สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

LITERATURE REVIEWS

GHB and Its Precursor

γ-Hydroxybutyric acid (GHB) itself is a naturally occurring derivative of the major CNS inhibitory neurotransmitter, γ-aminobutyric acid (GABA). The sodium salt of GHB has a molecular formula of $C_4H_7NaO_3$, and a molecular weight of 126.09. GHB appears to have no legitimate use as an industrial chemical. Interestingly, γ-butyrolactone (GBL) and 1,4-butanediol, two GHB precursor molecules, are used extensively in chemical manufacturing. GBL is known by the chemical names γ-hydroxybutyric lactone, 2(3H)-furanone dihydro, 4-butyrolactone, dihydro-2(3H)-furanone, 4-butanolide, and tetrahydro-2-furanone, and 1,4-butanediol has a synonym of 1,4-tetramethylene glycol (Kerrigan, 2001a). GBL and 1,4-butanediol are both chemical precursors and metabolic precursors of GHB (Teter and Guthrie, 2001). They have been marketed as "legal" alternatives to the FDA-banned GHB. GHB has been studied outside the United States as a potential treatment for narcolepsy, sleep apnea, opiate withdrawal and alcohol withdrawal and dependence (Galloway et al., 1997).

In addition to GBL and 1,4-butanediol, the most recent precursor to have appeared is tetrahydrofuran (THF) (Nicholson and Balster, 2001). A recent survey of internet sites relating to GHB indicates a potential in the clandestine manufacture of GHB. Recently, increasing inquires regarding the synthesis of 1,4-butanediol and THF have also appeared (Morris, 2000).

THF is one of the GHB-related chemicals (Engel, 2000; Wisotsky, 1999). There are many references for the synthesis of GBL from THF by chemical experiments (Rhodium, 2003; The Good Reverend Drone, 2003). Imbenotte et al. (2003) analysed the serum and urine from a woman who ingested THF by ^{1}H nuclear magnetic resonance (^{1}H NMR) and could quantitate GHB in the same analysis. The presence of THF and GHB was confirmed by GC/MS analyses. This could indicate that GHB could have been produced from THF (Cartigny et al., 2001)

Endogenous GHB in the Brain

GHB is a short-chain fatty acid that occurs naturally in the mammalian brain at a concentration of 1-4 μM (Maitre, 1997). The primary precursor of GHB in the brain is believed to be γ-aminobutyric acid (GABA). It is synthesized by a specific pathway that transforms GABA into succinic semialdehyde via GABA-transaminase (GABA-T) activity; then succinic semialdehyde (SSA) is converted into GHB by a NADPH-dependent enzyme succinic semialdehyde reductase (SSR). GHB can be reconverted back to SSA via GHB dehydrogenase, and the GHB-derived SSA can be converted back to GABA. SSA can also be metabolized by succinic semialdehyde dehydroganase (SSADH) to succinic acid which then enters the Krebs cycle (Figure 2-1) (Kerrigan, 2001a).

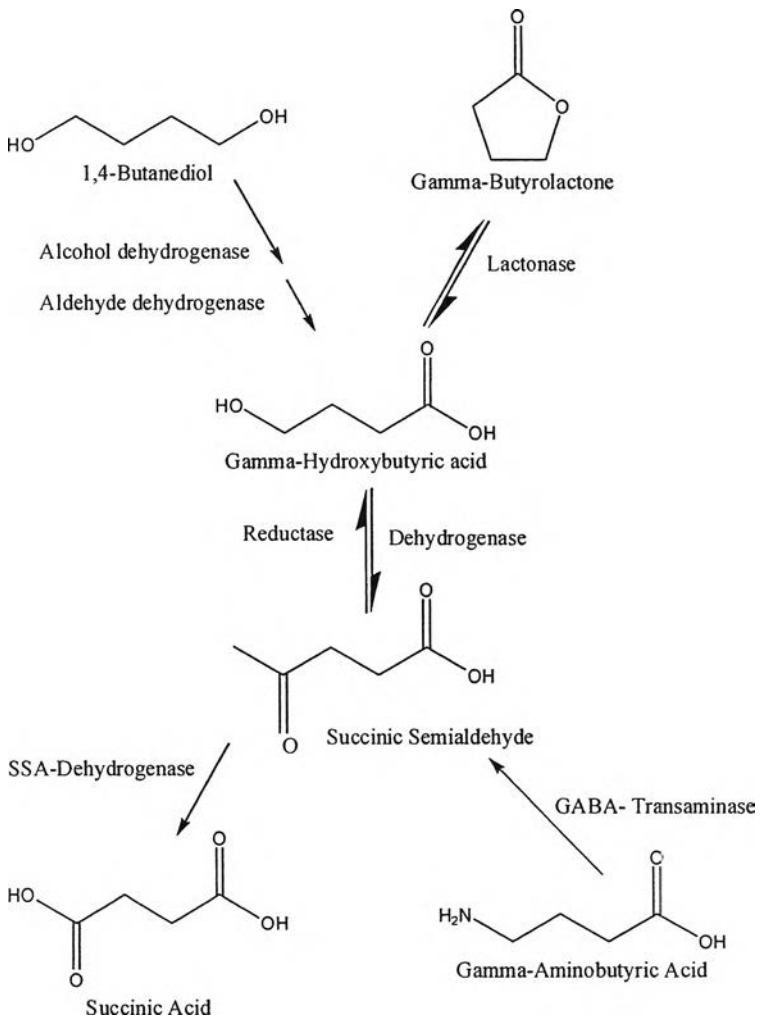

FIGURE 2-1 The Biotransformations of GHB and Its Precursors (Kerrigan, 2001a).

GHB has many properties that suggest this compound might play a role in the brain as a neurotransmitter or neuromodulator. These characteristics include a discrete, subcellular anatomical distribution for GHB and its synthesizing enzyme in neuronal presynaptic terminals. SSR is present in GABA-containing neurons, which suggests that GHB and GABA might co-localize in certain inhibitory nerve terminals (Hedou et al., 2000) and raises the possibility of a GHB-derived pool of GABA.

The presence of a GHB receptor is suggested by specific high-affinity [^{3}H] GHB binding sites that occur in the brain, with the highest density found in the hippocampus, followed by the cortex and then the thalamus. The binding kinetics of these sites correlate with the physiological concentrations of GHB in the brain because there is a high affinity GHB binding site with a dissociation constant (K_d) of 30-580 nM and a low affinity site with a K_d of ~2.3-16.0 μM. GHB is released by neuronal depolarization in a Ca^{2+}- dependent fashion and a Na^+- dependent GHB uptake system has been demonstrated in the brain (Maitre, 1997).

GHB also binds to the $GABA_B$ receptor (Mathivet et al., 1997; Snead, 1996). The relevance of this remains unknown but suggests that some of the pharmacologic actions of GHB are mediated by the $GABA_B$ receptor.

The $GABA_B$ receptor is a heterodimer in which the $GABA_{B(1)}$ receptor dimerizes with the $GABA_{B(2)}$ receptor to form a functional $GABA_B$ receptor. The $GABA_B$ receptor couples to various effector systems through a signal-transducing G protein. Presynaptically, activation of $GABA_B$ autoreceptors (located on GABA-containing neurons) and heteroreceptors (located on other neurotransmitter-releasing neurons) has been reported to inhibit neurotransmitter release through inhibition of Ca^{2+} channels. Postsynaptically, $GABA_B$ receptor activation produces slow inhibitory postsynaptic potential via G-protein-coupled inward rectifying K^+ channels (GIRK) (Bowery et al., 2002).

Many of the pharmacological and clinical effects of exogenously administered GHB are mediated via the $GABA_B$ receptor, where GHB might act both directly as a partial agonist and indirectly through GHB-derived GABA. GHB appears to be a weak $GABA_B$ receptor agonist (Mathivet et al., 1997), with an affinity for the $GABA_B$ receptor in the millimolar range (Lingenhoehl et al., 1999), which is

well above the 1-4 μM physiological concentrations of GHB in the brain (Maitre, 1997). Therefore, the high concentrations of GHB in the brain that would increase following exogenous administration could activate $GABA_B$ receptors.

Alternatively, GHB could activate the $GABA_B$ receptor indirectly via its conversion to GABA (Maitre, 1997). This hypothesis could explain the high concentration of GHB required to produce $GABA_B$ receptor-mediated effects because high micromolar to low millimolar concentrations of GHB are required to produce enough GHB-derived GABA to activate $GABA_B$ receptors. The possibility of GHB-derived GABA playing a role is favored when the concentration of GHB is high, as would be the case in GHB abuse and toxicity (Wong, Gibson, and Snead, 2004).

There is evidence to support the hypothesis that the GHB receptor is not the same as the $GABA_B$ receptor. $GABA_B$ receptor agonists do not displace [^{3}H]GHB binding and the ability of GHB to displace [^{3}H]baclofen binding is weak. GHB and its antagonist NCS-382 do not compete for [^{3}H]GABA binding in autoradiographic binding assays on rat brain sections (Wong et al., 2004).

Pharmacokinetics of GHB

In laboratory animals, as well as in humans, GHB is rapidly absorbed, freely crosses the blood-brain barrier with an onset of action within 15 min. In humans, the free fraction of GHB in plasma has been shown to be 0.99, indicating a lack of significant plasma protein binding. The half-life of GHB is 22-28 min after an oral dose of GHB 25 mg/kg, the half-life is slightly longer with higher doses. Less than 2% of a GHB dose is eliminated unchanged in the urine. Owing to the short half-life, there is no accumulation of GHB with repeated dosing and GHB doses of up to 100 mg/kg are no longer detectable in the blood from 2-8 hours or in the urine after 8-12 hours. In summary, it has been suggested that regardless of the dose given, the elimination of GHB is so rapid, even in those with compromised liver function, that GHB is completely eliminated within 4-6 hours after ingestion (Teter and Guthrie, 2001).

Once ingested, GBL and 1,4-butanediol are rapidly metabolized to GHB (Figure 2-1), and thus have similar pharmacologic and toxic effects as GHB. GBL is converted to GHB *in vivo* by a rapidly acting lactonase found in blood and

liver. Animal studies indicate the half-life of the conversion of GBL to GHB in plasma is approximately 1 min (Kerrigan, 2001a). GBL given per orally, intraperitoneally or intravenously is capable of producing higher concentrations of GHB in rat brain and blood than an equimolar amount of GHB administered by the same route (Guidotti and Ballotti, 1970). It was noted that GHB blood concentrations fell more rapidly after GHB than GBL administration. Roth and Giarman (1966) suggested that GBL was found to concentrate more in lean muscle than did GHB. This depot pool of drug appears to be metabolized more slowly because it is not readily accessible to enzymes involved in its biotransformation and its slow release back into the blood. This tissue, in turn, may act as a reservoir for GBL and extend its duration of action.

Dose-Related Effects of GHB

The primary dose-related effects of GHB are related to central nervous system depression. With GHB, very small dosage differences can cause greatly different effects. The difference between the dose required to produce sleep and the dose required to produce coma or death is usually less than one gram. The average dose used is between one and four grams. For a person weighing 140 lbs., two grams usually results in relaxation, decreased heart rate and respiration and motor impairment. Two to four grams will usually bring about severe intoxication. Five to six grams will usually lead to coma, while six grams will normally result in respiratory arrest. In a small person, two grams may be enough to produce coma (Goss, 2001).

In terms of mg/kg dose, 10 mg/kg GHB is capable of producing amnesia and hypotonia of the skeletal muscles resulting from the depression of neurons in the spinal cord. At 20-30 mg/kg, GHB promotes a normal sequence of rapid eye movement (REM) and non-REM (slow-wave) sleep, which lasts from 2-3 hours. At 40-50 mg/kg intravenously or orally, GHB produces a state of somnolence, which appears within 5-15 min. Anesthesia is associated with doses of 50 mg/kg, and doses higher than 50 mg/kg have been associated with coma, as well as decreased cardiac output, respiratory depression, and seizures. These effects are more pronounced with the co-ingestion of CNS depressants, particularly ethanol (Teter and

Guthrie, 2001). The pharmacological and clinical actions of GHB are summarized in Table 2-1.

TABLE 2-1 Pharmacological and Clinical Effects of GHB (Bernasconi et al., 1999).

GHB Doses	Physiological responses
• **Physiological and toxicological effects of single doses in rats**	
5-30 mg/kg i.p.	Hyperthermia
150 mg/kg i.p.	Anxiolytic activity
200-400 mg/kg i.p.	Sedation, absence-like seizures
400-750 mg/kg i.p.	Hypothermia, loss of righting reflex, apnea
500-1600 mg/kg i.p.	Coma
1700 mg/kg i.p.	Respiratory depression, LD_{50}
• **Clinical and Toxicological effects of single doses in humans**	
10 mg/kg p.o.	Anxiolysis, amnesia, hypotonia
20-30 mg/kg i.v. (50-60 mg/kg p.o.)	Unconsciousness, anaesthetic adjuvant
60-100 mg/kg p.o.	Coma, epileptic seizures, respiratory depression

Toxicological Analysis of GHB

Routine toxicological screening tests do not detect GHB-related compounds. Because of the efficient transformation of GHB's precursors GBL and 1,4-butanediol *in vivo*, toxicological analyses are commonly targeted towards GHB. *In vitro* conversion of GHB to GBL has been readily used for toxicological analyses. GBL is more amenable to conventional methods of extraction and gas chromatography compared with GHB, which is considerably more polar and less volatile. Acidification of the specimen results in lactonization, after which a solvent such as methylene chloride or chloroform can be used to extract GBL. GHB can be analysed directly using either liquid-liquid or solid-phase extraction techniques. Due to its small size and polar moieties, extracts tend to be non-specific, containing many other endogenous carboxylates and polar molecules. Derivatization is necessary prior to chromatographic analysis. Silylation of the hydroxyl and carboxylate moieties using common derivatizing agents is widely used. Derivatization of GHB increases

thermal stability, reduces volatility and increases the molecular weight, enhancing confidence in mass spectral identification (Kerrigan, 2001b).

Early gas chromatographic analyses were developed for the measurement of endogenous GHB in tissues. Samples were heated in the presence of acids, converting GHB to GBL, and were capable of measuring GHB concentrations as low as 1.45±0.22 nmoles/g (0.183±0.028 mg/L) of brain tissue in the rat (Roth and Giarman, 1970). Subsequent gas chromatography-mass spectrometry (GC-MS) assays were developed specifically for measuring GHB in human plasma and urine. This technique permitted the measurement of plasma and urine concentrations as low as 0.2 and 0.1 mg/L, respectively, but also required conversion of GHB to GBL (Ferrara et al., 1993). GC-MS assays allowing direct measurement of GHB in urine and blood with detection limits of 0.5-2 mg/L without GBL conversion have been developed (Elian, 2001; LeBeau, Miller and Levine, 2001; Villain et al., 2003). A high-performance liquid chromatography assay also has the advantage of being able to measure both GHB and GBL (Ciolino et al., 2001; de Vriendt et al., 2001). This may be useful for measuring drugs in the overdose setting when GBL has been ingested and partially metabolized to GHB, resulting in both drugs being present. Cartigny et al. (2001) and Imbenotte et al. (2003) have applied nuclear magnetic resonance (NMR) spectroscopy to identify and quantitate GHB directly in serum and urine.

Clinical Management of GHB Intoxication

Management of acute GHB intoxication focuses on alleviating symptoms and providing support. Airway management, mechanical ventilation, prevention of aspiration, and measures to counter bradycardia are commonly required. Extubation following 2-6 hours for mechanical ventilation is common. Due to the rapid absorption of GHB, gastric lavage or activated charcoal may be ineffective. There are no widely accepted antidotes to GHB, although physostigmine, a cholinesterase inhibitor, has shown some promise as a reversal agent. The opiate and GABA antagonists, Narcan and Flumazenil, are ineffective. Spontaneous recovery from GHB overdose is common, usually within a few hours (Kerrigan, 2001b).

In the rare human inborn error metabolism known as Succinic Semialdehyde dehydrogenase (SSADH) deficiency or γ-hydroxybutyric aciduria,

GABA is primarily catabolized to GHB. In essence, these children have chronic GHB poisoning with very elevated blood, urine and cerebrospinal fluid GHB concentrations as well as clinical resembling acute GHB intoxication (hypotonia, ataxia, seizures) (Hogema et al., 2001).

In an effort to explore potential pre-clinical therapeutic modalities for γ-hydroxybutyric aciduria, the mice that lack the gene encoding SSADH (SSADH $^{-/-}$) were developed. This mutant animal is characterized by inordinately elevated concentrations of brain GHB and GABA, which make it a potential model of chronic GHB abuse. During a critical period from postnatal days 16 to 22 SSADH$^{-/-}$ mice exhibit ataxia and develop generalized tonic-clonic seizures that lead to rapid death. Therapeutic intervention with the $GABA_B$ receptor antagonist CGP-35348 increased survival to 36.4 % of animals. However, treatment with the GHB receptor antagonist NCS-382 was much more effective, resulting in a survival rate of 61.5% (Gupta et al., 2002). These data suggest that both the $GABA_B$ receptor and GHB receptor are involved in the pathogenesis of CNS manifestations of GHB ingestion.

$GABA_B$ and GHB Receptor Antagonists

CGP-35348, a Selective $GABA_B$ Receptor Antagonist

The theory of using CGP-35348 to block GHB interaction with the $GABA_B$ receptor is supported by prior observations of CGP-35348's electrophysiological and pharmacological profiles in animals. Olpe et al. (1990) showed in the receptor binding assays that CGP-35348 is a centrally active and selective $GABA_B$ receptor antagonist with a median inhibitory concentration (IC_{50}) of 34 μM. They demonstrated that CGP-35348 was effective in antagonizing membrane hyperpolarization induced by baclofen (a $GABA_B$ receptor agonist) in the rat spinal cord and hippocampus.

Respiratory depression and apnea are the principal life-threatening effect of GHB overdose. Delpierre and Jammes (1997) theorized that hypoventilation by resistive load breathing can result from inhibition of the central respiratory drive by stimulation of $GABA_B$ receptors. If valid, ventilatory depression should be inhibited or decreased by $GABA_B$ receptor blockade. To test this assumption, the ventilatory effects of a $GABA_B$ receptor antagonist (CGP-35348) were evaluated in two groups of urethane anesthesized rabbits, breathing either through an inspiratory

resistive load (IRL group) or not (control group). CGP-35348 did not modify baseline ventilation in the control group. On the other hand, it partially reversed IRL-induced hypoventilation through a higher respiratory rate and central inspiratory drive. These data suggest that hypoventilation resulting from an acute increase in the work of breathing could be improved by $GABA_B$ receptor antagonism with CGP-35348.

NCS-382, a Selective GHB Receptor Antagonist

In addition to $GABA_B$ receptors, high affinity GHB-specific receptors have been identified (Tunnicliff, 1997). Maitre et al. (1990) demonstrated the antagonism effect of NCS-382 at the GHB receptor with an IC_{50} value of 130-200 nM. GHB receptor blockade may be a potential mechanism for inhibiting GHB toxicity. In particular GHB has been extensively demonstrated in animals to induce EEG and behavioral changes that resemble absence seizures (Maitre et al., 1990). They demonstrated that the numerous epileptiform spikes produced by GHB in Wistar rats were completely abolished by pretreatment with NCS-382 (2.3 mmol/kg, i.p.).

In addition to seizures, GHB is well known to induce sedation, sleep, and catalepsy following administration in animals. NCS-382 has been used by Schmidt et al. (1991) to inhibit these GHB manifestations in laboratory animals. They discovered that NCS-382 (0.83-2.50 nmol/kg) diminished the sedative, anesthetic, and cataleptic effects of GHB in a dose-dependent manner with respect to the grasping test, swimming test, chimney test, and cork test.

GHB Addiction and Withdrawal

The recent studies reported addictive properties of GHB in humans and GHB withdrawal symptoms (Craig et al., 2000; Galloway et al., 2000). The clinical presentation of GHB withdrawal ranges from mild clinical anxiety, agitation, tremors, and insomnia to profound disorientation, increasing paranoid with auditory and visual hallucination, tachycardia, elevated blood pressure, and extraocular motor impairment (Craig et al., 2000). Studies in rodents are in corcordance with the clinical data. After repeated treatment with GHB for 14 days, mice developed tolerance to the hypolocomotion effects of the drug (Itzhak and Ali, 2002). Fattore et al. (2000) and Martellotta et al. (1997) have evaluated that GHB possesses rewarding properties by means of conditioned place preference and intravenous self-administration.

GHB and Dopamine

Many experiments have firmly established that GHB perturbs: (1) firing rates of dopaminergic neurons, (2) dopamine release, (3) dopamine synthesis, and (4) levels of dopamine and its major metabolites. However, depending on such factors as GHB doses and routes of administration, animal species, brain latencies and techniques of measure, and types of anesthesia, GHB appears to either inhibit or stimulate dopamine functions (Feigenbaum and Howard, 1996, Howard and Feigenbaum, 1997). Most studies have shown that administration of high doses of GHB to unanesthetized rats causes a selective and reversible inhibition of the firing rate of dopaminergic cells in the nigrostriatal and mesolimbic pathways, which leads directly to a reduced basal release of dopamine. Consequently, dopamine concentration in the synaptic cleft dropped, resulting in a loss of activation of presynaptic dopamine autoreceptors. Due to this reduction of negative feedback, intraneuronal synthesis and accumulation of dopamine increase (Feigenbaum and Howard, 1996). In contrast, several authors have reported an enhancement of dopamine release following administration of GHB. Some studies indicate that a time-dependent stimulation of dopamine release ensues the initial inhibition, or that low and high doses of GHB have opposite effects. Other investigations show that dopamine release is inhibited in awake animals, but stimulated in anesthetized animals or is reduced in some brain structures but enhanced in others (Feigenbaum and Howard, 1996; Maitre, 1997).

GHB and GABA Neurotransmission

At low doses, systemically administered GHB produces pharmacological responses specifically mediated by GHB receptors and blocked by GHB receptor antagonist. However, most of the behavioral and biochemical responses induced by GHB occur only when brain levels increase more than 50-200 fold their endogenous values. At these high doses, GHB specific binding sites are probably saturated, and GHB likely interacts with other systems. GHB at about 5-10 mg/kg i.p. increased and at high doses (160-320) mg/kg i.p. decreased the spontaneous firing rate of prefrontal cortex neurons recorded in urethane-anesthetized rats. The selective GHB receptor antagonist NCS-382 blocked the effects of low doses, but left those of high doses unchanged (Godbout et al., 1995). Numerous data also suggest that GHB interacts with $GABA_B$ receptors. The biochemical, EEG and behavioral effects of

high doses of GHB are reproduced or potentiated by $GABA_B$ receptor agonists such as baclofen, and reversed by $GABA_B$ receptor antagonists such as CGP-35348 or CGP-36742 (Bernasconi et al, 1999). *In vivo* reports suggest that GHB can also modulate $GABA_A$ responses: the $GABA_A$ receptor agonists muscimol exacerbated GHB-induced absence seizures, whereas the antagonist flumazenil, attenuated the anxiolytic effect of GHB (Schmidt-Mutter et al, 1998). The fact that high doses of GHB mimic GABA-mediated responses could be explained by: (1) GHB mediated control of GABA release, (2) the re-transformation of GHB into GABA or (3) GHB is a structural analogue of GABA that could bind to GABA receptors (Mathivet et al., 1997).

Tetrahydrofuran (THF)

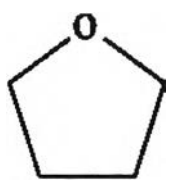

FIGURE 2-2. The Chemical Structure of Tetrahydrofuran.

Chemical and Physical Properties

Tetrahydrofuran (THF, CAS registry number: 109-99-9; C_4H_8O) (Figure 2-2) is an organic solvent belonging to a group of cyclic ethers. Synonyms of tetrahydrofuran are 1,4-epoxybutane; diethylene oxide; tetramethylene oxide; oxolan. THF is a colorless, volatile, highly flammable liquid at room temperature with a characteristic ether-like odor and a pungent taste. THF is miscible with water, alcohols, ketones, esters, ethers, and hydrocarbons. It has a melting point of -108.5°C, and a boiling point of 66°C. In contact with molecular oxygen, THF may undergo auto-oxidation to explosive hydroperoxides. The formation of peroxides can be prevented by the addition of radical inhibitors or storage under an inert atmosphere (National Occupational Health and Safety Commission, 2003).

Uses

THF is used as a reaction medium in Grignard and metal hydride reactions; in the synthesis of butyrolactone, succinic acid, and 1,4-butanediol diacetate; in the fabrication of articles for packaging, transporting, and storing of foods; as a solvent for dyes and lacquers; and as a chemical intermediate in polymerization solvent for fat oils, unvulcanized rubber, resins, and plastics. THF is also an indirect food additive when it is in the contact surface of materials intended for use in food processing (National Toxicology Program, 1998).

Pharmacokinetics

THF is readily absorbed through the lungs and the skin. THF blood levels in laboratory animals reach their highest values about 1 hour after the oral administration, and has a half-life of about 5 hours. Ratios of tissue levels to blood are 1.5-2 in the adipose tissue and kidney, and fairly equal in the brain, liver, spleen, and muscle (Hara, Nagata, and Kimura, 1987). No published experiments concerning the metabolism of THF exist (Moody, 1991).

Fujita and Suzuoki (1973) conducted a serie of experiments on the tetrahydrofurfuryl mercaptan moiety which demonstrated the saturated furan ring can be cleaved by oxidative metabolism. Kawalek and Andrews (1980) showed that incubation of THF with liver 9,000 x g supernatant from rats pretreated with Aroclor or Phenobarbital resulted in formaldehyde generation, a common product of mixed function oxidase reactions.

General Toxicity

Reports of animal studies document irritation of the skin and mucous membranes, including the eyes, nose and upper respiratory tract, as the predominant effect from lower exposures (about 100-200 ppm). High acute doses (about 25,000 ppm) produced anesthesia with delayed induction and recovery periods, accompanied by a fall in blood pressure and strong respiratory stimulation. The margin of safety between anesthesia and death is small. Other effects recorded are those of damage to liver, kidney and lung after prolonged exposures to levels of THF>1,000 ppm. Toxic manifestations varied somewhat with the route of exposure with irritation of upper respiratory tract observed with inhaled THF and inflammation of the gastrointestinal tract following oral ingestion (Hazardous Substances Databank, 2002).

Acute and Subchronic Toxicity

Malley et al. (2001) performed acute and subchronic neurotoxicological evaluations of THF by inhalation in rats. Evaluations included clinical observations, motor activity assessments, and a battery of functional test designed to reveal nervous system dysfunction. Acute exposure concentrations were 0, 500, 2500, or 5,000 ppm for 6 hours. During exposure to 2,500 and 5,000 ppm, rats had a diminished or absent startle response to a punctuate auditory alerting stimulus. Following exposure to 5,000 ppm, male and female rats were lethargic, exhibited abnormal gait or mobility, and splayed rear feet. Lethargy and splayed rear feet were also observed in female exposed to 2,500 ppm. Males exposed to 5,000 ppm had a lower incidence of palpebral closure, higher incidences of slow or absent righting reflex, and a biphasic pattern of reduced motor activity followed by increased motor activity. Females exposed to 5,000 ppm had increased incidences of palpebral closure in the open-field, increased incidences of slow or absent righting reflex, and decreased motor activity. During the 114-week subchronic exposure series, daily THF exposure concentrations were 0, 500, 1500, or 3,000 ppm, and neurobehavioral evaluations occurred on non-exposure days at approximately monthly intervals. Diminished startle responses to an auditory alerting stimulus were observed during exposure to 1500 or 3000 ppm; however, repeated exposure did not cause additional neurobehavioral or pathological effects. This pattern of effects is suggestive of transient sedation.

Chhabra et al. (1990) studied the subchronic toxicity of THF vapors in rats and mice. Groups of 10 rats and mice of each sex were administered THF vapor by whole body inhalation for 13 weeks at exposure concentrations of 0, 66, 200, 600, 1800, and 5,000 ppm. They showed that THF causes narcosis in rats and mice. Although minimal exposure related effects were seen in the liver of both species, morphologic changes were present only in mice. The body weight and survival were not affected by THF exposure, except in male mice at 5,000 ppm concentration which had reduced weight and three death. Rats of both sexes exposed to 5,000 ppm were ataxic and mice exposed to 1,800 or 5,000 ppm appeared to be in a state of narcosis. At 5,000 ppm, decreased in thymic and spleen weights in rats and mice of both sexes and increased in liver weights in both sexes of mice and in female rats were observed. A minimal to mild centrilobular hepatocytomegaly occurred in male and female mice exposed to 5,000 ppm THF. Atrophy of the uterus and degeneration of the X zone in

the adrenal cortex occurred in female mice exposed to 5,000 ppm THF. THF exposure of rats was associated with minimal to mild acanthosis and inflammation in the fore-stomach.

Moody (1991) summarized the literature concerning the lethality of THF that most of the studies employed rats and mice, with only slight species differences. In the comparative studies, rats were consistently slightly less susceptible to the lethal effects of THF, and in one of these studies, guinea pigs were equivalent to mice. These provided estimates for acute LD_{50} of 2-3 g/kg and 20-70 mg/L for oral and inhalation routes of exposure, respectively. Data pertaining to the toxicity of THF in humans is quite limited. The probable oral lethal dose in human is 50-500 mg/kg. Severe occipital headaches were reported in the testing for pharmacological properties of THF and among technicians performing animal experiments (Hazardous Substances Databank, 2002).

Genetic Toxicity

No induction of mutation was observed in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 treated with THF, with or without liver S9 metabolic activation enzymes (National toxicology program, 1998).

Carcinogenicity

Chhabra et al. (1998) carried out carcinogenesis studies of THF in groups of 50 male and 50 female F344/N rats and B6C3F1 mice. This study showed that there was some evidence of carcinogenic activity of THF in male F344/N rats due to increased incidences of adenoma or carcinoma (combined) of the kidney at the 600 and 1,800 ppm exposure levels. There was clear evidence of carcinogenic activity in female $B6C3F_1$ mice based on increased incidences of hepatocellular neoplasms at the 1,800 ppm exposure level. THF was not carcinogenic in female rats or male mice exposed at 200, 600, or 1,800 ppm.

Effect of THF on Drug-Metabolizing Enzymes

The inhibitory effect of THF on xenobiotic metabolizing enzymes was examined in *in vitro* systems. Ullrich, Weber, and Wollenberg. (1975) first demonstrated that THF inhibited specifically the O-dealkylation of 7-ethoxycoumarin by liver microsomal cytochrome P450 from Sprague-Dawley rats pretreated with

ethanol at a concentration of 10^{-2} M, THF blocked 80% of this enzyme activity. THF produced a pronounced ligand-type optical difference spectrum in microsomes from ethanol-pretreated female rats, and these microsomes also showed evidence of a new low-spin cytochrome P450 species in the presence of THF. Microsomes from female controls were significantly more sensitive to inhibition of O-dealkylation than those from males, providing evidence that well-known gender differences of drug metabolism in rats may be due to differences in the pattern of microsomal cytochrome P450 species in males and females. Draper, Madan, and Parkinson (1997) reported THF inhibited coumarin 7-hydroxylase (CYP2A6) activity in human liver microsomes. Elovaara, Pfaffli, and Savolainen. (1984) conducted an inhalation study in rats to determine THF effect on activities of drug metabolizing enzymes. Adult male rats were exposed to THF vapor at 200, 1000, or 2,000 ppm, 6 hours per day, 5 days per week, for 2 to 18 weeks. THF enhanced 7-ethoxycoumarin O-deethylase activity in liver and kidneys microsomal preparations. The exposure also caused inhibition of alcohol and formaldehyde dehydrogenase activities in liver at the highest dose. Muscle acetylcholine esterase activity was also increased showing possible effects on the myoneural junction.

Human Exposures

Human exposure to THF is primarily from occupational exposure relating to its use as a solvent for resins, adhesives, printers' ink, and coatings. Approximately 90,000 workers in 3,000 plants are exposed from industries related to chemical and allied products and electric, gas and sanitary services. Electricians, agricultural and biological technicians, electric power lineman, and cable lineman are potentially exposed to THF. When used as a solvent, significant amounts of THF may potentially be released into the environment and cause worker exposure (Hazardous Substances Databank, 2002). Garnier et al. (1989) reported chronic occupational exposure to THF can cause nausea, headache, blurred vision, dizziness, dyspnea, epigastric pain, and increase in liver enzyme activity. Ong et al. (1991) studied the biological monitoring of 58 workers in a videotape manufacturing plant by analysis of environmental air, blood, alveolar air, urine and blood. After exposure to a concentration of 200 mL/m^3 (590 mg/m^3), the extrapolated concentration of THF was 2.38 mg/L (33 μmol/L) in blood and 8.07 mg/L (111.9 μmol/L) at a specific

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จุฬาลงกรณ์มหาวิทยาลัย

gravity of 1.018 in urine. The correlation between exposure to THF and its concentration in exhaled breath and blood was low (r = 0.61 and 0.68 respectively). Urinary THF concentration corrected for specific gravity correlated well with THF concentration in air (r = 0.88), and uncorrected urinary THF concentration gave a similar result (r = 0.86). The data show that unmetabolized THF in urine correlates better with environmental exposure than THF in blood or exhaled breath. Laboratory methodological considerations are also in favor of determining urinary THF as it is non-invasive; however, THF was not detected in the urine collected the next morning (15 hours post-exposure). This suggests that either the excretion of THF is rapid or it is converted to other compound(s). Cases of acute exposure to high concentrations of THF are extremely rare. An acute THF poisoning case described deep, hypotonous and unreactive coma, with bilateral mydriasis, and respiratory depression (Cartigny et al., 2001). In Thailand, there was a woman who died and seven others became seriously ill from drinking La Sante wine in a birthday party in Pattaya. The Medical Science Department and the Food and Drug Administration (FDA) tested the drink, the blood and urine of the victims and found three chemical substances: acetonitrile, or methyl cyanide, an industrial solvent that irritate the skin and respiratory system and can cause death by suffocation; tetrahydrofuran, a potentially lethal anesthetic; and butyrolactone, a solvent used in printer's ink that can cause heart failure (Nation multimedia group, 2003), and is also a precursor for narcotic drug production (Kaopatumtip, 2003). Currently, there are no antidotes to treat THF toxicity.

Evidences for the Biotransformation of THF to GHB

Cartigny et al. (2001) and Imbenotte et al. (2003) reported the investigation by ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy of biological fluids in a case of intentional poisoning with THF. THF concentrations were 813 and 850 mg/L in serum and urine, respectively. The presence of GHB was detected and GHB concentrations were 239 and 2,977 mg/L in serum and urine, respectively. The molecular concentration ratio of GHB to THF was 0.20 in serum and 2.44 in urine, which could indicate that GHB could have been produced from THF and a metabolic link between the two compounds may exist. This metabolic reaction needs an oxidative metabolism leading to the formation of an acidic compound, lactic acid.

Bernhardt and Diekmann (1991) performed studies to elucidate the pathway for THF metabolic degradation by *Rhodococcus ruber,* a bacterial strain.

Rhodococcus ruber can hydroxylate THF at the carbon-2-position, resulting in 2-hydroxy tetrahydrofuran, which either isomerizes to 4-hydroxybutyraldehyde or can be oxidized to γ-butyrolactone; both derivatives can then lead to GHB. In fact, the isomerization of 2-hydroxytetrahydrofuran might occur spontaneously and not require an enzyme (Bjerke, 2002).

Kohlweyer et al. (2000) and Thiemer, Andreesen, and Schrader (2001, 2003), described the isolation and characterization of an organism, *Pseudonocardia sp.* strain K1., degraded THF; cloned and characterized a gene cluster involved in THF degradation. The activity of the initial oxidation of THF seems to be rather labile; however the NADH-dependent cytochrome c reductase activity in crude extracts from THF was detected. A preliminary characterization of this reductase induced during growth on THF indicated that it might be a multicomponent monooxygenase. Spectroscopic studies indicated that it contains an iron-sulfur cluster and a flavin cofactor. A gene cluster involved in the utilization of THF by *Pseudonocardia sp.* strain K1 was cloned and sequenced. The genes designated as *thmADBC*. The only reaction within the proposed THF degradation pathway that should be catalyzed by a monooxygenase is the initial hydroxylation of THF to 2-hydroxy-tetrahydrofuran. Two alcohol dehydrogenases oxidize 2-hydroxytetrahydrofuran to γ-butyrolactone and 4-hydroxybutyrate (GHB) to succinate semialdehyde (Thiemer et al., 2001).

Other proposed pathways of THF metabolic degradation concerned the metabolic activation of Ftorafur or 1-(tetrahydro-2-furanyl)-5-fluorouracil, an anticancer agent presents the tetrahydrofuran moiety. Au and Sadee (1980) and El Sayed and Sadee (1982, 1983) demonstrated the existence of 2 separate metabolic pathways of Ftorafur (FT) activation to 5-fluorouracil (FUra), one that is mediated by hepatic microsomal enzymes and one that occurs in the soluble enzyme fraction of the 100,000 x g supernatant. The metabolite GBL or GHB can only be observed in the 10,000 x g supernatant and 100,000 x g supernatants but not in the microsomal pellet preparation. The formation of GBL or GHB through the action of soluble enzymes can occur by any of the 3 pathways described which leads to chemically labile intermediates that spontaneously cleave to FUra and to GBL or GHB (C-2′ oxidation) or to succinaldehyde (C-5′ oxidation). Another potential pathway of Ftorafur activation involves C-2′ hydrolytic cleavage which produces FUra and 4-

hydroxybutanal. Furthermore, succinaldehyde and 4-hydroxybutanal can undergo enzymatic conversion to GBL or GHB. Three possible activation pathways of Ftorafur is shown in Figure 2-3.

The formation of GBL or GHB from succinaldehyde in the soluble enzyme preparation could occur by either one of the 2 reaction sequences described in Figure 2-3 : (a) reversible reduction to 4-hydroxybutanal followed by oxidation; or (b) oxidation to succinic semialdehyde followed by reversible reduction to GBL or GHB.

The formation of 5-fluorouracil from Ftorafur in the microsomal pellet was thought to be mediated by cytochrome P450 requiring NADPH and O_2. The hypothetical product of microsomal oxidation at the C-5′ position of Ftorafur is 5′ hydroxyftorafur, which was expected to be chemically unstable and to cleave spontaneously to 5-fluorouracil and succinaldehyde (Figure 2-4) (El Sayed and Sadee, 1982). It has been suggested that hepatic microsomal cytochrome P450 plays a role in Ftorafur activation to 5-fluorouracil. SKF 525-A prevented the formation of succinaldehyde in microsomal incubations. In addition, incubation of Ftorafur in the absence of NADPH did not yield succinaldehyde. These results provided strong evidence that microsomal cytochrome P450 was responsible for activation of Ftorafur to 5-fluorouracil and succinaldehyde according to the scheme shown in Figure 2-4.

El Sayed and Sadee (1983) observed higher level of GBL/GHB in 10,000 x g supernatant incubations of Ftorafur than in 100,000 x g supernatant (soluble enzymes) incubations. It is therefore possible that succinaldehyde generated from microsomal enzymes in the 10,000 x g medium is further metabolized to GBL/GHB by soluble enzymes that are also present. To test this hypothesis, they incubated succinaldehyde with 10,000 g and 100,000 g supernatant fractions, approximately 50-60% of the added succinaldehyde was converted to GBL/GHB within 1 h. These results demonstrate that GBL/GHB, which is detectable in plasma after Ftorafur administration to animals and patients, is generated via two separate pathways, i.e. the soluble enzyme pathway of yet unknown mechanism and the microsomal pathway.

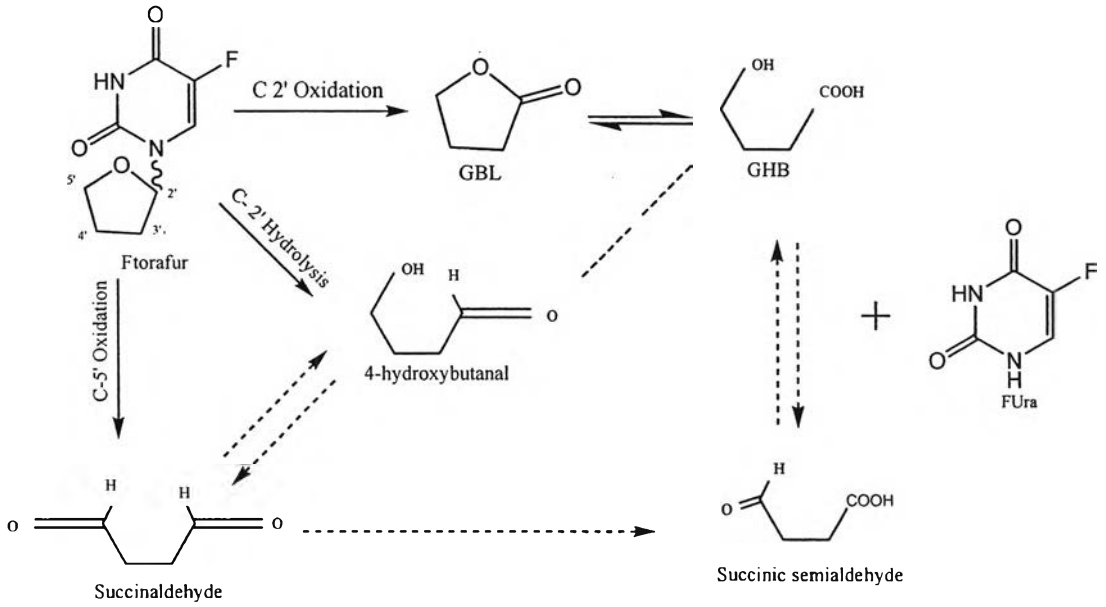

FIGURE 2-3 Three Possible Activation Pathways of Ftorafur (FT) that Lead to 5-Fluorouracil (FUra) and the Expected Products of the Tetrahydrofuran Moiety. Potential interconversion among these products is also indicated (El Sayed and Sadee, 1982).

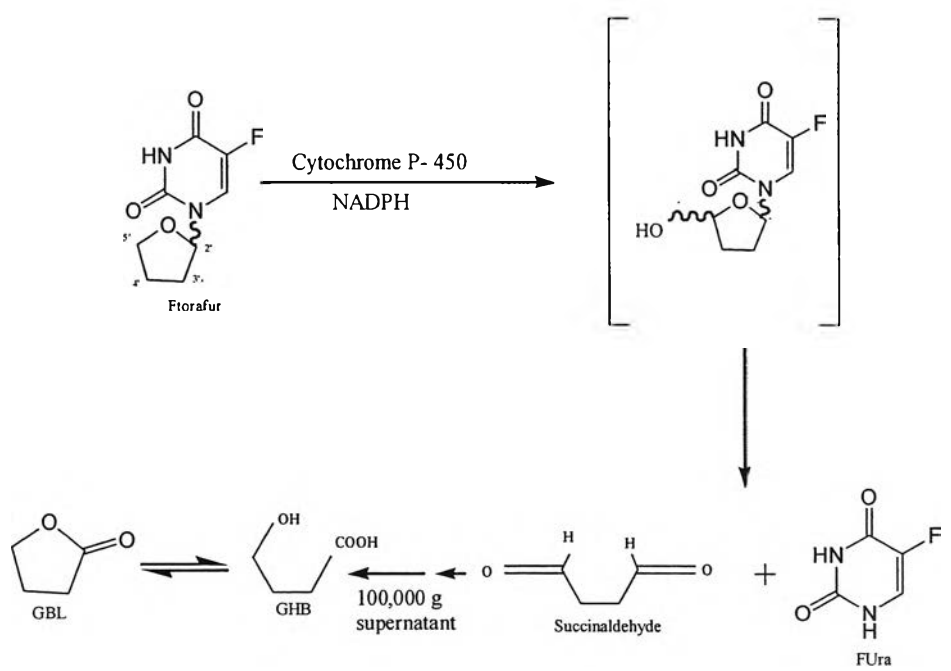

FIGURE 2-4 Proposed Activation Pathway of Ftorafur to 5-Fluorouracil (FUra) by the Microsomal Enzymes and the Major Metabolite of Succinaldehyde (El Sayed and Sadee, 1982)

The last proposed pathway of THF metabolic degradation was supported by Fujita and Suzuoki (1973)'s work. They reported the enzymatic studies on the metabolism of the tetrahydrofurfuryl mercaptan moiety of thiamine tetrahydrofurfuryl disulfide and stated the oxidative cleavage of the tetrahydrofuran. The tetrahydrofuran ring was first hydroxylated by liver microsomes in the presence of NADPH and O_2. They used [^{35}S] methyl tetrahydrofurfuryl sulfone as a substrate with rat liver homogenates and identified the reaction product as methyl 5-hydroxytetrahydrofurfuryl sulfone by infrared, nuclear magnetic resonance and mass spectra. Methyl 5-hydroxytetrahydrofurfuryl sulfone thus formed was further oxidized to 4-hydroxy-5-methylsulfonylvaleric acid by incubation with the hepatic cytosol. In the second reaction microsomes were no longer necessary. These results indicated that the tetrahydrofuran ring was first hydroxylated at the 5-position by microsomes and further cleaved to the straight chain fatty acid via the intermediate formation of either an aldehyde form or a γ–lactone in the presence of cytosol.

Evidence for the Biotransformation of GBL to GHB

The depressant activity of GBL has been attributed to its rapid *in vivo* hydrolysis to GHB by specific lactonases or by non-specific enzymatic alkaline hydrolysis (Fishbein and Bessman, 1966; Lettieri and Fung, 1978; Roth and Giarman, 1966), and therefore, GBL can be classified as a prodrug of GHB. The role of endogenous GBL is more obscure as lactonase activity has been described in the periphery but not in the brain cell (Fishbein and Bessaman, 1966). However equilibrium between GBL and GHB in brain could be the result of a chemical rather than an enzymatic process. The inter-conversion of GBL to GHB in aqueous solutions is pH dependent with solutions at pH 7 or above favoring the formation of GHB from GBL, with true equilibrium established at pH 2 (Ciolino et al., 2001). Guidotti and Ballotti (1970) confirmed the results of Giarman and Roth (1964) which suggested that GHB was the active substance as a sedative after GBL administration. They showed a good correlation between blood and brain GHB concentrations, and the loss of righting reflex after GBL administration, while similar correlation was not obtained with GBL levels. There was no modification in animal behavior, 3 min after oral or i.p. administration of GBL, when the cerebral levels of GBL were 4 or 20 times higher than GHB. On the contrary, CNS depression became evident 15 min after i.p.

and 30 min after oral administration of GBL or GHB when the GHB cerebral levels were 3 to 20 times greater than GBL concentration. Further, Roth and Giarman (1966) found that GBL when injected endocisternally or in given areas of the brain produced no central effects in the monkey and rabbit respectively while CNS effects appeared after injection of GHB. Bourguignon, Schmitt, and Didier (2000) demonstrated in an *in vitro* assay that unlike GHB, GBL did not displace [^{3}H]GHB from GHB binding sites suggesting that an open form is necessary for recognition at the receptor site.

Guidotti and Ballotti (1970) further demonstrated that GBL given orally, i.p. or i.v. is capable of producing higher concentrations of GHB in brain and blood than an equimolar amount of GHB administered by the same route. As compared to GHB that shows capacity limited transport *in vitro* (Arena and Fung, 1980) and slower absorption *in vivo* with increasing doses (Lettieri and Fung, 1979), GBL shows a higher intestinal flux *in vitro* and rapid *in vivo* absorption. This may account for GBL's increased oral hypnotic activity over GHB. The different speed with which the GHB and the lactone traverse the gastric and blood-brain barrier was explained by the fact that GHB penetrates the cells as a weak electrolyte (pK_a= 4.73), while GBL, an uncharged cyclic molecule with a low molecular weight may easily cross the cell membrane through the pores. Also, they found that the blood concentrations of GHB fell more rapidly after GHB administration than GBL. This, they thought may be because the lactone could be taken up from the blood into lean body mass more rapidly and efficiently than GHB. This depot pool of drug appears to be metabolized more slowly because it is not readily accessible to enzyme involved in biotranformation and its slow release back into the blood is capable of maintaining blood levels of drug at higher plateau. Snead (1991) validated the concept of using GBL in GHB model of generalized absence seizures as it was inactive by itself but provided rapid onset of bilaterally synchronous spikes and wave discharges in the rat, which correlated with the rapid appearance of GHB in the brain in the GBL treated animals. These experiments suggest that the pharmacologically active form of GBL is GHB and that it is longer acting and more potent than GHB.

Since THF is believed to act as a GHB/GBL precursor and no published experiments concerning the neuropharmacological properties of THF exist, these studies were therefore designed to investigate the central nervous system effects of THF in comparison to GBL utilizing a number of neurobehavioral tests including righting reflex test, rotarod test, locomotor activity test, open-field test, elevated plus

maze test, Y-maze test, Morris water maze test, open-space swimming test, tolerance test, and conditioned place preference test. The involvement of GABA and GHB receptors in the neuropharmacological effects of THF was clarified using specific receptor antagonists including picrotoxin ($GABA_A$ receptor antagonist), flumazenil ($GABA_A$ receptor antagonist), CGP-35348 ($GABA_B$ receptor antagonist), and NCS- 382 (GHB receptor antagonist).