University of Alberta

Neurochemical Studies on Clozapine and Lamotrigine

by

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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 Psychiatry

Edmonton, Alberta

Spring 2002

University of Alberta

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Title of Thesis: Neurochemical Studies on Clozapine and Lamotrigine

Degree: Master of Science

Year this Degree Granted: 2002

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Dated: December 21 2001

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Faculty of Graduate Studies and Research

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DEDICATION

To Jay

Thank you for your wonderful sense of humor, and your never-ending optimism and encouragement.

ABSTRACT

This thesis represents the results of an investigation into the neurochemistry of clozapine (CLOZ) and lamotrigine (LTG). CLOZ is an atypical antipsychotic agent that is often used in patients who do not respond to conventional antipsychotic treatment. Recently, CLOZ therapy augmented with LTG has been used for treatment of some otherwise refractory patients. However, little is known about the pharmacokinetic interactions between these two drugs. Human liver microsomes were used to investigate the metabolism of CLOZ in the presence of various inhibitors as well as LTG. The metabolism of isoclozapine (ISOCLOZ), an isomer of CLOZ, was also investigated. An animal study was designed to investigate the effects of acute coadministration of LTG and CLOZ on the levels of the drugs themselves in brain and liver and the levels of amino acids and spermidine in the brain.

In human liver microsomes LTG, only at the higher doses tested, exhibited a significant inhibition of CLOZ metabolism. A mass spectrometric study confirmed that ISOCLOZ, like CLOZ, undergoes N-demethylation and N-oxidation by human liver microsomes. LTG alone had no effects on any amino acid levels tested while CLOZ caused a significant increase in brain levels of glutamate, aspartate and alanine. CLOZ and LTG, alone or in combination, had no effect (at 1 hour and at 6 hours) on rat brain levels of spermidine.

ACKNOWLEDGEMENTS

To Dr. Andy Greenshaw and Dr. Glen Baker for giving me the opportunity to work at the Neurochemical Research Unit. I am grateful to Dr. Glen Baker for his guidance throughout the course of this program. Special thanks to Gail Rauw and Jordyce Van Muyden for their technical assistance throughout, and to Véronique Tanay for her assistance with the animal study presented in this thesis. I would also like to thank Dr. Jean-Michel Le Mellédo for giving me the opportunity to work with him. I am also indebted to Guifeng Jiang for her expertise and help with the mass spectrometry, and to Dr. Ron Coutts for helping me with the mass spectrometry fragmentation figures included in this thesis.

I am very grateful for the financial support provided by the Canadian Institutes of Health Research, the Davey Endowment and the Stanley Foundation, and the Province of Alberta Graduate Scholarship.

To Dr. Deborah James for her keen interest and caring in the lives of students I am greatly indebted. I have been fortunate to meet some great friends during this program. To Eva, Richelle, Rob and Mia thank you very much for your strength and laughter, it was appreciated and needed.

Most importantly I thank my parents Alan and Pierrette, as well as Carrina and Martin who were always interested and gave me constant encouragement. And especially to Junan who reminds us all how terribly exciting each little thing we learn and each new discovery should be.

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List of Abbreviations

ALA	Alanine
ASN	Aspargine
ASP	Aspartate
AMIT	Amitriptyline
β-ALA	β-Alanine
CLOZ	Clozapine
CNO	Clozapine N-oxide
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
EAA	Excitatory amino acid
ECD	Electron-capture detector
EDTA	Ethylenediaminetetraacetate
FLUVOX	Fluvoxamine
FMO	Flavin-containing monooxygenase
GABA	γ-Aminobutyric acid
GC	Gas chromatograph
GLN	Glutamine
GLU	Glutamate
GLY	Glycine
HPLC	High performance liquid chromatography
ID	Internal diameter
ISOCLOZ	Isoclozapine

KETO	Ketoconazole
L-DOPA	L-3,4-Dihydroxyphenylalanine
LTG	Lamotrigine
MS	Mass spectrometry
NADP	β-Nicotinamide adenine dinucleotide phosphate
NdM	N-Desmethylclozapine
NMDA	N-Methyl-D-aspartate
ODS	Octadecylsilane
OPT	o-Phthalaldehyde
PBS	Phosphate buffered saline
PCP	Phencyclidine
PET	Positron emission tomography
PFBC	Pentaflourobenzoyl chloride
PFC	Prefrontal cortex
SCOT	Support-coated open tubular
SEM	Standard error of the mean
SER	Serine
SSRI	Selective serotonin reuptake inhibitor
TAUR	Taurine
THF	Tetrahydrofuran
UV	Ultraviolet
WCOT	Wall-coated open tubular

1. INTRODUCTION

1.1 SCHIZOPHRENIA

According to DSM-IV criteria (American Psychiatric Association, 1994), schizophrenia is a disturbance that must last at least 6 months and includes the following symptoms: delusions, hallucinations, disorganized or catatonic behavior, disorganized speech, or negative symptoms. Positive symptoms include delusions and hallucinations, exaggerations in language and communication, and behavior distortions such as greatly disorganized, agitated or catatonic behavior. Negative symptoms include affective flattening, alogia (restrictions in productivity of thought or speech), avolition (restrictions in the initiation of goal-directed behavior), anhedonia (lack of pleasure) and attentional impairment (Harrison, 1999).

Several different hypotheses have been proposed in order to explain the biological mechanisms involved in schizophrenia. The neurodegenerative hypothesis focuses on the excitotoxic effects of glutamate (GLU) (Stahl, 2000). The neurodevelopmental hypothesis focuses on problems during fetal development that later become apparent (Bunney and Bunney, 1999). In schizophrenics several hypotheses involving different neurotransmitter systems have been put forward, the dopamine hypothesis being the predominant one (Olney and Farber, 1995). However, research in recent years has suggested a multifactorial view of schizophrenia, with the amino acids GLU and γ-aminobutyric acid (GABA) (see Figure 1) also being involved (Carlsson *et al.*, 2001). The complexity of schizophrenia as well as the complexity of the

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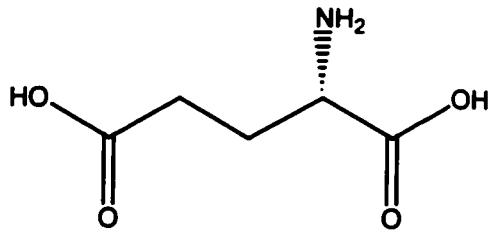


b)

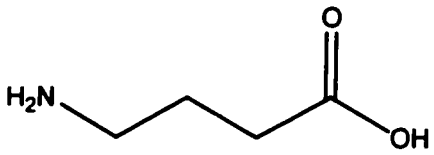


Figure 1. Structural formulae for a) GLU and b) GABA

neurotransmitter systems involved does not allow for a simple explanation involving only one system. It is therefore likely that several of these hypotheses intertwine; this can be seen with the popular dopamine hypothesis and the more recent glutamate hypothesis.

Although dopamine is the neurotransmitter which has received the most attention in schizophrenia research for many years, two amino acid neurotransmitters, GABA and GLU, have also been a focus of interest in recent years. GABA, by binding to its $GABA_A$ receptor, exerts an inhibitory effect on action potentials through its modulation of the chloride channel associated with that receptor. These channels remain in a prolonged open and activated state, allowing the influx of chloride ions and thereby reducing the resting membrane potential (Deutch and Roth, 1999; Marek and Aghajanian, 1999). GLU is a precursor to GABA production as well as the primary excitatory neurotransmitter in the central nervous system (Deutch and Roth, 1999; Stahl, 2000). Once released from its presynaptic nerve terminal it interacts with various receptors, including the N-methyl-D-aspartate (NMDA) receptor (Deutch and Roth, 1999; Marek and Aghajanian, 1999).

1.1.2 Glutamatergic Neurotransmission

GLU synthesis: The amino acid GLU is mainly used as a building block for protein synthesis in the body, but it is also an excitatory neurotransmitter which is synthesized from glutamine by glutaminase in the mitochondria. It is then stored in synaptic vesicles until its release during neurotransmission. Glutamine is found in glial cells adjacent to neurons. In these glial cells GLU is

converted to glutamine via the enzyme glutamine synthetase; glutamine can then enter the neuron for conversion into GLU that is then used as a neurotransmitter (Deutch and Roth, 1999; Stahl, 2000).

GLU removal: GLU is removed from the synaptic cleft by two transporter pumps, a presynaptic GLU transporter and a transport pump located on the nearby glial cells. Both transporter pumps remove GLU from the synapse and transport it into either the neuron or the glial cell (Stahl, 2000) respectively.

GLU receptors: There are several GLU receptors, including ionotropic receptors [NMDA-, *alpha*-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)-, and kainate-receptors] and multiple metabotropic GLU receptors (Stahl, 2000). The NMDA, AMPA, and kainate receptors are thought to be linked to ion channels whereas the metabotropic receptors, several of which have been identified, are G protein-linked (Stahl, 2000).

The NMDA receptor has several binding sites surrounding the calcium ion channel that can act as allosteric modulators of the receptor channel. These include a glycine site, a phencyclidine (PCP) site, a polyamine site, a site for zinc and another for magnesium (Stahl, 2000). The NMDA receptors surround cation channels and generally involve the modulation of the intracellular flux of Na^{+} and Ca^{++}, at the same time modulating the extracellular movement of K^{+} across the channel (Burnstein, 1995). Once GLU interacts with this receptor it can induce rapid neuronal firing, resulting in intermittent depolarization (Meldrum, 1991; Burnstein, 1995).

1.1.3 Schizophrenia Hypotheses

The dopamine hypothesis of schizophrenia is based on the idea of dopaminergic dysfunction, where there is a hyperactivity of dopamine in the brain. This hypothesis originated from the observation that several successful antipsychotic drugs are dopamine D_2 receptor antagonists and that dopamine-releasing agents like amphetamine cause psychosis (Harrison, 1999). Direct support for this hypothesis has primarily involved two lines of evidence. Firstly, using positron emission tomography (PET), it was found that the synthesis of labeled dopamine, from radiolabeled L-3,4-dihydroxyphenylalanine (L-DOPA), is increased in the brain of drug-naïve schizophrenic patients when compared to controls (Carlsson *et al.*, 2001). Secondly, again using PET studies, it was shown that following an amphetamine challenge the release of dopamine in the basal ganglia was elevated in drug-naïve schizophrenic patients when compared to controls and the elevation was correlated with positive psychotic symptoms (Carlsson *et al.*, 2001). Many of the known antipsychotics block dopamine D2 receptors, and drugs that mimic dopamine may cause psychotic symptoms, supporting the idea of increased dopamine activity in schizophrenia (Ossowska *et al.*, 2000).

There are however, several observations that detract from the dopamine hypothesis as an explanation for schizophrenia. Results from many studies involving dopamine levels in the brain show that there is a wide interpersonal variation within schizophrenics, suggesting that higher levels may apply to a sub-group of schizophrenics but cannot be generalized to the whole population. Stress

caused by the imaging procedure is also a factor that is known to affect dopamine levels and could produce abnormal values (Carlsson *et al.*, 2001). The results could also be secondary to a dysfunction in a different neurotransmitter system such as GLU (Carlsson *et al.*, 2001). Finally, many drugs aimed at blocking dopamine receptors have not been very successful at treating all the symptoms of schizophrenia, leading researchers to focus on other transmitter systems.

GABA is an inhibitory amino acid that has been implicated in the pathophysiology of schizophrenia. It is thought that in schizophrenic patients there is a decrease in the inhibitory influence of GABAergic neurons on the dopaminergic pathway (Brunello *et al.*, 1995). Studies have also found a decrease in the expression of mRNA for glutamic acid decarboxylase (GAD), the enzyme responsible for the synthesis of GABA, in patients with schizophrenia (Byne *et al.*, 1999). In limbic and prefrontal cortical regions of post mortem schizophrenic brains a reduction of GABAergic neurons has been observed as well as an increase in the number of $GABA_A$ receptors (Carlsson *et al.*, 2001).

There are several lines of research that have focused on the role of GLU in schizophrenia. Opposing GLU hyperfunction and GLU hypofunction hypotheses have been put forward. Support for GLU hyperfunction is based on morphological differences observed in schizophrenic brains when compared to controls. Neuronal loss and shrinkage in the cortex and hippocampus have been reported (Harrison, 1999) and this pattern of neuronal loss has been interpreted by some to indicate a process of prolonged cell death involving GLU excitotoxicity. Lamotrigine (LTG), an anticonvulsant which decreases GLU release, has been

found to reduce the psychotic effect of ketamine, an NMDA receptor antagonist (Anand *et al.*, 1997). Others believe that GLU activity is decreased in schizophrenia based on the similarity between NMDA receptor antagonist-induced psychosis and schizophrenia. It is also possible that the two theories of the role of GLU can be combined. It is likely that decreased GLU function could be present after an increase in GLU has caused cell death (Byne *et al.*, 1999).

Ketamine and PCP block NMDA receptors by binding to a site within the calcium channel of the receptor; as a result the flow of ions in the channel is blocked and the effect of NMDA agonists, such as GLU, is antagonized (Do *et al.*, 1995). Both PCP and its derivative ketamine facilitate the efflux of GLU, which may cause an increase in the activation of non-NMDA receptors (Duncan *et al.*, 1999; Moghaddam *et al.*, 1997). This results in some regions showing inhibition whereas others show great excitation. The excitation is thought to be due to a disruption in the inhibitory neural circuits, possibly involving the reduced activity of the GABAergic system (Duncan *et al.* 1999).

The GLU hypofunction hypothesis proposes that the hyperactivity of dopaminergic neurons leads to an enhanced inhibition of GLU release (Byne *et al.*, 1999; Olney, 1992). Kim *et al.* (1980) proposed the GLU hypofunction hypothesis after discovering low levels of cerebrospinal fluid GLU in schizophrenic patients. Tsai and colleagues (1995) found that postmortem analysis of schizophrenic brains indicated decreased GLU levels in the prefrontal cortex as well as hippocampus. Whether these decreased levels are due to drug action or the disease itself remains controversial. A decrease in cortical GLU is

further supported by the work of Sherman *et al.* (1991) showing that in synaptosomes prepared from brains of schizophrenic patients there is a decrease in the amount of GLU released, regardless of whether release was induced by a specific NMDA receptor agonist or nonspecifically by veratridine.

The NMDA receptor antagonist model is one model used by researchers that attempts to investigate the role of GLU and the GLU NMDA receptor in schizophrenia. The convergence of GLU, GABA and dopamine systems in the striatum, nucleus accumbens, and prefrontal cortex is thought to play an important role in relation to the NMDA antagonist model of schizophrenia (Byne *et al.*, 1999). Increases in NMDA receptors have been detected in several brain areas in schizophrenic patients (Olney and Farber, 1995; Ossowska *et al.*, 2000). It is thought that the decreased amount of GLU transmission leads to an upregulation of the glutamatergic NMDA receptors. An alternate interpretation could be that the upregulation of these receptors is due to the use of antipsychotics, and not to due the disease itself. It is also possible that NMDA receptor antagonists affect GABAergic neurons, thereby reducing inhibition of glutamatergic neurons and resulting in an increase in glutamatergic transmission as well as that of dopamine (Byne *et al.*, 1999; Olney and Farber, 1995).

Another theory for the role of GLU in the biological basis of schizophrenia proposes a dysfunction of GLU neurotransmission where an excessive release of GLU causes an increase in the amount of calcium entering cells. This causes a series of excitotoxic events, including the activation of certain enzymes that lead to overproduction of free radicals. These free radicals can then

lead to the destruction of the neuron by destroying vital organelles and membranes (Stahl, 2000). The effects of NMDA receptor antagonism are not yet well understood. Some studies have found that the result of blocking NMDA receptors is an excessive release of GLU in the cerebral cortex (Anand *et al.*, 1997; Moghaddam *et al.*, 1997), and not a decrease in GLU as others have suggested. GLU uptake is increased in the prefrontal cortex of patients with schizophrenia, which suggests a decrease in glutamatergic transmission, yet at the same time there is a reduction in the glutamatergic uptake in the basal ganglia (Tsai *et al.*, 1995). It is likely that the effects of NMDA receptor antagonism are different depending on what region of the brain one is investigating.

NMDA receptor antagonists, such as PCP and ketamine, have been found to induce neurodegenerative changes in the corticolimbic region of the rat brain and to produce psychotic behaviors in humans (Olney and Farber, 1995). These effects can be inhibited by dopamine antagonists and GABA agonists (Olney and Farber, 1995). Moghaddam *et al.* (1997) conducted a series of biochemical studies investigating the action of the NMDA antagonist ketamine. Subanesthetic doses of ketamine had a biphasic effect on GLU outflow in the prefrontal cortex; low doses increased extracellular GLU levels whereas anesthetic doses decreased these GLU levels. Increased GLU efflux was not seen in the striatum. It is thought that the increased GLU levels in the prefrontal cortex may be due to the disinhibition of GABAergic neurons, leading to enhanced firing of glutamatergic neurons (Moghaddam *et al.*, 1997). The decrease of GLU at anesthetic doses was expected, and could be due to inhibition of other neuronal systems post-synaptic

to the NMDA receptors (Moghaddam *et al.*, 1997). Therefore it was suggested that part of the effect of ketamine and phencyclidine (PCP) might be due to the activation of glutamatergic neurotransmission at non-NMDA receptors, rather than a glutamatergic deficiency (Moghaddam *et al.*, 1997). Moghaddam and Adams (1998) followed up this hypothesis by testing a metabotropic receptor agonist in rats to see if it would antagonize the behavioral effects of PCP. They found that not only did the agonist do this but it also caused an antagonism of the PCP-induced increase in GLU. Recently, however, Ossowska *et al.* (2000) found that antipsychotic drug action did not seem to involve non-NMDA receptors, because an agonist of the group II glutamate metabotropic receptors did not reverse the effects of PCP.

Another hypothesis of schizophrenia, linked to the role of GLU, is that it is a neurodevelopmental and neurodegenerative disorder. It is suggested that abnormalities in fetal brain development during the early stages of neuronal selection and migration could lead to schizophrenia. The symptoms of schizophrenia do not appear until adolescence. At this time the normal function of restructuring the brain's neuronal processes is occurring. It is possible that the problems of neuronal migration and selection, which occurred during fetal development, are finally seen in adolescence. Women who have had trauma or pathogens in their second trimester of pregnancy have a higher risk of their child developing schizophrenia than women who have not had these complications (Bunney and Bunney, 1999; Stahl, 2000). Some researchers suggest that during the developmental period hyperfunction of GLU would cause excitotoxic

processes, leading to a possible pattern of abnormal brain development, later resulting in the development of the schizophrenic psychosis (Olney, 1992). The excitotoxicity would result in a degeneration of neurons containing the glutamate NMDA receptors, thereby leading to the future GLU hypofunction because of the deficiency in NMDA receptors.

Olney and Farber (1995) have hypothesized that NMDA receptors tonically drive GABAergic neurons that in turn synapse onto glutamatergic neurons. The unmodulated excitation produced by the decreased inhibition from GABAergic neurons to glutamatergic neurons, which extend from the thalamus to the cortex, may be accentuated with the completion of myelination that occurs in late adolescence and early adulthood (Bunney and Bunney, 1999). If there was NMDA receptor hypofunction, this could lead to a decrease in GABA release followed by an increase in GLU release. For example, a decrease in GABAergic transmission in the thalamus would increase GLU release from the terminals of thalamocortical neurons (Byne *et al.*, 1999). This phenomenon would allow for certain brain regions to have higher glutamatergic neurotransmission than others.

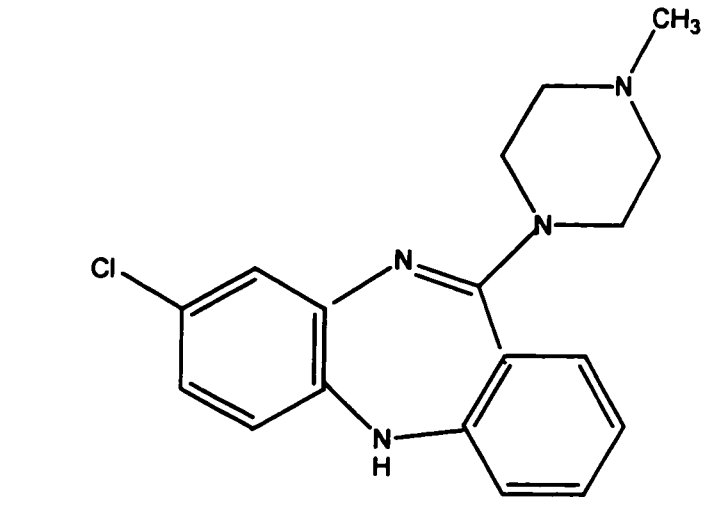
1.2 CLOZAPINE (CLOZ)

CLOZ is notably different from various typical antipsychotics. It is successful in treating the core symptoms of schizophrenia, both positive and negative. CLOZ also has the benefit of having reduced risk of extrapyramidal symptoms, as well as decreasing the risk of tardive dyskinesia (Burns, 2001). It is also more effective than most antipsychotics in treating treatment-resistant

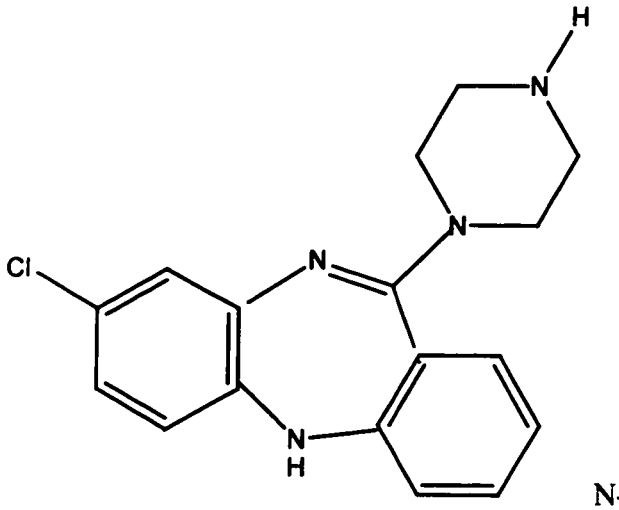
schizophrenic patients (Meltzer, 1992; Ashby and Wang, 1996; and Stahl, 1999). It is for these reasons that CLOZ has been named an atypical antipsychotic drug. Although these attributes should indicate that CLOZ would be the first-line choice in the treatment of schizophrenia, this is not the case due to the risk of developing agranulocytosis in anywhere from 0.5-2.0% of people taking the drug. Agranulocytosis is potentially fatal, and therefore it is required that patients undergo regular blood monitoring. CLOZ serum concentrations are variable from patient to patient and depend on a number of factors such as age, gender, body weight and smoking (Dettling *et al.*, 2000). CLOZ is metabolized primarily to N-desmethylclozapine (NdM) (active metabolite) and to clozapine N-oxide (CNO) (see Figure 2).

1.2.1 Mechanism of Action

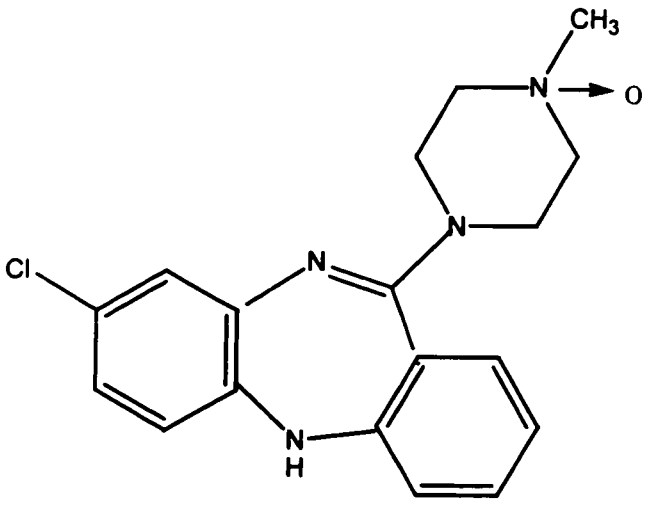
There are several mechanisms of action that are attributed to CLOZ. One mechanism is the serotonin-dopamine antagonism that is thought to contribute significantly to CLOZ's success as an antipsychotic agent in the treatment of schizophrenia. CLOZ binds to all the dopamine receptors, with the greatest affinity for the D4 receptor subtype, as well as to receptors for serotonin (5HT 1 and 5HT 2), acetylcholine, histamine and norepinephrine (Guitton *et al.*, 1998; Michel and Trudeau, 2000). Early on, the D1 receptor antagonistic properties of CLOZ were thought to be primarily responsible for its therapeutic effects; however, selective D1 antagonists developed subsequently were not effective in



Clozapine



N-Desmethylclozapine



Clozapine N-oxide

Figure 2. Structural formulae of CLOZ and its metabolites NdM and CNO.

treating symptoms of schizophrenia. A balance between a high affinity for 5HT2A receptors combined with a lower affinity for D2 receptors may account for the therapeutic actions of CLOZ (Duncan *et al.*, 1999), but other transmitter systems are also thought to play a role.

There continues to be controversy about the relative importance of CLOZ D_2 receptor blockade and 5-HT_2 receptor blockade in the therapeutic actions of CLOZ. Kapur *et al.* (2001) recently used isoclozapine (ISOCLOZ), a positional isomer of CLOZ (transposition of the chlorine from the 8-position to the 2-position) (Figure 3) to make this point. ISOCLOZ in *in vitro* studies was found to have a much higher affinity for, and a much slower dissociation from, the dopamine D_2 receptor than CLOZ. ISOCLOZ showed equal affinity to CLOZ on the major receptors thought to be involved in the actions of CLOZ (5-HT_2, 5-HT_{1A}, M_1, D_1, and D_4). They tested both drugs for their ability to cause catalepsy in relation to dopamine D_2 occupancy, their ability to induce FOS production, and their effect on prolactin levels. All results indicated that ISOCLOZ had a profile similar to typical antipsychotics such as haloperidol, rather than its atypical isomer CLOZ. The authors concluded that the affinity for the dopamine D_2 receptor was a crucial difference in atypical antipsychotic efficiency.

Recently, the NMDA receptor has been implicated in CLOZ's mechanism of action in what is known as the GLU hypothesis of schizophrenia. Support for

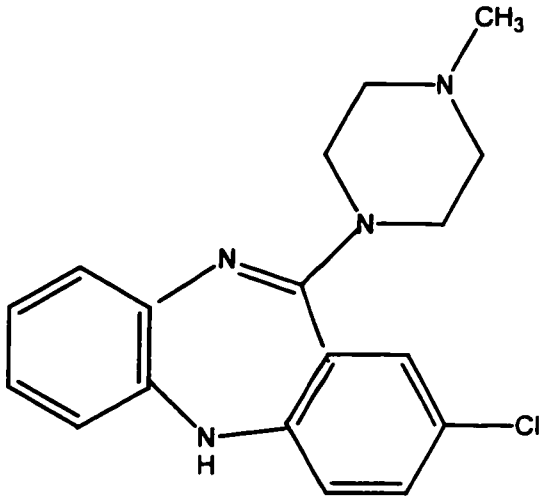


Figure 3. Structural formula of ISOCLOZ.

this hypothesis is found in the use of drugs such as ketamine and PCP that are NMDA receptor antagonists and produce behaviors and symptoms similar to those seen in schizophrenic patients (Olney and Farber, 1995; Ashby and Wang, 1996; Malhotra *et al.*, 1997; Duncan *et al.*, 1999). Schizophrenics also experience a heightened response to ketamine and PCP when compared to controls (Byne *et al.*, 1999). It is believed that CLOZ increases the release of GLU in the nucleus accumbens as well as in the prefrontal cortex (Malhotra *et al.*, 1997). When studying the effect of CLOZ on ketamine-induced psychosis, Malhotra *et al.* (1997) found that the psychotic effect of ketamine was blunted in the presence of CLOZ, suggesting some link between its mechanism of action and the NMDA receptor.

1.2.2 CLOZ and GLU

CLOZ has been found to interact with the NMDA receptor (Ossowska *et al.*, 2000). Chronic treatment with CLOZ in rats increased the number of NMDA receptors in different cortical areas (Ossowska *et al.*, 2000). This observation suggests that there is a potential dysfunction in the glutamatergic system that is potentially alleviated by an increase in NMDA receptors. CLOZ did not influence the PCP-induced decrease in prepulse inhibition, a model of the sensorimotor gating deficit in schizophrenia (Ossowska *et al.*, 2000). Using the stimulation of the somatosensory cortex as a measure of GLU transmission, Lidsky *et al.* (1993) found that CLOZ inhibited the corticostriatal response, indicating an anti-glutamatergic effect of CLOZ. CLOZ has been found to displace MK-801, a

noncompetitive NMDA receptor antagonist, in the cortex and striatum (Lidsky *et al.*, 1993; Ashby and Wang, 1996). CLOZ at the same time is said to reverse behaviors in rats that are mediated by PCP, suggesting that it can act as an agonist at the NMDA receptor (Brunello *et al.*, 1995).

Microdialysis in rat prefrontal cortex (PFC) showed that CLOZ, at a minimum dose of 25mg/kg, caused a significant increase in extracellular GLU levels over time (140 minutes) (Daly and Moghaddam, 1993). Because the response was not immediate (60 minutes for a significant effect), it was assumed that the effect is through indirect mechanisms such as interaction with the GABAergic system. GLU efflux was not significantly changed in the rat striatum; the reason for the regional selectivity remains to be determined.

A study involving the effect of ketamine in schizophrenic patients without CLOZ and later with CLOZ found that CLOZ blunted the positive symptoms induced by ketamine (Malhotra *et al.*, 1997). This study was consistent with other investigations into the interaction between CLOZ and the NMDA receptor. It therefore seems that CLOZ does act through the NMDA receptor in a clinically relevant way.

1.2.3 CLOZ and GABA

CLOZ, as well as its desmethyl metabolite, decreases GABA-mediated chloride influx in brain vesicles, thereby antagonizing GABAergic transmission (Wong *et al.*, 1996; Michel and Trudeau, 2000). Chronic CLOZ treatment in rats antagonized $GABA_A$ receptor function, but only at high doses (Wong *et al.*, 1996). The same study found that CLOZ did not significantly antagonize GABA

binding to the GABA receptor. The authors therefore speculated that CLOZ did not interact directly with the $GABA_A$ receptor, and that it was unlikely that GABA antagonism played a significant role in the therapeutic effect of CLOZ. In contrast, a more recent study by Michel and Trudeau (2000) investigating the effect of CLOZ on GABAergic neurons from the ventral tegmental area in culture showed that there was a dose-dependent antagonism of the inhibitory currents produced. The doses of CLOZ given were representative of the physiological concentrations that may be found in the brain after usual doses of CLOZ in humans, and the decrease seen in GABAergic transmission was also considered physiologically significant. *In vivo* microdialysis has shown that CLOZ increases GABA release in the rat ventral striatum (Brunello *et al.*, 1995). The interaction of CLOZ at dopamine receptors is thought to cause the increase of GABA release in the striatum. Several studies have found that chronic, but not acute, administration of CLOZ increases GABA turnover in the nucleus accumbens, globus pallidus, substantia nigra and caudate nucleus (Ashby and Wang, 1996). CLOZ has low affinity for the GABA receptors, and is not thought to block GABA uptake or alter GABA metabolism (Ashby and Wang, 1996). Though most studies point towards some interaction between CLOZ and the GABAergic system, none has yet been able to show this as being significantly relevant to its antipsychotic action.

1.2.4 CLOZ and polyamines

Polyamines play a role in the regulation of cell growth and differentiation, metabolic pathways and cell membrane functions. The polyamine spermidine (Figure. 4) is known to interact at the polyamine site of the NMDA receptor and therefore could be of interest when looking at possible effects of NMDA receptor dysfunction in schizophrenia. Spermidine functions as an allosteric modulator of the NMDA receptor, and under pathological conditions such as brain trauma the production of polyamines is dramatically increased (Cooper *et al.*, 1996). This increase, if excessive, is thought to mediate the excitotoxic events that lead to neuronal damage (Cooper *et al.*, 1996). In the literature there is a discrepancy concerning the involvement of the NMDA receptor in the neurotoxic effects of polyamines (Segal and Skolnick, 2000). Due to reports of cellular membrane abnormalities found in schizophrenic patients, there have been suggestions that polyamines may be implicated in the pathology of schizophrenia (Ramchand *et al.*, 1994). Polyamines affect membrane components such as phospholipids and proteins by disrupting their functions and modulating enzyme activity involved in the production of different lipids, such as phospholipids and glycerolipids (Ramchand *et al.*, 1994). These lipids are needed for cell membrane production and growth. Higher levels of polyamines, specifically spermine and spermidine, have been found in fibroblasts of schizophrenic patients when compared to controls (Ramchand *et al.*, 1994). The patients were undergoing treatment with antipsychotics, so the authors could not conclude that the higher levels were due to the drugs or were inherent in the patients. Das *et al.* (1989) had previously

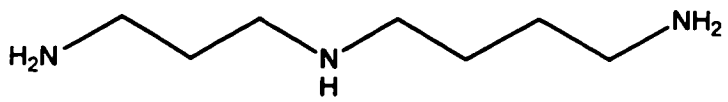


Figure 4. The chemical structure for spermidine.

reported that polyamine levels were increased in the blood of schizophrenic patients. Again the authors could not discern whether this was a drug-induced effect or not.

1.2.5 CLOZ and LTG

The combination of LTG and CLOZ has been proposed as a treatment for refractory schizophrenics because LTG appears to enhance the beneficial effects of CLOZ (Dursun and Devarajan, 2001). LTG decreases GLU release and has been shown to reduce the effects of ketamine as well as possibly reducing the risk of seizure while taking CLOZ (Anand *et al.*, 2000; Dursun and Devarajan, 2001). In a preliminary study, Dursun *et al.* (1999) examined the effect of adding LTG to schizophrenic patients receiving CLOZ treatment. The patients were partial responders to CLOZ and had been taking that medication for at least 16 months. The addition of LTG caused a significant improvement in their Brief Psychiatric Rating Scale scores that were monitored over a period of 24 weeks. Therefore it is thought that LTG may be a successful augmentation strategy for CLOZ. On the basis of this rather successful preliminary study Kossen and colleagues (2001) decided to give a patient LTG in addition to the CLOZ treatment that the patient was already taking. The patient had been treated with CLOZ for three years and responded partially to this treatment. CLOZ plasma levels over these three years had been stable at 300 – 500 μg/L. When LTG treatment began the CLOZ plasma level was at 350 μg/L. After two weeks the patient complained of dizziness and

sedation, and there was no clinical improvement over the two-week period. The patient's CLOZ plasma level at two weeks was found to be 1020 μg/L.

1.3 LTG

LTG (Figure 5) was first introduced as an adjunct treatment for partial seizures in Europe in 1991, but its therapeutic role has since expanded greatly (Ferrier, 1998; Matsuo, 1999). LTG is rapidly and completely absorbed after oral administration, and its protein binding is approximately 55% (Ferrier, 1998). It is metabolized in the liver and in humans its major metabolite is the 2-N-glucuronide; no active metabolites are produced (Guberman and Bruni, 1999). LTG has an elimination half-life of 25-30 hours when used as monotherapy (Ferrier, 1998). The coadministration of LTG and hepatic metabolic enzyme-inducing drugs such as phenytoin, phenobarbital and carbamazepine results in a significant increase in LTG clearance (Burnstein, 1995). LTG is not thought to induce cytochrome P450 (CYP) enzymes or to have any interactions with other drugs (Burnstein, 1995; Ferrier, 1998; Messenheimer, 1995)(but see the case report by Kossen *et al.* 2001discussed above). Side effects include headache, nausea, dizziness, and occasionally sedation. A skin rash occurs in approximately 5% of the population treated with this drug (Ferrier, 1998).

Interest in other therapeutic uses for LTG was stimulated by the observed mood elevation and quality of life improvement unrelated to seizure control seen in epileptic patients (Xie and Hagan, 1998). Several studies suggest LTG's

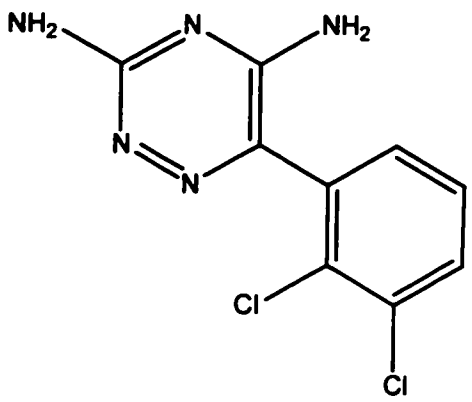


Figure 5. Structural formula of LTG.

effectiveness in helping treat mania in bipolar disorder, as well as possible antidepressant and antipsychotic properties (Berk, 1999).

1.3.1 LTG and Schizophrenia

Results vary depending on the study, but one common finding is that LTG has several possible mechanisms of action that may contribute to its efficiency as an anticonvulsant drug. The multiple mechanisms of action suggest that LTG could be therapeutic for dysfunctions in addition to epilepsy.

Some studies have investigated the potential therapeutic effect of LTG with regard to schizophrenia. Because of the hyperglutamatergic effect of NMDA receptor antagonists, such as ketamine and phencyclidine, they have been used as models of schizophrenia in order to study the effects of NMDA receptor dysfunction. Some preliminary studies have suggested that drugs that inhibit GLU release, such as LTG, reduce the responses to ketamine (Anand *et al.*, 1997). In healthy controls, Anand *et al.* (1997) found in their preliminary study that the effect of ketamine given after administration of LTG was blunted. Erfurth *et al.* (1998) reported that three female patients diagnosed with schizoaffective disorder and who were treated with LTG as a monotherapy experienced considerable mood stabilization as well as remission of paranoid symptoms. The effect was seen at doses that led to serum concentrations of greater than 10mg/L; only limited therapeutic effectiveness was seen at serum levels of 5mg/L (Erfurth *et al.*, 1998).

1.3.2 Mechanism of action

LTG, like some other anticonvulsants, blocks voltage-gated sodium and calcium channels, therefore reducing the high frequency repetitive firing of action potentials seen in epilepsy. LTG is also thought to decrease, or inhibit, the release of the excitatory transmitter GLU, and thereby decrease the excitability of overactive neurons (Cunningham and Jones, 2000; Ferrier, 1998). Excessive release of GLU can cause an increase in the influx of calcium and sodium into postsynaptic neurons (Anand *et al.*, 2000). Decreased GLU release can be accomplished through several mechanisms, including blocking sodium channels and calcium channels, decreasing potassium efflux and agonism at presynaptic metabotropic GLU receptors (Grunze *et al.*, 1998; Anand *et al.*, 2000). Since LTG is still a fairly new drug, there have been a limited number of studies focusing on its mechanism of action.

LTG is not thought to interfere with the normal physiological release of excitatory and inhibitory neurotransmitters. Rather, it is under pathological conditions that LTG exerts its ability to suppress repetitive firing of neurons that are triggered by GLU neurotransmission (Xie and Hagan, 1998). Electrophysiological studies have found that LTG inhibited GLU and GABA release caused by veratrine, a sodium channel opener (Leach *et al.*, 1986; Xie and Hagan, 1998). Therefore, it is thought that LTG acts by suppressing pathological events while not affecting normal neurotransmission (Leach *et al.*, 1986; Xie and Hagan, 1998).

In patients suffering from epilepsy, Eriksson and O'Connor (1999) found that CSF levels of LTG did not reflect the ability of this drug to inhibit GLU release in the CNS. The authors suggested that LTG was inhibiting GLU release only during ischemic and veratrine-induced GLU release. Results of this study could have been affected by the fact that LTG was being used as an add-on treatment, and therefore the results may not have directly shown the effect of LTG but an interaction with the other drugs that were being used in the treatment as well.

On the other hand, some evidence suggests that LTG may increase the release of GABA, a major inhibitory neurotransmitter (Hassel *et al.*, 2001; Cunningham and Jones, 2000). Hassel and colleagues (2001), in a study in rats showed that chronic treatment caused an increase of GABA levels in the hippocampus of the rat, while acute treatment did not cause any changes in GABA levels. In Eriksson and O'Connor's (1999) study with patients suffering from epilepsy, they found that LTG did not influence GLU or GABA levels in CSF or plasma.

1.4 CYTOCHROME P450 (CYP) ENZYMES

The cytochrome P450 (CYP) system is responsible for the metabolism of drugs and a number of endogenous substances (e.g. steroids). Metabolism occurs mainly in the liver, but also in the gut and other tissues (Baker *et al.*, 1998; Stahl, 2000). The system biotransforms drugs so that they can be excreted. Cytochrome P450 enzymes are heme-containing proteins found in the endoplasmic reticulum

and mitochondria (Baker *et al.*, 1998). Generally the drugs are converted to metabolites that are less pharmacologically active than the parent drug, and more water-soluble (Baker *et al.*, 1998; Stahl, 2000). It is also possible that the parent drug is converted to an equally or more active compound called an active metabolite. The CYP proteins make up a family of enzymes which function in the oxidative metabolism of drugs (Baker *et al.*, 1998). Pharmacokinetic drug-drug interactions can be caused by coadministration of drugs that are metabolized by the same CYP enzyme or by inhibiting or inducing a particular CYP enzyme (Baker *et al.*, 1998). It is important to understand these interactions as the drug concentrations when drugs are coadministered can potentially differ from when they are given alone, causing either more pronounced or decreased drug effects. Genetic polymorphism of certain CYP enzymes may result in differences in an individual's capability of metabolizing drugs and other compounds. Variations in plasma levels of patients receiving a particular dose of CLOZ may be the result of such polymorphism of certain CYP enzymes (Eiermann *et al.*, 1997).

There is a great diversity in the CYP enzymes; there are at least 14 different gene families of CYP enzymes in humans (1, 2, 3, 4, 5, 7, 8, 11, 17, 19, 21, 24, 27, and 51) (Nelson *et al.*, 1996). Families 1 through 3 have the greatest role in the drug metabolism. CYP enzymes 4,5,7,8,24,27,and 51 are involved in the metabolism and biosynthesis of substrates such as bile acids, eicosanoids and vitamins. The remaining families play a role in the synthesis of steroids from cholesterol (Baker *et al.*, 1998). The designation of CYP enzymes is based on their family number, followed by the subfamily letter and the individual gene

number. A number of similar CYP enzymes involved in drug metabolism are found in both rat and human, although they differ somewhat in their structure (Fang, 2000). The levels of CYP enzymes are generally lower in the brain than in the liver.

CLOZ is metabolized primarily to NdM (active metabolite) and to CNO (Figure 6). There have been reports of large variations in the concentrations of CLOZ and its metabolites between individuals. Several studies indicate that CYP1A2 and CYP3A4 are the major enzymes catalyzing formation of NdM and that CYP3A4 contributes to the production of clozapine-N-oxide (Eiermann *et al.*, 1997; Fang, 2000; Raaska and Neuvonen, 2000). It has also been shown that the flavin-containing monooxygenase (FMO) hepatic enzyme, specifically FMO3, is also involved in the formation of clozapine-N-oxide (Fang *et al.*, 1998). Like CLOZ, NdM is a potent $5HT_{1C}$ receptor antagonist and has similar affinity for D_2 and $5HT_2$ receptors (Guitton *et al.*, 1996). In the rat brain, it has been shown that, in contrast to the situation with human liver microsomes, the major metabolite of CLOZ is CNO and that NdM is a minor metabolite (Fang, 2000).

1.5 ANALYTICAL PROCEDURES

1.5.1 General Principles of Chromatography

Chromatography is a term used to describe a variety of separation techniques based on the sample partitioning between a mobile phase, which can be either liquid or gas, and a stationary phase, which can be either solid or liquid

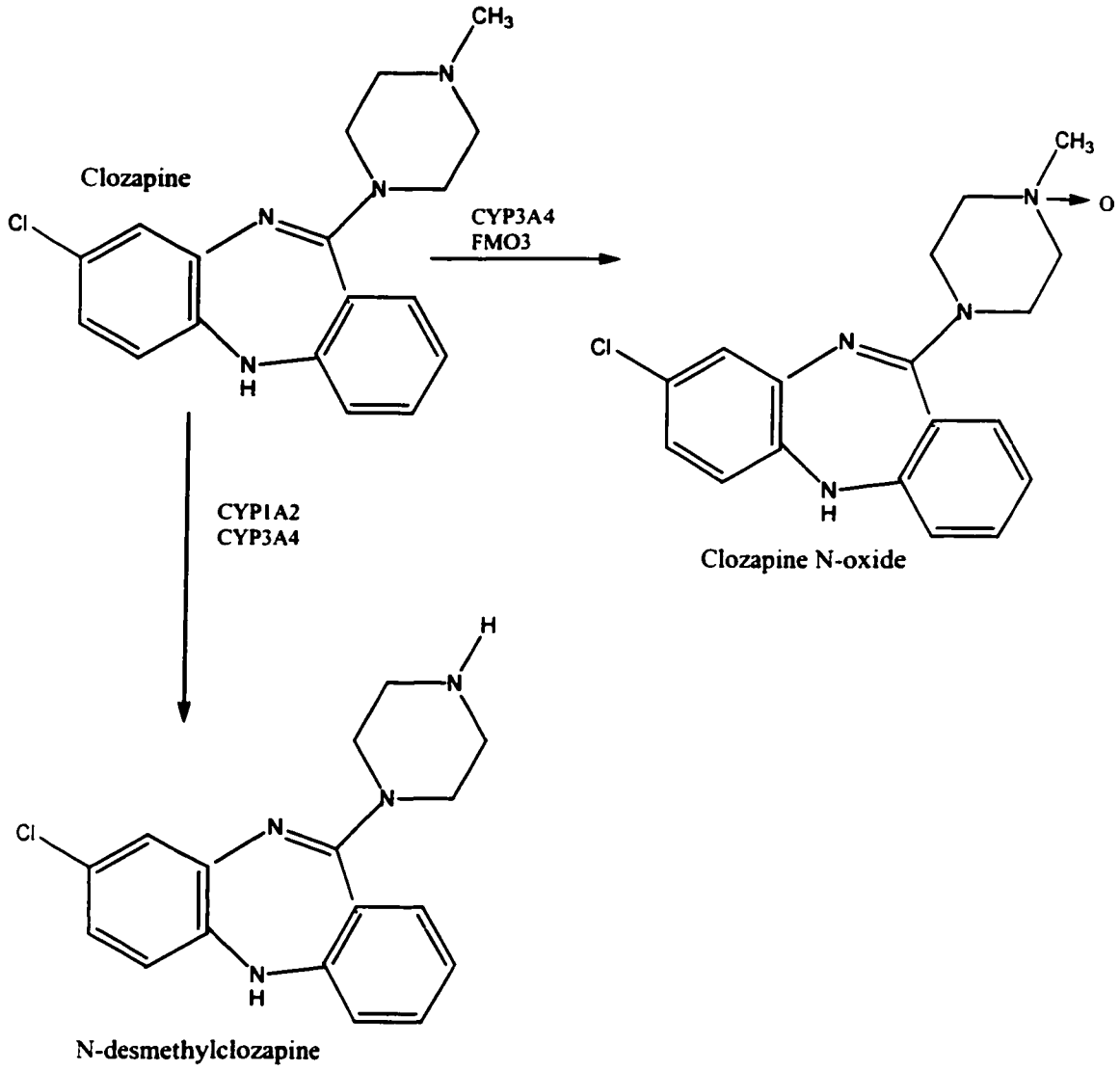


Figure 6. Enzymes proposed to be responsible for catalyzing the 2 major metabolic routes of CLOZ (adapted from Fang *et al.*, 1998).

(Holman *et al.*, 1993). The two major types of chromatography are gas chromatography and liquid chromatography, classified according to their mobile phase.

1.5.2 High Performance Liquid Chromatography (HPLC)

The basic principle behind the mechanism of HPLC is that a mobile phase is pumped through an attached column at high pressure. The pump can be regulated to allow an isocratic flow or a gradient flow of the mobile phase. Isocratic flow maintains the same mobile phase composition, whereas gradient flow can change mobile phase composition in a continuous or stepwise fashion (Burtis *et al.*, 1987; Holman *et al.*, 1993). The sample to be analyzed is introduced into the mobile phase before it enters the column. Once in the column, the differential equilibrium between the sample, the mobile phase and the column causes compounds to separate (Feldman *et al.*, 1997). The column effluent can then be analyzed using a variety of detector systems.

Liquid chromatography systems can be either normal phase or reverse phase systems depending on the polarity of the mobile and stationary phases involved. When the stationary phase that is more polar than the mobile phase, this is classified as a normal phase system (Burtis *et al.*, 1987; Holman *et al.*, 1993), whereas a separation system in which the mobile phase is more polar than the stationary phase is called reverse phase chromatography (Holman *et al.*, 1993). In this system polar analytes will elute first; this is significant because many biological compounds are polar and therefore reverse phase chromatography has

become more popular. The stationary phase consists of an organic moiety that is covalently bound to the surface of a support particle that is usually silica (Burtis *et al.*, 1987; Holman *et al.*, 1993). The organic moiety can be either polar or non-polar. Non-polar organic groups include octadecylsilane (ODS or C-18) and polar organic groups include cyanopropyl (CN), aminopropyl (NH2), and glycidoxypropyl (diol) silanes (Burtis *et al.*, 1987).

In the drug metabolism studies presented in this thesis, an ultraviolet detector was used to detect compounds as they eluted from the HPLC column. A wavelength is chosen that corresponds to the maximum absorption of the compound of interest (Feldman *et al.*, 1997). The light passes through a flow cell to be absorbed by the compound as it elutes from the column. The absorbance of the eluate is plotted over time, and the changes in absorbance are directly related to the concentration of the compound present in the effluent (Feldman *et al.*, 1997). The absorbance changes are represented as peaks that are recorded as a chromatogram.

In the study on amino acid levels in rat brain after administration of LTG and/or CLOZ, a fluorometric detector was used for detection after derivatization of the amino acids with o-phthalaldehyde (OPT). Fluorescence detection can be used to detect compounds that are naturally fluorescent or compounds that are converted to fluorescent derivatives (Baker *et al.*, 1985). Ultraviolet (UV) radiation is produced by a light source at a particular wavelength and is directed onto the sample to be analyzed. Light emitted from the sample results in light of only a specified wavelength reaching the detector (Baker *et al.*, 1985). The

number of photons emitted is proportional to the concentration of the fluorescent substance in the sample (Baker *et al.*, 1985).

1.5.3 Gas Chromatography

Gas chromatography is used to separate compounds that are volatile, or made volatile (Laios and Wu, 1996). Gas chromatography can be gas-solid chromatography, with a solid stationary phase, or gas-liquid, with a nonvolatile stationary phase (Laios and Wu, 1996). The carrier gas can be an inert gas such as nitrogen, helium or argon (Burtis *et al.*, 1987). The type of gas depends on the type of detector used in the instrument. The instrument can be operated at a constant temperature or programmed to run at different temperatures if the sample has compounds with different volatilities.

The sample is injected through a septum, and must be injected as a gas. This can be accomplished by keeping the injection port at high temperatures so as to vaporize the sample components. The vaporized sample can then be taken onto the column by the carrier gas. Volatile compounds will move quickly through the column, whereas compounds with higher boiling points will move more slowly through the column (Laios and Wu, 1996). The effluent passes through a detector that produces a signal that is proportional to the amount of volatile compounds present (Laios and Wu, 1996). The types of columns used are generally made of glass or stainless steel. Two types of columns exist, either packed or capillary columns. Packed columns are filled with inert particles such as glass beads coated with a non-volatile stationary phase. These columns have internal diameters (I.D.)

of 1-4mm (Burtis *et al.*, 1987). Capillary-wall coated open tubular columns have I.D.s of 0.2-0.5mm (Burtis *et al.*, 1987). A liquid phase can be deposited directly on the inner glass of the column (wall-coated open tubular columns; WCOT), or onto a thin layer of solid support material (support-coated open tubular columns; SCOT).

Several types of detectors can be used for GC work. In this study the detector used was the electron-capture detector (ECD). This detector is used to detect compounds that contain halogen atoms, ketone groups, or nitro groups (Burtis *et al.*, 1987). In an ECD a radioactive isotope releases beta particles that collide with the carrier gas molecules, producing low-energy electrons (Burtis *et al.*, 1987). These electrons are collected on electrodes and produce a small but constant measurable current (Burtis *et al.*, 1987). As sample compounds that have the ability to capture the low-energy electrons pass through the column, they cause a decrease in this current (Burtis *et al.*, 1987). The concentrations of the compounds are therefore inversely proportional to the current.

1.5.4 Derivatization

Derivatization is a procedure that chemically alters a sample so that it is more suitable for separation and/or detection using the GC. It usually involves the replacement of the active polar groups (-NH, -OH, -SH) through acylation, acetylation, alkylation, or condensation (Coutts *et al.*, 1985). Derivatization is utilized to produce a more volatile compound, to increase sensitivity to the detector being used, and/or to attain better separation using the GC (Burtis *et al.*,

1987). Pentafluorobenzoyl chloride (PFBC) was used to increase the sensitivity of spermidine to the ECD in this study.

1.5.5 Mass spectrometry

A mass spectrometer (MS) can be used for the identification and quantification of compounds as well as for structural information and molecular weight determination of a compound (Laios and Wu, 1996). In an MS the sample is first volatized and then ionized to form charged molecular ions and fragments that are separated according to their mass-to-charge ratio (m/z) (Durden, 1985; Laios and Wu, 1996). The sample is then measured by a detector that gives the intensity of the ion current for each species (Durden, 1985).

1.6 Objectives of the Research Described in this Thesis

1. To investigate possible effects of LTG on CLOZ metabolism by human liver microsomes. Although this drug combination is now being used clinically, there is a lack of information about whether or not there is a possible pharmaceutical interaction between the two drugs.
2. To study the effects of acute coadministration of LTG and CLOZ on the following in the rat:
    a. Brain and liver levels of the drugs themselves
    b. Brain levels of several amino acids, including GABA and GLU.
    c. Brain levels of spermidine, a polyamine that acts at a binding site on the NMDA receptor.
3. To develop assay procedures for: CLOZ, ISOCLOZ and their metabolites; LTG; and the polyamine spermidine in order to carry out the studies mentioned above.

In order to investigate mechanisms of action of psychiatric drugs, experiments are often conducted in rats. However, many such experiments in rats often neglect to consider possible pharmacokinetic interactions between drugs when two or more drugs are being administered concomitantly. The importance of considering such interactions in rats has recently been illustrated by experiments in the Neurochemical Research Unit with selective serotonin reuptake inhibitor (SSRI) antidepressants, which are strong inhibitors of CYP enzymes (Kennedy *et al.*, 2001). Coadministration of fluoxetine with desipramine (Goodnough and Baker, 1994) or amphetamine (Sills *et al.*, 1999a) resulted in marked increases in rat

brain levels of desipramine and amphetamine compared to values obtained with these drugs administered alone. Similarly, coadministration of sertraline and amphetamine produced an increase in brain levels of amphetamine compared to the situation when amphetamine was administered alone (Sills *et al.*, 1999b). These interactions were apparent even after acute administration. It was decided to conduct a similar experiment with CLOZ and LTG. It was also important to determine, under the same conditions, whether these drugs affected brain levels of GABA, and GLU. As described above in the Introduction, both drugs may have actions on GABA and GLU; however, there is a lack of information about their effects on brain levels of these amino acids. Similarly, there is no information available in the literature on the effects of CLOZ and LTG on polyamines in rat brain.

2. MATERIALS

2.1 Instrumentation and Apparatus

High Performance Liquid Chromatography for Drug Metabolism Studies in Microsomes

A HPLC Waters 2690 Separations Module (Waters Associates, Milford, MA) with a Waters 2487 dual wavelength absorbance detector (Waters Associates, Milford, MA) was used. A Phenomenex Hypersil CN (250 x 4.6mm I.D., 5 micron particle size) was used for the microsome work, whereas a Phenomenex (Phenomenex, Torrance, CA) Phenosphere CN (250 x 4.6mm I.D., 5 micron particle size) was used for the rat brain and liver work.

High Performance Liquid Chromatography for Measurement of Amino Acids in Rat Brain

A HPLC Waters 2690 Separations Module (Waters Associates, Milford, MA) with a Waters 474 Fluorescence Scanning detector was used. A Spherisorb ODS2 column C18, (250 x 4.6mm I.D., 5 micron particle size) was used for the separation of amino acids in rat brain.

Data processing was carried out using a Pegasus Pentium 2, 400 MHz computer (Pegasus Computer Company, Edmonton, AB) with Waters Millenium software.

Gas Chromatography with ECD for Measurement of Spermidine in Rat Brain

Analysis of spermidine was carried out using a Hewlett Packard (HP) Model 5890A gas chromatograph equipped with a ^{63}Ni ECD and linked to a HP 6890 Series injector and automatic sampler and a HP 3396 Series II integrator (Hewlett Packard, Palo Alto, CA). A fused HP5 silica capillary column (25m x 0.32mm) coated with 1.05μm film thickness of 5% phenylmethylsilicone was employed. The carrier gas was helium and the make-up gas was argon-methane (95:5) (Praxair Canada Inc, Edmonton, AB).

2.2 Human Liver Microsomes

Human liver microsomes were obtained from lot number HHM 205 purchased from the International Institute for the Advancement of Medicine and Science (IIAMS) In vitro Technologies Inc. (Baltimore, MD). The microsomes were stored at –80°C until needed.

2.3 Equipment

Balance:

A Mettler AE 160 electronic balance (Mettler Instrument Corporation, Highstown, NJ) was used to weigh tissue and chemicals.

Centrifuges:

A Sorvall GLC-2B General Laboratory Centrifuge (Dupont Instruments, Wilmington, DE) was used to process samples that required low g values (1000g).

A Beckman Model J-21B Centrifuge (Beckman Instruments Inc., Palo Alto, CA) was used at 10,000 rpm for microfuge tubes.

A Beckman Coulter Allegra 21R Centrifuge (Beckman Coulter Inc., Palo Alto, CA) was used at 10,000 rpm for samples of larger volumes.

Filter Apparatus for mobile phase preparation:

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

Glassware Cleaning:

All glassware was rinsed with tap water and washed in a dishwasher (Miele Electronic 6715) with Sparkleen (Fisher Scientific Co.). Test tubes were sonicated (ultra-sonic cleaner, Mettler Electronics) in a 2-5% solution of Decon 75 concentrate (BDH chemicals) before rinsing with distilled water in the dishwasher. All glassware was then air-dried in a mechanical convection oven (Model 28, Precision Scientific Group, Chicago, USA).

pH Meter:

The pH values of solutions were adjusted using an Accumet pH Meter 915 equipped with an Accumet probe (Fisher Scientific, Nepean, ON).

Tissue Homogenizer:

Brain and liver samples were homogenized using a TRI-R STIR-R Model S63C Variable Speed Laboratory Motor (TRI-R Instruments, Rockville Center, NY) equipped with a Teflon pestle and a glass grinding tube.

Vortex Mixer:

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

Vortex Shaker:

An IKA Vibrax-VXR with a Typ VX2 attachment (Janke and Kunkel, Staufen, Germany) was used for shaking samples in test tubes.

Waterbath:

A Fisher Isotemp Waterbath (Fisher Scientific, Nepean, ON) was used and set at a temperature of 37 °C.

Water Still:

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

2.4 Chemicals

Chemical	Supplier
Acetonitrile	Fisher Scientific (Nepean, ON)
Alanine	Aldrich Chemical Company (Milwaukee, WIS)
β-Alanine	Aldrich Chemical Company
γ-Aminobutyric acid	Sigma (St. Louis, MO)
Amitriptyline	Sigma
Ammonium acetate	Fisher Scientific
Ammonium hydroxide	Fisher Scientific
Ascorbic acid	Fisher Scientific
Aspargine	Sigma
Aspartate	Sigma
Clozapine	Novartis (Basel, Switzerland)
Clozapine N-oxide	Novartis
N-Desmethylclozapine	Novartis
Dimethyl sulfoxide	Fisher Scientific
Ethyl acetate	Fisher Scientific
Ethylenediaminetetraacetate, disodium salt	Fisher Scientific
Fluvoxamine maleate	Solvay Dulphar (Hanover, Germany)
Formic acid	Fisher Scientific
Glacial acetic acid	Fisher Scientific

Glucose 6-phosphate	Sigma
Glucose 6-phosphate dehydrogenase	Sigma
Glutamate	Sigma
Glutamine	Sigma
Glycine	Sigma
Isoclozapine	Dr. S. Kapur (Toronto, ON)
Ketoconazole	Sigma
Lamotrigine	GlaxoSmithKline Pharma (Hertforshire, UK)
Magnesium chloride	Sigma
Methanol	Fisher Scientific
β-Nicotinamide adenine dinucleotide phosphate	Sigma
Perchloric Acid, 60%	Fisher Scientific
o-Phthalaldehyde (Fluroaldehyde reagent)	Pierce Chemicals (Rockford, IL)
Potassium phosphate dibasic	Fisher Scientific
Potassium phosphate monobasic	Fisher Scientific
Serine	Sigma
Sodium hydroxide	Fisher Scientific
Sodium phosphate monobasic	Fisher Scientific
Sodium phosphate tribasic, 12-hydrate crystal	JT Baker Inc (Phillipsburg, NJ)
Spermidine	Sigma
Taurine	BDH Biochemical (Poole, England)

Tetrahydrofuran	Fisher Scientific

3. METHODS

3.1 Microsomal studies:

General experimental methods for the analysis of CLOZ, CNO, NdM, ISOCLOZ (and its proposed metabolites) and LTG in microsomes are described below.

3.1.1 Preparation of Solutions and Incubation Mixtures

Stock solutions were made up for all the drugs used in the microsome experiments. LTG, ketoconazole (KETO), ISOCLOZ, and CLOZ were prepared as 10mM stock solutions in methanol. Fluvoxamine (FLUVOX) was prepared as a 10mM solution using deionized water. For CNO and NdM 1mg/mL stock solutions were made in methanol. Stock solutions were subsequently stored at 4°C for up to one month.

NADPH-GENERATING SYSTEM:

In a final concentration of 100µl the NADPH-generating system consisted of 1.3mM β-nicotinamide adenine dinucleotide phosphate, 3.3mM glucose 6-phosphate, 0.4 U/mL glucose 6-phosphate dehydrogenase, and 3.3mM $MgCl_2$ in a 0.1M potassium phosphate buffer (pH 7.4).

POTASSIUM PHOSPHATE BUFFER:

Monobasic solution: 1.3609g in 100mL ddH_2O

Dibasic solution: 1.7418g in 60mL ddH_2O

The monobasic solution was added to the dibasic solution to make a final solution of pH 7.4 which was then transferred to a volumetric flask and ddH_2O was added to the 100mL line.

INHIBITORS

KETO was added to the incubation mixture at concentrations of 5, 10, 20, and 40 μM. FLUVOX and LTG were added at concentrations of 50, 100, 200, and 400 μM.

INCUBATION CONDITIONS:

0.1 mL reaction mixtures containing 10μl of microsomal preparation, 25 μl of an NADPH-generating system, 100μl of CLOZ solution in a 0.1M potassium phosphate buffer and 15μl of 0.1M potassium phosphate buffer (pH 7.4).

3.1.2 Incubation Procedure

All tubes were pre-incubated for 15 minutes in the absence of CLOZ in a 37°C water bath. CLOZ or ISOCLOZ (final concentration 100μM) was then added and the incubation continued for another 30 minutes. The tubes were then placed on ice and 50μl of ice-cold acetonitrile was added to terminate metabolism. The tubes were left on ice for 10 minutes and then centrifuged at 4°C at 10,000 rpm. A portion (20μl) of the supernatant was injected on the HPLC for analysis.

CLOZ Metabolism Over Time

CLOZ was incubated alone for 15, 30, 45, 60 or 120 minutes to examine its metabolism over time.

ISOCLOZ Metabolism Over Time

ISOCLOZ was incubated alone for 15, 30, 45, 60, or 120 minutes to determine if its metabolism was similar to that of CLOZ over time.

CLOZ Incubated with Inhibitors

The metabolism of CLOZ to NdM and CNO was investigated in the presence of several inhibitors. KETO (a potent inhibitor of CYP3A4), FLUVOX (an inhibitor of CYP1A2 and CYP3A4) and LTG were used to examine their effect on the production of CLOZ metabolites. These were incubated at different concentrations with 100μM of CLOZ. The concentrations of the drugs used were 5, 10, 20 and 40 μM for KETO and 50, 100, 200, and 400μM for LTG and FLUVOX.

3.1.3 Calibration Curves

With each assay a calibration curve was also run along with the samples. These consisted of CLOZ (0-100μM), NdM (0-6.4μM), and CNO (0-11.6μM). The standards were prepared by adding the appropriate amount of CLOZ and the metabolites to the NADPH-generating system and the 0.1N phosphate buffer. In the case of the ISOCLOZ assay, it was used under the same concentrations as

CLOZ was in the other assays. These samples were then injected on the HPLC under the same conditions as the samples being analyzed.

3.1.4 Analysis by Mass Spectrometry of ISOCLOZ and its Metabolites

After incubating ISOCLOZ for 120 minutes under the conditions described above, the supernatant was then diluted ten times in 50% methanol/deionized water containing 1% formic acid.

3.1.5 Instrumentation

All experiments were performed on an Applied Biosystem/MDS Sciex QSTAR Pulsar I mass spectrometer (Concord, ON), equipped with a turbo ionspray source. Analyte solutions were introduced into the source by a 1mL gas tight syringe from Hamilton (Reno, NV) and an integrated syringe pump. MS/MS spectra were acquired with a collision energy of 51eV. Analyst QS software was used for spectrum collection and data analysis.

3.2 Analysis of CLOZ and LTG in Rat Brain.

3.2.1 Solution Preparation

A stock solution of a 1mg/mL amitriptyline (internal standard for the assay) was made in deionized water; 1mg/mL stock solutions of LTG, CLOZ, CNO, and NdM were made in methanol and all were stored at 4°C for up to one month.

10% Na_3PO_4•12 H_2O: 23.15g were dissolved in 100mL ddH_2O.

1N Perchloric acid ($HClO_4$) with disodium ethylenediaminetertaacetate (EDTA) and ascorbic acid: 54.4mL of concentrated $HClO_4$ was added to a 500mL volumetric flask containing about 100mL of deionized water. It was then brought to volume with deionized water, and a stir bar was added. The solution was stirred while adding 44mg of ascorbic acid and 500mg EDTA until dissolved. The stock solution was diluted as needed to make 0.1N solution. In experiments using liver, 4N perchloric acid was prepared and then diluted to make a 0.4N solution. The final concentration of EDTA and ascorbic acid in all cases were 10mg % and 50μM, respectively.

Mobile phase: The mobile phase was a 50% acetonitrile and 50% ammonium acetate buffer (10mM) solution with a pH of 5.4 that was attained using glacial acetic acid.

3.2.2 Animal Experiments

Male Sprague-Dawley rats weighing 200-250g were injected with either LTG (2.5mg/kg), CLOZ (10mg/kg), LTG (2.5mg/kg) and CLOZ (10mg/kg), or with vehicle [dimethyl sulfoxide (DMSO): phosphate buffered saline (PBS), 1:1]. The animals were sacrificed by decapitation either 1 hour or 6 hours after injection. Whole brains and livers were removed and immediately placed on dry ice and then frozen at –80°C until used for analysis.

3.3 Assay Procedure for LTG and CLOZ

Rat brains were homogenized in 5 volumes of ice-cold 0.1N $HClO_4$ (which contained 10mg% EDTA and 50μM ascorbic acid). Livers were homogenized in

5 volumes of 0.4N $HClO_4$ containing EDTA and ascorbic acid at the same concentrations. The internal standard amitripyline (AMIT) (5μg) was added to the homogenates. The homogenates were centrifuged at 10,000 rpm at 4°C for 15 minutes. A portion (2mL) of the clear supernatant was then placed into a test tube and basified with 400 μl of 10% Na_3PO_4. To each tube 3mL of ethyl acetate was added. The tubes were capped, shaken vigorously for 5 minutes using a vortex-shaker and then centrifuged at 1,000g for 5 minutes. The ethyl acetate layer was then removed and transferred to smaller screw-capped tubes and taken to dryness under nitrogen. The sample was then reconstituted in 250μl of acetonitrile and 20μl was used for injection on the HPLC.

3.3.1 Calibration Curves

Calibration curves were run with each assay and were prepared using naïve rat brain or liver homogenate. The curves for rat brain were run at concentrations of 0-500ng/mL for CNO, 0-2μg/mL for NdM, 0-4μg/mL of CLOZ, and 0-1.5μg/mL of LTG. These were the same curves run using livers with the exception that the LTG curve ran from 0-2.0μg/mL. To each standard the appropriate amount of each compound was added along with 5μg/mL of internal standard (AMIT). The standard homogenates were then processed in the same manner as the samples described above.

3.3.2 Instrumentation

HPLC analysis

All standards and samples were analyzed using the Waters Alliance 2690XE Separations Module system, with a Waters 2487 Dual wavelength Absorbance

Detector set at 254nm and a sensitivity setting of 0.05 absorbance units full scale (AUFS). The column used for microsome samples was a Phenomenex Hypersil 5CN (250 x 4.6mm I.D., 5 micron particle size) column. The rat brain and liver samples were analysed using a Phenomenex Phenosphere 5CN column (250 x 4.6mm I.D., 5 micron particle size). These columns were coupled to a CN guard column that was packed with the same material as the analytical column. Data were collected and processed using Waters Millenium software (Waters Associates, Milford, MA). The mobile phase was a 50% acetonitrile and 50% ammonium acetate buffer (10mM) solution with a pH of 5.4 that was attained using glacial acetic acid. The mobile phase was pumped at a flow rate of 0.8mL/min. A run time of 50 minutes was used as all peaks had retention times of under 40 minutes. All analyses were carried out isocratically at a column temperature of 30°C. Samples were held at 4°C.

3.4 Analysis of GABA and GLU in Rat Brain

3.4.1 Solution preparation:

All standards were prepared as 1mg/mL stocks in deionized water. An intermediate standard consisted of 40μg GLU, 20μg GABA, serine, glutamine; 10μg aspartate, taurine; 5μg glycine, alanine; and 2μg aspargine, β-alanine, in 866μl 20% methanol. The intermediate stock was then added to appropriate volumes of 20% methanol to obtain the standard curve desired.

Mobile Phases: Mobile phase A consisted of 900mL of (NaH_2PO_4) (0.08M), 240mL of methanol, 20mL of acetonitrile, and 10mL of tetrahydrofuran (THF) (stored under nitrogen). The mobile phase was brought to a pH of 6.2 with sodium hydroxide (NaOH) (10N) and then filtered and degassed under vacuum with 0.2μm filters. Mobile phase B consisted of 1340mL NaH_2PO_4 (0.04M) with 1110mL of methanol, and 60mL THF. It was then adjusted to a pH of 6.2 using NaOH (10N) and filtered and degassed under vacuum with 0.2μm filters.

3.4.2 Assay Procedure for Amino Acids in Rat Brain

Rat brains were homogenized in 5 volumes of deionized water and frozen at –80°C until used for analysis. The homogenates were then thawed on ice. A portion (100μl) of homogenate was added to 400μl of ice-cold methanol in a microfuge tube. This mixture was then rehomogenized and left on ice for 10 minutes. The microfuge tubes were then centrifuged for 4 minutes at 4°C at 11 000 rpm. A portion (50μl) of the supernatant was removed and added to 450μl of ice-cold deionized water. Then 5μl was injected on the HPLC system.

3.4.3 Calibration Curves

The calibration curve for GABA ran from 0-5μg/mL, and from 0-10μg/mL for GLU.

3.4.4 Instrumentation

HPLC analysis

All standards and samples were analyzed using the Waters Alliance 2690XE Separations Module system, with a Waters 474 fluorescence detector set at gain

10. The excitation wavelength was set at 260nm and the emission wavelength was set at 455nm. A precolumn Waters μBondapak C18 guard column was used. The column used was a Waters Spherisorb ODS2, C18, (250 x 4.6mm I.D., 5 micron particle size) held at a temperature of 30°C. Samples were maintained at 4°C prior to injection. OPT, obtained as fluoraldehyde reagent (Pierce Chemicals, Rockford, IL), was replaced daily from a fresh stock which was stored under nitrogen. OPT reagent (5μl) was added to 5μl of sample standard or blank which was taken up and held in the loop for 1.5minutes before injecting onto the column. Two mobile phases were used in a gradient flow. The flow was initially set at 60% mobile phase A and 40% mobile phase B at a rate of 0.5ml/min. The gradient was then shifted to 100% B for 10 minutes. At 12 minutes the flow rate was increased to 0.7mL/min. At 20 minutes the flow was increased to 1mL/min. At 40 minutes the gradient was returned immediately to initial conditions, and was then run for at least 20 minutes to condition the column. The total run time was 60 minutes.

3.5 Analysis of Spermidine in Rat Brain

3.5.1 Solution Preparation

Spermidine was prepared as 1mg/mL stock solution in water.

3.5.2 Assay procedure for spermidine in rat brain

A modification of the procedure of Wong (1985) was utilized. Half brains were homogenized in 5 volumes of deionized water, and frozen at –80°C until

used for analysis. In a test tube, 1mL of homogenate was added to 1mL of deionized water, and basified with 250µl of 10% Na_3PO_4. After vortexing the tubes, 5mL of derivatizing solution (each 5mL toluene contained 2.5µl PFBC) was added to the test tube to produce nonpolar compounds to be extracted into organic solvent. The test tubes were then capped, shaken and centrifuged at 1000g to separate the organic and aqueous phases. The organic layer was removed and dried under nitrogen. The sample was then reconstituted in 300µl toluene and 2µl was then injected on the GC ECD for analysis.

3.5.3 Calibration Curves

Calibration curves were run with each assay and were prepared using water instead of brain homogenate. The spermidine standard curve was run from 0- 40µg.

3.5.4 Instrumentation

Analysis of spermidine was carried out using a Hewlett Packard (HP) Model 5890A gas chromatograph equipped with a ^{63}Ni ECD and linked to a HP 3396 Series II integrator (Hewlett Packard, Palo Alto, CA). A fused silica capillary column (25m x 0.32mm) coated with 1.05 µm film thickness of 5% phenylmethylsilicone was employed. The carrier gas was helium (flow rate 2mL/min) and the make-up gas was argon-methane (95:5) (Praxair Canada Inc, Edmonton, AB) at a flow rate of 60mL/min. The oven temperature was set at an initial temperature of 105°C for 0.5min. The temperature was increased by 20°

per minute to 295°C. The injector temperature was set at 250°C, and the detector temperature was set at 325°C.

3.6. Statistical Analysis

Main treatment effects for microsomal studies were determined by a one-way analysis of variance (ANOVA) of means using SPSS software. When significant ($p<0.05$) main effects were reached, *post hoc* multiple comparisons with Tukey's test was employed to identify individual group differences.

Main treatment effects for animal studies were determined by a 2 x 2 x 2 (LTG, CLOZ, time) univariate ANOVA of means using SPSS software. If there was a significant main effect reached, then a 2 x 2 (LTG, CLOZ) univariate ANOVA was employed to determine differences at one time point.

4 RESULTS

4.1 Metabolism of CLOZ and ISOCLOZ by human liver microsomes and confirmation of structures of metabolites by mass spectrometry

4.1.1 Calibration Curves

Calibration curves were generated by plotting the peak height of CLOZ, CNO, or NdM on the y-axis versus the concentration of that compound on the x-axis. Calibration curves were linear and consistently had r^2 values of greater than 0.99. Typical calibration curves are shown in figures 7-9. A Typical chromatogram of a standard is shown in figure 10.

4.1.2 Production of Metabolites of CLOZ Over Time

Mean levels of CNO and NdM production over time are shown in table 1. These values are plotted graphically in figure 11.

4.1.3 Production of Metabolites of ISOCLOZ Over Time

Mean levels of ISOCLOZ N-oxide and N-desmethylisoclozapine production over time are shown in table 2. These values are plotted graphically in figure 12.

4.1.4 Mass spectrometry of ISOCLOZ Metabolites

The MS trace of a CLOZ standard is shown in figure 13a, and the MS trace of ISOCLOZ is shown in figure 13b. The proposed mass fragmentation of CLOZ is shown in figure 14 (only the major fragments are shown). Since CLOZ and ISOCLOZ have the same structure except for the placement of the chlorine atom,

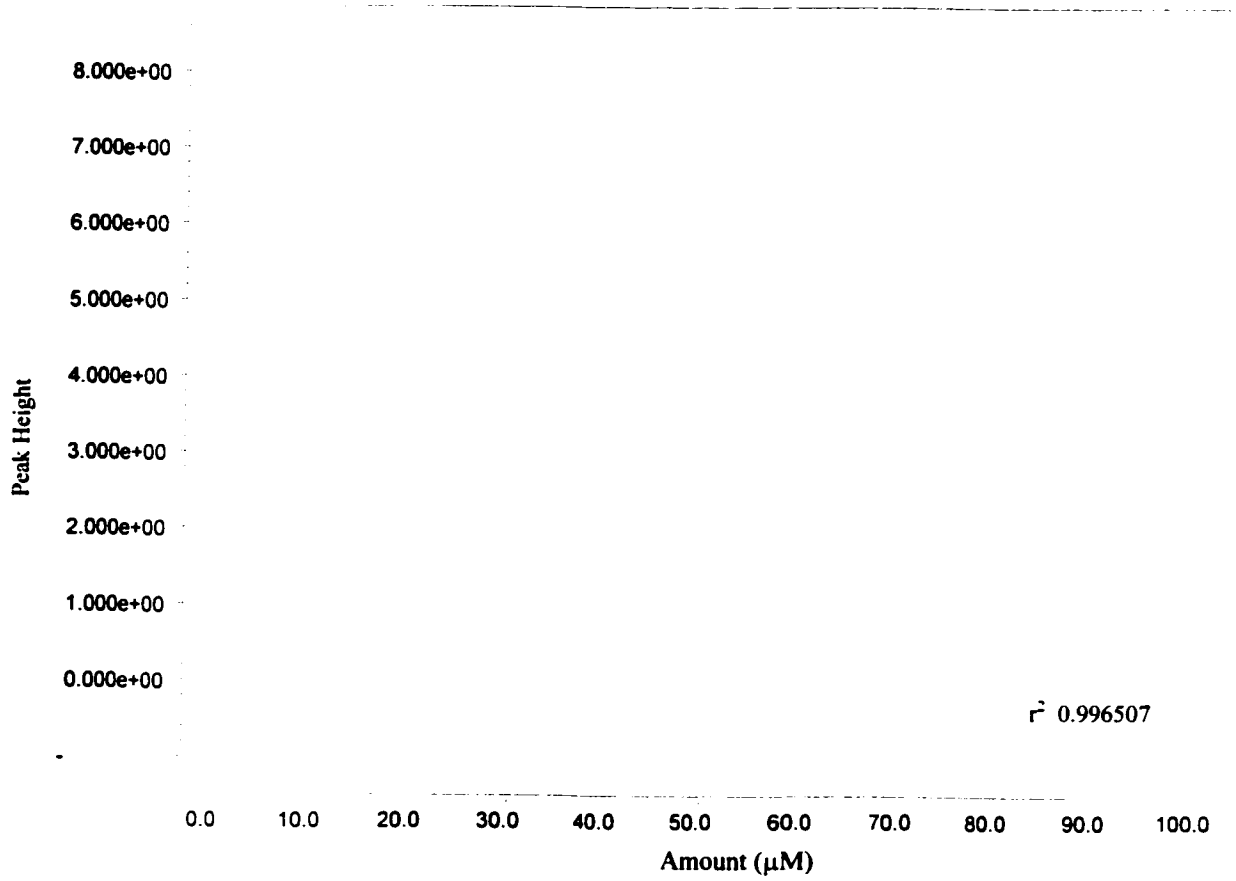


Figure 7. Typical calibration curve for CLOZ added to human liver microsomes

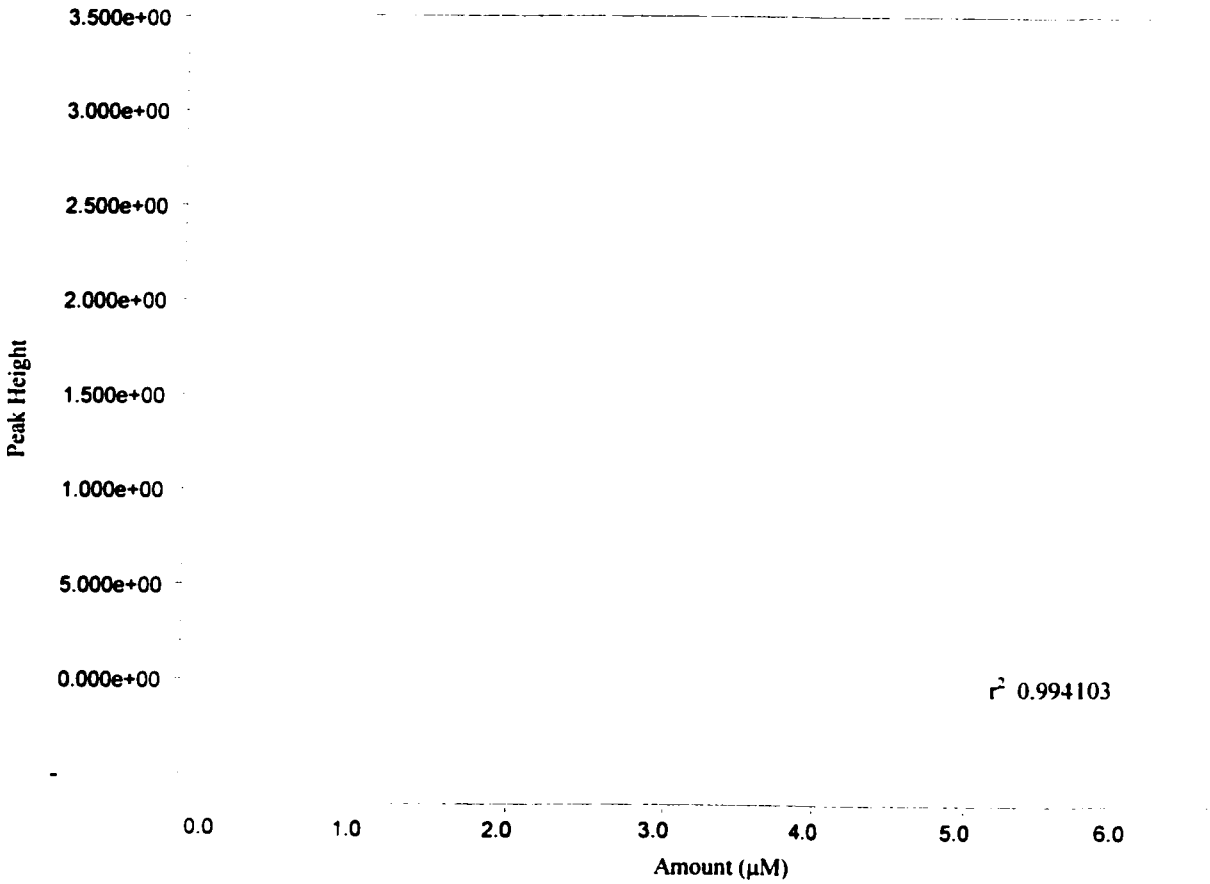


Figure 8. Typical calibration curve for the quantification of NdM added to human liver microsome samples

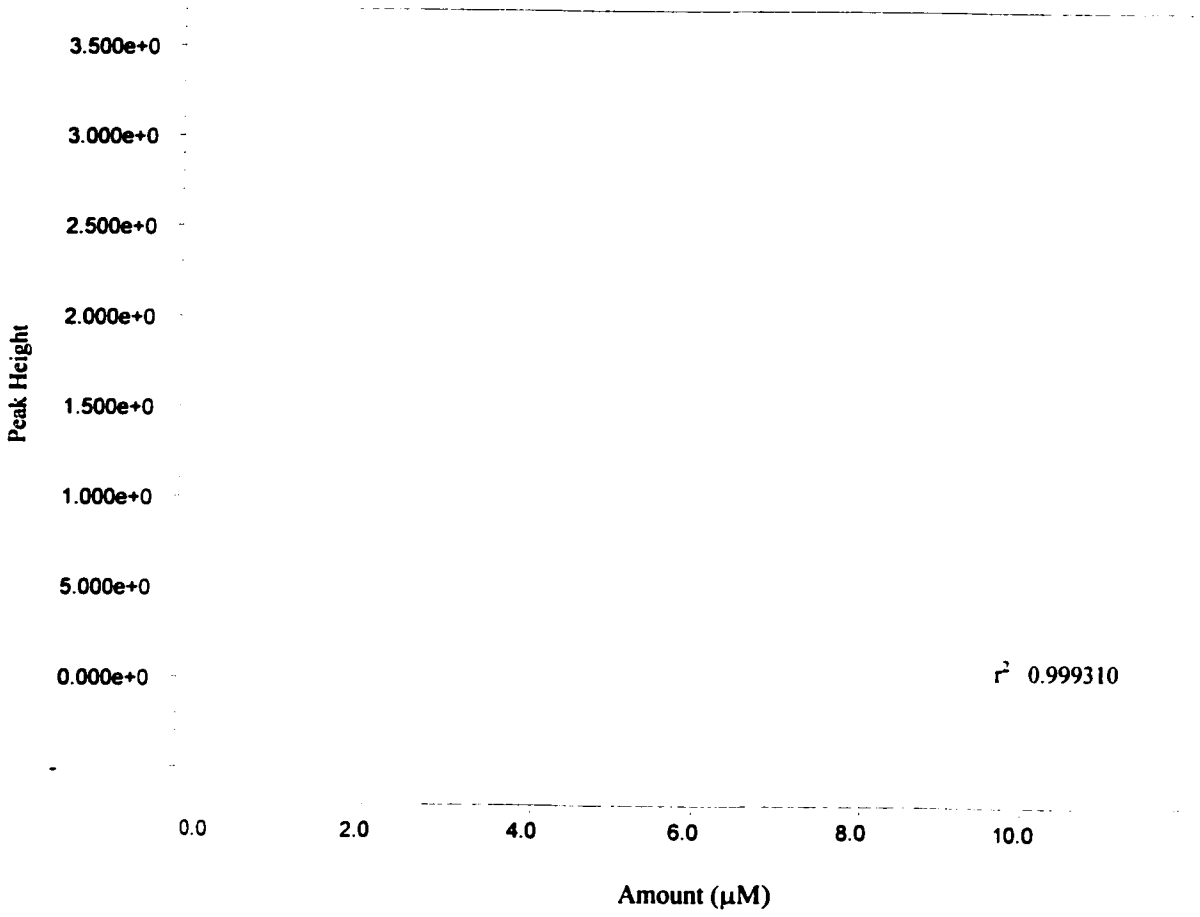


Figure 9. Typical calibration curve for the quantification of CNO added to human liver microsome samples

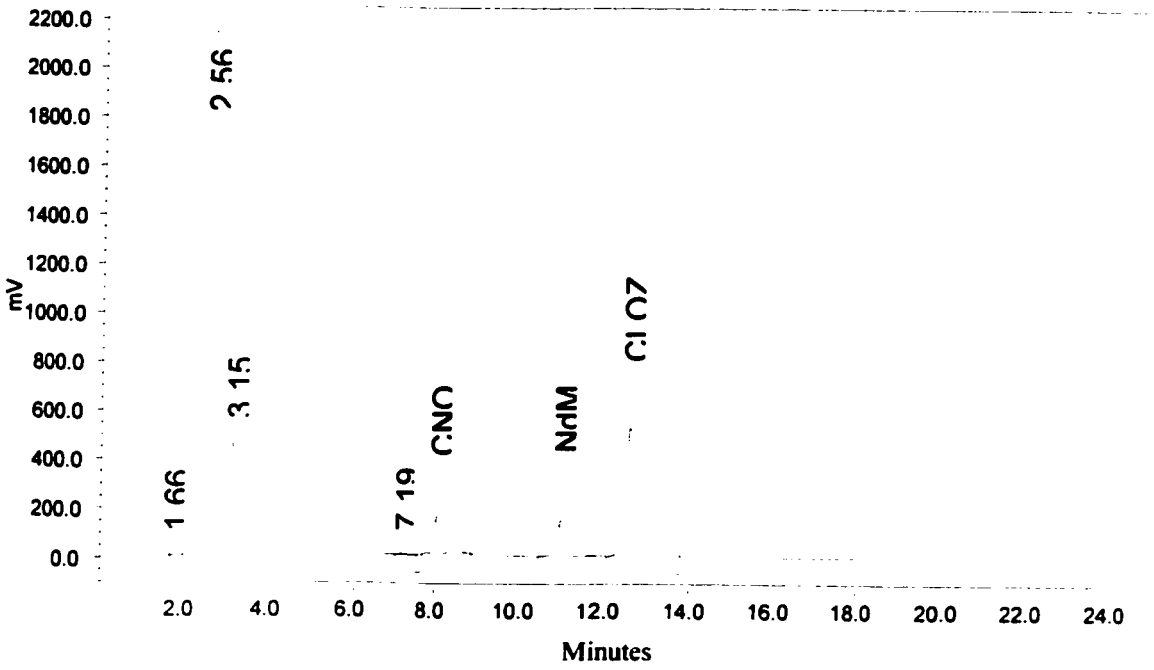


Figure 10: HPLC trace of standards added to human liver microsomes: 50μM CLOZ, 5.8μM CNO, and 3.2μM NdM.

Time (min)	CNO (μM)	NdM (μM)
15	5.668 +/- 0.29	1.742 +/- 0.055
30	8.065 +/- 0.53	2.415 +/- 0.14
60	10.235 +/- 0.90	3.303 +/- 0.15
120	14.875 +/- 1.43	4.681 +/- 0.14

Table 1: Production of metabolites of CLOZ over time by human liver microsomes containing 100μM CLOZ. Values represented are means +/- SEM (n=3).

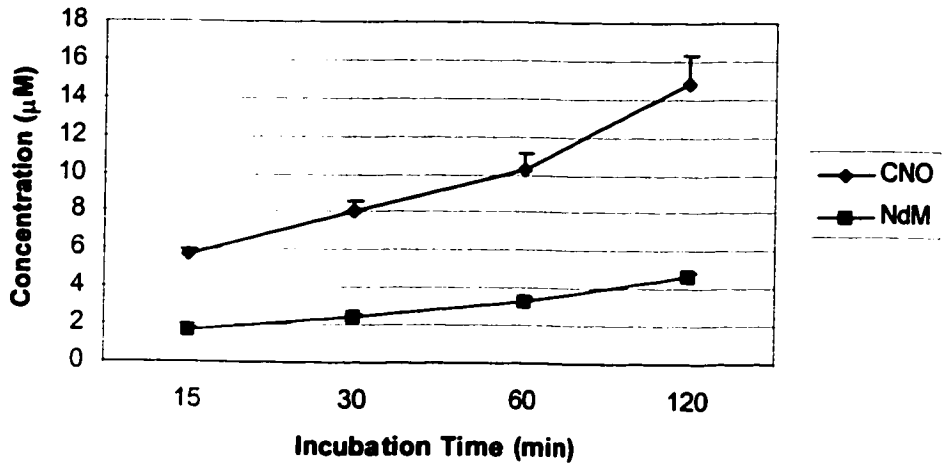


Figure 11. Graphic representation of CNO and NdM formation over time by human liver microsomes containing CLOZ (100µM). Values represent means +/- SEM(n=3); error bars are not shown for errors less than 5%.

Time (min)	IsoCNO (μM)	NdMiso (μM)
15	3.5553 +/- 0.15	2.2447+/- 0.029
30	4.7827 +/- 0.33	2.9267+/- 0.067
60	7.6847 +/- 0.27	3.8867+/- 0.052
120	9.571 +/- 0.30	4.553+/- 0.022

Table 2: Production of metabolites of ISOCLOZ over time by human liver microsomes containing 100μM ISOCLOZ. Values represented are means +/- SEM (n=3).

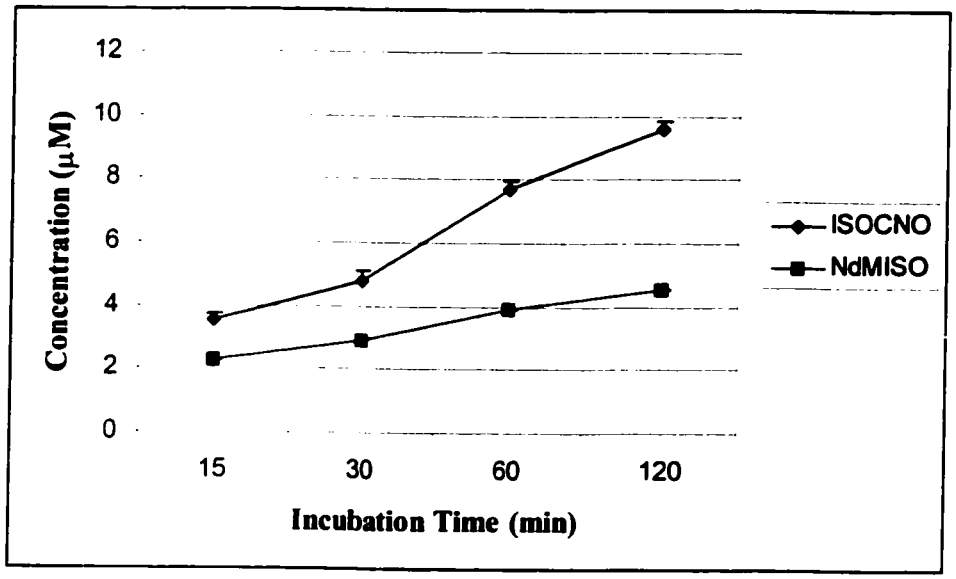
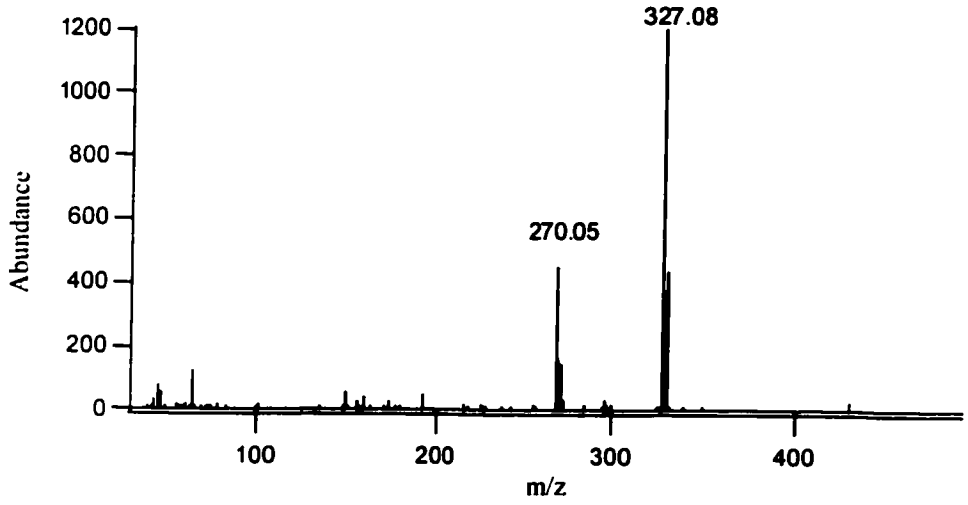


Figure 12. ISOCLOZ metabolism over time by human liver microsomes containing 100μM ISOCLOZ. Values represent means +/- SEM (n=3). Error bars less than 5% are not shown.

a)



b)

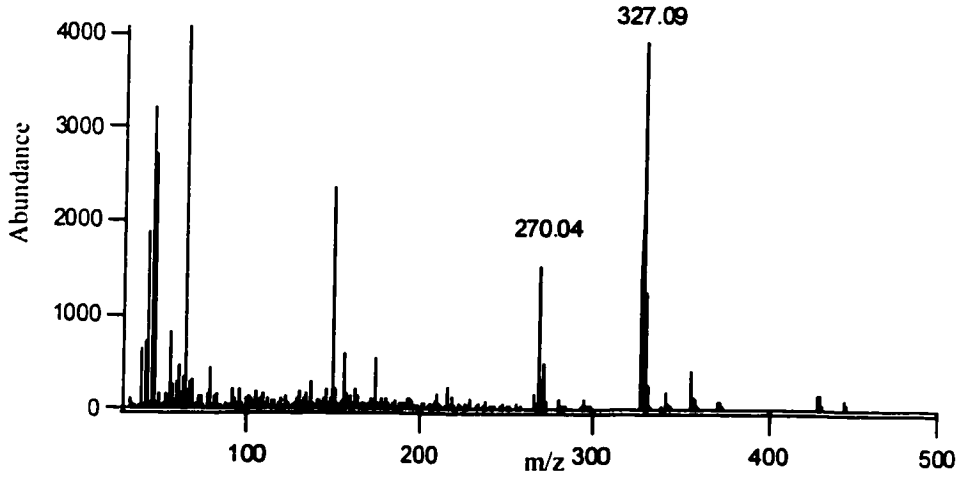


Figure 13. Mass spectrum analysis of a) a CLOZ standard and b) an ISOCLOZ standard (MH^+= 327/329 in each case).

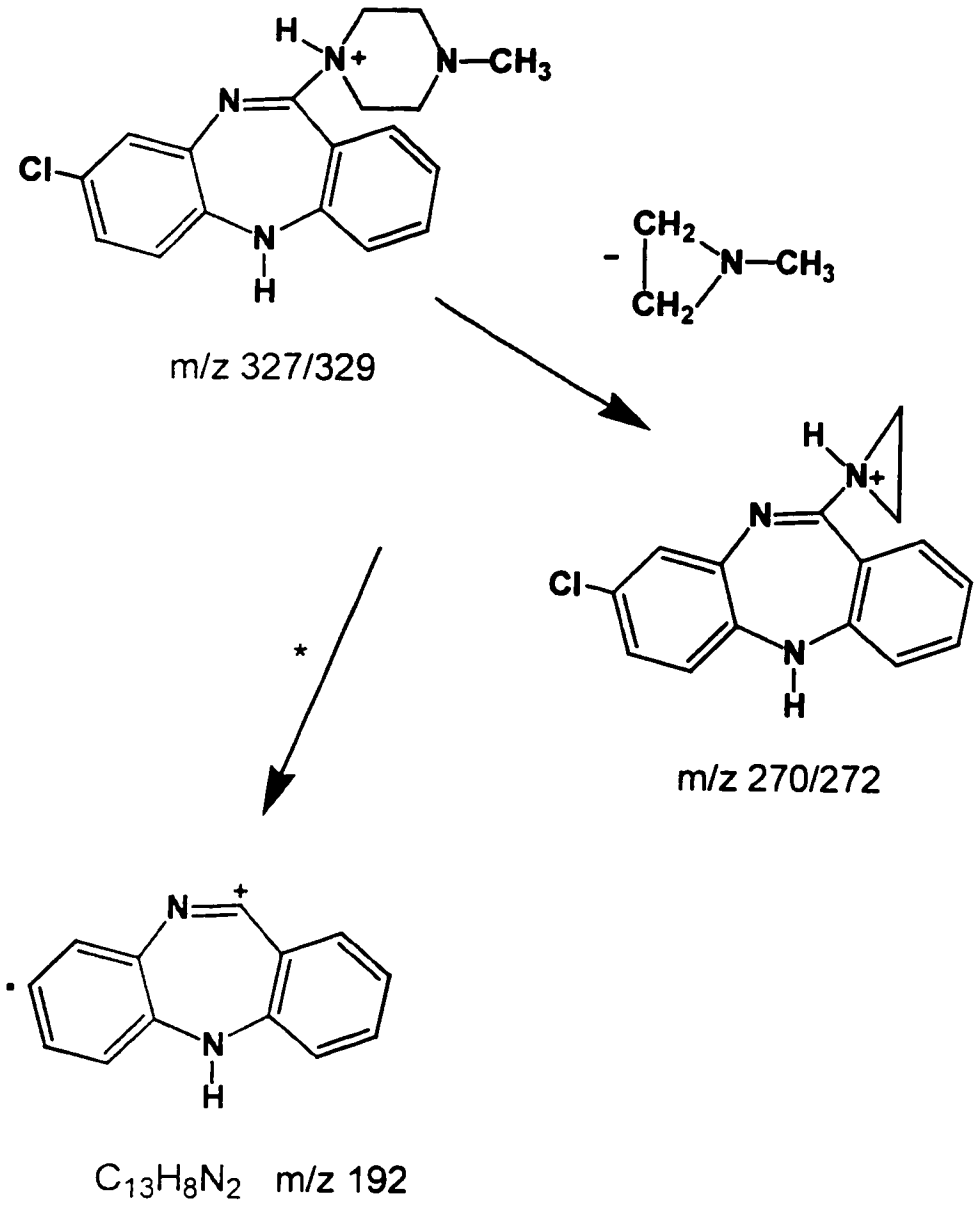


Figure 14. The proposed mass fragmentation of CLOZ; only the major fragments are shown. *This is speculative since odd electron ions are rarely formed from even electron ions.

they have the same molecular weight and would be expected to have very similar mass fragmentation patterns. The same would apply to the metabolites of both drugs if ISOCLOZ, like CLOZ, undergoes N-demethylation and N-oxidation. The MS trace of a standard with the two main CLOZ metabolites NdM and CNO is shown in figure 15a, and the trace of ISOCLOZ and its metabolites extracted from a microsomal preparation is shown in figure 15b. The proposed mass fragmentations of CNO and NdM are shown in figures 16 and 17.

4.2 Effects of inhibitors on production of NdM and CNO from CLOZ by human liver microsomes.

4.2.1 Levels of Metabolites of CLOZ in the Presence of KETO

Mean levels of CNO and NdM production in the presence of KETO are presented in table 3. These values are shown graphically in figure18. A significant inhibition ($p < 0.05$) of the production of both CNO and NdM was observed at the lowest concentration of KETO used, but this degree of inhibition did not increase with increasing concentrations of KETO, i.e. the inhibition was not dose-dependent.

4.2.2 Levels of Metabolites of CLOZ in the Presence of FLUVOX

Mean levels of CNO and NdM production in the presence of FLUVOX are presented in table 4. These values are shown graphically in figure 19. There was no significant effect of FLUVOX on the production of CNO. A significant effect of FLUVOX on the production of NdM was found ($p < 0.05$) at the lowest

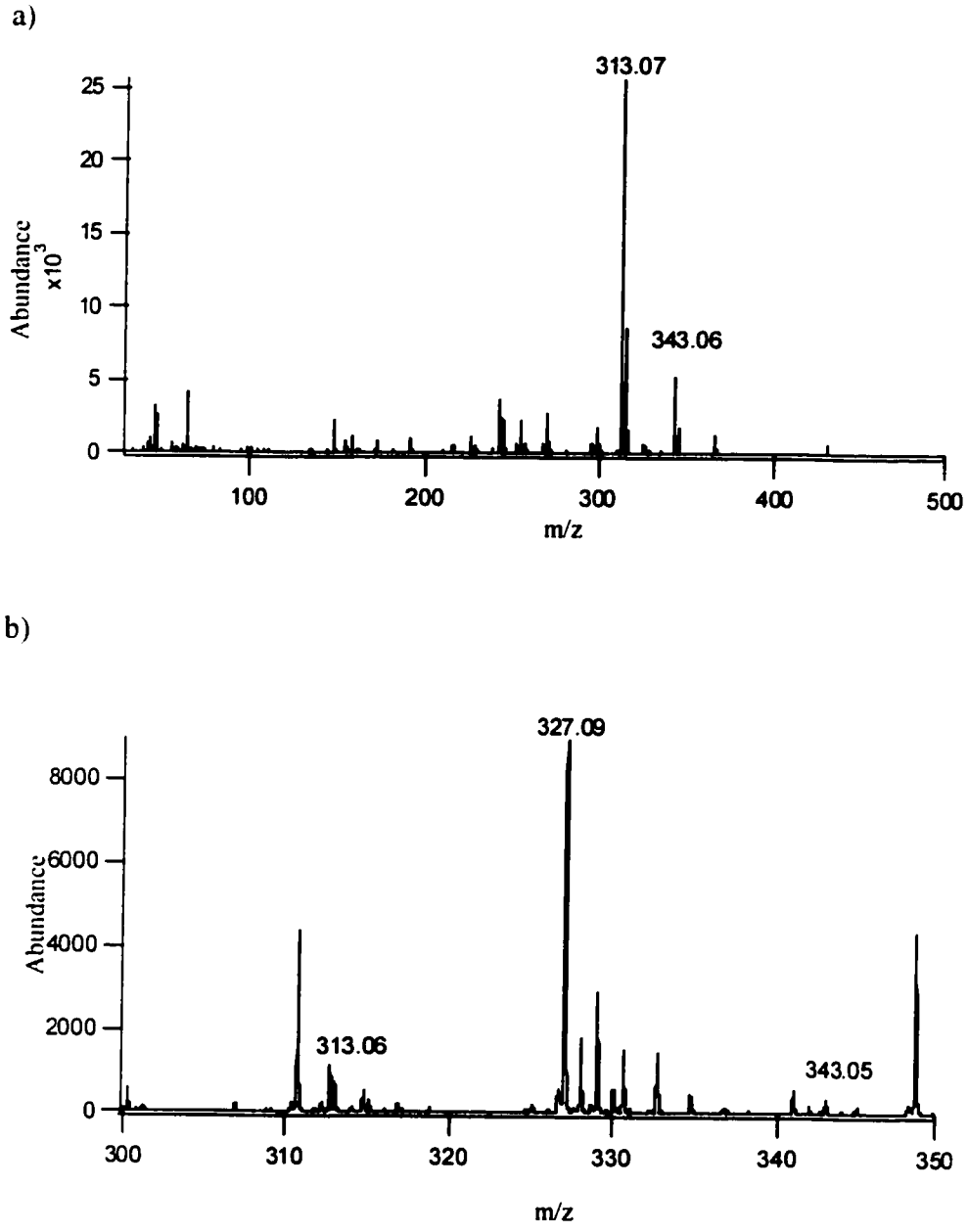


Figure 15. Mass spectrum analysis of a) a CNO (MH^+= 343/345) and NdM (MH^+= 313/315) standard and b) a microsomal preparation of ISOCLOZ and its metabolites.

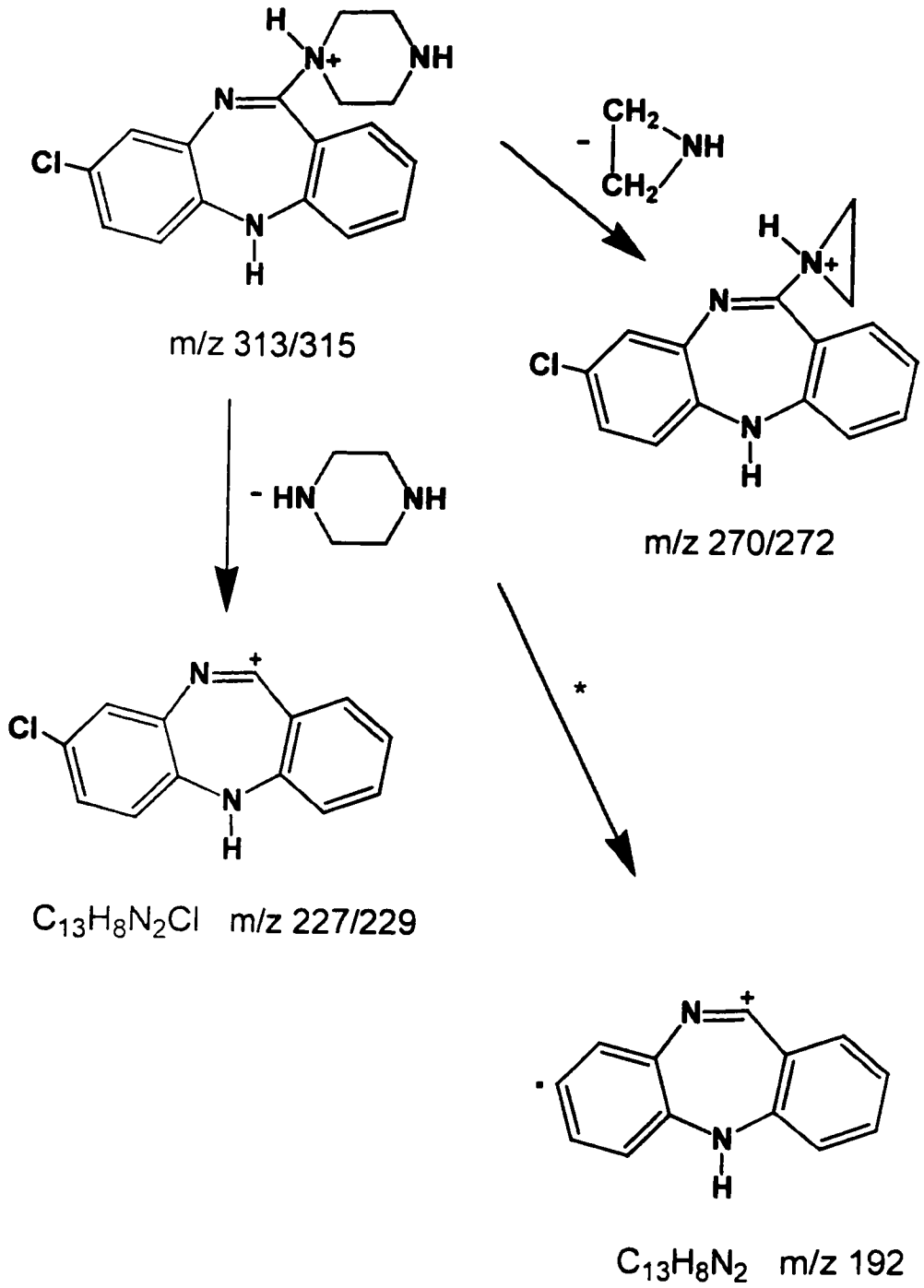


Figure 16. The proposed mass fragmentation of NdM. *See footnote to Figure 14.

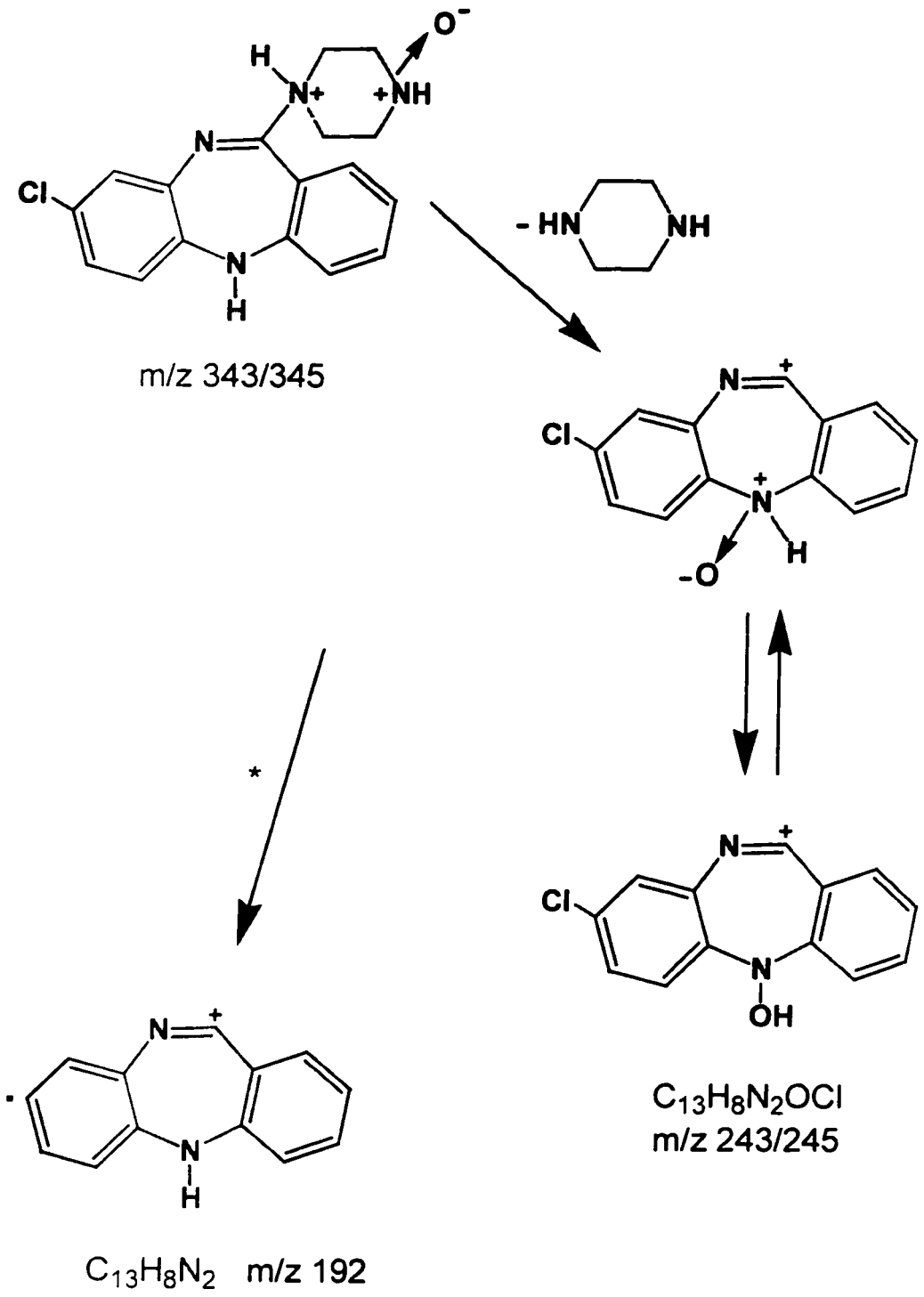


Figure 17. The proposed mass fragmentation of CNO. The oxygen appears to have migrated to another nitrogen during fragmentation. * See footnote to Figure 14.

KETO concentration (μM)	CNO (% of control)	NdM (% of control)
0	100	100
5	56.68 +/- 3.40	62.51+/-2.86
10	51.22+/-2.91	54.21+/-1.95
20	49.88+/-2.63	51.93+/-1.66
40	54.06+/-5.41	50.65+/-8.15

Table 3: Inhibition of CNO and NdM production from CLOZ by human liver microsomes in the presence of KETO. Values are means +/- SEM (n=5).

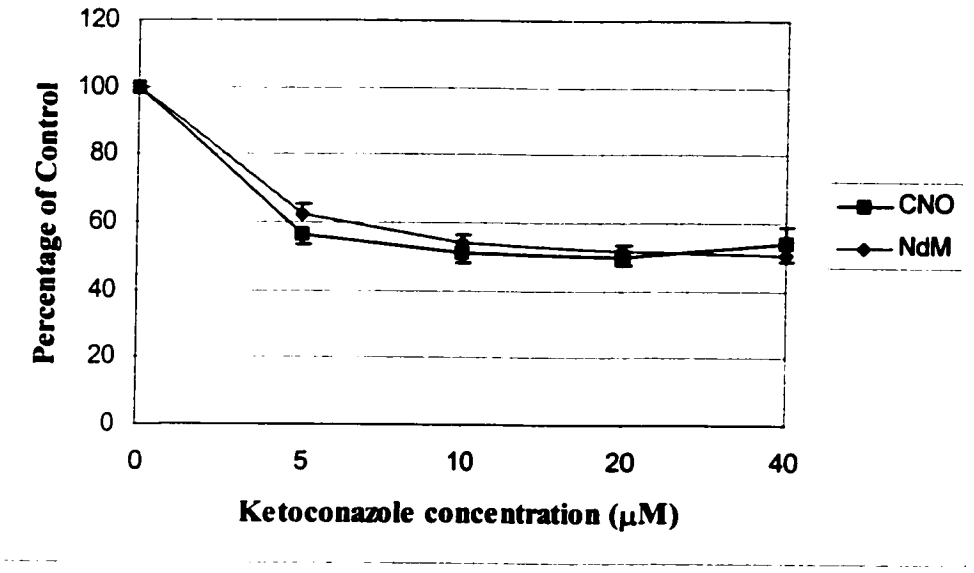


Figure 18. Graphic representation of inhibition of CNO and NdM production from CLOZ (100μM) by human liver microsomes in the presence of KETO. Values represent means +/- SEM (n= 5). A significant effect was seen at the lowest concentration of KETO for both CNO ($p<0.0001$) and NdM ($p<0.0001$); this effect was not increased by increasing the concentration of KETO.

FLUVOX concentration (μM)	CNO (% of control)	NdM (% of control)
0	100	100
50	87.77+/-6.043	45.71+/-1.591
100	82.68+/-4.256	40.49+/-0.9455
200	80.64+/-4.995	33.35+/-1.541
400	79.8+/-6.137	23.84+/-1.834

Table 4: Inhibition of CNO and NdM production by human liver microsomes containing 100μM CLOZ in the presence of FLUVOX. Values are means +/- SEM (n=5).

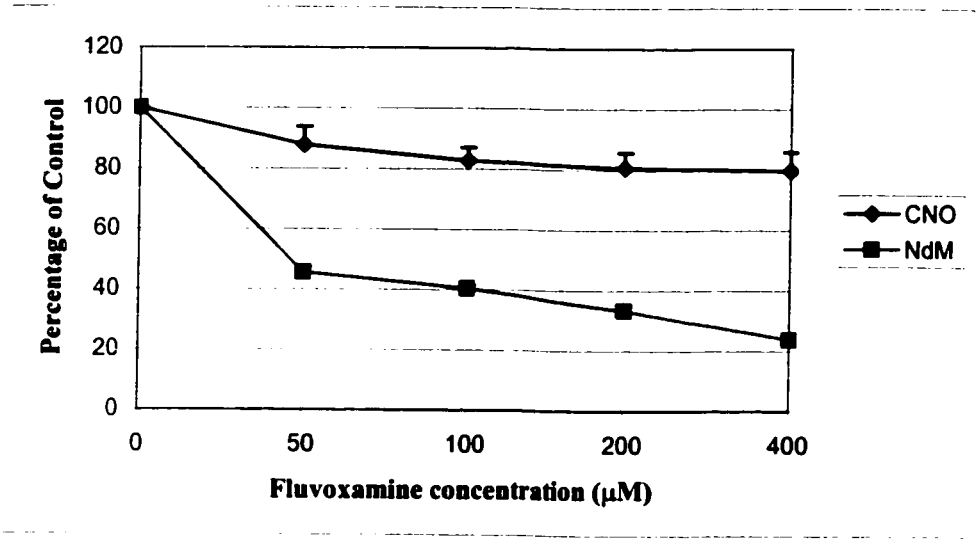


Figure 19. Inhibition of CNO and NdM production from CLOZ by human liver microsomes in the presence of FLUVOX. Values represent means +/- SEM (n=5). A significant dose-dependent inhibition of NdM by FLUVOX was seen ($p<0.0001$) with the exception of between 50 and 100μM concentrations of FLUVOX, whose effects were not significantly different from each other.

concentration tested. The inhibition was dose-dependent, with significant differences between all concentrations except between the doses of 50 and 100μM.

4.2.3 Levels of CLOZ Metabolites in the Presence of LTG

Mean levels of CNO and NdM production in the presence of LTG are presented in table 5. These values are shown graphically in figure 20. LTG caused a significant inhibition of CNO production ($p<0.05$) at the higher doses tested (200 and 400μM). The inhibition of CNO production was dose-dependent. LTG caused significant inhibition of NdM production ($p<0.05$) at 400μM.

4.3 Effects of LTG and CLOZ on each other's levels in rat brain

4.3.1 Calibration Curves

In these types of experiments, it is usual just to measure levels of the parent drugs since a pharmacokinetic interaction will show up as a change in levels of the parent drugs (Goodnough and Baker, 1994; Sills *et al.*, 1999a,b). If marked changes in either parent drug are observed, then levels of the metabolites can be pursued. Calibration curves for LTG and CLOZ, were generated by plotting the peak height ratio of LTG or CLOZ to a fixed amount of AMIT (internal standard) on the y-axis versus the concentration of each compound on the x-axis. Calibration curves were linear and consistently had r^2 values greater than 0.99. Typical calibration curves for LTG, and CLOZ are shown in figures 21 and 22. A typical chromatogram is shown in Figure 23.

LTG concentration (μM)	CNO (% of control)	NdM (% of control)
0	100	100
50	91.44+/-5.81	96.98+/-6.231
100	84.79+/-6.308	91.45+/-8.694
200	69.27+/-1.88	82.65+/-3.571
400	59.75+/-3.892	63.82+/-1.903

Table 5: Inhibition of CNO and NdM production by human liver microsomes containing 100μM CLOZ in the presence of LTG. Values are means +/- SEM (n=5).

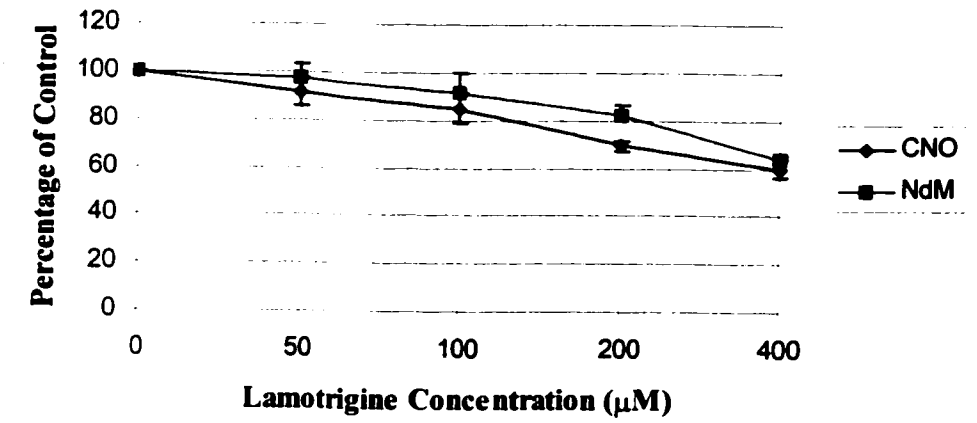


Figure 20. Graphic representation of inhibition of CNO and NdM production from CLOZ by LTG. Values represent means +/- SEM (n=5). A significant effect of LTG on CNO production ($p<0.0001$) was found at LTG concentrations of 200 and 400 μM. LTG at 400μM also significantly inhibited production of NdM ($p<0.0001$).

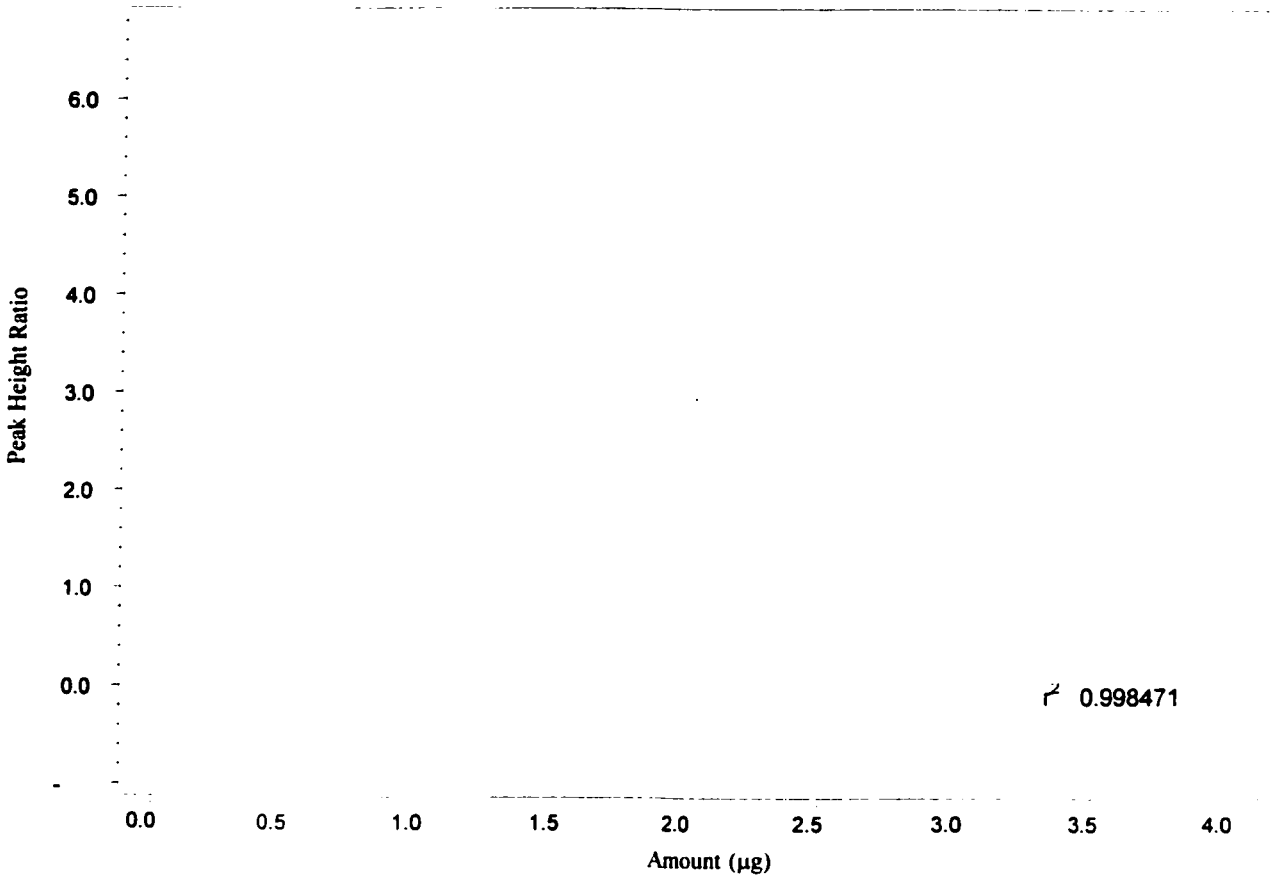


Figure 21. Typical calibration curve for the quantification CLOZ in rat brain extracts.

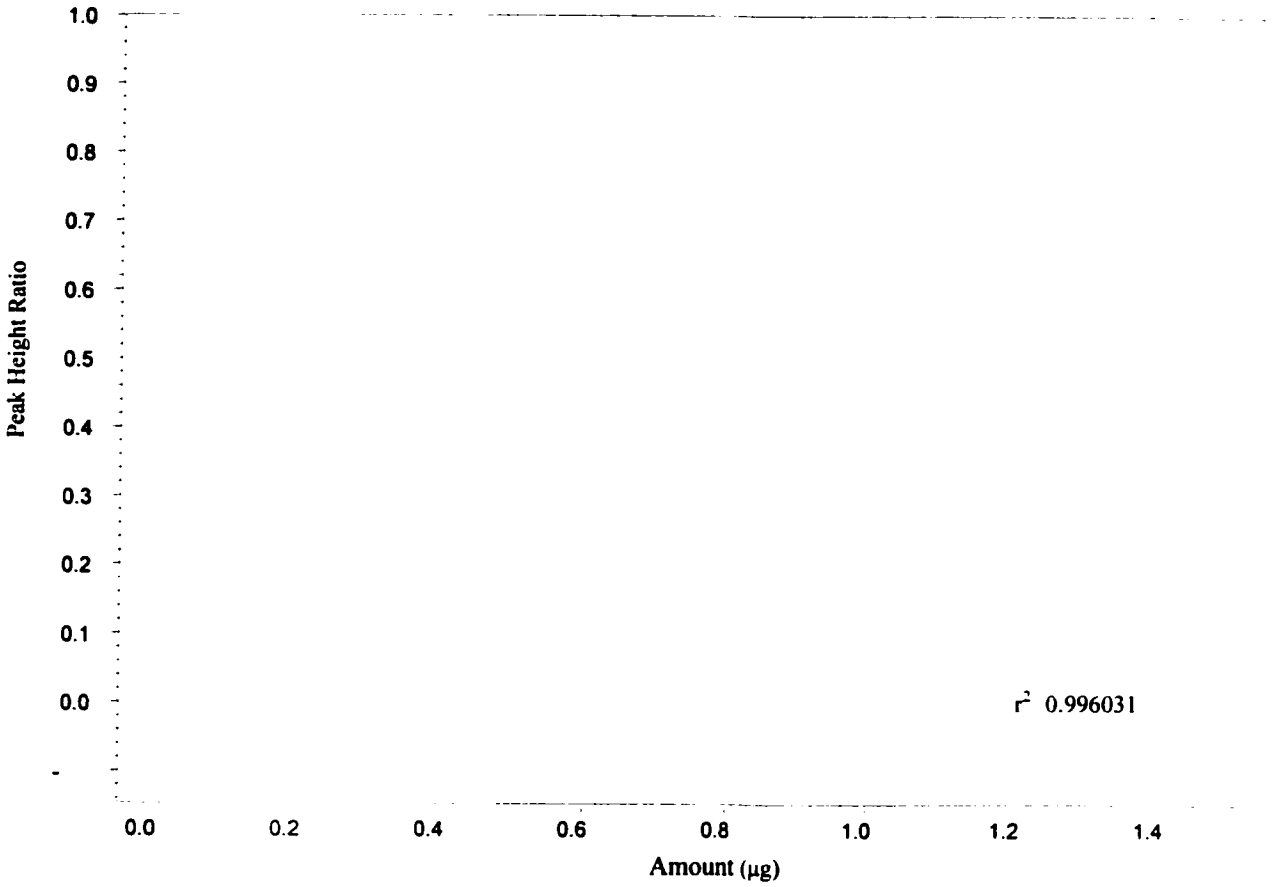


Figure 22. Typical calibration curve for the quantification of LTG in rat brain extracts.

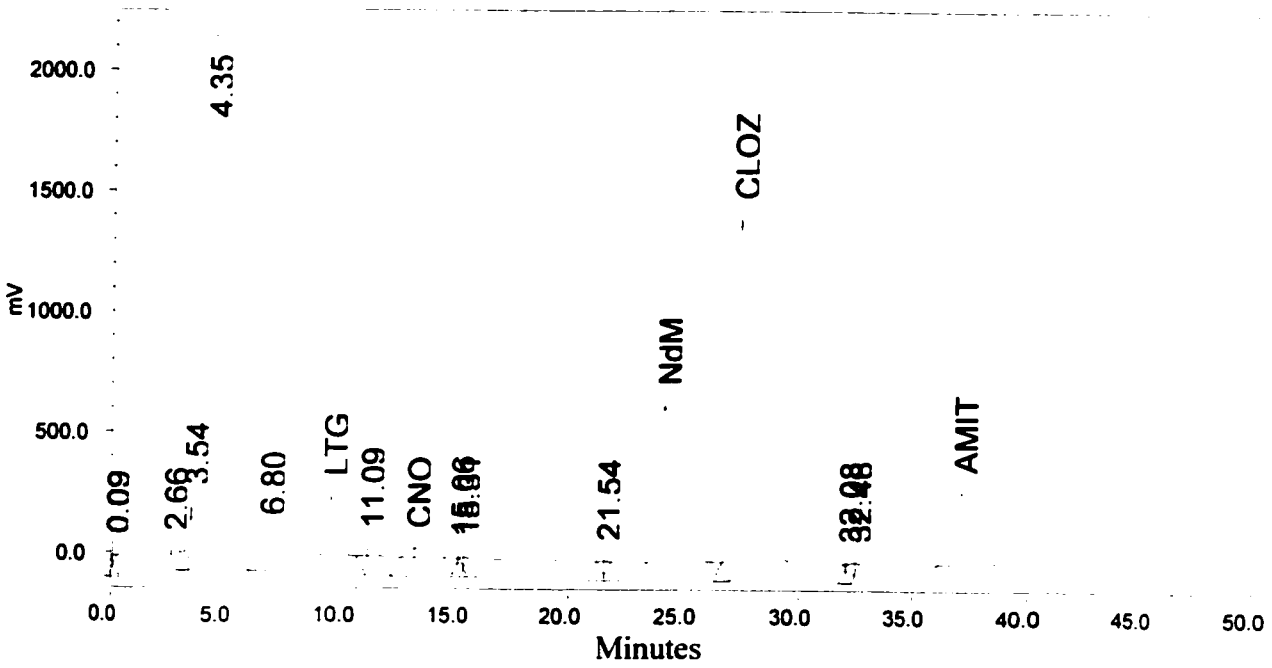


Figure 23. Typical HPLC trace of standards of CLOZ, NdM, CNO, and LTG added to rat brain extracts. Concentrations shown are as follows: 1.5 μg LTG, 0.5μg CNO, 2μg NdM, 4μg CLOZ, and 5μg AMIT.

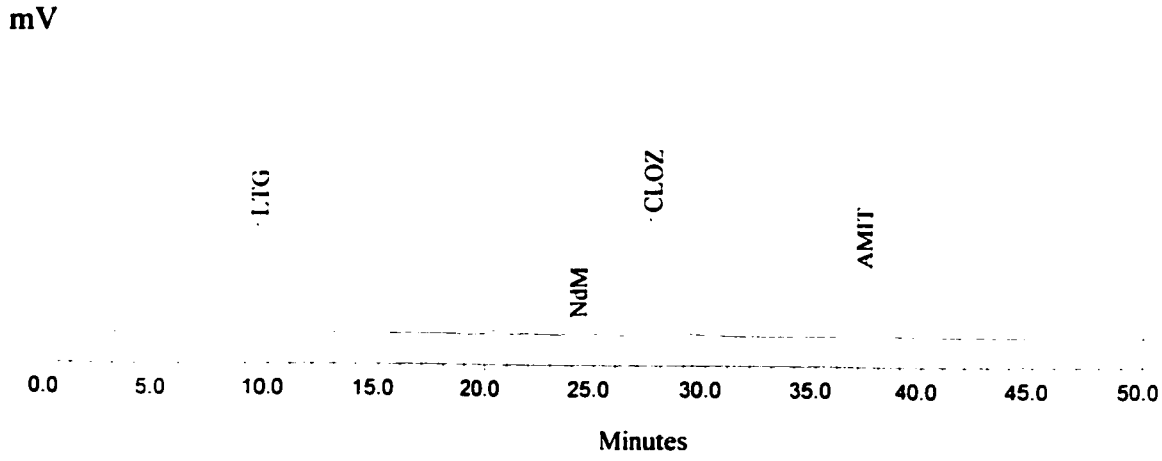


Figure 24. Typical HPLC trace from brain extract from a rat treated with LTG (2.5mg/kg) and CLOZ (10mg/kg) i.p.1 hour prior to death. AMIT was added as internal standard. No peak for CNO was detected.

4.3.2 LTG and CLOZ Levels

A typical chromatogram of a treated sample is shown in figure 24. Mean levels of LTG and CLOZ in rat brain and liver samples are presented graphically in figures 25-26. CLOZ levels in brain and liver were not altered by the presence of LTG at either time interval. LTG levels were increased in the coadministration group but only by 16.1%, and this increase was not significant.

4.4 Analysis of GABA and GLU levels from rats treated with LTG and/or CLOZ

4.4.1 Calibration curves

Calibration curves were generated by plotting peak heights for GABA and GLU on the y-axis versus the concentration of that compound on the x-axis. A typical calibration curve for GABA is shown in figure 27, and a typical calibration curve for GLU is shown in figure 28. A typical chromatogram for a standard is shown in figure 29.

4.4.2 GABA and GLU levels in rat brain

A chromatogram of brain extract from a rat treated with CLOZ is shown in figure 30. A chromatogram of brain extract from a rat treated with CLOZ and LTG is shown in figure 31. Mean levels of GABA and GLU in rat brain are shown graphically in figures 32-35. Levels of GLU were found to be significantly higher

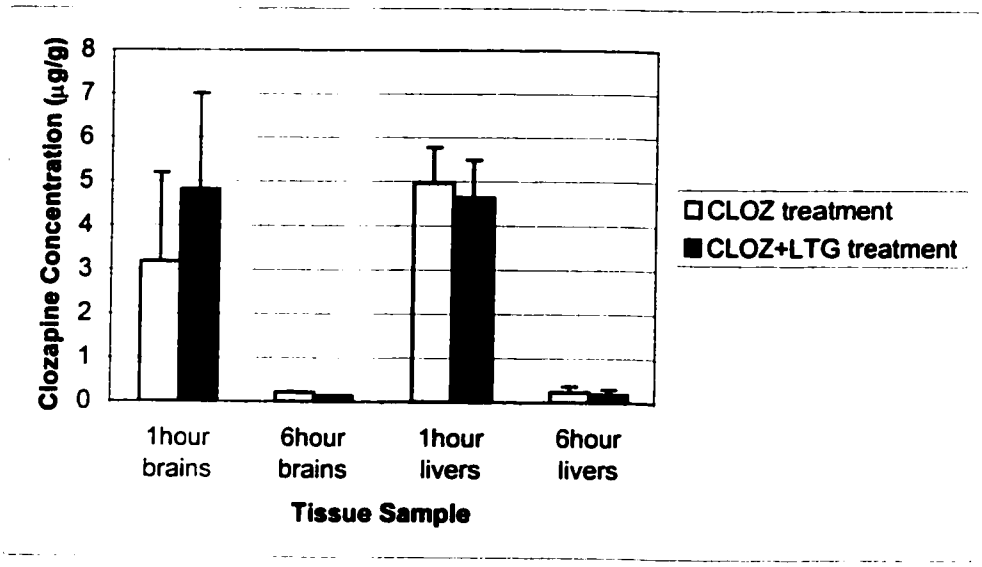


Figure 25. CLOZ levels in brain and liver from rats treated with CLOZ (10mg/kg) and LTG (2.5mg/kg) i.p. Values represent means +/- SEM (n= 5 for 1 hour samples, n=6 for 6 hour samples).

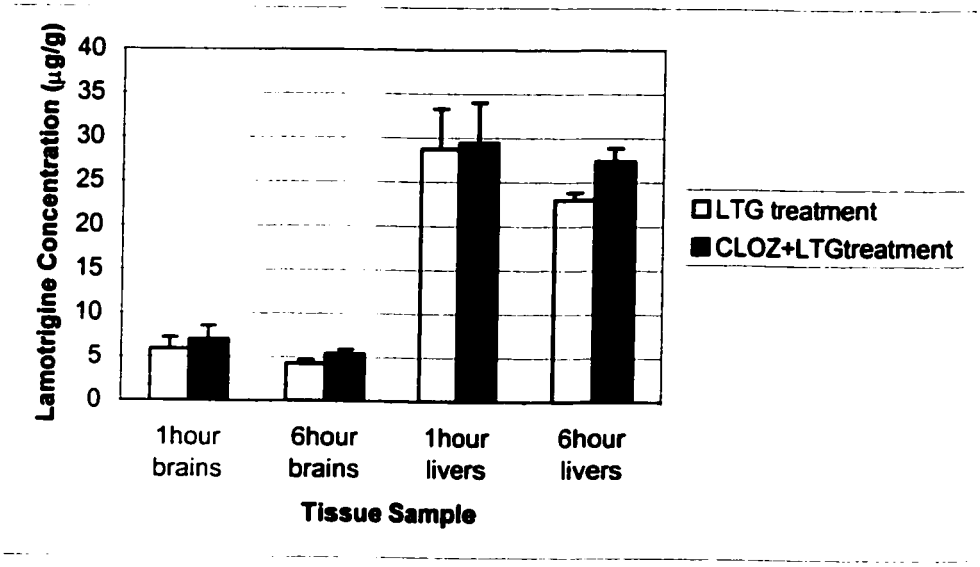


Figure 26. LTG levels in brain and liver from rats treated with CLOZ (10mg/kg) and LTG (2.5mg/kg) i.p. Values represent means +/- SEM (n= 5 for 1 hour samples, n=6 for 6 hour samples).

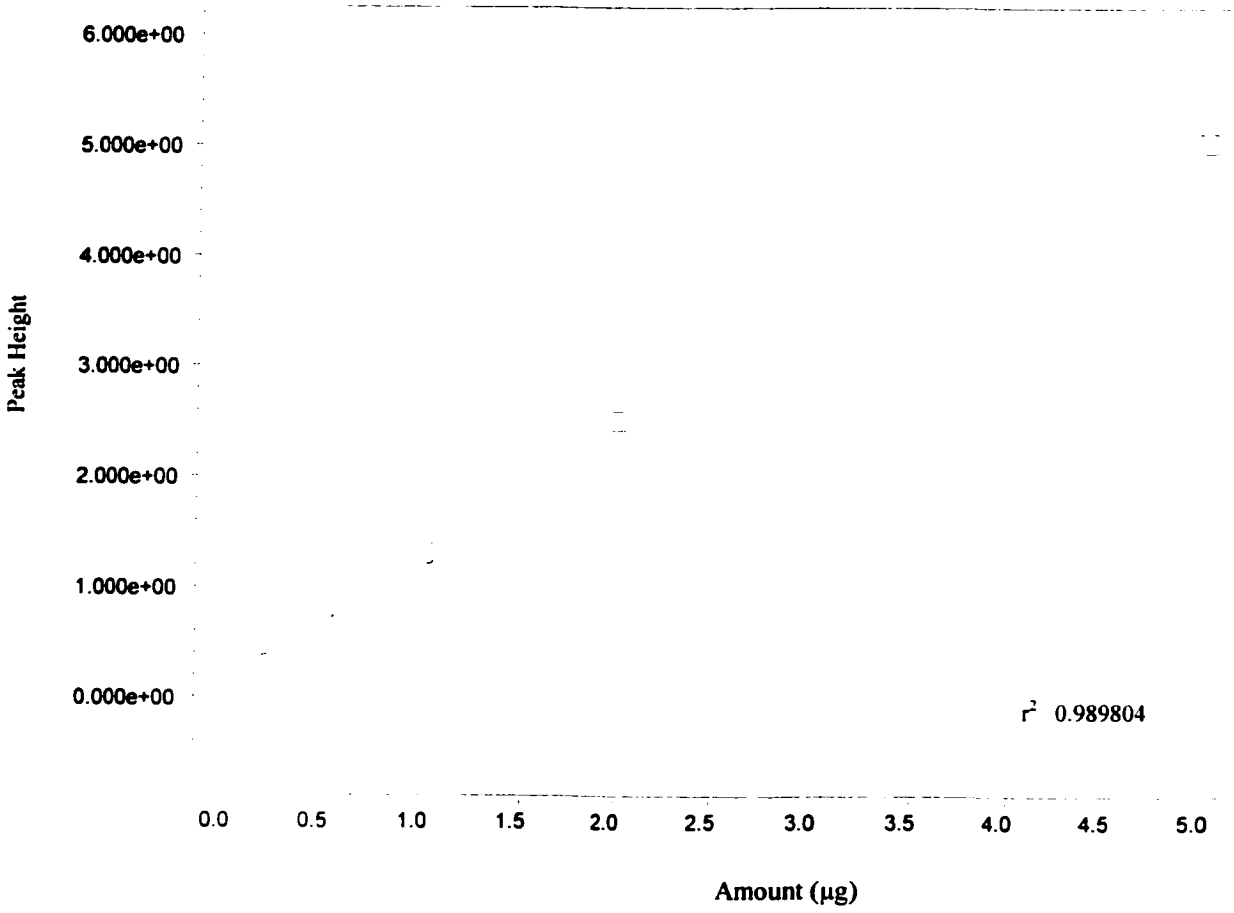


Figure 27. Typical calibration curve for the quantification of GABA in rat brain.

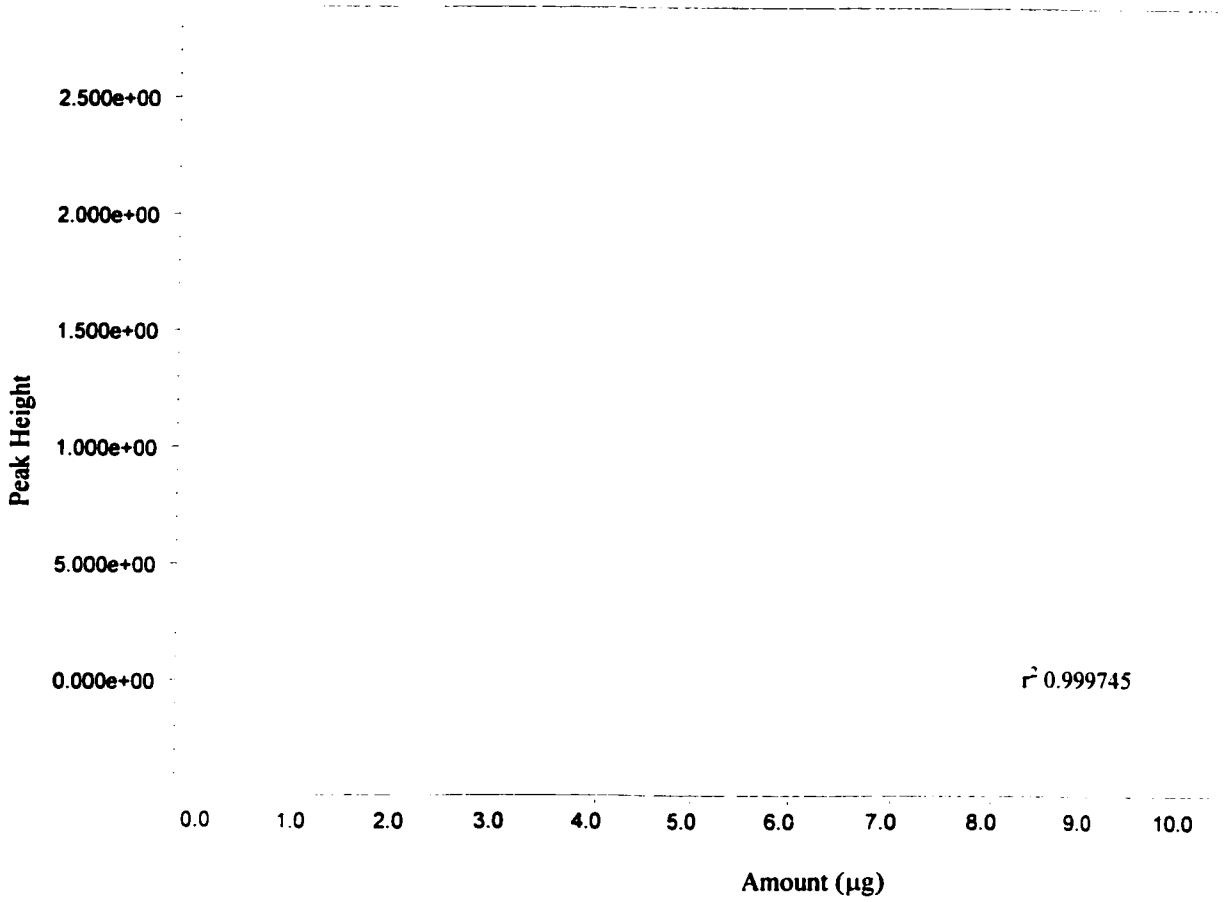


Figure 28. Typical calibration curve for the quantification of GLU in rat brain.

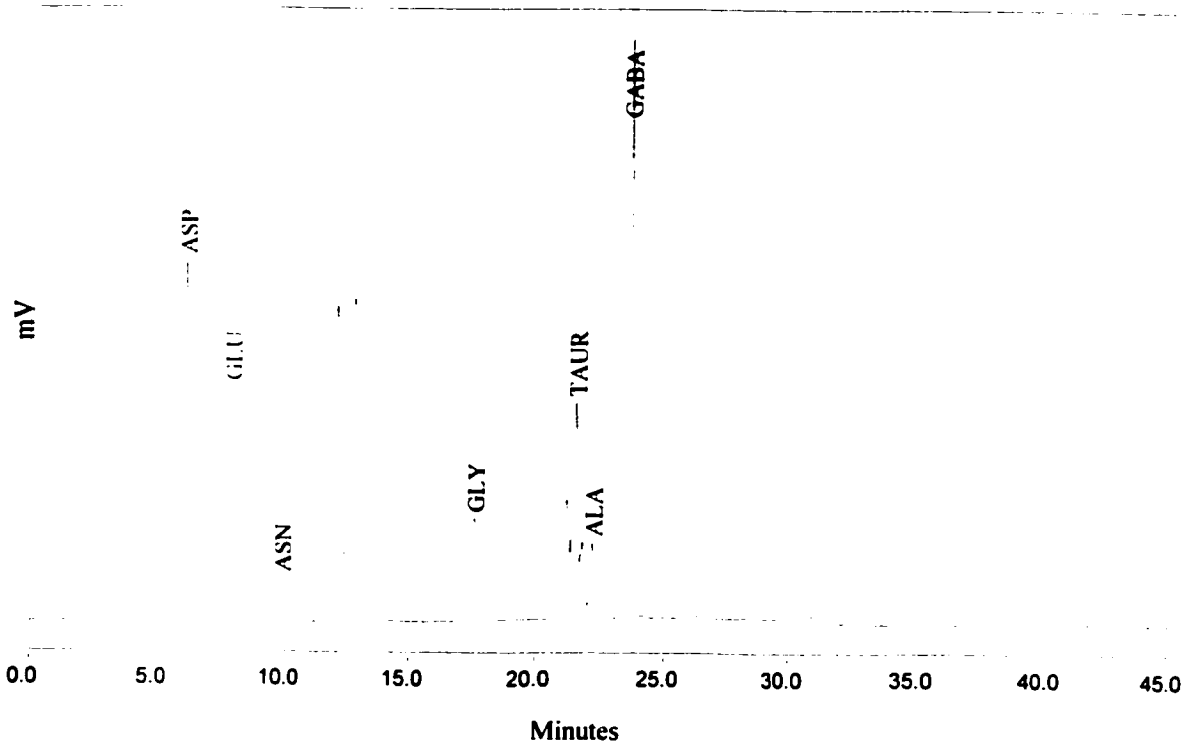


Figure 29. Typical HPLC trace of a standard solution of amino acids. Concentrations of glutamate and GABA are 2μg/mL and 1 μg/mL respectively. Abbreviations: aspartate (ASP), glutamate (GLU). asparagine (ASN), glycine (GLY), taurine (TAUR), alanine (ALA), and γ-aminobutyric acid (GABA).

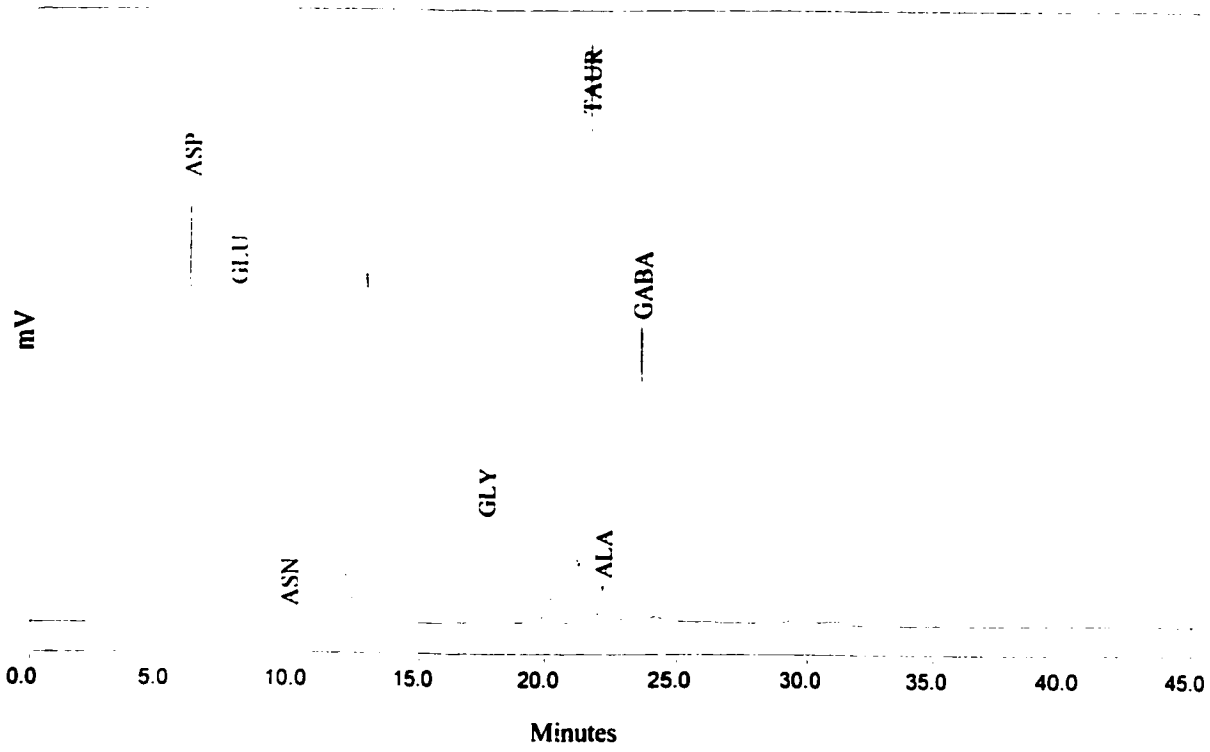


Figure 30. Typical HPLC trace of a brain extract from a rat treated with CLOZ (10mg/kg) 1 hour prior to death.

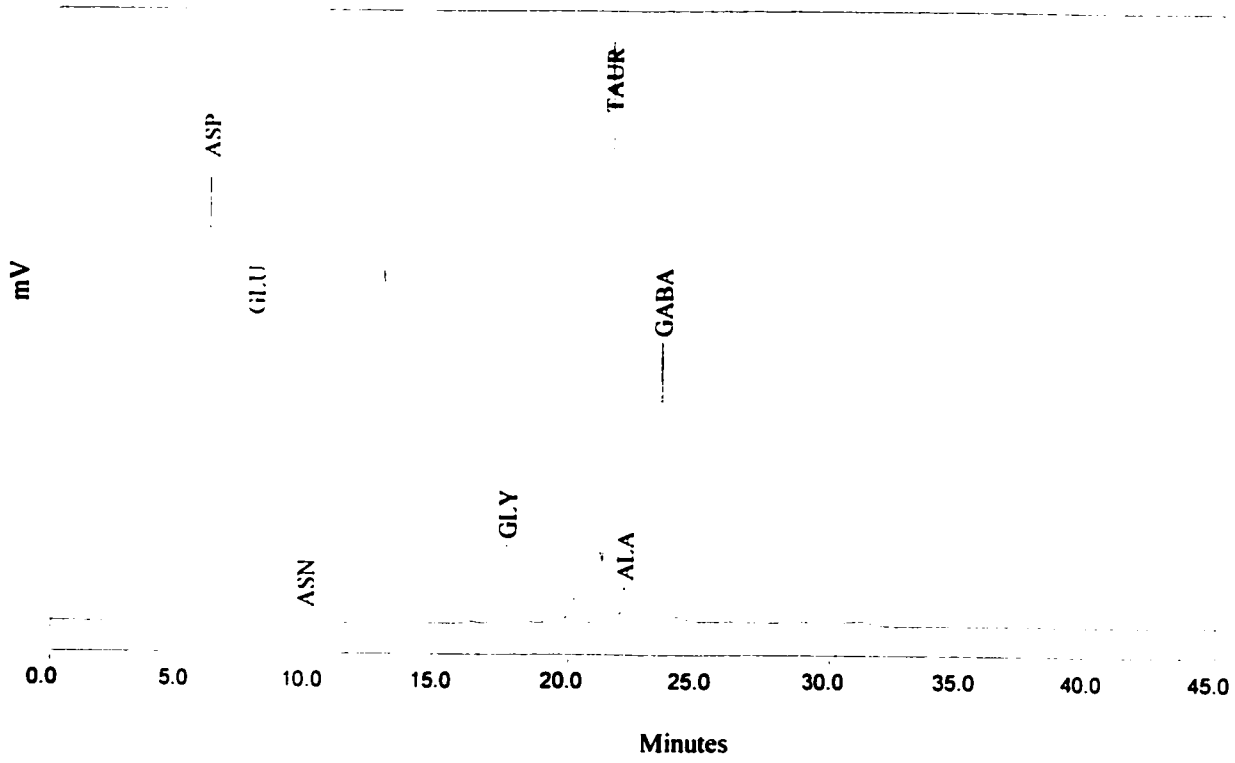


Figure 31. Typical HPLC trace of a brain extract from a rat treated with CLOZ (10mg/kg) +LTG (2.5mg/kg) 1 hour prior to death.

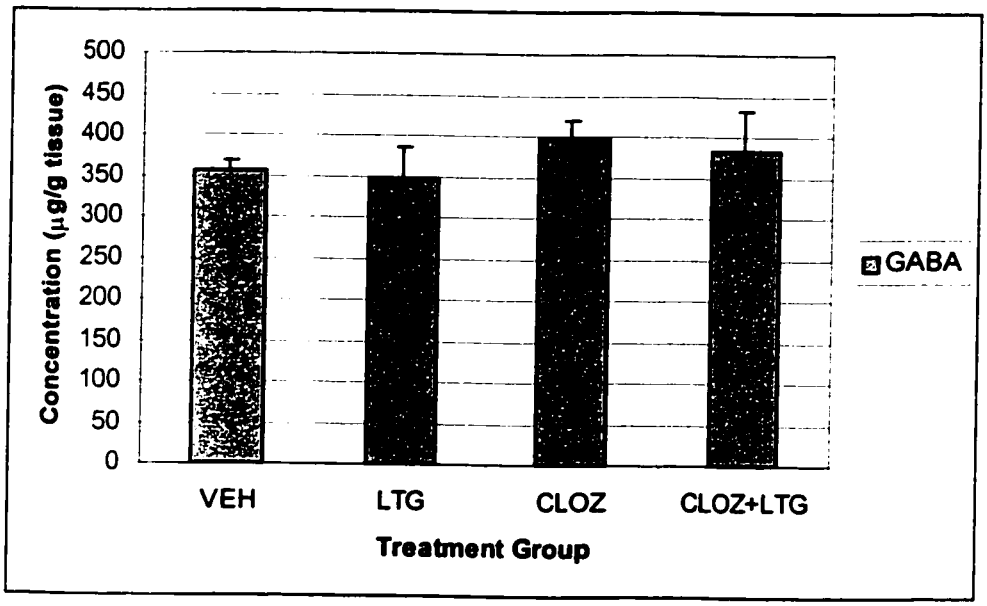


Figure 32. GABA levels in the brains of rats sacrificed 1 hour after injection. Values are means +/- SEM (VEH, n=3; 2.5mg/kg LTG, n=5; 10mg/kg CLOZ, n=5; 10mg/kg CLOZ+ 2.5mg/kg LTG, n=5).

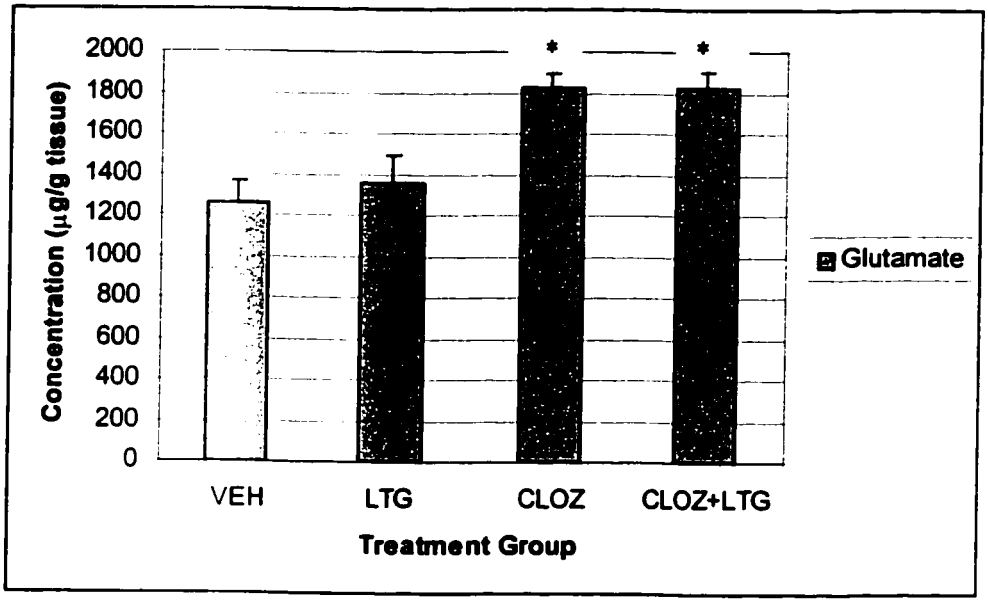


Figure 33: Glutamate levels in the brains of rats sacrificed 1 hour after injection. Values are means +/- SEM (VEH, n=3; LTG, n=5; CLOZ, n=4; CLOZ+LTG, n=5). The CLOZ treatment group and the CLOZ+LTG treatment group were significantly different from the VEH and LTG treatment groups ($p<0.0001$). Doses of the drugs are as in Figure 32.

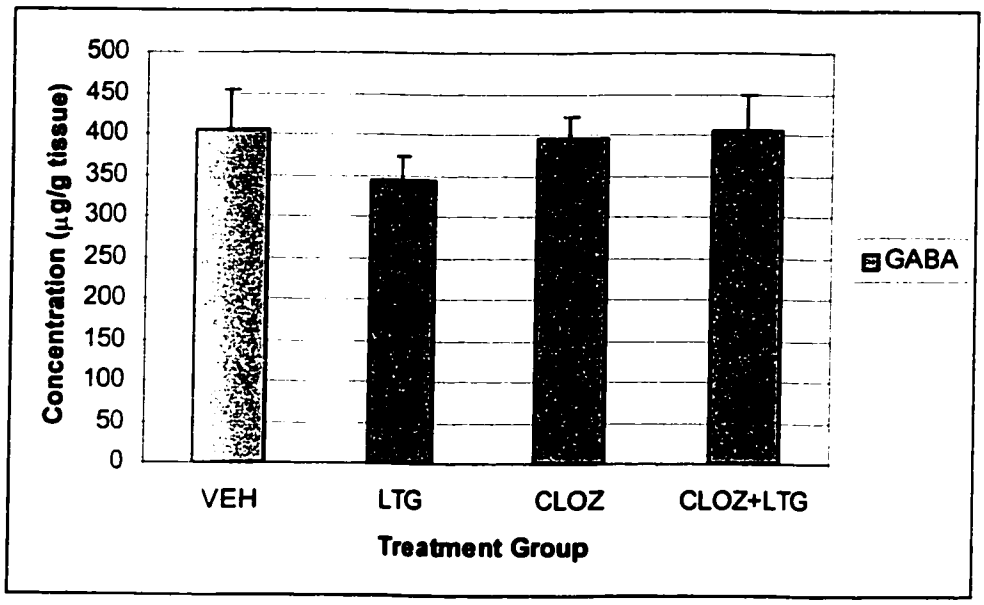


Figure 34. GABA levels in the brains of rats sacrificed 6 hours after injection. Values are means +/- SEM (VEH, n=3; LTG, n=5; CLOZ, n=5; CLOZ+LTG, n=5). Doses of the drugs are as in Figure 32.

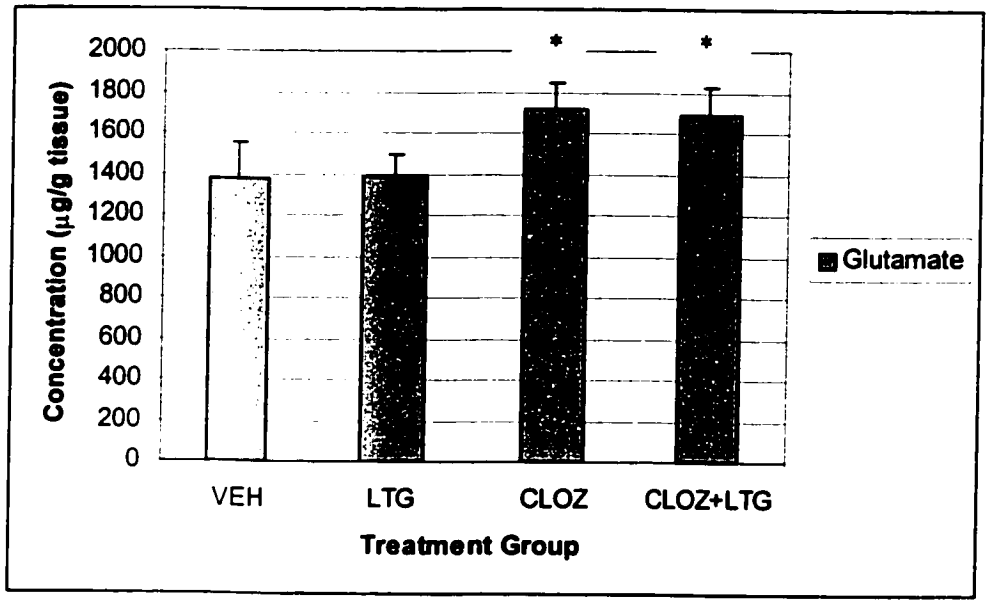


Figure 35. Glutamate levels in the brains of rats sacrificed 6 hours after injection. Values are means +/- SEM (VEH, n=3; LTG, n=5; CLOZ, n=5; CLOZ+LTG, n=5). The CLOZ treatment group and the CLOZ+LTG treatment group were significantly different from the VEH and LTG treatment groups (p=0.015). Doses of the drugs are as in Figure 32.

in brains of rats treated with CLOZ and CLOZ+LTG than in brains of rats treated with LTG or VEH ($p<0.001$ at 1 hour, and $p=0.015$ at 6 hourS). Levels of ASP and ALA were also found to be significantly higher in brains 1 hour after treatment with CLOZ and CLOZ+LTG when compared to LTG or VEH ($p=0.001$ and $p=0.005$ respectively). There was a 30.7% increase in ASP 1 hour after CLOZ treatment, and a 33.9% increase 1 hour after administration of CLOZ+LTG. There was a 38.2% increase in ALA 1 hour after CLOZ administration and a 35.1% increase 1 hour after CLOZ+LTG treatment. There were no significant differences in GABA, ASN or GLY levels among groups at either time interval.

4.5 Analysis of spermidine levels in rats treated with LTG and/or CLOZ

4.5.1 Calibration Curves

Calibration curves were generated by plotting the peak height for spermidine on the y-axis versus the concentration of spermidine on the x-axis. Calibration curves were linear and consistently had r^2 values greater than 0.99. A typical calibration curve is shown in figure 36.

4.5.2 Spermidine Levels in Rat Brain

A GC trace of a sample is shown in figure 37. Mean levels of spermidine in rat brain 1 hour after injection are shown graphically in figure 38, and mean values in rat brain 6 hours after injection are shown graphically in figure 39. Levels of spermidine were not found to be significantly different from the mean value obtained from vehicle brain samples.

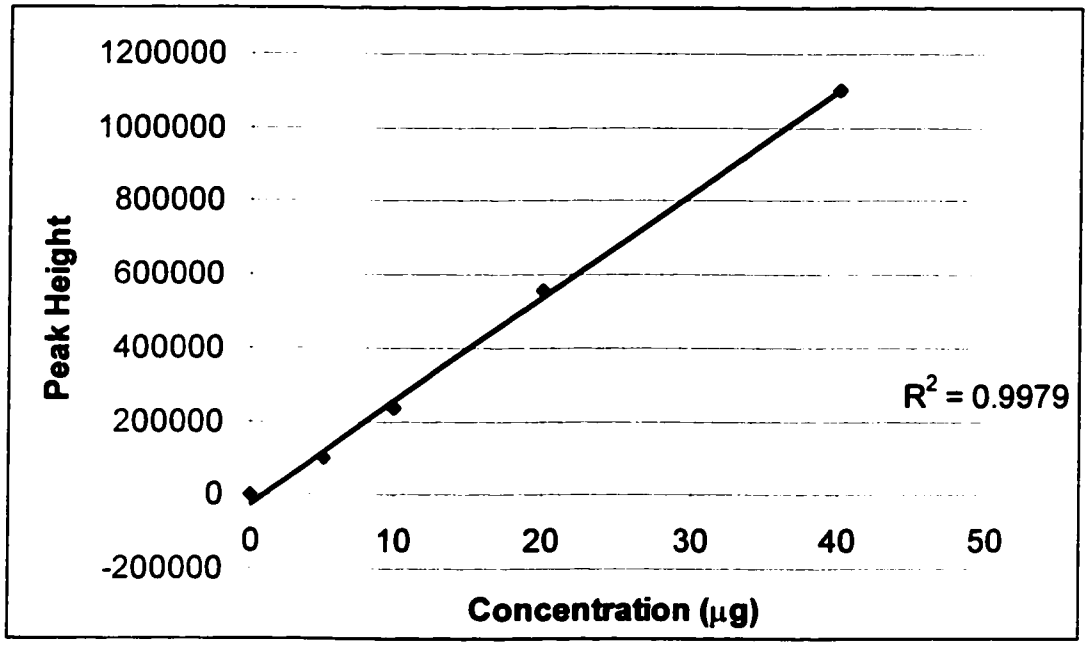


Figure 36. A typical calibration curve for the quantification of spermidine in rat brain.

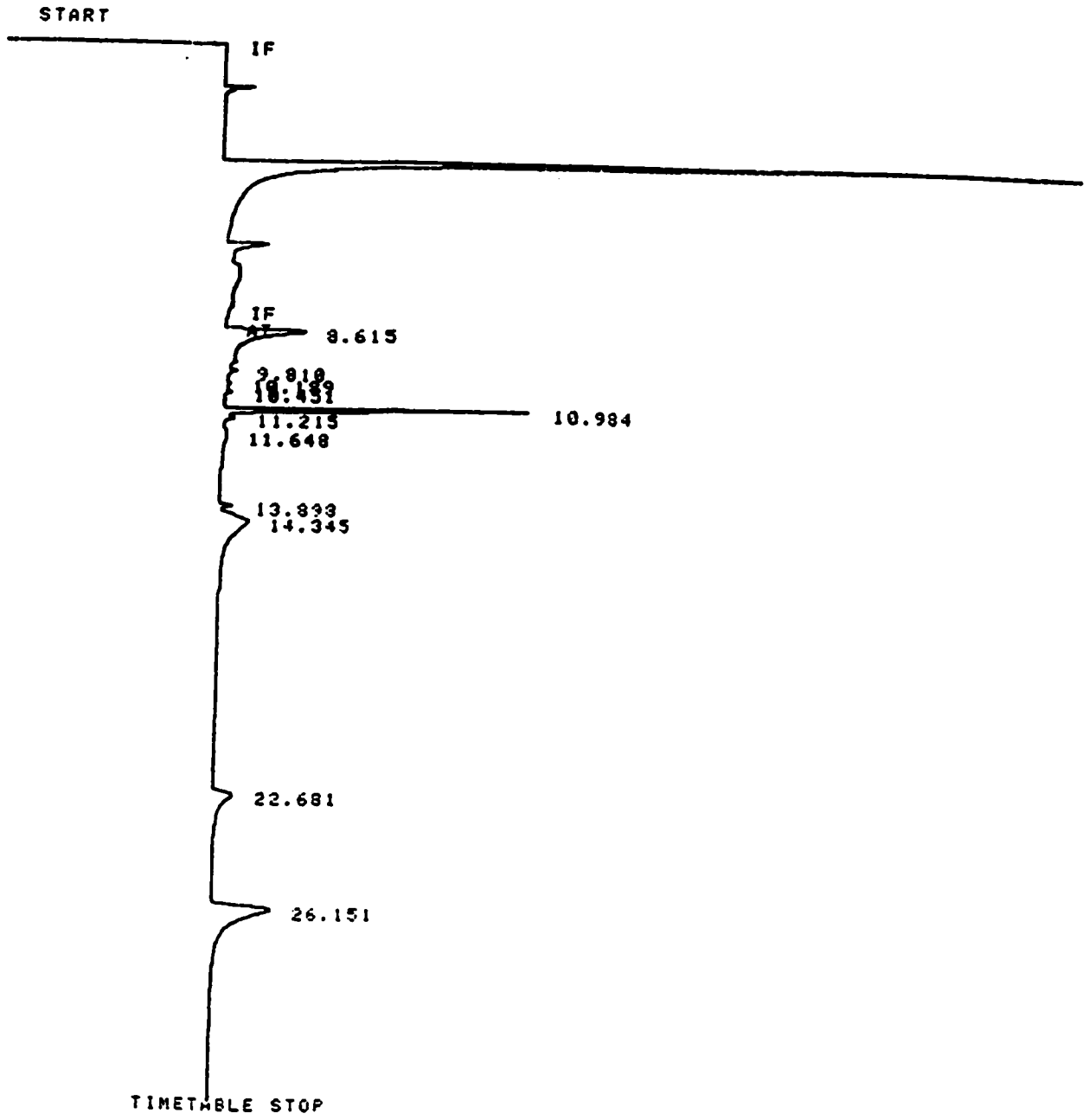


Figure 37. A GC trace of a rat brain sample for the analysis of spermidine. The retention time for spermidine is at 26.151 minutes.

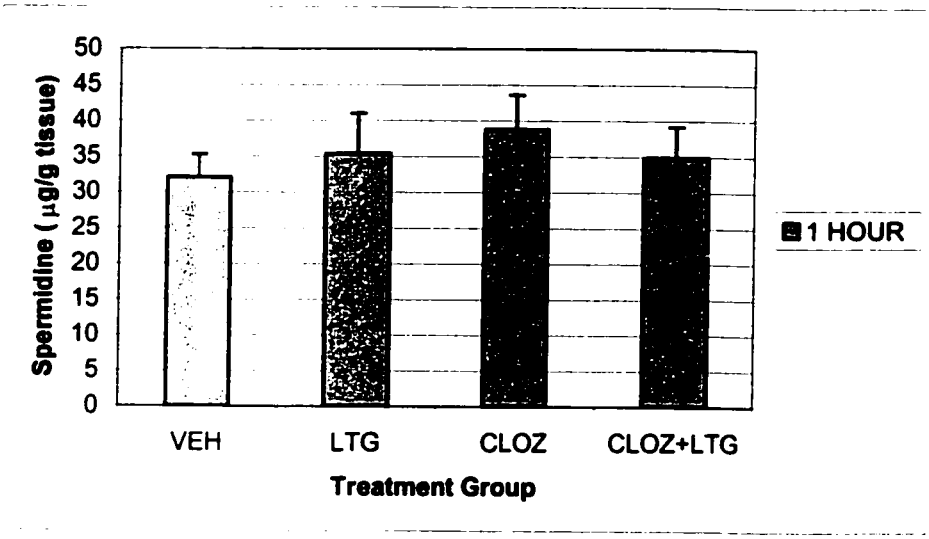


Figure 38. Spermidine concentrations in rat brain tissue 1 hour after treatment with LTG and/or CLOZ. Doses are as described in Figure 32. The values represent means +/- SEM (n=5-6). None were significantly different from vehicle. The vehicle groups at 1 hour and 6 hours were not different from one another and were pooled for the purpose of the analysis.

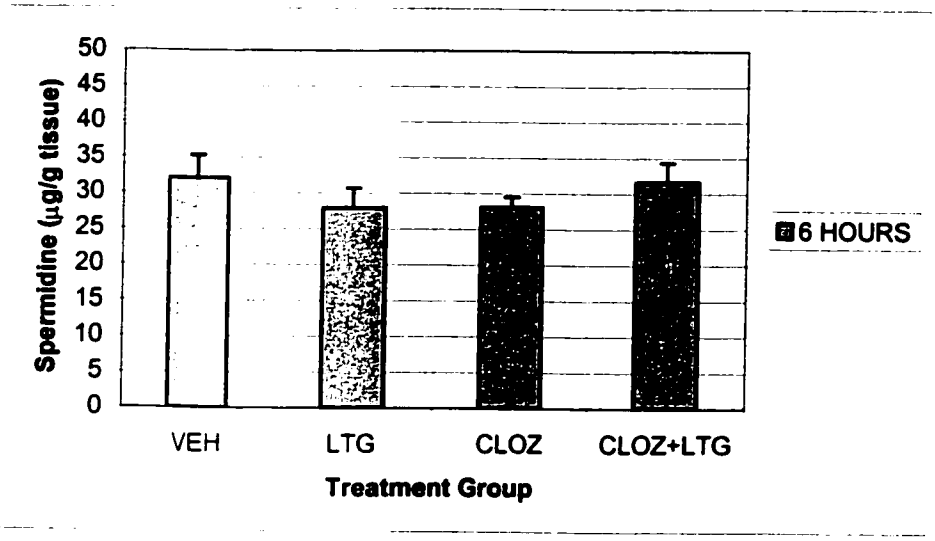


Figure 39. Spermidine concentrations in rat brain tissue 6 hours after treatment with LTG and/or CLOZ. Doses are as described in Figure 32. The values represent means +/- SEM (n=5-6). None were significantly different from vehicle. The vehicle groups at 1 hour and 6 hours were not different from one another and were pooled for the purpose of the analysis.

5. DISCUSSION

5.1 Comments on Assay Procedure Development

5.1.1 LTG, CLOZ, ISOCLOZ, CNO, and NdM Analysis

The assay for the analysis of LTG, CLOZ, ISOCLOZ, CNO and NdM was a modification of the procedure described by Fang *et al.* (1998) for analysis of CLOZ, CNO and NdM in microsomes. Peaks with good chromatographic properties were produced and there were no interfering peaks. The calibration curves were reproducible and linear. The method was also shown to be applicable to extracts of rat brain and liver, where it was used for analysis of LTG and CLOZ. The results in this thesis indicate that the method is also applicable to analysis of ISOCLOZ and its metabolites in microsomes. In another set of experiments that are not part of this thesis it was demonstrated, in samples sent to the Neurochemical Research Unit by Dr. Kapur of the Center for Addiction and Mental Health, that the procedure was also applicable to the analysis of ISOCLOZ in striatum of rat brain. To our knowledge, the results in this thesis represent the first report of analysis of metabolites of ISOCLOZ.

5.1.2 Amino Acid Analysis

The assay for the analysis of amino acids in rat brain is a simple procedure that is reproducible and produces good HPLC traces with no interfering peaks. The assay has been used extensively to analyze amino acid levels in various studies (Parent *et al.*, 2001). The calibration curves were consistently linear and reproducible.

5.1.3 Spermidine Analysis

The GC assay for the analysis of spermidine is simple, rapid and produces a derivative with good chromatographic properties and no interfering peaks. The calibration curves were consistently linear. The results were reproducible and the rat brain values obtained were similar to those of other studies (Shaw and Pateman, 1973; Morrison *et al.*, 1995). Extractive derivatization was conducted under aqueous conditions using PFBC as the derivatizing reagent. PFBC has been used extensively in the past for the analysis of various amines and phenols under similar conditions (Baker *et al.*, 1986; Nazarali *et al.*, 1987; Rao *et al.*, 1987; Salsali *et al.*, 2000). Wong et al., (1985) applied extractive derivatization with PFBC to analysis of spermidine in food products and, as shown in this thesis, the procedure can be adapted successfully to the analysis of spermidine in brain tissue.

5.2 The Effect of FLUVOX, KETO, and LTG on The Metabolism of CLOZ in Human Liver Microsomes

The metabolism of CLOZ and ISOCLOZ over a time period of 120 minutes indicated that the degree of metabolism was very similar between the two. More of the N-oxide metabolite was produced in both cases than the N-desmethyl metabolite. The traces that were produced from these incubations showed that ISOCLOZ and CLOZ had very similar retention times, as did the metabolites of CLOZ and the proposed metabolites of ISOCLOZ.

The effects of various inhibitors on the formation of the metabolites of CLOZ were investigated. The CYP1A2 inhibitor FLUVOX and the CYP3A4 inhibitor KETO had significant inhibitory effects on the demethylation of CLOZ. KETO was more potent than FLUVOX or LTG at inhibiting the formation of CNO and NdM, but this inhibition did not increase markedly from 5 to 40μM KETO, indicating that CYP enzymes other than CYP3A4 are also contributing to the formation of these metabolites; this finding is in agreement with the findings of Fang *et al.* (1998). Neither FLUVOX nor LTG had a strong effect on CNO formation, even at concentrations as high as 400 μM. FLUVOX had a much stronger inhibitory effect than LTG on NdM formation, although neither drug completely inhibited this metabolic pathway even at a concentration as high as 400 μM.

These findings seem to support those of Fang *et al.* (1998), who reported that FMO3 as well as CYP3A4 contributed to the metabolism of CLOZ to CNO while both CYP1A2 and CYP3A4 contributed to the formation of NdM. Pirmohamed *et al.* (1995), who also worked with human liver microsomes *in vitro*, reported that production of NdM was inhibited by both KETO and furafylline (a CYP1A2 inhibitor). *In vivo* studies, as well as case reports, indicate that coadministration of drugs that are metabolized by CYP1A2 or CYP3A4, and inhibit or induce these enzymes, influence plasma CLOZ levels in patients and healthy subjects (Centorrino *et al.*, 1994; Hiemke *et al.*, 1994; Jerling *et al.*, 1994; Eiermann *et al.*, 1997). In other investigations plasma levels of CLOZ in patients were shown to be correlated to N_3-demethylation of caffeine (a measure of

CYP1A2 activity) and the clearance of CLOZ in patients was reported to be enhanced by carbamazepine, an inducer of CYP3A4 (Raitasuo *et al.*, 1993; Jerling *et al.*, 1994).

The fact that LTG did not affect the metabolism of CLOZ except at concentrations of at least 200μM, and even then to a modest extent in human liver microsomes, suggests that LTG would not exert an important effect on CLOZ metabolism in the clinical situation. Plasma levels of LTG at usual therapeutic doses are 1-4μg/mL (3.9-15.6μM) (Castel-Branco *et al.*, 2001), much lower than the concentrations which caused inhibition in the studies reported in this thesis. The liver levels may well be higher than this, but there is no information currently available on liver/plasma ratios of LTG in humans. Liver/plasma ratios for the SSRI antidepressants have been reported to be 12-27 (Preskorn, 1996), and if the ratios for LTG were in the upper range of these values, levels attained in liver could be in the highest range tested in the present project. Phenothiazine antipsychotics have a much higher liver/plasma ratio than the SSRIs (Seeman *et al.*, 1980) and if similar ratios are applied to LTG, then concentrations of 200-400μM or considerably higher could be attained in humans. There is very little information available on possible pharmacokinetic interactions between CLOZ and LTG in the clinical situation. Kossen et al. (2001), in a recent case report in which a patient had LTG added to his CLOZ dose for two weeks, reported that the CLOZ plasma levels were tripled after the two week period. The authors could provide no logical explanation for this observation since LTG is unlikely to displace CLOZ from protein binding (LTG is only 55% protein bound itself) and

LTG metabolism is thought to be primarily via glucuronidation (Kossen et al., 2001; Gillis 2001). However, anticonvulsants that induce CYP1A2 enzymes (e.g. phenytoin, phenobarbital, and carbamazepine) do increase the plasma clearance and reduce the elimination half-life of LTG (Gillis, 2001); therefore it is possible that LTG is a substrate for CYP1A2 and thus at high concentrations could compete with CLOZ for metabolism by this enzyme. Kossen *et al.* (2001) did not measure levels of LTG and did not measure CLOZ plasma levels at time intervals less than 2 weeks after coadministering LTG. As described later in this discussion, the concentration of LTG attained in rat brain after a dose of LTG (2.5mg/kg) similar to clinical doses (3.4mg/kg) gave a LTG level of approximately only 23μM. Parsons *et al.*, (1995) reported on the administration of LTG to rats p.o. at doses similar, on a mg/kg basis to the usual clinical dose, and obtained brain to plasma ratios in the range of only 1.6-1.9.

The effects of CLOZ on LTG metabolism were not investigated in the present study due to the lack of availability of authentic standards of the metabolites of LTG.

5.3 Metabolism of ISOCLOZ by human liver microsomes and confirmation of structures of metabolites by mass spectrometry

ISOCLOZ has become of interest because of a recent study by Kapur *et al.* (2001) that indicated that ISOCLOZ has a behavioral and receptor binding profile similar to typical antipsychotics such as haloperidol, rather than its atypical isomer CLOZ. To my knowledge the results in this thesis represent the first report

of ISOCLOZ's metabolism. The combined HPLC-mass spectrometry method has demonstrated unambiguously that like CLOZ, ISOCLOZ undergoes both N-demethylation and N-oxidation.

ISOCLOZ and CLOZ have the same molecular weight, and, as shown in figures 13a and 13b, the same mass fragmentation. If the previously unidentified peaks seen in the microsomal extracts after incubation with ISOCLOZ are indeed the N-oxide and N-desmethyl metabolites, parallel to the situation with CLOZ, they would be expected to have the same molecular weight and very similar mass fragmentations to CNO and NdM (for which authentic standards were available), respectively. This did turn out to be the case, confirming that ISOCLOZ, like CLOZ, undergoes metabolism by N-oxidation and N-demethylation.

5.4 Effects of LTG and CLOZ on each other's levels in rat brain and liver

At the doses and time intervals used there was no effect of LTG on the levels of CLOZ in rat brain or liver. Similarly, LTG levels remained the same between the LTG treatment group and the LTG + CLOZ treatment group, indicating that there was no effect of CLOZ on LTG metabolism. The ratio of doses of the drugs used in the rat study were based on the preliminary study by Dursun *et al.* (1999) in humans where LTG was added on to CLOZ treatment in a group of schizophrenic patients.

As mentioned previously in this thesis, drug-drug interactions involving CYP enzymes are usually detectable by changes in levels of the parent drugs at short time intervals after administration of the drugs of interest (Goodnough and

Baker, 1994; Sills *et al.*, 1999a,b). Changes would have suggested a pharmacokinetic interaction and prompted a study of metabolites. Since no change in brain levels of LTG or CLOZ were observed in the presence of the coadministered drug, pursuing a comprehensive drug metabolism study was not considered. It should be mentioned here that rat CYP enzymes are not identical to human CYP enzymes (Fang, 2000). However, they are very similar, and metabolic routes such as hydroxylation and N- and O-demethylation also occur commonly in rats. It is also important to do metabolism studies in rats since most of the investigations on the possible mechanisms of action of psychiatric drugs are done in rats, often without considering metabolism or possible drug-drug interactions.

The findings here with both human liver microsomes and rat tissues represent the first demonstration, to our knowledge, that there is unlikely to be a relevant pharmacokinetic interaction between LTG and CLOZ except at high concentrations of LTG (in excess of 200μM). However, the case report of Kossen *et al.* (2001) indicates that a comprehensive clinical study on such an interaction, which would include measurements of plasma levels of both LTG and CLOZ, is warranted.

5.5 Analysis of GLU and GABA levels in rat brain after treatment with LTG and/ or CLOZ

The levels of GABA and GLU were found to be unchanged after acute treatment with LTG in rats. Several other studies have reported different results

when looking at the effect of LTG on GLU and GABA. Most of these studies have used models of epilepsy to show the effect of LTG. Hassel *et al.* (2001) found that chronic LTG treatment in rats caused an increase in GABA in the hippocampus but not in other brain regions; they also found the levels of GLU remained unchanged. Because my study examined whole brain, it is possible that regional increases in GABA levels were masked. Chronic studies were not conducted here because of the lack of availability of sufficient quantities of LTG. Several studies examining the mechanism of action of LTG have reported that under normal conditions LTG will not affect GLU levels but will cause a decrease in GLU levels only under pathological conditions such as those used in models of epilepsy (Leach *et al.*, 1986; Xie and Hagan, 1998). This could also explain why I saw no change in the levels of GLU and GABA.

Brain ALA, GLU and ASP levels were not increased by administration of LTG alone, but were increased compared to controls in the CLOZ and CLOZ+LTG treatment groups. In a microdialysis study, Daly and Moghaddam (1993) found that CLOZ, but not haloperidol, increased extracellular levels of GLU and ASP in the pre-frontal cortex of rats. These researchers suggested that this effect could be due to a direct action of CLOZ on excitatory amino acid (EAA) receptors or by an indirect action on receptors that normally modulate EAA release. ALA, like GLY, is a coagonist at the NMDA receptor, but is weaker than GLY in this regard (Thomson, 1989).

5.6 Analysis of spermidine levels in brains treated with LTG and/or CLOZ

The spermidine levels in rat brain did not differ significantly among treatments. Neither LTG nor CLOZ appear to exhibit any effect on spermidine concentrations in rat brain when given acutely. Spermindine is known to be an agonist at a modulatory site on the NMDA receptor and has been reported to reverse working memory deficits in rats caused by blockade of hippocampal muscarinic receptors and class I metabotropic glutamate receptors (Kishi *et al.*, 1998a,b). Ramchand *et al.* (1994) suggested that an altered polyamine metabolism might be involved in the pathophysiology of schizophrenia. The results in the study reported here indicate that CLOZ and LTG, at least after acute administration do not alter the brain levels of spermidine; thus it seems unlikely that either drug affected this polyamine's metabolism at the time interval tested here. This supports the hypothesis that the increases in polyamine levels seen in other studies that examined schizophrenic patient levels were inherent in these patients and not due to the effects of the drugs used for treatment (Das *et al.*, 1989; Ramchand *et al.*, 1994), although chronic studies would have to be conducted in rats in order to state definitely that a similar pattern occurred in this species. It is also of interest that administration of LTG, which reduces release of GLU from nerve terminals, has no effects, at least in the acute situation, on levels of spermidine, an amine which has a binding site on an important receptor for GLU, namely the NMDA receptor (Ientile *et al.*, 1999; Sharma and Reynolds, 1999; Stastny *et al.*, 1999; Segal and Skolnick, 2000; Worthen *et al.*, 2001).

6. CONCLUSION

6.1 Summary

- It was shown that a previously reported assay for analysis of CLOZ, NdM, and CNO in human liver microsomes could be adapted for analysis of ISOCLOZ and its metabolites, and LTG in the same preparation. The assay was also shown to be applicable to the analysis of CLOZ and LTG in brain and liver of the rat.
- Experiments with various inhibitors in human liver microsomes and studies in rat brain and liver after coadministration of CLOZ and LTG indicated no significant pharmacokinetic interaction between these two drugs, except at the higher doses (200μM and 400μM) of LTG tested.
- A combined HPLC-MS technique was utilized to demonstrate for the first time, that ISOCLOZ, like CLOZ, undergoes N-demethylation and N-oxidation.
- At 1 and 6 hours after administration, CLOZ and LTG had no effect on rat brain levels of GABA. LTG alone had no effects on GLU or GABA levels while CLOZ caused a significant increase on brain levels of GLU.
- CLOZ and LTG, alone or in combination, had no effect (at 1 hour and at 6 hours) on rat brain levels of spermidine.

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