LABORATORY-BASED SIMULTANEOUS DETECTION OF RESIDUAL XYLAZINE AND SEDATIVE-HYPNOTICS FROM DRUGS-SPIKED BEVERAGES FOR FORENSIC INVESTIGATION

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LIST OF SYMBOLS

±	More or less
≈	Approximately
2C-C	2-(4-Chloro-2,5-dimethoxyphenyl)ethan-1-amine
CO_2	Carbon dioxide
H_2SO_4	Sulphuric acid
H_3O+	Hydronium ion
NaCl	Sodium chloride
pA	Peak area
pH	Potential of hydrogen
m/z	Mass-to-charge
NaH_2PO_4	Monosodium dihydrogen orthophosphate
Na_2HPO_4	Disodium monohydrogen orthophosphate
R^2	Linear regression
$[Ru(bpy)_3]^{2+}$	Tris(bipyridine)ruthenium (II) chloride,
T	Time
v/v	Volume per volume
v/v/v	Volume per volume per volume
w/v	Weight per volume

LIST OF UNITS

%	Percent
°C	Degree celsius
µg	Microgramme
µg/mL	Microgramme per millilitre
µL	Microlitre
µL/min	Microlitre per minute
µm	Micrometre
µmol/L	Mircomole per litre
AL	Attolitre
cm	Centimetre
eV	Electron volt
g/cm^3	Gramme per cubic centimetre
g	Gramme
kg	Kilogramme
M	Molar
mM	Millimolar
mg	Milligramme
mg/L	Milligramme per litre
mg/mL	Milligramme per millilitre
min	Minute
mL	Millilitre
mL/min	Millilitre per minute
mm	millimetre
Mol/L	Mole per litre
N	Normal

ng	Nanogramme
nL/min	Nanolitre per minute
nm	Nanometre
ppm	Parts per million
ppmw	Parts per million by weight
pptv	Parts per trillion by volume
rpm	Revolutions per minute
s	Seconds

LIST OF ABBREVIATIONS

% Recovery/ies	Percentage recovery/ies
%RSD	Percentage of relative standard deviation
µPAD	Microfluidic paper-based analytical device
ADME	Absorption, distribution, metabolism, and excretion
AdSV	Adsorptive stripping voltammetry
AET	Alcohol Education Trust
ATR-FTIR	Attenuated total reflectance-Fourier transform infrared
ATS	Amphetamine type stimulant
BCG	Bromocresol green
BDDE	Boron doped diamond electrode
BSTFA	O-Bis(trimethylsilyl) trifluoroacetamide
C^4D	Conductivity detector
CDSA	Controlled Drugs and Substances Act
CE	Capillary electrophoresis
CEN	European Committee for Standardisation
CFVE	carbon fibre veil electrode
CNS	Central nervous system
CW/DBV	Stableflex Carbowax/Divinylbenzene
Cy5	Cyanide dye
CZE-DAD	Capillary zone electrophoresis-diodearray detector
DAI	Direct aqueous injection
DEA	Drug Enforcement Administration
DEP/MS	direct electrospray probe/mass spectrometry
DESI/MS	desorption electrospray ionisation/mass spectrometry
DFCs	Drug-facilitated crimes
DFSA	Drug-facilitated sexual assault
DI	Direct ionization/ desorption
DLLME	Dispersive liquid-liquid microextraction
DMSO	Dimethyl sulfoxide
DPV	Differential pulse voltammetry
d-SPE	dispersive solid phase extraction

ECD	electron capture detector
ECL	Electrochemiluminescence
e.g.	Exempli grati- for example
ELCD	Eelectrolytic conductivity detector
EME	Eelectro membrane extraction
ESI	Electrospray ionisation
ESSI	Electrosonic spray ionisation
et al.	et alia- and others
EWA	Early Warning Advisory
FID	Flame ionisation detector
FTIR	Fourier transform infrared
hC-dots	Hydrophobic C-dots
HPLC-PDA	High-performace liquid chromatography-photodiode array detector
HS-GC-FID	Head space-gas chromatography-flame ionisation detector
FDA	Food and Drug Administration
FIA	Flow injection analysis
FPD	Flame photometric detector
GABA	Gamma-aminobutyric acid
GBL	Gamma butyrolactone
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionisation detector
GC-MS	Gas chromatography-mass spectromerty
GHB	Gamma hydroxybutyrate
GNPs/SPCE	Nanoplatelets-modified screen-printed carbon electrode
HF-LLME	Hollow fiber supported liquid phase microextraction
HPTLC	High-performance thin layer chromatography
i.e.	id est- that is
i.d.	Internal diameter
IR	Infrared
IS	Internal standard
LC	Liquid chromatography
LC-DED	Liquid chromatography-dual electrode detector
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDA	Linear discriminant analysis

LDS-DLLME	Low density solvent dispersive liquid-liquid extraction
LLE	Liquid-liquid extraction
LLE-LTP	Liquid-liquid extraction-low temperature partitioning
LOD	Limit of detection
LOQ	Limit of quantitation
LPME	Liquid phase microextraction
LSD	Lysergic acid diethylamide
6-MAM	6-Monoacetylmorphine
MECC	Micellar electrokinetic chromatography
MDA	Misuse of Drug Act
MDMA	3,4-Methylenedioxymethamphetamine
MEPS	Microextraction by packed sorbents
MPFS	Multipumping flow system
MS	Mass spectrometry
NADA	National Anti-Drug Agency
NFC	Not from concentrated
NIST	National Institute of Standards and Technology
NMDA	N-methyl-D-aspartate
NPD	Nitrogen phosphorus detector
NPS	New psychoactive substance
NRS	National Statistic of Scotland
OTC	Over-the-counter
P2P	Benzyl-methyl ketone
PCs	Principal components
PCA	Principal component analysis
P-CE	Portable capillary electrophoresis
PCP	phencyclidine
PDA	Photodiode array detector
PDDAC/DS	Poly (diallyldimethylammonium chloride)/dextransulphate
PID	Photoionisation detector
PS/MS	Paper spray/mass spectrometry
PTR-MS	Proton transfer reaction mass spectrometry
QC	Quality control
QC_{low}	Low quality control

QC_{medium}	Medium quality control
QC_{High}	High quality control
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RI	Refractive index
RP-HPLC	Reverse phase high-performance liquid chromatography
RPLC	Reverse phase liquid chromatography
SAMHSA	Substance Abuse and Mental Health Services Administration
SBSE	Stir-bar sorptive extraction
SCX	Strong cation exchange
SDME	Single drop based liquid phase microextraction
SDS	Sodium dodecyl sulphate
SPCE	Screen-printed carbon electrode
SPE	Solid phase extraction
SPEs	Screen-printed electrode
SPME	Solid phase microextraction
SUSMP	Standard for the Uniform Scheduling of Medicines and Poisons
TCD	Thermal conductivity detector
TD-ESI/MS	Thermal desorption electrospray ionisation/mass spectrometry
TELISA	Thermometric enzyme-linked immunosorbent assay
TLC	Thin layer chromatography
UA-DLLME	Ultrasonic assisted-dispersive liquid-liquid extraction
UA-LDS-DLLME	Ultrasonic assisted-low density solvent dispersive liquid-liquid extraction
UNODC	United Nations Office on Drugs and Crime
UV	Ultraviolet
UV-VIS	Ultraviolet-visible
VOCs	Volatile organic compounds
WHO	World Health Organisation
ZWT	Zebrafish water tank

LIST OF APPENDICES

PENGESANAN SERENTAK SISA XILAZIN DAN SEDATIF-HIPNOTIK BERASASKAN MAKMAL DARIPADA MINUMAN YANG DIMASUKKAN DADAH BAGI PENYIASATAN FORENSIK

ABSTRAK

Jenayah yang disebabkan dadah (DFCs) melumpuhkan mangsa melalui pengambilan sejenis atau kombinasi dadah. Xilazin, ketamin dan benzodiazepin seperti diazepam dan nimetazepam merupakan dadah yang kerap digunakan dalam DFCs. Secara konvensional, sampel biologi dikumpulkan daripada mangsa dan dianalisis untuk membuktikan penggunaan dadah. Walau bagaimanapun, metabolisme pesat dengan kelewatan dalam analisis boleh menjejaskan pengesanan dadah tersebut. Oleh itu, minuman dimasukkan dadah dicadangkan sebagai bukti dalam penyiasatan DFCs. Kajian ini bertujuan untuk membangunkan strategi-strategi analisis untuk mengesan secara serentak xilazin dan sedatif-hipnotik (diazepam, ketamin dan nimetazepam) dalam minuman yang dimasukkan dadah dalam bentuk cecair, titisan dan kering. Dalam kajian ini, ujian kimia, spektroskopi inframerah transformasi Fourier-pantulan keseluruhan dikecilkan (ATR-FTIR), kromatografi gas (GC) dan kromatografi cecair berprestasi tinggi (HPLC) telah digunakan dan diaplikasikan untuk mengesan dadah sasaran daripada sampel minuman yang dimasukkan dadah. Ujian Janovsky membolehkan pengesan diazepam (≈ 5 µg), ketamin (≈ 1 mg), dan nimetazepam (≈ 4 µg). Xilazin tidak dapat dikesan menggunakan ujian kimia. ATR-FTIR digabungkan dengan analisis kemometrik membolehkan diskriminasi xilazin dan sedatif-hipnotik (diazepam, ketamin dan nimetazepam), termasuk sampel yang diperoleh dalam bentuk kering. Kaedah GC-FID telah dibangunkan [had pengesanan (LOD) (xilazin: 80 ng/mL; ketamin: 80 ng/mL; nimetazepam: 160 ng/mL); lineariti

(R^2>0.99), kejituan (%RSD ≤7.19); kadar pemulihan (% pemulihan: 92.84% – 103.48%)] untuk mengesan dadah sasaran dalam minuman dimasukkan dadah. Dengan menggunakan kaedah GC yang dibangunkan bersama dengan prosedur pengekstrakan mikro cecair-cecair serakan (DLLME), kadar pemulihan yang memuaskan dicapai untuk sampel minuman berbentuk cecair (51.03% – 97.13%), titisan (48.27% – 95.91%) dan kering (44.38% – 92.71%) yang dimasukkan dadah. Kaedah HPLC juga dibangunkan [LOD (xilazin: 39.1 ng/mL; diazepam: 78.1 ng/mL; ketamin: 156.3 ng/mL; nimetazepam: 78.1 ng/mL); linearity (R2>0.99), kejituan (%RSD ≤7.81); kadar pemulihan (% pemulihan: 95.31% - 106.56%)] dan digunakan untuk mengesan dadah sasaran dalam sisa minuman dimasukkan dadah. Pemulihan yang baik dapat dicapai untuk sampel yang dimasukkan dadah dalam bentuk cecair (64.21% – 105.43%), titisan (49.45% – 81.67%) dan kering (47.24% – 88.27%). Namun, hanya 13.33% ketamin dapat diperolehi daripada air mineral yang wujud dalam bentuk kering. Kesimpulannya, kaedah analisis telah berjaya dibangunkan untuk mengesan sisa xilazin dan sedatif-hipnotik (diazepam, ketamin dan nimetazepam) secara serentak daripada minuman yang dimasukkan dadah. Kajian ini bermanfaat kepada pihak penguatkuasaan undang-undang dalam penyiasatan DFCs, terutamanya dalam penentuan sama ada ubat sedatif-hipnotik telah digunakan.

LABORATORY-BASED SIMULTANEOUS DETECTION OF RESIDUAL XYLAZINE AND SEDATIVE-HYPNOTICS FROM DRUGS-SPIKED BEVERAGES FOR FORENSIC INVESTIGATION

ABSTRACT

Drug facilitated crimes (DFCs) involve the incapacitation of a victim due to the administration of a single drug or a combination of drugs. Xylazine, ketamine and benzodiazepines such as diazepam and nimetazepam are also the frequently used drugs in DFCs. Conventionally, biological samples are collected from a victim and analysed to provide evidence of drug administration. However, the rapid metabolism of many such drugs together with delays in analysis can compromise the detection of such drugs. Therefore, drugs-spiked beverages were suggested as evidence during DFC investigation. The present study was aimed to establish the analytical strategies for simultaneous detection of xylazine and sedative-hypnotics (diazepam, ketamine and nimetazepam) in drugs-spiked beverages appearing in liquid, droplet, and dry forms. In this study, chemical tests, attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy, gas chromatography (GC) and high-performance liquid chromatography (HPLC) detection were used and applied for the detection of the target substances from drugs-spiked beverage samples. Janovsky test allowed for the detection of diazepam (≈ 5 µg), ketamine (≈ 1 mg) and nimetazepam (≈ 5 µg). Xylazine could not be detected using the chemical tests. ATR-FTIR in couple with chemometric analyses enabled the discrimination of xylazine and sedative-hypnotics (diazepam, ketamine and nimetazepam), including those samples recovered from the dry beverage remains. GC method was established [limit of detection (LOD) (xylazine;

80 ng/mL; ketamine: 80 ng/mL; nimetazepam: 160 ng/mL); linearity (R^2>0.99), precision (%RSD <7.20); accuracy (% recovery: 92.84 – 103.48%)] to detect target substances in spiked beverages remains. Satisfactory recoveries were achieved from drugs-spiked liquid (51.03% – 97.13%), droplet (48.27% – 95.91%) and dry (44.38% – 92.71%) samples through the application of developed GC method upon dispersive liquid-liquid microextraction (DLLME) procedure. A HPLC method was also established [LOD (xylazine: 39.1 ng/mL; diazepam: 78.1 ng/mL; ketamine: 156.3 ng/mL; nimetazepam: 78.1 ng/mL); linearity (R^2>0.99), precision (%RSD <7.82); accuracy (% recovery: 95.31% - 106.56%)] and applied to detect target substances in spiked beverage remains. Good recoveries were achieved from the drug-spiked samples in liquid (64.21% – 105.43%), droplet (49.45% – 81.67%) and dry (47.24% – 88.27%) forms. However, only 13.33% of ketamine was able to be recovered from mineral water appearing in dry form. To conclude, analytical methods were successfully developed to simultaneously detect residual xylazine and other drugs of abuse (diazepam, ketamine, and nimetazepam) from spiked beverages. This study is beneficial to the law enforcement authorities during DFCs investigation, especially the determination of whether a sedative-hypnotic drug had been used.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

1.1.1 Drug Facilitated Crimes

Drug facilitated crimes (DFCs) refer to malicious act involving the incapacitation of victims using alcohol or drugs (Pépin, 2014). Consequently, the victim's security and safety are threatened (LeBeau, 2009). Drug-facilitated sexual assault (DFSA) is frequently reported DFCs apart from abduction, burglary, and robbery (Shbair & Lhermitte, 2010). Fast-acting sedative-hypnotics which could affect the central nervous system (CNS) by controlling the bodily functions and movement are commonly used to commit DFCs (Rocca *et al*., 2016).

Historically, majority of alcoholic beverages often serve as an ideal medium for criminal to commit proactive or opportunistic DFC and DFSA cases (Anderson *et al*., 2017; Wells, 2001). Ethanol was used as agent to commit DFCs alone or together with other substances (Staub & Spörri, 2014), particularly for its euphoria effect (Wasserman, 2021). Other than that, addition of drugs associated with incapacitation capabilities, as well as memory loss (amnesia) into beverages have become one of the infamous tactics for DFC and DFSA offences among criminals (Gharedaghi *et al*., 2018). Such cases frequently happen in parties or nightclubs where majority of the victims are youngsters (Djezzar *et al*., 2014).

1.1.2 Drink Spiking

Drink spiking is a deliberate act of introducing alcohol or drugs into someone's beverage without their knowledge or consent (Nicole & Jarryd, 2021). Drink spiking can happen anywhere where beverages are served such as pubs, parties, nightclubs, restaurant, and even private homes. It is illegal and the punishment is dependent on the type of substance used and the seriousness of the offence (Nottinghamshire Police, 2022).

DFCs *via* drink spiking is not a new practice but could date back to late 1890s, where it was first described as "slipping a Mickey" (Shbair & Lhermitte, 2010). "Mickey" refers to beverages that spiked with incapacitating agent prior to victim's knowledge for the incapacitation purpose. "Slipping a Mickey" was a term originated from a Chicago saloon, Mickey Finn who was alleged of using knock-out drops (chloral hydrate) to incapacitate and robbed his customer (Parkin & Brailsford, 2009). Thereafter, the victims were found to be awaked later while experiencing amnesia.

Despite it being reported that drink spiking cases are on the rise; yet there is no empirical evidence supporting the statement (Carly, 2021). Drink spiking incidents are hard to detect due to a high number of under-reporting (Beynon *et al*., 2008). Victim did not realise that they have been spiked, anecdotal reporting, and even perceived as an urban legend are some causes resulted in difficulty of drink spiking to be detected (Beynon *et al*., 2005). In the United Kingdom, the incident of drink spiking is now becoming common and can be occurred to anyone (Matthew, 2022). A prospective study claimed that only nine plausible drink spiking cases were spotted among the 97 alleged drink spiking cases (Quigley *et al*., 2009). In a survey to study the drink spiking

incidents among university students, only 7.8% of them knew they were spiked (Swan *et al.*, 2017). According to a survey by Alcohol Education Trust (AET), 94 out of 747 people had been spiked with majority among females (15%) as compared to males (7%) (Matthew, 2022). Among the 94 people, only 8% of them made report on the incident, suggesting the underreporting of criminal cases related to drink spiking.

1.1.3 Drugs used in Drink Spiking

To date, more than 50 drugs were suspected to be used in drink spiking, including recreational drugs [ketamine, methamphetamine, lysergic acid diethylamide (LSD), cannabinoids, amphetamines and its derivative, opioids, cocaine, as well as phencyclidine (PCP)], sedative-hypnotics (benzodiazepines), gamma hydroxybutyrate (GHB), over-the-counter (OTC) and prescription medications (Shbair *et al*., 2010a; Shbair *et al*., 2010b]. As a drug for drink spiking, they often possess certain properties, including (1) tasteless, odourless and colourless; (2) dissolve easily in beverages; (3) produce rapid onset effects at a low dosage; (4) short half-live; (5) availability, either from workspace (paramedic, veterinary and medical), prescription, from OTC or street as well as online accessibility; as well as (6) capable of causing anterograde amnesia would be additional benefits as drugs used in drink spiking (Sonone *et al*., 2021). Such properties of drugs could initiate the desired effects with ease without the knowledge by victim (Beynon *et al*., 2005).

In this study, drugs which potentially reported in DFCs [xylazine and sedative-hypnotics (diazepam, ketamine and nimetazepam)] were investigated. Xylazine was reported as a novel substance used for committing DFCs in our neighboring countries, *i.e* Singapore (Elena, 2016) and Thailand (Krongvorakul *et al*., 2018). Note that the

administration of xylazine by human can cause hazardous consequences including death (Ruiz-Colón *et al*., 2014). However, analytical method capable to detect xylazine in relation to DFCs application is yet to explore. Meanwhile, ketamine was also selected in this study as it is commonly used drugging agent based on literatures (Albright *et al*., 2012; Gahlinger, 2004; Wille *et al*., 2013). It is actively used together with xylazine to induce anaesthesia in animals legitimately. Nevertheless, ketamine was also reported to be used together with xylazine (Fatih *et al*., 2004; Forrester, 2016; Liu *et al*., 2007).

It is well-known that benzodiazepines are often used to commit DFCs. (Rocha *et al.*, 2021; Vincenti *et al.*, 2021; Yen *et al.*, 2020). Presently, there are more than 2,000 of benzodiazepines are marketed (Jennifer, 2021). In this study, only two benzodiazepines, namely diazepam, and nimetazepam were selected. Diazepam is a prescription substance (Paula *et al.*, 2018; Magrini *et al.*, 2016; Sarin *et al.*, 1998). Legitimately, it is used together with both ketamine and xylazine in veterinary applications. However, diazepam had been reported where it was used together with ketamine in DFSA related cases (Wille *et al.*, 2013). Erimin-5 with the active ingredients composing of diazepam, or nimetazepam, are the two most common benzodiazepines abused (Mail *et al.*, 2021). It was noted that there are two benzodiazepines listed under the Dangerous Drug Act 1952, namely flunitrazepam and nimetazepam. In recent profiling study, the presence of flunitrazepam was hardly detected by the law enforcement authorities; but nimetazepam remains the substance under the benzodiazepines which frequently reported, probably due to relatively cheap price and ease of accessibility in black market (Mail *et al.*, 2021). It had also been used in DFCs (Yen *et al.*, 2020). Hereby, xylazine and other three sedative-hypnotics

(diazepam, ketamine and nimetazepam) were chosen as the target substances in this study.

1.2 Problem Statement

A significant rising in reports of DFCs globally threatened the safety of public. DFCs are criminal deeds committed when the victims were subjected to incapacitation effects of a substance. In most circumstance, substances were to be detected from biological samples such as blood, and urine when investigating DFCs (UNODC, 2011). Nevertheless, false negative results might be arisen owing to the nature of substance used in DFCs (high metabolism following administration, resulting in quick elimination from body) and others factors such as delays in reporting and sample collection. Restriction on the successful detection from these collected samples might cause the difficulties in DFCs and DFSA conviction (Busardò *et al.*, 2019; UNODC, 2011).

To overcome these limitations, previous studies had suggested the use of drugs-spiked beverages as the important forensic evidence in investigating DFCs (Acikkol *et al.*, 2009; Piergiovanni *et al.*, 2018; Yen *et al.*, 2020; Zhai *et al.*, 2014). During forensic investigation, the drugs-spiked beverages residue might appear as liquid, droplet or dry conditions (Albright *et al.*, 2012). Liquid sample represents the instance where a residual volume of more than 2 mL appeared in the drinking glass, frequently due to the incomplete consumption of the beverages by the victims. Droplet samples can be encountered when the small volume of spiked beverages, usually less than 1 mL, are left unattended in the drinking glass for few hours. Such condition appears where the small volume tends to stick to the inner wall or the bottom of the

drinking glass, as well as a slightly delay on the sampling collection allowed for evaporation of beverages samples. Complete consumption of beverages by victims or delayed in forensic analysis of collected sample might lead to the third scenario where the evidence is left in the form of totally dry residue.

Previously, detection strategies had been suggested to detect substances used in drink spiking predominantly appearing in liquid form; however, the detection of substances from residual beverages in the form of droplet and completely dried was lacking. On most occasions, qualitative determination of drugs-spiked droplet and dry sample was performed as for the detection of GHB (Gorecho III, 2011), ketamine (Albright *et al.*, 2012; Lygrisse *et al.*, 2009), flunitrazepam (D'Aloise & Chen, 2012) and diazepam (Øiestad *et al.*, 2014). Despite positive identification was possible through qualitative analyses; however, it is crucial to quantify the drugs spiked in the beverages. Quantification of these drugs is important to evaluate whether the drugs found in the beverage residue is due to deliberately addition for malicious intention or contaminated from unexpected sources (Øiestad *et al.*, 2014). This study provides laboratory-based detections of residual xylazine and other sedative-hypnotics (diazepam, ketamine, and nimetazepam) spiked in beverage remain appearing liquid, droplet, and dry forms. A combination of analytical methods could serve as a preventative action for the purpose of legal prosecution in the future, promoting a greater social well-being.

1.3 Aim and Objectives

This study was aimed to simultaneously determine residual xylazine and sedative-hypnotics (diazepam, ketamine, and nimetazepam) from drugs-spiked beverages appeared as liquid, droplet, and dry forms *via* laboratory-based analytical method for forensic investigation. To achieve the aim, the objectives of the study are set as follows:

1. To screen the presence of residual xylazine and sedative-hypnotics from drugs-spiked dry beverages *via* Marquis, Simon, Froehde and Janovsky tests.
2. To detect the presence of residual xylazine and sedative-hypnotics from drugs-spiked dry beverages *via* attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy.
3. To develop a gas chromatography (GC) method to simultaneously detect the residual xylazine and sedative-hypnotics from drugs-spiked beverages.
4. To develop a high-performance liquid chromatography (HPLC) method to simultaneously detect the residual xylazine and sedative-hypnotics from drugs-spiked beverages.
5. To assess the effectiveness of established analytical techniques in detecting the presence of residual xylazine and sedative-hypnotics from drugs-spiked beverages appearing in liquid, droplet, and dry forms.

1.4 Scope of Study

Among the substances which potentially spiked into the various beverages to facilitate crimes, xylazine and sedative-hypnotics (ketamine, diazepam and

nimetazepam) were chosen as the four substances of interest in this study. Xylazine is emerged as a novel substance used in DFCs which might cause hazardous consequences. Ketamine is commonly reported substances in DFCs, and likely to be abused together with xylazine. Diazepam and nimetazepam are two benzodiazepines commonly abused in Malaysia and reported as substances used in DFCs.

The design of this study shall be reflected in the routine forensic analysis in Malaysia settings. GC and LC are the two detection strategies commonly utilised by the Department of Chemistry, Malaysia for drug testing. Prior to the instrumental analysis, chemical spot tests are always carried out to narrow down the possible classification of suspected drugs. ATR-FTIR is also another readily available instrument in the forensic laboratories, suggesting its possible screening for the presence of drugs in relation to DFC. In view of this, four detection strategies were explored and established in this study.

During the drink spiking incidents, beverage of varying matrices suspected to have been spiked with hypnotic-sedatives could be potentially collected from crime scene. In this study, the representative beverages from alcoholic and non-alcoholic beverages were selected as the target substrates. The choices of the beverage were selected based on the previous literatures (Section 2.5) and availability in the local market. Meanwhile, only one type of glassware commonly seen in restaurant and drinking bar was selected to prepare the drugs-spiked droplet and dry samples, avoiding the variations due to the usage of different glassware.

1.5 Significance of Study

This study would be beneficial to the law enforcement agencies, particularly from the aspect of emergency medicine and forensic intelligence. The novelty of this study would be the establishment of detection strategies for simultaneous trace level detection of four xylazine and sedative-hypnotics (diazepam, ketamine and nimetazepam) for forensic investigation. The strategies suggested would also allow for good recoveries of the target substances, in addition to the qualitative detection of their presences to prove the occurrence of crimes.

Establishment of readily available analytical methods to simultaneously detect xylazine and sedative-hypnotics (diazepam, ketamine, and nimetazepam) could aid the emergency medicine settings where the studied substances intoxication was suspected. The ability to identify the substance that cause intoxication is of great importance to enable the physician to apply suitable treatments in suspected intoxication. Note that the target substances in this study, particularly xylazine could be fatal if taken excessively or in combination with other drugs of abuse especially with benzodiazepines and ketamine.

This study also proposed the appropriate sample processing protocol to collect and analyse the residual sedative-hypnotics spiked in the beverages for DFCs investigation. In real case scenario, the amount of sedative-hypnotics found in the drugs-spiked beverages might not be always ideal and adequate for analyses due to certain uncontrolled factors *i.e* environmental contamination or loss of samples due to delayed of sampling. The establishment of appropriate collection and analysis strategies could aid in maximising the recovery of sedative-hypnotics from spiked

beverages residues regardless of the physical appearance. Residual beverages might not be always present in liquid form whenever drink spiking cases occurred but also as droplet or completely dry. Therefore, such evidence shall not be overlooked as a source for the detection of suspected spiked drugs.

1.6 General Approaches of Study

Figure 1.1 illustrates the general approach used in this study. Generally, this study was divided into four major parts where the first two parts involved the screening assays using four conventional chemical spot tests (*i.e.* Marquis, Simon, Froehde, and Janovsky Test) (Chapter 3) and ATR-FTIR spectroscopy technique (Chapter 4). Subsequently, GC (Chapter 5) and HPLC (Chapter 6) methods were established as confirmatory assays to detect the xylazine and sedative-hypnotics (diazepam, ketamine and nimetazepam).

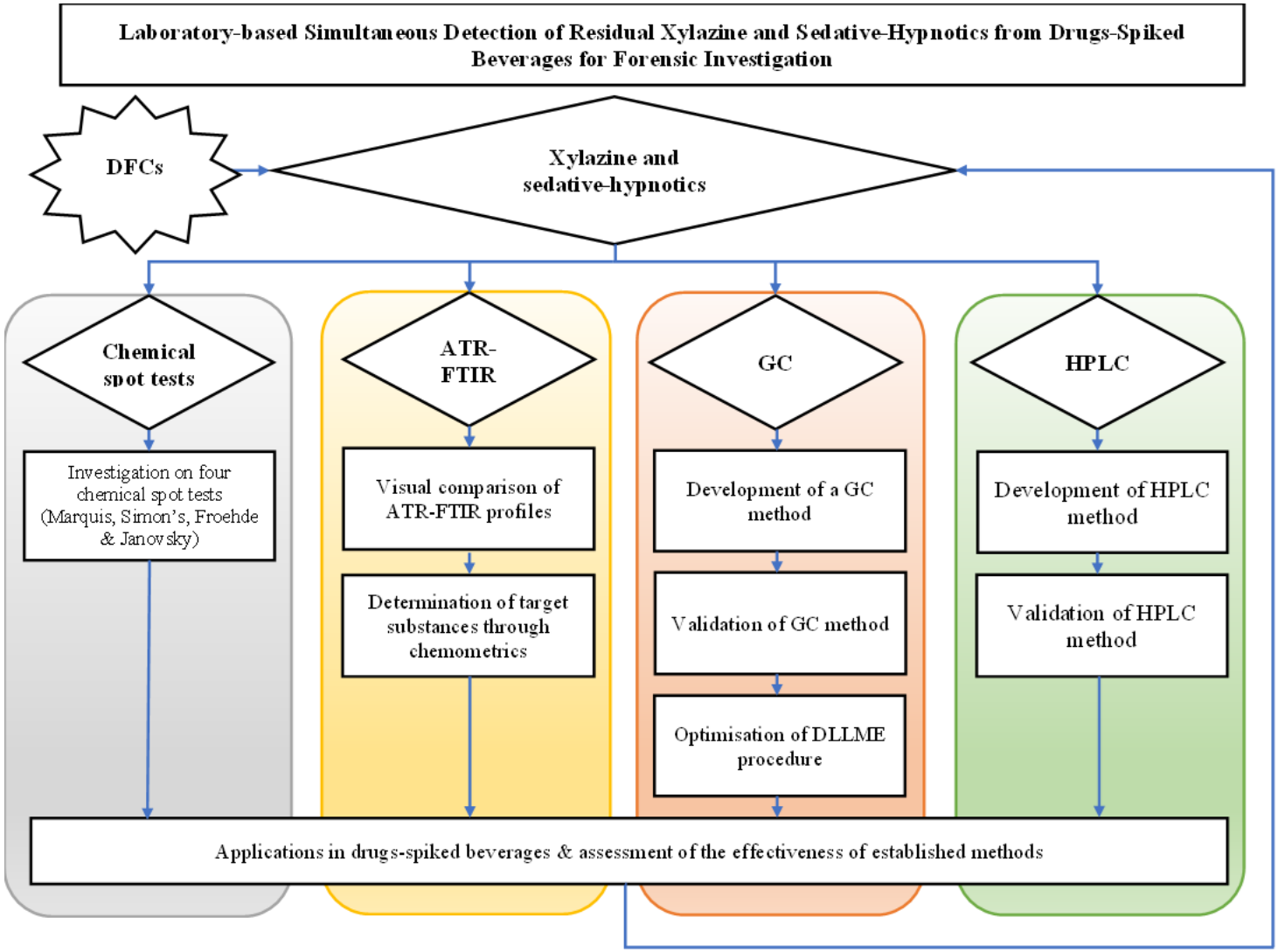

Figure 1.1: General approach used in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

World Health Organisation (WHO) defines drug as a chemical substance that capable to change the normal function when taken by living organisms into their body (WHO, 2021). Drug is recognised as a substance by pharmacopeia to diagnose, cure, mitigate, treat, and prevent diseases (FDA, 2017). Drugs are usually produced for medical treatment, but they are also involved in non-medical use, potentially affecting the societies from physical and mental perspectives. In this Chapter, non-medical use of drugs globally as well as in Malaysia were described. Then, the use of beverage as a medium for committing crime was highlighted. Subsequently, sample preparation procedures used to prepare drugs-spiked beverages prior to instrumental detection was discussed. Among the sample preparation procedures, DLLME used in this study, and its principle as well as the factors that affect extraction efficiency of DLLME were described. Finally, detection strategies used to detect drugs from spiked beverages were also discussed.

2.2 Non-medical Use of Drugs

Abuse of drugs refers to the non-therapeutic administration of drugs either persistently or occasionally to induce the positive psychoactive effects (FDA, 2019). Misuse of drugs refers to the extra-labeled usage of drugs or the drug itself is used for purposes other than prescribed by physicians (FDA, 2019). Despite the abuse and misuses of drugs shared a different terminology; however, they are collectively known as non-medical use of drugs by FDA (2020).

The non-medical use of drugs has surged since last decade even though such practice had affected the well-being including prosperity, health, and happiness (Smith *et al.*, 2013). A total of 35.8 million people originated from the United States aged 12 or older are associated with the non-medical use of drugs in 2019 (SAMHSA, 2020). In 2017, 4.5 million Canadian aged 15 or older initiated the use of illicit drugs, reporting a 2% increase since 2015 (Government of Canada, 2019). A decrease of new drugs abusers for approximately 13% was reported in Singapore in 2020 when compared with the previous year. Nevertheless, the number of drugs abusers in Singapore is remained significantly higher, achieving 38% of the total population (Central Narcotics Bureau, 2020).

In Malaysia, the number of drugs addicts reported in Malaysia remained static over the past three years after achieving a peak value at 30,844 in 2016 as shown in Figure 2.1 (AADK, 2021a). The number of new drugs addicts remained static over the years from 2017 to 2019. It was also reported that an increase in relapse drugs addicts within the same duration.

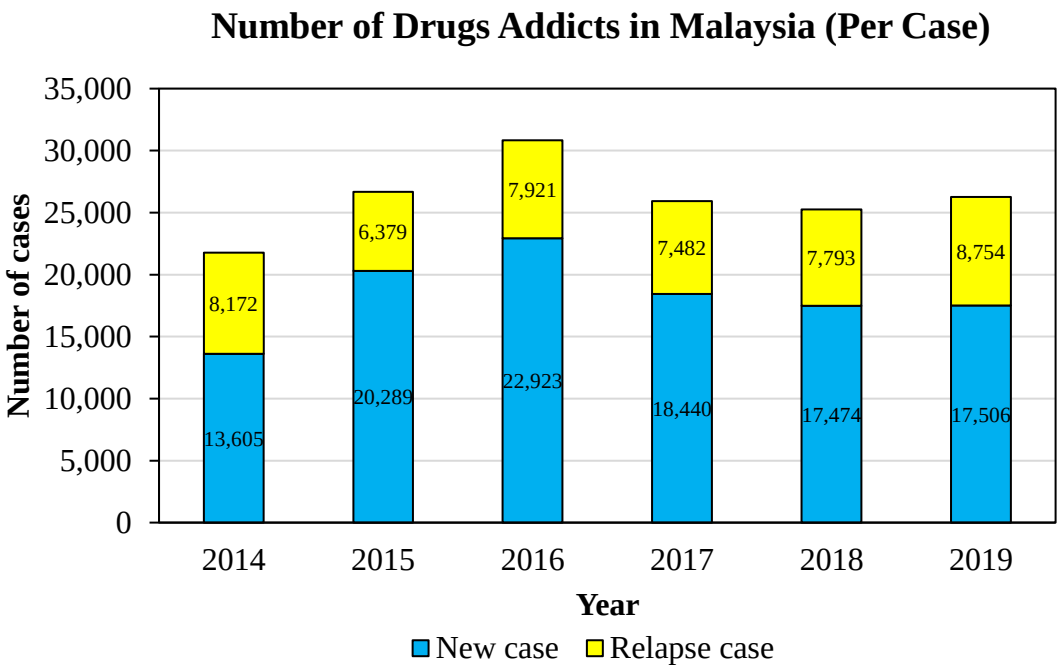

Figure 2.1: Number of drug addicts in Malaysia from year 2014 to 2019.

Marijuana (48.2 million) remained the highest illicit drugs used followed by pain reliever (9.7 million), hallucinogens (6.0 million), tranquilizer (5.9 million), cocaine (5.5 million), stimulant (4.9 million), inhalant (2.1 million), methamphetamine (2.0 million), and heroin (745,000) in the United States according to a survey conducted by SAMHSA (2020). Meanwhile, cannabis is the highest illicit drugs used by Canadian in 2017 (Government of Canada, 2019). In Singapore, stimulants, particularly methamphetamine continued as most popular drugs encountered, accounted for 79% of the total seized drugs, followed by new psychoactive substance (NPS) (9%), cannabis (7%) and other drugs (5%) consisting of cocaine, ketamine, and LSD (Central Narcotics Bureau, 2020).

In Malaysia, methamphetamine persisted as the most used illicit drugs in 2019 (Figure 2.2), contributing to 58.08% among the seized illicit drugs (AADK, 2021b). Opiates at approximately 28.54% remained the second highest abused illicit drugs of

the country. Amphetamine type stimulant (ATS) excluding methamphetamine and marijuana made up 10.33% and 2.71%, respectively. 0.33% of other miscellaneous drugs such as hallucinogens, inhalants, dissociative, kratom leaves, benzodiazepines, Erimin-5, and psychotropic pills totaled the seized illicit drugs in Malaysia.

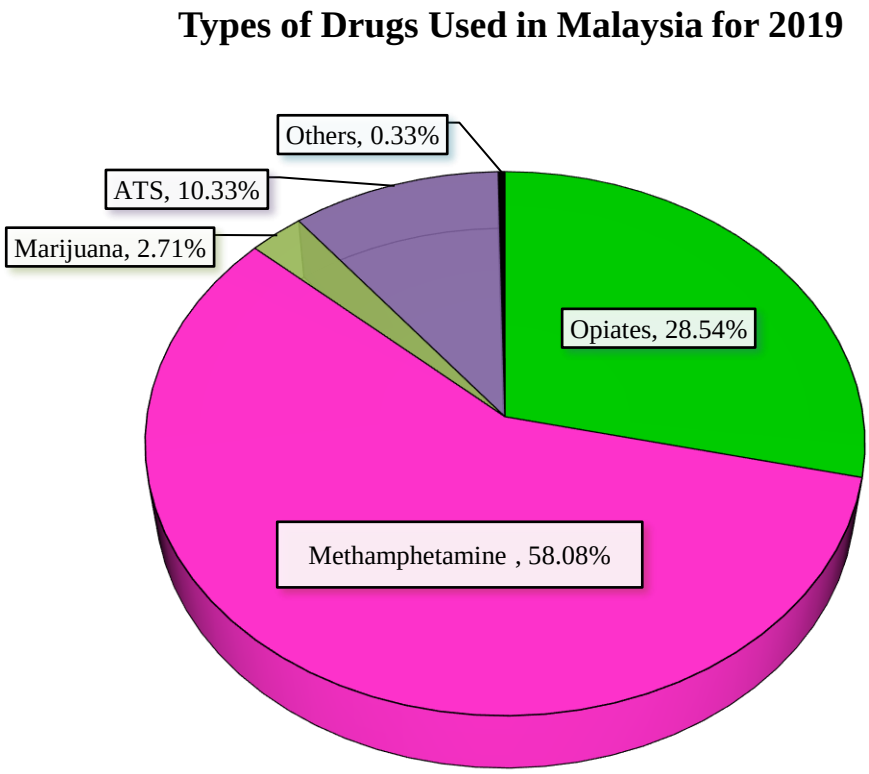

Figure 2.2: Types of drugs used in Malaysia in 2019.

Legislation and enactment were introduced, amended, and enforced by countries across the world to combat the non-medical use of drugs, especially matters regarding to the clandestine manufacturing, plant cultivation, trafficking, distribution, and sale of illicit drugs. In the United States, activities related to illicit drugs are regulated by the Controlled Substance Act. CDSA is the primarily legislation used by Canada to regulate drugs-related criminal offences (Ahmad, 2020). In Australia, the legal status of a drug is determined by the Poisons Standard (Australian Government,

2019). Misuse of Drug Act (MDA) lists the penalties for drugs-related offences in Singapore (Central Narcotics Bureau, 2020). Meanwhile, Dangerous Drugs Act 1952, Poison Act 1952, Dangerous Drugs (Special Preventive Measures) Act 1985 and Dangerous Drugs (Forfeiture Property) Act 1988 are four main legislations on drugs in Malaysia dealing in drug regulations (Kamarudin, 2007).

Dangerous Drug Act 1952 is the first enactment introduced in Malaysia to regulate the importation, exportation, manufacture, sale and use, possession, cultivation, the use of premise of specific drugs listed in the schedule of the Act. On the other hand, Poison Act 1952 regulates the use of drugs which do not covered by the Dangerous Drug Act 1952. Those drugs that listed under the Poison Act 1952 can be supplied and possessed by licensed professions. Dangerous Drugs (Special Preventive Measures) Act 1985 is a decree to detain and restrict preventively any person suspected to have involved in drug trafficking activities. Dangerous Drugs (Forfeiture Property) Act 1988 is enacted to make provisions on property-related offence, as well as seizure and forfeiture of property, in connection with activities related to the Dangerous Drug Act 1952.

2.3 Target Sedative-hypnotics

2.3.1 Xylazine

Xylazine (also known as N-(2,6-dimethylphenyl)-5,6-dihydro-4H-1,3-thiazin-2-amine) was firstly introduced in the Federal Republic of Germany by Farbenfabriken Bayer in 1962s (Greene & Thurmon, 1988). The structure of xylazine (Figure 2.3) is similar to tricyclic antidepressant, phenothiazine and clonidine with a pKa of 6.94 Xylazine was marketed as Anased®, Rompun®, and Sedazine® in the form of readily

water injectable solution or crystalline powder (Bayramoglu *et al.*, 2016; Hoffmann *et al.*, 2001). Xylazine is highly soluble in lipid and possesses high-volume of distribution. In other words, xylazine is a lipophilic substance which is capable to cross the blood-brain barrier easily to cause an effect in CNS.

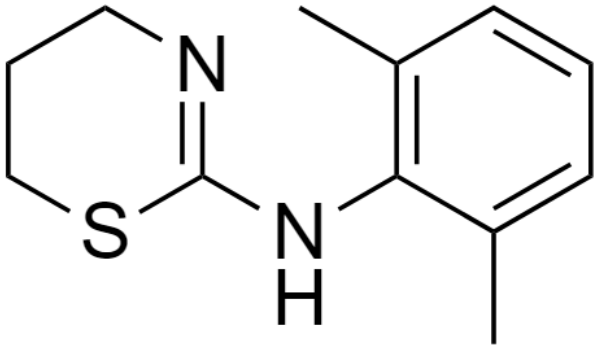

Figure 2.3: Chemical structure of xylazine.

Legitimately, xylazine could be used as non-narcotic sedative in veterinary field. Nevertheless, xylazine was prohibited to be used as human medicine, animal doping substance as well as animal food industries. Xylazine is not a controlled substance under the Drug Enforcement Administration (DEA). It can be prescribed under a physician's prescription in Australia and Canada. In Thailand, xylazine is listed as the control substance. Meanwhile, xylazine is categorised as Class B Poison under Malaysia Poison Act 1952 where dispensation of xylazine was only possible by a related registered practitioner.

In veterinary science, xylazine is applied as sedative, analgesic, muscle relaxant, immobilisation agent and sporadically as cat's emetic agent (Dart, 1999; Mittleman *et al.*, 1998; Plumb, 2008). As a veterinary α-agonists, xylazine requires a

minute amount to initiate effects and dose-dependent (Dart, 1999). In addition, profound analgesic and anaesthetic effects can be initiated when xylazine is used together with other drugs such as ketamine and diazepam (Dart, 1999).

Pharmacokinetics of xylazine in animal are well-established where xylazine was proven to demonstrate a high absorption, distribution, metabolism, and excretion (ADME) profile in animals. The pharmacokinetics of xylazine in human were poorly understood as it is not allowed for human administration. The plasma half-life of xylazine in human was estimated as 4.9 hours (Hoffmann *et al.*, 2001) while the effects of xylazine in human could end up to 72 hours (Velez *et al.*, 2006). Although the typical dosage of xylazine required to induce an effect in human remained unclear; yet literature suggested that 40 to 2400 mg of xylazine was responsible to induce toxicity in human. Inhalation, intramuscular, intravenous, oral, smoking, sniffing, and subcutaneous are approaches reported for non-medical use of xylazine human apart from accidental ocular exposure (Ruiz-Colón *et al.*, 2014).

Xylazine tends to metabolise considerably through cytochrome P450 route *via* *N*-*S*-dealkylation in the liver instead of eliminating in parent form *via* urine (Mulders *et al.*, 2016). At the early stage, 2,6-dimethylanline was identified as the major metabolites of xylazine in different species. Since then, the xylazine metabolism has been actively researched in rats and horses/equines (Lavoie *et al.*, 2013; Meyer & Maurer, 2013; Spyridaki *et al.*, 2004). In human, metabolites of xylazine from urine of both human and rats following exposure was analysed and compared by Meyer & Maurer (2013). Their experiment suggested that xylazine exhibited a similar metabolism route in both human and rat due to the formation of same metabolites of

xylazine. On the other hand, Matos *et al.* (2020) investigated the metabolism of xylazine in human by using zebrafish water tank (ZWT) method. 4-hydroxylated and oxygenated derivatives were two major metabolites discovered in their work (Matos *et al.*, 2020). Both metabolites were also suggested as target substances in detecting misuse of xylazine (Matos *et al.*, 2020). Nonetheless, such information is vital for developing analytical methods for detecting the misuse of xylazine and its metabolites from toxicology aspects.

Initially, xylazine was clinically test as drug for hypertension for human; later, it was prohibited for human administration due to compelling CNS depressant effects (Bayramoglu *et al.*, 2016). Nonetheless, non-medical use of xylazine by human as suicidal agent, recreational drug, adulterants to other drugs, and DFCs was found increased since 2000s. Recreational use of xylazine have been reported in Canada, New York, Puerto Rico, Philadelphia, Puerto Rico, and Texas. A total of 43 xylazine intoxication cases have been reported up to 2013, according to a review by Ruiz-Colón *et al.* (2014). Xylazine was also reported as an adulterant for heroin/diacetylmorphine (Reyes *et al.*, 2012; Torruella, 2011), with an aim to induce synergistic pharmacological effects when taken together (Ruiz-Colón *et al.*, 2014; Torruella, 2011). Due to its sedative effect, xylazine had been used to committed DFCs (Andresen-Streichert *et al.*, 2017; Elena, 2016; Jeanous, 2019; Krongvorakul *et al.*, 2018).

2.3.2 Diazepam

Diazepam (Figure 2.4) was first patented in 1959 and became the 117th most prescribed medicine in the United States in 2019. With a molecular formula of

$C_{16}H_{13}ClN_2O$ and chemical name of 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, diazepam belongs to the benzodiazepine family with anxiolytic properties. In the market, diazepam was available under the name of Valium®, Vazepam®, Valrelease®, Dizac®, Diaz Intensol® and Diastat® in the form of tablet or liquid (FDA, 2017). Diazepam exists as yellow white or solid white crystals with odorless and taste slightly bitter. In terms of solubility, diazepam was freely soluble in chloroform, soluble in alcohol and slightly soluble in water. The pKa of diazepam is reported as 3.4 (Manallack, 2007).

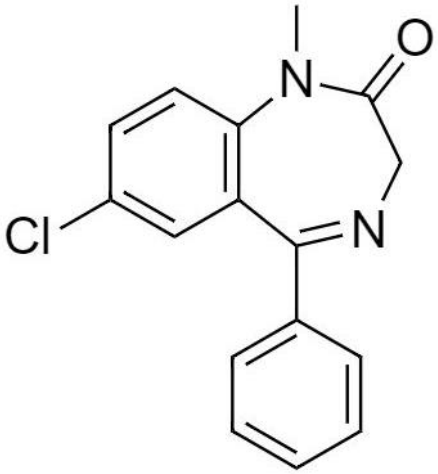

Figure 2.4: Chemical structure of diazepam

Diazepam is scheduled as controlled substances in certain countries. For instance, diazepam is a schedule IV substance under the Controlled Substances Act 1970 in United States and Controlled Drugs and Substances Act (CDSA) in Canada, respectively. In Australia, diazepam is listed as prescription only medicine under Standard for the Uniform Scheduling of Medicines and Poisons (SUSMP) with granted accessibility under the valid prescription. Diazepam is scheduled as poison under Poison Act 1952 in Malaysia.

Legally, diazepam is commonly used for the treatment of anxiety, and insomnia (Lance & Brian, 2000). Nonetheless, diazepam was also reported as a pre-medication for initiate anxiolysis, amnesia and sedation prior to medical procedures such as endoscopy (Bråthen *et al.*, 2005). In 2020, Valtoco® (diazepam nasal spray) is approved by Food and Drug Administration (FDA) for treatment of epilepsy in the form of nasal spray (FDA, 2020). Diazepam can also be prescribed for treating convulsive disorders, detoxification from alcohol and other substances and muscle spasticity.

Oral, intravenous, intramuscular and suppository are commonly reported routes of administration of diazepam. Following administration, diazepam took less than 30 min to produce the effects with peak response occurred between 15 and 60 min depending on the mode of administration. It was reported to has a half-life of 30 to 56 hours. Due to its high lipophilic nature, diazepam readily passes through the placenta and blood-brain barrier, and subsequently undergoes metabolism in liver primarily through cytochrome P450 enzyme system. In brief, demethylation, hydroxylation and glucuronidation are the main metabolic pathways undergone by diazepam (Rouini *et al.*, 2008). Desmethyldiazepam (nordiazepam) forms part of the main metabolite of diazepam while other minor metabolites of diazepam include temazepam and oxazepam (Rouini *et al*., 2008).

It was noted that diazepam is labelled as a substance with high risk of misuse, causing substance dependence and death (UNODC, 2020). According to a survey done by the Substance Abuse and Mental Health Services Administration (SAMHSA), the number of visits to emergency department due to non-medical use of diazepam had

been increased from 15,619 cases in 2004 to 21,118 cases in 2011 (SAMHSA, 2011). It was also reported as the substance accounted for 27% of drugs-related death in 2014, according to the National Statistic of Scotland (NRS) (NRS, 2016). In addition of recreational use of alone, diazepam was reported to be abused together with opioids and, also as a substance used in DFCs.

2.3.3 Ketamine

Ketamine (Figure 2.5), named after the ketone and amine group presence in its structure, was firstly synthesised by Calvin Steven in 1962. Also recognised as “K”, “Kit-kat”, “Special K” and 2-chlorphenyl-2-methylamino-cyclohexanone, ketamine belongs to a derivative PCP. Ketamine exists as white crystalline powder with a molecular weight of 237.73 g/mol. Due to its high lipid properties, ketamine can rapidly penetrate the blood-brain barrier, causing a rapid onset of effect (Atlee, 2007). The pKa of ketamine is reported as 7.4.

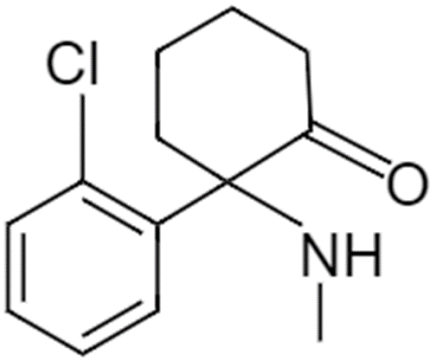

Figure 2.5: Chemical structure of ketamine.

The first human trial involving ketamine was reported in 1964 (Domino & Warner, 2010). Ketamine was approved for human administration in earlier years and used by the wounded soldiers during the Vietnam War. Since then, its medical

expedition was started to broaden. While the medical value of ketamine kept expanding, it had been misused as recreational drugs and led to the call by DEA in 1973 to regulate its misuse. In addition to its recreational use, ketamine was reported to have involved in DFCs (Albright *et al.*, 2012; Yehia *et al.*, 2020) Based on the survey of Early Warning Advisory (EWA) of United Nations Office on Drugs and Crime (UNODC), ketamine was used as incapacitation agent in three of the 32 DFSA cases occurred between August to December 2021 (UNODC EWA, 2021).

At presence, ketamine is listed as substance in Schedule III by DEA. It is also enlisted as a controlled substance by different countries due to its abuse potential. For instance, ketamine was a Schedule I substance in Controlled Drugs and Substance Act enacted by Canada government. In Australia, ketamine is categorised as Schedule 8 under the Poison Standard. In other word, ketamine is considered as a therapeutic substance with high possibly of abuse and addiction. Meanwhile, ketamine is placed under Schedule 10 of the Drug and Cosmetics Act India in 2013. Ketamine is regulated according to the Dangerous Drug Act 1952 Malaysia.

Ketamine is non-competitive antagonist of N-methyl-D-aspartate (NMDA) receptor, capable of causing cataleptic trance-like condition showing deep analgesia, unconsciousness, and amnesia. Ketamine is commonly administered orally, intravenously, intramuscularly, smoking and snorting. Following administration, ketamine can be distributed rapidly with a distribution half-life of 7 to 11 minutes (Copeland & Dillon, 2005). Metabolism of ketamine mainly occurred in the liver. It is metabolised into norketamine with the aid of CYP3A4 and CYP2B6 isoenzymes (Li *et al.*, 2011). Later, the norketamine would further convert into 5,6-

dehydronorketamine and 5-hydroxynorketamine, leading to elimination (UNODC, 2014).

Legitimately, ketamine is used to induce and maintain anaesthesia in patients diagnosed with asthma and acute bronchial constriction (Gao *et al.*, 2016). It is also reported as one of the among very few substances allowed to induce anaesthesia during caesarean sections (Margaret, 2015), in addition to anti-inflammation and anti-depressant treatments (Gao *et al.*, 2016). Apart from human treatment, ketamine is also widely applied in veterinary science as anaesthetic (Plumb, 2008).

Upon the intravenous administration of ketamine by human, adverse effects such as floating sensation, and numbness in their limbs with emergence delirium effects are reported (Domino & Warner, 2010). Accordingly, ketamine was proposed to be used together with sedatives, including diazepam and propofol to reduce these adverse effects (Domino & Warner, 2010). As a dissociative anaesthetic, it is capable to display mood controlling and rewarding characteristics (Liu *et al.*, 2016).

2.3.4 Nimetazepam

Nimetazepam or 1-methylnitrazepam is an intermediate-acting benzodiazepine derivative with strong anxiolytic, sedative, hypnotic, skeletal muscle relaxant and anticonvulsant characteristics. Nimetazepam was firstly introduced by Hoffmann-La Roche in 1964 and marketed as Erimin® and Lavol®. Figure 2.6 illustrates the chemical structure of nimetazepam with a molecular weight of 295.3 g/mol. The pKa of nimetazepam is reported as 2.53 (Inotsume & Nakano, 1980). It is practically insoluble