Kingston University London

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Development of an analytical method for the detection of benzodiazepines, under section 5A, in blood samples of UK motorists

The thesis is submitted in partial fulfilment of the requirements of Kingston University for the degree of:

Masters by research in Chemistry

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Abstract

A method was validated for the analysis of six benzodiazepines listed under Section 5A of the Road traffic Act. The benzodiazepines selected were diazepam, oxazepam, temazepam, clonazepam, lorazepam and flunitrazepam in line with ISO 17025 guidelines. The method can be utilised for casework analysis of blood samples in road traffic toxicology for all six benzodiazepines for assessing if a motorist's blood sample is above the *per se* limits. In addition, the method was validated in line with the criteria and general guidelines stipulated in document FSR-C-133 and specifications for methods to use for Section 5A of the Road Traffic Act. The method has met all requirements detailed by FSR-C-133 including minimum number of calibrators, QC's, along with validated parameters of linearity, accuracy, precision, percentage recovery, matrix effect and robustness. The developed method has an accuracy of ±10%, a %CV of ≤10%, linearity of ≥0.99 for all analytes. The LOQ was set at the first calibration point and the LOD was half the LOQ concentration. In all cases the LOD and LOQ displayed suitable ion ratio acceptance (≤10% of target) and accuracy (≤10% of target). The method displayed ion suppression in all analytes. The average ion suppression was -48%, this did not affect the methods accuracy or sensitivity as indicated by studies on LOD and LOQ. Further parameters tested included reproducibility with three separate extractions from three separate analyst on different days, in all three extractions the methods performance was unaffected. The analysis of all six benzodiazepines is possible, at concentrations typically detected in samples and cases found in road traffic incidents. Although a method was developed and validated for six benzodiazepines, there is scope to expand the method and add other less commonly detected benzodiazepines. Moreover, the method was validated to take into account a variety of storage conditions; room temperature, refrigerated and frozen and then analysed to ascertain whether the method was suitable in casework study where samples are not analysed on the same day. The results indicate that the blood samples are stable and all fall within ±20% of their detected concentrations.

Contents

List of Abbreviations

APCI- Atmospheric Chemical Ionisation
BZD- Benzodiazepines
BZ1- Benzodiazepine 1
BZ2- Benzodiazepine 2
CNS- Central Nervous System
CV%- Coefficient of variance percent
DRE- Drug Recognition Experts
DRUID- Driving under the influence of drugs, alcohol and medicine
ESI- Electrospray
FIT- Field Impairment Test
GABA- *gamma*-Aminobutyric acid
GC-MS- Gas Chromatography-Mass Spectrometry
HC- Hippocampus
HPLC- High Pressure Liquid Chromatography
LC-MS- Liquid Chromatography-Mass Spectrometry
LD50- Lethal dose 50
LLE- Liquid-Liquid Extraction
LOD- Limit of Detection
LOQ- Limit of quantitation
MRM- Multiple Reaction Monitoring
NA- Nucleus Accumbens
PFC- Prefrontal Cortex
PPT- Protein Precipitation
QC- Quality Control
RSD- Relative Standard Deviation
SPE- Solid Phase Extraction
SRM- Selected Reaction Monitoring
VTA- Ventral Tegmental Area
WS(A)- Working Solution A
WS(E)- Working Solution E

1 Introduction

1.1 Origin of Benzodiazepines

The unintentional discovery of chlorodiazepoxide represents a significant starting point in the history of central nervous system depressants (Gerecke, 1983). First founded by L.H Sternbach and L.O Randall in 1957, the compound gave rise to an array of moieties with similar therapeutic properties. Chlordiazepoxide (marketed as Librium) was initially accepted with high praise by the medical community, resulting in an exponential growth in patient demand and by the late 70's it was the most prescribed drug in the world (Wick, 2013). However, the therapeutic merits of the chlorodiazepoxide drug came under increasing scrutiny as the potential for addiction and dependence was realised (Sternbach, 1979).
With the efficacy to treat a wide array of medical conditions, benzodiazepines are a family, or class, of drugs that are predominantly used to treat sleeping disorders, anxiety, muscle spasms and epilepsy seizures (O.H. Drummer, 2001). Benzodiazepines are subdivided into different classes based on their activity as a depressant, sedative or hypnotic. The most widely used and prescribed benzodiazepine is diazepam (Valium). Other drugs of similar use but of different structural compounds that will also be investigated are oxazepam (Serax), lorazepam (Ativan), flunitrazepam (Rohypnol), clonazepam (Klonopin) and temazepam (Restoril). These drugs are typically controlled and only available with a valid prescription, however, their cost and ease of availability has made them a widely encountered drug of abuse.

1.2 Chemical structure of BZD

The core structure of a benzodiazepine consists of a bicyclic heterocyclic compound having a benzene nucleus fused to a seven-membered ring containing two nitrogen atoms (Qadir, 2015). The variation in R groups attached to the general structure, allow for increased efficiency of GABA (gamma-aminobutyric acid), a neurotransmitter that decreases neuron excitation, leading to reduced propagation of nerve signals. Sternbach *et al*. discovered that the N-Oxide and the methyamino group in positions 4 and 2 respectively hindered the efficacy of the compound. Structural relationships between the core drug and the target receptor has allowed the development of drugs with higher selectivity to the target receptor resulting in increased potency or activity. By interchanging these groups through oxidation or reduction it was discovered compounds with varying degrees of potency could be formulated (Sternbach, 1968).

Interchanging the following groups affect benzodiazepine binding sites and their intrinsic activity in relation to $GABA_A$ receptors. Consequently, this determines agonist, antagonist and inverse agonist behaviour. The agonists allosterically modulate GABA binding to the receptor, which in turn applies a positive cooperative effect; thus there is an increase in frequency of chloride channel openings (Wang, Han and Xue, 2006). This allows a greater flow of chloride ions into the neuron and ultimately leads to membrane hyperpolarisation and decreased neuron excitability. The resultant physiological effect is a reduction in brain/central nervous system activity and a decrease in anxiety (Sorensen *et al.,* 2017).

In position 1, polarity is the most important concept when deciding on the constituent; the size of the substituent is important *(Wang, Han and Xue, 2006)*. Only the methyl group increases binding potential and any larger substituent will decrease it. At position 2 a carbonyl group is present which is critical for the pharmacological activity; this functional

group acts as the receptor binding motif via histidine residue proton donation (So and Karplus, 1996). One of the few chemical substitutions that can effectively replace this carbonyl group and retain receptor activity is the addition of a halogen in position 2. This substitution results in a significant increase in drug potency; conversely a reduction in affinity for the GABA receptor occurs when a halogen is substituted into position 4. Groups at position 3 are responsible for the pharmacokinetic activity of the drug. For position 3 any substituent bigger than a methyl or hydroxyl group (such as ethyl or carboxyl) will contribute to a loss of efficacy. In addition, highly polar groups are directly conjugated resulting in direct excretion of the drug or metabolite thereby reducing the duration of action. Positions 4 and 5 require the unsaturated double bond for activity and any saturation decreases activity. (Maddalena, 1995). By contrast, position 5 requires an aromatic ring for optimal action; such as the phenyl group present in diazepam. Activity can be increased by substitution on the phenyl group with an electronegative compound, such as fluorine. However, stoichiometric positioning is also a significant factor in determining drug potency, as the group has to be substituted in the ortho position; as para substitution will decrease efficacy. For position 7 research has shown that an electro-negative group is integral for optimal activity and cannot be compromised. The general rule of the thumb is the higher the electronegativity and lipophilicity of the group attached at position 7, the more potent the drug will be; due to increased affinity of the ligand for the binding site *(Wang, Han and Xue, 2006).* Another avenue of variation is the introduction of additional rings, primarily attached to the diazepine ring (Gerecke, 1983). Research undergone has shown positions 6,8 and 9 should not be substituted with any other groups as loss of activity may occur.

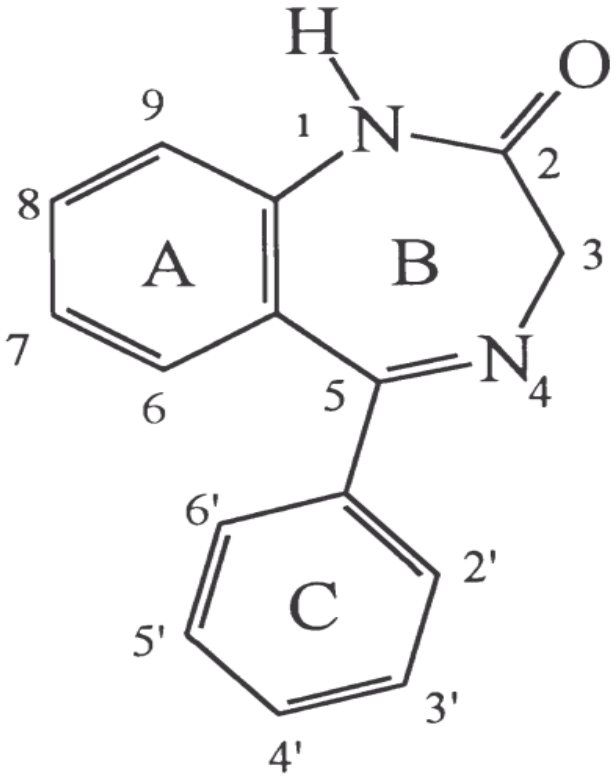


Figure 1. An illustration of the classic benzodiazepine with its assigned numbering system (Wang, Han and Xue, 2006).

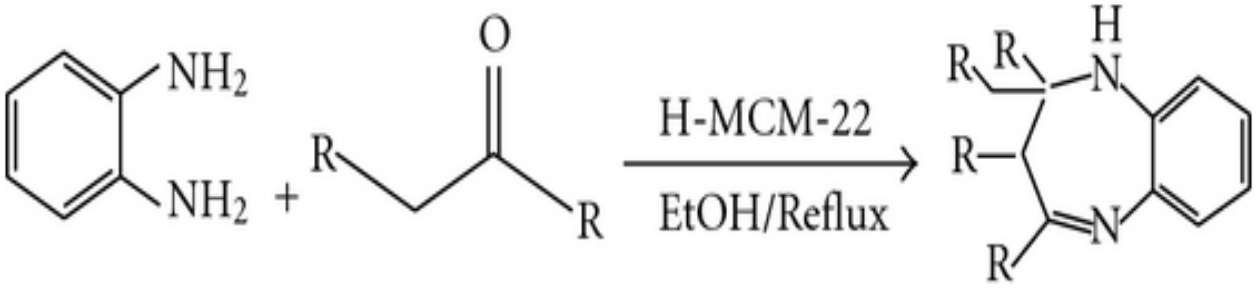


Figure 2. Synthesis of 1,5-benzodiazepines using H-MCM-22 zeolite catalysts at room temperature. (Majid, Khanday and Tomar, 2012)

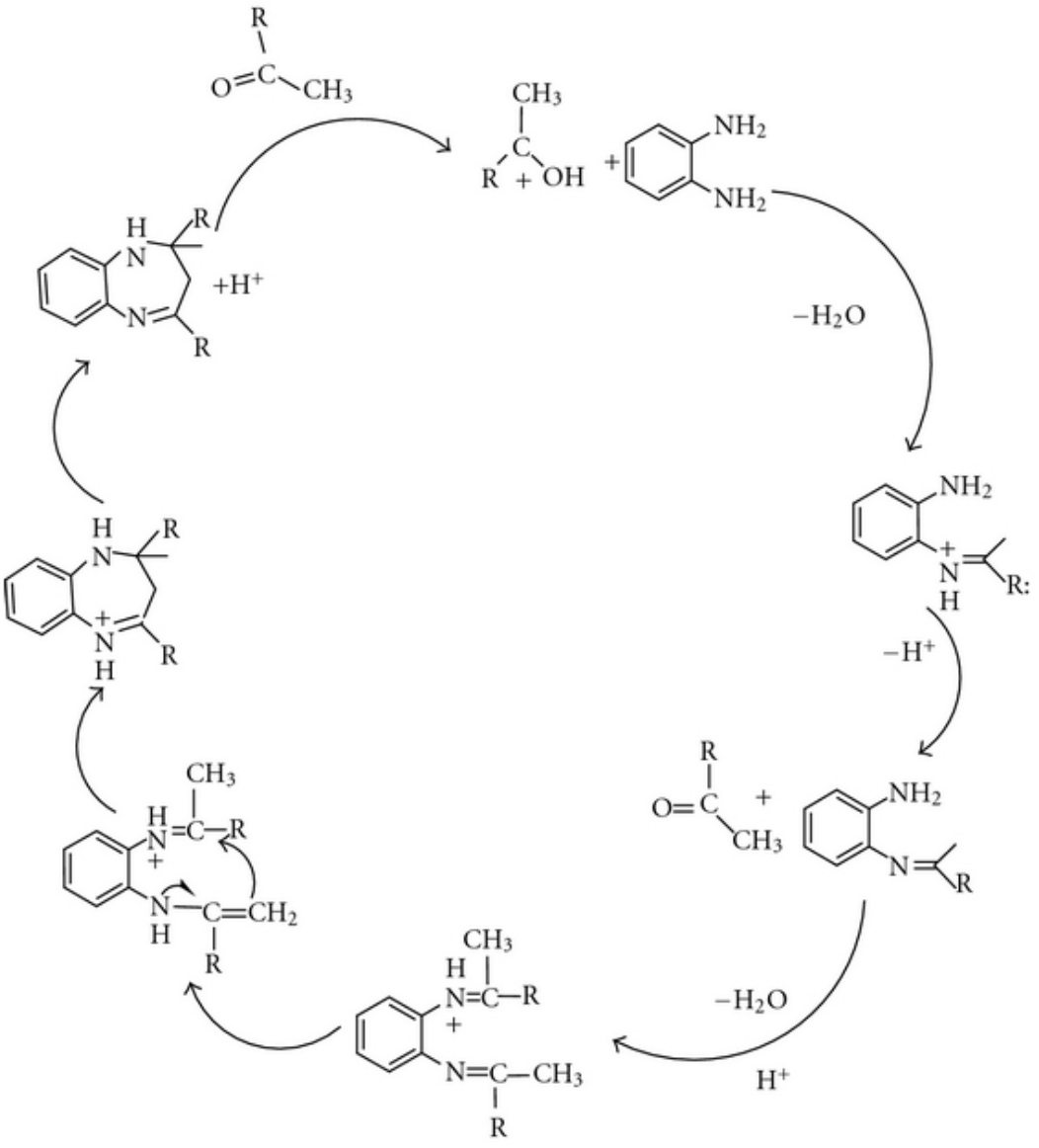


Figure 3. Reaction mechanism of the synthesis of 1,5-benzodiazepines using H-MCM-22 catalyst at room temperature (Shobha et al., 2010).

1.2 Pharmacology of Benzodiazepines

Benzodiazepines are often categorised on the elimination half-life of the drug based on their pharmacokinetic parameters. Categories are approximate, since half-life may vary from individual to individual based on height, weight and underlying health conditions. There may be some overlap where medium acting benzodiazepines have longer durations due to active metabolites that are formed from the pro-drug (Greenblatt, Shader, Divoll and Harmatz, 1981). The drugs in question on this research project are all solely medium half-life and long half-life. In addition, all drugs in this project are outlined in Section 5A of the road traffic act. Although this is not an exhaustive list of benzodiazepines, all those included are most commonly found in UK motorists.

Table 1: List of benzodiazepines, their therapeutic use, range of half-life and average daily dose (Baselt, 2020).

Name of drug	Usage: Anxiolytic or Hypnotic	Half-life (hours)	Typical daily dose (mg)
Medium half-life:			
Oxazepam	Anxiolytic	4-15	30mg maximum
Temazepam	Hypnotic	8-22	10-30mg
Lorazepam	Anxiolytic	10-40	4mg maximum
Flunitrazepam	Hypnotic	16-35	0.5-2mg
Diazepam	Anxiolytic	20-100	2-40mg
Clonazepam	Anxiolytic/epilepsy treatment	30-40	2-8mg

Medium-acting benzodiazepines have a net biochemical half-life of 8-24 hours (Moffat, Osselton, Widdop and Watts, 2011). Active metabolites are less common and pharmacological activity is typically derived from the parent compound alone. Their shorter half-life and faster clearance results in drug accumulation in the body being less prevalent. During multiple dose treatment a steady state condition is quickly attained where the maximum desired effect of the drug is achieved without the effect of over sedation with hypnotics due to the build-up and residual effects of prior doses (Greenblatt et al., 1979).

Long-acting benzodiazepines have a half-life of 24 hours or longer (Moffat, Osselton, Widdop and Watts, 2011). They commomly have a rapid onset of action and are clinically effective over a short term (Furukawa *et al.,* 2002); 2-4 weeks (Kennedy and O'Riordan, 2019). This is due to either the parent compound or one of the pharmacologically active metabolites. A direct result is drug accumulation when patients are taking multiple doses and accumulation may occur at different rates based on half-life and how fast drugs are cleared from the body. Accumulation has both advantages and disadvantages, where a

single dose may be advantageous for those suffering from anxiety and insomnia. In addition, a missed dosage may not have such an adverse effect where the pre-medicated symptoms arise (Greenblatt, Shader, Divoll and Harmatz, 1981). However, longer acting benzodiazepines may have the effect of over sedation and anterograde amnesia, which are α1 mediated (van Rijnsoever, 2004).

Although all benzodiazepines work in a similar way and have almost identical functionality, it is noteworthy that potency differs hugely. As a result, the potency of equivalent doses between different benzodiazepines can vary as high as 20-fold. This difference in potency should be taken into consideration when switching between different benzodiazepines. For example, when switching from lorazepam to diazepam to reduce withdrawal effects (Ashton, 2002).

Depending on the dosage, medical condition and underlying health complications, the route of administration of benzodiazepines can vary. Predominantly, the drug is taken orally as a whole tablet or capsule with the active ingredient inside. In medical settings the drug can be taken intravenous, intramuscular or in rectal gel form. However, it can also be dissolved in drinks before drinking or is crushed up into a fine powder before snorting it through the nasal cavity. The latter route is not used medically but is more common among recreational users as it achieves the user desired effect rapidly.

Once administered orally benzodiazepines are readily absorbed into the circulatory system. On the contrary, when administered intramuscularly, uptake and absorption of some drugs are often erratic and pain at the injection site may occur. Benzodiazepines and their metabolites are highly protein bound and have a highly lipophilic nature exhibiting a large volume of distribution (0.7-21/Kg) (Nilsson, 1991). The more lipophilic a benzodiazepine, the faster the rate of absorption and thus a faster clinical onset. Most benzodiazepines are metabolised via oxidation by the cytochrome P450(CYP)3A (Phase 1) (Griffin et al., 2013). Active metabolites are further metabolised via polymorphic cytochrome CYP3A and CYP2C19 isoforms (Kim *et al.,* 2017). During (Phase 2) active metabolites are metabolised to glucuronide conjugates (Kim *et al.,* 2017) and almost entirely excreted via urine (Griffin et al., 2013). The cytochrome P450 and its enzymes act as inhibitors or inducers which in turn affect its metabolism and clearance from the body. Although not completely characterised, studies suggest inducers increase enzyme activity by increasing enzyme synthesis, thereby increasing metabolism and clearance time. However, inhibitors will directly inhibit the activity of cytochrome P450 (Meyer, 2012) resulting in slower metabolism and clearance and the possibility of drug accumulation as represented by longer-acting benzodiazepines. Benzodiazepines produce their effect by binding to the central benzodiazepine receptors which are situated at the post and presynaptic membranes (Burt and Kamatchi, 1991). GABA is the most common of neurotransmitters found in the CNS and is present in high concentrations in the cortex and limbic region (Wu and Sun, 2014, Xu et al., 2011); responsible for regulating memory, emotion and stimulation. GABA has 3 different sub-units assigned letters A-C; $GABA_A$ is the binding motif that interacts with benzodiazepines. This can be further broken down into receptor complexes containing 2 α subunits, 2 β subunits and 1 γ subunit. Each α subunit contains histidine residues H101, H101, H126, and H105, commonly denoted as 1, 2, 3 and 5 respectively (Griffin et al., 2013). These residues have a high affinity for benzodiazepines which potentiate the binding and subsequent pharmacological effects. Each $GABA_A$ receptor has two binding sites. Benzodiazepine binding sites can be located at the interface between α and β subunits where they join

together and create a pocket (Kelly et al., 2002). $GABA_A$ receptors act as a ligand gated ion channel. In the mammalian central nervous system, GABA works as a key inhibitory neurotransmitter in opposition to glutamate, the excitatory neurotransmitter (Gordon *et al.,* 1990). When activated GABA is able to control the Cl^- concentration in its pores and augments chloride currents. Benzodiazepines increase GABA responses by lengthening the average channel opening time by a factor of five, while no change is made in the single channel current amplitude (Charles *et al.,* 1991). Consequently, chloride ions are able to flow through the GABA channel for a longer period of time after being exposed to benzodiazepines or barbiturates.

On the principle of α, β and γ subunits having different isoforms indicated with a numbering system akin to their different properties such as affinity for different benzodiazepines; benzodiazepine receptors are also categorised numerically. The BZ1 receptor contains α1 isoforms (SIEGHART, 1992). Due to this, benzodiazepines binding to the BZ1 receptor elicits effects of sedation and amnesia. The majority (60%) of $GABA_A$ receptors contain the BZ1 receptor which holds α1 isoforms which elucidates why anterograde amnesia is such a common side effect of benzodiazepines (Mattila-Evenden *et al*., 2001). In addition, glutamate is one of the major neurotransmitters involved in memory formation and consolidation. When $GABA_A$ is over activated in cases of benzodiazepine use, the ability for glutamate to carry out its function is drastically hindered.

BZ2 receptors hold the α2 isoform which bring about the effects to reduce anxiety and increase muscle relaxation (Sieghart, 1992) (Kaufmann *et al*., 2003). The variation of clinical effects brought about by different benzodiazepines are due to the drugs binding to BZ receptors with varying degrees of affinity as well as discrepancies in α subunit isoform amounts in different $GABA_A$ receptors as well as the binding site locality within the central nervous system (Crestani et al., 2001).

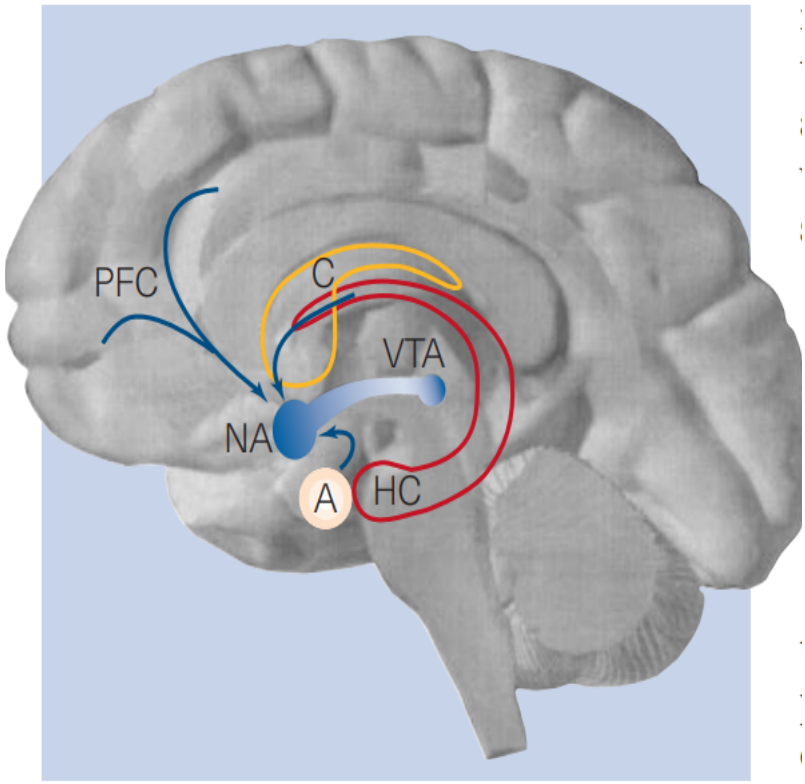


Figure 4. An illustration of the parts of the brain and limbic system affected by drugs of abuse. The limbic dopamine system is located in the ventral tegmental area (VTA) of the midbrain. This projects onto the nucleus accumbens (NA). The amygdala (A), prefrontal cortex (PFC) and hippocampus (HC) send excitary neurons to the nucleus accmbens. (Robbins and Everitt, 1999)

All of the drugs housed under the benzodiazepine class of drugs have similar effects on the user causing impaired judgment, loss of motor control, lack of coordination, decreased reaction time, dizziness, loss of memory while under the influence, confusion, drowsiness, respiratory depression with higher doses, slurred speech.

All of the aforementioned reactions can last anywhere from 4-12 hours or more and because of these adverse effects, driving under the influence of benzodiazepines is dangerous and becoming an increasingly bigger problem (Barker *et al.,* 2004). These drugs inhibit psychomotor and cognitive function which in turn affects reaction time and driving skills significantly (Linnoila et al., 1990, Stone et al., 2015). Meta-analysis research studies suggest road traffic collisions are 60-80% more likely with benzodiazepine use (Dassanayake et al., 2011). When used alongside alcohol the danger is even more prevalent as both drugs are central nervous system depressants which have a synergetic effect on GABA receptors and increase toxicity of each other when taken together.

1.3 Epidemiological Prevalence of Benzodiazepines

Research and surveys indicate that benzodiazepines are one of the most commonly prescribed drugs in the world, with approximately 16 million prescriptions in 2015 (HSCIC, 2016). An estimated 500,000 to 1 million of prescribed patients are thought to be dependent on benzodiazepines (Ashton, 2004). The use of prescription medicine in general, has steadily increased in the last decades as a result of, but not entirely, the changing age demographics; with an increasing number of the population now considered elderly. Also, the use of anxiolytic and hypnotic drugs may have always been excessive and inappropriate, based on dependence and habituation (Lader, 1991). However, the prevalence of abuse is of increasing concern. In the UK, participants of surveys who said they had driven under the influence of drugs at least once or twice in the last 12 months; 31 percent admitted to being under the influence of sedative drugs in that period (Daamen *et al*., 2012). In a separate study by Oliver *et al*. 2006, biological samples were tested for 3,616 people who had been pre-screened for drug driving. Of these 75 percent were found to have drugs in their system with the most common drug found to be benzodiazepines.

A study carried out by Simon *et al.* (1996), found that 3 factors determine length of use and consequently likelihood of addiction. The patient's age, the use of high potency drugs (such as clonazepam) and dosage of drugs in the initial prescription (Simon *et al*., 1996). This is further verified by another study in the United States in 1997 which found that age, frequent or daily use as well as prescriptions provided by other than a general practitioner were all associated with long term use; an average of 13 years (Isacson, 1997). Research suggests that young adults involved in frequent non-medical use are more likely to be addicted. A study of 356 participants aged 18-28, who had used benzodiazepines at least once in the last 90 days, elucidated that 12.6% met the dependence criteria (Kurtz, Buttram and Surratt, 2016). In contrast, medical use, especially long term, is most prevalent among females over the age of 50 (Dupont, 1988). Benzodiazepines with stronger hypnotic properties are considered of higher risk due to likelihood of increased dependency from continuous use. Moreover, those on higher dosages are more prone to dependence. This is evident from the fact when withdrawing primary care studies suggest gradual dose reduction over at least a period of 10 weeks to effectively obtain long term abstinence (Denis *et al.,* 2013).

Recreational benzodiazepine use is becoming more and more widespread and although the size of the population is not definitive, estimates suggest that 200,000 people use benzodiazepines illicitly in the UK. At least half of amphetamine, cocaine and opiate users as well as alcoholics take benzodiazepines concurrently with their drug of choice. (Ashton, 2002; Ashton 2004). Reasons for this are to bring users down from stimulant drugs such as cocaine or to reduce the withdrawal effects of other drugs. Some users say illicit drug effects are augmented when taken with benzodiazepines (Ashton, 2005), such as in the case of opiates where both are CNS depressants resulting in a synergetic potentiating effect. Some users will take benzodiazepines as the primary recreational drug, usually on high doses and short acting time via means of intravenous injection (Ashton, 2002).

In the United States in 2008 there were more than 270,000 visits to the emergency department from illicit benzodiazepine use, of which 40% involved alcohol (Substance Abuse and Mental Health Services Administration (SAMHSA), 2011). This number further increased to 426,000 people in 2011, of which 24.2% involved alcohol (Marmorstein, 2011). In addition to the aforementioned figures, there are an estimated 85 million people who are legally prescribed benzodiazepines for outpatients with anxiety and mood disorders only. It is evident the prevalence of benzodiazepine use in the United States is rife (Moloney, Konrad and Zimmer, 2011). In a Canadian study, approximately 8.4% of the total population used at least one benzodiazepine in 2006 with 3.5% of the prescriptions exceeding 100 days use (Cunningham, Hanley and Morgan, 2010).

The availability of these drugs are common place and sources of illicit benzodiazepines predominantly come from general practitioner prescriptions or thefts from chemists and pharmaceutical warehouses as well on the internet through black market websites (Ashton, 2004; Grzybowski, 2004).

Although benzodiazepines are considered to have a high LD50 when compared to other CNS depressants such as barbituates, tolerance grows exponentially in regard to their sedative activity. Although some patients suffering from insomnia report continual hypnotic efficacy of the drug, this may be due to the prevention of rebound insomnia; a withdrawal effect of benzodiazepines. The majority of hypnotic benzodiazepine users will gradually increase their dosage, at times to over the recommended daily prescribed limit, due to increased tolerance where a higher dose is required for the same effect provided by the initial prescription. Increased tolerance is also evident as it is commonplace for individuals suffering from sleeping disorders to take two or more benzodiazepines daily and concomitantly (Morin, Bélanger, Bastien and Vallières, 2005; Poyares, Guilleminault, Ohayon and Tufik, 2004). For their anticonvulsant and muscle relaxant effects, the potency of benzodiazepines are significantly reduced after two months of constant use (Gerecke, 1983), suggesting that for long term treatment of muscle spasticity and seizures, benzodiazepines are not appropriate. Although slower than sedative tolerance builds up, which takes a matter of days or weeks of continual use (Ashton, 2005). Research has shown that cognitive impairment is likely and long-term users of benzodiazepines suffer from shortfalls in all 12 of the cognitive domains which include attention, memory, learning and visuo-spatial capability to name a few (Barker, 2004). In terms of anxiolytic effects, tolerance build up is slow and can take a few months. Even so, research shows dosage escalation is common, where multiple benzodiazepines are taken following the first losing efficacy. A previous study indicated over a quarter of participants were taking two anxiolytic benzodiazepines (Ashton, 1987).

Furthermore, clinical observation indicates chronic benzodiazepine use to have no effect in controlling anxiety disorders, but in some cases amplifying it (Ashton, 2005).
Public Health England found that between 2017 and 2018, 1.4million adults (3% of the adult population) had received and dispensed one or more prescriptions for benzodiazepines. This is not inclusive of illicit use. In the UK alone, an estimated 500,000 to a million patients who are prescribed benzodiazepines are dependent on them (Ashton, 2004). A large population of the number are elderly and female who regularly take hypnotic benzodiazepines and their usage continues to grow (Curran *et al.,* 2003).

Studies show the likelihood of dependence increases with long term use of benzodiazepines, higher dosage usage and usage of short acting benzodiazepines. Key indicators of dependence become evident during dosage reduction or complete cessation of use. Another means of inferring dependence is through benzodiazepine users other drug habits. People with drug and alcohol dependence are more likely to develop addiction to benzodiazepines (Johansson, 2003).

Withdrawal symptoms of benzodiazepine use includes but are not limited to palpitations, perceptual distortions, panic attacks, anxiety, agitation, muscular pain, weight loss, restlessness, sweating, sensory hypersensitivity, cognitive decline and severe sleep disturbance. Those on a higher dosage are more likely to experience more severe and sometimes life threating withdrawal symptoms such as seizures and psychotic reactions (Petursson, 1994). Specific studies show that all subjects suffer from some of the constellation of withdrawal symptoms mentioned above; namely anxiety, restlessness, agitation and sleep disturbance (Petursson and Lader, 1981).

Research shows that withdrawal is often protracted with numerous cases indicating that patients can take up to 3-4 years to fully recover and are often left with underlying symptoms directly attributed to benzodiazepine use; such as tinnitus (Ashton, 2002). In the UK alone it is estimated that over a 100,000 people would be willing to engage in services to help discontinue use from benzodiazepine drug dependency (Davies, Rae and Montagu, 2017).

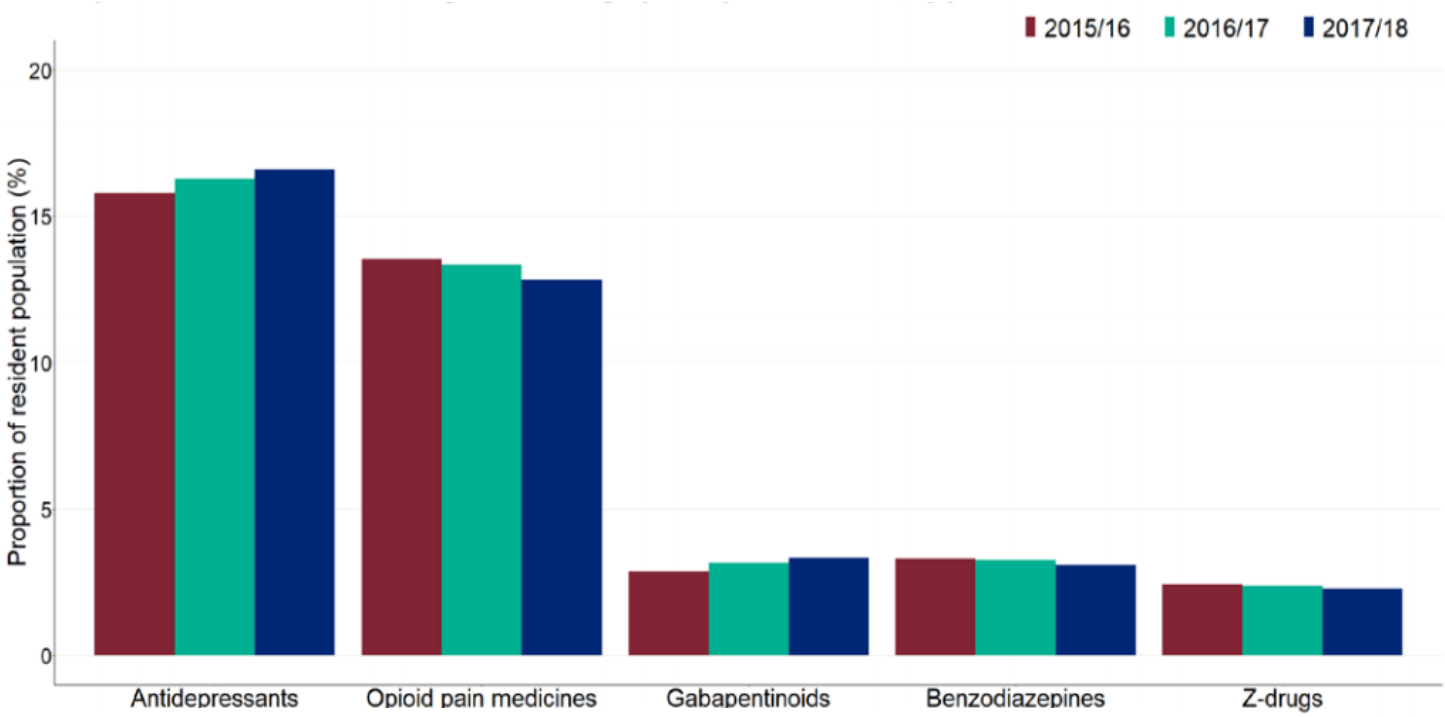
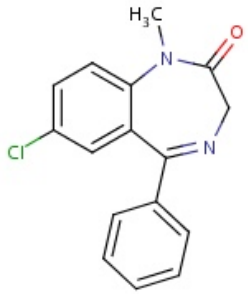


Figure 5. Demonstrates the proportion of adults in England receiving a prescription between the years 2015-2018 by year and class of medicine (Public Health England, 2019)

1.5 Diazepam



Diazepam, the second benzodiazepine approved for human usage (Baselt, 2020) is one of the most widely prescribed benzodiazepines (Griffin et al., 2013). The structural makeup accounts for its long acting, medium potency. At position 1 an alky group is present and the specific structure and size allowing for maximal agonist activation. The essential carbonyl group required for pharmacological activity via receptor binding is present at position 2. At position 3 a small hydrogen substituent is present to allow for maximum efficacy. Position 4 to 5 has the unsaturated double bond to also allow for maximum effectiveness. Position 5 has the phenyl ring for peak action. At position 7 a highly electronegative chloride atom is present giving its strong sedative properties.

Dosages vary and are dependent on patient to patient health conditions, but typical daily intake is between 2 and 40mg (Baselt, 2020). Diazepam absorbs relatively quickly and peak plasma concentrations are seen anywhere between 15 and 90 minutes following administration, however this can be slowed via the intake of fatty meals. Diazepam has an average distribution volume of 0.7-2.6L/Kg (Baselt, 2020). It metabolises in the liver to N-desmethyldiazepam, also known as nordiazepam, as well as the metabolites oxazepam and temazepam (Wolff *et al.,* 2003). The primary metabolites are pharmacologically active and has a lengthy elimination half-life which may contribute to prolongation of clinical efficacy (Nilsson, 1991). In young adults the elimination phase occurs at a rate of 20-30mL/min, however this can be extended by multiple dosing (Griffin et al., 2013). Toxic effects of this drug and its metabolites can be seen when concentrations exceed 1500 µg/L (Moffat, Osselton, Widdop and Watts, 2011). As mentioned prior, anxiolytics tend to have a longer tolerance build-up of approximately a few months.

Diazepam has strong sedative properties and can cause anterograde memory deficits, severe drowsiness (Jongen, Vuurman, Ramaekers and Vermeeren, 2018) as well as impairment in perceptual speed resulting in driving too fast, coordination skills resulting in failure of lane observance (Dubois, Bédard and Weaver, 2008) and reaction time. In addition, flicker fusion discrimination and visual parameters are affected (Seppala et al, 1976); all integral for driving.

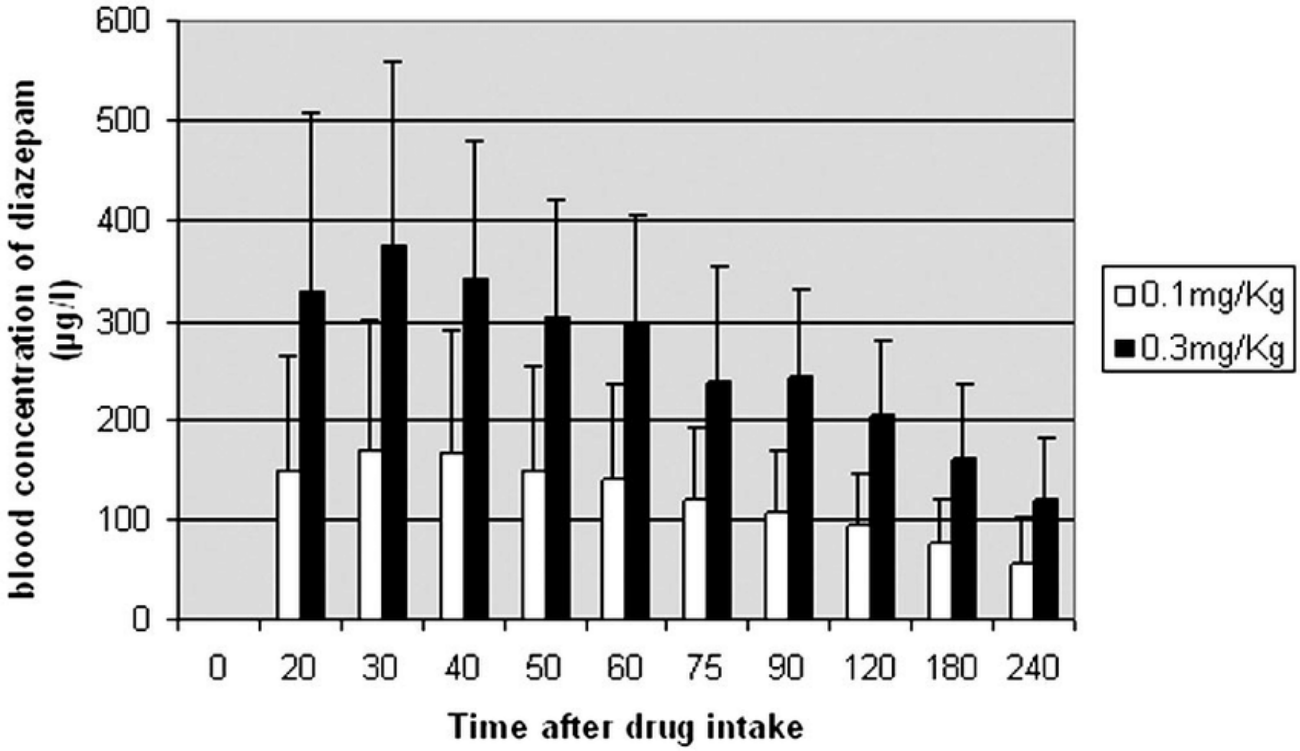
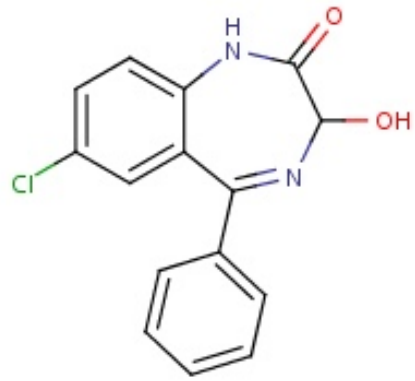


Figure 7. Mean blood concentration of diazepam as a function of the dosage (0.1 vs. 0.3 mg/kg) and time at which blood samples were collected: before intake of the drug (0) and at 20, 30, 40, 50, 60, 75, 90, 120, 180, and 240 min after intake of the drug. Vertical bars represent standard deviations (Boucart *et al*., 2007).

1.6 Oxazepam

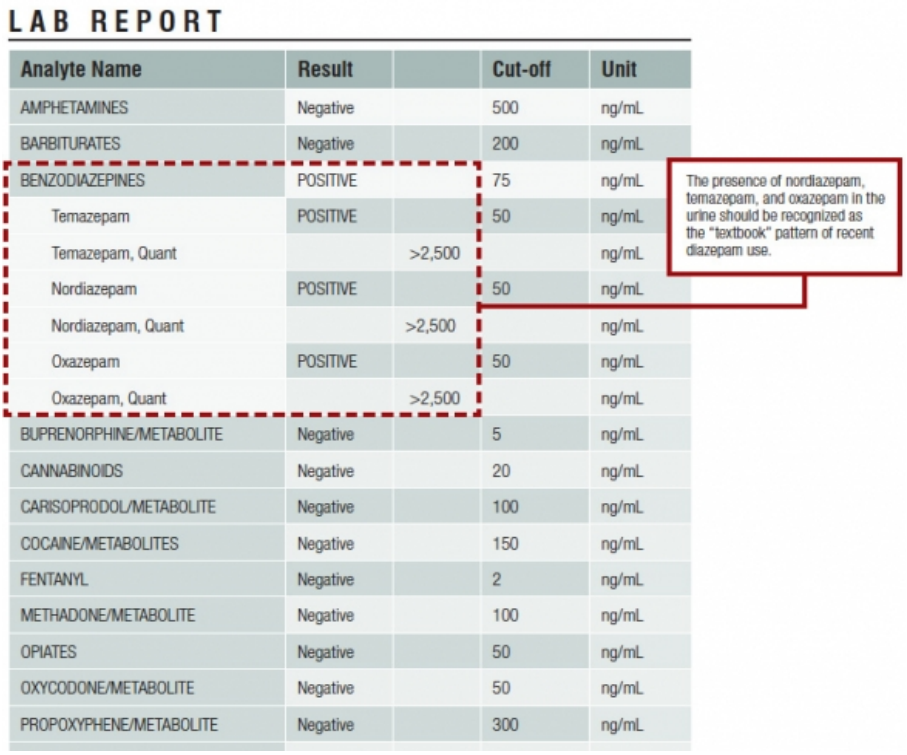


Oxazepam is a benzodiazepine derivative that has been used clinically as an antianxiety agent since 1965 (Baselt, 2020). At position 1 of the oxazepam structure a hydrogen is attached to the nitrogen which allows for good biological activity when consumed and may be suitable for people who do not require a drug as strong as the likes of diazepam. At position 2, the essential carbonyl group required for pharmacological activity is present. At position 3 a polar hydroxyl group being present allows the drug to provide therapeutic aid when a short acting dug is required and can be excreted quickly. Being highly polar the drug is excreted without phase 1 metabolism. Position 4 to 5 retains its unsaturated double bond in order to not lose its activity. At position 7, an electronegative chloro group covalently bonded is present which gives the compound its characteristic sedative properties.

Although a prescribed benzodiazepine in its own respect, it is a metabolite of various other benzodiazepines including but not limited to diazepam and temazepam. It is itself metabolised into glucuronide conjugates. Usually, prescribed as an anxiolytic, its effect on $GABA_A$ receptors also produces a hypnotic effect (Greenblatt, 2012). Typical doses vary significantly, but maximum daily doses are limited to 30mg (Baselt, 2020). Due to its short acting period once daily anxiolytic treatment is not viable and dosages must be sub-divided and given 3-4 times a day for anxiolytic effects to be therapeutically effective (Greenblatt, Shader, Divoll and Harmatz, 1981., Baselt, 2020). Peak absorption time is approximately 2-4 hours, and onset of effects at 3 hours. Distribution volume ranges from 0.7 to 1.6L/Kg (Baselt, 2020). it is noteworthy to mention, renal disease will increase the volume of distribution and extend half-life. Oxazepam is rapidly conjugated with glucuronic acid and excreted in the urine. Oxazepam glucuronide, an inactive metabolite, is found to a limited trace amount in serum and accounts for 61% of an oral dose in the 48 hour urine (Baselt, 2020). Following detailed meta-analysis of 26 studies on plasma-concentrations, the DRUID project interpreted that 330µg/L of oxazepam was the corresponding level of impairment caused by 50mg of alcohol per 100ml of blood.

Diazepam is extensively metabolised via oxidative pathways into nordiazepam and temazepam which are then both metabolised into oxazepam. As a result, compliance monitoring is a cause for concern. Patients who are prescribed diazepam often will not test positive for the pro-drug but rather one or multiple of the pharmacologically active metabolised agents. Patterns emerge when patients take diazepam frequently as opposed to a prescribed as needed basis. Those taking diazepam regularly and over the long term

commonly test for all three metabolites (see Figure 8). It is often the case that high levels of the metabolites are detected. Infrequent diazepam users generally show oxazepam as the only active metabolite and it is not uncommon to find trace levels of oxazepam (<300ng/mL) even several days after a dose of diazepam (see Figure 9) (Gunn, 2015).

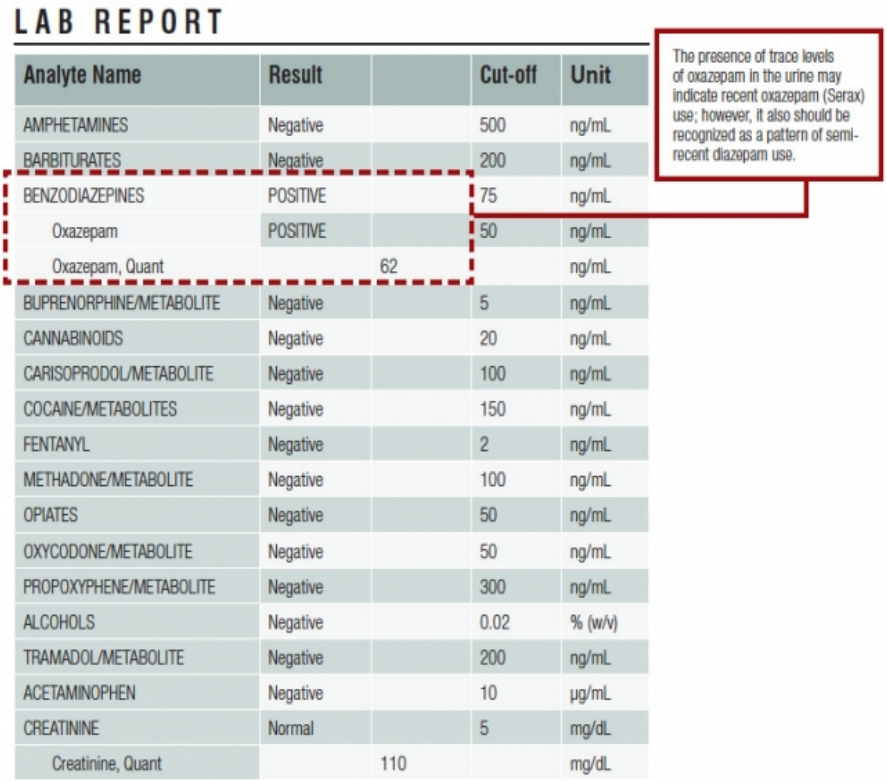


LAB REPORT

Analyte Name	Result		Cut-off	Unit
AMPHETAMINES	Negative		500	ng/mL
BARBITURATES	Negative		200	ng/mL
BENZODIAZEPINES	POSITIVE		75	ng/mL
Temazepam	POSITIVE		50	ng/mL
Temazepam, Quant		>2,500		ng/mL
Nordiazepam	POSITIVE		50	ng/mL
Nordiazepam, Quant		>2,500		ng/mL
Oxazepam	POSITIVE		50	ng/mL
Oxazepam, Quant		>2,500		ng/mL
BUPRENORPHINE/METABOLITE	Negative		5	ng/mL
CANNABINOIDS	Negative		20	ng/mL
CARISOPRODOL/METABOLITE	Negative		100	ng/mL
COCAINE/METABOLITES	Negative		150	ng/mL
FENTANYL	Negative		2	ng/mL
METHADONE/METABOLITE	Negative		100	ng/mL
OPIATES	Negative		50	ng/mL
OXYCODONE/METABOLITE	Negative		50	ng/mL
PROPOXYPHENE/METABOLITE	Negative		300	ng/mL

The presence of nordiazepam, temazepam, and oxazepam in the urine should be recognized as the "textbook" pattern of recent diazepam use.

Figure 9. tabulates a toxicology report for all three active matabolites present in the body from a frequent diazepam user (Gunn,2015).



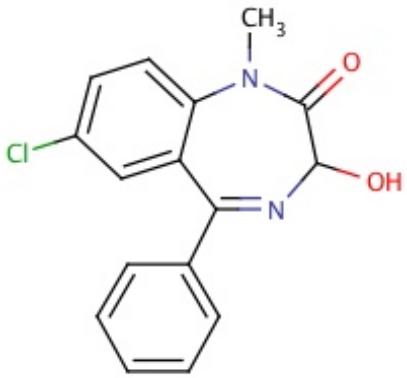
LAB REPORT

Analyte Name	Result		Cut-off	Unit
AMPHETAMINES	Negative		500	ng/mL
BARBITURATES	Negative		200	ng/mL
BENZODIAZEPINES	POSITIVE		75	ng/mL
Oxazepam	POSITIVE		50	ng/mL
Oxazepam, Quant		62		ng/mL
BUPRENORPHINE/METABOLITE	Negative		5	ng/mL
CANNABINOIDS	Negative		20	ng/mL
CARISOPRODOL/METABOLITE	Negative		100	ng/mL
COCAINE/METABOLITES	Negative		150	ng/mL
FENTANYL	Negative		2	ng/mL
METHADONE/METABOLITE	Negative		100	ng/mL
OPIATES	Negative		50	ng/mL
OXYCODONE/METABOLITE	Negative		50	ng/mL
PROPOXYPHENE/METABOLITE	Negative		300	ng/mL
ALCOHOLS	Negative		0.02	% (w/v)
TRAMADOL/METABOLITE	Negative		200	ng/mL
ACETAMINOPHEN	Negative		10	µg/mL
CREATININE	Normal		5	mg/dL
Creatinine, Quant		110		mg/dL

The presence of trace levels of oxazepam in the urine may indicate recent oxazepam (Serax) use; however, it also should be recognized as a pattern of semi-recent diazepam use.

Figure 10. tabulates a toxicology report for a semi-recent diazepam dosage where only trace levels of oxazepam are detected (Gunn, 2015).

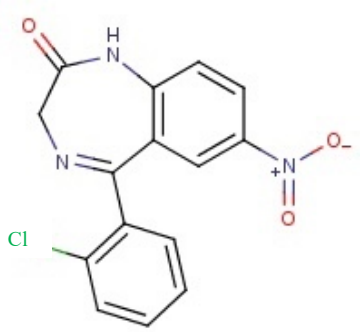
1.7 Temazepam



Temazepam has been used clinically as a hypnotic drug since 1979 (Baselt, 2020). At position 1 of the temazepam moiety an alky group is present allowing for maximal activity. The keto group as present in the majority of benzodiazepines can also be found in the temazepam structure giving it its characteristic properties. At position 3 a polar hydroxyl group being present allows for short action of the drug and can be excreted quickly via urine. Temazepam shows a short phase 1 reaction via a demethylation reaction forming oxazepam before being excreted. Position 4 to 5 has the unsaturated double bond to also allow for maximum effectiveness. At position 7, a chloride group is present allowing for good potency, however, not as potent as clonazepam and flunitrazepam which have the nitro groups in the same position.

As a hypnotic drug, temazepam is prescribed to aid sleep disorders as well as those suffering withdrawal from Class A drugs. Typical daily doses are 10-20mg with a peak absorption rate of 2-3 hours and a half-life of 8-13 hours. Peak plasma concentrations in the elderly given a 10mg dose averaged at 305µg/L, while in young healthy men averaged at 668µg/L with a 20mg dose (Baselt, 2020). Although a medium acting benzodiazepine, a common residual side effect can leave minor sedation up to 24 hours after consumption but does not significantly impair driving ability (Verster et al., 2004). Overdose may produce loss of consciousness and mild to moderate respiratory depression (Baselt, 2020). Fatal cases of temazepam, solely due to this drug, have been recorded in femoral blood concentrations at 10,000µg/L Akin to oxazepam, temazepam is a metabolite of other benzodiazepines such as diazepam and is itself demethylated to oxazepam and then further into glucuronide conjugates. The major metabolite of temazepam is the O-conjugate of temazepam (90%) and the minor being O-conjugate of N- desmethyl temazepam (7%). Excretion of temazepam was found to occur via urine (80%) and the faeces (12%)(Schwarz, 1979).

1.8 Clonazepam

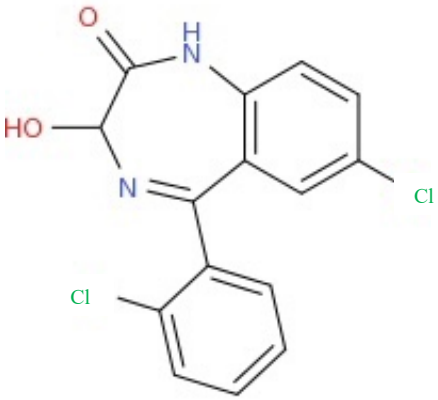


Clonazepam is a benzodiazepine derivate that was approved for use as an anticonvulsant in the US in 1975 Baselt, 2020). At position 1 a hydrogen atom is present attached to the nitrogen atom. At position 2, the essential carbonyl group required for pharmacological activity is present. There is no hydroxyl group present at position 3 that can be seen in oxazepam, temazepam and lorazepeam. As a result clonazepam is a long action duration drug. Position 4 to 5 has the unsaturated double bond to also allow for maximum effectiveness. Position 5 has the phenyl ring for peak action. In addition to this the aromatic ring has a electron withdrawing chloride group substituted at the ortho position to further increase its potency. Position 7 has an NO_2 group with electron withdrawing effects stronger than that of the halogens. As a result, the potency of the drug is even further increased. Out of the drugs in question clonazepam has the highest potency and is the longest acting in the body, without any active metabolites, with a half-life of up to 40 hours.

Daily dosage ranges from 0.5-2mg up to 4 times a day (Baselt, 2020). Entirely absorbed after oral administration this benzodiazepine sees greatest plasma concentration between 1 and 4 hours, with an onset of action in less than 1 hour. Distribution volume is typically 1.5-4.4L/Kg (Baselt, 2020). It is completely eliminated from the body at a typical range of 30 and 40 hours. Peak blood concentrations of clonazepam range from 6.5–13.5µg/L and were reached within 1–2 hours following a single 2 mg oral dose of clonazepam in healthy adults. (Riss, Cloyd, Gates and Collins, 2008). An acute clonazepam dose of 14 to 32mg taken orally produced a plasma concentration of 69µg/L which eludes toxicity symptoms such as antaxia (Welch *et al.,* 1977). Plasma level of more than 80µg/L is considered to be toxic while the therapeutic level ranging between 10 µg/L and 50µg/L. Coma-tose lethal doses were found to occur at 1000µg/L (Regenthal *et al.,* 1999)

With regard to driving, Clonazepam affects psychomotor performance by impairing flicker fusion, reaction times and memory (van der Meyden, 1989) and therefore, being in charge of a motor vehicle may increase risk of road traffic accident.

1.9 Lorazepam



A 3- hydroxy benzodiazepine, structurally related to oxazepam and temazepam has been used clinically as a anti-anxiety agent since 1971 (Baselt, 2020). At position 1 of the lorazepam structure a hydrogen atom is present attached to the nitrogen atom. At position 2, the essential carbonyl group required for pharmacological activity is present. At position 3 the hydroxyl group providing high polarity, similarly to oxazepam and temaepam, allows for short action of the drug and can be excreted quickly. Being highly polar the drug is excreted without phase 1 metabolism. Lorazepam is one of the strongest and most potent drugs on the market with a maximum half-life of up to 24 hours.

Pure lorazepam is a white powder that is almost completely insoluble in water and oil. For medical use lorazepam is prescribed mainly as tablets or a solution to inject via deep intramuscular injection or intravenous injection where absorption is rapid and complete. In some medical settings the drug can also be prescribed as a sublingual tablet which can reach peak drug concentration in 60 minutes (Fox *et al.,* 2011), or an oral solution or a skin patch. Dosage varies from patient to patient, however a maximum daily dose of 4mg is given with onset starting within 30 minutes. Peak absorption takes 2 hours (Greenblatt, 2012) with a distribution volume of 0.9-1.3L/Kg (Baselt, 2020)
Plasma concentrations for this benzodiazepine are typically measured between 300-600µg/L and toxic effects are seen at concentrations above 1500µg/L (similar to that of Diazepam). This correlates to an approximate administration of a 100-200mg dose. Lorazepam metabolises to the inactive glucuronide and 50% is excreted within 24 hours.

Common side effects of lorazepam are weakness and disorientation. Psychomotor skills are significantly impaired due to slowed reaction time and impaired flicker fusion and the magnitude is more extreme than that of diazepam (Clarkson et al., 2004).

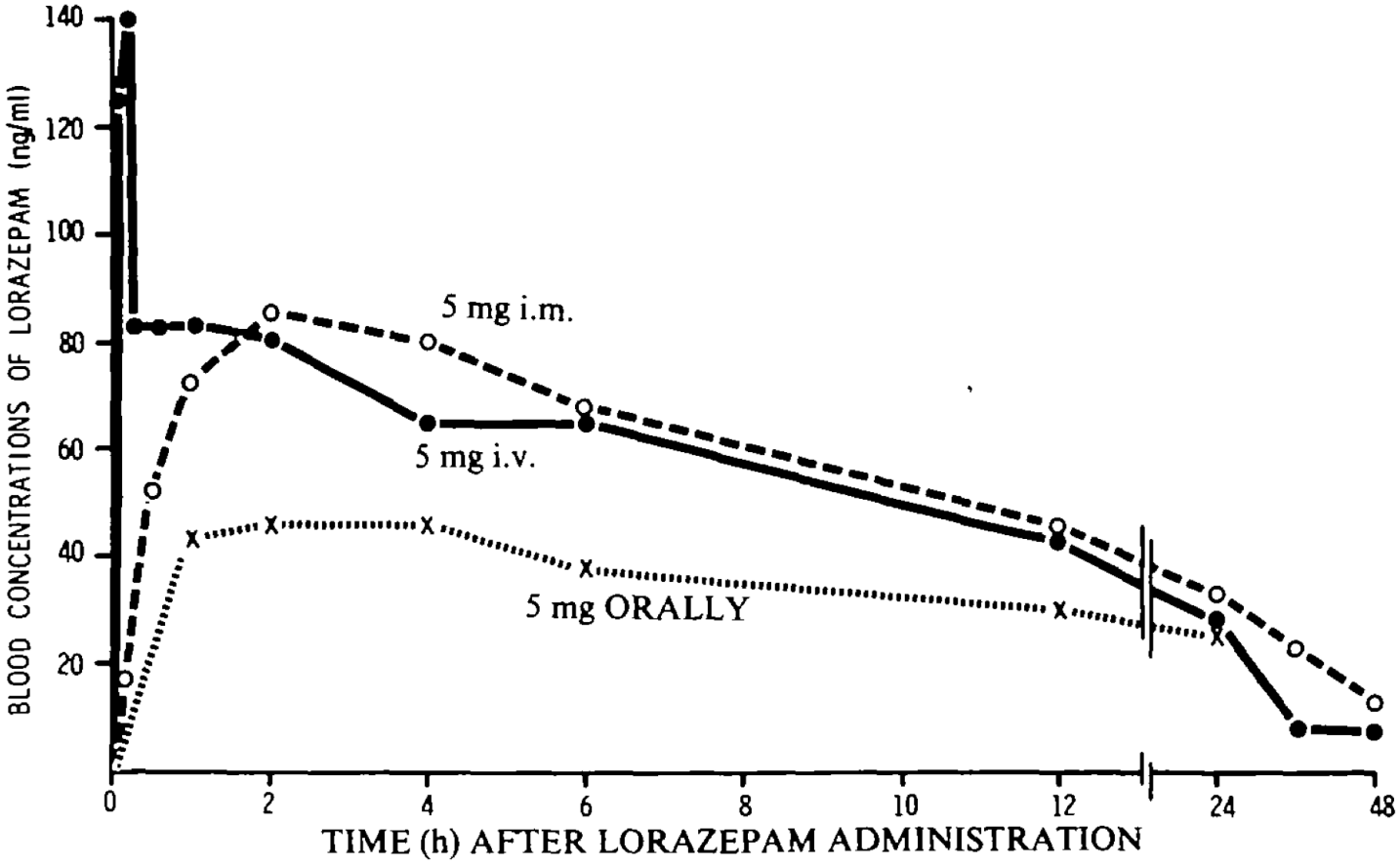
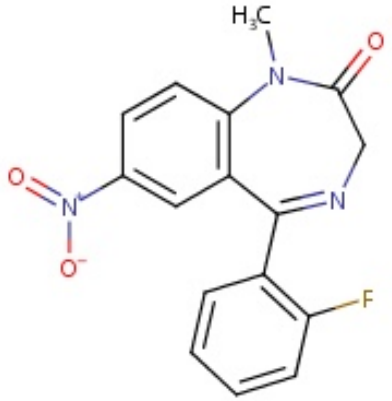


Figure 14. Comparison of blood concentration following intramuscular, intravenous and oral administration of 5mg single dose of Lorazepam. Note: ng/ml= µg/L (Elliot, 1976).

1.10Flunitrazepam



Chemical structure of Flunitrazepam- At position 1 a hydrogen atom is present attached to the nitrogen atom. At position 2, the essential carbonyl group required for pharmacological activity is present. Position 4 to 5 has the unsaturated double bond to also allow for maximum effectiveness. Position 5 has the phenyl ring for peak action. In addition to this the aromatic ring has a fluoro group substituted at the ortho position to further increase its potency. In position 7, the NO_2 group is highly electronegative and in dire need of electrons giving it strong electron withdrawing properties, thereby increasing its efficacy, giving the compound its strong characteristic sedative properties.

Flunitrazepam is used primarily to aid severe insomnia, as a pre medication for surgical procedures and/or for inducing anaesthesia. In addition to this, since the early 1990's it has also been used to reduce the depression that drugs such as methamphetamines and cocaine cause because of the muscle relaxation and anxiety reduction effects (Gambi et al., 1999). Dosages are typically in the range of 0.5-2mg due to its highly potent nature. Flunitrazepam is considered a medium acting benzodiazepine, however some studies may suggest it long acting due to its upper limit of half-life being 35 hours. Pharmacokinetic characteristics allow the drug to be rapidly metabolised to desmethyl flunitrazepam and 7-aminoflunitrazepam which are active and inactive respectively (Moffat, Osselton, Widdop and Watts, 2011). Peak plasma concentrations occur an hour after oral administration of a single dose while patients who administer the drug daily for a month or more, of continuous use can expect to see peak concentrations after 3 hours (Moffat, Osselton, Widdop and Clarke, 2004). The common concentration range for someone using this drug therapeutically at night is between 5 and 20μg/L and between 10 and 50μg/L for those who are arrested for DUI (Jones, Holmgren and Kugelberg, 2007; Robertson and Drummer, 1998; Baselt, 2008). Furthermore, some studies propose that high doses of flunitrazepam causes aggression in some indivduals (Bramness et al., 2006). This fact alone indicates the dangers of driving under the influence of flunitrazepam given its powerful hypnotic properties and being prescribed to use at night before sleep.

All of the above drugs used in conjunction with alcohol can increase the intoxication of alcohol. Not used as commonly in the UK compared to other European countries, between

the years 2001 and 2003, Norway was suffering a flunitrazepam epidemic. The number of suspected drug drivers under the influence of said drug increased three fold and number of cases peaked at approximately 2100 cases (Bramness, Skurtveit and Mørland, 2006).

1.11 DRUID report

The Driving under the influence of drugs, alcohol and medicines (DRUID) report is a research project undertaken in Europe and is a compilation of work which comprises of detailed information on research projects undertaken, epidemiological research, policing, classification of drugs and medicine, rehabilitation following conviction and the withdrawal of driving licences. In addition, information is present on dissemination and guidelines. (Schulze, 2013). The DRUID report findings suggest that the most frequently found substances that cause serious injury, following alcohol and THC, are benzodiazepines (alcohol serious injury range:14.1-30.2%, THC serious injury range: 0.5-2.3%, Benzodiazepines serious injury range: 0.0-2.3%) (Schulze, 2013). In addition to this, the study also found that accidents causing death was second highest for benzodiazepines, following alcohol (alcohol causing death range: 15.6-38.9%, benzodiazepines causing death range: 0.0-5.2%) (Schulze, 2013).

Based on roadside tests and experimental studies, it was found that both benzodiazepines and medicinal opioids were most prevalent in middle aged and older female drivers (generally > 35) (Olfson, King and Schoenbaum, 2015, Gerlach, Wiechers and Maust, 2018) . However, it is note-worthy to mention that the studies in the report which suggest serious injury or death by use of benzodiazepines is most prevalently found in male drivers aged >35 and most were detected with benzodiazepines in conjunction with other psychoactive substances such as alcohol and illicit drugs (Schulze, 2013).

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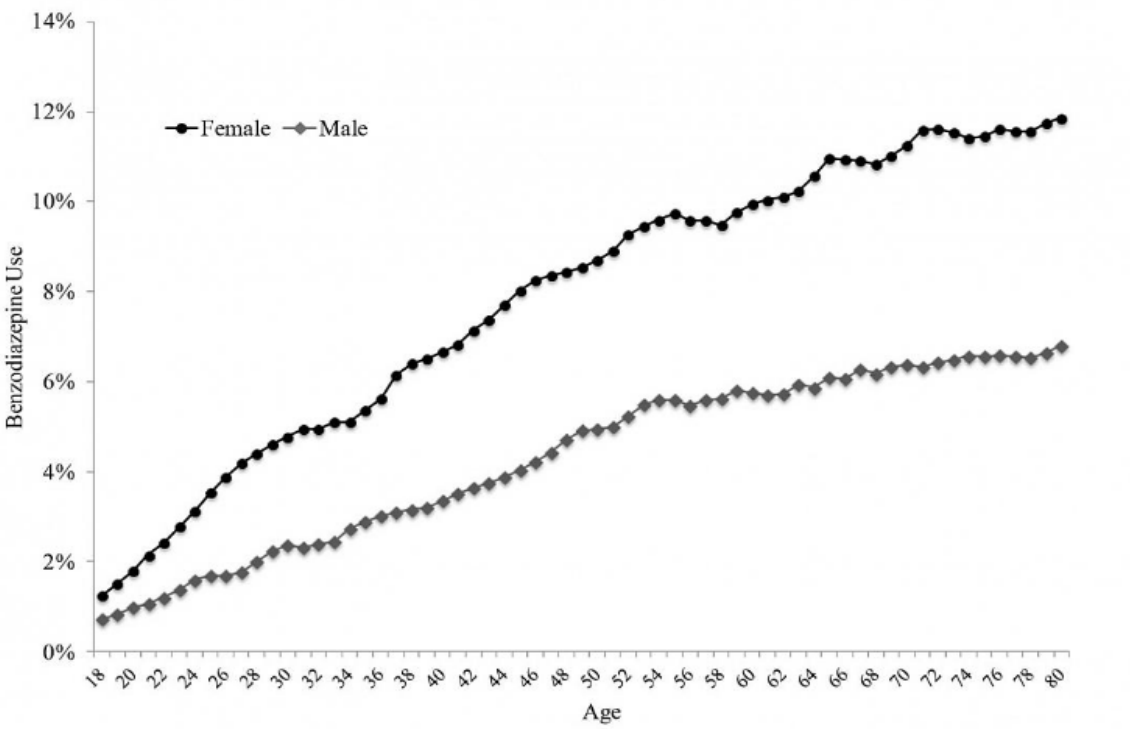


Figure 16. Graph exemplifying the percentage of the population who use benzodiapeines by sex and age. Data Source: IMS LifeLink Information Assets-LRx Longitudinal Prescription Database, 2008, IMS Health Incorporated.

Meta-Analysis is the examination of data from numerous different independent studies on the same subject which are collated and analysed to determine overall trend. In the DRUID project, 605 different publications were taken into account for medicines and illicit drugs and the results suggest that with increasing dosages, certain drugs (especially anxiolytics, antidepressants and sedatives) caused a high degree of impairment and negatively impacted motor skills (Schulze et al., 2012). In the study, degree of impairment was given a whole number integer with 0 being no impairment and going all the way up to 571 for the benzodiazepine lorazepam. All other drugs fell in between this range. The greatest impairment of a substance is a crucial factor when estimating the danger associated with use of that substance; however, consideration must be made to maximum impairment being of short or long duration. As a result, for each substance that had been the subject of sufficient experimental studies, the project used the impairment function to construct an impairment curve, showing the degree of impairment over time. Once the curve was constructed, the 'area under the curve' was calculated to capture both the level of impairment and the duration. To exclude minor effects, a line was drawn to represent impairment equivalent to 0.3 g/l BAC. The final measure of the danger of a substance, the 'degree of impairment', indicates the area between the approximation curve and the minor impairment line, thus capturing in a single parameter both the intensity (magnitude of impaired effects) and duration of impairment (DRUID, 2013).

In the case of diazepam, the most commonly prescribed benzodiazepine, a dosage of 5mg predicted a degree of impairment of 17. If this dosage is doubled to 10mg the predicted degree of impairment increases exponentially to 57, also the case at 20mg where degree of impairment increases to 171 signifying the rapid loss of driving skills with increases in dosage. Interestingly, however, the benzodiazepine temazepam which comes under the class of drug hypnotic and sedative causes 0 impairment from a 10mg dose. This increases to 40 when dosage is doubled to 20mg. The study suggests that lorazepam and flunitrazepam cause the most concern when looking at medicinal drugs as a 2mg dose of lorazepam causes a degree of impairment of 418 which dramatically increases to 571 (the highest number in the listing) when dosage is increased by a fractional 0.5mg. For a 2mg dosage of flunitrazepam, it is even higher with a degree of impairment of 461 in comparison to 418 for the same dosage of lorazepam (Schulze et al., 2012).

The DRUID project also evaluated roadside screening devices to determine if enforcement officials can confidently use these systems to screen motorists for drug use and subsequently convict drug-drivers. All devices screened oral fluid as it provides the most accurate information on recent drug use in both practical and analytical terms (Schulze, 2013). In addition to this, clinical signs indicating drug use such as uncoordinated movement and bloodshot eyes were appraised to see if these indicators are an effective means of pre-screening suspected drivers. This was done due to the time and monetary cost of doing roadside oral tests. In addition to this, in the UK roadside screenings for benzodiazepines are not carried out as oral fluid cannot confirm that the individual is impaired from driving at that moment in time, only a blood sample can confirm this (Drummer, 2009). The evaluation focused on aspects relating to the above-mentioned points in addition to hygiene aspects as well as an officer's view of ease of use of the device. Of 13 devices used the results showed that 8 of them were cost beneficial.

The approach the board took was setting a specific limit for each of the drugs based on potency and its overall effect on driving skills at different concentrations. As a result, any

driver found guilty of exceeding said limit could be prosecuted without the prerequisite that he/she was impaired from being in charge of a motor vehicle. However, due to certain medicinal drugs, primarily benzodiazepines, being prescribed so widely for both short- and long-term use (at least a million people prescribed in 2005) (Ashton, 2005); considerations had to be made. The board coordinating the Wolff report evaluated epidemiological and experimental research studies, in relation to blood drug concentrations and driving behaviour. Considerations were made taking into account practical and ethical implications. In contrast to this, the board had not only the prescribed patients to consider, when setting limits, but also the illicit use of benzodiazepines. According to the 'World Drug Report' of 2011, benzodiazepine seizures had increased by more than 50 percent in 2011 alone; implying these drugs are being sold outside the channels of health authorities and through illicit means often in the form of illegal online pharmacies (Wolff *et al*., 2013). In turn, making as close to true estimations on the extent of illicit benzodiazepine use is problematic.

Findings from the vast drug drive research compiled in the Wolff report found that those using benzodiazepines posed a 62% increase in risk of road traffic accidents compared to those who do not take benzodiazepines. Reviews suggest this value can increase to 290% (Engeland, Skurtveit, & Morland, 2007) In addition to this, findings also suggest that long acting benzodiazepines will further increase the risk of road traffic accidents in comparison to their short acting counter parts highlighting an increased need and awareness for those driving following benzodiazepine ingestion (Barbone et al, 1998). One study carried out by Orriols (2009) which evaluated 3 French national databases over a 3-year period (2005-2008) comprising of 72,000 drivers found that those with prescriptions for 3 or more benzodiazepines posed a higher risk of accident as well as those taking anxiolytic benzodiazepines over hypnotics. Those taking anxiolytics were more at risk of causing road traffic accidents regardless of half-life (Dassanayake et al, 2011). Another study by Thomas (1998) had findings highlighting that those aged 65 or over would be an increased hazard to the road when taking longer acting benzodiazepines and consuming them in larger amounts. The National institute of Forensic Toxicology analyses 818 Danish drivers who were suspected of driving under the influence of drugs or alcohol and found that benzodiazepines were detected in impaired drivers at supra therapeutic concentrations in blood as opposed to levels that would be seen under normal prescribed levels.

After evaluating all of the data and research the panel for the Wolff report recommended threshold limits for those benzodiazepines which posed a greater risk of road traffic accidents, especially when taken above therapeutic guidelines. The threshold recommended for diazepam was 550µg/L, clonazepam 50µg/L, lorazepam 100µg/L, flunitrazepam 300µg/L. Special consideration was taken when setting limits for oxazepam (300µg/L) and temazepam (1000µg/L) based on the understanding that these drugs are common metabolites of other benzodiazepines.

1.13Fixed driving limits England and Wales

The Wolff report provides detailed information on driving under the influence of an array of drugs and is supported by the department of transport. The panel in charge of assembling data, results and findings brought together expert advice on the matter. Prior to 2020, impairment had to be proven via means of a Field Impairment Test (FIT) test. This method was deemed unsuitable as certain tasks during the test were too difficult and could result in a conviction even if those suspected were not drug impaired. Blood concentration analysis has been proposed as a more effective method (O'Keefe, 2013) due to allowing broader spectrum testing and better correlation in blood and pharmacological effect over other analysis of urine or saliva samples (Langel et al., 2014). Time delay, storage conditions and degradation are also important factors to consider. Due to the need of a professionally trained medic to withdraw a sample of blood, there is often a time delay between a drugged driver being stopped, assessed, arrested, taken back to the station and then a sample of blood taken. This is likely to have an effect on blood concentration levels as the benzodiazepines metabolised; with emphasis on the shorter acting drugs. Furthermore, a study by (Melo, Bastos and Teixeira, 2012) found that freezer conditions allowed stability of concentration for 6 months. However, at positive temperatures such as fridge or room temperature, significant degradation took place. Analyte degradation before analysis if often a result of chemical or physical decomposition due to drug instability. An example of this is diazepam is prone to hydrolyisis while flunitrazepam is associated with substantial degradation in a biological sample within a day when exposed to sunlight (Benhamou-Batut, 1994). Thus, incorrect storage conditions will significantly impact final blood concentration levels calculated. As a result, a new approach with significant improvement on efficiency is required.

1.14 Section 5A of Road Traffic Act

The effects of benzodiazepines on driving have been recorded extensively, demonstrating that they contribute to an increased risk of road traffic collisions and research supports this view (Dassanayake *et al*., 2011). Section 5A came into force on 2nd March 2015 and has specified new concentration limits for controlled drugs, when driving. The drugs are broadly categorised into two groups; medicinal drugs and drugs of abuse. The medicinal drugs have been given a relatively high limit utilising a risk threshold methodology in order to not dissuade patients from using prescribed medication. For the drugs of abuse a zero-tolerance approach has been utilised which only take into account levels for accidental exposure (Rooney *et al.,* 2016). Rooney *et al* (2016) found that the majority of drivers caught under the influence of drugs had concentrations of benzodiazepines under the threshold stipulated in Section 5A. Of 545 samples, those found driving under the influence of diazepam (limit: 550µg/L) 38.2% of those were found to be over the limit with a mean concentration of 619µg/L. In relation to oxazepam (limit: 300µg/L) 14.7% of samples were found to be over the limit with a mean concentration of 173µg/L. With regard to temazepam (limit: 1000µg/L) only 5.8% of samples were found to be over the limit with a mean of 280µg/L. In terms of lorazepam, this was found in less than 10 cases and did not warrant inclusion in this study. Irrespective of this, under the new legislation an automatic defence is included for motorists who can prove their benzodiazepine intake is in accordance with their prescription.

Table 2: List of drugs stipulated in Section 5a of the Road Traffic Act and their respective limits in blood. (Forensic Science Regulator FSR-C-133. 2016)

Controlled Drug	Legal Limit (µg/L)	FSR expanded uncertainty (99.7% confidence level (%)	Common reporting threshold (µg/L)
Amphetamine	250	20	314
Benzoylecgonine	50	20	64
Clonazepam	50	20	64
Cocaine	10	35	17
Delta-9-Tetrahydrocannabinol	2	30	3
Diazepam	550	20	689
Flunitrazepam	300	25	402
Ketamine	20	20	27
Lorazepam	100	25	135
Lysergic Acid Diethylamide	1	45	2
Methadone	500	25	668
Methylamphetamine	10	40	1
Methylenedioxymethamphetamine	10	25	15
6-Monoacetylmorphine	5	35	8
Morphine	80	25	108
Oxazepam	300	20	377
Temazepam	1000	20	1257

There are approximately 20 million prescriptions issued every year for the drugs covered in the offences under Section 5a; as surveyed by the department of transport. A 2016 study collected blood samples from drivers during 2010-2012 and found that benzodiazepines are the second most common drug in driving under the influence of drugs cases in the United Kingdom (Rooney *et al.,* 2016). Due to the public health risk associated with these drugs, it is imperative to have strict guidelines on prescribed use and zero tolerance for recreational use. To further support the need for such an approach, a study suggests Drug recognition experts (DRE) observations are least likely (after alcohol) to spot CNS depressant use (41%) (Talpins and Hayes, 2004). It is noteworthy to mention, DRE do not operate in the UK, however, they use a similar Field Impairment Test (FIT) which both include the modified romberg balance, the walk and turn, the one leg stand, and the finger to nose test. Anyone arrested for these offences can offer a medical defence stipulating the drug was lawfully prescribed, supplied or purchased over the counter for medical purposes and was taken in accordance with advice given by the prescriber.

1.15 Chromatographic Analytical Techniques

The combination of chromatographic analytical techniques has been intensively researched and sought after for decades due to its accurate, sensitive and highly specific nature. Due to certain limitations and irreconcilability issues regarding the existing mass spectrometer ion sources of the era and the need for a continuous liquid stream; progress in the field was very limited. Nonetheless, research continued, and methods were built but the unreliability and temperamental nature of these first-generation methods meant uptake in clinical settings were minimal. This changed in the 1980's when analytical chemist John Fenn heavily contributed to and developed the electrospray ion source (Fenn et al., 1989). By the 1990's, reliability and performance drastically improved as well as the price of the technology resulting in a huge number of laboratories, albeit mainly biochemistry centres, taking on this new technology. Although its primary major use was previously in the field of biochemical genetics and analysis of neonatal blood (Rashed et al., 1997), Liquid Chromatography- Mass Spectrometry (LC-MS) has become a standard in many fields owing to its ability to analyse a broad range of biological molecules over its previously developed counterpart; GC-MS. In addition to this, the steady increase in requiring LC separations in laboratories has also contributed to its success as LC-MS is more specific and can handle complex matrices better than LC alone (Ho et al. 2003). Added to this, one study found that although GC-MS has been the gold standard for drug analysis for a long time, LC-MS provides specific advantages such as ease and speed of sample extraction as well as shorter run times (Perez et al., 2016). Research suggests that studies where it is possible to use GC–MS with cold EI improves all the central performance aspects of GC–MS. This includes improving sample identification via the provision of enhanced molecular ions combined with improved mass spectral isomer and structural information (Kachhawaha et al., 2017).

1.16 High Performance-Liquid Chromatography

Chromatography has many forms and is an indispensable separation technique for the analysis of one or more components from a mixture that contains simple and/or complex molecules. It is a method based on the principle where molecules in a mixture are applied through the surface of a solid whereby the stationary phase separates from each other while moving with the aid of the mobile phase. Factors effecting the separation are the molecular characteristics such as adsorption, partition and affinity among their molecular weights (Goswami, 2015).

A form of column chromatography which utilises high pressure to push a sample (the analyte) which is dissolved in a solvent (mobile phase) through a preconditioned column with a chromatographic packing material (stationary phase). HPLC separates the individual components of a sample on the basis of retention time. Retention time and elution time is dependent on the properties and nature of the analyte, mobile phase and stationary phase. When moving through the column, the analytes which have the strongest attraction for the stationary phase will elute slowest; thereby taking longer to pass through the column and resulting in a longer retention time. Conversely, those analytes with weakest interactions with the stationary phase will elute fastest with a shorter retention time. This process can be done via two methods. Gradient or isocratic elution. The latter ensures the makeup of the mobile phase remains constant for the whole process. However, the former changes the composition of the mobile phase, depending on user preference, allowing for dissociation from the stationary phase favouring analyte conditions (Olga and Karin 2017).

1.17 Ionisation Sources of Mass Spectrometers

Mass spectrometry relies on the principle of recognising an analyte molecule by charging it to an ionised state (either positive or negative ionisation mode) followed by analysis of the charged molecule. Many different mass spectrometer technologies exist which induce the ionisation of a parent molecule such as electron impact, chemical ionisation, fast atom bombardment, thermospray and electrospray (ESI) to name but a few (Keshishian *et al.,* 2007). The two most common are ESI and Atmospheric Chemical Ionisation (APCI).
The ESI technique is amongst the most popular of the ionisation techniques as it is appropriate for a wide range of drug types and can produce multiple high molecular liquid samples are pushed through a metal capillary while being sustained at a voltage of between 2 and 5kV. The capillary tubing is positioned at an off-axis angle to the entrance of the mass spectrometer machinery in order to minimise contamination. The resulting formulation of highly charged droplets are then evaporated off via heat and dry nitrogen. Residual charged molecules then desorb from the sprayed droplets and are transferred through small apertures through the rest of the mass spectrometer which has a high vacuum (Kebarle, 2000). The use of ESI has specific advantages over other ion sources such as the limited fragmentation occurring due to only a small amount of energy required for the analyte. Therefore, we consider this method a 'soft' ionisation source. The primary advantage of this is further fragmentation can be made to occur if required by increasing voltage which works via increased collisions with nitrogen (Pitt, 2009). Furthermore, ESI is a process which is easy to use and interface with other analytical techniques such as HPLC.

1.18 Triple Quadrupole Mass Spectrometer

Triple Quadrupole mass spectrometer consist of three separate segmentations, Q1, Q2 and Q3. The first quadrupole, Q1, is an efficient mass filter which allow for specific m/z to pass through while diverting all others to waste. These m/z are the downstream products of the ionised parent molecule from the ES ion source. Specificity is high for the triple quad due to its utilisation of two mass filters consecutively where Q1 identifies and selects the parent ion. Q2 is known as the collision cell where ions from the former quad are selected and collided with a noble gas in order to produce fragment ions. The third quadrupole, Q3, selects the fragment m/z. In order for optimal efficiency the triple quad requires two parameters optimised for each of the target metabolites. Once parameters are established each metabolite is measured using a 'selected reaction monitoring' (SRM) scan event where the parent ion produces a specific fragment ion at that specific collision energy. When multiple metabolites are involved an MRM approach is used which also takes into account multiple product ions for one parent ion. The main advantages of MRM are high sensitivity, due to the two MS steps, as well good linear dynamic range; owing to the efficacy of the quadrupoles and ion detectors (Crutchfield, Lu, Melamud and Rabinowitz, 2010).

1.19 Method Development

Due to a high usage of benzodiazepines worldwide, there is a large body of research focused on the development of innovative, highly sensitive, and accurate methods to analyse this drug class and their inactive metabolites. The analysis of benzodiazepines in biological fluids is vital in clinical assays as well as in forensics and toxicological studies (Qriouet et al., 2019). Analytical method development and validation can significantly impact cost efficiency as well as the research time taken. It is an ongoing process that runs parallel to the research, by means of monitoring process efficiency so relevant adjustments can be made. The overall goal of method development is to ensure that the method goals are feasible before ultimately being subjected to a validation programme. During method validation ensuring parameters of the method are clearly defined and limitations and capabilities of the method are detailed. In the scope of this project, the office for Forensic Science Regulator have stipulated the requirements, common approach, analysis and reporting of the concentrations of certain drugs in relation to offences under Section 5A Road Traffic Act. This guidance comes in the form of a document titled 'The analysis and reporting of Forensic Specimens In relation to S5A Road Traffic Act 1988 FSR-C-133 *Draft* (2016)*'* to aid analysis of samples. The use of limits guidance is stipulated in the document 'Section 5A Road Traffic Act 1988 Use of limits FSR-G-221 (2020)', while the validation parameters and guidance can be found in 'Validation FSR-G-201 (2020)'. Laboratories undertaking analysis of blood samples must also comply with quality standards as follows: It shall be accredited to ISO 17025 [A]. The analysis of blood samples shall be specifically listed in the scope of accreditation. It shall comply with the codes of practice and conduct. The laboratory should comply with the guidance on forensic toxicology issued by the United Kingdom and Ireland association of Forensic Toxicologists.

Method development involves numerous stages and can take a number of months before it is fully complete. The procedure typically includes sampling, sample preparation, analysis, calibration and data evaluation and reporting. This process of bioanalysis can take time due to the complex nature of matrices, such as blood and plasma, and require rigorous sample preparation prior to injection into an analytical instrument. Information about the sample's chemical properties, reactivity, volatility, polarity and stability should all be investigated and compiled before carrying out field work. Once this is done a suitable sample preparation technique has to be devised which give the cleanest extractions with the highest efficiency. Alongside this, internal standards which can effectively compensate for matrix effects must be chosen in order to achieve accurate results. Analyte concentrations also need to be known to decide upper and lower limits of detection and quantification.

Although a vast array of extraction techniques are well established in the process of method development and validation, such as Protein Precipitation (PPT), Solid phase microextraction (SPME), Stir bar sorptive extraction (SBSE), Microextraction by packed sorbent (MEPS), the most common extraction techniques are still Liquid- liquid extraction (LLE) and Solid phase extraction (SPE) due to their universal applicability in most areas of study.
Solid phase extraction works through the mixing of the sample (in the liquid phase) and an adsorbent (in the stationary phase). The first step will require conditioning of the stationary phase column where the site of retention is wetted via means of an organic solvent. Following

this, in order to improve extraction yield, washing is required whereby interferences that are weakly attached are removed and cleaned out. During the elution phase it is recommended to use a solvent that will elute the metabolites of interest at the lowest flow rate possible, so any interferences more strongly retained on the inside of the column are not also eluted (Qriouet et al., 2019).

SPE is the most widely used sample preparation method given its quick process, high reproducibility rates and the ability to extract compounds that are generally difficult to extract. Disadvantages of SPE are its high cost and its effect on the environment due to the use of plastics.

LLE is commonly used for any preparation involving aqueous and biological samples (El-Beqqali and Abdel-Rehim, 2016; Højskov, Heickendorff and Møller, 2010). During sample preparation an aqueous sample containing the analyte and an immiscible organic solvent are mixed to transfer the analyte into the organic solvent (Ashri and Abdel-Rehim, 2011). This method has proven to give good recovery rates. There are some disadvantages of this procedure such as time consumption and a high environmental cost due to large amounts of toxic solvents being used. As well as this, LLE is not suitable for the extraction of multiple analytes with varying degrees of polarity from the same sample (Moein, El Beqqali and Abdel-Rehim, 2017). However, as a simple low-cost extraction procedure, it is still commonly used either as the sole extraction technique or a sample preparation precursor to SPE.

1.20 Method Validation

Method validation has a crucial role and is the most important step in regulatory affairs to ensure the quality of the applied method is sound, not only at the time of development but continuously after long periods of use. For the analytical method to be validated for road traffic toxicology, it must accurately detect and measure the benzodiazepines of study well within the range, legislated under Section 5a. Parameters for method validation are required to be in line with the requirements and recommendations set out in the Forensic Science Regulator FSR-C-133 document and must include method accuracy (the closeness of the calculated value to the true value) and acceptance criteria, method precision (closeness of repeated measurements) [Coefficient of Variation (CV) or Relative Standard Deviation (RSD)] and method sensitivity (Limit of Detection, Upper and Lower Limits of Quantification); LOD is the lowest concentration of the analyte that can be reliably differentiated from background noise while LOQ is the lowest concentration that can be reliably quantified. Method selectivity is the techniques ability to identify the compound of interest in the presence of other similar components in the biological sample (such as other benzodiazepine drugs) and other related potential interferences. A further requirement for rigorous method validation is the quantification of matrix effects, these chiefly refer to ion suppression and enhancements. Matrix effects is defined as the interference in a signal due to impurities in the biological sample. In the case of blood samples this can be plasma proteins, minerals or vitamins that may elevate or suppress the ionisation signal. Matrix effect studies which quantify the level of matrix effect can be quantified by using either post column infusion or post extraction spike, the former is a qualitative analysis that predicts where on the chromatographic run an interference may occur while the latter is a quantitative

assessment on the level of suppression/enhancement. Recovery of each drug and acceptance criteria. Stability of each drug (ensure that the drug of interest is not compromised throughout the analysis process). This includes stability pre-analysis and post analysis storage (including any freeze/thaw cycles if appropriate). Calibration method and acceptance criteria, where weighted curves are used for calibration the laboratory must be able to experimentally justify the weighting used. Robustness (effect of minor variations, for example variation in analyst, calibration standard, operating temperature) (Forensic Science Regulator FSR-C-133, 2016).

1.21 Objectives

Currently there is a lack of methods that can reliably analyse a range of benzodiazepines. As a result, the development and validation of a method that can quantify benzodiazepines, namely diazepam, oxazepam, temazepam, clonazepam, lorazepam and flunitrazepam in a single run would with a short run time would be a benefit for casework laboratories. These benzodiazepines have been chosen as they have reliable reference materials and are amongst the most predominantly detected and commonly abused of this class of drugs in the UK. In addition to this, the aforementioned benzodiazepines are all stipulated in Section 5A of the road traffic act.

- The criteria which the method will be validated will be in line with ISO 17025 recommendation for method validation.
- Method development will be carried out by doing scouting runs to find ions.
- Validation experiments carried out will be accuracy, precision, linearity, reliability, repeatability, LOD, LOQ and specificity.
- This research project will assess matrix effects, recovery rates as well as stability parameters.
- Results shall be reported in units of micrograms per litre where applicable to facilitate comparison against the legal limits as recommended by FSR-C-133.

2 Materials and Methods

2.1 Reagents and Chemicals

The following reagents and chemicals were employed in this project; LCMS grade water (Fisher Scientific), Formic acid (Sigma Aldrich), LCMS grade acetonitrile (Fisher Scientific), Acetic Acid (Fisher Scientific), HPLC grade methanol (Fisher Scientific), HPLC grade methanol (Fisher Scientific), ethyl acetate (Fisher Scientific) , acetyl acetate (Fisher Scientific), distilled water.
Cerilliant solutions: Diazepam 1.0mg/mL, Oxazepam 1.0mg/mL, Temazepam 1.0mg/mL, Lorazepam 1.0mg/mL, Clonazepam 1.0mg/mL and Flunitrazepam 1.0mg/mL; internal standards, Diazepam-D5 100µg/mL , Oxazepam-D5 µg/mL, Temazepam-D5 µg/mL, Lorazepam-D5 µg/mL, Clonazepam-D8 µg/mL, Flunitrazepam-D8 µg/mL.

2.2 Reagent Preparation

The following reagents were made and used for sample preparation and extraction.
Internal standard (working solution (WS) E)
To a 2.5mL volumetric flask add diazepam-D5 100µg/mL (100µL) , oxazepam-D5 µg/mL (50µL), temazepam-D5 µg/mL (125µL), lorazepam-D5 µg/mL (20µL), clonazepam-D8 µg/mL (10µL), Flunitrazepam-D8 µg/mL (50µL) and then make up to 1mL using methanol.

Table 3: Preparation of Internal standards (working solution E)

Internal standard	Added volume of internal standard (mL)	Volume of Methanol made up to (mL)	Final concentration (µg/L)
Temazepam	2.5	5	500
Oxazepam	1	5	200
Lorazepam	0.4	5	80
Flunitrazepam	1	5	200
Diazepam	2	5	400
Clonazepam	0.2	5	40

Solution for calibrator (WSA)
To a 5mL volumetric flask add diazepam 1.0mg/mL (50µL), oxazepam 1.0mg/mL (50µL), temazepam 1.0mg/mL (125µL), lorazepam 1.0mg/mL (20µL), clonazepam 1.0mg/mL (10µL) and flunitrazepam 1.0mg/mL (50µL). Make the volume up to 5mL using methanol.

Table 4: Preparation of Drug analytes (working solution A)

Analyte	Added volume of analyte (mL)	Volume of Methanol made up to (mL)	Final concentration (µg/L)
Temazepam	125	5	25000
Oxazepam	50	5	10000
Lorazepam	20	5	4000
Flunitrazepam	50	5	10000
Diazepam	100	5	20000
Clonazepam	10	5	2000

Borate buffer (pH 9)
Two solutions A and B made up. Solution A: to a beaker add boric acid (6.18g) and potassium chloride (7.46g) and then dissolve into 100mL milli-Q water. Solution B: dissolve sodium carbonate (10.6g) into 100mL milli-Q water. From solution A remove 63mL and from solution B remove 37mL and place into separate beaker. Stir through thoroughly.

2.3 Preparation of calibration line and quality control samples

Table 5: *Volumes of WS used for calibrators and quality controls to spike blood for extraction*

Calibrator /QC	Volume	Working solution to use	Volume	Working solution to use	Final Concentration diazepam (µg/L)	Final Concentration Oxazepam (µg/L)	Final Concentration Temazepam (µg/L)	Final Concentration Clonazepam (µg/L)	Final Concentration Lorazepam (µg/L)	Final Concentration Flunitrazepam (µg/L)
Cal 1	10µL	A	30 µL	E	200	100	250	20	40	100
Cal 2	20µL	A	30 µL	E	400	200	500	40	80	200
Cal 3	30µL	A	30 µL	E	600	300	750	60	120	300
Cal 4	40µL	A	30 µL	E	800	400	1000	80	160	400
Cal 5	50µL	A	30 µL	E	1000	500	1250	100	200	500
Cal 6	60 µL	A	30 µL	E	1200	600	1500	120	240	600
QC low	15µL	A	30 µL	E	300	150	375	30	30	150
QC high	35µL	A	30 µL	E	700	350	875	80	80	350

2.4 Sample preparation and extraction

Sample preparation
Samples, calibrators, blanks and quality control (QC) standards had a starting volume of 1 mL of whole blood in 15mL conical *centrifuge tubes*. Internal standard (30 µL) from WSE was added to all samples, calibrators, quality controls and vortexed thoroughly for ≈1min. Following this WSA was used to add 10 µL, 20 µL, 30 µL, 40 µL, 50 µL, 60 µL, 15 µL, and 35 µL to each separate conical centrifuge tube before being vortexed for ≈1min each. Borate buffer (145 µL) was then added to each tube before being vortexed for a further 5 mins. Ethyl acetate (1.5 mL) was added to each tube before centrifuging at 3500 rpm for 5 mins. Following this, the supernatant was removed and ≈1 mL was placed in a Agilent vial. The liquid was evaporated via use of nitrogen gas in a sample contactor at 40°C before adding 250 µL with mobile phase B. After sample preparation LLE extraction was performed as described below.

2.5 Instrument and laboratory equipment

LC; Agilent 1260 Infinity Binary Pump
MS: Agilent Triple Quad LC/MS 6430
Column measurements: Synergi 4 µm Fusion-RP 80 A LC Column 150x2mm
15mL conical Centrifuge tubes from Fisher Scientific, utilised in sample preparation
5mL LABCO vials
Pipettes: VWR positive displacement pipette 1 -10 µl, VWR positive displacement pipette 10 -100 µl, VWR positive displacement pipette 100 -1000 µl.

2.6 Instrument parameters

The parameters for ion source were: Positive electrospray, gas temperature at 350°C, gas flow at 12L/min, nebulizer 25 psi, capillary +4000V. EMV+ 400.
The parameters for LC: Mobile phase A 1.0L water, 1mL 0.1% formic acid, 0.15g acetyle acetate. Mobile phase B 1mL 0.1% formic acid, 250mL acetonitrile. Temperature 20°C, stop time for LC pump 10.6 min, injection with needle wash (acetonitrile as wash solvent), injection volume 20µl, injection draw position -1.6mm, injection draw speed and eject speed 200µl/min, auto sampler temperature 20°C.

3. Results

3.1 Development of an analyte library

Post column infusion with a syringe pump at the speed of 5µL/min and MassHunter Optimizer software was used to construct the analyte database. The settings for the ion source were the same for all analytes, as described in the method section. All analytes were infused individually with the software settings as described in Table 6. All samples were made in 50:50 (mobile phase A, water 0.1% formic acid and mobile phase B, acetonitrile 0.1% formic acid mix) for infusion at a concentration of 100ng/mL. The optimiser software identifies the parent ion and its respective optimum fragmentation energy and collision energy for the as well as quantifier ion transitions (MRM). The results of all compounds precursor ions and product ions and energies are presented in Table 7.

Table 6: Optimizer parameters used for infusion benzodiazepines

Parameter	Value
Optimization dwell time	20ms
Fragmentor course range	60-180
Collision energy range	10-70
Low mass cut-off	50m/z

3.2 LC Gradient Development

The development of the LC separation method was based on literature research of methods that previously analysed benzodiazepines. The Phenomenex C18 Synergi Fusion (Reverse Phase) column was selected; this was chosen as it displayed good retention of polar analytes and is aqueous stable with TMS capping that ensure column durability and good peak resolution. The solvents used consisted of a Aqueous mobile phase A (water 0.1% formic acid) and an organic mobile phase B (acetonitrile 0.1% formic acid), with conditions for the isocratic gradient described in (Table 8). The gradient achieved adequate separation for the compounds, with the exception of the IS of Clonazepam and Flunitrazepam, to provide sufficient co-elution the run time was extended from 0.35 to 0.45mL/min. The final optimised chromatography method is described in Table 9.

Table 7: Compounds and precursor ions and product ions.

Analyte name	Parent ion m/z	Qualifier ions m/z	Ion ratios
Diazepam	285.1	193.1 154.1 222.2	48.2 83.15
Diazepam-D5	290.1	198.2	
Lorazepam	321	275.2 194 229	68.95 54.7
Lorazepam-D4	325.1	297	
Oxazepam	287.1	241.1 269.1 103.9	68.2 36.85
Oxazepam-D5	292.1	246.2	
Clonazepam	316.1	270.2 214.1 241.2	18.45 30.4
Clonazepam-D4	320.1	274.2	
Temazepam	301.1	255 283.3 177.1	32.35 20.55
Temazepam-D5	306.1	260.3	
Flunitrazepam	314.1	268.2 239.2 183	37.55 23.15
Flunitrazepam-D7	321.1	275.2	

3.3 Method Optimisation

Following testing of different LC gradients, an optimal gradient was found. Optimisation of the method was then carried out to accomplish accurate identification and quantifications of all drugs in a single runtime. Ion source optimisation was achieved through testing of different flow rates and nebuliser pressures, both of which affected sensitivity. Reconstitution volume was set at a volume of 250µL in order to provide enough volume for injection and reinjection if required. The reconstitution liquid used was mobile phase B (acetonitrile, 0.1% formic acid), throughout the duration of the project as it attained the best peak resolution.

***Table 8**: Original LC Gradient Elution Method Parameters*

Time (min)	Mobile Phase A	Mobile Phase B	Flow rate (mL/min)	Max pressure (kPA)
0.00	70	30	0.35	500
1.00	70	30	0.35	500
2.50	50	50	0.35	500
6.00	50	50	0.35	500
6.50	70	30	0.35	500
8.00	70	30	0.35	500

***Table 9**: Amended LC Gradient Elution Method Parameters*

Time (min)	Mobile Phase A	Mobile Phase B	Flow rate (mL/min)	Max pressure (kPa)
0.00	70	30	0.45	500
1.00	70	30	0.45	500
2.50	50	50	0.45	500
6.00	50	50	0.45	500
6.50	70	30	0.45	500
8.00	70	30	0.45	500

During the method development stages co-elution issues arose with clonazepam and flunitrazepam internal standard. Temperature, pressure and run-time parameters were changed to no affect. Following this the flow rate was amended from 0.35µl/min to 0.45µl/min which rectified the issue.

3.4 Method Validation Plan

Table 10: Validation table (Forensic Science Regulator FSR-C-133, 2016)

Criteria	Experiments to be conducted	Required performance criteria	Result
Accuracy	This will be assessed using the Quality Control (QC) results obtained from repeatability and reproducibility batches. All batches contain QC samples at two concentrations. A low and high concentration contained within the calibration curve. Accuracy will be assessed if the measured concentration is within 20% of the spiked concentration.	Expecting that the measured concentration will be within 20% of the QC concentration.	Pass for all analytes.
Precision	This will be assessed using the QC results obtained from repeatability and reproducibility batches. A minimum of 5 different extractions are assessed, each contains QC samples at 2 concentrations (low and high). CV% value will be assessed on the five different extractions.	The %CV is not expected to exceed 10%.	Pass for all analytes.
Linearity (Calibration model)	Linearity will be demonstrated by running a 6 point calibration curve, minimum 5 separate extractions to be assessed.	It is expected that the R^2 of the curves will be equal or greater than 0.99 for all analytes	Pass for all analytes
LOD	Blank matrix is spiked with analytes at 50% concentration of the LOQ and run alongside extracted QCs and calibrators.	The LOD will be the lowest concentration at which the S/N ratio is greater or equal to 3:1 and achieves identification criteria (retention time, mass spectral ion ratios based on calibration curve of the run)	Pass for all analytes.
LOQ	The LOQ for the analytes will be the lowest calibrator. Accuracy will be assessed, and all ion ratios must be within 20% of the target.	It is expected that no significant difference between calculated value and expected value will be found. Signal to noise ratio will be above or equal to 10:1, identification	Pass for all analytes.

		criteria (retention time, mass spectral ion ratios based on calibration curve of the run) will be met.	
Specificity	Drugs in question will be all analysed in separate samples to ascertain whether the method specifically picks up each separate drug. Drugs will be analysed in multi drug samples and in individual drug spiked samples.	The method is expected to discriminate drugs in both sample types on the basis of retention time and ion ratios. There should be no cross identification of drugs in either single or multi drug samples.	Pass for all analytes.
Repeatability	A sample will be extracted on two separate occasions and the concentrations measured and compared with each other.	No significant difference is expected analytes to be found for the concentration the difference should not be 7.5%	Pass for all analytes.
Reproducibility	This will be assessed by preparing 3 separate exactions on 3 separate days by 3 separate analysts. Each batch will contain a calibration curve consisting of 6 points and QC`s at 2 different concentrations.	The measured concentration for QC samples passes the accuracy criteria.	Pass for all analytes.
Matrix effect (ME)	Post extraction addition approach is used in which two sets of samples are compared. Set 1 consists of samples spiked with analytes and their internal standards in 1mL of LCMS grade methanol and afterwards reconstituted. Set 2 consists of samples spiked with analytes and their internal standards before the evaporation of the elution solution following extraction of blank blood.	ME%=(C/Ax100) The ion suppression or enhancement will be established at low and high concentrations. The values should not exceed 25% however if the values exceed 25% impact on LOD, LOQ. Bias is evaluated, all 3 values are suitable, pass the required criteria.	All analytes have matrix effect higher than 25%, however the LOQs, bias is not affected for any of the analytes.
Recovery (RE)	Pre spiked and neat sample sets are compared. Set 1(A) consists of samples spiked with analytes and their internal standards in	RE%=C/Ax100 Recovery will be established at low and high concentrations. It	All analytes have a recovery above 45%, and the average was 56.04%, however

	1mL of LCMS grade methanol and afterwards reconstituted. Set 3 (C) consist of blank blood samples spiked with analytes and their internal standards followed by extraction procedure.	is expected that the recovery is above 80% or if not it does not affect the bias of LOQs	the LOQs, bias is not affected for any of the analytes.
Stability	Stability is tested by analysing the same blood sample, spiked with analytes, on two different occasions, on day one and then after a week. Stability of extracted samples is tested by extracting samples and keeping them in the refrigerator/freezer for two weeks.	The blood sample concentration should be similar (not different by more than 20%) on both days when analysed. The extracted samples after being kept in the refrigerator/freezer should still pass bias and linearity criteria.	Pass for all analytes.

3.5 Method Validation Results

Accuracy

Accuracy is the closeness of the calculated value to the true value. The accuracy is determined as a percentage by comparing the calculated concentration to the expected concentration. All benzodiazepines were expected to be within ±20% of the target concentration. Five runs are examined with 2 duplicates of low and high concentration QCs. The accuracy results can be viewed in Tables 11-12. The accuracy of analytes, which had their respective deuterated internal standards was in the range of 20%. The method can quantify all analytes with an acceptable accuracy.

Table 11: *Low QC values for all six benzodiazepines*

Analyte	**Low QC Value**	**Target Concentration (µg/L)**	**Accuracy (%)**	
Diazepam	331.80	300	110.60	Pass
Oxazepam	166.88	150	111.25	Pass
Temazepam	417.53	375	111.34	Pass
Clonazepam	34.41	30	114.71	Pass
Lorazepam	66.07	60	110.11	Pass
Flunitrazepam	164.22	150	109.48	Pass

Table 12: *High QC values for all six benzodiazepines*

Analyte	**High QC Value**	**Target Concentration (µg/L)**	**Accuracy (%)**	
Diazepam	760.57	700	108.65	Pass
Oxazepam	379.12	350	108.32	Pass
Temazepam	938.55	875	107.26	Pass
Clonazepam	69.81	70	99.73	Pass
Lorazepam	166.44	140	118.88	Pass
Flunitrazepam	394.76	350	112.78	Pass

Precision

Precision of the method shows the closeness of repeated measurements, the agreement between results when the method is used repeatedly. Precision was assessed using the QC results obtained from repeatability and reproducibility batches. 5 different extractions were assessed, each contains QC samples at 2 concentrations (low and high). CV% value was assessed on the five different extractions. The between day precision is calculated by looking at all values for the QC (n=10), finding the standard deviation for them, diving it by the mean of QC (n=10) and multiplying by 100. The %CV value was below 20% for all analytes.

Table 13: %CV for QC1 and QC2 between days for all six benzodiazepines

Analyte	**%CV QC1 Between days**	**%CV QC2 Between days**
Diazepam	3.89	1.62
Oxazepam	4.81	0.85
Temazepam	5.34	1.10
Clonazepam	2.45	3.79
Lorazepam	6.79	2.63
flunitrazepam	4.50	0.90

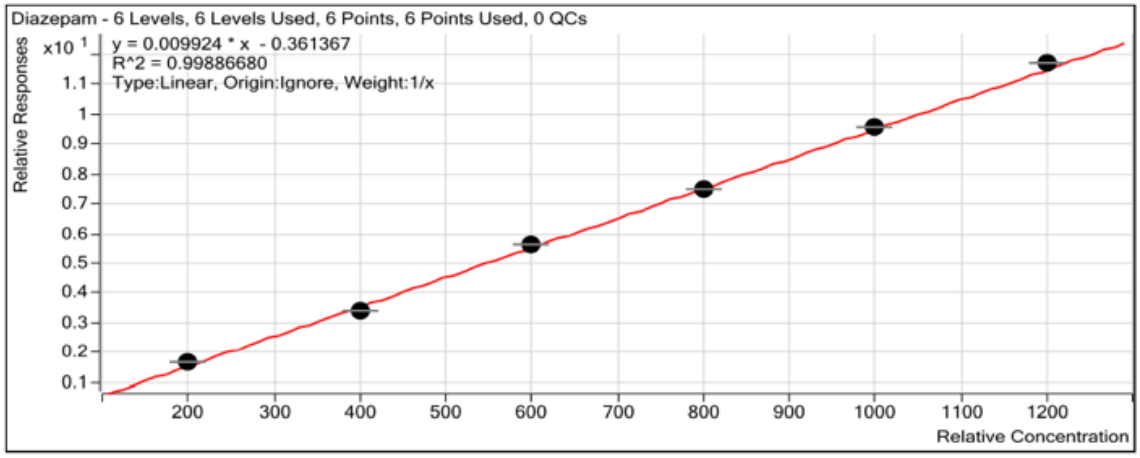
Linearity

Linearity is a theoretical mathematical model utilised to highlight the relationship between the response of an analyte and the concentration. This was tested by running a six-point calibration curve on 5 separate days with 5 separate extractions. A weighing factor of 1/x was utilised for all analytes. The method successfully passed the criteria achieving greater than 0.99 for R^2 for all analytes.

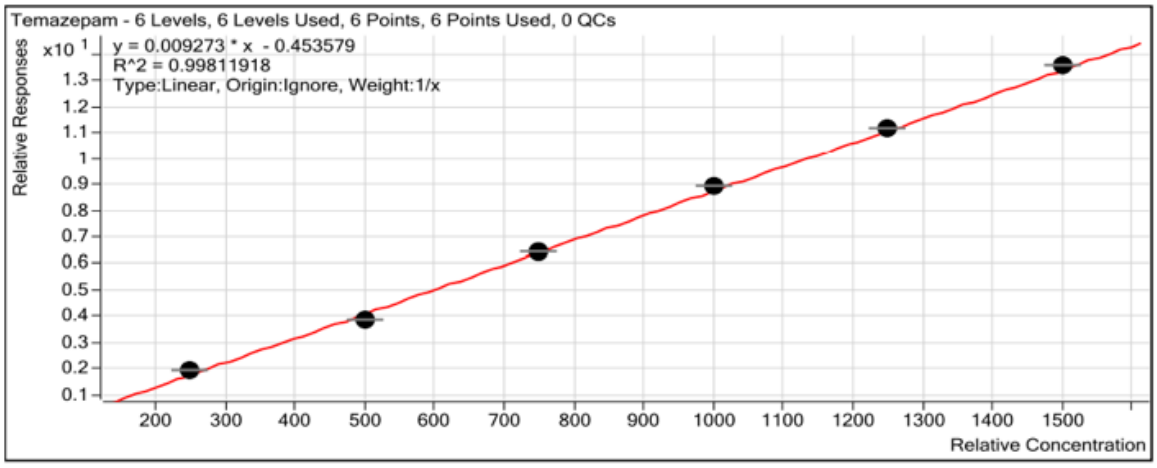
Table 14: The linearity is displayed in table 14.

Analyte name	**Linearity R^2day 1**	**Linearity R^2 day 2**	**Linearity R^2 day 3**	**Linearity R^2 day 4**	**Linearity R^2 day 5**
Diazepam	0.9988	0.9921	0.9947	0.9940	0.9980
Oxazepam	0.9991	0.9901	0.9953	0.9955	0.9987
Temazepam	0.9981	0.9904	0.9952	0.9950	0.9969
Clonazepam	0.9936	0.9949	0.9936	0.9960	0.9929
Lorazepam	0.9969	0.9910	0.9934	0.9945	0.9939
Flunitrazepam	0.9912	0.9927	0.9917	0.9950	0.9946

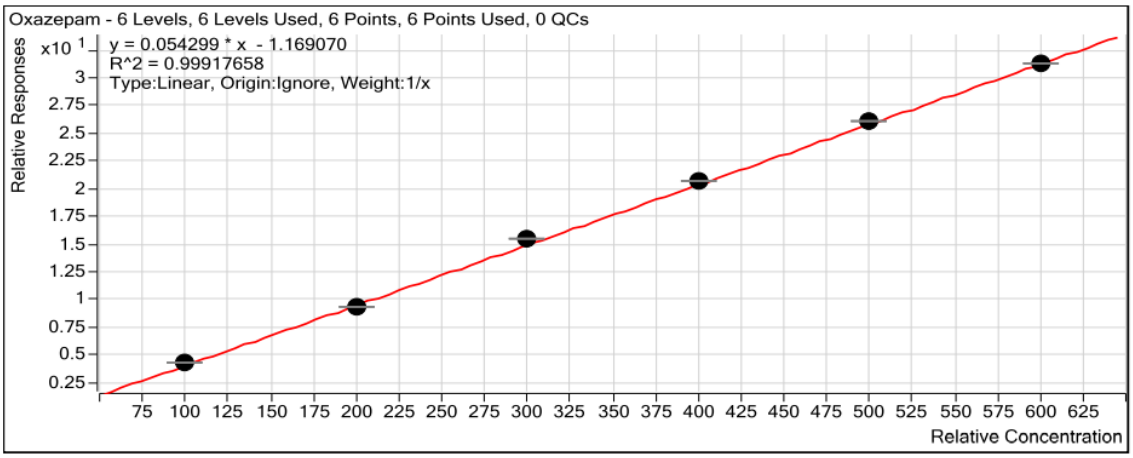
Target Compound *Diazepam*



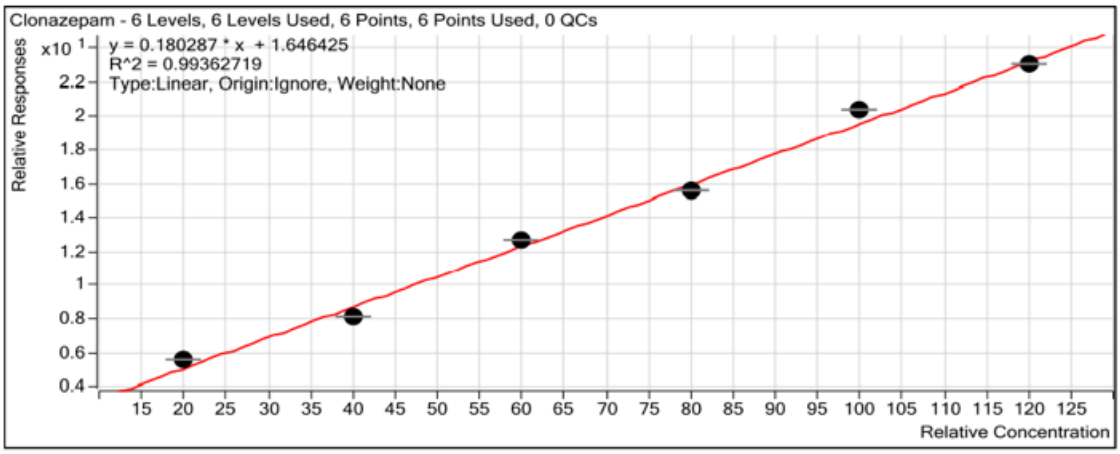
Target Compound *Temazepam*



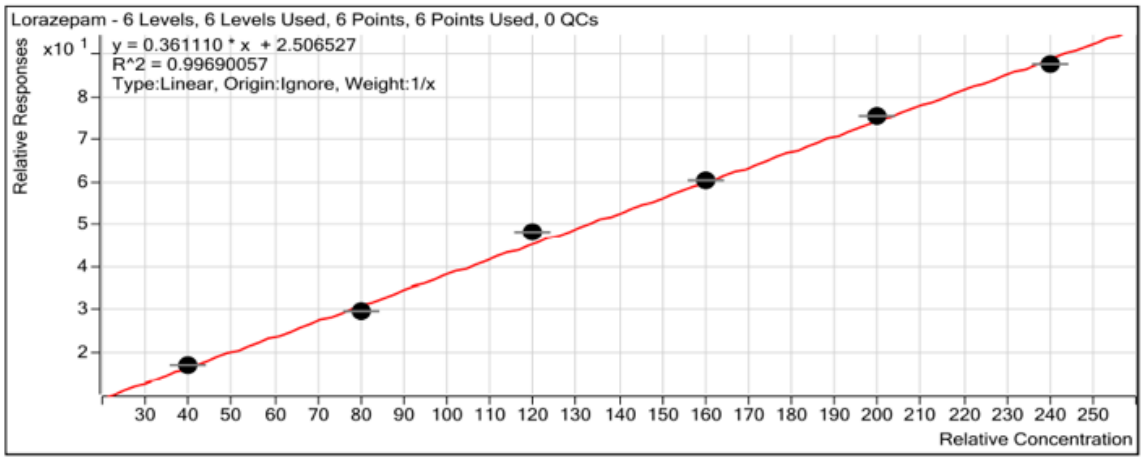
Target Compound *Oxazepam*



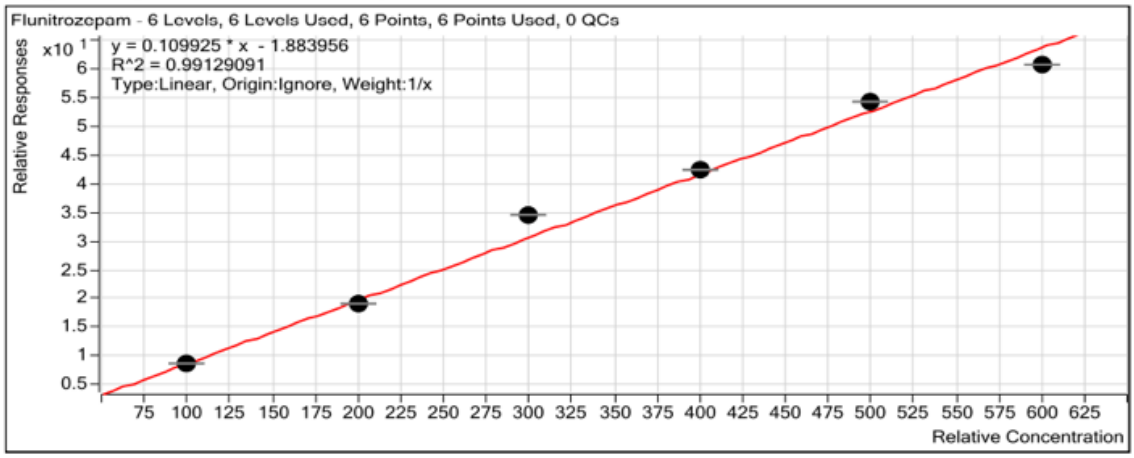
Target Compound *Clonazepam*



Target Compound *Lorazepam*



Target Compound *Flunitrozepam*



LOD

LOD is the lowest concentration of an analyte that can reliably be distinguished from background noise. Samples for LOD testing were prepared at half the concentration of Calibrator 1 (LOQ) for all analytes. Samples were then analysed and added to a calibration curve before analysing the signal to noise ratio, the ion ratio and retention time. Although quantitative values were generated, the values fall outside the calibration curve and therefore accuracy of the quantitation cannot be assured.

Table 15: LOD result summary. Three LODs were analysed on different days

Analyte name	**Concentration***	**Accuracy%**	**Signal to noise ratio**	**Ion ratio**	**Retention time (min)**
Diazepam	101.2862	101.28	838.981	Pass	3.986
	100.1056	100.10	1224.878	Pass	4.005
	100.9832	100.98	948.531	Pass	3.991
Oxazepam	52.4442	104.88	177.217	Pass	2.611
	51.1246	102.24	379.953	Pass	2.632
	52.2850	104.57	336.037	Pass	2.630
Temazepam	132.5015	106.00	7500.841	Pass	3.550
	122.1375	97.71	2601.956	Pass	3.550
	132.2375	105.79	5091.713	Pass	3.558
Clonazepam	10.9114	109.11	38.224	Pass	3.973
	9.4758	94.75	33.493	Pass	3.286
	10.7889	107.88	151.267	Pass	3.478
Lorazepam	20.7126	103.56	251.821	Pass	2.875
	20.8786	104.39	39.058	Pass	2.865
	21.4784	107.39	403.751	Pass	2.875
Flunitrazepam	54.3658	108.73	1378.516	Pass	3.675
	48.8126	97.62	900.210	Pass	3.632
	54.5560	109.10	3000.152	Pass	3.824

*These concentrations are outside the calibration range and are a qualitative only.

LOQ

LOQ is the lowest concentration that can be reliably quantitated and in the scope of this project, the lowest calibrator in each run was considered was assigned to be the LOQ. In all cases ion ratio was assessed to be within 20% of target and signal to noise ratio was above 3:1. The results are detailed in Table 16:

Table 16: *LOQ result summary. Three LOQs were analysed on different days*

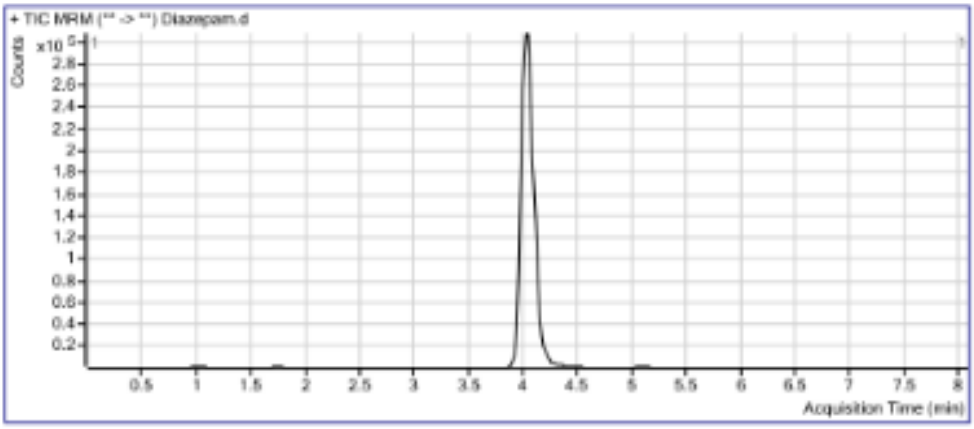
Analyte name	**Concentration***	**Accuracy%**	**Signal to noise ratio**	**Ion ratio**
Diazepam	199.9922	100.00	199.9922	Pass
	205.8465	102.92	1386.537	Pass
	207.4889	103.74	1555.468	Pass
Oxazepam	100.3690	100.37	661.625	Pass
	106.1678	106.16	483.797	Pass
	102.0265	102.02	1053.291	Pass
Temazepam	235.3445	94.14	2981.174	Pass
	260.9773	104.39	5615.713	Pass
	262.6250	105.05	1815.365	Pass
Clonazepam	21.4754	107.38	11.141	Pass
	19.6973	98.48	204.528	Pass
	22.2647	111.32	408.901	Pass
Lorazepam	37.0240	92.56	100.247	Pass
	16.8768	84.38	321.066	Pass
	20.2546	101.27	342.845	Pass
Flunitrazepam	98.6815	98.68	2625.596	Pass
	109.3795	109.37	1610.146	Pass
	110.1695	110.16	1555.776	Pass

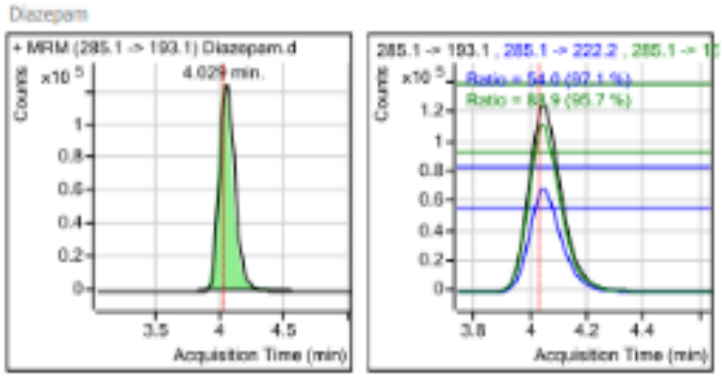
Specificity

Specificity is the capacity of a method to detect the target analyte without detecting other non-target analytes. Samples were spiked with the analytes in separate samples and results were compared to other benzodiazepines to see differentiation.

Table 17: Ion ratios pass rate for all six benzodiazepines

Analyte name	Ion ratio
Diazepam	Pass
Oxazepam	Pass
Temazepam	Pass
Clonazepam	Pass
Lorazepam	Pass
Flunitrazepam	Pass





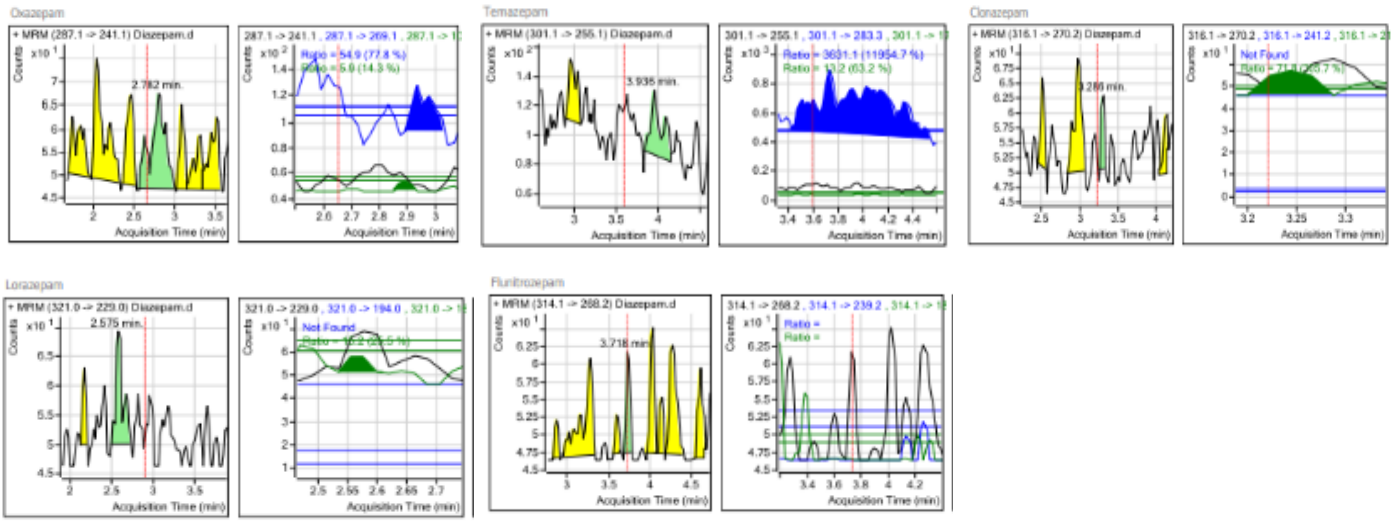


Figure 18. Representative chromatograms showing ion ratios for diazepam in selectivity experiment.

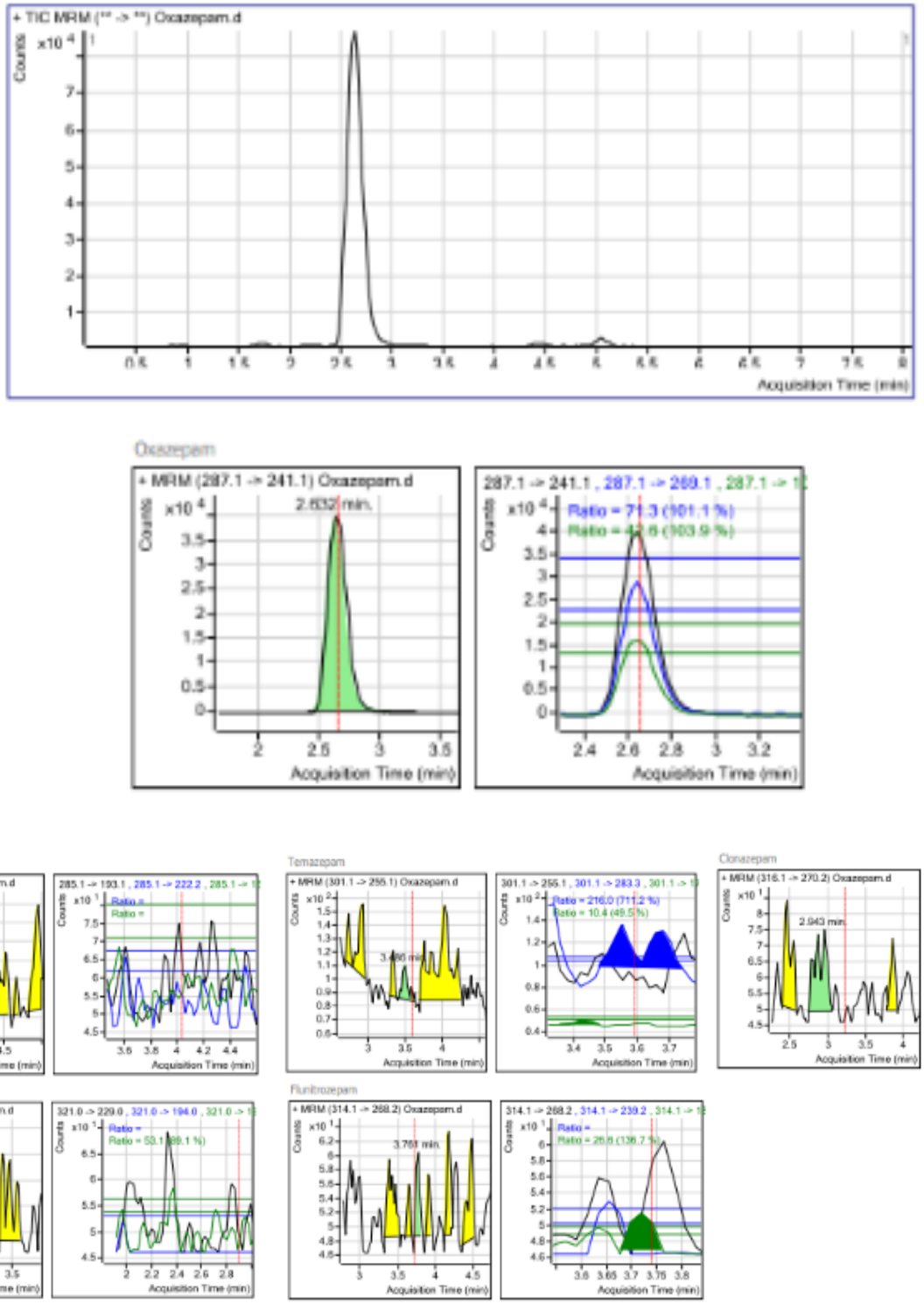


Figure 19. Representative chromatograms showing ion ratios for oxazepam in selectivity experiment

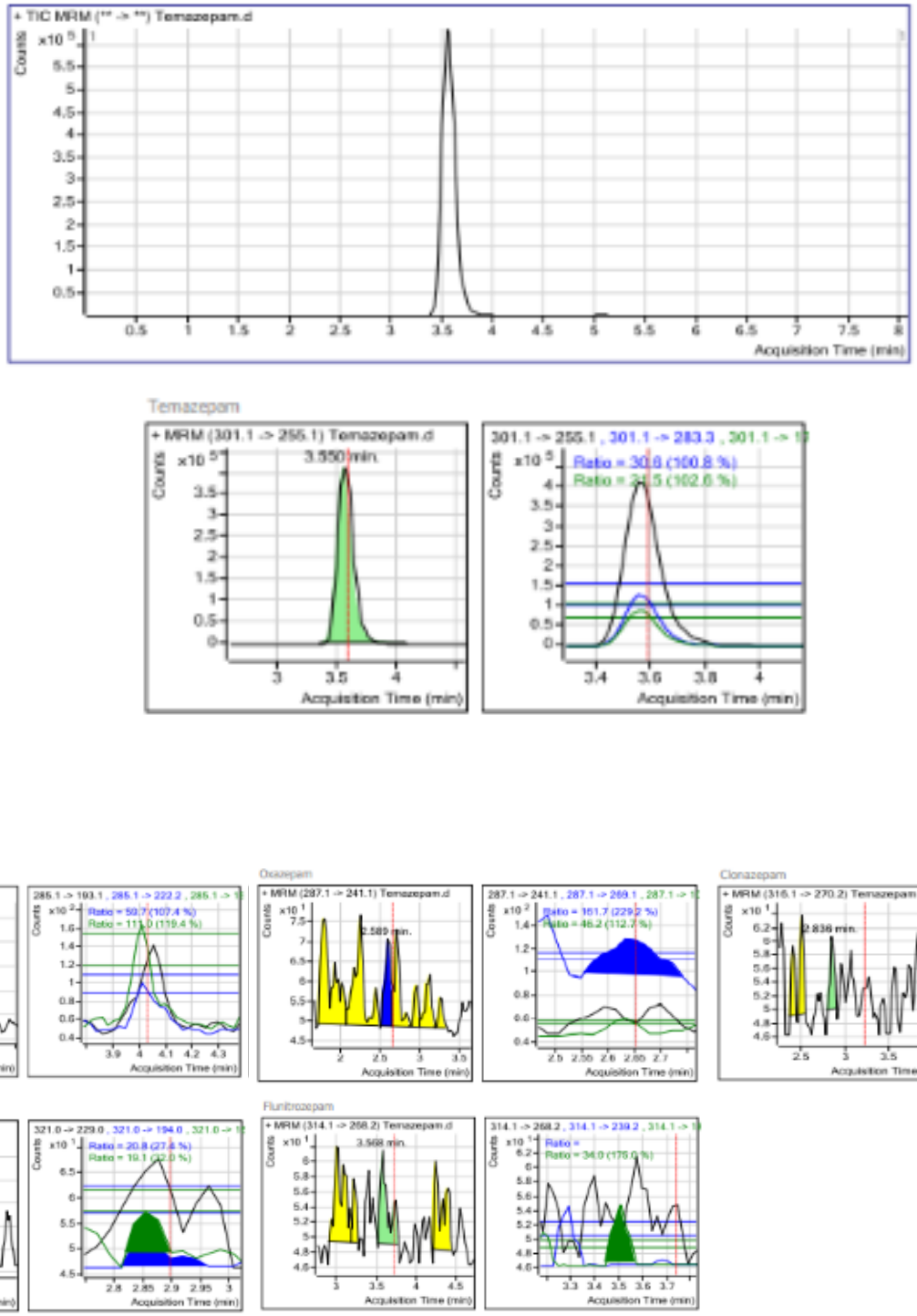


Figure 20. Representative chromatograms showing ion ratios for temazepam in selectivity experiment

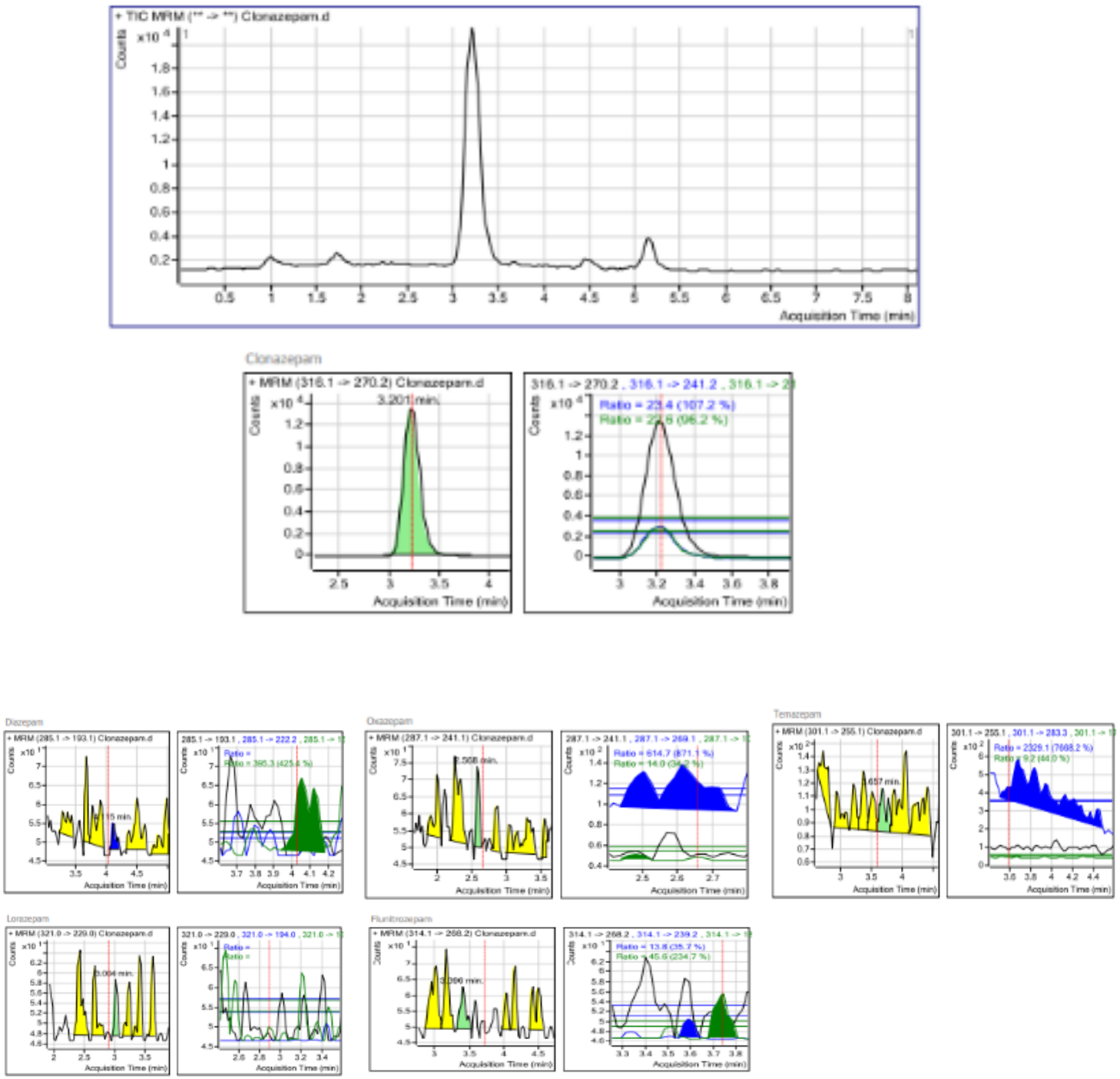
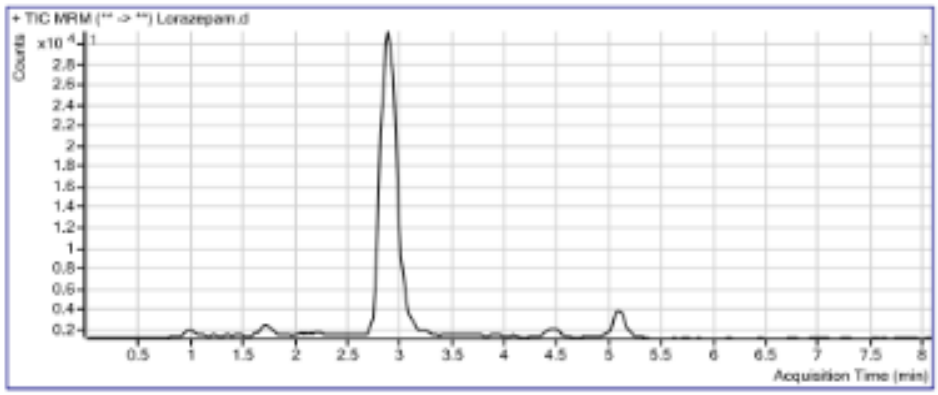
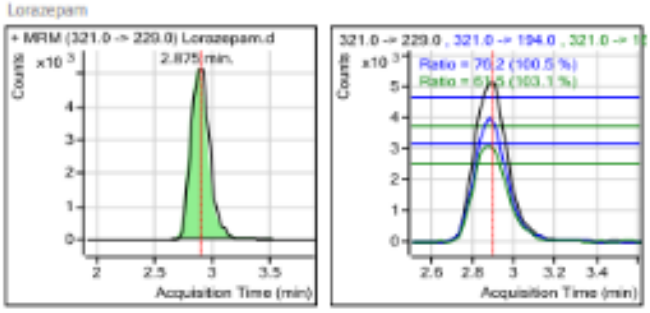


Figure 21. Representative chromatograms showing ion ratios for clonazepam in selectivity experiment





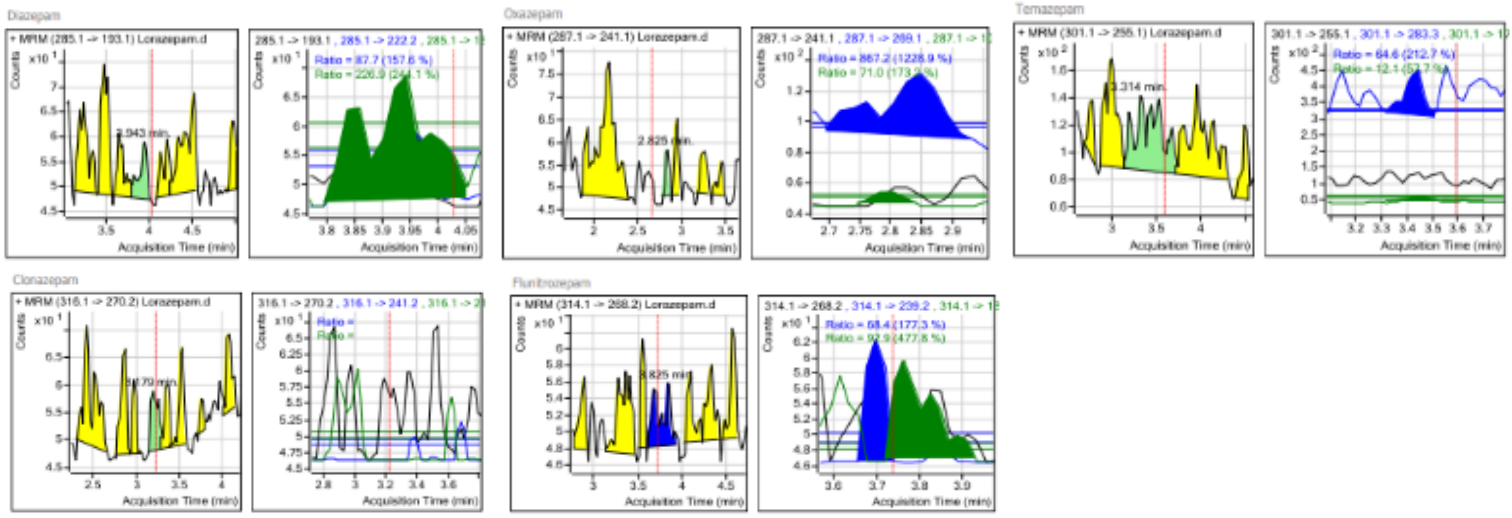
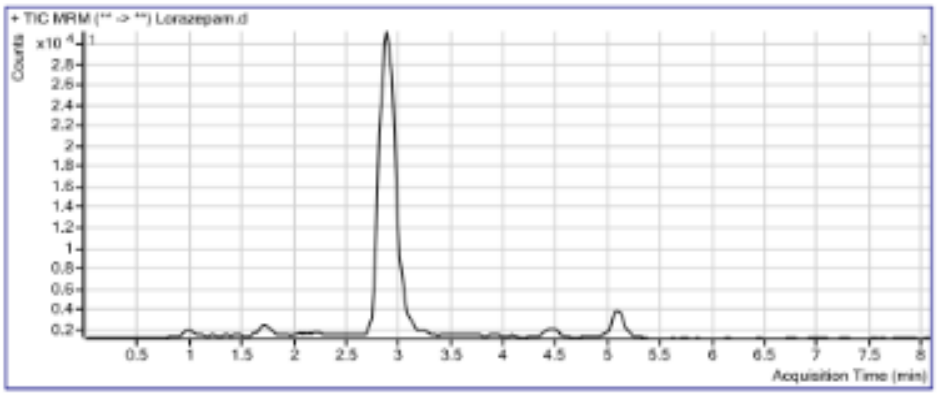
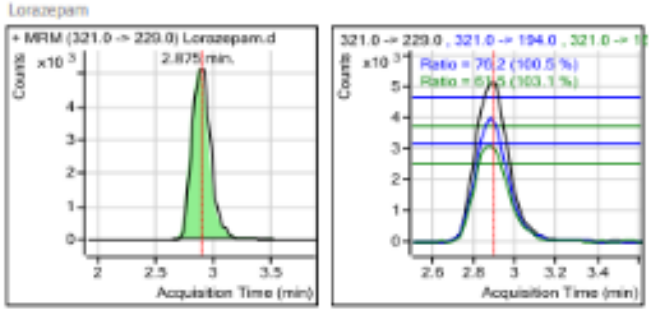


Figure 22. Representative chromatograms showing ion ratios for lorazepam in selectivity experiment





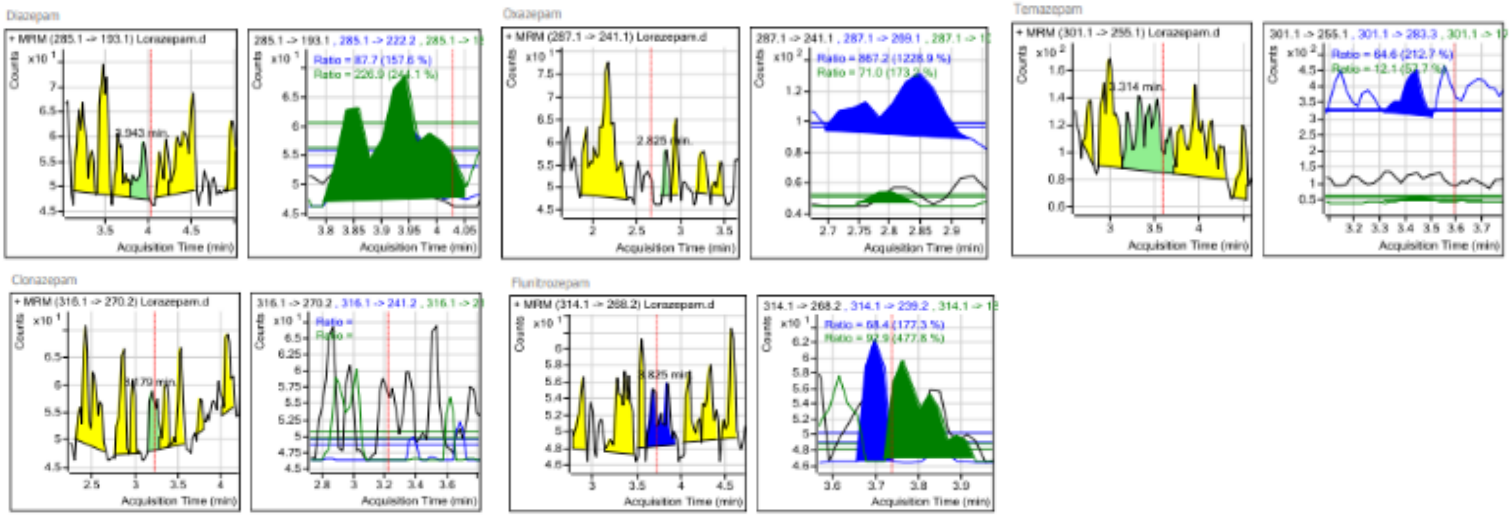


Figure 23. Representative chromatograms showing ion ratios for flunitrazepam in selectivity experiment

Repeatability

Repeatability was tested to investigate how effectively the method operated when testing the same samples on different days and how close the results were grouped together. Known concentrations of the samples were spiked at Cal 3 and extracted and then preparation of calibration curve and QC's on the following days 25th July 2020 and then 28th July 2020. All analytes were expected not to differ by more than 10%. the results can be seen in table 18.

Table 18: Repeatability sample values on two different days of analysis

Analyte name	Sample value on first run (µg/L)	Sample value on second run (µg/L)	Difference (%)
Diazepam	615.7126	598.7650	2.75%
Oxazepam	307.2864	301.4842	1.88%
Temazepam	752.9748	743.6387	1.24%
Clonazepam	64.0456	59.8750	6.51%
Lorazepam	127.5014	117.0398	8.21%
Flunitrazepam	299.3687	308.6824	3.11%

Reproducibility

Reproducibility was assessed by carrying out separate extractions by more than one analyst on separate days within the same laboratory. Three different analysts extracted calibration curves and QC.

Table 19: *Results for reproducibility experiments carried out by three different analysts*

	Analyte	Linearity	QC1 Concentration (µg/L)	Accuracy (%)	QC2 Concentration (µg/L)	Accuracy (%)
Analyst 1	Diazepam	0.9959	317.1743	105.72	710.1832	101.35
	Oxazepam	0.9955	158.3692	105.57	354.3216	101.23
	Temazepam	0.9950	401.1996	106.98	882.7542	100.88
	Clonazepam	0.9960	34.7680	115.89	75.6470	108.06
	Lorazepam	0.9945	60.8565	101.42	148.9203	106.37
	Flunitrazepam	0.9940	165.9730	103.67	358.3360	102.38
Analyst 2	Diazepam	0.9994	296.2797	98.760	711.3950	101.62
	Oxazepam	0.9992	141.4143	94.270	356.7039	101.91
	Temazepam	0.9991	356.7946	101.94	870.3755	99.47
	Clonazepam	0.9945	29.8724	99.574	71.5555	102.22
	Lorazepam	0.9920	51.9544	86.590	142.8324	102.02
	Flunitrazepam	0.9993	150.6073	100.40	357.8981	107.39
	Diazepam	0.9947	319.3679	106.45	710.2393	101.46

Analyst 3	Oxazepam	0.9953	158.7116	105.80	355.2901	101.51
	Temazepam	0.9952	401.2165	106.99	882.1594	100.81
	Clonazepam	0.9936	33.8957	112.98	74.5301	106.47
	Lorazepam	0.9934	62.1495	103.58	148.5742	106.09
	Flunitrazepam	0.9917	163.7352	109.16	366.0928	104.59

Recovery

Recovery is measured by comparing extracted samples to unextracted controls as a percentage and calculated by comparing analyte ion peak areas with the mathematical formula B/A x 100. An unextracted set of low, medium and high calibrators were compared with that of an extracted set in order to determine the amount of analyte recovered from the matrix. This is demonstrated in tables 20–25 showing percentage recovery for all six benzodiazepines. Recovery for all analytes is above 45%. The average recovery of the six benzodiazepines in this method is 56.72%. The sensitivity and LOQs for all analytes passed the required criteria of the method.

Table 20: *Recovery of Diazepam from blood/benzodiazepine matrix*

Calibrator concentration	**Unextracted peak area (A)**	**Extracted peak area (B)**	**Recovery percentage (B/A x100)**	**Average recovery percentage (%)**
Cal 1	1111718	518686	46.65	48.48
Cal 3	3773493	1911544	50.65	
Cal 5	6224236	2996537	48.14	

Table 21: *Recovery of Oxazepam from blood/benzodiazepine matrix*

Calibrator concentration	**Unextracted peak area (A)**	**Extracted peak area (B)**	**Recovery percentage (B/A x100)**	**Average recovery percentage (%)**
Cal 1	212294	118716	55.92	49.39
Cal 3	736334	345635	46.93	
Cal 5	1320903	598541	45.31	

Table 22: *Recovery of Temazepam from blood/benzodiazepine matrix*

Calibrator concentration	**Unextracted peak area (A)**	**Extracted peak area (B)**	**Recovery percentage (B/A x100)**	**Average recovery percentage (%)**
Cal 1	568068	385961	67.94	55.84
Cal 3	1741739	892811	51.25	
Cal 5	2934300	1418118	48.32	

Table 23: *Recovery of Clonazepam from blood/benzodiazepine matrix*

Calibrator concentration	Unextracted peak area (A)	Extracted peak area (B)	Recovery percentage (B/A x100)	Average recovery percentage (%)
Cal 1	5500	3131	56.92	46.59
Cal 3	18286	7388	40.40	
Cal 5	28989	12303	42.44	

Table 24: *Recovery of Lorazepam from blood/benzodiazepine matrix*

Calibrator concentration	Unextracted peak area (A)	Extracted peak area (B)	Recovery percentage (B/A x100)	Average recovery percentage (%)
Cal 1	21886	15328	70.03	54.08
Cal 3	79698	35801	44.92	
Cal 5	129130	61060	47.28	

Table 25: *Recovery of Flunitrazepam from blood/benzodiazepine matrix*

Calibrator concentration	Unextracted peak area (A)	Extracted peak area (B)	Recovery percentage (B/A x100)	Average recovery percentage (%)
Cal 1	34942	31240	89.40	85.96
Cal 3	107610	90182	83.80	
Cal 5	289303	245031	84.69	

Matrix effect

Matrix effects is the interference in a signal due to impurities in the biological sample. A quantitative technique is utilised to analyse matrix effect, also known as ion suppression or enhancement. An unextracted set of triplicates of low, medium and high calibrators were compared to that of a post extraction spiked set of triplicates to determine the effects of interfering compounds in the matrix other than the analytes in question. This is then compared and contrasted with the mathematical formula (C/A x 100)-100. Tables 26–31 demonstrate ion enhancement (+ME value) or suppression (-ME value) for each analyte in the blood/benzodiazepine matrix. Overall, all analytes experienced ion suppression. Our results suggest that this method has an ion suppression present in all 6 benzodiazepines. The average ion suppression across all analytes was -48.43%. This has not affected the sensitivity of the method or its accuracy as both LOQ/LOD and QC validation indicate no loss in performance.

Table 26: *Matrix effects of Diazepam in blood/benzodiazepine mixture*

Calibration concentration	**Unextracted in MeOH peak area (A)**	**Post spiked peak area (C)**	**Matrix effect percentage (C/A x 100)-100**	**Average matrix effect percentage (%)**
Cal 1	1111718	626562	-43.45	-48.75
Cal 3	3773493	2064813	-45.28	
Cal 5	6224236	3644348	-41.44	

Table 27: *Matrix effects of Oxazepam in blood/benzodiazepine mixture*

Calibration concentration	**Unextracted in MeOH peak area (A)**	**Post spiked peak area (C)**	**Matrix effect percentage (C/A x 100)**	**Average matrix effect percentage (%)**
Cal 1	212294	105248	-50.42	-48.75
Cal 3	736334	373979	-49.21	
Cal 5	1320803	705002	--46.62	

Table 28: *Matrix effects of Temazepam in blood/benzodiazepine mixture*

Calibration concentration	**Unextracted in MeOH peak area (A)**	**Post spiked peak area (C)**	**Matrix effect percentage (C/A x 100)**	**Average matrix effect percentage (%)**
Cal 1	568068	281107	-50.51	-46.88
Cal 3	1741739	913195	-47.56	
Cal 5	2934300	1685418	-42.56	

Table 29: *Matrix effects of Clonazepam in blood/benzodiazepine mixture*

Calibration concentration	Unextracted in MeOH peak area (A)	Post spiked peak area (C)	Matrix effect percentage (C/A x 100)	Average matrix effect percentage (%)
Cal 1	5500	2215	-59.72	-48.15
Cal 3	18286	7706	-57.85	
Cal 5	28989	15715	-45.78	

Table 30: *Matrix effects of Lorazepam in blood/benzodiazepine mixture*

Calibration concentration	Unextracted in MeOH peak area (A)	Post spiked peak area (C)	Matrix effect percentage (C/A x 100)	Average matrix effect percentage (%)
Cal 1	21886	10403	-52.46	-49.92
Cal 3	79698	37380	-53.09	
Cal 5	129130	72053	-44.20	

Table 31: *Matrix effects of Flunitrazepam in blood/benzodiazepine mixture*

Calibration concentration	Unextracted in MeOH peak area (A)	Post spiked peak area (C)	Matrix effect percentage (C/A x 100)	Average matrix effect percentage (%)
Cal 1	24942	29017	-16.95	-48.15
Cal 3	107610	10058	-90.65	
Cal 5	289303	182686	-36.85	

Stability

The stability of samples was tested by analysing three different storage conditions and then compared to extraction concentrations detected on the same day. Storage was tested without any refrigeration and being left out at room temperature for a week. Second, testing was done on samples which had been kept in a refrigerator for a week as well as samples stored in a freezer for a week before being thawed and then analysed. Our results show good stability in all storage conditions tested with all the analytes being within 20% of target concentration.

Table 32: *Stability results and concentration differences under different storage conditions*

Analyte	Storage conditions	Sample	Target concentration (µg/L)	Concentration (µg/L)	Accuracy (%)
Diazepam	Same day Extraction	QC 1	300	317.6719	105.89
		QC 1 Duplicate	300	322.2032	107.40
		QC 2	700	692.3871	98.91
		QC 2 Duplicate	700	698.0715	99.72
		QC Mid	600	526.5944	87.76
	Room temperature (After one week)	QC 1	300	317.8975	105.96
		QC 1 Duplicate	300	321.7242	107.24
		QC 2	700	683.9212	97.70
		QC 2 Duplicate	700	695.9866	99.42
		QC Mid	600	525.2711	87.54
	Refrigerated (After one week)	QC 1	300	325.8018	108.60
		QC 1 Duplicate	300	323.7268	107.90
		QC 2	700	694.4567	99.20
		QC 2 Duplicate	700	691.6591	98.80
		QC Mid	600	526.2544	87.70
	Freeze (After one week)	QC 1	300	329.7309	109.91
		QC 1 Duplicate	300	324.3609	108.12
		QC 2	700	696.6868	99.52
		QC 2 Duplicate	700	709.9952	101.42
		QC Mid	600	528.1242	88.02
Oxazepam	Same day Extraction	QC 1	150	154.9266	103.28
		QC 1 Duplicate	150	162.1916	108.12
		QC 2	350	351.8320	100.52
		QC 2 Duplicate	350	363.1384	103.75
		QC Mid	300	268.6969	89.56

	Room temperature (After one week)	QC 1	150	156.4390	104.29
		QC 1 Duplicate	150	158.7860	105.85
		QC 2	350	352.8720	100.82
		QC 2 Duplicate	350	350.4305	100.12
		QC Mid	300	267.0870	89.02
	Refrigerated (After one week)	QC 1	150	160.2048	106.80
		QC 1 Duplicate	150	158.6292	105.75
		QC 2	350	344.1014	98.31
		QC 2 Duplicate	350	357.3620	102.10
		QC Mid	300	265.2709	88.42
	Freeze (After one week)	QC 1	150	162.3410	108.22
		QC 1 Duplicate	150	159.7793	106.51
		QC 2	350	347.5310	99.29
		QC 2 Duplicate	350	356.2248	101.77
		QC Mid	300	264.9128	88.30
Temazepam	Same day Extraction	QC 1	375	396.8175	105.81
		QC 1 Duplicate	375	395.9059	105.57
		QC 2	875	841.4997	96.17
		QC 2 Duplicate	875	866.1335	98.98
		QC Mid	750	658.9219	87.85
	Room temperature (After one week)	QC 1	375	397.4713	105.99
		QC 1 Duplicate	375	398.8590	106.36
		QC 2	875	849.2170	97.05
		QC 2 Duplicate	875	867.4366	99.13
		QC Mid	750	646.4684	86.19
	Refrigerated (After one week)	QC 1	375	397.2482	105.93
		QC 1 Duplicate	375	397.5186	106.00
		QC 2	875	852.8401	97.46
		QC 2 Duplicate	875	857.6100	98.01
		QC Mid	750	647.4198	86.32
	Freeze (After one week)	QC 1	375	399.3173	106.48
		QC 1 Duplicate	375	396.8619	105.82
		QC 2	875	846.0722	96.69
		QC 2 Duplicate	875	883.0615	100.92
		QC Mid	750	651.5089	86.86

Clonazepam	Same day Extraction	QC 1	30	33.0078	110.02
		QC 1 Duplicate	30	32.2289	107.42
		QC 2	70	71.7408	102.48
		QC 2 Duplicate	70	74.7768	106.82
		QC Mid	60	54.2921	90.48
	Room temperature (After one week)	QC 1	30	32.3361	107.78
		QC 1 Duplicate	30	31.5375	105.12
		QC 2	70	69.8852	99.83
		QC 2 Duplicate	70	71.2684	101.81
		QC Mid	60	50.9017	84.83
	Refrigerated (After one week)	QC 1	30	34.2808	114.26
		QC 1 Duplicate	30	33.5208	111.73
		QC 2	70	68.6232	98.03
		QC 2 Duplicate	70	73.3859	104.83
		QC Mid	60	53.3943	88.99
	Freeze (After one week)	QC 1	30	31.9713	106.57
		QC 1 Duplicate	30	33.1376	110.45
		QC 2	70	68.8668	98.38
		QC 2 Duplicate	70	70.2148	100.30
		QC Mid	60	55.9665	93.27
Lorazepam	Same day Extraction	QC 1	60	70.0735	116.78
		QC 1 Duplicate	60	69.6753	116.12
		QC 2	140	145.0638	103.61
		QC 2 Duplicate	140	148.3126	105.93
		QC Mid	120	109.5451	91.28
	Room temperature (After one week)	QC 1	60	67.3974	112.32
		QC 1 Duplicate	60	65.0422	108.40
		QC 2	140	139.9475	99.96
		QC 2 Duplicate	140	151.9030	108.50
		QC Mid	120	107.4706	89.55
	Refrigerated (After one week)	QC 1	60	65.9516	109.91
		QC 1 Duplicate	60	68.2853	113.80
		QC 2	140	138.1092	98.64
		QC 2 Duplicate	140	140.2633	100.18
		QC Mid	120	107.6931	89.74

	Freeze (After one week)	QC 1	60	68.9484	114.91
		QC 1 Duplicate	60	63.2523	105.42
		QC 2	140	138.8051	99.14
		QC 2 Duplicate	140	140.2633	100.18
		QC Mid	120	107.6931	89.74
Flunitrazepam	Same day Extraction	QC 1	150	155.2555	103.50
		QC 1 Duplicate	150	158.4345	105.62
		QC 2	350	334.0062	95.43
		QC 2 Duplicate	350	351.1647	100.33
		QC Mid	300	256.8462	85.61
	Room temperature (After one week)	QC 1	150	158.3729	105.58
		QC 1 Duplicate	150	160.9795	107.31
		QC 2	350	347.4074	99.25
		QC 2 Duplicate	350	355.8647	101.67
		QC Mid	300	262.3890	87.46
	Refrigerated (After one week)	QC 1	150	160.7179	107.14
		QC 1 Duplicate	150	162.4765	108.31
		QC 2	350	345.7210	98.77
		QC 2 Duplicate	350	354.6792	101.33
		QC Mid	300	270.4361	90.14
	Freeze (After one week)	QC 1	150	163.6993	109.13
		QC 1 Duplicate	150	158.5633	105.70
		QC 2	350	342.6982	97.91
		QC 2 Duplicate	350	353.4756	100.99
		QC Mid	300	265.8388	88.61

4. Discussion

A method that could effectively detect and quantify six benzodiazepine analytes was developed. After method development phases of the project, the analytical technique was subject to validation procedures to ensure the reliability, effectiveness and general applicability of the method for casework purposes. This method was successfully validated for all six benzodiazepines included in Section 5A of the Road Traffic Act.

Pre-method development literature research was carried out in order to identify the required ion transitions for the selected benzodiazepines. An array of previous studies researching benzodiazepines have utilised the same ion fragmentation pathways as the ones chosen in this project. The sample preparation, extraction and separation technique were based on methodology described by Simonsen *et el.,* however, during the method development stages, modifications were made to improve recovery and efficiency.

Following the successful method development for the column where scouting runs and optimisation of the gradient was carried out to ensure that there was no co-elution of analytes; the validation procedure was started. During the method development stages co-elution occurred between flunitrazepam and clonazepam. Modifications were first made to temperature and pressure without resolving the issue. Following this flow rate was changed from 0.35µL/min to 0.45µL/min which rectified the problem and separated the occurrence of both drugs. The parameters tested for the quantitative method are as follows: accuracy (bias), precision, linearity (calibration model) repeatability, reproducibility, specificity, LOD (limit of detection), LOQ (limit of quantitation), matrix effects and recovery. The FSR-C-133 document has been published by the Forensic Science regulator to establish requirements for, and a common approach to, the analysis and reporting of the concentrations of certain drugs in relation to offences under Section 5A of the Road Traffic Act 1988. This is with regard to but not limited to quality standards, analytical requirements as well as unit requirements. The criteria for the method should fall in line with UKAS (United Kingdom Accreditation Service) guidelines for optimal practice in ISO 17025 accreditation. Preceding method validation experiments on aforementioned parameters, a validation plan was created which outlined the criteria to be met for each method specification. All experiments undergone were performed on whole blood.

The data collected indicates that the validated method is a reliable and accurate method to be employed in forensic toxicology and in line with Section 5A of the road traffic act testing specifications. The accuracy of results for all six benzodiazepine analytes passed the validation criteria. This highlights the methods ability to efficiently quantitate results.

One common characteristic found in studies done in relation to benzodiazepines is long duration of the method run times, typically 20-35 minutes. Smink *et al.,* 2004, devised a LC-MS method for the detection of the six benzodiazepines listed in this study, however, in comparison to this method which completes the analysis in less than 10 minutes, the method validated by Smink *et al.,* 2004 required over 20 minutes to complete analyte quantification. From a casework perspective a longer run time is typically disadvantageous as it permits fewer sample analysis and longer instrument usage, thereby being less cost effective. The validated method in this research project is ideal for large volume testing of road traffic samples whereby the variety of benzodiazepines encountered is relatively narrow. A 2017 study by Rooney *et al.,* indicated that over 90% of benzodiazepines encountered in road

traffic toxicology consist of diazepam and its two primary metabolites oxazepam and temazepam. The inclusion of the aforementioned metabolites are vital to be included in the study due to its prevalence from not only diazepam metabolism but also from their prescribed usage in their own right. By contrast, this method would need analyte expansion for use in criminal or post-mortem cases as the more common drugs of abuse such as the (non) benzodiazepine drugs like zolpidem and zopiclone. This can be seen in the works of ElSohly et al., (2006) who successfully validated a method for the detection of 22 benzodiazepines. One disadvantage of detecting more benzodiazepines is the much longer run-times in excess of 25 minutes.

Accuracy was assessed by measuring two QC's high and low concentrations, this method successfully detected all concentrations to within 20% of the target. Results were as expected with all the drugs being within the ±20% concentration range. All drugs except lorazepam were within ±15%. Precision was tested using repeatability and precision batches to calculate percentage coefficient of variance. All drugs passed the ±10% threshold with the highest value being lorazepam QC 1, as was seen in accuracy, with a value of 6.79%. LOD and LOQ's all passed their criteria in terms of accuracy, S/N ratio and ion ratios. The method was able to accurately detect the target analyte without interference from other non-target analytes. It should however be stated that the LOD and the LOQ for this method are both at comparatively high concentrations, this is in line with the requirements of the calibration curve to be within a close concentration of the fixed limits of Section 5a. This highlights why this method is specifically useful as a road traffic testing tool and would not be suitable for casework samples such as DFSA where sub ng/ml detection limits are required.

Matrix affects the concentration of the analytes by causing interference in a signal due to impurities in the biological sample that may elevate or suppress the ionisation signal, it is a vital to ascertain the degree of this effect. This was done by preparing three sets of low, medium and high calibrators containing all analytes. Set A consisted of a non-extracted methanol solutions spiked with concentrations equivalent Calibrators 1, 3, and 5 and their respective internal standards before being evaporated and reconstituted with 250µl of mobile phase B. Set B was prepared using calibrators 1, 3, and 5 and extracted as per the protocol with 1ml of blank blood. Set C was prepared using calibrators 1, 3, and 5 in blank blood, however, the calibrators were spiked post- extraction. Matrix effects were calculated using ME%=C/Ax100 as described by Matuszewski *et al*. 2003. The mean matrix effect ion suppression detected for all analytes was -48.43%.

A further parameter investigated was extraction efficiency, this was done by calculating the percentage recovery of the sample preparation. Recovery was calculated by RE%=B/Ax100 (Matuszewski *et al.* 2003). The recovery percentages were all above 80% in the Simonsen *et al.* 2010 study, while in this research project, the recovery percentages were averaged 50.87% for all analytes except for flunitrazepam in which 85.96% was recovered. To account for this, a deuterated internal standard was used. This is because the ratio of ion responses between the analyte and its respective internal standard is not affected by the matrix effect (Matuszewski *et al.* 2003). Any future research may benefit from optimising the extraction procedure and yield a higher recovery percentage. Recovery percentage is a limitation of this method as other research methods had percentages on average greater than 60% (Smink *et al*., 2004, Quintela *et al.,* 2006). However, as this method is typically detecting higher concentrations of benzodiazepines a lower percentage recovery is not likely to have affected it adversely. It may be that alterations in centrifugation and pH could increase percentage recovery to that described in the Simonsen *et al* study. In addition, future work would benefit

from research with other parameters tested. A study by Gunn *et al*, (2010) did not use a buffer during the extraction process to avoid matrix effects caused by buffering salts in MS.

This developed and validated method was used to determine the effects of temperature on the stability of benzodiazepines in blood. This is critical in casework environments as samples may be stored incorrectly at police stations, laboratories or during transfer. This research investigated what degradation of each individual benzodiazepine occurs when stored in different conditions. The blood collected for sample analysis in drug cases, in the UK, are held in road traffic vials with preservatives of sodium fluoride and potassium oxalate as anticoagulants. Samples in this study were stored at room temperature, refrigerated and freezer conditions. All samples were within 20% of the expected concentration highlighting good stability.

A method for the analysis of benzodiazepines in road traffic toxicology is required for the new law which came into force on the 2nd of March 2015, not only due to the high usage of these drugs in society but also due to risks posed when driving under the influence of benzodiazepines. All benzodiazepines can potentially cause impairment while driving by negatively impeding cognitive as well as psychomotor performance. This validated method can be utilised for reliable quantification of these analytes.

The introduction of *per se* limits in 2015 has radically changed the landscape of road traffic toxicology in England and Wales. Critically there has been a significant increase in the number of samples coming into forensic toxicology labs to be analysed for driving under the influence of drugs. Following the introduction of *per se* limits, the Driver and Vehicle Licensing Agency (DVLA) showed an increase in drug driving convictions (Freedom of Information Request 5687 Drug Driving). In the first three quarters of 2014 (prior to introduction of *per se* limits) there were a total of 805 convictions involving drug driving. In the first three quarters of 2016 (following the introduction of fixed limits) there were a total of 7,800 convictions involving drug driving (Freedom of Information Request 4976 Drug Driving).While individual police agencies have also reported significant increases in drug driving arrests, South Yorkshire police recorded 456 drug driving arrests in 2015 compared to 13 in 2014. During the period of March 2015 to January 2016 Cheshire police recorded 530 arrests compared to 70 in 2014. The North Wales arrest rate for March 2014 until March 2015 was 32 compared with 224 from March 2015 to March 2016 (Freedom of Information request 2016/238 Drug Driving).

This increase in drug driving sample submission after the introduction of fixed limits has previously been seen in other European countries such as Norway, where the introduction of *per se* limits resulted in a 20% increase in number of DUID samples taken (Vindenes, V *et al.,* 2014). While in Sweden a 10-fold increase was seen after the introduction of fixed limits (Jones A.W, 2005). However, at this moment in time the roadside screening procedure is only approved for cocaine and THC (Home Office of GB). A recent study by LGC (TIAFT 2019) indicated that cannabis and cocaine now account for over 85% of road traffic samples, this is a contrast to a 2017 (Rooney *et al* 2017) study which indicated that prior to the introduction of Section 5a benzodiazepines were the second most prevalent drug encountered after cannabis. Numerous studies have shown that there is a high reported usage of benzodiazepines in motorist in the UK and Ireland, it is unlikely that this trend has changed even if the detection methodology has (Rooney *et al.,* 2016, Officer. J, 2016, Cosbey, S.H, 1986, Fitzpatrick et al., 2006).

Due to the lack of road-side screening for the majority of drugs listed in section 5A, those impaired by drugs, will experience a time delay between incident and sampling. The average time until sampling was found to be 3.23 hours after incident in 2010-12. A strong correlation is evident between the sampling time and the final drug concentration detected (Hartman *et al.* 2016). Given the already high limits in place for benzodiazepines, significant improvements must be made in sampling time in order to achieve increased efficiency and convictions for driving while impaired. It should be noted that a significant number of benzodiazepines that are detected by forensic laboratories following the introduction of *per se* limits are the result of poly drug use, typically cannabis and cocaine users who have are also taking benzodiazepines. With the low limits set for cocaine and high limits for benzodiazepines, recreational drugged drivers are more likely to be over the limit for cocaine and under the limit for benzodiazepines. As a result, limits set in place for benzodiazepines should be reconsidered.

Although the introduction of Section 5a has vastly improved the ability of police forces to achieve prosecution in drugged driving cases due to stronger evidence via means of drug concentration analysis, it does contain several regulations and limits that could be improved on. In particular there is serious consideration that the medicinal drug limits are not be fit for purpose. The limits for certain benzodiazepines such as temazepam and diazepam are in excess of concentrations that cause impairment. A 1997 study by Druid and Holmgren found blood concentrations ranging from 10-50µg/L in a group of 130 intoxicated drivers. Another study found average flunitrazepam concentrations to be at 18µg/L (Jones *et al.,* 2007). However, the limits set out in Section 5A are significantly higher at 300µg/L. A study by Thatcher *et al.,* 2003 found 14 people arrested for erratic driving which displayed slow and slurred speech as well as poor coordination found blood concentrations of lorazepam ranging between 10-320µg/L (average, 70). Another study of 22 impaired drivers found average lorazepam blood concentration levels at 30µg/L. The limit set in section 5A of the road traffic act is set at 100µg/L. Another significant factor in is the expanded uncertainties stipulated in 'The Analysis and reporting of forensic specimens in relation to S5a Road Traffic Act'. This provision states all analytes in that are tested under Section 5a have an analytical uncertainty subtracted from their measured concentration, it is the result after this subtraction that is utilised for court. The uncertainty subtractions for benzodiazepines are as high as 20%. For temazepam a 20% subtraction is performed on all samples, thereby effectively making the temazepam limit 1200µg/L, a concentration in excess of those previously reported in impairment cases (Baselt, 2020). Given the high limits set for benzodiazepine drugs, Section 5a, is not the most effective legislation for the drug drive prosecution of benzodiazepines. Instead a charge of impairment under Section 4 of the road traffic should be used. The latter of which is an impairment offence with drug concentration used alongside manner of driving and a field impairment test. Section 5A has outlined a new offence of driving, attempting to drive or being in charge of a motor vehicle on public roads if the concentration of a specified drug in blood is above the prescribed limits. This has been designed to mirror provisions and laws set out for alcohol. Used in conjunction with other legislation pertaining to driving while impaired may be more effective.

In the scope of this project, the office for Forensic Science Regulator have specified the requirements, common approach, analysis and reporting of the concentrations of drugs in relation to offences under Section 5A Road Traffic Act. This guidance can be found in the document titled 'The analysis and reporting of Forensic Specimens In relation to S5A Road Traffic Act 1988 FSR-C-133 *Draft* (2016)*'* to aid analysis of samples. The use of limits guidance is stipulated in the document 'Section 5A Road Traffic Act 1988 Use of limits FSR-

G-221 (2020)', while the validation parameters and guidance can be found in 'Validation FSR-G-201 (2020)'. The laboratory should comply with the guidance on forensic toxicology issued by the United Kingdom and Ireland association of Forensic Toxicologists. In order for accreditation for Forensic service providers (FSP's) for the analysis of drugs in relation to Section 5a of the Road Traffic Act, a minimum standard of validation must be met for all methods which comply with quality standards as outlined by ISO 17025. The analysis of blood samples shall be specifically listed in the scope of accreditation. It shall comply with the codes of practice and conduct (Ibrahim, 2017). The guidance provided for the validation indicates that prior to method validation and analysis of sample, the LC-MS shall be calibrated using a multi-point calibration curve (a minimum of a 5 point calibration curve with 2 QCs) and the acceptance criteria for the calibration correlation coefficient should be equal to or greater than 0.99. it is also stated that isotopically labelled deuterated standards should be used for analytes. In addition, an appropriate system suitability check must be determined for the parameters of mass spectrometer tuning, mass calibration, sensitivity (peak of the low calibration standard or signal to noise ratio), chromatography (peak asymmetry, peak resolution, retention time stability). Following this, for method validation to be achieved, precision and recovery shall be established for each drug in whole blood matrix. Recovery estimates relevant to the whole blood matrix and drugs under the investigation shall be determined by the use of spiking experiments. Precision should be estimated using analysis of variance to give a total error of standard deviation. Whole blood matrix must also be used for estimating a Limit of Detection (LOD) and this must be done for each individual drug (Dft, 2015).

5.Conclusion

This project successfully produced a validated method for the detection of six benzodiazepines in whole blood in line with the legislative criteria of Section 5a of the Road Traffic Act. It has been validated for diazepam, oxazepam, temazepam, clonazepam, lorazepam and flunitrazepam. Low as well as high concentration benzodiazepines can be measured simultaneously, with low detection limits, low limits of quantitation and fulfils satisfactory validation parameters. Recovery was tested and all analytes had a recovery of greater than 45%. Assessing matrix effects indicated that average ion suppression was -48.43% for all analytes. Stability was assessed and all analytes passed the validation criteria. This method can reliably be used for the analysis of casework samples in line with the *per se* limits set out in Section 5a of the Road Traffic Act.

6. Bibliography

Addona, T. A., S. E. Abbatiello, et al. (2009) "Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma." Nat Biotechnol 27:633-41. Abstract

Ashri, N. and Abdel-Rehim, M., 2011. Sample treatment based on extraction techniques in biological matrices. *Bioanalysis*, 3(17), pp.2003-2018.

ASHTON, H., 1987. Benzodiazepine Withdrawal: outcome in 50 patients. *Addiction*, 82(6), pp.665-671.

Ashton, H., 2002. *Benzodiazepines - How They Work And How To Withdraw - Medical Research Information From A Benzodiazepine Withdrawl Clinic*. University of Newcastle.

Ashton CH. Benzodiazepine abuse. In: Caan E, de Belleroche J, editors. Drink, drugs and dependence. London: Routledge; 2002. pp. 197–212.

Ashton H. Benzodiazepine dependence. In: Haddad P, Dursun S, Deakin B, editors. Adverse syndromes and psychiatric drugs. Oxford: Oxford University Press; 2004. pp. 239–260.

Ashton, H., 2005. The diagnosis and management of benzodiazepine dependence. *Current Opinion in Psychiatry*, 18(3), pp.249-255.

Barbone F, McMahon AD, Davey PG, Morris AD, Reid IC, McDevitt DG, et al., Association of road-traffic accidents with benzodiazepine use, The Lancet, 1998, vol. 352(9137): pp. 1331-6

Baselt, R., 2020. *Disposition Of Toxic Drugs And Chemicals In Man*. Edition.12

Barker, M., 2004. Persistence of cognitive effects after withdrawal from long-term benzodiazepine use: a meta-analysis. *Archives of Clinical Neuropsychology*, 19(3), pp.437-454.

Barker, M., Greenwood, K., Jackson, M. and Crowe, S., 2004. Cognitive Effects of Long-Term Benzodiazepine Use. *CNS Drugs*, 18(1), pp.37-48.

Bramness, J., Skurtveit, S. and Mørland, J., 2006. Flunitrazepam: Psychomotor impairment, agitation and paradoxical reactions. *Forensic Science International*, 159(2-3), pp.83-91.

Boucart, M., Waucquier, N., Michael, G. and Libersa, C., 2007. Diazepam impairs temporal dynamics of visual attention. *Experimental and Clinical Psychopharmacology*, 15(1), pp.115-122.

Burt, DR., Kamatchi, GL., 1991. GABAA receptor subtypes: from pharmacology to molecular biology. *FASEB J.*, 5(14), pp 2916-23.

Charles F. Zorumski, M.D., and Keith E. Isenberg, M.D. 1991. Insights into the structure and function of GABA-Benzodiazepine Receptors: Ion Channels and Psychiatry. *Am J Psychiatry*, 148 (2) pp.162-173.

Choice Reviews Online, 2011. Substance Abuse and Mental Health Services Administration (SAMHSA). 49(03), pp.49-1507-49-1507.

Clarkson, J., Gordon, A. and Logan, B., 2004. 'Lorazepam and Driving Impairment', Journal of Analytical Toxicology, 28(6), pp.475-480.,
Cosbey, S.H., *Drugs and the impaired driver in Northern Ireland: an analytical survey.* Forensic Sci Int, 1986. **32**(4): p. 245-58.

Crestani, F., Löw, K., Keist, R., Mandelli, M., Möhler, H. and Rudolph, U., 2001. Molecular Targets for the Myorelaxant Action of Diazepam. *Molecular Pharmacology*, 59(3), pp.442-445.
Crutchfield, C., Lu, W., Melamud, E. and Rabinowitz, J., 2010. Mass Spectrometry-Based Metabolomics of Yeast. *Methods in Enzymology*, pp.393-426.

Cunningham, C., Hanley, G. and Morgan, S., 2010. Patterns in the use of benzodiazepines in British Columbia: Examining the impact of increasing research and guideline cautions against long-term use. *Health Policy*, 97(2-3),

CURRAN, H., COLLINS, R., FLETCHER, S., KEE, S., WOODS, B. and ILIFFE, S., 2003. Older adults and withdrawal from benzodiazepine hypnotics in general practice: effects on cognitive function, sleep, mood and quality of life. *Psychological Medicine*, 33(7), pp.1223-1237.

Daamen AP, Penning R, Brunt T, Vester JC, Cocaine In Drug Abuse and Addiction in Medical Illness, Editors Vester JC, Brady K, Galanter M, Conrod P, Springer, New York, 2012. ISBN 987-1-4614-3374-3, Chapter 11, pp. 151-162.

Dassanayake, T., Michie, P., Carter, G. and Jones, A., 2011. Effects of Benzodiazepines, Antidepressants and Opioids on Driving. *Drug Safety*, 34(2), pp.125-156.
Davies, J., Rae, T. and Montagu, L., 2017. Long-term benzodiazepine and Z-drugs use in England: a survey of general practice. *British Journal of General Practice*, 67(662), pp.e609-e613.

Denis, C., Fatseas, M., Lavie, E. and Auriacombe, M., 2013. Pharmacological interventions for benzodiazepine mono-dependence management in outpatient settings. *Cochrane Database of Systematic Reviews*,.

Drummer, O., Gerostamoulos, J., Batziris, H., Chu, M., Caplehorn, J., Robertson, M. and Swann, P., 2004. The involvement of drugs in drivers of motor vehicles killed in Australian road traffic crashes. *Accident Analysis & Prevention*, 36(2), pp.239-248.

Drummer O.H, M. Odell, The Forensic Pathology of Drugs of Abuse, Arnold, London, UK, 2001

Dupont, R., 1988. Abuse of Benzodiazepines: The Problems and the Solutions. *The American Journal of Drug and Alcohol Abuse*, 14(sup1), pp.1-69.

El-Beqqali, A. and Abdel-Rehim, M., 2016. Molecularly imprinted polymer-sol-gel tablet toward micro-solid phase extraction: I. Determination of methadone in human plasma

utilizing liquid chromatography–tandem mass spectrometry. *Analytica Chimica Acta*, 936, pp.116-122.

ELLIOTT, H., 1976. METABOLISM OF LORAZEPAM. *British Journal of Anaesthesia*, 48(10), pp.1017-1023.

ElSohly, M., Gul, W., ElSohly, K., Avula, B. and Khan, I., 2006. LC-MS-(TOF) Analysis Method for Benzodiazepines in Urine Samples from Alleged Drug-Facilitated Sexual Assault Victims. *Journal of Analytical Toxicology*, 30(8), pp.524-538.

Engeland A, Skurtveit S, Morland J, Risk of road traffic accidents associated with the prescription of drugs: a registry-based cohort study, Annals of Epidemiology, 2007, vol. 17(8): pp. 597-602.

F. Benhamou-Batut, F. Demotes-Mainard, L.Labat, G. Vancon J. Pharm. Biomed. Anal., 12 (1994), pp. 931-936

Fenn, J., Mann, M., Meng, C., Wong, S. and Whitehouse, C., 1989. Electrospray ionization for mass spectrometry of large biomolecules. *Science*, 246(4926), pp.64-71.
Fitzpatrick, P., et al., *Drinking, drugs and driving in Ireland: more evidence for action.* Inj Prev, 2006. **12**(6): p. 404-8.

Accreditation of Forensic Service Providers (FSPs). The Analysis of Drugs in support of Section 5A of the Road Traffic Act 1988 (as amended). 2015.

Forensic Science Regulator 2020. Annual report. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/868052/20200225_FSR_Annual_Report_2019_Final.pdf [accessed on 02 Feb 2021]

Forensic Science Regulator 2020. Section 5A Road Traffic Act 1988 Use of limits FSR-G-221. Issue 3. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/914258/221_S5A_Analysis_-_Use_of_Limits__v3.0.pdf [accessed on 02 Feb 2021

Forensic Science Regulator 2020. For forensic science providers and practitioners in the criminal justice system. Issue 5. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/880708/Codes_of_Practice_and_Conduct_-_Issue_5.pdf [Accessed on 02 Feb 2021]

Forensic Science Regulator 2016. The analysis and reporting of Forensic Specimens In relation to S5A Road Traffic Act 1988 FSR-C-133 *Draft*

Forensic Science Regulator 2020. Validation FSR-G-201. Issue 2. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/920449/201_-_FSR-G-201_Validation_Guidance_Issue_2.pdf [Accessed on 02 Feb 2021]

Fox C, Liu H, Kaye AD. Manchikanti L, Trescot AM, Christo PJ, et al, eds. *Clinical Aspects of Pain Medicine and Interventional Pain Management: A Comprehensive Review*. Paducah, KY: ASIP Publishing;; 2011. Antianxiety agents; pp. 543–552. In.
Freedom of Information Request 5687 Drug Driving., H.o.D.S.P.a.F.t.D.V.L. Agency, Editor.

Freedom of Information Request 4976 Drug Driving. Head of Data Sharing Policy Freedom of Information and Publication Scheme Unit. Driver and Vehicle Licensing Agency.

Freedom of Information request 2016/238 Drug Driving. Freedom of Information and Publication Scheme Unit, North Wales Police.

Furukawa TA, Streiner DL, Young LT. Antidepressant and benzodiazepene for major depression. Cochrane Database Syst Rev 2002; 1: CD001026.

Gambi, F., Conti, C., Grimaldi, M., Giampietro, L., De Berardis, D. and Ferro, F., 1999. Flunitrazepam a Benzodiazepine Most used among Drug Abusers. *International Journal of Immunopathology and Pharmacology*, 12(3), p.205873929901200.

Gerecke, M., 1983. Chemical structure and properties of midazolam compared with other benzodiazepines. *British Journal of Clinical Pharmacology*, 16(S1), pp.11S-16S.

Gerlach, L., Wiechers, I. and Maust, D., 2018. Prescription Benzodiazepine Use Among Older Adults. *Harvard Review of Psychiatry*, 26(5), pp.264-273.

Gordon, C., Arup, G., 1990. Modeling the benzodiazepine receptor binding site by the general three-dimensional structure-directed quantitative structure-activity relationship method. *Molecular Pharmacology*, 37, pp.725-734

Goswami, J., 2015. Different Separation or Experimental Techniques for Clinical Chromatography: Small Review. *Journal of Chromatography & Separation Techniques*, 06(07).

Greenblatt, D., Allen, M., Locniskar, A., Harmatz, J. and Shader, R., 1979. Lorazepam kinetics in the elderly. *Clinical Pharmacology & Therapeutics*, 26(1), pp.103-113.

Greenblatt, D., Shader, R., Divoll, M. and Harmatz, J., 1981. Benzodiazepines: a summary of pharmacokinetic properties. *British Journal of Clinical Pharmacology*, 11(S1), pp.11S-16S.

Griffin, 3., Charles E et al. (2013) 'Benzodiazepine pharmacology and central nervous system-mediated effects', The Ochsner Journal, 13(2), pp. 214-223.

Grzybowski, S., 2004. The black market in prescription drugs. *The Lancet*, 364, pp.28-29.

Gunn, J., 2015. Understanding the toxicology of diazepam. *Focus on screen*, 12:1

Hartman, R.L., et al., *Effect of Blood Collection Time on Measured Delta9-Tetrahydrocannabinol Concentrations: Implications for Driving Interpretation and Drug Policy.* Clin Chem, 2016. **62**(2): p. 367-77.

Health and Social Care Information Centre. 2016. *Prescriptions dispensed in the community, England 2005–2015* (HSCIC), https://www.gov.uk/government/statistics/prescriptions-dispensed-in-the-community-england-2005-to-2015 (accessed 16 Jan 2020).

Ho CS, Lam CWK, Chan MHM, Cheung RCK, Law LK, Lit LCW, et al. Electrospray ionisation mass spectrometry: principles and clinical applications. Clin Biochem Rev. 2003;24:3–12.

Home Office of Great Britain. Preliminary Drug Testing Devices: A Guide to Type Approval Procedures for Preliminary Drug Testing Devices Used for Transport Law Enforcement in Great Britain.

Højskov, C., Heickendorff, L. and Møller, H., 2010. High-throughput liquid–liquid extraction and LCMSMS assay for determination of circulating 25(OH) vitamin D3 and D2 in the routine clinical laboratory. *Clinica Chimica Acta*, 411(1-2), pp.114-116.

Ibrahim. S. The new ISO 17025 requirement. 2017.

Isacson, D., 1997. Long-term benzodiazepine use: Factors of importance and the development of individual use patterns over time—A 13-year follow-up in a Swedish community. *Social Science & Medicine*, 44(12), pp.1871-1880.

J. Pitt, J., 2009. Principles and Applications of Liquid Chromatography-Mass Spectrometry in Clinical Biochemistry. *Clin Biochem Rev.*, 30(1), pp.19–34.

Johansson, B., 2003. DEPENDENCE ON LEGAL PSYCHOTROPIC DRUGS AMONG ALCOHOLICS. Alcohol and Alcoholism, 38(6), pp.613-618.

Jones, A., Holmgren, A. and Kugelberg, F., 2007. Concentrations of Scheduled Prescription Drugs in Blood of Impaired Drivers: Considerations for Interpreting the Results. *Therapeutic Drug Monitoring*, 29(2), pp.248-260.

Jones, A.W., Driving under the influence of drugs in Sweden with zero concentration limits in blood for controlled substances. Traffic Inj Prev, 2005. **6**(4): p. 317-22.

Jongen, S., Vuurman, E., Ramaekers, J. and Vermeeren, A., 2018. Comparing the effects of oxazepam and diazepam in actual highway driving and neurocognitive test performance: a validation study. *Psychopharmacology*, 235(4), pp.1283-1294.

Kachhawaha, A., Nagarnaik, P., Jadhav, M., Pudale, A., Labhasetwar, P. and Banerjee, K., 2017. Optimization of a Modified QuEChERS Method for Multiresidue Analysis of Pharmaceuticals and Personal Care Products in Sewage and Surface Water by LC-MS/MS. *Journal of AOAC INTERNATIONAL*, 100(3), pp.592-597.

Kaufmann, W., Humpel, C., Alheid, G. and Marksteiner, J., 2003. Compartmentation of alpha 1 and alpha 2 GABAA receptor subunits within rat extended amygdala: implications for benzodiazepine action. *Brain Research*, 964(1), pp.91-99.

Keshishian, H., T. Addona, et al. (2007) "Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution." Mol Cell Proteomics 6:2212-29. Abstract

Kebarle, P., 2000. A brief overview of the present status of the mechanisms involved in electrospray mass spectrometry. *Journal of Mass Spectrometry*, 35(7), pp.804-817.
Keshishian, H., Addona, T., Burgess, M., Kuhn, E. and Carr, S., 2007. Quantitative, Multiplexed Assays for Low Abundance Proteins in Plasma by Targeted Mass Spectrometry and Stable Isotope Dilution. *Molecular & Cellular Proteomics*, 6(12), pp.2212-2229.

Kelly, M., Smith, A., Banks, G., Wingrove, P., Whiting, P., Atack, J., Seabrook, G. and Maubach, K., 2002. Role of the histidine residue at position 105 in the human α5 containing GABAAreceptor on the affinity and efficacy of benzodiazepine site ligands. *British Journal of Pharmacology*, 135(1), pp.248-256.

Kennedy, K. and O'Riordan, J., 2019. Prescribing benzodiazepines in general practice. *British Journal of General Practice*, 69(680), pp.152-153.

Kim Do-Hyung., Cho Ji-Yoon., Chae Soo-In, Kang Bo-Kyung, An Tae-Gil, Shim Wang-Seob, Noh Young Su, Hwang Se Jung, Chung Eun Kyoung and Lee Kyung-Tae. 2017. Development of a simple and sensitive HPLCMS/MS method for determination of diazepam in human plasma and its application to a bioequivalence study. *Transl Clin Pharmacol, 25(4), pp173-178.*

Kurtz, S., Buttram, M. and Surratt, H., 2016. Benzodiazepine Dependence among Young Adult Participants in the Club Scene Who Use Drugs. *Journal of Psychoactive Drugs*, 49(1), pp.39-46.

Lader, M., 1991. History of benzodiazepine dependence. *Journal of Substance Abuse Treatment*, 8(1-2), pp.53-59.

Ledingham, D., 1999. Drugs and driving: a retrospective study of the analyses of blood and urine specimens submitted to the Lothian and Borders Police Forensic Laboratory. *Journal of Clinical Forensic Medicine*, 6(3), pp.133-140.

Langel K, Gjerde H, Favretto D, Lillsunde P, Oiestad EL, Ferrara SD, et al. Comparison of drug concentrations between whole blood and oral fluid. Drug Test Anal. 2014;6(5):461–71

Maddalena DJ, Johnston GAR. 1995. Prediction of receptor properties and binding affinity of ligands to benzodiazepine/GABAA receptors using artificial neural networks. *J Med Chem*; 38:715–724

Majid, S., Khanday, W. and Tomar, R., 2012. Synthesis of 1,5-Benzodiazepine and Its Derivatives by Condensation Reaction Using H-MCM-22 as Catalyst. *Journal of Biomedicine and Biotechnology*, 2012, pp.1-6.

Marmorstein, N., 2011. Associations between subtypes of major depressive episodes and substance use disorders. *Psychiatry Research*, 186(2-3), pp.248-253.

Mattila-Evenden, Johan Franck, Ulf, M., 2001. A study of benzodiazepine users claiming drug-induced psychiatric morbidity. *Nordic Journal of Psychiatry*, 55(4), pp.271-278.
Meyer, U., 2012. Cytochrome P450 enzymes. *Drug Metabolism and Drug Interactions*, 27(1), pp.1-2.

Moein, M., El Beqqali, A. and Abdel-Rehim, M., 2017. Bioanalytical method development and validation: Critical concepts and strategies. *Journal of Chromatography B*, 1043, pp.3-11.

Moffat, A., Osselton, M., Widdop, B. and Watts, J., 2011. *Clarke's Analysis Of Drugs And Poisons*. London and Chicago: Pharmaceutical Press.

Moffat, A., Osselton, M., Widdop, B. and Clarke, E., 2004. *Clarke's Analysis Of Drugs And Poisons 2004*. [London]: Pharmaceutical Press.

Moloney, M., Konrad, T. and Zimmer, C., 2011. The Medicalization of Sleeplessness: A Public Health Concern. *American Journal of Public Health*, 101(8), pp.1429-1433.
Morin, C., Bélanger, L., Bastien, C. and Vallières, A., 2005. Long-term outcome after discontinuation of benzodiazepines for insomnia: a survival analysis of relapse. *Behaviour Research and Therapy*, 43(1), pp.1-14.

National Highway Traffic Safety Administration. Drugs and Human Performance Fact Sheets. Available from: https://www.wsp.wa.gov/breathtest/docs/webdms/DRE_Forms/Publications/drug/Human_Performance_Drug_Fact_Sheets-NHTSA.pdf [Accessed on 02 Feb 2021]

Neutel, C., 1995. Risk of traffic accident injury after a prescription for a benzodiazepine☆. *Annals of Epidemiology*, 5(3), pp.239-244.

Nilsson, A., 1991. Pharmacokinetics of benzodiazepines and their antagonists. *Baillière's Clinical Anaesthesiology*, 5(3), pp.615-634.
Officer, J., *Trends in drug use of Scottish drivers arrested under Section 4 of the Road Traffic Act--a 10 year review.* Sci Justice, 2009. **49**(4): p. 237-41.

O' Keefe, M., 2013. Investigation Into Field Impairment Tests And An Evaluation Of Their Validity And Reliability As Clinical Tests Of Drug-Related Impairment Of Driving Ability. *Edinburgh Research Archive, Edinburgh Medical School Thesis and Dissertation*. Edinburgh.
Olfson, M., King, M. and Schoenbaum, M., 2015. Benzodiazepine Use in the United States. *JAMA Psychiatry*, 72(2), p.136.

Olga, E. Petrova and Karin, Sauer., 2017. High Performance Liquid Chromatography (HPLC)- based detection and quantitation of celluar c-di-GMP. *Methods Mol Biol*, 1657, pp. 33-43.

Orriols L, Salmi LR, Philip P, Moore N, Delorme B, Castot A, Lagarde E, The impact of medicinal drugs on traffic safety: a systematic review of epidemiological studies, Pharmacoepidemiology and Drug Safety, 2009, vol. 18(8): pp. 647-658.

PETURSSON, H., 1994. The benzodiazepine withdrawal syndrome. *Addiction*, 89(11), pp.1455-1459.

Petursson, H. and Lader, M., 1981. Withdrawal from long-term benzodiazepine treatment. *BMJ*, 283(6292), pp.643-645.

Perez, E., Knapp, J., Horn, C., Stillman, S., Evans, J. and Arfsten, D., 2016. Comparison of LC–MS-MS and GC–MS Analysis of Benzodiazepine Compounds Included in the Drug Demand Reduction Urinalysis Program. *Journal of Analytical Toxicology*, 40(3), pp.201-207.

Pitt, J., Eggington, M. and Kahler, S., 2002. Comprehensive Screening of Urine Samples for Inborn Errors of Metabolism by Electrospray Tandem Mass Spectrometry. *Clinical Chemistry*, 48(11), pp.1970-1980.

Poyares, D., Guilleminault, C., Ohayon, M. and Tufik, S., 2004. Chronic benzodiazepine usage and withdrawal in insomnia patients. *Journal of Psychiatric Research*, 38(3), pp.327-334.

Qadir, M., 2015. Recent Structure Activity Relationship Studies of 1,4-Benzodiazepines. *Open Journal of Chemistry*, pp.008-012.

Qriouet, Z., Qmichou, Z., Bouchoutrouch, N., Mahi, H., Cherrah, Y. and Sefrioui, H., 2019. Analytical Methods Used for the Detection and Quantification of Benzodiazepines. *Journal of Analytical Methods in Chemistry*, 2019, pp.1-11.

Quintela, O., Sauvage, F., Charvier, F., Gaulier, J., Lachâtre, G. and Marquet, P., 2006. Liquid Chromatography–Tandem Mass Spectrometry for Detection of Low Concentrations of 21 Benzodiazepines, Metabolites, and Analogs in Urine: Method with Forensic Applications. *Clinical Chemistry*, 52(7), pp.1346-1355.

Rashed, M., Bucknall, M., Little, D., Awad, A., Jacob, M., Alamoudi, M., Alwattar, M. and Ozand, P., 1997. Screening blood spots for inborn errors of metabolism by electrospray tandem mass spectrometry with a microplate batch process and a computer algorithm for automated flagging of abnormal profiles. *Clinical Chemistry*, 43(7), pp.1129-1141.

Regenthal R, Krueger M, Koeppel C, Preiss R*., 1999* Drug levels: therapeutic and toxic serum/plasma concentrations of common drugs. *J Clin Monit Comput. 15(7-8):529-44*

Riss, J., Cloyd, J., Gates, J. and Collins, S., 2008. Benzodiazepines in epilepsy: pharmacology and pharmacokinetics. *Acta Neurologica Scandinavica*, 118(2), pp.69-86.

Robertson, M. and Drummer, O., 1998. Stability of Nitrobenzodiazepines in Postmortem Blood. *Journal of Forensic Sciences*, 43(1), p.16081J.

Robbins, T. and Everitt, B., 1999. Drug addiction: bad habits add up. *Nature*, 398(6728), pp.567-570.

Rooney, B., et al., *Drugged Drivers Blood Concentrations in England and Wales Prior to the Introduction of Per Se Limits.* J Anal Toxicol, 2016.

Schulze, H., 2013. Recommendations of EU-project DRUID (Driving under influence of Drugs, Alcohol and Medicines). *European Journal of Public Health*, 23(suppl_1).

Schwarz HJ., 1979. Pharmacokinetics and metabolism of temazepam in man and several animal species. *British Journal of Clinical Pharmacology*. 8 (1): 23S–29S

Shobha, D., Chari, M., Selvan, S., Oveisi, H., Mano, A., Mukkanti, K. and Vinu, A., 2010. Room temperature synthesis of 1,5-benzodiazepine and its derivatives using cage type mesoporous aluminosilicate catalysts. *Microporous and Mesoporous Materials*, 129(1-2), pp.112-117.

Simon, G., Vonkorff, M., Barlow, W., Pabiniak, C. and Wagner, E., 1996. Predictors of chronic benzodiazepine use in a health maintenance organization sample. *Journal of Clinical Epidemiology*, 49(9), pp.1067-1073.

So S-S, Karplus M. Genetic neural networks for quantitative structure-activity relationships: Improvements and application of benzodiazepine affinity for benzodiazepine/GABAA receptors. J Med Chem 1996;39:5246–5256

SOFT 2006. Forensic Toxicology laboratory guidelines. Available from: http://www.the-ltg.org/data/uploads/guidelines/soft-guidelines_2006.pdf [Accessed 02 Feb 2021]

Sørensen Andreas T., Marco Ledri., Miriam Melis., Litsa Nikitidou Ledri., My Andersson., and Merab Kokaia., 2017. Altered Chloride Homeostasis Decreases the Action Potential Threshold and Increases Hyperexcitability in Hippocampal Neurons. *eNeuron, 4(6)*

Sternbach LH, Randall LO, Banziger R, Lehr H. 1968. Structure activity relationships in 1,4 benzodiazepine series. In: Berger A, Decker M, Drug affecting central nervous system. editors Inc. New York: 237-264.

Sternbach, L., 1979. The benzodiazepine story. *Journal of Medicinal Chemistry*, 22(1), pp.1-7.

SIEGHART, W., 1992. PHARMACOLOGY OF BENZODIAZEPINE RECEPTORS: AN UPDATE. *Clinical Neuropharmacology*, 15, pp.523A-524A.

SMINK, B., BRANDSMA, J., DIJKHUIZEN, A., LUSTHOF, K., GIER, J., EGBERTS, A. and UGES, D., 2004. Quantitative analysis of 33 benzodiazepines, metabolites and benzodiazepine-like substances in whole blood by liquid chromatography?(tandem) mass spectrometry. *Journal of Chromatography B*, 811(1), pp.13-20.

Stone, B., Correa, K., Brown, T., Spurgin, A., Stikic, M., Johnson, R. and Berka, C., 2015. Behavioral and Neurophysiological Signatures of Benzodiazepine-Related Driving Impairments. *Frontiers in Psychology*, 6

Storm, D. (2013) 'Tablets', Leviathan, 15(1), pp. 82-84.

Talpins S. K., Hayes C. (2004). *Drug Evaluation and Classification (DEC) Program: Targeting Hardcore Impaired Drivers*. Alexandria, VA: American Prosecutors Research Institute

TIAFT Oral Presentation, 2019, Dr Matthew Christopher, “The Profile of Road Traffic Toxicology since the introduction of per se limits”.

TIAFT. Laboratory Guidelines. Available from: http://www.tiaft.org/data/uploads/documents/tiaft-sta-laboratory-guidelines.pdf [Accessed 02 Feb 2021]

TIAFT. Recommendations on sample collection. Available from: http://www.tiaft.org/data/uploads/documents/tiaft-sta-recommendations-on-sample-collection.pdf [Accessed 02 Feb 2021]

van der Meyden, C., Bartel, P., De Sommers, K., Blom, M. and Pretorius, L., 1989. 'Effects of clobazam and clonazepam on saccadic eye movements and other parameters of 44 | P a g e psychomotor performance', European Journal of Clinical Pharmacology, 37(4), pp.365- 369.

van Rijnsoever, C., 2004. Requirement of 5-GABAA Receptors for the Development of Tolerance to the Sedative Action of Diazepam in Mice. *Journal of Neuroscience*, 24(30), pp.6785-6790.

Verster, J., Veldhuijzen, D. and Volkerts, E., 2004. 'Residual effects of sleep medication on driving ability', Sleep Medicine Reviews, 8(4), pp.309-325.

Verstraete, A., 2005. Oral fluid testing for driving under the influence of drugs: history, recent progress and remaining challenges. *Forensic Science International*, 150(2-3), pp.143-150.

Vindenes, V., et al., *Drugged driving arrests in Norway before and after the implementation of per se law.* Forensic Sci Int, 2014. **245**: p. 171-7

Wang, Q., Han, Y. and Xue, H., 2006. Ligands of the GABAA Receptor Benzodiazepine Binding Site. *CNS Drug Reviews*, 5(2), pp.125-144.

Welch TR, Rumack BH, Hammond K. 1977. Clonazepam overdose resulting in cyclic coma. *Clin Toxicol. 10(4):433-6.*

Wolff K, Brimblecombe R, Forfar JC, Gilvarry E, Johnston A, Morgan J, Osselton MD, Read L, Taylor D 2013. DRIVING UNDER THE INFLUCENCE OF DRUGS. Report from the Expert Panel on Drug Driving

Wick, J., 2013. The History of Benzodiazepines. *The Consultant Pharmacist*, 28(9), pp.538-548.

Wolff, K., Rostami-Hodjegan, A., Shires, S., Hay, A., Feely, M., Calvert, R., Raistrick, D. and Tucker, G., 2003. The pharmacokinetics of methadone in healthy subjects and opiate users. *British Journal of Clinical Pharmacology*, 44(4), pp.325-334.

Wu, C. and Sun, D., 2014. GABA receptors in brain development, function, and injury. *Metabolic Brain Disease*, 30(2), pp.367-379.