DEVELOPMENT OF A PORTABLE ELECTROCHEMICAL SENSOR IN DETECTING XYLAZINE FOR FORENSIC INVESTIGATION

KASRIN SAISAHAS

UNIVERSITI SAINS MALAYSIA

2022

DEVELOPMENT OF PORTABLE ELECTROCHEMICAL SENSOR IN DETECTING XYLAZINE FOR FORENSIC INVESTIGATION

by

KASRIN SAISAHAS

Thesis submitted in fulfilment of the requirements

for the degree of

Doctor of Philosophy

May 2022

ACKNOWLEDGEMENT

This thesis became a reality with the kind support and help of many people. I would like to express my sincere thanks to all of them. It is a genuine pleasure to express my deep sense of gratitude to my supervisor, Associate Professor Dr Ahmad Fahmi Lim bin Abdullah, and co-supervisor, Dr Chang Kah Haw for giving me the opportunity to work in my area of interest and for their help, advice, suggestions, and support through the whole duration of this work. They had provided insight, suggestion, and encouragement that have showed me the correct path and mindset to have as a researcher. I would like to express the deepest appreciation to my field supervisor, Associate Professor Dr Warakorn Limbut from the Division of Health and Applied Sciences, Faculty of Science, Prince of Songkhla University (PSU), Hat Yai, Songkhla for his help, advice, support, as well as the knowledge and supervision in electrochemistry during my research in the Department of Applied Science, PSU. I would also like to specially thank for "Subaru Group" in the analytical laboratory, PSU, especially Asamee, Kritsada, Kiattisak, Jenjira and Cheng Ho, for all exceptional assistance and profusely thank Lee Khai and Sarah for their help and encouragement. I am also grateful to the Universiti Sains Malaysia for providing the USM RUI grant (1001/PPSK/8012322) that have funded my research throughout the course of my study. Lastly, I am greatly indebted to my family they have been my strongest inspiration. I wish to record my deep gratitude to the love and support they have offered throughout my life. I hope this thesis and all my hard work will made them proud.

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LIST OF SYMBOLS

C	Concentration
D	Diffusion coefficient
E_p	Peak potential
E_{pa}	Oxidation potential
E_{pc}	Reduction potential
F	Faraday's constant
I	Current
I_{pa}	Anodic peak current
I_{pc}	Cathodic peak current
n	Number of electrons
pk_a	Acid dissociation constant
R_{ct}	Chart transfer resistance
t	Time
υ	Scan rate

LIST OF UNITS

%	percent
µA	microampere
µg	microgram
µL	microlitre
cm^2	square centimetre
g	gram
kg	kilogram
mg	milligram
min	minute
mL	millilitre
mol	mole
ng	nanogram
s	second
V	volt

LIST OF ABBREVIATIONS

4-NP	4-nitrophenol
AdSV	Adsorptive stripping voltammetry
AE	Auxiliary electrode
AgMCs	Silver microcubics
ANI	Aniline monomer
AuNPs, GNP	Gold nanoparticles
CE	Counter electrode
Cl-DNB	1-choloro-2,4-dinitrobenzene
CNTs	Carbon nanotube
CS	Chitosan
CV	Cyclic voltammetry
DNB	Dinitrobenzene
DNT	2,4-dinitrotoluene
DPV	Differential pulse voltammetry
EG	Exfoliated graphite
EIS	Electrochemical impedance spectroscopy
ePAD	Electrochemical paper-based analytical device
FE-SEM	Field Emission Scanning Electron Microscopy
FT-IR	Fourier-transform infrared spectroscopy
GCE	Glassy carbon electrode
GC-MS	Gas chromatography-mass spectrometry
GCMs	Glassy carbon microspheres
GLC	Gas liquid chromatography

GNPs	Graphene nanoplatelets
GNSs	Graphene nanosheets
GO	Graphene oxide
GR	Graphene
HPLC	High-performance liquid chromatography
HPLC-MS	High-performance liquid chromatography with mass spectrometry
HPLC-UV	High-performance liquid chromatography with UV-detection
LC-MS	Liquid chromatography mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LSV	Linear sweep voltammetry
LTT	Low-tack transfer tape
MCNT	Multiwalled carbon nanotubes
Nf	Nafion
NG	Nitroglycerin
PAA	Polyacrylic acid
PdNFs	Palladium nanoflowers
PdNPs	Palladium nanoparticle
PGr	Porous graphene
PM	Poly(melamine)
P(o-PDA-co-ANI)	Poly(o-phenylenediamine-aniline)
PVA	Poly vinyl alcohol
RE	Reference electrode
SPCE	Screen printed carbon electrode

SWAdSV	Square wave adsorptive stripping voltammetry
SWSV	Square wave stripping voltammetry
TNT	2,4,6-trinitrotoluene
TNB	Trinitrobenzene
UHPLC-QTOF	Ultra-HPLC with quadrupole-time of flight mass spectrometry
WE	Working electrode
YOH	Yohimbine

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PEMBANGUNAN SENSOR ELEKTROKIMIA MUDAH ALIH DALAM PENGESANAN XILAZIN BAGI PENYIASATAN FORENSIK

Abstrak

Xilazin merupakan suatu ubat bukan narkotik dan penyalahgunaannya telah dilaporkan dalam memudahcarakan kes-kes rompakan dan rogol disebabkan ciri-ciri tanpa warna, tanpa bau dan tanpa rasanya. Xilazin berkemungkinan membawa ancaman serius kepada masyarakat dan komuniti, terutamanya apabila melibatkan sempadan negara yang mempunyai status undang-undang berbeza. Justeru, kajian ini bertujuan untuk membangunkan sensor elektrokimia yang mudah, kos rendah, mudah alih, sensitif, bertindak balas cepat dan tanpa pra-pemprosesan untuk membantu penyiasatan forensik. Pertama sekali, suatu sensor elektrokimia telah direka dan dibangunkan berdasarkan elektrod karbon bercetak skrin (SPCE) yang diubahsuai dengan nanoplatelet graphene (GNPs), dan seterusnya ditentukan dengan voltametri pelucutan adsorptif (AdSV). Mikroskop elektron pengimbas dan voltammetri berkitar telah digunakan untuk mencirikan morfologi permukaan dan tingkah laku elektrokimia xilazin pada GNPs/SPCE. Pemuatan GNP untuk pengubahsuaian, pH elektrolit, potensi pengumpulan dan masa pengumpulan pada AdSV juga telah dioptimumkan. Dalam keadaan optimum, sensor ini memberi bacaan yang linear pada julat kepekatan antara 0.4 - 6.0 μg mL^{-1} dan 6.0 - 80.0 μg mL^{-1}. Had pengesanan (LOD) dan had kuantitatif (LOQ) masing-masing ditentukan pada 0.1 μg mL^{-1} dan 0.4 μg mL^{-1}. Kebolehulangan yang baik (3.57 - 6.85%) juga dilaporkan. Aplikasi GNPs/SPCE pada minuman yang ditambahkan xilazin juga menunjukkan pemulihan semula yang baik antara 80.8 ± 0.2 dan 108.1 ± 0.3%. GNPs/SPCE telah ditambahbaikkan dengan pembangunan suatu peranti analitikal elektrokimia berasaskan kertas (ePAD). ePAD

telah direka menggunakan pencetak pemotong lancing dan pita pemindahan lapisan rendah untuk menghasilkan templat pelitup tiga elektrod. Dakwat graphene seterusnya disalut ke atas kertas tersebut dengan teknik percetakan skrin dan ditambahbaik dengan polianilin nano berbentuk karang (PANI). Sensor PANI/ePAD tersebut telah diuji dengan menganalisis xilazin melalui AdSV. Keadaan rekaan dan operasi bagi PANI/ePAD juga telah dioptimumkan. PANI didapati telah membekalkan permukaan efektif yang lebih luas dan menggalakkan pemindahan caj antara xilazin dan permukaan elektrod. Kelelurusan telah dicapai dalam julat antara 0.2 - 5.0 μg mL^{-1} dan 5 - 100 μg mL^{-1}, dengan LOD dan LOQ masing-masing pada 0.06 μg mL^{-1} dan 0.22 μg mL^{-1}. Sensor PANI/ePAD tersebut juga telah menunjukkan kebolehulangan yang baik (1.52 – 4.79%) and pemulihan semula yang baik antara 85.0 ± 3.0% dan 105.0 ± 2.0% untuk penentuan xilazin dalam sampel minuman. Sensor elektrokimia mudah alih bagi pengesanan xilazin telah berjaya dibangunkan pertama kali untuk menguji kehadiran xilazin. Sensor tersebut boleh menyokong analisis forensik dengan membantu prosedur penyaringan dan pemantauan.

DEVELOPMENT OF A PORTABLE ELECTROCHEMICAL SENSOR IN DETECTING XYLAZINE FOR FORENSIC INVESTIGATION

Abstract

Xylazine is a non-narcotic medication and its misuses have been reported in facilitating robbery and rape cases due to its colourless, scentless, and tasteless nature. It potentially poses severe threat to the society and communities, especially when dealing with borders of countries having different legal status. Therefore, this study was aimed to develop simple, low cost, portable, sensitive, fast response and without pre-treatment electrochemical sensor to facilitate forensic investigation. Firstly, an electrochemical sensor was fabricated and developed based on graphene nanoplatelets (GNPs) modified screen-printed carbon electrode (SPCE), and subsequently determined by adsorptive stripping voltammetry (AdSV). Scanning electron microscope and cyclic voltammetry were used to characterise the surface morphology and electrochemical behaviour of xylazine on GNPs/SPCE. The loading of GNPs for modification, pH of electrolyte, accumulation potential and accumulation time of AdSV were also optimised. Under the optimal conditions, the sensor provided linear readings at the concentration range of 0.4 – 6.0 μg mL^{-1} and 6.0 – 80.0 μg mL^{-1}. The limit of detection (LOD) and the limit of quantitation (LOQ) were determined as 0.1 μg mL^{-1} and 0.4 μg mL^{-1}, respectively. Good reproducibility (3.57 - 6.85%) was also reported. Application of GNPs/SPCE on xylazine-spiked drinks also demonstrated good recoveries between 80.8 ± 0.2 and 108.1 ± 0.3%. GNPs/SPCE was further improved with development of an electrochemical paper-based analytical device (ePAD). The ePAD was fabricated using a craft cutter printer and low-tack transfer tape to create the three-electrode template mask. Graphene ink was then coated on the

paper by screen-printed technique and further improved with nano coral-like polyaniline (PANI). The PANI/ePAD sensor was tested by analysing xylazine through AdSV. The fabrication and operating conditions for PANI/ePAD were optimised, where PANI was found to have provided a larger effective surface area, promoting the charge transfer between xylazine and electrode surface. Linearity was obtained in the ranges of 0.2 - 5.0 μg mL^{-1} and 5 - 100 μg mL^{-1}, with LOD and LOQ of 0.06 μg mL^{-1} and 0.22 μg mL^{-1}, respectively. PANI/ePAD sensor also showed good reproducibility (1.52 – 4.79%) and good recoveries between 85.0 ± 3.0% and 105.0 ± 2.0% for the determination of xylazine in beverage samples. The portable electrochemical sensors for the detection of xylazine were successfully developed for the first time to test for the presence of xylazine. They could serve to support the forensic analysis, aiding the screening and monitoring procedures.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Xylazine, with a chemical name of N-(2,6-dimethyl phenyl)-5,6-dihydro-4H-1,3-thiazin-2-amine, is a non-narcotic drug, marketed only as veterinary sedative and analgesics (Ruiz-Colón et al., 2014). It was firstly synthesised by Farbenfabriken Bayer in 1962 for hypertensive treatment (Greene and Thurmon, 1988, Ruiz-Colón et al., 2014). As a derivative of clonidine, it is similar to phenothiazines, tricyclic antidepressants, and clonidine (Ruiz-Colón et al., 2014). Clinical studies presented the effect of this drug as an exorbitant depressant on the central nervous system and respiratory depression of human, potentially leading to conditions such as bradycardia, hypotension, and transient hyperglycaemia (Carruthers et al., 1979, Krongvorakul et al., 2018). Due to its negative health implications, the Food and Drug Administration (FDA) does not approve xylazine for human use (Ruiz-Colón et al., 2014, Forrester, 2016). Presently, xylazine is restricted to veterinary use only for analgesic, anaesthetic, and sedative purposes in cattle, sheep, goats, horses, cats, and primates. However, it was also reported to be smuggled for the use facilitating criminal activities (Spyridaki et al., 2004, Santonastaso et al., 2014).

Xylazine is a potent alpha2-adrenergic agonist that mediates *via* the stimulation of central alpha-2 (α2) receptors (Barroso et al., 2007). The release of norepinephrine and dopamine in the central nervous system is reduced by α2 stimulation, resulting in sedation, muscle relaxation, and decreased perception of pain stimuli (Samanta et al., 1990, Stillwell, 2003, Ruiz-Colón et al., 2014). The activities of this drug may also

relate to cholinergic, serotonergic, dopaminergic, α-1-adrenergic, histaminergic, or opiate mechanisms (Shi et al., 2016). In animals, there are a variety of known side effects, including the transient hypertension, hypotension, and respiratory depression (Swindle et al., 2002).

Xylazine can be administered intravenously, intramuscularly, and subcutaneously (Grant and Upton, 2004). Upon administrated into human, xylazine might cause of drowsiness, diarrhoea, muscle relaxation, and pain relief (Ruiz-Colón et al., 2014). It was noted that this drug acts mainly on the central nervous system and depending on the drug dosage, it might also lead to depression, sleep, muscle weakness and lowered respiratory rate (Samanta et al., 1990). The effects of xylazine shall begin within a few minutes and last up to four hours (Gallanosa et al., 1981, Greene and Thurmon, 1988, Velez et al., 2006). In certain instances, the drug might cause high blood pressure at first which subsequently decreased and remained constant causing arrhythmia (Samanta et al., 1990).

In Malaysia, xylazine cases have not been reported thus far on its abuse; however, there are several cases in Thailand and Singapore. In Thailand, xylazine is a controlled drug and must be labelled on the label and related document, stating with "required a veterinarian prescription and use under supervision by a therapist with a license" (ThaiFDA, 2013). In addition, the production, import, and distribution activities require license and are only allowed for authorised personnel. All the documentation must also be kept properly and at place. Trading xylazine from a veterinary medical store must only be sold to those who have a veterinarian's prescription. Note that if the licensee violates the regulations, he or she will be fined

from 2,000 to 10,000 baht (ThaiFDA, 2013). In Singapore, the introduction of xylazine into drinking water was reported claiming as love potion (Chong, 2016).

Apart from the legal way to obtain the xylazine, there are drug smuggling activities reported in the black market, online, or in various illegal stores. Indirectly, it causes the abuse of xylazine, particularly for the commission of crime. To date, the prevalence of xylazine misuse in Malaysia remains unclear. However, many cases in Thailand were found involving the abuse of xylazine which can be categorised as drug-facilitated crimes or DFCs. United Nations (UNODC, 2011) defined DFCs as "*criminal acts carried out by means of administering a substance to a person with the intention of impairing behaviour, perceptions or decision-making capacity*". In such cases, xylazine was mixed into the drinks by the perpetrators to be consumed by the victim, and after the victim becomes unconscious, they will rob or rape the victim (Online, 2013, Reporters, 2013).

It is vital for the law enforcement authority and the scientific community to pay the most serious attention in any potential occurrence of victimisation in DFC related crimes, particularly in drug-facilitated sexual assaults (DFSA) especially on the vulnerable groups such as women and children. Note DFSA on victims occurs when drugs are used, unknowingly by the victim, to compromise the victim's ability to consent to any unwilling sexual activity, or to make the victim unable to resist from subsequent assault (Jurado, 2015).

As stated by Madea and Mußhoff (2009), the drugs used in DFC shall be able to provide the property of sedation, induce sleep, alter the victim's behaviour, and

create a helpless state that the criminal can deliberately exploit. More importantly, the drugs shall be odourless and tasteless and must dissolve readily in drinks (Madea and Mußhoff, 2009). Based on these, xylazine becomes a good candidate for DFSA besides alcohol, gamma-Hydroxybutyrate (GHB), benzodiazepines and ketamine. Therefore, to cope with the xylazine cases that may have occurred or might potentially occur in Malaysia due to the border between countries, the properties of the drug, its crime scene detection and subsequent forensic laboratory analysis shall be initiated to prepare and avoid drug problems that may arise if a related case is found in the country.

1.2 Problem statement

Xylazine had been misused in robbery and rape cases, but it is not the target drug to be detected by law enforcement agencies. In other words, the drug is not tested and detected in any forensic evidence submitted to the forensic laboratories, unless specifically requested, and therefore, its misuse remains unclear, especially in Malaysia. It was noted that xylazine can be accessibility with appropriate license, and thus its existence in the local market and among the drug abusers must be given attention.

Today, the situation of xylazine is very similar to ketamine many years ago, which is also a veterinary drug but had been extensively abused in Malaysia and Thailand as a recreational drug or a drug to facilitate another crime. Ketamine had been misused and reported in many drug-facilitated crimes. In view of this, xylazine also demonstrates the potential to pose a severe threat to the society and communities, particularly against the victims in DFC and particularly DFSA among the vulnerable groups, and in the current situation when dealing with borders of countries having

different legal status of the substance concern. Besides routine biological sample analysis using urine, hair or blood in toxicological analysis, the initial analysis of first responders and crime scene investigators on any potential physical evidence and leftover residues are also the key for successful forensic investigation in DFC and DFSA investigation and prosecutions. Analysis of this drug, therefore, shall be investigated, screened, and analysed in a forensic science context, aiding the investigation.

Literatures reported on the analysis of xylazine using various instrumental techniques, including liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC–MS), as well as the high-performance liquid chromatography with UV detection (HPLC-UV) (Doran and Bradbury, 2015). In general, methods of DFSA drugs analysis are reliable and acceptable if it is validated with proper quality assurance system in place in the laboratory (UNODC, 2011). Nonetheless, many existing methods also possess certain limitations and disadvantages especially in biological samples and trace samples. Generally, these methods require technical experts, involve complicated and time-consuming sample preparation, need larger space for instrumentation, as well as expensive and costly maintenance. It is also worth noting that the future trend of forensic investigation service, as a result of increasing public demand and shrinking funding, various strategies such as quick screening for investigative intelligence during scene investigation and sample collection, at-the-point sample/evidence determination using portable kits and instruments, as well as possible readily-user friendly testing scheme, preferably *in-situ* without the need of trained technical personnel, could become the trend in future forensic service investigation and service delivery.

For the above reasons, a quick and innovative method for the detection of xylazine shall be established. The method shall be easy to use, simple to construct, low cost, portable, sensitive, fast response, and pre-treatment step-free, serving as is a good option to the forensic applications. Electrochemical approaches have attracted great attention in the sensor development due to their intrinsic remarkable advantages, including its simple, high sensitivity, fast analysis, and cost-effective properties (Yang et al., 2015, de Araujo et al., 2018, Mendes et al., 2019). For electroanalytical methods, screen-printed carbon electrode (SPCE) in sensor devices was used owing to its cheap, easy to use and suitable for on-site analysis (Radu et al., 2013, Lima et al., 2018). In this study, novel electrochemical sensor for the detection of xylazine was developed, assisting the forensic analysis.

1.3 Aim and objectives

The aim of this study is to develop novel electrochemical sensing system for the detection of xylazine in forensic applications. To accomplish the aim, the objectives of the study are defined as follows:

i. To fabricate and develop an electrochemical sensor on modified SPCE for detection of xylazine.

ii. To improve and modify the electrochemical sensor into cost-effective and portable electrochemical paper-based analytical device (ePAD) for on-site application in detecting xylazine.

iii. To investigate the electrochemical behaviours and determination of xylazine in beverage samples using the developed electrochemical sensors.

1.4 Research scope

This study involved two phases for the development of portable electrochemical sensor to detect the presence of xylazine in drug-spiked beverage samples.

In Phase I, a portable electrochemical sensor was fabricated and developed based on GNPs modified screen-printed carbon electrode (SPCE), and subsequently determined by AdSV. The experiments in Phase I included the following:

- SPCE modification with GNPs.
- Electrode characterisation.
- Optimisation of GNPs/SPCE, i.e. the effect of amount of GNPs on modified SPCE, pH of electrolyte, effect of scan rate, accumulation potential and accumulation time.
- Determination of analytical performance of GNPs/SPCE, i.e. linearity, limit of detection (LOD), limit of quantification (LOQ), selectivity and reproducibility.
- Drug-spiked sample analyses.

In Phase II, the electrochemical sensor was further improved based on an ePAD modified PANI. The experiments in Phase II included the following:

- Synthesis of PANI.
- Fabrication and modification of ePAD.
- Electrode characterisation.
- Optimisation of PANI/ePAD, i.e. effect of amount of PANI on modified ePAD, pH of electrolyte, effect of differential pulse parameter, accumulation potential, and accumulation time.

- Determination of analytical performance of electrochemical sensor, i.e. linearity, LOD, LOQ, selectivity and reproducibility.
- Drug-spiked sample analyses.
- Fabrication of portable drug sensor for xylazine detection.

1.5 Significance of study

Abuse of illicit drugs continues to threaten the society, economy, and nation worldwide, and particularly in DFC and DFSA, simply because the most vulnerable group in our society often becomes the victims. Recently, misuse of xylazine had been reported in many countries, and therefore, the development of a simple, fast, and accurate detection technique would allow the screening of possible existence of xylazine in forensic cases, including both Malaysia and Thailand.

The potential to apply and utilise a sensor on-site, particularly at a scene of crime, could act as the first line screening procedure towards the detection of xylazine. This is particularly important during monitoring and seizure, where huge number of suspected samples are involved. Apart from that, the sensing device could also serve for the purpose of rapid diagnosis of potential xylazine related cases. This research provides a method for the detection of xylazine, complementing both field testing and laboratory analysis by the electrochemical technique.

The successful development of the novel electrochemical sensor in detecting the presence of xylazine shall also be applied for the detection of other drugs of abuse. It could prove the suitability and efficiency of the sensing device to be applied for

forensic investigation and analysis, perhaps, the proof of concept can be further explored for multi-compounds analysis in miniaturised form.

1.6 Thesis organisation

The first chapter of the thesis covers the background of the study, problem statement, aim and objectives, research scope and significance of study. In Chapter 2, the first part reviews the chemical and physical properties of xylazine, while the second part provides information on the misuse of xylazine, including the legal status, case reports, as well as forensic significance of detecting xylazine. The last part of Chapter 2 also describes on the electrochemistry in forensic science, including the electrochemical techniques along with their applications in forensic science. Chapter 3 explains the materials and methods of this study. In short, the methodologies are described in two separate sections, namely the development of GNPs/SPCE and development of PANI/ePAD. Chapter 4 covers the results and discussion on the sensor development along with their applications for detecting xylazine in spiked beverage samples. Lastly, Chapter 5 concludes the study, and outlines the limitations and future recommendations for improvements to this work.

CHAPTER 2

LITERATURE REVIEW

2.0 Introduction

As a potential candidate for its abuse and misuse, either as drug of abuse for self-administration purposes or as potential substance in drug-facilitated crimes particularly for drug-facilitated sexual assaults, analytical study of xylazine and its properties as well as the method of its detection and identification for forensic purposes shall be initiated.

This chapter provides literature review on xylazine especially on its chemical and physical data as well as its toxicokinetic in Section 2.1. Literature review on misuse of xylazine, specifically on its legal status, cases involving xylazine and the forensic significance of xylazine detection was covered in Section 2.2. As this study focused only the electrochemical detection of xylazine (i.e., a part of research project that also covers chromatography study on xylazine by other study), reviews on electrochemical techniques such as voltammetry, amperometry and electrochemical impedance spectroscopy was covered in Section 2.3.1.

It is important to innovate and improve the ability of electrochemical detection method and therefore, in Section 2.3.2, a review on modification and improvement of electrochemical sensor focusing on the areas of electrode modification, carbon nanomaterials and conducting materials were briefly conducted. Section 2.3.3 focused on portable electrochemical sensor, which is an important aspect for forensic development of any detection strategies due to the nature of forensic setting and crime

scene requirements. Section 2.3.4 reviewed some electrochemical sensors in forensic applications such as in drugs testing, explosive material testing, gunshot residues testing as well as forensic food investigations. Finally, a section of review on forensic significance in using chemical sensors for forensic testing was presented in Section 2.3.5.

2.1 Xylazine

As described in section 1.1, xylazine was developed in early 1962 as an antihypertensive agent (Greene and Thurmon, 1988, Ruiz-Colón et al., 2014). Xylazine is also nicknamed as the "zombie drug". Due to it depressing effect on the central nervous system, it causes people to drift in and out of consciousness even when standing, giving them a zombie-like appearance (NYState, 2016). Clinical investigations discovered that this drug introduced the central nervous system depression, respiratory depression, bradycardia, hypotension, and even deadly effects in humans. Therefore, it was restricted to be used in human, with approval only for veterinary uses (Greene and Thurmon, 1988, Ruiz-Colón et al., 2014).

Xylazine acts similarly as clonidine in term of its action mechanism. Being an α2 adrenergic receptor agonist, it causes bradycardia and temporary hypertension followed by hypotension (Gallanosa et al., 1981, Wong et al., 2008). Symptoms such as sedation and decreased cardiac output are resulted by the inhibited release of norepinephrine (Gallanosa et al., 1981, Wong et al., 2008). FDA approved the drug only in veterinary use, exclusively for sedative, analgesic, and centrally acting muscle relaxant (Samanta et al., 1990, Silva-Torres et al., 2014a).

The administration of xylazine into the body can be either by intravenous, intramuscular, subcutaneous injection (Grant and Upton, 2004). Intravenous injection is more common as it allows for quick access to the circulatory system. With full dosage bioavailability, distribution via the circulatory system directly transports the xylazine to organs including heart, lungs, liver, and kidney (Tesfamariam and DeFelice, 2007, Silva-Torres et al., 2014a).

2.1.1 Chemical and physical data of xylazine

Xylazine is a methylbenzene or specifically 1,3-dimethylbenzene which is substituted by a 5,6-dihydro-4H-1,3-thiazin-2amine at position 2. Figure 2.1 illustrates the chemical structure of xylazine. Collectively, it is a methylbenzene, a 1,3-thiazin and a secondary amino compound. The physicochemical properties of xylazine retrieved from PubChem database are shown in Table 2.1.

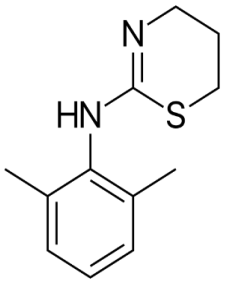

Figure 2.1: Xylazine structure

Table 2.1: Physical and chemical properties of xylazine (PubChem).

Characteristics	Xylazine
Formula	$C_{12}H_{16}N_2S$
CAS number	7361-61-7
Molecular mass	220.33 g/mol
Melting Point	140 °C
Colour	Colourless

2.1.2 Toxicokinetic of xylazine

Xylazine is a medication used by veterinarians to stun animals or as an anaesthetic for animals before surgery. The routes of xylazine administration in animals include intravenous, intramuscular and, subcutaneous, with the amount of xylazine to be used vary depending on the species of animals (Silva-Torres et al., 2014a). Pharmaceutical formulations availability of xylazine are either in 20, 100, or 300 milligrams (mg). The range of dosage for intramuscular administration are from 0.25 – 4.0 mg per pound, where else intravenously, it is at 0.5 mg per pound (Ruiz-Colón et al., 2014). This dosage could promote analgesia effect for 15-30 minutes, and the sedative effect might be able to proceed for 1-2 hours (Paddleford and Harvey, 1999), though the analgesic effects vary by species. The reported dosage rates required to induce sedative effects toward various animals are shown in Table 2.2 (Gross and Tranquilli, 1989).

Table 2.2: Xylazine doses required for animals by species.

Species	**Xylazine (mg/kg bodyweight)**	
	Intravenous	**Intramuscular**
Horses	0.5 to 1.1	1.0 to 2.0
Cattle	0.03 to 0.10	0.10 to 0.20
Sheep	0.05 to 0.10	0.10 to 0.30
Goats	0.01 to 0.50	0.05 to 0.50
Swine	-	2.0 to 3.0
Dogs	0.5 to 1.0	1.0 to 2.0
Cats	0.5 to 1.0	1.0 to 2.0
Birds	-	5.0 to 10.0

The pharmacokinetics of xylazine have been established in various animal species. To summarize, xylazine is rapidly absorbed, metabolised, and eliminated (Spyridaki et al., 2004, Velez et al., 2006). In 1981, Garcia-Villar and co-worker have studied the pharmacokinetics of xylazine in sheep, dog, cattle, and horse following intravenous xylazine administration (Garcia-Villar et al., 1981). In all the four species, the distribution half-life ($t_{1/2\alpha}$) was very short (1.21–5.97 minutes) and the elimination half-life ($t_{1/2\beta}$) were varied from 23 to 49 minutes. These values demonstrated that the xylazine level would decrease to a not detectable level within a few hours. Xylazine's bioavailability in intravenous after intramuscular administration were reported to be ranged from 17 to 73% in sheep, 52 to 90% in the dog, and 40 to 48% in the horse. The peak plasma concentrations could be reached within 12-14 minutes for all species. (Garcia-Villar et al., 1981).

On the metabolism of xylazine, there is limited information. In rats, Duhm and co-workers performed study using radio-labelled xylazine. They found that after intravenous injection (0.2-1.0 mg/kg), xylazine was rapidly disseminated to various tissues and 70% of the drug was eliminated in urine via kidney and 30% via faeces with a biological half-life of about 2-3 hours (Duhm et al., 1969). Moreover, only 8% of the activities corresponded to the unchanged form of the drug (Duhm et al., 1969). Similar pharmacokinetics would be expected in human, but prolonged effects had been noted in some patients administered with xylazine (Velez et al., 2006). Based on information from literatures, the dosages known to produce toxic effects in humans were varied from 40 mg up to 2400 mg. Table 2.3 describes several xylazine intoxication cases reported in human.

Table 2.3: Cases of xylazine intoxication in human.

Gender	Age	Usage*	Route	Dose (mg)	Concentration (mg/L)	References
Male	34	S	Intramuscular	1000	-	(Carruthers et al., 1979)
Female	20	S	Oral	400	-	(Gallanosa et al., 1981)
Female	39	A	Intramuscular	-	Serum: 0.03 Urine: 1.70	(Lewis et al., 1983)
Male	36	S	Intravenous	-	Serum: 0.2 Urine: 7.0	(Poklis et al., 1985)
Female	29	-	Intramuscular	40 (0.73 mg/kg)	-	(Spoerke et al., 1986)
Female	37	S	Intramuscular	2400 (22 mg/kg)	-	
Female	29	-	Intravenous	-	-	
Male	19	S	Subcutaneous	200	-	(Samanta et al., 1990)
Female	23	H	-	-	liver: 42 mg/kg kidney: 28 mg/kg brain: 19 mg/kg	(Mittleman et al., 1998)
Male	33	H	-	-	liver: 0.26 mg/kg kidney: 0.15 mg/kg brain: 0.16 mg/kg	
Male	27	S	Intramuscular	1500 (13 mg/Kg)	serum: 4.6 urine: 194 stomach: 446	(Hoffmann et al., 2001)

* A: accidental intoxication; H: homicide; S: suicide attempt.

Recently, a study on metabolism of xylazine in urine was investigated by Meyer and Maurer (2013). All metabolites identified in the rat urine could also be detected in human urine with similar relative abundances. The metabolic pathways were hypothesised as demonstrated in Figure 2.2. They included the conversion of N-

dealkylation to 2,6-dimethylaniline, hydroxylation followed by the dehydration to oxothiazine metabolites, N,S-di dealkylation to 2,6-dimethylphenylthiourea, S-oxidation to a sulfone, aromatic ring hydroxylation to two isomeric metabolites, followed by glucuronidation or sulfation (Meyer and Maurer, 2013). In rat urine after a low dose as well as in human urine after an overdose, the hydroxy metabolites were predominantly detected using the standard urine screening approaches by GC–MS and LC–MS (Meyer and Maurer, 2013).

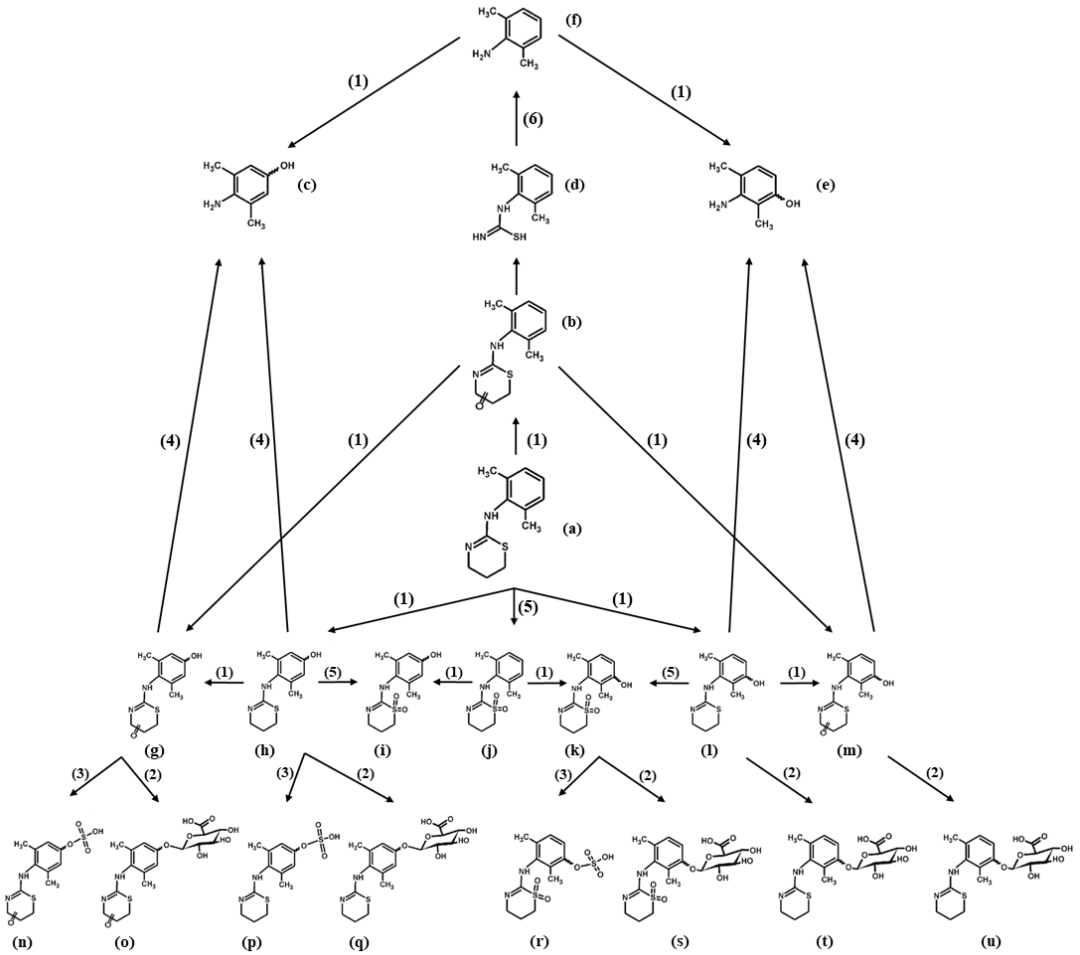

Figure 2.2: Proposed metabolic pathways of xylazine (Meyer and Maurer, 2013).

***(1)**: Hydroxylation, **(2)**: Glucuronidation, **(3)**: Sulfation, **(4)**: N,S-di-dealkylation, **(5)**: S-oxidation, **(6)**: N-dealkylation, **(a)**: Xylazine, **(b)**: Xylazine-M (oxo-), **(c)**: HO-2,6-dimethylaniline isomer 1, **(d)**: Xylazine-M (N-thiourea-2,6-dimethylaniline), **(e)**: HO-2,6-dimethylaniline isomer 2, **(f)**: 2,6-dimethylaniline, **(g)**: Xylazine-M (HO-oxo-) isomer 1, **(h)**: Xylazine-M (HO-) isomer 1, **(i)**: Xylazine-M (sulfone-HO-) isomer 1,

(j): Xylazine-M (sulfone, **(k)**: Xylazine-M (sulfone-HO-) isomer 2, **(l)**: Xylazine-M (HO-) isomer 2, **(m)**: Xylazine-M (HO-oxo-) isomer 2, **(n)**: Xylazine-M (HO-oxo-) isomer 1 sulfonate, **(o)**: Xylazine-M (HO-oxo-) isomer 1 glucuronide, **(p)**: Xylazine-M (HO-) isomer 1 sulfonate, **(q)**: Xylazine-M (HO-) isomer 1 glucuronide, **(r)**: Xylazine-M (sulfone-HO-) isomer 2 sulfonate, **(s)**: Xylazine-M (sulfone-HO-) isomer 2 glucuronide, **(t)**: Xylazine-M (HO-) isomer 2 glucuronide, **(u)**: Xylazine-M (HO-oxo-) isomer 2 glucuronide.

In human, xylazine causes drowsiness, diarrhoea, muscle relaxation and pain relief (Stillwell, 2003, Ruiz-Colón et al., 2014). It primarily affects the central nervous system and, depending on the dosage, causes exhaustion, sleepiness, muscle weakness, and reduction in respiratory rate (Samanta et al., 1990, Stillwell, 2003, Ganapathy et al., 2018). The effects of xylazine could be attenuated, blocked, and reversed with α2-adrenergic antagonist yohimbine (Hoffmann et al., 2001, Torruella, 2011). In the last decade, xylazine has become a popular recreational drug (Meyer and Maurer, 2013, Ruiz-Colón et al., 2014, Silva-Torres et al., 2014a) and widely used to adulterate illicit drugs such as cocaine, heroin, and speedball (a mixture of cocaine and heroin) (Silva-Torres et al., 2014b). However, the toxicity and effects of xylazine in combination with heroin and/or cocaine or other drugs in humans remained unexplored due to the restriction of its administration to humans (Spoerke et al., 1986, Silva-Torres et al., 2014b).

2.2 Misuses of xylazine

As with other common drugs of abuse such as Rohypnol, ecstasy, GHB, ketamine, and potentially xylazine, drug abuse is a serious public health and social issue. Many drugs have long been misused and therefore, their analytical studies and testing methods were well developed and validated, much such analytical wealth of information might still be absent for xylazine. As described in previous sections,

xylazine is not approved for human uses; however, it had, to some extent, though not widely yet, been misused as recreational drugs by drug abusers, for suicide or in accidental exposure, as well as to facilitate a crime.

Xylazine is an emerging adulterant with fentanyl in fatal drug intoxications, which has public health, safety, and criminal investigative implications (Nunez et al., 2020). This drug was also combined with opioid or other drugs such as cocaine and heroin to either increase or decrease the drug effects, as well as to raise the drug's value when sold on the street (Elejalde et al., 2003, Wong et al., 2008, Nunez et al., 2020). Detection of xylazine may help forensic pathologist distinguish illicit drug from those prescribed drugs, and law enforcement agents to trace the illicit drugs back to a specific drug supplier (Nunez et al., 2020).

2.2.1 Legal status of xylazine

Xylazine, an animal tranquilizer not authorised by the FDA for human use, had been misused for recreational purposes (Ruiz-Colón et al., 2014). It was classified as a schedule III-controlled substance in 1997 (NYState, 2016). Xylazine is becoming an increasingly popular recreational drug. The use of xylazine by human was started in Puerto Rico and had been spread to the continental United States (Detox, 2021). Moreover, heroin dealers use xylazine to enhance the effects of their products (NYState, 2016).

In Malaysia, xylazine is listed as Group B poison according to the Malaysia Poison Act 1952. Due to their similar effects upon administration, it has demonstrated potential as another “ketamine scenario”. Ketamine was classified as a dangerous drug

under the ACT 234 Dangerous drug ACT 1952 (revised 1980) of Malaysia. Recreational uses of ketamine in Malaysia were reported to be in the increase, especially outside of nightclubs and nightlife scenes (Li et al., 2011).

As for Thailand, xylazine is scheduled as a controlled medicine according to the Food and Drug Administration, Ministry of Health [Notification of the Ministry of Public Health (edition 45) B.E. 2557 (2014)]. The Ministry of Health, Thailand announced drug status for α2-adrenergic agonist groups (such as xylazine, detomidine, medetomidine, dexmedetomidine, and romifidine etc.) to be shifted from dangerous drugs to controlled drugs. Based on the Drugs Act, B.E. 2510 of Thailand, only the first-class veterinary practitioners could prescribe xylazine and it must be use under supervision from a therapist. It is compulsory to include information about the drug on its label and accompanying document as "Only sold by prescription of a first-class veterinary practitioner and used under the supervision of a therapist". A fine ranged from 2,000 to 10,000 baht (60 to 300$) could be imparted to those who breach the legislation under the ministerial regulations section 26(7) of Thailand. However, the rampant drug smuggling in the black market, online, or in various illicit businesses has led to xylazine misuses and criminal activities.

2.2.2 Case reports on xylazine misuses

There have been case reports on the misuses of xylazine in literatures, including both suicide and accidental exposure cases. Additionally, it had been used as recreational drugs by drug abusers or as adulterants to other illicit substances. More recently, xylazine spiking into food or drinks to facilitate criminal activities demands

serious attention from the law enforcement authorities. The following section described several selected cases indicating the current situation of this drugs.

Case 1: The clinical course and laboratory results of a patient who had self-administered overdose of xylazine was reported. The patient, a 34-year-old man with equestrian affiliations, had easy access to xylazine supplies. He was fully aware of the limits imposed by veterinary authorities and xylazine's sedative characteristics, as he later claimed that the administration of drugs had successfully treated five or six episodes of sleeplessness and serious depression with modest intramuscular doses. During the occurrence of the case, he was discovered unconscious in the evening of his admittance. The patient was found to be in deep comatose, apnoeic, and areflexic. He was also transferred to intensive care unit (ICU) at the university hospital where he was intubated and placed on a respirator. Supportive measures included endotracheal intubation, intravenous fluid therapy, bladder catheterisation, and central venous pressure monitoring had been implemented (Carruthers et al., 1979).

Case 2: A 27-year-old farmer who attempted suicide by injecting xylazine intramuscularly was discovered unconscious with constricted pupils and no response to pain or light stimulation. His initial heart rate was determined to be 88 beats per minute, and his blood pressure was 180/100 millimetres mercury (mmHg) during the medical investigation. The attempted suicide was due to conflict situation in his family (Hoffmann et al., 2001).

Case 3: A healthy 18-year-old male with history of drug misuse involving a variety of drugs (cocaine and amphetamines) arrived at the emergency room 15 hours

after intentionally inhaling xylazine as an agent of drug abuse. After inhalation, he experienced dizziness, gait instability, palpitations and two episodes of syncope with bradycardia and hypotension. Physical examination indicated an arterial pressure of 90/60 mmHg, a heart rate of 45 beats per minute, disorientation, dysarthria, dysmetria, ataxia, reactive pupils of intermediate size, and no other abnormal characteristics in the patient (Elejalde et al., 2003).

Case 4: A 14-year-old boy had unintentionally injected a narcosis arrow into his left thigh while cleaning up the deer park. It was found that the arrow contained xylazine and ketamine and had incompletely been absorbed into his body. The patient was intubated and ventilated after losing his consciousness. However, he was cardiorespiratory stable (blood pressure 130/90 mmHg, heart rate 55 per minute). The patient was extubated after 4 hours in the ICU and had suffered dizziness, nausea, and vomiting (Meyer et al., 2013).

Case 5: In 2013, a case was reported in Ramathibodi Hospital, Thailand where an asset of patients who was waiting to be treated in the hospital was robbed. The robber used xylazine mixed into water and brought to those awaiting to be served in the hospital. When the victims got unconscious, the property of the victims were stolen.

Case 6: In 2020, contaminated xylazine was found in runners' drinking water bottles. Case in detail, a young runner posted a warning message mentioning that he was poisoned in Nonthaburi City Hall Park while jogging. On that day, he left his water bottle at the starting point and returned to that point upon jogging. He

emphasised that his drinking bottle was not opened but he experienced stiffness on his tongue and subsequently lost consciousness not long after drinking the water. When the victim awoke, he found himself taken to the hospital. He suspected that he was poisoned by criminals where the water bottle had a hole pierced with possibly a syringe. Later, it was confirmed that the water within the bottle was contaminated with xylazine.

Reviews on the misuses of xylazine was also published in the literatures. Reyes et al. (2012) described the reports on the prevalence of xylazine in various previously published studies. Several deaths were connected to the use of xylazine. The authors had suggested the effects of xylazine as a drug abuse is of significant concern to the public (Reyes et al., 2012).

In 2020, reports had been established in some areas of the United States regarding the prevalence of xylazine in the black market and increasing purchasing activities of the drugs. According to the Drug Enforcement Administration (DEA), frequency of deaths involving xylazine overdose has grown in Philadelphia during the last three years since 2017. Two urban countries in Ohio had also issued public warnings in 2019 concerning the distribution of xylazine medication batches. Additionally, in one overdose fatality report in Dayton, the public health officials detected existence of daze-inducing mixture, and the officials near Columbus had connected xylazine to three fatality cases (Keppler, 2020).

There were more than 50 cases reported so far when summing up states in some areas of the United States with overdose fatalities in 2019. To be exact, 71 cases were

discovered involving xylazine out of a total of 1,200 deaths (Keppler, 2020). According to a literature review by Ruiz-Colón et al. (2014), 43 cases of xylazine intoxication in human were reported from the years 1966 to 2013 in which 21 cases were non-fatal scenarios while 22 cases resulted in fatalities. 17 from the 43 reported cases were associated with use of xylazine as adulterant in recreational drugs such as heroin and cocaine. Therefore, the develop of analytical technique for xylazine can help and support forensic work and law enforcement authorities.

2.2.3 Forensic significance of detecting xylazine

Reviews of literature for xylazine detection had been variously published. Most of the research focused on detecting xylazine for veterinary proposes such as quantitation of xylazine in blood, urine, or animal tissue as well as the effects of xylazine alone or combined with another drug in animals. In cases involving xylazine, the established analytical methods have predominantly been chromatographic methods, including gas liquid chromatography (GLC) (Poklis et al., 1985), GC-MS (Spyridaki et al., 2004), HPLC with UV-detection (HPLC-UV) (Niedorf et al., 2003), HPLC with mass spectrometry (HPLC-MS) (Doran and Bradbury, 2015), ultra-HPLC with quadrupole-time of flight mass spectrometry (UHPLC-QTOF) (Gao et al., 2015), and LC-MS (Zheng et al., 2013).

Over the years, the electrochemical method has garnered attention to be applied in forensic scenarios. Moreover, the available of portable sensors for on-site analysis of forensic samples have seen rapid interest and growth, resulting in the development of presumptive tests for screening of samples (de Araujo et al., 2018, Pereira de Oliveira et al., 2018). In a forensic investigation, xylazine could be associated for use

in drug-facilitated sexual assaults and suicide attempts. There were research reports regarding the detection of xylazine, including by Mendes et al. (2019) who described an electrochemical study and the development of a sensitive and accurate electroanalytical method for quantification of xylazine through the analysis of artificial urine samples. To the best of our knowledge, no portable electroanalytical sensor had been described to quantify xylazine for on-field analysis. Thus, the development of a portable electrochemical sensor for xylazine detection for the first time is therefore proposed in this study.

In forensic investigations, various scientific methods are utilised to process and offer scientific evidence to the court or as an investigative tool. The selections of analytical techniques, procedures, and processes are based on the evaluation of several factors, including the ability of the analytical method to analyse a target compound contained within a particular sample matrix, as well as its sensitivity, selectivity and robustness which are the key analytical performance characteristics. In some instances, these requirements often involve a combination of techniques and procedures rather than a single approach. This is especially true during crime scene investigation where screening procedures are carried out on-site and the subsequent confirmatory analysis is undertaken in the forensic laboratory (Shaw and Dennany, 2017). Therefore, electrochemical analytical method is worth to be studied and developed as alternative to support the confirmatory analysis due to its easy analytical processes, time and cost savings, ability to analyse with little or no pre-treatment procedure, and able to be easily miniaturised and develop into a portable device or kit (Mendes et al., 2019, Noviana et al., 2020).