Influence of Early Postmortem Interval on the Detection of Hepatic Diazepam Concentrations in Male Albino Rats

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Abstract

Background: Background: The increasing misuse of benzodiazepines (BZDs) in drug-facilitated crimes (DFCs) has become a serious concern for forensic experts, healthcare professionals, and legal authorities. These drugs, which are commonly prescribed for anxiety and sleep disorders, are also used to commit crimes such as sexual assault and robbery. Their sedative and memory-blocking effects render them particularly dangerous. **So, the aim of the present work** is to study the effect of postmortem interval on diazepam concentration in liver tissue after acute oral lethal dose administration in male albino rats, using Gas Chromatography- Mass Spectrometry (GC-MS). **The study was conducted on** 56 adult male albino rats divided randomly to two groups 28 rats for each and diazepam group is further subdivided into 4 subgroups (a, b, c, and d) 7 animals for each subgroup. Group I: control group received 1ml distilled water. Group II: rats received lethal dose of diazepam. After death of diazepam animals, control animals were sacrificed at the same time and immediate dissection was done. The livers were extracted for detection of diazepam. The control group showed no considerable change in liver diazepam. As regards liver tissue diazepam of group II, a non-significant elevation was detected in the subgroups II-b, II-c and II-d as compared to the subgroup II-a.

Keywords: postmortem interval, forensic toxicology, diazepam, GC-MS.

Introduction

Estimation of the Postmortem Interval

The postmortem interval (PMI), the time passed since death is the period between the death of a person and the analysis of his body after death **(1).** In a forensic investigation, the precise time since death determination is a critical and challenging issue **(2).**

The time since death estimation is one of the main tasks of the forensic pathologists **(3).**

The PMI has important implications that affect both civil and criminal matters. For example, it plays a fundamental role in homicide cases where a miscalculation can have significant negative effects on the outcome of an investigation **(4).** PMI is a challenge of utmost importance in the daily criminal practice. Traditional methods face limitations in accuracy and reliability, especially for the advanced stages of decomposition **(5).**

In forensic practice, PMI analysis belongs to a specific branch called mortuary science, a specialty concerned with the analysis of the macroscopic and microscopic changes of the body after death**(6).**

From the point of view of criminal law, precise time of death calculation (postmortem clocking) aids in setting the murder time, and verifying witnesses' statements. It is also of crucial importance for forensic investigators, particularly when they are gathering evidence that can support or deny the stated actions

of suspects in crime **(7).**

As death progresses, a series of early changes occur in the body that results in a marked change in the physical nature and/or appearance of the body before the obvious, recognizable decompositional changes become apparent. These changes have traditionally been used in PMI estimates and can be a source of confusion if not recognized **(8).**

Throughout the history of forensic medicine, PMI has been one of the most common and sensitive investigative problems. The importance of accurate PMI in the context of a medico-legal death investigation cannot be understated given its utility and application to investigative determinations including the inclusion or exclusion of suspects, determination of time of assault versus time of death, and identification of the primary victim. However, despite its importance, the question of estimating the time interval after death is often answered with a low degree of accuracy compared to certainty rates in other forensic disciplines **(9).**

The postmortem period is determined in different ways depending on the condition of the body and the circumstances in which it was found **(10).**

Several researchers have proposed classifying PMI into different stages based on time, particularly referring to early and late stages. In particular, some authors have described the early postmortem period (ePMI) as that occurring within 24–36 hours after death, in which there are supravital reactions characterized by metabolic processes that determine tissue reactions (i.e., livor mortis, rigor mortis) (**11).**

In forensic practice, it is known that, the methods mainly used to define the ePMI are represented by the classic triad of postmortem changes. It consists of the thermometric method based on the application of the Henssge nomogram; rigor mortis evaluation, with reference to the maneuver of cadaveric rigidity reversal; and the main hypostases' findings analysis **(12).**

Traditional methods for estimating PMI rely on postmortem changes in the body, including early changes involving physical processes (e.g., cooling of the body and rigor), metabolic processes (suprabiotic reactions), autolysis (loss of selective membrane permeability, diffusion), and physical and chemical processes (rigor mortis). They produce relatively reliable estimates of time since death for the early postmortem period, typically only within the first 24 hours after physiological death **(13).**

Many methods have been proposed in the past for PMI estimation, however nowadays methods, especially for estimation of late PMI are far from being accurate **(14).**

The longer the period of time between death and post-mortem examination, the more difficult and important it becomes to estimate the time period after death, as it is an essential prerequisite for a comprehensive investigation of the case. In some cases, there may be a period of survival between the initial injury and the actual death itself. Understanding the relationship between such events and being able to clearly discern the time since death is crucial to the medico-legal investigation of death **(9).**

This necessitates a dual approach to investigative practices surrounding the estimation of PMI. First, pre- and post-mortem changes and injuries such as wound pattern analysis, infection, or other injuries must be evaluated to determine whether they have any impact on survival time in relation to time since death. Second, post-mortem changes must be evaluated to provide a preliminary estimate of time since death **(3).**

In general, postmortem changes occur in a predictable order of increasing stages of deterioration. However, there are significant differences due to a wide range of influencing factors arising from the environment and the human body itself. Changes caused by the human body itself are referred to as intrinsic factors while changes caused by the environment are considered extrinsic factors **(12).**

Due to the medico-legal restrictions on the evaluation of classic postmortem changes, methods from various fields, such as chemistry and biochemistry, histopathology and immunohistochemistry, molecular biology, and entomology, have been used to evaluate PMI. At the same time, tissues and organs, especially the heart, liver, kidneys, blood, and other body fluids, have been the subject of research **(15).**

Postmortem Forensic Toxicology

Forensic toxicology is a branch of toxicology in the service of forensic science. It focuses on explaining the results in the medico-legal context. In legal toxicology, qualitative and quantitative analyses are performed in order to explain the potential role of the compound in a case under investigation. In fact, a criminal toxicologist contributes to the creation of each of the causes and methods of poisoning or death through the analysis of the different fluids and tissues obtained from the examination of the autopsy **(16).**

Forensic toxicology is the study and practice of the application of toxicology to the purposes of the law. The field of forensic toxicology can be divided into three distinctly separate areas: post-mortem forensic toxicology, human-performance forensic toxicology, and forensic urine drug testing. The forensic toxicologist determines the absence or presence of volatiles and other drugs and chemicals in a variety of biological specimens. In post-mortem forensic toxicology, the forensic toxicologist aids in establishing the cause and manner of intoxication or death through the analysis of various fluids and tissues obtained during autopsy. In human-performance forensic toxicology, the forensic toxicologist is responsible for evaluating the role of drugs in the modification of human behavior, usually applied to traffic safety and the operation of a motor vehicle. In forensic urine drug testing, the forensic toxicologist is responsible for demonstrating prior use or abuse of selected drugs through the analysis of urine. Results from these tests are usually applied to the workplace setting **(17).**

Forensic medicine and forensic toxicology are unique among all other medical fields due to their basic legal impact, especially in civil and criminal issues **(18).**

Postmortem forensic toxicology is a crucial field within forensic science that analyzes biological samples to detect and quantify drugs, alcohol, and other toxic substances in deceased individuals, playing a vital role in determining causes of death, investigating potential homicides, and often more difficult than in other forms of forensic toxicology due to the variable and degraded identifying substance abuse trends, particularly in the context of rising substance-related fatalities driven by the increasing misuse of illicit and prescription drugs **(19).**

Apparent accidental deaths may turn out to be suicides or natural deaths occurring in circumstances that suggest an accident. The primary goal for the toxicology laboratory is to determine whether substances are present in the deceased in sufficient quantities to contribute to or cause death **(20).**

Therefore, postmortem toxicology is an important ancillary analysis informing coronial investigations which may help in the determination of both cause and manner of death. However, difficulties arise in the interpretation of postmortem drug levels. In a postmortem setting, difficulties in toxicological interpretation are further confounded by several physiological and environmental variables which may change postmortem drug levels. After death, a series of changes ensue that collectively influence the distribution of compounds in the body over time. As such, a postmortem drug concentration may not be an accurate representation of the drug concentration that was present during life **(21).**

More than 2,000 different benzodiazepines (BZD) have been synthesized. These drugs have been implicated in sudden and unexplained deaths particularly when co-ingested with ethanol. Interpretation of forensic postmortem toxicological data can be very hard and should be done with a thorough knowledge of the case history, including its autopsy results, reports from the scene, and available medical history. Experienced forensic toxicologists rely on their own case experience as well as the unique circumstances of each case under examination. Even armed with detailed toxicological data, it is still difficult to pinpoint the cause of death when multiple agents are ingested at the same time **(22).**

Drug testing is routinely performed in postmortem samples as part of an autopsy. Initially, toxicological analysis helps the coroner, medical examiner, or equivalent establish evidence of drug use. Alternatively, toxicological data may help disprove drug use as a cause of death. This is important in forensic autopsies because pathological examination alone will not reveal evidence of drug use. Drug use can only be confirmed by appropriate toxicology procedures performed on a properly collected specimen(s). Clearly, in cases of sudden or unexplained death, evidence of drug use may help

determination the cause of death, or at the very least, point to evidence suggesting drug abuse, overuse, or even suicide **(23).**

Quantitative determination of toxic, potentially toxic drugs or toxins in different body fluids and tissues in postmortem toxicology plays a major role in the interpretation process that contributes towards the determination of the cause of death **(24).**

There are great difficulties related to the interpretation of the levels of the postmortem drug. For instance, pharmacokinetic and pharmacodynamic factors, including the redistribution of the postmortem, diffusion, and site-to-site variability in drug levels, different drug properties and metabolism, bacterial activity, genetic polymorphisms, tolerance, resuscitation efforts, underlying conditions and the toxicity profile of cases (i.e. mono or mixed toxicity). Many investigative matrices have been developed as possible indicators of postmortem redistribution, but have a limited practical value. Recent research has focused on developing databases for the levels of the peripheral postmortem drug for a variety of types of cases to increase the ability to transfer to real life and improve interpretations. As such, guidelines and practices will continue to develop while enhancing our understanding of these phenomena **(25).**

Advances in analytical techniques and the interpretation of postmortem forensic toxicology results have significantly enhanced our understanding of death causes, emphasizing the importance of diverse methodologies, demographic considerations, and the pivotal role of toxicological analysis in clarifying death circumstances. This underscores need for continued research and improvement in forensic toxicology to tackle increasingly complex cases **(26) (27) (28).**

The integration of omics technologies and advanced analytical tools in forensic toxicology, including genomics, proteomics, metabolomics, and transcriptomics, is revolutionizing the field by elucidating how the body responds to drugs and poisons. When combined with high-resolution mass spectrometry (HRMS), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and nuclear magnetic resonance (NMR) spectroscopy, these tools can help differentiate between therapeutic drug use, overdose, and poisoning. Personalized interpretation through genomic analysis examines an individual's genetic makeup to determine how they metabolize drugs, enabling experts to assess whether drug levels are indicative of overdose. Proteomics and biomarker discovery identify protein markers that change in response to toxin exposure, indicating drug-related deaths and specific organ damage. Metabolomics for toxicological profiling examines the end products of cellular processes, revealing changes that suggest overdose or poisoning, even at therapeutic drug levels **(29).**

The development of sensitive and precise analytical methods has enhanced the detection and quantification of substances in biological samples, leading to more accurate determinations of cause and manner of death. The integration of advanced data analysis and artificial intelligence has also improved the interpretation of toxicological results. However, the field faces challenges such as the complexity of interpreting results in individual cases, the influence of polydrug use and environmental factors, and the rapid emergence of new synthetic drugs **(29).**

Despite the recognized importance of comprehensive toxicological screening, gaps remain in identifying novel substances and standardizing postmortem toxicological protocols **(30).**

Benzodiazepines

Benzodiazepines (BZD, BDZ, BZs) are a class of psychoactive drugs known for their depressant effect on the central nervous system (CNS). They quickly diffuse through the blood–brain barrier to affect the inhibitory neurotransmitter GABA and exert sedative effects. GABA is the most common neurotransmitter in the CNS, and BZDs primarily work on the GABA-A receptor subunit **(31).** Given their lipid solubility, BZDs have a high volume of distribution in the body, which translates to higher tissue concentrations than blood. After exerting their effect, BZDs are metabolized primarily by the liver and excreted by conjugation, so they should be used in caution in the elderly, smokers, and those with liver disease or damage **(32).**

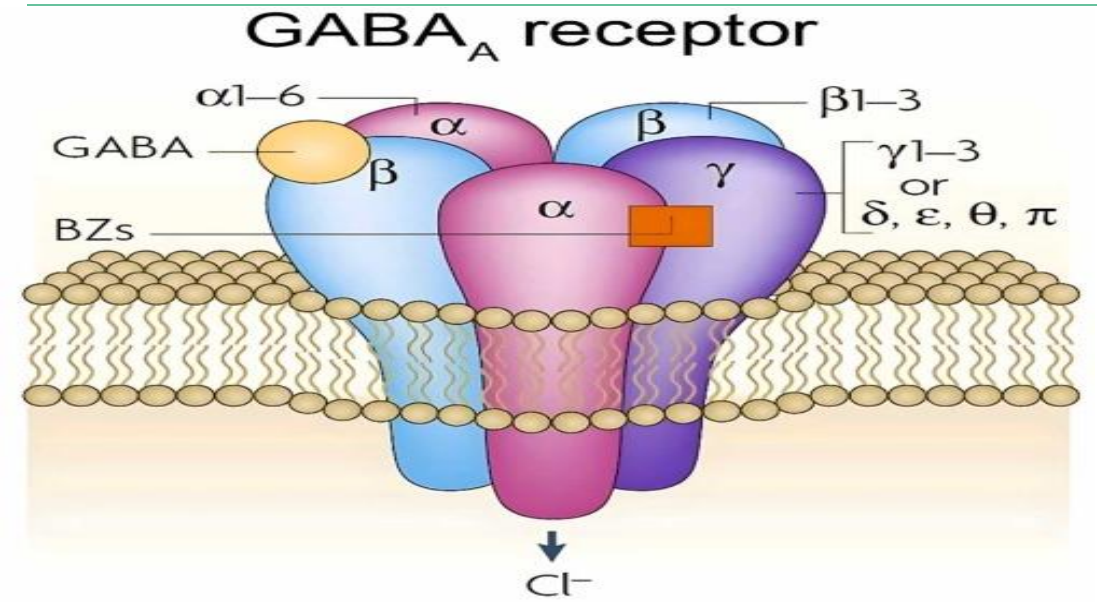

Fig. (1): Schematic diagram showing the binding sites of GABA-A receptors for GABA and benzodiazepines **(Jacob et al., 2008).(33).**

BZDs can be placed into one of three groups by its elimination half-life, or the time it takes for the body to eliminate half of the dose **(34).** Some BZDs have long-acting active metabolites, such as diazepam and chlordiazepoxide, which are metabolised into desmethyldiazepam. Short-acting compounds have few residual effects if taken before bedtime, rebound insomnia may occur upon discontinuation, and they might cause daytime withdrawal symptoms such as next day rebound anxiety with prolonged usage. Examples are brotizolam, midazolam, and triazolam. Intermediate-acting compounds have some residual effects in the first half of the day if used as a hypnotic. Rebound insomnia, however, is more common upon discontinuation of intermediate-acting BZDs than longer-acting BZDs. Examples are alprazolam, estazolam, flunitrazepam, clonazepam, lormetazepam, lorazepam, nitrazepam, and temazepam. Long-acting compounds have a risk of accumulation in the elderly and in individuals with severely impaired liver function, but they have a reduced severity of rebound effects and withdrawal. Examples are diazepam, clorazepate, chlordiazepoxide, and flurazepam **(35).**

Due to the rapid onset and immediate symptom relief of BZDs , they are used for those struggling with sleep, anxiety, spasticity due to CNS pathology, muscle relaxation, and epilepsy. Their sedative effect aids in sleep and insomnia disorders by reducing sleep onset latency. Their CNS depressant effects potently reduce anxiety and abort acute-onset panic and anxiety attacks **(36).** Benzodiazepines are also incredibly effective at rapidly aborting convulsant activity in those with epilepsy or other seizure disorders **(37).**

BZDs are most commonly used for panic disorder and generalized anxiety disorder (GAD) regarding its indications for anxiety. Specifically, temazepam is commonly used for insomnia, clonazepam is commonly used for anxiety and seizures, lorazepam is commonly used for catatonia and seizure abortion when used intramuscularly or intravascularly, and diazepam is commonly used for anxiety, muscle spasms and rectally for seizures **(38).**

BZDs have a detrimental effect, known as anterograde amnesia in the CNS, which alters memory formation and increases memory loss **(39).** Patients with cardiovascular disorders have increased prevalence of depression and anxiety. BZDs reduce the autonomic hyperactivity of cardiac cells, which helps manage coronary heart disease by suppressing hypertension and myocardial ischemia **(40).**

As a result of CNS depression by BZDs use, the muscle of the upper airway relaxes, leading to airway obstruction, which can worsen in patients with obstructive sleep apnea **(41).** BZDs can be used to treat anxiety disorders and panic attacks in patients with gastrointestinal symptoms. It reduces gastric

secretion, which will ultimately help improve the signs and symptoms of peptic ulcers and cause relaxation of smooth muscle **(42).**

Adverse events may occur if BZDs are not well tolerated, with common manifestations including CNS effects such as sedation, dizziness, confusion, and memory impairment, including anterograde amnesia; respiratory effects such as hypoventilation and, in severe cases, respiratory arrest; cardiovascular effects including hypotension and bradycardia; gastrointestinal disturbances such as nausea and constipation; and musculoskeletal effects including weakness and ataxia **(43).**

Benzodiazepines are associated with an increased risk of suicide due to aggression, impulsivity, and negative withdrawal effects **(44).**

Moreover, BZDs are often used in drug-facilitated crimes (DFC), including robberies and sexual assaults, because of their sedative effect and ability to induce amnesia. Their use can also make victims more compliant. The DFC model involves covertly administering drugs by slipping drugs into drinks/foods in order to make the unconscious victim easy prey for the perpetrator. In these cases, crime scene analysis is very important because it can help to clarify cases of sexual assault or robbery **(45).**

Diazepam

Introduction

Diazepam is a drug from the BZDs class that is a long-acting BZD with rapid onset. It is CNS depressants and produces a sedative effect by affecting the signals transmission to brain cells. Diazepam has the potency to be misused as it is highly effective, easy to obtain, and cheap. Its misuse is to replace illegal drugs such as opiates, tranquilizers, and neuroleptics. Addiction can be caused due to drug abuse of diazepam. According to the Drug Enforcement Administration (DEA), more than 20 million people abuse BZDs, including diazepam. BZDs misuse can contribute to sudden death, and more than 7,900 people die from overdoses involving diazepam each year. Drug analysis is essential for drug therapy monitoring and to determine drug abuse easily **(46).**

Diazepam is the most typical hypnotic drug in the family of BZDs. It has been widely used to treat CNS disorders such as anxiety, epilepsy, and alcohol withdrawal since the 1960s. However, many other major problems emerged, such as drug dependence and drug abuse, which finally increases the number of forensic cases, such as suicide, drug-driving, sexual assaults, and robbery **(47).**

Physico-chemical characteristics:

●Origin of the substance:

It is Synthetic in origin, Its method of synthesis is Benzoyl chloride reacts with p-chloroaniline to produce 2-amino-5-chlorobenzophenone. This is converted to the oxime with hydroxylamine. After cyclization with chloroacetyl chloride and ring enlargement with alkali treatment, 7-chloro-1,3-dihydro-5-phenyl-2H-1,4- benzodiazepin-2-one-4-oxide is reduced and methylated to diazepam **(48).**

Chemical structure

- Chemical name:

7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one. Alternative, 7-Chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2(1H)-one. Molecular formula, $C_{16}H_{13}ClN_2$. Molecular weight, 284.76 **(49).**

Main brand names, main trade names

Diazepam as the only active substance:

Diazeplex, Diazepam, Relanium, Stesolid, Valium, Others

Combination products:

Aneurol, Ansium, Calmaven, Diaceplex, Edym Sedante, Gobanal, Pacium, Pertranquil, Reladon, Tepazepam, Tropargal, Vincosedan **(49).**

physical properties:

The drug diazepam occurs as a pale yellow-white crystalline powder without a distinctive smell and slightly bitter taste. Diazepam is moderately lipophilic which means that it tends to dissolve more

readily in lipid-based environments, such as chloroform, acetone, ethanol and ether **(50).**
The pH of diazepam is neutral (i.e., pH = 7). Diazepam has a shelf life of five years for oral tablets and three years for IV/IM solutions. Diazepam is stored at room temperature (15–30 °C). The solution for parenteral injection is kept so that it is protected from light and kept from freezing. The oral forms are stored in air-tight containers and protected from light **(51).**

- Chemical properties :
The core chemical structure of BZDs is the link between a benzene ring and a diazepine ring; these drugs act as depressants by lowering the levels of neurotransmission **(52).**
Diazepam has anticonvulsant properties. BZDs act via micromolar BZDs binding sites as calcium channel blockers (CCBs) and significantly inhibit depolarization-sensitive calcium uptake in rat nerve cell preparations**(53).** Diazepam inhibits acetylcholine release in mouse hippocampal synaptosomes. This has been found by measuring sodium-dependent high-affinity choline uptake in mouse brain cells in vitro, after pretreatment of the mice with diazepam in vivo. This may play a role in explaining diazepam's anticonvulsant properties **(54).**
Diazepam binds with high affinity to glial cells in animal cell cultures, and at high doses has been found to decrease histamine turnover in mouse brain via diazepam's action at the benzodiazepine-GABA receptor complex **(55).** Diazepam also decreases prolactin release in rats **(56).**

- Pharmacokinetics :
Diazepam can be administered orally, intravenously (it is always diluted, as it is painful and damaging to veins), intramuscularly (IM), or as a suppository and recently as nasal route **(57).**

Absorption
Diazepam is absorbed rapidly following oral administration; with peak plasma concentrations generally being achieved within 1.0 hour (range 0.08 to 2.5 hours) **(58)**. Its rate of absorption is decreased by food and antacids. It is almost complete with bioavailability near 1.0 **(59).**

After oral administration, diazepam is almost completely absorbed (99 %), this is useful for the treatment of many acute cases such as anxiety, insomnia or seizures. It reaches its maximum concentration (Cmax) 30–90 minutes after total administration (Tmax). The average time to achieve peak plasma concentrations is 1 to 1.5 hours. Absorption is slowed when administered with a meal. There is a rise in the mean time to achieve peak concentrations to approximately 2.5 hours in the presence of food **(60).**

Distribution
The distribution volume has been measured to range from 0.7 to 2.6 L/kg **(59) (61).** In human volunteers, the plasma protein binding level of diazepam is greater than 95% **(65) (59).** The concentration in the CSF correlates with the plasma free fraction **(64).**
Patients with hypoalbuminemia may suffer from CNS effects secondary to an increased free fraction of diazepam. Due to its high lipid solubility, Diazepam passes rapidly into the brain, and other well perfused organs, and is afterwards redistributed to muscle and adipose tissue. Diazepam has minimal enterohepatic circulation , it crosses the placental barrier to the fetus and is present in breast milk **(60).**

Metabolism
Diazepam is mostly metabolized in the liver by the microsomal enzymes CYP2C19 and CYP3A4 enzymes to several active metabolites, mainly desmethyldiazepam (nordiazepam). Other minor active metabolites include oxazepam and temazepam. The average half-lives of oral diazepam and desmethyldiazepam are about 46 and 100 hours, respectively **(60).**
Diazepam is primarily metabolized by CYP3A4 and CYP2C19 to the major active metabolite, desmethyldiazepam. Approximately 2% of Europeans, 13% of East Asians, and as much as 57% of

Oceanians have reduced or absent CYP2C19 enzyme activity poor metabolizers. The FDA-approved drug label for diazepam gel states that "the marked inter-individual variability in the clearance of diazepam reported in the literature is probably attributable to the variability of CYP2C19". However, the most recent drug label for oral formulations (tablet and liquid) only briefly discusses CYP2C19 in the context of potential drug interactions **(66).**

It is extensively metabolized in the liver to nordiazepam (active metabolite) via CYP2C19 and CYP3A4 and to temazepam via CYP3A4. Both are hydroxylated to oxazepam by CYP3A4 and / or CYP2C19, respectively. Temazepam and oxazepam are minor active metabolites. Oxazepam is glucuronized by UDP-glucuronosyltransferase (UGT) enzymes (UGT2B15, UGT2B7 and UGT1A9) , and is excreted in the urine. Its elimination half-life (t1/2), is 24–48 h **(60).**

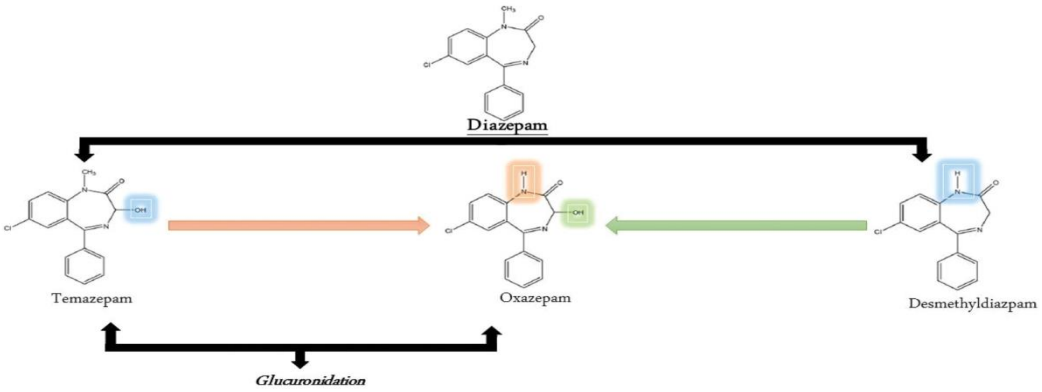

Fig. (2): The metabolic pathway of Diazepam **(67) (68)**

-Elimination

Diazepam undergoes hepatic metabolism and is eliminated from the kidney. Therefore, urine is a conventional specimen used to document diazepam-related crimes. In addition, due to the convenience and non-invasive nature of its collection, testing for diazepam abuse in urine is also required for multiple reasons, including legal and workplace policies. Studies have reported a great value in urine levels of metabolites of diazepam due to their longer detection window. More importantly, published detection methods for diazepam in urine always involve hydrolysis of the glucuronide metabolites and focus on diazepam as well as on its active metabolites nordiazepam, temazepam, and oxazepam. These methods have many limitations, including the possibility of incomplete hydrolysis due to competitive inhibition of the enzyme, reduction reactions of oxazepam or oxazepam glucuronide (OG) to nordiazepam, conversion of temazepam to diazepam during enzymatic hydrolysis, and decomposition of benzodiazepine molecules to benzophenones during acid-catalyzed hydrolysis **(47).**

Urinary excretion of diazepam is primarily in the form of sulphate and glucuronide conjugates, and accounts for the majority of the ingested dose **(59) (61) (62).** There is some evidence that the disposition of diazepam is slowed by chronic dosing and by plasma desmethyldiazepam levels **(63).** There is some evidence for species differences in biliary excretion. However, studies by **(64) (65) (63)** suggest that biliary excretion of diazepam is probably clinically unimportant in man. The initial distribution is followed by a prolonged terminal elimination($t_{1\backslash 2}$ ~ 48 hours). Additionally, the terminal elimination $t_{1\backslash 2}$ of the active metabolite N-desmethyldiazepam is up to 100 hours **(60).**

- Mechanism of action:

The pharmacological effects of diazepam are mediated through two main types of receptors: the GABAA receptor and the transporter protein (TSPO). Diazepam is a targeted drug acting on the GABAA receptor, a positive variant modulator. The GABAA receptor is a pentamer composed of three subunits of αβγ in a 2:2:1 ratio, arranged counterclockwise and assembled into a ring-like pentameric complex with a chloride channel in the middle as in (Fig. (1). Diazepam acts on GABAA receptors, enhances the action of GABA and GABAA receptors, regulates the opening of chloride channels, increases the entry of Cl-into the cell, and causes hyperpolarization and inhibitory postsynaptic potentials in the cell membrane. Diazepam acts on the alpha subunit of the GABAA receptor, which includes six subtypes (α1-6). According to the different affinities of benzodiazepines binding to GABAAR, they are divided into sensitive and insensitive GABAAR. GABAAR containing α1, α2, α3, and α5 subunits are sensitive, while α4 and α6 subunits belong to insensitive GABAAR. This is because the replacement of histidine with arginine in the α4 and α6 subunits causes it to lose its affinity **(69).**

Diazepam is not the only drug to target these GABAA receptors. Drugs such as flumazenil also bind to GABAA to induce their effects **(70).**

Different subtypes of GABAR play different roles in the CNS. GABAARs containing the α1 subunit mediate sedative, amnesic, and partially, anticonvulsant effects of diazepam is through receptor binding within the cortex, thalamus and cerebellum. GABAARs containing the α2 subunit mediate anxiolytic and, to a large extent, myorelaxant effects. GABAARs containing α3 and α5 subunits also contribute to myorelaxant effects. GABAAR containing the α2 and α5 subtypes are the most promising targets for addressing schizophrenia symptoms. GABAARs expressing the α5 subunit were shown to modulate the spatiotemporal memory of benzodiazepines, associated with addiction **(71) (72) (73).** The beta subunit is associated with sedative, ataxic, and narcotic drug effects **(74) (75).**

Diazepam is not selective for multiple subunits of the GABAAR and therefore has different effects and side effects. Additionally, Shisa7 is an auxiliary subunit of GABAAR, and endogenous Shisa7 co-localizes with gephyrin, interacts with GABAARs, regulates GABAAR transport to synapses, and modulates channel dynamics and pharmacology. Shisa7 was found to enhance diazepam-induced currents in the α2β3γ2/Shisa7 complex and to play a key role in regulating tonic inhibition in hippocampal neurons **(76) (77).** With deeper understanding of the mechanism of action of diazepam and the receptors, a variety of drugs targeting the GABAAR subunits have been developed in recent years, although the associated side effects were not resolved **(78).**

Some new subunit-specific GABAA receptor modulators that may have new indications, such as analgesia, schizophrenia, cognitive enhancement, depression, and stroke have been developed **(73).** The mechanism of action of diazepam is largely unknown, and thanks to the emerging technological developments in electron cryo-microscopy, the current introduction of high-resolution electron cryo-microscopy has revealed the molecular basis of diazepam-regulated receptors, which is important for the understanding of GABAAR biology and the development of diazepam pharmacology. The search for new and effective targets and drug development is of great significance **(79) (80) (81) (82) (83).**

Specifically, the allosteric binding within the limbic system leads to the anxiolytic effects . While within the spinal cord and motor neurons is the primary mediator of the myorelaxant effects seen with diazepam. Mediation of the sedative, amnestic, and anticonvulsant effects of diazepam is through receptor binding within the cortex, thalamus and cerebellum **(60).**

- Uses /Indications:

Diazepam is an anxiolytic BZDs. It is a fast acting, long-lasting BZDs. Off-label (non-FDA approved) use for diazepam includes sedation in the ICU and short-term treatment of spasticity in children with cerebral palsy **(60).**

Diazepam has become a commonly used drug for treatment of acute repetitive epileptic seizures and febrile convulsions in children. Considering the advantages of rectal administration of diazepam **(84).**

Diazepam is used in the treatment of anxiety, insomnia, panic attacks and symptoms of acute alcohol

withdrawal. It is also used as a premedication for inducing sedation, anxiolysis, or amnesia before certain medical procedures (e.g., endoscopy). In 2020, it was approved for use in the United States as a nasal spray to interrupt seizure activity in people with epilepsy. Diazepam is the most commonly used BZDs for "tapering" BZDs dependence due to the drug's comparatively long half-life, allowing for more efficient dose reduction. Benzodiazepines have a relatively low toxicity in overdose **(50).**

BZDs, such as diazepam, a pharmacological agent used as an anxiolytic, muscle relaxant, and anticonvulsant, has recently gained interest for its antitumor properties **(85).** Diazepam can regulate basic cellular processes such as apoptosis, proliferation, and angiogenesis, which are indispensable to cancer development **(86).** These effects are thought to be mediated through the interaction of the albumin with GABAA receptors, mitochondrial channels, and calcium signaling pathways **(87).** A series of studies have recently shown the promise of repurposing existing drugs to treat cancer, including diazepam. Inhibition of cancer cell proliferation and modulation of GABAA receptor by modulating diazepam have been shown to be promising anticancer activities **(88) (89).** Additionally, it has been attributed to effect functions regarding mitochondrial function and calcium ion channels **(90).** These findings suggest that diazepam may be a complementary or alternative therapeutic strategy for cancer that would increase treatment dose intensity and reduce toxicity. Diazepam appears to exhibit selective cytotoxic effects toward different cancer cell lines, including glioblastoma, melanoma, and breast cancer **(91).** In addition to this, its unique ability to make chemotherapy drugs more effective while lowering stress and increasing anxiety in chemo patients is a multifaceted therapeutic approach **(92).** However, the specific mechanisms by which these drugs work and potentially apply to the clinic still remain to be determined. Diazepam presents some limitations in cancer therapy, including the potential for tumor-promoting effects, drug interactions, and immune suppression. Additionally, its sedative properties and risk of dependence should be carefully considered, which highlights the importance of exploring alternative treatments with fewer side effects and greater efficacy in oncology **(93) (94) (95).**

- Drug Interaction :

Synergistic CNS depressant effects are observed when diazepam is administered with ethanol and other CNS depressants. Concomitant administration of CNS depressants is very common and is almost always present in the presence of coma greater than grade II **(96)**.

Metabolic interaction :

Diazepam does not induce or inhibit hepatic enzyme activity, nor does it alter the metabolism of other agents. There is no evidence of induction or inhibition that would significantly alter the metabolism of diazepam with chronic treatment. There is a suggestion that digoxin serum levels can be altered by diazepam therapy **(49).**

As diazepam is primarily dependent on hepatic metabolism for elimination, numerous agents that either induce or inhibit hepatic cytochrome P450 pathways or conjugation can alter the rate of diazepam metabolism. With many interactions it is not clear whether the interaction is maintained with chronic therapy. These interactions would be expected to be most significant with chronic diazepam therapy, and their clinical significance is variable. The following lists includes most of the reported interactions (however, the possibility of interactions between diazepam and any substance known to alter hepatic metabolism should be considered) **(49).**

Inhibitors of diazepam metabolism:

Cimetidine, Oral contraceptives, Disulfiram, Erythromycin, Isoniazid, Probenicid, Propranolol, Fluvoxamine, Imipramine, Fluoxetine and Ciprofloxacin.

Inducers of diazepam metabolism:

Rifampin, Phenytoin, Carbamazepine, Phenobarbital and Cigarette smoking **(97) (98) (99) (100) (62) (49) (101).**

Dynamic interaction :

The major dynamic interactions with diazepam involve the synergistic increase in CNS depression associated with other CNS depressant agents, including ethanol, non-benzodiazepine sedative-hypnotics, barbiturates, drugs with CNS anticholinergic effects such as the antihistamines and tricyclic antidepressants, and opioids. These interactions increase synergistically the CNS depression, respiratory depression, and hemodynamic depression produced by each agent involved **(49).**

The anticonvulsant action of diazepam antagonizes the pro-convulsant activity of certain agents, such as cocaine and strychnine **(49).**

Drug Interactions:

Potent inhibition of the CYP2C19 enzyme by certain drugs (fluoxetine and chloramphenicol) and CYP3A4 enzymes by certain medications (ketoconazole, protease inhibitors, erythromycin) may cause increased levels of diazepam. Inducers of CYP2C19 (rifampicin and prednisone) and CYP3A4 (carbamazepine, topiramate, phenytoin, rifampin, or barbiturates) may cause lower diazepam levels **(60).**

- Adverse effects :

The duration of treatment should be as short as possible, as long-term use of diazepam increases the risk of developing tolerance dependence and adverse drug reactions (ADRs). The most common ones are fatigue, muscle weakness, and ataxia. Other less frequent ADRs involve the CNS (e.g., confusion, dizziness or headache), the gastrointestinal system (e.g., constipation, nausea, elevated transaminases and alkaline phosphatase or changes in salivation), the cardiovascular system (i.e., hypotension), psychiatric and paradoxical reactions (e.g., aggressiveness, sleep disturbances or hallucinations), the urogenital system (e.g., incontinence or urinary retention), and skin reactions **(102).**

Serious adverse effects of diazepam include:

Suicidality, Dependency and abuse, Withdrawal symptoms, Respiratory depression, Cardiovascular collapse, Bradycardia, Hypotension, Syncope and Paradoxical CNS stimulation **(60).**

Common adverse effects of diazepam include:

Sedation, Fatigue, Confusion, Anterograde amnesia, Depression- Ataxia, Irritability, Dis-inhibition, Local injection site reaction, Headache, Tremor, Dystonia, Urinary retention, Incontinence, Nausea, Constipation, Diplopia, Libido changes, Rash, Menstrual irregularities and ALT and/or AST elevation **(60).**

- High Risk Population / Use in Specific Patient Populations :-

Patients with Renal Impairment:

Diazepam description should be with caution in the elderly especially with renal impairment, as this people is at high risk of diazepam accumulation and its major metabolites. It is recommended to limit its dose to the smallest effective amount. Also they may suffer from paradoxical agitation which is manifested as hyperactivity, aggressive behavior, irritability, anxiety, and hallucinations, so diazepam must be discontinued in this case **(60).**

Patients with Hepatic Impairment:

In mild to moderate cirrhosis, the mean $t_{1\backslash 2}$ of diazepam and the volume of distribution are increased. In addition, average clearance decreases by almost half. Mean $t_{1\backslash 2}$ is also increased with hepatic fibrosis by approximately 90 hours, chronic active hepatitis by 60 hours, and acute viral hepatitis to about 74 hours **(60).**

Prenancy and Breastfeeding Considerations:
Diazepam is classified as category D, indicating positive evidence of human fetal risk. Still, the benefits from use in pregnant women may be acceptable despite the risk. It's use in pregnancy correlates with an increased risk of congenital malformations, premature birth, low birth weight, and other neurodevelopmental abnormalities. Diazepam can cross the placental barrier, and use during pregnancy may result in neonatal withdrawal soon after birth. Neonatal withdrawal symptoms include high-pitched cry, hypertonia, tremors, irritability, feeding difficulties, sleep/wake disturbances, gastrointestinal and autonomic disturbances, respiratory problems, and failure to thrive. Its onset could be anywhere from the first days of life to the first few weeks. (Floppy infant syndrome) may result in the last trimester of pregnancy due to using of diazepam, characterized by hypotonia, hypothermia, lethargy, respiratory distress, and suckling difficulties. Diazepam and its metabolites are excreted in breast milk and may influence the nursing baby. Breastfeeding should be discontinued in cases where diazepam doses are high or when repeated administration is necessary. However, when a single dose of diazepam is needed for a procedure or seizure, the physician should advise the mother to wait six to eight hours before resuming breastfeeding, especially in premature infants**(60).**

Contraindications include :
hypersensitivity to diazepam, Age under six months, severe respiratory insufficiency, myasthenia gravis, sleep apnea syndrome and severe hepatic insufficiency, acute narrow-angle glaucoma, but it is permissible in patients with open-angle glaucoma receiving appropriate therapy **(103).**

- Toxicity:
The toxicity associated with long-standing BZDs use in older persons is a critical issue **(104).**

Diazepam Overdose
The therapeutic index of BZDs is very high, making them relatively safe medications. However, the potential of overdose from diverted diazepam always occurs when combined with opioids, alcohol, or other centrally acting agents. Toxicity in adults frequently involves the co-ingestion of other CNS depressants, which work synergistically to increase toxicity. In the case of single-agent diazepam overdose, are very rarely fatal and symptoms manifest as CNS depression. In mild overdose, lethargy, drowsiness, and confusion are common symptoms. In severe cases, symptoms manifest as ataxia, diminished reflexes, hypotonia, hypotension, respiratory depression, coma, and death (rarely) **(105).**

Other symptoms may include:
Bluish-colored lips and fingernails, vision problems (blurred vision, double vision), breathing is slow, labored, or stopped, Confusion, depression, dizziness, drowsiness, lack of alertness, excitability, hiccups, rapid side-to-side movement of the eyes, rash, stomach upset, tiredness, tremor and weakness and uncoordinated movement **(106).**

Potential for Diazepam Abuse and Dependence
Diazepam is a Schedule IV controlled substance with potential for abuse. Dependence and tolerance can occur in patients who are prone to addiction, undergo long-term treatment, or those who take high doses. Therefore, these individuals should be under close supervision; once an individual becomes dependent, the risk of developing withdrawal symptoms increases. Signs of benzodiazepine withdrawal include tremors, rebound anxiety, cognitive disturbances, mood disturbances, psychosis, agitation, irritability, insomnia, sweating, headache, confusion, muscle pain, abdominal pain, and vomiting. With long-term use and abrupt discontinuation, hallucinations and seizures are possible **(103) (107).**

Propylene Glycol Toxicity :

Propylene glycol toxicity is a rare toxidrome associated with parenteral intake of diazepam. Propylene glycol is a common diluent used in intravenous diazepam suspensions. Large doses or prolonged intravenous infusion of diazepam can result in accumulation of propylene glycol and subsequent anion gap metabolic acidosis. Signs of propylene glycol toxicity include the development of serum hyperosmolality, hemolysis, cardiac arrhythmias, hypotension, lactic acidosis, seizures, acute kidney injury, and multiple organ failure **(108).**

Treatment of Toxicity (Management) :

- General principles:

The mainstay treatment for acute benzodiazepine toxicity is supportive care, which may include endotracheal intubation to provide definitive airway management. Single-dose or multi-dose activated charcoal, hemodialysis, or whole bowel irrigation play no role in managing benzodiazepine toxicity. Flumazenil is a nonspecific competitive antagonist at the benzodiazepine receptor that can reverse benzodiazepine-induced sedation and it is reserved for cases with severe respiratory or cardiovascular complications and should not replace the basic management of the airway and respiration **(109).**

- Decontamination:

Gastric lavage is not routinely indicated following benzodiazepine overdose.
Emesis is contraindicated because of the potential for CNS depression.
Activated charcoal can be given orally **(110) (111).**

- Enhanced elimination:

Renal and extracorporeal elimination methods are not effective **(110) (111).**

- Antidote treatment:

It should be used in limited cases. Flumazenil is a nonspecific competitive antagonist at the BZDs receptor that can reverse BZDs-induced sedation. However, in most cases, the risks of flumazenil usually excel the benefits in acute toxicity, and thus flumazenil is not recommended for routine reversal of this sedative agent. It can cause seizures and cardiac dysrhythmias, especially paroxysmal supraventricular tachycardia. Flumazenil can precipitate acute withdrawal syndromes in those with chronic BZDs dependence. If a patient with a chronic dependence is given flumazenil, it can lower their seizure threshold and potentially cause life-threatening seizures. The treatment of seizures, which typically involves BZDs, would be rendered useless, as the flumazenil has blocked the BZDs receptors. So it can be safely prescribed to non-habituated users of benzodiazepines. This habitually occurs in children with accidental ingestion or after procedural anesthesia **(109).**

The initial intravenous dose in adults of 0.3 to 1.0 mg may be followed by further doses if needed. If there is no clinical improvement to 2 mg of flumazenil within 5 to 10 minutes indicates that the major cause of CNS depression or coma is not BZDs poisoning. The patient regains consciousness within 15 to 30 seconds after injection of flumazenil, but it is rapidly metabolized than diazepam so CNS depression can be recurred and the patient must be well monitored after initial response to flumazenil therapy. If toxicity recurs, further bolus doses may be administered or an infusion commenced at a dose of 0.3 to 1.0 mg/hour**.** In children , it should be repeated each minute until the child is conscious. Continuous parenteral infusion should be administered at a rate of 0.1 to 0.2 mg/hour **(112).**

Differential Diagnosis:

Differential Diagnosis of BZDs toxicity includes alcohol and opiate toxicity,
hypoglycemia, hyponatremia or hypernatremia and Stroke **(109).**

Prognosis

Prognosis depends on the speed of diagnosis and treatment **(109).**

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