A mechanism that would produce this fragment is provided in Figure 6b. b) Two fragment ions that derive from the pentafluoropropionyl group are present in both spectra in low abundance. These are the ions $(CF_3CF_2)^+$, m/z 119, and $(CF_3)^+$, m/z 69, which are of diagnostic importance. c) The spectrum of PFP-SER contains an $(M-15)^+$ fragment of low abundance [m/z values (% RA): 436 (9.81%), 438 (6.74%), 440 (1.00%)]. Its formation is explained in Figure 6b.

3) Hydrocarbon fragments: the spectra of PFP-SER and PFP-DMS contain numerous fragment ions of identical mass. The most important of these have m/z values of 204, 203, 202, 129, 128, 115 and 101. None of them contains chlorine, and, since they are present in both PFP derivatives, the C_2F_5CONR side chain (R=H or CH_3) must also be absent. These fragments, therefore, are hydrocarbons. Fragmentation pathways that explain the formation

of ions of m/z 204, 203 and 202 are presented in Figure 6a. Appropriate structures for the ions 129, 128, 115 and 101 are suggested in Figure 6c.

4.2 **Rat brain tissue studies**

4.2.1 **Standard curves**

Calibration curves constructed for SER and DMS using spiked brain homogenate from drug-naïve rats were linear over the range of 50-2000ng, with r^2 values > 0.99 obtained consistently (Figure 7).

4.2.2 **Time course experiment**

The time study showed that as post-injection sacrifice time was prolonged, a decrease in SER brain levels and corresponding increase in levels of its metabolite were observed. The brain levels (ng/g) of SER and DMS for rats (n=5) treated with 20mg/kg SER were (mean ± SEM): 1 hour post-injection; SER 3520 ± 532 and DMS 612 ± 82; 3 hours post-injection; SER 3017± 379 and DMS 913±135; 6 hours post-injection; SER 1497±212 and DMS 1409±130 (Figure 8).

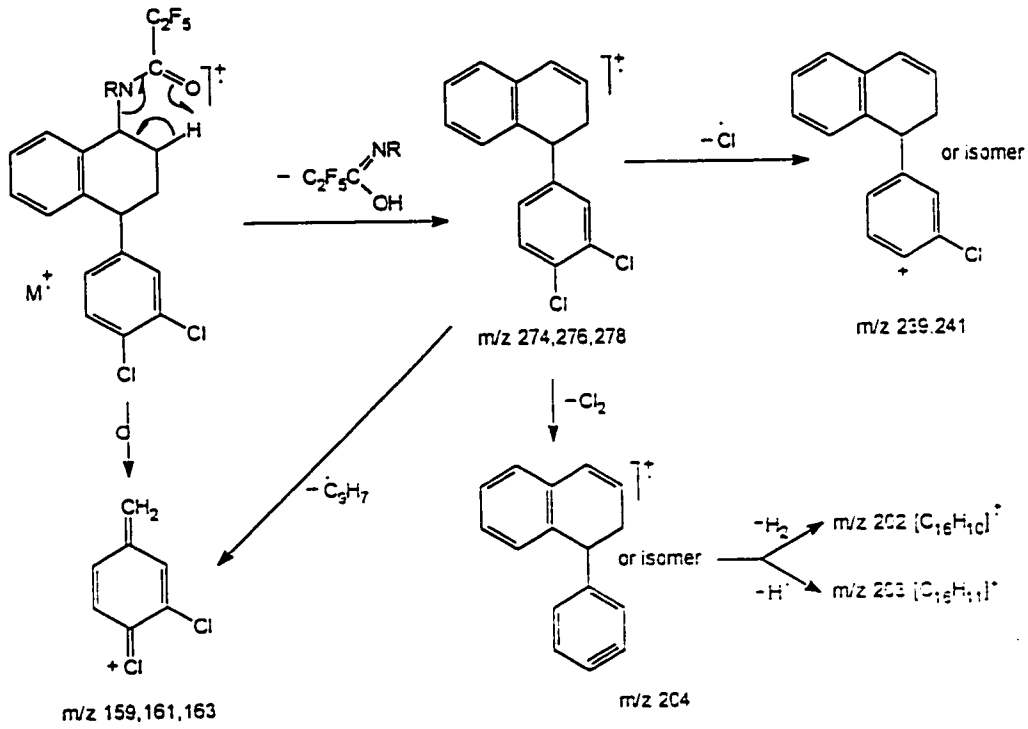


Figure 6a: Proposed electron impact mass spectrometric fragmentation pattern for the derivatives of SER and DMS.

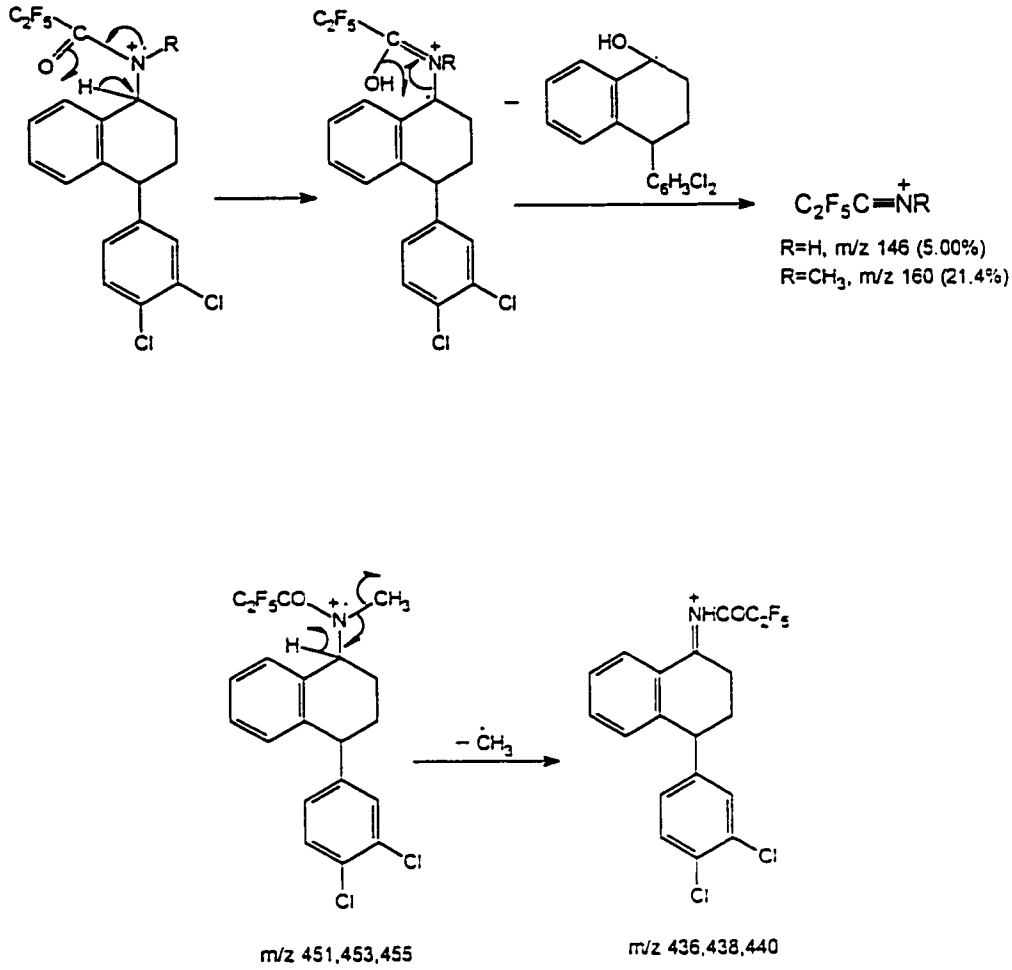


Figure 6b: Proposed electron impact mass spectrometric fragmentation pattern for the derivatives of SER and DMS.

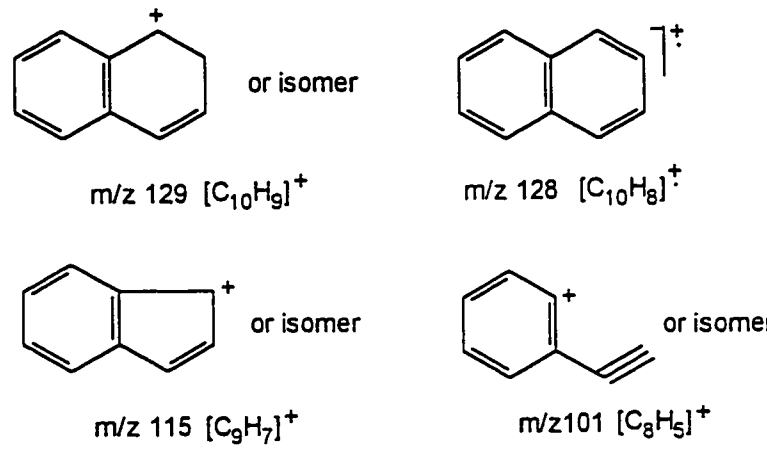


Figure 6c: Proposed electron impact mass spectrometric fragmentation pattern for the derivatives of SER and DMS.

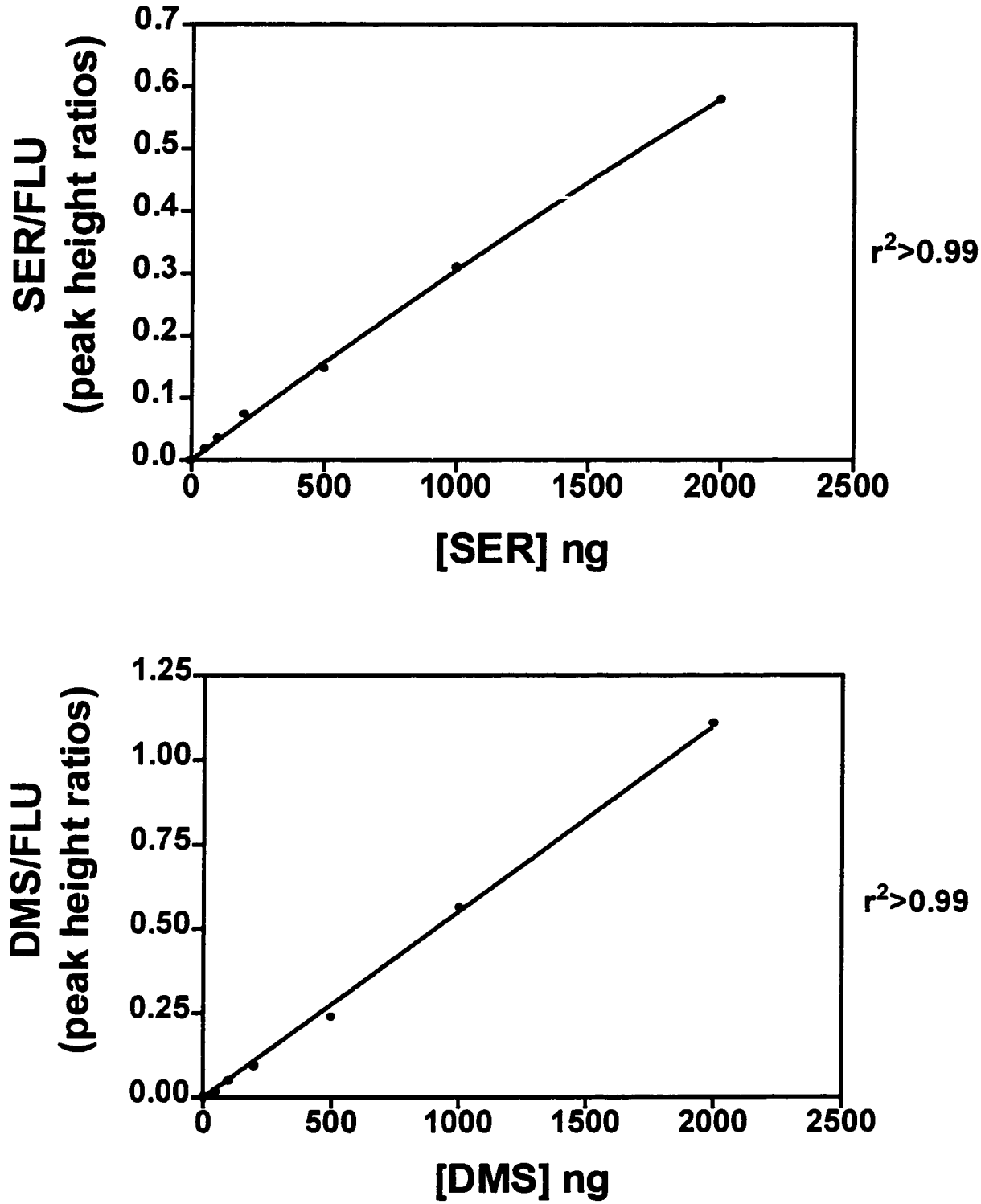


Figure 7: Typical calibration curves obtained using the assay procedure described for analysis of SER and DMS in rat brain homogenate. The ratio on the y-axis represents the peak height ratio of derivatized SER (A) or DMS (B) to a fixed amount of derivatized I.S.; the concentration of SER (A) or DMS (B) is shown on the x-axis.

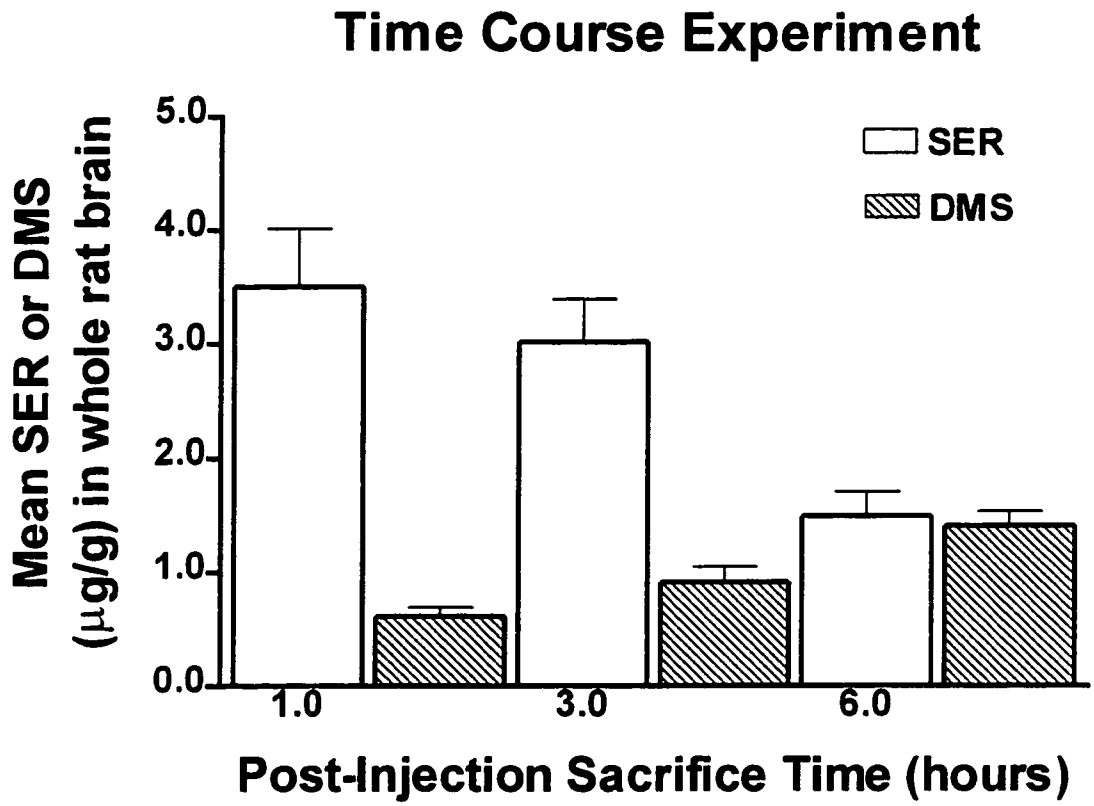


Figure 8: Levels of SER and DMS determined in whole brain of rats treated with SER (20 mg/kg), and sacrificed 1, 3, or 6 hours later. The results represent means ± SEM (n=5).

4.2.3 **Dose-concentration experiment**

As expected, brain levels of both SER and DMS decreased as the dose of SER administered to the rats was lowered (Figure 9). The ratio of SER to DMS also decreased as the dose of SER was decreased from 20mg/kg to the lower doses.

4.3 **Human plasma samples**

4.3.1 **Precision and recovery**

The reproducibility of the assay was determined using naïve human plasma (1ml) spiked with 200ng of SER and DMS (n=6). The within day mean coefficients of variation were: SER (8.33%) and DMS (9.52%). Mean recoveries of SER and DMS were determined by comparing these samples (carried through the assay procedure) to tubes containing only the equivalent amounts of SER and DMS and reacted directly with PFPA. The mean recoveries of SER and DMS were determined to be 62% and 50% respectively (n=6).

4.3.2 **Standard curves**

Calibration curves constructed using spiked plasma extracts from drug-naïve individuals were linear over the range of 50-2000ng for both SER and DMS, with r^2 values > 0.99 consistently obtained (Figure 10).

4.3.3 **Patient samples**

The plasma levels of SER and DMS in the patients are summarized in Table 2.

4.4 ***In vitro* microsomal enzyme experiments**

4.4.1 **Standard curves**

Calibration curves constructed using spiked control microsomal protein preparations (lacking significant CYP activity) were linear over the range of 0.25 – 20 μM of DMS, with r^2 values > 0.99 consistently obtained (Figure 11).

4.4.2 **Time course experiment**

SER (100 μM) was incubated with human hepatic microsomes for 5, 10, 20, 30, 45 or 60 min and the assay procedure was performed as described in section 3.6.3 "*In vitro* microsomal enzyme studies". The time course for the formation of DMS is shown in Figure 12. Subsequent experiments used 20 min incubation periods based upon the observation that this was within the incubation time period where the metabolite formation rate was linear.

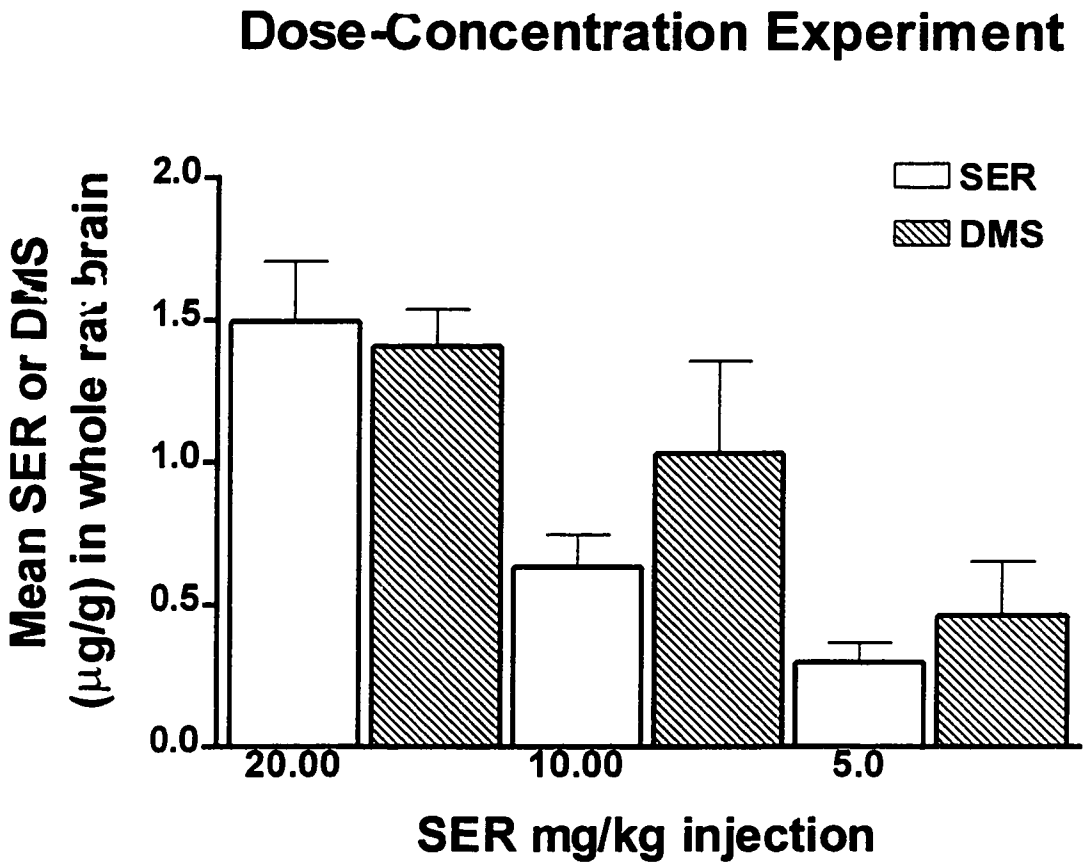
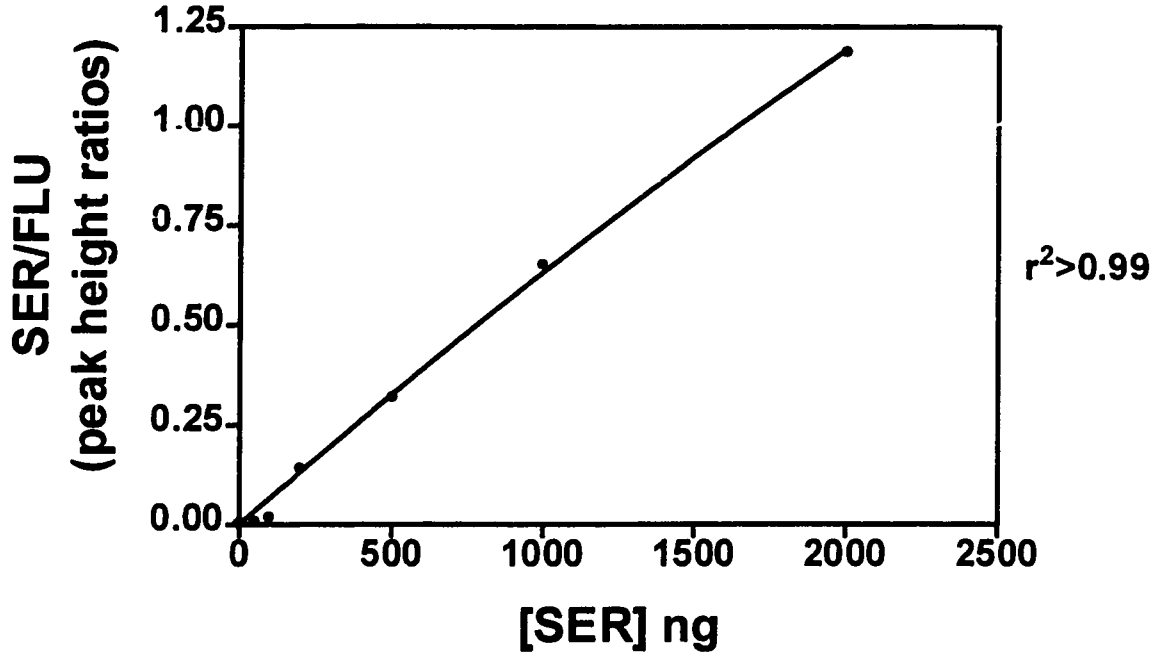


Figure 9: Levels of SER and DMS determined in whole brain of rats treated with either 5, 10 or 20 mg/kg SER, and sacrificed 6 hours later. The results represent means±SEM (n=5).



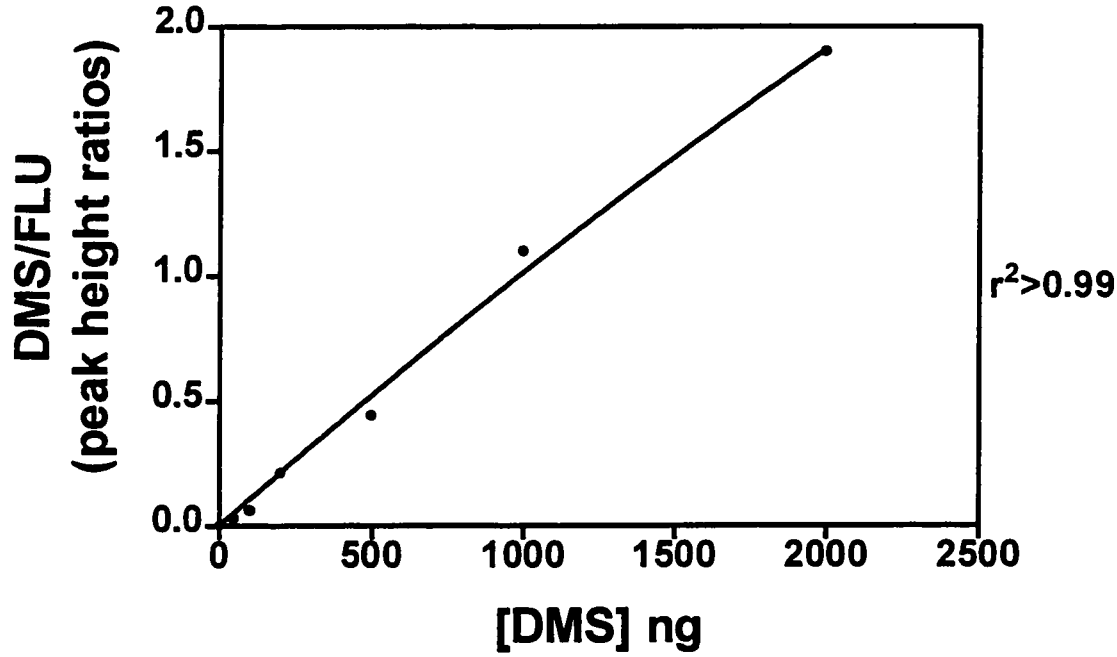


Figure 10: Typical calibration curves obtained using the assay procedure described for analysis of SER and DMS in human plasma samples.

Patient	1	2	3
Gender	Female	Female	Female
Dose Administered (Time Admin.)	100mg Zoloft® (SER) (evening)	200mg Zoloft® (SER) (evening)	300mg Zoloft® (SER) (evening)
Coadministered Medications	Risperidol 4mg	Unknown	Clonazepam 0.5mg
Time Sample Drawn	Afternoon	Morning	Afternoon
SER Levels (ng/ml plasma)	65	88	494
DMS Levels (ng/ml plasma)	104	190	798

Table 2: Plasma levels of SER and DMS in patient samples.

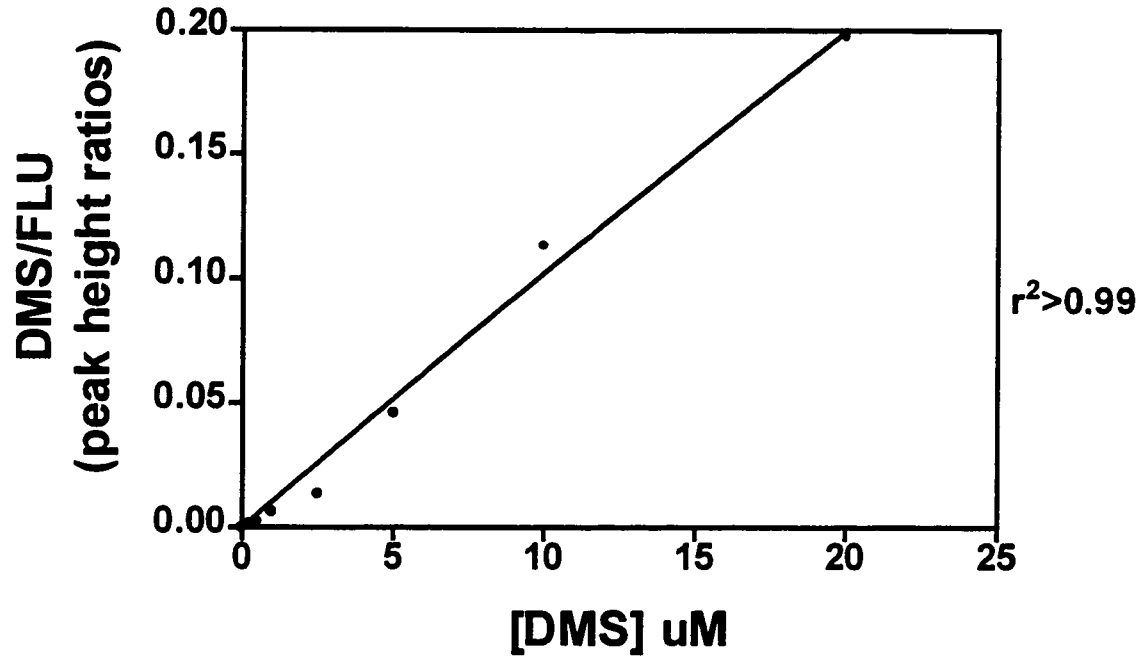


Figure 11: Typical calibration curve obtained using the assay procedure described for analysis of DMS in microsomal protein preparations.

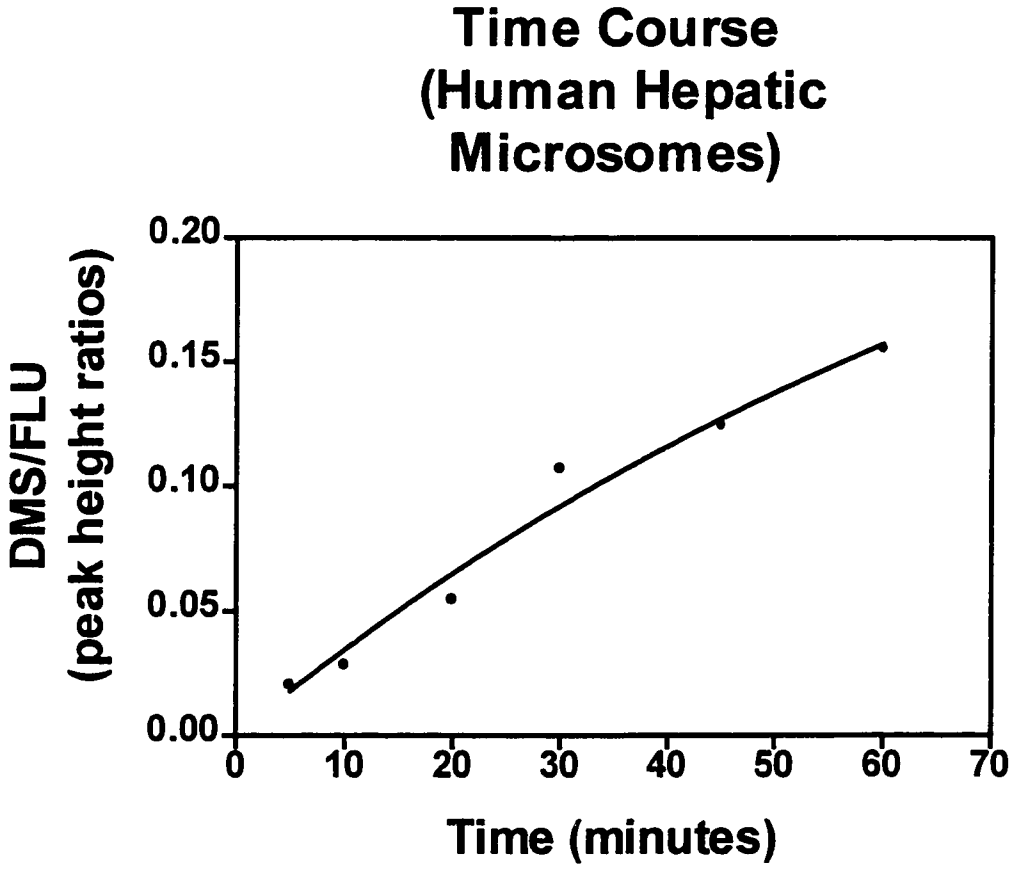


Figure 12: Time course for the formation of DMS in human hepatic microsomal preparations using a SER concentration of 100μM.

4.4.3 **Dose-concentration experiment**

Individual microsomal samples were incubated with SER at the following concentrations: 25, 50, 100, 150, 200 or 400μM for 20 min and the assay procedure was performed as described in section 3.6.3 "*In vitro* microsomal enzyme studies". The concentration curve for the formation of DMS is shown in Figure 13. Microsomal enzymes appear to be approaching saturation at SER concentrations above 200μM. SER at 100μM was used for the inhibition studies based upon the observation that this was a concentration at which DMS formation was in the linear range. Although initially the SER concentration of 400μM was used in the correlation studies, for consistency and to more accurately represent pharmacologically relevant concentrations, 100μM was used when the experiment was repeated.

4.4.4 **Correlation experiments**

The rate of formation of DMS was correlated with the activities of the CYP enzymes for each of the characterized HHM preparations in the panel. Any enzyme producing a significant correlation was implicated as having a role in the metabolite's production. Using a substrate concentration of 100μM, CYP4A ($r= 0.52$, $p= 0.039$), CYP3A4 ($r= 0.700$, $p= 0.0025$) and CYP2A6 ($r= 0.82$, $p= 0.001$) appear to be involved in the formation of DMS (Figure 14). The same enzymes

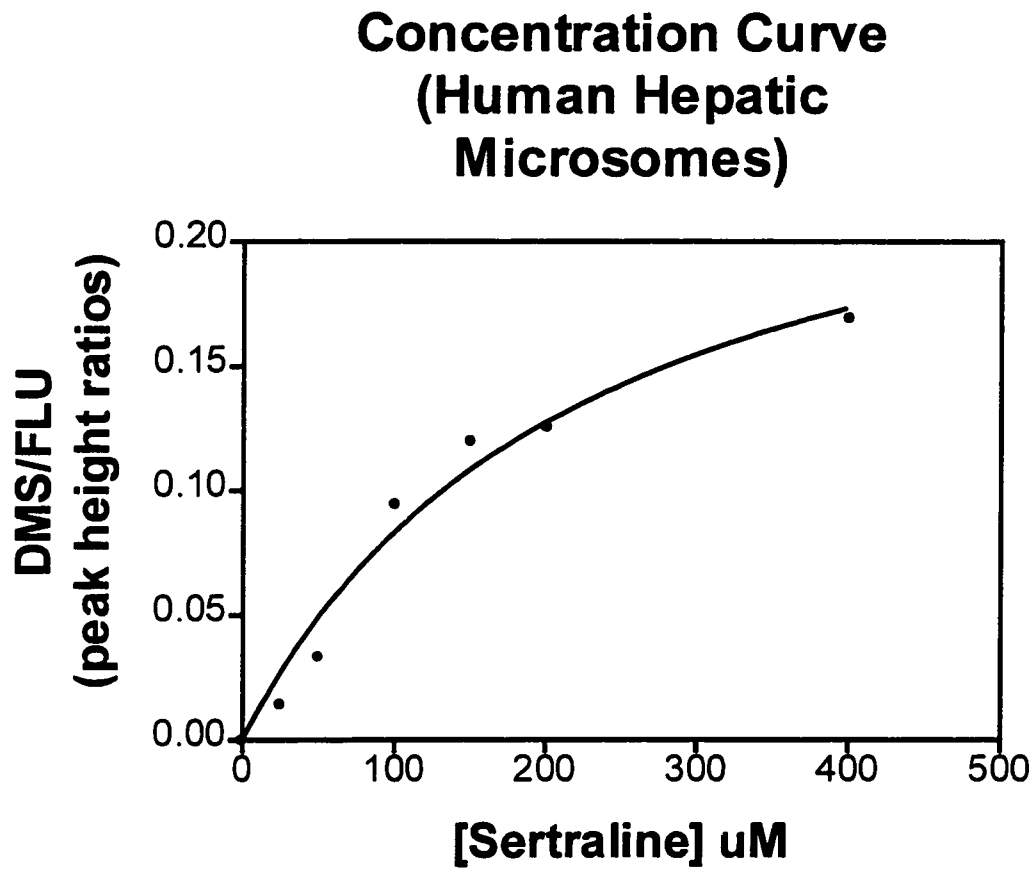


Figure 13: Concentration curve for the formation of DMS in human hepatic microsomal preparations.

were identified as being involved in DMS formation when the substrate concentration was increased to 400μM (data not shown).

4.4.5 **Inhibition experiments**

To investigate further the role of specific CYP enzymes in the formation of DMS, human liver microsomes were pre-incubated with known inhibitors of specific CYP enzymes before the addition of SER. FLV was chosen as it is a known, potent inhibitor of CYP3A4, as well as of CYP1A2 and CYP2C19 (Hansten and Horn, 1996; Nemeroff et al., 1996; Preskorn, 1996a; Ereshefsky et al., 1996). Ketoconazole, an antifungal agent, was used as it is also a known potent inhibitor of CYP3A4 and of CYP2A6 (Bertz and Granneman, 1997). The antibiotic erythromycin is a specific inhibitor of CYP3A4 (Hansten and Horn, 1996). Presently, no selective inhibitors of CYP4A have been identified (Spatzenegger and Jaeger, 1995). Quinidine was used as an potent inhibitor of CYP2D6 and a positive control (Preskorn, 1996a); at the concentration used (10μM), quinidine has been shown in our laboratories to produce almost complete inhibition of CYP2D6 under the incubation conditions used here (Rotzinger et al., 1998).

Further, with the knowledge that, theoretically, all drugs which are substrates for a particular isozyme can also act as competitive inhibitors of the metabolism of other drugs by that enzyme (DeVane, 1994); coumarin (Merkel et al., 1994) and nicotine (Bertz and Granneman, 1997) were included in the study as substrates/competitive inhibitors of CYP2A6.

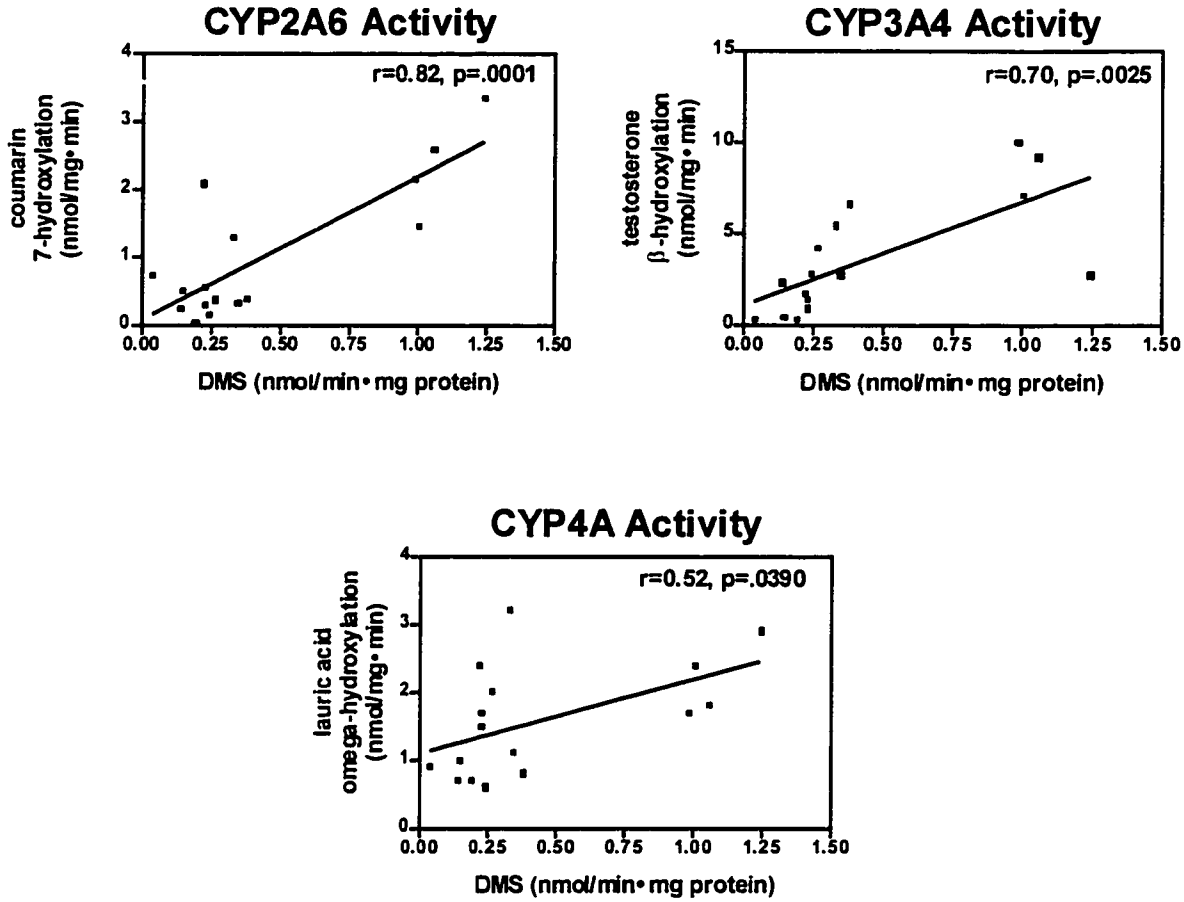


Figure 14a: Correlations of DMS production from SER (100μM) and enzyme activity in HHM from a panel of 16 human livers characterized for activities of CYP2A6, CYP3A4, and CYP4A.

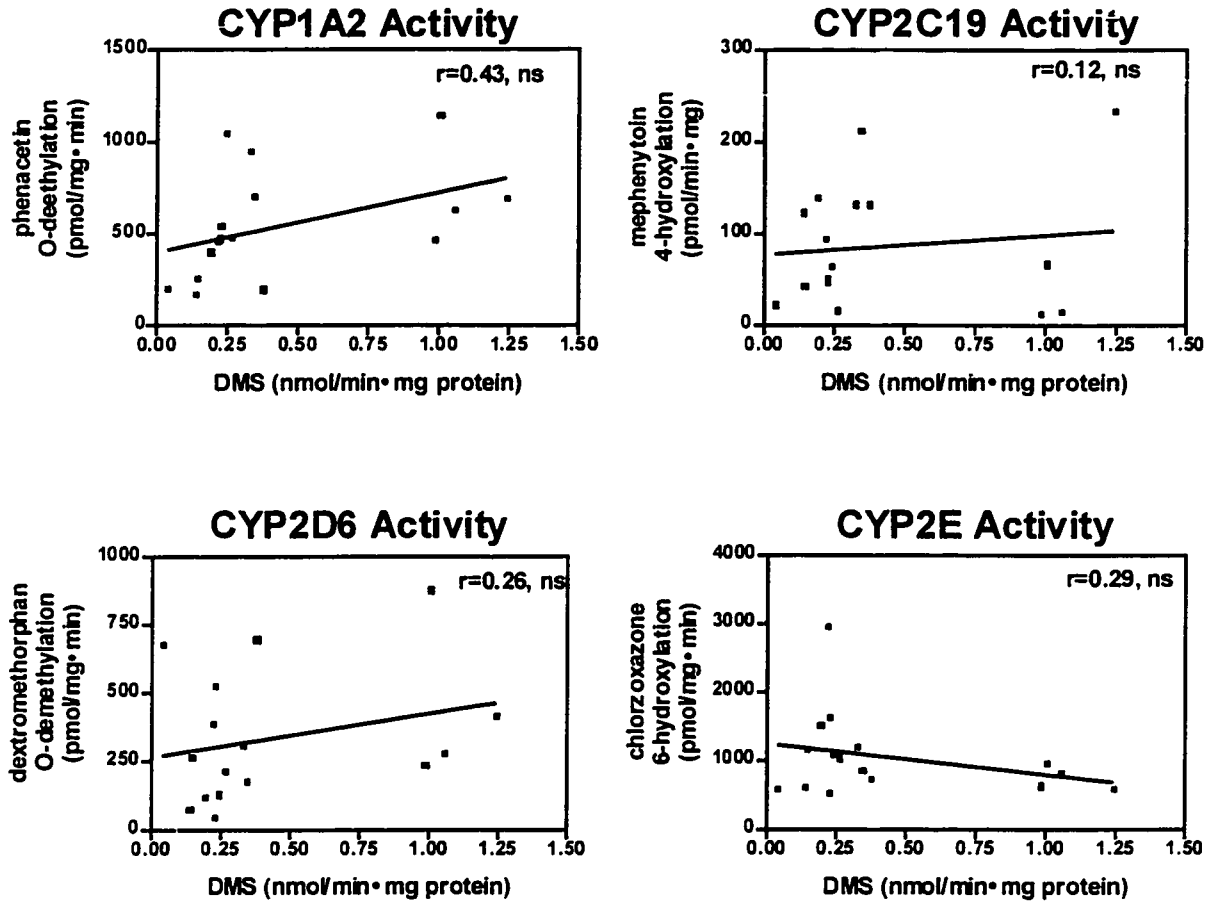


Figure 14b: Correlations of DMS production from SER (100μM) and enzyme activity in HHM from a panel of 16 human livers characterized for activities of CYP1A2, CYP2C19, CYP2D6, and CYP2E.

The inhibition experiments demonstrated that as the concentrations of the inhibitors increased in the microsomal preparations, the formation of DMS decreased significantly relative to controls containing no inhibitors (figures 15 and 16). Quinidine did not significantly inhibit SER N-demethylation at concentrations (under 100 μM) it would be expected to if CYP2D6 was involved in the metabolism of SER.

4.4.6 **Experiments with individual CYP enzymes**

DMS (1.47μM) was produced when microsomes expressing the individual cDNA for human CYP3A4 were incubated with SER (100μM) for 20 min. Interestingly, the results for the individually expressed CYP2A6 did not corroborate the results from the experiments with the human liver microsome panel. Incubation of SER (100μM) with the control microsomes (from cells expressing no significant CYP activity) did not produce any DMS.

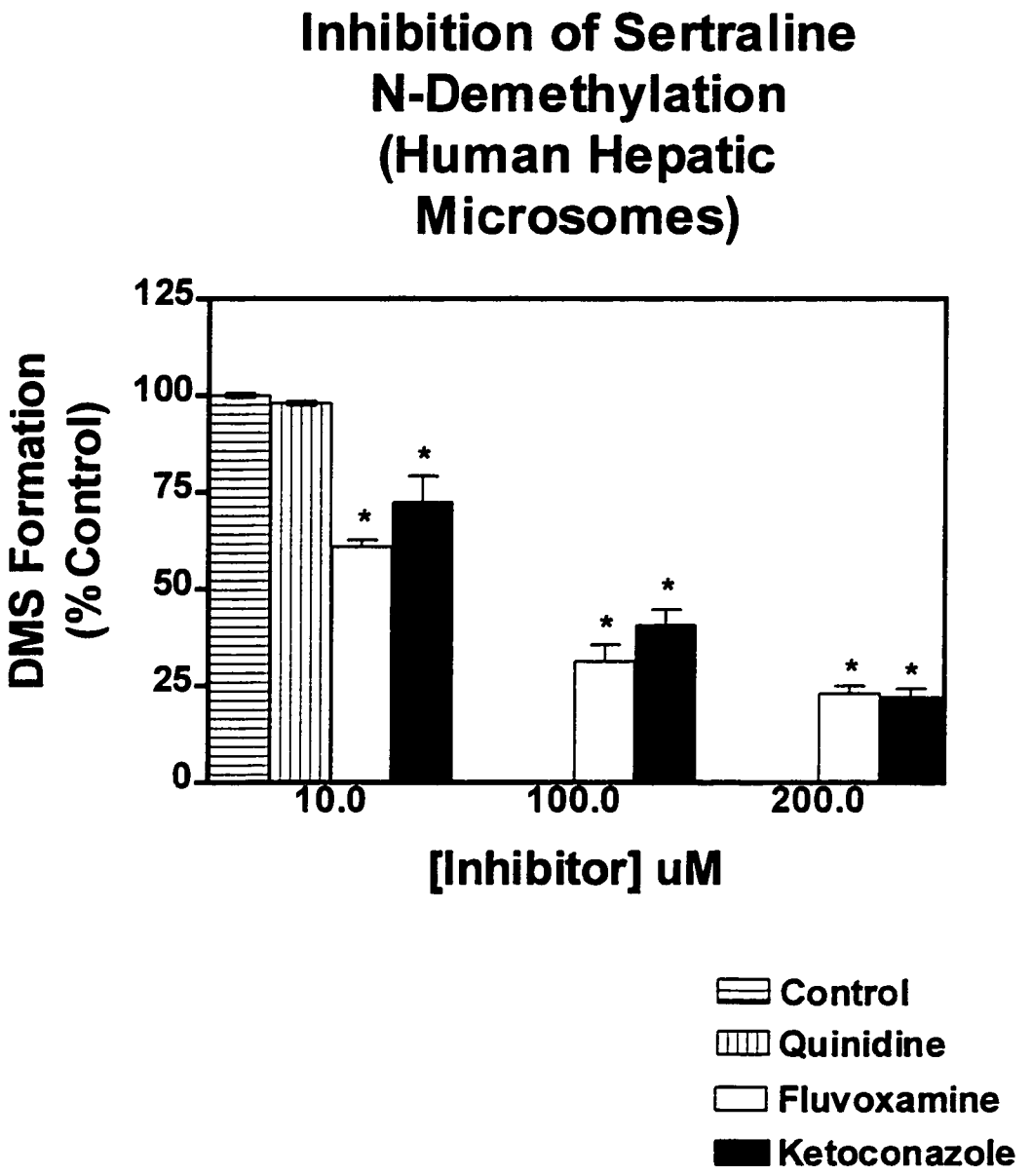


Figure 15: Inhibition of the metabolism of SER to DMS (% control) in HHM preparations in the presence of various concentrations of the inhibitors FLV and ketoconazole. Quinidine (10μM) was also included as a positive control. Data shown are means±SEM (n=3). * Signifies $p<0.05$. No inhibitor was present in the control.

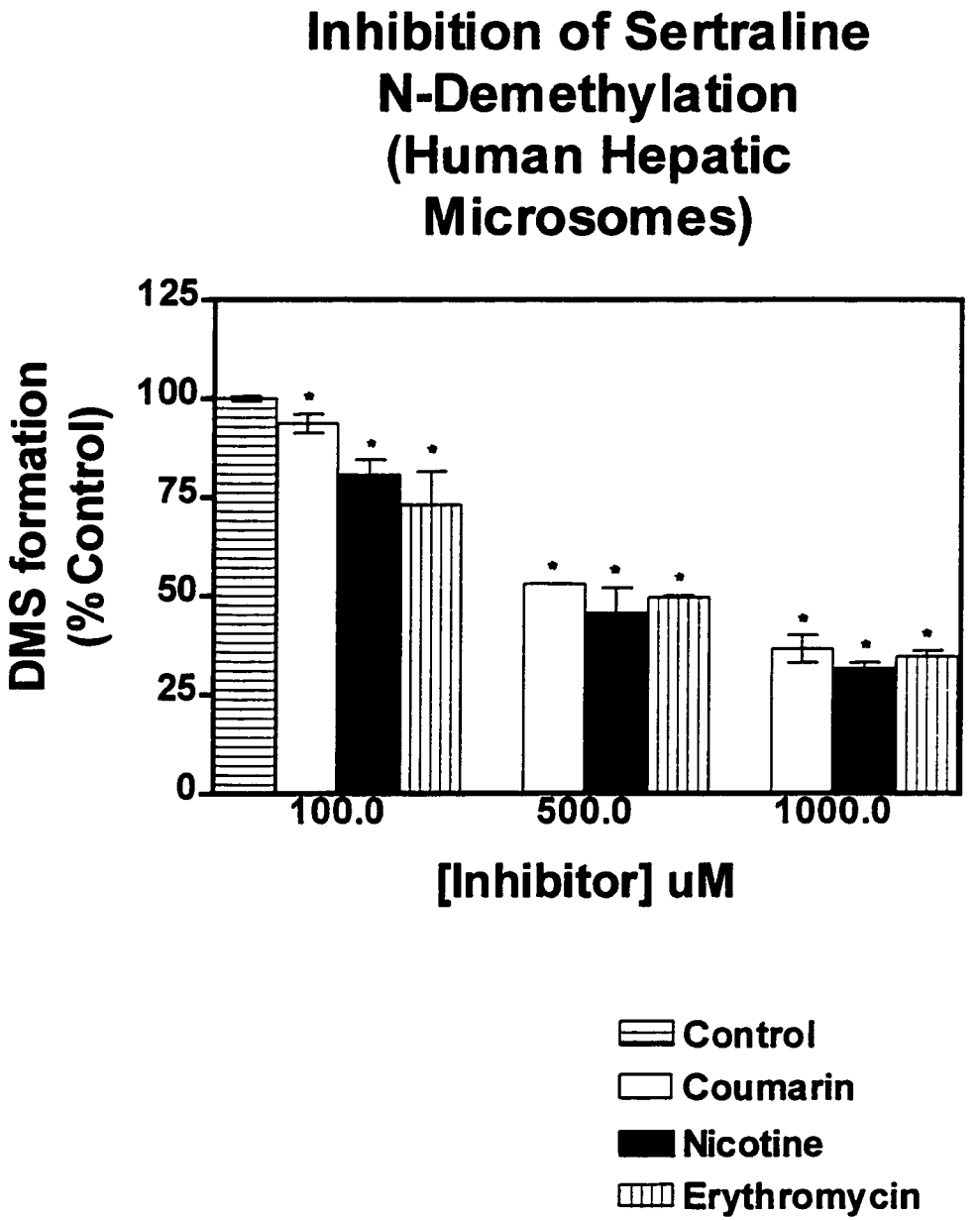


Figure 16: Inhibition of the metabolism of SER to DMS (% control) in HHM preparations in the presence of various concentrations of the inhibitors coumarin, nicotine and erythromycin. Data shown are means±SEM (n=3). * Signifies $p<0.05$. No significant inhibition was apparent at 10μM with any of these inhibitors. No inhibitor was present in the control.

5.0 DISCUSSION

5.1 Simultaneous quantification of SER and N-DMS

The novel assay procedure for SER and DMS reported in this thesis is relatively simple, produces derivatives with good chromatographic properties, and is suitable for analysis of brain tissue, plasma and microsomal incubates. In initial studies on the development of an assay for SER and DMS, extractive derivatization with pentafluorobenzoyl chloride and pentafluorobenzenesulfonyl chloride, two reagents which react readily with amines and have been used in the Neurochemical Research Unit to assay other amine-containing drugs (Rotzinger et al., 1997 and references contained therein; Rao et al., 1986; Paetsch et al., 1991) was attempted. However, when these reagents were reacted with brain homogenates, peaks which interfered with the analysis of SER and DMS (i.e. had similar retention times) were present. Initial experiments also determined that the assay for SER and DMS had to be conducted using brain homogenate rather than a supernatant obtained after homogenization of the brain in perchloric acid and subsequent centrifugation to precipitate protein. Although such a protein precipitation step is a useful cleanup procedure, most of the SER and DMS adhered to the protein pellet; a similar phenomenon has been observed in the Neurochemical Research Unit with the SSRI antidepressants FLU (Torok-Both et al., 1992) and FLV (Rotzinger et al., 1997).

In the present assay, utilizing PFPA as the derivatizing reagent, the derivatized DMS was found to be unstable over time, necessitating injecting the

samples on the gas chromatograph the same day as the extraction and derivatization procedure (if the assay extended into a second day, samples were stored at -80°C overnight after the extraction phase). Recoveries of SER and DMS were relatively low, but the calibration curves were linear (r^2 values consistently >0.99) and the assay was reproducible, so SER and DMS could be quantified routinely if samples were derivatized the same day they were injected into the gas chromatograph. Tremaine and Joerg's (1989) GC-ECD assay protocol for the quantification of SER in plasma (employing trifluoroacetic anhydride as the derivatizing reagent) exhibited an even lower recovery of SER (40%) than the present one, was applied only to plasma samples, and did not provide analysis of DMS.

The advantage of using of using a GC-ECD method is that the assay is sensitive to low ng levels in samples, equivalent to pg levels "on column". In addition, the equipment is relatively inexpensive, reliable and within the budget of most laboratories. The procedure developed in the present project is particularly useful for future studies of SER pharmacodynamics and pharmacokinetics as it provides analysis of both SER and DMS and is applicable to a variety of tissues and body fluids as well as cell line experiments.

5.2 **Rat brain tissue studies**

The *ex vivo* preliminary time course experiment confirmed the expected result that by 6 hours a substantial proportion of the parent compound had been

metabolized, with SER concentrations decreasing as DMS concentrations increased. The subsequent dose-concentration study indicated that the conversion of SER to DMS is, as expected, dose-dependent. At the 6 hour sacrifice time, DMS concentrations were found to be higher than SER at both the 5 and 10 mg/kg doses, and very nearly equal those of SER at 20 mg/kg.

These results are consistent with those noted in earlier research. Previously, Fuller *et al.* (1995), using an HPLC method to determine DMS and SER in rat brain tissue, reported a marked accumulation of DMS to the point that it was present in rat brain at similar concentrations at 6 hours and at higher concentrations than SER at 8 hours and thereafter after a single injection of SER at 10.0 mg/kg. Additionally, SER concentrations declined between 1 and 7 hours, whereas DMS concentrations increased during this time interval. The concentrations and ratios of SER/DMS obtained in the present study were very similar to those of Fuller *et al.* (1995).

As in humans, there appears to be a considerable amount of N-demethylation of SER in rats. These preliminary *ex vivo* studies have shown that SER conversion to DMS in rat brain is time- and dose-dependent. This assay could be further used in future animal studies of SER metabolism. An understanding of psychotropic drug metabolism and the ability to quantify both parent compounds and metabolites in animal models (particularly the rat) is essential as these models are frequently used in studies on the mechanisms of action of antidepressants. Currently however, studies in animal models are often

analyzed with little regard to or knowledge of the brain levels of the drugs or metabolites.

5.3 **Human plasma**

The plasma levels of SER in patients determined with this assay agree with literature values (Preskorn, 1996a). The DMS levels are also within the range expected; as the metabolite has a significantly longer half life than the parent compound, steady state levels of DMS exceed those of SER in human subjects (Finley, 1994). This assay, as modified for patient plasma samples, could readily be used for therapeutic drug monitoring (TDM) of SER and DMS and thus be useful for studying patient compliance (Eap and Baumann, 1996) and for future studies *in vivo* on drug-drug interactions involving the metabolism of SER (Brøsen, 1996).

5.4 ***In vitro*** **microsomal enzyme studies**

In vitro enzyme preparations are valuable tools in the search for the specific metabolic pathways involved in the metabolism of clinically used drugs. Harvey and Preskorn (1996a) have indicated some observations that should be present *in vitro* if a compound is metabolized by a given CYP isoform. They include: 1) In liver microsomes, a high degree of correlation should be observed between the rate of oxidation and the immunochemically determined level of the

CYP. 2) Where a model substrate for the CYP has been identified, there should be a strong, positive correlation between product formation rates of the compound in question and the model substrate, and the compound in question should be a competitive inhibitor of the model substrate's oxidation. 3) Oxidation should be inhibited by antibodies to the CYP or by established, specific, and potent inhibitors of the CYP. 4) The compound should be oxidized by the purified CYP or by the CYP cDNA expressed in an appropriate vector (Harvey and Preskorn, 1996a).

In the experiments using human liver microsomes described in this thesis, it was determined (on the basis of correlations between DMS formation and CYP activity in the microsomes) that the isozymes CYP4A, CYP3A4 and CYP2A6 may play a role in the N-demethylation of SER. No inhibitor of CYP4A is known at this time, but preliminary studies with known inhibitors of CYP3A4 (FLV, ketoconazole, erythromycin) and CYP2A6 (ketoconazole, coumarin, nicotine) showed that these drugs significantly inhibited the production of DMS from SER.

Incubations of SER with microsomes from cells containing individual cDNA-expressed enzymes were conducted with CYP3A4 and CYP2A6, but microsomes from cells expressing CYP4A were not commercially available. These studies confirmed that CYP3A4 was involved in the formation of DMS from SER, but CYP2A6 did not appear to be involved (as no DMS was produced following incubation with SER). Although microsomes from cells expressing individual CYP enzymes have greatly facilitated studies on drug metabolism, it must be remembered that these preparations are artificially produced. It may be

that their activity may rely markedly on some factors (eg. lipids, cytochrome b_5, reductases, the presence of other CYP enzymes) that may be present in a more physiological medium, such as human liver microsomes. In a recent paper, Fang et al. (1998) found similar discrepancies in studies on clozapine using human liver microsomes and individual cDNA-expressed CYP enzymes, and emphasized the importance of using multiple preparations in such studies and not relying entirely on the individual CYP isoforms expressed in microsomes outside of their usual environment.

As shown in the results section, FLV and ketoconazole are more potent inhibitors of SER metabolism to DMS than are nicotine or coumarin. Ketoconazole and related nitrogen heterocycles are among the most potent reversible inhibitors of the CYPs because they interact with both the hydrophobic substrate binding site and the ligand binding heme, interfering with both substrate and oxygen binding. They also bind with high affinity to both the oxidized and reduced forms of CYP (Halpert, 1995). The clinical relevance of the inhibition exhibited by nicotine and coumarin on SER metabolism will obviously depend upon the relative affinity of the enzyme (CYP2A6) for the compounds, the concentrations of SER and the inhibitors at the active site after actual doses and the relative role of the enzyme in the overall elimination of the compounds in relation to the other CYPs (Halpert, 1995). At present there is a scarcity of information available on CYP2A6 and its inhibition with regard to these factors, but it is unlikely that the concentration of CYP2A6 inhibitors used in the present study would be attained *in vivo* (Homsy et al., 1997).

A disadvantage of using *in vitro* methods to extrapolate to *in vivo* situations is that the substrate and/or inhibitor concentrations used *in vitro* may span a range that is not necessarily clinically relevant. Further, in order to predict whether an inhibitor presents a concern *in vivo,* plasma concentrations of the inhibitor (as seen in clinical practice) in combination with the liver:water partition ratio must also be considered (Glue and Banfield, 1996; Richelson, 1997), although often such ratio information is not available. Clinically, a drug-drug interaction will only be detected if one drug impedes a CYP enzyme to the degree that the change in the metabolism of the inhibited drug exceeds *intra-*patient variability in clearance. Even then, if the substrate has a broad therapeutic window its metabolic inhibition may have no clinically meaningful implications (DeVane, 1994; Glue and Banfield, 1996). However, as cases of serious and even fatal drug-drug interactions have occurred, it is of utmost importance to obtain an idea of the potential a drug possesses for CYP inhibition through *in vitro* research. In psychiatric practice, where polypharmacy is common, this information becomes even more critical.

To date, research on SER has in the main focused on its effects on the metabolism of other drugs (Glue and Banfield, 1996). Although some SSRIs are known to be potent inhibitors of the CYP system, SER is generally considered to have less of a clinically meaningful effect on this system (Preskorn, 1996a; Richelson, 1997), although this remains a matter of debate (Nemeroff et al., 1996). There is a paucity of information available on the CYP enzymes involved in SSRI metabolism, and to the best of our knowledge, this is the only study that

has examined the CYP enzymes that may be involved in SER metabolism. The effects of the inhibitors on SER N-demethylation seen in the present microsomal experiments reinforce the need to elucidate the relevance of these drug interactions in the clinical situation. If CYP2A6 plays a role in the metabolism of SER, the administration of this antidepressant to psychiatric patients who are also smokers should be investigated *in vivo*. As CYP3A4 is the most abundant CYP enzyme in the liver (Ereshefsky et al., 1996) and is known to be inhibited by many compounds (Preskorn, 1997), clinicians should also be aware of possible drug-drug interactions that may arise with SER and be on the lookout for a subsequent increase in adverse effects due to increases in SER blood levels. To date, researchers have been more concerned about the CYP-mediated effects of SSRIs on other drugs such as the TCAs rather that the CYP-mediated effects of other drugs on SSRI metabolism. This situation has arisen because some of the drugs which are substrates for CYP enzymes, e.g. the TCA antidepressants, have a narrow therapeutic index and increased levels such as those seen after metabolic drug-drug interactions may be toxic (e.g. cardiotoxic levels of TCAs) (Preskorn, 1996a). The adverse effects of SSRIs, on the other hand, are not generally life threatening (Hyman et al., 1995), and therefore researchers have not been as concerned about increases in levels of SSRIs caused by the inhibition of CYP enzymes. However, the SSRIs are not without adverse effects – they can result in initial anxiety, gastrointestinal side effects and sexual dysfunction (Hyman et al., 1995) and, if present in excessive quantities, particularily in the presence of other serotonergic drugs, can result in the life-

threatening serotonin syndrome (Skop et al., 1994; Harvey and Burke, 1995; Hegerl et al., 1998). The symptoms of serotonin syndrome include confusion, agitation, myoclonus, tremor, incoordination and fever (Hegerl et al., 1998). At present, there is no effective antidote for this syndrome; drug withdrawal is recommended, and in the case of SSRIs this can present another set of difficulties, including dizziness, paresthesia, asthenia, nausea, visual disturbances and headache (Coupland et al., 1996; Mallya et al., 1993; Louie, 1994). Thus, it is becoming obvious that there should be more information available about CYP-mediated metabolism of SSRIs.

Although much work remains to clarify the involvement of CYP isoforms in SER biotransformation, the studies described in this thesis provide a starting point for future research and demonstrate that the assay developed here for SER and DMS is an excellent one with which to conduct such research. As for all SSRIs, any increase in knowledge about the interactions of these frequently prescribed drugs with the CYP system *in vitro* may translate into the safer, more effective use of these antidepressants in the clinical setting in the future.

6.0 **CONCLUSION**

A rapid, sensitive and economical GC-ECD assay was developed for the simultaneous analysis of SER and its metabolite DMS. This assay has wider application than any of the other assays currently available for SER, not only providing for the simultaneous analysis of SER and its N-demethylated metabolite, but also for utilization in the analysis in rat brain, human plasma and incubates from *in vitro* drug metabolism studies with human liver microsomes and individual cDNA-expressed CYP enzymes. The preliminary findings with the assay reported in this thesis indicate that: 1) studies in rats should include measurements of both SER and DMS since marked levels of both are attained at the usual doses administered to rats in studies on possible mechanisms of action of SER; 2) SER and DMS can be measured routinely in small amounts (1ml) of human plasma; and 3) caution should be observed when coadministering SER with other drugs which are inhibitors of CYP3A4. Initial experiments reported in this thesis suggested that there may also be interactions with agents (e.g. nicotine) that inhibit CYP2A6, but additional future experiments involving comprehensive enzyme kinetics and plasma/liver ratio measurements for SER and potential inhibitors are required to confirm the potential clinical significance of such interactions.

7.0 **REFERENCES**

Agúndez J.A., Ledesma M.C., Ladero J.M. and Benitez J. (1995) Prevalence of CYP2D6 gene duplication and its repercussion on the oxidative phenotype in white populations. *Clin. Pharmacol. Ther.* **57**: 265 – 269.

Arranz F.J. and Ros S. (1997) Effects of comorbidity and polypharmacy on the clinical usefulness of sertraline in elderly depressed patients: An open multicentre study. *J. Affect. Dis.* **46**: 285 – 291.

Baker G.B., Coutts R.T. and LeGatt D.F. (1982) Gas chromatographic analysis of amines in biological systems, In: *Analysis of Biogenic Amines*, Baker G.B. and Coutts R.T. (eds.), Elsevier Scientific Publishing Company, New York, NY, pp. 109 –128.

Baker G.B. and Greenshaw A.J. (1989) Effects of long-term administration of antidepressants and neuroleptics on receptors in the central nervous system. *Cell. Mol. Neurobiol.* **9**:1 – 44.

Bakish D., Hooper C.L., West D.L., Miller C., Blanchard A. and Bashier F. (1995) Moclobemide and specific serotonin re-uptake inhibitor combination treatment of resistant anxiety and depressive disorders. *Hum. Psychopharmacol.* **10**: 105 – 109.

Baumann P. (1996) Pharmacokinetic-pharmacodynamic relationships of the selective serotonin reuptake inhibitors. *Clin. Pharmacokinet.* **31**: 444 – 469.

Benazzi F. (1996) Serotonin syndrome with moclobenmide-fluoxetine combination. *Pharmacopsychiat.* **29**: 162.

Bertz R.J. and Granneman G.R. (1997) Use of *in vitro* and *in vivo* data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin. Pharmacokinet.* **32**: 210 – 258.

Bieck P. and Antonin K. (1989) Tyramine potentiation during treatment with MAO inhibitors: brofaromine and moclobemide vs. irreversible inhibitors. *J. Neural Transm.* **28**: 21 – 31.

Blier P. and de Montigny C. (1994) Current advances and trends in the treatment of depression. *Trends Pharmacol. Sci.* **15**: 220 – 226.

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

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

Bourin M. and Baker G.B. (1996) Do G proteins have a role in antidepressant actions? *Eur. Neuropsychopharmacol.* **6**: 49 – 53.

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

Brøsen K. (1996) Drug-metabolizing enzymes and therapeutic drug monitoring in psychiatry. *Ther. Drug Monit.* **18**: 393 – 396.

Brown W.A. and Harrison W. (1995) Are patients who are intolerant to one serotonin selective reuptake inhibitor intolerant to another? *J. Clin. Psychiatry.* **56**: 30 – 34.

Chouinard G. (1992) Sertraline in the treatment of obsessive compulsive disorder: two double-blind, placebo-controlled studies. *Int. Clin. Psychopharmacol.* **7**(suppl 2): 37 – 41.

Compendium of Pharmaceuticals and Specialties: Fifteenth Edition (1997) Canadian Pharmaceutical Association. Ottawa, Ontario.

Coppen A.J. and Doogan D.P. (1988) Serotonin and its place in the pathogenesis of depression. *J. Clin. Psychiatry.* **49**(suppl 8): 4 – 11.

Coupland N.J., Bell C.J. and Potokar J.P. (1996) Serotonin reuptake inhibitor withdrawal. *J. Clin. Psychopharmacol.* **16**: 356 – 362.

Coutts R.T. and Baker G.B. (1982) Gas Chromatography, In: *Handbook of Neurochemistry, 2nd edn., Volume 2: Experimental Neurochemistry*, Lajtha A. (ed.), Plenum Press, New York, N.Y., pp. 429 – 448.

Coutts R.T., Baker G.B. and Nazarali A.J. (1985) Gas chromatography of amines and their metabolites in tissues and body fluids. In: *Neuromethods 2: Amines and their metabolites*, Boulton A.A., Baker G.B. and Baker J.M. (eds.), The Humana Press Inc., Clifton, N.J., pp. 45 – 85.

Coutts R.T., Bolaji O.O., Su P., and Baker G.B. (1994) Involvement of CYP2D6, CYP3A4, and other cytochrome P-450 isozymes in N-dealkylation reactions. *J. Pharmacol. and Toxicol. Methods.* **31**: 177 – 186.

Crewe H.K., Lennard M.S., Tucker G.T., Woods F.R. and Haddock R.E. (1992) The effect of selective serotonin re-uptake inhibitors on cytochrome P4502D6 activity in human liver microsomes. *Br. J. Clin. Pharmacol.* **34**: 262 – 265.

Damlouji N.F., Feighner J.P. and Rosenthal M.H. (1985) Recent advances in antidepressants. In: *Pharmacotherapy of Affective Disorders; Theory and Practice*, Dewhurst W.G. and Baker G.B. (eds). New York University Press, New York, N.Y. pp. 286 – 311.

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

DeVane C.L. (1995) Comparative safety and tolerability of selective serotonin reuptake inhibitors. *Human Psychopharmacol.* **10** (suppl): 185 – 193.

Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (1994) American Psychiatric Association, Washington D.C.

Doogan D.P. and Caillard V. (1988) Sertraline: a new antidepressant. *J. Clin. Psychiatry.* **49**: 46 – 51.

Duman R.S., Heninger G.R. and Nestler E.J. (1997) A molecular and cellular theory of depression. *Arch. Gen. Psychiatry.* **54**: 597 – 606.

Eap C.B. and Baumann P. (1996) Analytical methods for the quantitative determination of selective serotonin reuptake inhibitors for therapeutic drug monitoring purposes in patients. *J. Chromatogr. B.* **686**: 51 – 63.

Ereshefsky L., Riesenman C. and Lam Y.W.F. (1996) Serotonin selective reuptake inhibitor drug interactions and the cytochrome P450 system. *J. Clin. Psychiatry* **57** (suppl 8): 17 – 25.

Fang J., Coutts R.T., McKenna K.G. and Baker G.B. (1998) Elucidation of individual cytochrome P450 isoenzymes involved in the metabolism of clozapine. *Naunyn-Schmiedeberg's Arch. Pharmacol.* (in press).

Finley P.R. (1994) Selective serotonin reuptake inhibitors: Pharmacologic profiles and potential therapeutic distinctions. *Annals of Pharmacother.* **28**: 1359 – 1369.

Fuller R.W., Hemrick-Luecke S.K., Littlefield E.S. and Audia J.E. (1995) Comparison of desmethylsertraline with sertraline as a monoamine uptake inhibitor *in vivo*. *Prog. Neuro-Psychopharmacol. & Biol. Psychiat.* **19**: 135 – 149.

Garfinkel D. (1958) Studies on pig liver microsomes. 1. Enzymatic and pigment composition of different microsomal fractions. *Arch. Biochem. Biophysics.* **77**: 493 – 509.

Gentest Corporation, Product Inserts (1995 – 1998) Woburn, M.A., U.S.A..

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

Goldstein L., Barker M., Segall F., Asihene R., Balser S., Lautenbach D. and McCoy M. (1996) Seizure and transient SIADH associated with sertraline (letter). *Am. J. Psychiatry.* **153**: 732.

Goodman Gilman A., Rall T.W., Nies A.S. and Taylor P. (Eds.) (1993) *Goodman and Gilman's The Pharmacological Basis of Therapeutics: 8th edition.* McGraw-Hill Inc. New York, N.Y..

Goodnough D.B. and Baker G.B. (1994) 5-Hydroxytryptamine$_2$ and ß-adrenergic receptor regulation in rat brain following chronic treatment with desipramine and fluoxetine alone and in combination. *J. Neurochem.* **62**: 2262 – 2268.

Guengerich F.P. and MacDonald T.L. (1990) Mechanisms of cytochrome P-450 catalysis. *FASEB J.* **4**: 2453 – 2459.

Haider A., Miller D.R., Christenson J.L., Cushing A.G. and Staton D. (1995) Clinical effect of converting antidepressant therapy from fluoxetine to sertraline. *Am. J. Health-Syst. Pharm.* **52**: 1317 – 1319.

Halpert J.R. (1995) Structural basis of selective cytochrome P450 inhibition. *Ann. Rev. Pharmacol. Toxicol.* **35**: 29 – 53.

Hamelin B.A., Turgeon J., Vallée F., Bélanger P-M., Paquet F. and LeBel M. (1996) The disposition of fluoxetine but not sertraline is altered in poor metabolizers of debrisoquin. *Clin. Pharmacol. Ther.* **60**: 512 – 521.

Harvey A.T. and Burke M. (1995) Comment on: The serotonin syndrome associated with paroxetine, an over-the-counter cold remedy, and vascular disease. *Am. J. Emergency Med.* **13**: 605 – 606.

Harvey A.T. and Preskorn S.H. (1996a) Cytochrome P450 enzymes: Interpretation of their interactions with selective serotonin reuptake inhibitors. Part I. *J. Clin. Psychopharmacol.* **16**: 273 – 285.

Harvey A.T. and Preskorn S.H. (1996b) Cytochrome P450 enzymes: Interpretation of their interactions with selective serotonin reuptake inhibitors. Part II. *J. Clin. Psychopharmacol.* **16**: 345 – 354.

Hansten P.D. and Horn J.R. (1996) Substrates and inhibitors for selected cytochrome P450 isozymes. *Drug Interactions: A Clinical Perspective and Analysis of Current Developments: Quarterly Updates.* **16**: 205 – 206.

Hegerl U., Bottlender R., Gallinat J., Kuss H.J., Ackenheil M., and Möller H.J. (1998) The serotonin syndrome scale: first results on validity. *Eur. Arch. Psychiatry Clin. Neurosci.* **248**: 96 –103.

Heym J. and Koe B.K. (1988) Pharmacology of sertraline: a review. *J. Clin. Psychiatry.* **49**(suppl 8): 40 – 45.

Homsy W., Yan K., Houle J.M., Besner J.G., Gossard D., Pierce C.H. and Caillé G. (1997) Plasma levels of nicotine and safety of smokers wearing transdermal delivery systems during multiple simultaneous intake of nicotine and during exercise. *J. Clin. Pharmacol.* **37**: 728 – 736.

Hyman S., Arana G. and Rosenbaum J. (1995) *Handbook of Psychiatric Drug Therapy: Third Edition.* Little, Brown and Company, Boston.

Katzelnick D.J., Kobak K.A., Greist J.H., Jefferson J.W., Mantle J.M. and Serlin R.C. (1995) Sertraline for social phobia: A double-blind, placebo-controlled crossover study. *Am. J. Psychiatry.* **152**: 1368 – 1371.

Kenyon J. and Caldwell M. (Eds.) (1993) Sertraline: effective, safe and expensive in depression and obsessive-compulsive disorder. *Drugs Ther. Perspectives.* **1**:1 – 4.

Koe B.K., Weissman A., Welch W.M. and Browne R. (1983) Sertraline, 1S,4S-N-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-naphthylamine, a new uptake inhibitor with selectivity for serotonin. *J. Pharmacol. Exp. Ther.* **226**: 686 – 700.

Logan B.K., Friel P.N. and Case G.A. (1994) Analysis of sertraline (Zoloft®) and its major metabolite in postmortem specimens by gas and liquid chromatography. *J. Anal. Toxicol.* **18**: 139 – 142.

Louie A.K., Lannon R.A. and Ajari L.J. (1994) Withdrawal reaction after sertraline discontinuation. *Am. J. Psychiatry.* **151**: 450 – 451.

Mallya G., White K. Gunderson C. (1993) Is there a serotonergic withdrawal syndrome? *Biol. Psychiatry* **33**: 851 – 852.

Merck Index: Eleventh Edition (1989) Merck & Co. Inc.. Rahway, N.J.

Merkel U., Sigusch H. and Hoffmann A. (1994) Grapefruit juice inhibits 7-hydroxylation of coumarin in healthy volunteers *Eur. J. Clin. Pharmacol.* **46**: 175 – 177.

Montgomery S. (1995) Serotonin, sertraline and depression. *J. Psychopharmacol.* **9** (2, suppl): 179 – 184.

Moon C.A.L., Jago W., Wood K. and Doogan D.P. (1994) A double-blind comparison of sertraline and clomipramine in the treatment of major depressive disorder and associated anxiety in general practice. *J. Psychopharmacol.* **8**: 171 – 176.

Morinobu S., Nibuya M. and Dunman, R.S. (1995) Chronic antidepressant treatment down-regulates the induction of *c-fos* mRNA in response to acute stress in rat frontal cortex. *Neuropsychopharmacol.* **12**: 221 – 228.

Murdoch D. and McTavish D. (1992) Sertraline: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential in depression and obsessive-compulsive disorder. *Drugs.* **44**: 604 – 624.

Murray M. (1987) Mechanisms of the inhibition of cytochrome P-450-mediated drug oxidation by therapeutic agents. *Drug Metab. Rev.* **18**: 55 – 81.

Nelson D.R., Koymans L., Kamataki T., Stegeman J.J., Feyereisen R., Waxman D.J., Waterman M.R., Gotoh O., Coon J., Estabrook R.W., Gunsalus I.C. and Nebert D.W. (1996) P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogen.* **6**: 1 – 42.

Nemeroff C.B. (1998) The neurobiology of depression. *Sci. Amer.* **6**: 1-12.

Nemeroff C.B., DeVane C.L. and Pollock B.G. (1996) Newer antidepressants and the cytochrome P450 system. *Am. J. Psychiatry* **153**: 311 – 319.

Nutt D. (1995) The anxiety factor in depression. *J. Psychopharmacol.* **9** (2 suppl): 185 – 189.

Omura T. and Sato R. (1962) A new cytochrome in liver microsomes. *J. Bio. Chem.* **237**: PC1375-PC1376.

Otton S.V., Dafang W., Joffe R.T., Cheung S.W. and Sellers E.M. (1993) Inhibition by fluoxetine of cytochrome P450 2D6 activity. *Clin. Pharmacol. Ther.* **53**: 401 – 409.

Paetsch P.R., Baker G.B., Caffaro L.E., Greenshaw A.J., Rauw G.A. and Coutts R.T. (1991) An electron-capture gas chromatographic procedure for simultaneous determination of amphetamine and N-methylamphetamine. *J. Chromatogr. Biomed. Appl.* **573**: 313 – 317.

Parkinson A. (1996) Biotransformation of Xenobiotics, In: *Casarett & Doull's Toxicology; The Basic Science of Poisons, 5th Edition.* Klaassen C.D. (ed.), McGraw-Hill (Health Professions Division), New York, N.Y., pp. 113 – 186.

Perry P.J. (1996) Pharmacotherapy for major depression with melancholic features: relative efficacy of tricyclic versus selective serotonin reuptake inhibitor antidepressants. *J. Affect. Dis.* **39**: 1 – 6.

Poklis A. (1989) Gas Chromatography, In: *Clinical Chemistry: Theory, Analysis and Correlation*, Kaplin L.A. and Pesce A.J. (eds.), The C.V. Mosby Company, Toronto, ON, pp. 110 – 125.

Preskorn S.H. (1993) Recent pharmacologic advances in antidepressant therapy for the elderly. *Am. J. Med.* **94** (suppl 5A): 5A-2S – 5A-12S.

Preskorn S.H. (1994) Targeted pharmacotherapy in depression management: comparative pharmacokinetics of fluoxetine, paroxetine and sertraline. *Int. Clin. Psychopharmacol.* **9**: 13 – 19.

Preskorn S.H. (1996a) Reducing the risk of drug-drug interactions: A goal of rational drug development. *J. Clin. Psychiatry.* **57** (suppl 1): 3 – 6.

Preskorn S.H. (1996b) *Clinical Pharmacology of Selective Serotonin Reuptake Inhibitors (First Edition).* Professional Communications, Inc., Wichita.

Preskorn S.H. (1997) Clinically relevant pharmacology of the selective serotonin reuptake inhibitors. *Clin. Pharmacokinet.* **32** (suppl 1): 1 – 21.

Preskorn S.H. and Lane R.M. (1995) Sertraline 50mg daily: the optimal dose in the treatment of depression. *Int. Clin. Psychopharmacol.* **10**: 129 – 141.

Rao T.S., Baker G.B. and Coutts R.T. (1986) Pentafluorobenzenesulfonyl chloride as a sensitive reagent for the rapid chromatographic analysis of tranylcypromine in tissues and body fluids. *Biochem. Pharmacol.* **35**: 1925 – 1928.

Rapeport W.G., Coates P.E., Dewland P.M., and Forster P.L. (1996a) Absence of a sertraline-mediated effect on digoxin pharmcokinetics and electrocardiographic findings. *J. Clin. Psychiatry.* **57** (suppl 1): 16 – 19.

Rapeport W.G., Muirhead D.C., Williams S.A., Cross M., and Wesnes K. (1996b) Absence of effect of sertraline on the pharmacokinetics and pharmacodynamics of phenytoin. *J. Clin. Psychiatry.* **57** (suppl 1): 24 – 28.

Rapeport W.G., Williams S.A., Muirhead D.C., Dewland P.M., Tanner T. and Wesnes K. (1996c) Absence of a sertraline-mediated effect on the pharmacokinetics and pharmacodynamics of carbamazepine. *J. Clin. Psychiatry.* **57** (suppl 1): 20 – 23.

Rasmussen B.B., Mäenpää J., Pelkonen O., Loft S., Poulsen H.E., Lykkesfeldt J. and Brøsen K. (1995) Selective serotonin reuptake inhibitors and theophylline metabolism in human liver microsomes: potent inhibition by fluvoxamine. *Br. J. Clin. Pharmacol.* **39**: 151 – 159.

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

Riesenman C. (1995) Antidepressant drug interactions and the cytochrome P450 system: A critical appraisal. *Pharmacotherapy.* **15**(6 Pt 2): 84S – 99S.

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

Rogowsky D., Marr M., Long G. and Moore C. (1994) Determination of sertraline and desmethylsertraline in human serum using copolymeric bonded-phase extraction, liquid chromatography and gas chromatography-mass spectrometry. *J. Chromatogr. B.* **655**: 138 – 141.

Ronfeld R.A., Tremaine L.M. and Wilner K.D. (1997) Pharmacokinetics of sertraline and its *N*-desmethyl metabolite in elderly and young male and female volunteers. *Clin. Pharmacokinet.* **32** (suppl 1): 22 – 30.

Rosenbaum J.F. (1995) Managing selective serotonin reuptake inhibitor-drug interactions in clinical practise. *Clin. Pharmacokinet.* **29** (suppl 1): 53 – 59.

Rotzinger S., Fang J., Coutts, R.T. and Baker G.B. (1998) Human CYP2D6 and metabolism of m-chlorophenylpiperazine (mCPP). *Biol. Psychiatry* (in press).

Rotzinger S., Todd K.G., Bourin M., Coutts R.T. and Baker G.B. (1997) A rapid electron-capture gas chromatographic method for the quantification of fluvoxamine in brain tissue. *J. Pharmacol. Toxicol Methods* **37**: 129 –133.

Sanders-Bush E., Breeding M., Knoth K. and Tsutsumi M. (1989) Sertraline-induced desensitization of the serotonin 5-HT_2 receptor transmembrane signaling system. *Psychopharmacol.* **99**: 64 – 69.

Schmider J., Greenblatt D.J., Von Moltke L.L., Harmatz J.S. and Shader R.I. (1995) N-Demethylation of amitriptyline *in vitro:* role of cytochrome P-450 3A (CYP3A) isoforms and effect of metabolic inhibitors. *J. Pharmacol. Exp. Ther.* **275**: 592 – 597.

Schwaninger M., Schöfl C., Blume R., Rössig L. and Knepel W. (1995) Inhibition by antidepressant drugs of cyclic AMP response element-binding protein / cyclic AMP response element-directed gene transcription. *Mol. Pharmacol.* **47**: 1112 – 1118.

Shader R.I., Von Moltke L.L., Schmider J., Harmatz J.S., and Greenblatt D. (1996) The clinician and drug interactions – an update. *J. Clin. Psychopharmacol.* **16**: 197 – 201.

Shelton R.C., Manier D.H. and Susler F. (1996) cAMP dependent protein kinase activity in major depression. *Am. J. Psychiatry.* **153**: 1037 – 1042.

Skop B.P., Finkelstein J.A., Mareth T.R. (1994) The serotonin syndrome associated with paroxetine, an over-the-counter cold remedy, and vascular disease. *Am. J. Emerg. Med.* **12**: 642 – 644.

Sokolov S.T.H. and Joffe R.T. (1995) Practical guidelines for combination drug therapy of treatment resistant depression. *CNS Drugs.* **4**: 341 – 350.

Soucek P. and Gut I. (1992) Cytochromes P-450 in rats: structures, functions, properties and relevant human forms. *Xenobiotica* **22**: 83 – 103.

Spatzenegger M. and Jaeger W. (1995) Clinical importance of hepatic cytochrome P450 in drug metabolism. *Drug Metab. Reviews.* **27**: 397 – 417.

Stahl S.M. (1993) Serotonergic mechanisms and the new antidepressants. *Psychol. Med.* **23**: 281 – 285.

Tabor M.W. (1989) Chromatography: theory and practice. In: *Clinical Chemistry Theory, Analysis, and Correlation, 2nd Ed.,* Kaplan L.A. and Pesce A.J. (eds.) The CV Mosby Company, Toronto, ON., pp. 73 – 93.

Testa B. (1990) Mechanisms of inhibiton of xenobiotic-metabolizing enzymes. *Xenobiotica* **20**: 1129 – 1137.

Tilakaratne N., Yang Z., and Friedman E. (1995) Chronic fluoxetine or desmethylimipramine treatment alters 5-HT_2 receptor mediated *c-fos* gene expression. *Eur. J. Pharmacol., Mol. Pharmacol. Sect.* **290**: 263 – 266.

Tollefson G.D. (1995) Selective serotonin reuptake inhibitors. In: *Textbook of Psychopharmacology.* Schatzberg A.F. and Nemeroff C.B., eds. APA press, Washington D.C., pp 161 – 182.

Torok-Both G.A., Baker G.B., Coutts R.T., McKenna K.F. and Aspeslet L.J. (1992) Simultaneous analysis of fluoxetine and norfluoxetine enantiomers in biological samples by gas chromatography with electron capture detection. *J. Chromatogr. Biomed. Appl.* **579**: 99 – 106.

Toth M. and Shenik T. (1994) Antagonist-mediated down-regulation of 5-hydroxytryptamine type 2 receptor gene expression: modulation of transcription. *Mol. Pharmacol.* **45**: 1095 – 1100.

Tremaine L.M. and Joerg E.A. (1989) Automated gas chromatographic-electon-capture assay for the selective serotonin uptake blocker sertraline. *J. Chromatogr. Biomed. Appl.* **496**: 423 – 429.

Van Moffaert M., Bartholomé F., Cosyns P., De Nayer A.R. and Mertens C. (1995) A controlled comparison of sertraline and fluoxetine in acute and continuation treatment of major depression. *Human Psychopharmacol.* **10**: 393 – 405.

von Moltke L.L. Greenblatt D., Schmider J., Harmatz J.S. and Shader R.I. (1995) Metabolism of drugs by cytochrome P450 3A Isoforms: Implications for drug interactions in psychopharmacology. *Clin. Pharmacokinet.* **29** (Suppl 1): 33 – 44.

Zoloft Product Monograph. Zoloft (sertraline HCl) (1994) Pfizer Canada Inc., licensee.