Development and Validation of Analytical Protocol for Forensic Investigation of Benzodiazepines and other Psychoactive Compounds

" This thesis includes a standalone chapter; therefore, the literature review and the introduction will reflect this and will include topics that are not related to each other."

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Declaration of Authenticity and Author's Rights

'This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.'

Signed: Husein Kamal

Date: June, 2025

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By the name of Allah

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Husein Kamal

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of Tables

Chapter 2

Chapter 3

List of Abbreviations

ATS	Amphetamine -type stimulant
ATR FTIR	Attenuated total reflectance Fourier transform infrared
AC	Alternating current
ACMD	British Advisory Council on the Misuse of Drugs
ADHD	Attention-deficit/hyperactivity disorder
AMP	Amphetamine
APCI	Atmospheric pressure chemical ionisation
API-ESI	Atmospheric pressure ionisation combined with electrospray ionisation
AUCs	Areas under the curve
BDD	N,O-bis(trimethylsilyl)trifluoroacetamide
BZDs	Benzodiazepines
CI	Chemical ionization
CLB	Clobazam
CNS	Central nervous system
COD	Codeine
DAD	Diode-array detectors
DC	Direct current
DMCLB	N-desmethylclobazam
DoA	Drugs of abuse
DZP	Diazepam
EI	Electron impact
ESI	Electrospray ionization

FT-ICR	Fourier transform ion cyclotron resonance analyser
FTIR	Fourier transform infrared
GABA	gamma-aminobutyric acid
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HER	Heroin
HPLC	High-performance liquid chromatography
IUPAC	The International Union of Pure and Applied Chemistry
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-mass spectrometry
LLE	liquid–liquid extraction
LOD	Limit of detection
MALDI	Matrix-assisted laser desorption/ionisation
MAMP	Methamphetamine
MENA	The Middle East and North Africa
MOR	Morphine
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamie
NPS	Novel Psychoactive Substances
O-TMS	Oleate-TMS
PGB	Pregabalin
PPT	Protein precipitation
PTR	Proton transfer reaction ionisation

Q	Quadrupole mass analyser
QqQ	Triple quadrupole mass spectrometer
RSD%	Relative standard deviation
SIM	Selected ion monitoring
SO	Sodium oleate
SPE	Solid phase extraction
SRM	Selected reaction monitoring
SFC	Supercritical fluid chromatography
TOF	Time-of-flight mass analyzer
TQ-ESI	Triple quadrupole electrospray ionisation
UHPLC	Ultra-high-performance liquid chromatography

Abstract

The increasing workload in forensic laboratories in Kuwait often results in prolonged delays in processing samples, which can significantly affect the reliability of drug detection in both post-mortem cases and samples from living individuals. In light of this, an assessment of the stability of drugs, such as the benzodiazepines (BDZs), amphetamines, opioids and other controlled drugs like pregabalin and gabapentin which are commonly abused, becomes crucial in forensic toxicology to determine the effects of storage on the viability of these samples. Chapter two of this thesis addresses this issue by focusing on the development and validation of analytical methods to detect five benzodiazepines (alprazolam, bromazepam, clonazepam, flunitrazepam, and diazepam) that are commonly encountered during forensic investigations in Kuwait.

An HPLC method was designed and refined utilising International Council for Harmonisation guidelines to ensure the accuracy, repeatability, and robustness of the method. The developed method was applied to perform stability assessments on the chosen BDZs, revealing that the integrity of samples diminishes significantly after three weeks at room temperature, while refrigeration helps preserve sample integrity. These findings stress the importance of proper storage conditions to maintain sample validity during forensic investigations.

In Chapter three, the real-world application of the developed method was explored by utilising it to screen a collection of 48 urine samples collected from individuals under investigation for suspected drug-related offenses. This revealed a high prevalence of BDZ use, with over 93% of samples testing positive. The detection of flunitrazepam, in particular, highlights the method's sensitivity and potential role in supporting criminal investigations involving controlled substances.

Beyond this, the research study included the development of an HPLC-MS method which utilises a shorter column than the HPLC method, resulting in a significant reduction in analysis time required per sample while maintaining accuracy and precision. This method can also be extended to include metabolites, making it suitable for routine toxicological screening in forensic laboratories. Chapter four contains a stand-alone study conducted during the COVID-19 lockdown. The experiments in this study analysed a new oxazolidinone compound (PH-192) with anticonvulsant potential. The study developed a UHPLC-QToF-MS method that was successfully validated and applied to the analysis of the oxazolidinone compound (PH-192).

Lastly, the studies in Chapter 5 sought to incorporate a broader drug screening approach for the identification of a wide range of substances that are often abused in Kuwait, such as the amphetamine, codeine diazepam and the controlled anticonvulsant drug pregabalin. An HPLC-UV method was developed and validated, along with preliminary GC-MS analyses, to capture both UV-active compounds and compounds with limited UV activity. The GC-MS method requires further refinement; however, it shows strong potential for comprehensive toxicological screening.

In summary, this research contributes to forensic science by offering reliable, validated methods for drug detection, identification and stability evaluation utilising equipment that are readily available in forensic laboratories in Kuwait. The findings not only enhance local forensic capabilities but also provide a framework for broader applications in forensic toxicology worldwide.

Chapter 1 General Introduction and Literature Review

General Introduction

1.1 Forensics Overview of The Illicit Drug Menace in Kuwait

Despite a lack of comprehensive, accurate, and current data regarding the scale of drug abuse in Kuwait, anecdotal evidence coupled with what little data is available and newspaper reports estimates the number of drug addicts in the country to be between thirty-five to forty thousand, and the number of deaths attributed to drug overdoses were reported in a 2000 report to have increased tenfold compared with recorded deaths in the preceding decadee[1,2]. A 2017 study concluded that the abuse of illicit substances among older teenagers was on the increase having found that Hashish (marijuana) was the illicit drug most used by the teenagers, 3.7% of the respondents were active users while 5.3% claimed to have tried it at some point[3].

Illicit drugs are those that are illegal to possess or whose nonmedical use, where legally available, is prohibited by international law, such as cannabis in all its forms including the new synthetic cannabinoids, amphetamine-type stimulants (ATS) such as 3,4 methylenedioxymethamphetamine (MDMA), alkaloids such as cocaine, opioids such as heroin and morphine, depressant drugs including barbiturates such as pentobarbital, anticonvulsants such as pregabalin and benzodiazepines such as diazepam[4].

Although cannabis is the most confiscated illicit substance in Kuwait, methamphetamine has been reported to be the most abused illicit drug[5]. Deaths resulting from the abuse of benzodiazepines and heroin are reported to be the highest in the single drug category, while deaths resulting from the abuse of heroin-benzodiazepine combinations topped the multiple-drug use death category [5]. Illicit

drugs are also reported to be involved in most crimes in Kuwait, with some news reports claiming that drugs are involved in 65-73% of all crimes in Kuwait, which puts immense pressure on the legal system[6,7].

Illicit drug usage, including prescription medication misuse, as well as the unlawful use and trafficking of opioids and other psychoactive drugs, is a serious global issue[8,9]. The World Health Organisation (WHO) estimates that illicit drug use is responsible for about six hundred thousand deaths annually, with more male victims (420k) than females (160k)[10]. The Middle East and North Africa (MENA) area, notably Kuwait, faces significant issues regarding illicit drug use and trafficking[5]. Aside from cannabis in its different forms, including the new synthetic cannabinoids, drugs such as amphetamine, methamphetamine, morphine, codeine, diazepam, and pregabalin have been claimed to be among the most used[5]. These substances expose users to serious health hazards, such as cardiovascular disease and mental health difficulties, in addition to the high risk of addiction[11,12].

In a study from Kuwait, cannabis, including marijuana, was reported as the drug substance most often seized, ahead of heroin, opium, and cocaine[4]. Amphetamines, including methamphetamine tablets and powders, made up the largest amount of all the psychoactive substances seized. This translated into amphetamines being the most abused drug substances, followed by benzodiazepines, then cannabis, and lastly heroin. From postmortem specimens, segregated by sex, in suspected drug-related deaths, heroin, benzodiazepines, and methamphetamine are the most identified single drugs while heroin–benzodiazepines, cannabis–benzodiazepines, and cannabis–amphetamines were the most often the multiple-drug cocktail of detected[4]. Although there are significant challenges in addressing illicit drug use, a comprehensive

approach integrating legal, social, health and educational-based strategies can help mitigate the problems[13]. Further research and data collection are essential to inform interventions based on real-life evidence.

1.2 Separation Methods Utilised in High-Pressure Chromatography Mass Spectrometry Mediated Forensic Analysis

Law enforcement investigators often encounter a variety of samples during their investigations, which could be a complex mixture of substances such as blood, urine, tissue, fibres, along with the substances of forensic interest and substances made from other materials. These may be from crime scenes; materials seized from suspects or samples that are provided by suspects. These samples can additionally contain a complex mixture of chemical substances, making it a huge challenge to isolate and identify substances of forensic interest[14].

The analysis of these crime-related samples using high-performance liquid chromatography (HPLC) and HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) is a common and powerful technique in forensic and pharmaceutical science to address this challenge by combining two sophisticated methods[15,16].

HPLC separates samples into their constituent compounds based on their chemical properties, while mass spectrometry (MS), identifies and quantifies the individual compounds based on their mass-to-charge ratio.

1.2.1 High-Performance Liquid Chromatography (HPLC)

HPLC is a powerful, widely employed analytical technique, used for the separation, identification, and quantification of the components in samples that contain a mixture of substances[17,18]. HPLC is particularly effective for samples that contain substances

which may be thermally unstable or non-volatile, which makes it a valuable tool in various industries such as the pharmaceutical and food industries, environmental sciences, forensics and research[19–21].

HPLC is routinely utilised for the separation, identification, and quantification of the components in complex mixtures, which is essential for the analysis of unknown substances, such as those found at crime scenes, which may include drugs and their metabolites (drug identification such as cocaine, heroin, amphetamines), poisons, toxins and toxic industrial chemicals (toxicology), explosives or biological samples such as blood, urine, and tissue samples[18,22–24].

1.2.1.1 Basic Principles of HPLC

HPLC is based on the principle of chromatography, where the components of a mixture are separated based on their interactions with two phases: a stationary phase and a mobile phase [25,26].

In HPLC, a sample or analyte flows, at high pressure in a solvent, referred to as the mobile phase, through a column filled with chromatographic packing material, referred to as the stationary phase[27,28].

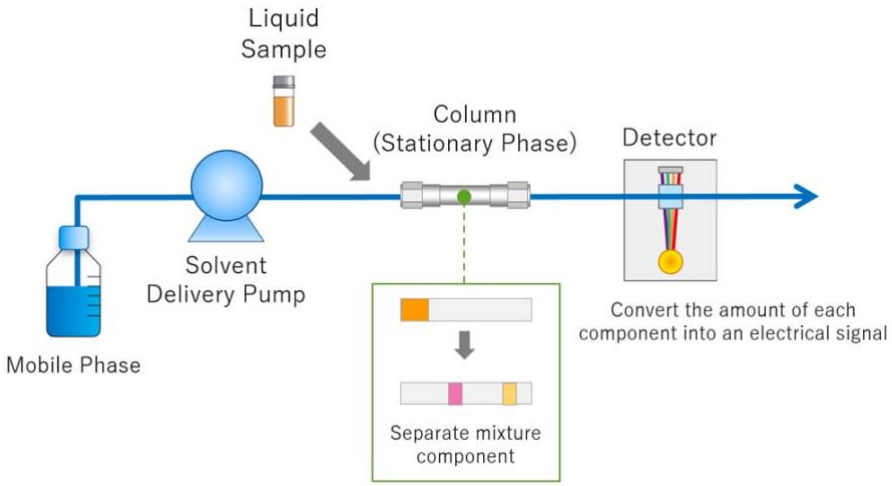


Figure 1. 1 Overview of the HPLC process[29].

The stationary Phase is a porous solid material with a large surface area with which the column is packed, and which interacts with the compounds in the mixture as they pass through [21,30]. The choice of the stationary phase significantly determines the outcome of the chromatographic process. It influences the selectivity, separation efficiency, and the resolution achievable during the process[21,30].

1.2.1.2 Types of Stationary Phases in HPLC

There are two main types of HPLC columns based on the stationary phase they contain: normal-phase and reversed-phase. In normal-phase HPLC, the stationary phase is nonpolar, and the mobile phase is polar. In reversed-phase HPLC, the stationary phase is nonpolar, and the mobile phase is polar.

1.2.1.3 Normal Phase

In Normal Phase (NP-HPLC), the stationary phase is made of polar material (silica or alumina)[31]. Based on their relative affinities for the stationary phase the compounds in the sample are retained to various extents due to their polarities as they interact with

the stationary phase resulting in a separation based on the differential in affinity for the stationary phase. This is technique is often used for the separation of compounds with polar functional groups, such as alcohols, amines, and acids.

1.2.2 Reversed phase

In reversed-phase HPLC (RP-HPLC), the stationary phase is non-polar and hydrophobic made from silica particles bonded with non-polar alkyl chains such as cyanopropylsilyl- (CN), *n*-octylsilyl- (C_8), and *n*-octadecylsilyl- (C_{18}), in order of decreasing polarity[31]. Non-polar compounds are retained longer than polar compounds due to differential hydrophobic interactions, resulting in a separation as the mobile phase flows through the column. This technique is widely used for the separation of non-polar and moderately polar compounds like small organic molecules, steroids, and lipids. Reversed-phase HPLC is used in about 75% of all HPLC separations due to its superior reproducibility and broad applicability[32,33]. The mobile phase is an aqueous blend of water mixed with a polar organic solvent, usually acetonitrile or methanol. The most used stationary phase is C_{18}–bonded silica.

1.2.2.1 Hydrophilic-Interaction Chromatography (HILIC)

Hydrophilic-interaction chromatography (HILIC) is often viewed as a variant of NP-HPLC [23,34]. The addition of water, less than 20%, to aprotic solvents such as acetonitrile used as the mobile phase in NP-HPLC, which is normally 100% organic containing only trace amounts of water present in the mobile phase or polar stationary phase, can facilitate the elution of polar compounds which would ordinarily be strongly retained on the column in NP-HPLC. HILIC may be run in either isocratic or gradient mode with water efficiently facilitating the elution of polar analytes by

increasing the polarity of the mobile phase by increasing the polarity (water content) of the mobile phase with analytes eluted in order of increasing polarity.

1.2.2.2 Hydrophobic-Interaction Chromatography (HIC)

Hydrophobic-interaction chromatography (HIC) is a variant of RP-HPLC used to separate large biomolecules, such as proteins, because it is necessary to avoid contact with denaturing solvents [35,36]. HIC utilises the intrinsic hydrophobic attraction of large molecules for moderately hydrophobic C_8 and C_{18} stationary phases [36]. High salt concentrations in water encourage the proteins to be retained (salted out) in the mobile phase. Gradients are typically run by gradually reducing the salt concentration, enabling the elution of the biomolecules in order of increasing hydrophobicity.

1.2.2.3 Historical Development of HPLC

Liquid chromatography (LC) was first reported by the Russian botanist, Mikhail S. Tswett, in the early 1900s for the separation of compounds extracted from plant materials [37]. Extracts from plant leaves were introduced onto open glass columns filled with particles of powdered chalk (calcium carbonate) or alumina, followed by the addition of pure solvents to the column. As the sample of extracts moved downwards under gravity through the column, different coloured bands separated due to the difference in the rate of movement of the different components of the mixture. Compounds that were more strongly attracted to the particles packing the columns moved more slowly, whereas those with a stronger attraction to the solvent travelled faster. This process was given the name *chromatography* by Tswet, from the Greek words *chroma*, meaning colour, and graph[38]. Liquid chromatography (LC), in its various forms, is one of the most powerful techniques used in analytical chemistry today [38,39].

The acronym HPLC was coined by the late Prof. Csaba Horváth in his 1970 Pittcon paper, which highlighted that high pressure could be used to generate the flow required for liquid chromatography in packed columns. The early pumps only had a pressure capability of up to 500 psi (35 bar)[39]. Following great technological leaps during the 1970s, HPLC instruments were developed that could produce up to 6,000 psi (400 bar) of pressure, with improved injectors for sample handling, detectors, and columns integrated into the systems. Further advances in the technology over the years have resulted in columns with increasingly smaller particle sizes and even higher operating pressures. While the acronym HPLC has remained, the name of the technique was changed to high-performance liquid chromatography[40]. High-performance liquid chromatography is now one of the most powerful tools in analytical chemistry [39]. It has enabled the separation, identification, and quantification of the compounds present in samples that can be dissolved in a solvent. Compounds in trace concentrations, as low as the parts per trillion (ppt) or nanogram per litre ngL^{-1} range, can easily be identified[41]. Further advances in instrumentation and column technology made since 2004 have enabled very significant increases in resolution, speed, and sensitivity in HPLC. Columns with smaller particles, as low as 1.7-micron-diameter, and instrumentation with specialised capabilities designed to deliver mobile phases at up to 15,000 psi (1,000 bar) have enabled new levels of performance, resulting in new systems specifically created to perform ultra-performance liquid chromatography, now known as UPLC technology[42].

Ongoing research into newer columns containing even smaller 1-micron-diameter particles and instruments capable of operating at 100,000 psi (6,800 bar), offers a glimpse of what may be possible in the future[43].

1.2.2.4 Components of the HPLC System

HPLC systems mainly consist of different components, which can be configured to suit the requirements of the specific separation to be undertaken. The main components of a basic HPLC system would include[44]:

The solvent reservoirs contain the mobile phase (solvents), which are pumped through the system.

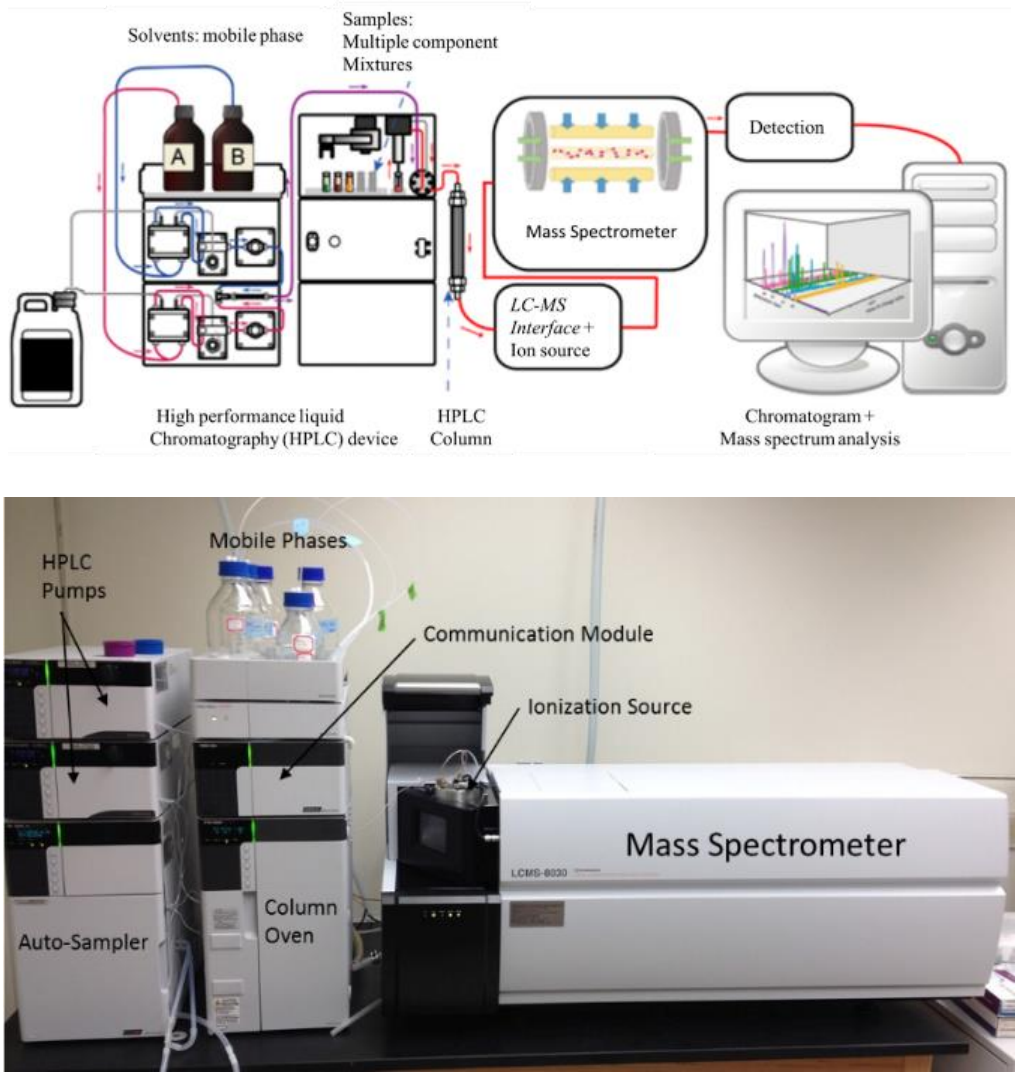


Figure 1. 2 Top: Schematic depiction of a HPLC/MS system[45], Bottom: A modern HPLC /MS system[46].

The Infusion Pumps blend and move the mobile phase through the system at a constant pressure and flow rate. There are two elution methods that commonly used in HPLC analysis: isocratic elution and gradient elution[47]. During isocratic elution, the composition of the mobile phase remains constant over the entire duration of the analytical cycle. This method has been found to be most useful for samples made up of a few components and which have little difference in their properties. In gradient elution, the composition of the mobile phase is varied constantly over the duration of the analytical cycle, controlled by the infusion pumps. Solvent parameters such as the polarity of the solvent, ionic strength, and pH are carefully varied over the analysis cycle to achieve the desired separation. Gradient elution protocols are typically used for the analysis of complex samples, such as samples with many components and/or large differences in the properties of the components of the sample. Gradient elution methods can shorten analysis times, improve the resolution and peak shapes of the sample components. It can also help to increase the sensitivity of detection but is often associated with baseline drifts and reduction of reproducibility.

The injector introduces the sample mixture into the mobile phase stream.

The Column is usually tubular and made of suitable materials such as metal or glass and packed with the stationary phase material. The separation occurs as the sample travels through the column. The Detector provides a measure of the separated components as they elute off the column.

The Data Management System or computer processes and records the signals from the detector and generates a chromatogram, which is used for analysis.

Other components, such as online degassers, auto samplers, pre/guard columns, column temperature controllers, and fraction collectors can be integrated into the system if required.

HPLC is a versatile and powerful technique that allows for precise and accurate analysis of complex mixtures. Its adaptability and reliability have made it a cornerstone in modern analytical laboratories. It is used in forensics, the pharmaceutical industry for the analysis of active pharmaceutical ingredients, and impurities, in the food and beverage industry for the detection of vitamins, contaminants and preservatives, in biochemistry for the analysis of proteins, peptides and other biomolecules, as well as in environmental testing for the monitoring of pollutants and contaminants in air, soil and water[24,48–50].

1.2.2.4.1 Common Detectors used in HPLC Analysis

Several different detectors are used for HPLC. HPLC detectors are vital for detecting, analysing, and quantifying the compounds contained in a sample. The selection of the most appropriate detector is critical for a successful analysis and is based on several factors and criteria, including the detection limit and sensitivity level, type and nature of the sample to be measured, the expected results, must be taken into consideration when deciding on a detector. Since the first use of UV and fluorescence detectors in 1958, the field has grown rapidly, and more powerful and diverse detection systems are continually being developed.

1.2.2.4.2 UV-Visible Detectors

UV-Visible detectors are among the most widely used detectors in HPLC [24]. The detection principle of UV-Visible spectroscopy is based on the chromatographically

specific absorption of ultraviolet or visible light by analytes, followed by their quantification. The operation of UV-Visible detectors is based on the absorption properties of the analyte. Therefore, UV-Visible absorption detection is dependent on the chemical structure of individual compounds. Detectors should therefore be sensitive and flexible in providing both qualitative and quantitative data. UV-Visible detectors are generally based on two configurations: single-wavelength and diode array detectors[24,50]. UV-Vis has a history of routine applications, including pharmaceutical, environmental, flavour and fragrance, chemical, petrochemical, amongst others, that also support applications for automated qualitative and quantitative analysis.

1.2.2.4.3 Single-Wavelength Detectors:

Single-wavelength detectors are the most widely used and commonly employed detectors in HPLC systems. These fixed single-wavelength detectors are excellent for the measurement of analytes, and they are easy to install, cost-effective, with high sensitivity. Single-wavelength UV-Vis detectors are made up of a light source, monochromator, flow cell, and photodetector that operate by measuring the absorbance of the analytes at a particular wavelength. These detectors can generally operate over the range of 190-900 nm and can be used for analysis in the viscous to non-viscous UPLC to preparative flow ranges with flow rates from less than 0.1 to greater than 100 $mLmin^{-1}$. Single-wavelength UV-Vis detectors are limited to investigation of the absorbance of the eluting analytes at a single wavelength and are restricted to only eluents that are optically transparent within the measurement path of the flow cell located in the instrument. This type of UV-Vis detector is useful for pharmaceutical testing, aflatoxins in food analysis, monitoring of industrial effluents,

and process control of analytes of interest, among others[51]. These flow cells typically have a path length of 5-10 mm, with an increase in the sensitivity of the detector as the flow cell path length increases due to more time available for sample-analyte interactions to obtain an absorbance signal with less error. [51].

Current single-wavelength UV-Vis detectors have been optimised for high efficiencies during separations where retention factors, selectivity, and efficiencies are important. Modern detectors today have the advantage of using deuterium lamps to correct possible baseline drift, a lamp lifetime of more than one year, and are optimised to work with high sensitivity and an extended measuring range. The usage of these detectors is limited to specific wavelengths, such as automatic dual wavelengths, which show one wavelength regardless of the detection of other species at a different wavelength, which limits possible interference of analyte matrix waiting time between separation and analysis for preparing the column, and more sensitive options to promote a more accurate quantitation.

1.2.2.4.4 Diode Array Detectors:

A diode array detector (DAD) can measure multiple wavelengths simultaneously [52]. Compared to single-wavelength detectors, DADs are flexible and can measure wavelengths every few milliseconds [52]. The detection response times exhibited by UV-Visible detectors are in the order of microseconds and are therefore faster when compared to other detectors, such as flame ionisation and thermal conductivity detectors, which are in the order of milliseconds.

DADs are extremely versatile and can provide full wavelength data for multiple analytes in a single run. They operate based on the use of an array of photodiodes that can detect light over a range of wavelengths[53]. Scanning monochromators can capture spectral information, typically from 200 to 800 nm, though the ranges do vary. This range of detectors is useful for providing high-quality 3D and 2D contour plots, aiding in spectral purity determination and simultaneous multi-wavelength monitoring. DADs are often applied for newer method development in which full spectral information is desirable. The ability to tune diode array devices to monitor whole traces is particularly informative and instrumental in assay validation. DADs are more often used for research and are often gateway detectors of the laboratory from the training bench to the process. A major drawback of a diode array it is an expensive and high-maintenance instrument.

1.2.3 Gas Chromatography

Gas chromatography (GC) was discovered in the 1950s. It is used for the separation of compounds that are volatile or can be easily vaporised without decomposition. In GC, the mobile phase used is an inert carrier gas such as hydrogen or helium and a thin layer of liquid or a polymer adsorbed on an inert solid support material and packed into long glass or metal capillary tubes, several meters in length, is used as the stationary phase. In this technique, the separation of the components of the sample occurs between the gas and the liquid stationary phases. Similar to LCMS, GC can be coupled to a mass spectrometry system (GC-MS) to optimise the quality of the data obtained. Both GC and GC-MS are commonly used techniques in forensic sciences and have diverse applications, including the identification and quantification of volatile compounds in a diverse range of fields such as monitoring in environmental

sciences, forensics, food analysis, and the pharmaceutical industry. GC affords a high-resolution separation and a significant improvement in peak resolution, utilising the controlled temperature feature that it supports. GC has also been utilised to facilitate the identification of the unknown materials by cross-referencing and matching with online libraries utilising electron impact ionisation mass spectra. Additionally, an inert gas carrier is used as the mobile phase therefore, there is no solvent background noise, which is an advantage over LC-MS. GC-MS however, is limited by the fact that the samples to be analysed must be volatile, which restricts its use. GC-MS is also not suitable for the direct analysis of aqueous samples, which require solvent extraction or pre-concentration before analysis. A review of the recent advances in GC and GC-MS approaches in forensic sciences can be found in the report by Gould *et al*.[54]

1.2.4 Analytical Methods Used for HPLC Coupled with Tandem Mass Spectrometry (HPLC-MS/MS)

The combination of chromatography and mass spectrometry (MS) is a technique that has drawn much interest over the years. The combination of gas chromatography with mass spectrometry (GC-MS) was first described in 1958 and was available commercially in 1967[55,56]. The combination of liquid chromatography with mass spectrometry (LC-MS) was however, more complex and occurred in stages over several years[56,57]. High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) is a powerful analytical technique that enhances the specificity and sensitivity of sample analysis by combining the separation power of HPLC with the detection power of mass spectrometry[58,59]. It is frequently employed in the analytical sciences to identify and quantify chemical compounds in complex samples from various sources such as crime scenes or research laboratories. This technique is

particularly valuable due to its high sensitivity, precision, and ability to analyse a wide range of substances, including drugs, explosives, toxins, and biological markers, even in trace amounts[60,61]. HPLC-MS is routinely used for confirming the identity of unknown substances, quantifying trace amounts of drugs, toxins, and other chemicals, and analysing complex biological matrices such as blood or tissues[60,62–64].

1.2.5 Basic Operating Principles

The HPLC portion of the system serves as the separation tool, where a sample is dissolved in a solvent and passed through a chromatographic column under high pressure. Different components in the sample are separated based on their chemical properties as they interact with the column material, allowing individual compounds to be isolated. Once separated, the compounds enter the mass spectrometer, where they are ionized and detected based on their unique mass spectra, providing specific identification and quantification. In tandem MS (MS/MS), ions are fragmented in multiple stages. Molecules are initially ionized to create charged particles.

The ions pass through the first mass filter, where they are sorted by mass-to-charge ratio (m/z). The selected ions are then fragmented into smaller ions. These fragments are then passed through the second mass filter, where they are analysed to give a highly specific mass spectrum. The resulting mass spectra data are then processed and interpreted to provide structural information about the sample to facilitate its identification and quantification with high precision.

HPLC-MS/MS is a highly sensitive tool for detecting drug metabolites, even in extremely low concentrations, making it an ideal tool for toxicology or doping analysis. HPLC-MS/MS can also be used to detect trace amounts of chemicals such as illicit drugs, toxins, or other hazardous substances, even when present in extremely

low quantities. HPLC-MS/MS is widely used in the screening of biological samples, such as blood, hair, or tissue samples, for drugs and poisons, even in cases where concentrations are extremely low.

High-performance liquid chromatography-mass spectrometry (HPLC-MS) is a powerful analytical technique frequently employed in analytical sciences to identify and quantify chemical compounds in complex samples collected from various sources. This technique is particularly valuable due to its high sensitivity, precision, and ability to analyse a wide range of substances, including drugs, explosives, toxins, and biological markers, even in trace amounts.

1.2.6 Mass Spectrometry (MS):

The analysis of molecules based on their relative molecular mass is achieved by the analytical technique called Mass spectrometry. Mass spectrometry provides both qualitative and quantitative information about an analyte and works by the formation of ions, which are either positively or negatively charged in the gas phase, which are then accelerated in a detector while being subjected to either an electric or magnetic field, which facilitates their separation based on the differences in mass to charge ratio (*m/z*) of the individual ions[65]. This technique is helpful for the development of analytical protocols due to its high sensitivity and specificity, especially when used in conjunction with various chromatography platforms such as gas chromatography and liquid chromatography.

1.2.7 Mass Spectrometry Ionisation Methods:

In MS, several different ionisation methods are used to create the charged molecules that can then be analysed by the mass spectrometer. These Ionisation methods include techniques such as electron ionisation (EI), chemical ionisation (CI), electrospray

ionisation (ESI), matrix-assisted laser desorption/ionisation (MALDI) and atmospheric pressure chemical ionisation (APCI).

1.2.7.1 Electron ionisation (EI):

Electron ionisation (EI), one of the oldest ionisation techniques used in mass spectrometry, was first described in 1918 and was initially referred to as either Electron impact Ionisation or Electron bombardment ionisation[66–68]. EI works by bombarding solid or gas phase atoms with high-energy electrons to form ions and often results in extensive fragmentation. EI is a hard ionisation technique which, like ESI, is compatible with a variety of separation techniques, which has led to its use in applications such as GC-MS and LC-MS[69]. The extensive fragmentation obtained in the mass spectra with EI is one of its important advantages, because it is an aid to structure elucidation [70]. Diagnostic fingerprints can be obtained from the fragments that make up the spectra, which can be compared with spectral libraries utilising mass/intensity correlations. These mass spectral libraries, which are constantly updated and have been built up over decades since the introduction of EI-MS. There were more than 270,000 spectra available in the NIST 2017 library[71,72]. The EI-MS ionisation technique is very popular with diverse applications in a broad range of research disciplines, including archaeology, environmental sciences, forensics, agriculture, pharmaceuticals, food and biological sciences[69,73–76].

1.2.7.2 Electrospray Ionisation (ESI):

Electrospray Ionisation (ESI) works by applying an electric charge to a stream of the sample solution to assist the transfer of ions from the solution to the gas phase. Protonation converts both charged species and neutral molecules in the solution stream into positive ions, allowing for highly sensitive analysis[65]. Polar solvents, often

with an organic modifier to promote ionisation, are normally used to make up the sample, facilitating its transportation through a channel connected to a high electric potential, charged aerosol droplets are formed between the mass spectrometer and the needle. The charge on the aerosol droplets is derived from the high electric potential applied to the needle (1-4 kV). The charged droplets evaporate in a stream of warm nitrogen, becoming smaller and increasing the electrostatic repulsion between the individual ions within each droplet. The increasing electrostatic repulsion eventually leads to the breakup of the droplets, producing naked ions (Figure 1,3). The electric field applied between the needle and ion transfer capillary causes the ions to move towards the ion transfer capillary and are thus transferred to the high vacuum region of the mass analyser, where their charge to mass ratios (*m/z*) are determined[77].

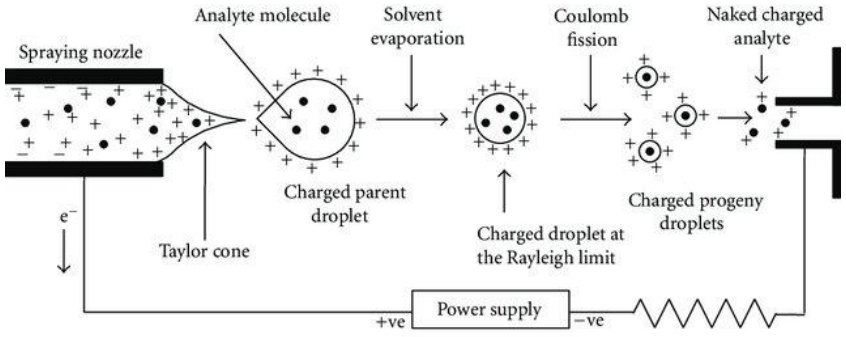


Figure 1. 3 Schematic depicting the ionisation process in Electrospray Ionisation ESI[78].

In ESI, there are two possible ion modes, negative ion electrospray ionisation (NIESI) and positive ion electrospray ionisation (PIESI). PIESI provides more sensitivity in comparison to NIESI. Compounds like amino acids, which have both positive and negative charges, can be detected in either mode. In ESI, however, there is a tendency to form ions with adducts from the mobile phase. Adducts of methanol, ammonia, calcium, acetonitrile and potassium are often formed in the positive mode, while

chloride, acetic acid and formic acid adducts are formed in the negative mode from the mobile phase or buffers added to improve the chromatography. High percentages of organic solvents in the mobile phase can facilitate efficient ionisation of compounds in ESI because it aids aerosol formation and solvent evaporation, which leads to the formation of gas phase ions[79].

ESI is a relatively simple way to ionize non-volatile solutions that allows sensitive direct detection of samples. It can be used for the analysis of inorganic substances, organometallic ion complexes and biomacromolecules. In ESI, high molecular weight molecules usually carry multiple charges, with the distribution of charged states accurately quantifying the molecular weight. This provides both an accurate molecular mass and vital structural information. ESI offers multiple ionisation modes to choose from (positive ion mode and negative ion mode). ESI is effectively combined with a variety of chromatographic techniques to enable analysis of complex samples.

The advantages offered by ESI make it the most used mass spectrometric technique with liquid chromatography.

1.2.7.3 Chemical ionisation (CI):

1.2.7.4 Chemical ionisation (CI) was first introduced in 1966 by Burnaby Munson and Frank H. Field. CI operates by the reaction of ionized reagent gas molecules, which are ionised by EI, with the molecules of the analyte in the gas phase to form ions of the analyte. CI is therefore regarded as a soft ionisation technique80–83. CI results in less ionisation than seen by the direct ionisation of EI. The extent of ionisation can also be controlled by the choice of reagent gas. Similarly to EI, fragmentation is useful for structure elucidation of molecular structures of unknown compounds70. Like other ionisation techniques CI can similarly be coupled to a variety of separation techniques like GC (GC-CI-MS), HPLC (LC-CI-MS) and capillary electrophoresis but it however limited to use with volatile compounds only. Other variants of CI include Atmospheric Pressure Chemical Ionisation (APCI), where the reagent gas used is usually water, charge-exchange Chemical Ionisation which unlike CI produces of a radical cation with an odd number of electrons and negative chemical ionisation (NCI), where the analyte must be capable of the production of negative ions84–86Matrix Assisted Laser Desorption/ionisation (MALDI).

The ionisation technique matrix assisted laser desorption/ionisation (MALDI), was discovered in the 80s and uses lasers, (UV or IR), in conjunction with crystalline compounds, referred to as the matrix, which can absorb laser energy and transferring it to ionise large molecules with little fragmentation of the molecule[87–90].

Although MALDI-TOF, (for TOF see section 1.4.5 below), mass spectrometry is still a valuable tool, especially for the rapid identification of microbial species, its widespread use is waning due to modern advancements in other mass spectrometry techniques[91,92]. MALDI is a static technique where the sample is co-crystallised onto

a plate with the matrix. It is an extremely effective technique for the ionisation of highly phosphorylated molecules, which in many cases are suppressed during ESI-MS. MALDI can be coupled with chromatographic techniques such as LC and size exclusion chromatography and has a wide range of applications in chemistry, biochemistry, materials, biological and medical sciences[93].

1.2.8 Ion Separation and Detection Methods

Mass spectrometry determines the mass of analytes via a process made up of four major steps (Figure 4). The sample is first introduced into the mass spectrometer either as a liquid or a gaseous sample. In step two, the sample undergoes an ionisation process, which produces ions from the sample. In the third step, the ions produced from the sample pass into the mass analyser, where they are separated based on their mass-to-charge ratio (*m/z*). In the fourth and final step, the ions are physically detected by a detector based on their ion current. The full process of sample analysis is illustrated below (Figure 1.4)[94].

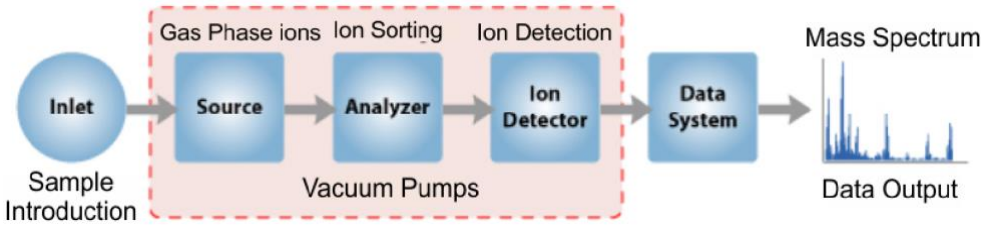


Figure 1. 4 Mass spectrometer operating process[94].

1.2.9 Quadrupole systems

A single quadrupole instrument in combination with a high-quality chromatographic system, can provide high-quality data about complex systems. The reasonable cost of these HPLC-MS systems is an additional advantage for them when making a choice of system; however, they do not produce high resolution/accurate mass measurements

or fragmentation patterns[95]. Tandem MS triple quadrupole instruments are therefore the workhorses mainly used to produce fragments in the analysis of drugs and their metabolites because of their accuracy and high sensitivity. The resolution of these instruments is, however, limited to approximately 0.5 amu, which is one of their major disadvantages.

1.2.10 Ion traps

Ion trap mass analysers or quadrupole ion traps are another type of mass analyser that operates by storing the ions generated from the analytes in an ion trap before detection [96]. They have a lower mass resolution compared to other types of detectors but are cheap to manufacture and can perform multiple fragmentation, MS/MS experiments on the stored ions, which affords useful structural information alongside the molecular ion [97].

1.2.11 Time-of-flight analysers (TOF analyser)

For time-of-flight analysers, the charge-to-mass ratio (*m/z*) of the ions is determined utilising the measurements of the time taken by the ions to arrive at the detector with the aid of the electric field of known strength. TOF instruments were initially plagued with poor resolution due to fluctuations in the kinetic energies displayed by the ion population, which was largely rectified by the introduction of the reflectron, which significantly improved the ability to focus the ions[98,99]. Quadrupole time of flight (QTOF) instruments are recommended for the routine analysis of samples for metabolomics because this hybrid instrument can provide both accurate mass data and fragmentation information. The resolution of these instruments is dependent on the length of their flight tubes, which is a limiting factor. Very sensitive methods have been developed by combining MALDI with TOF (MALDI-TOF)[100,101].

1.2.12 Fourier transform ion cyclotron resonance analyser (FT-ICR)

Fourier transform ion cyclotron resonance analyser (FT-ICR), first introduced in 1974 by Comisarow and Marshall, is a highly sensitive technique that gives the highest mass resolution of all ion detection systems[102,103]. It has a mass accuracy of less than 1 ppm, but this can only be achieved when internal standards are used[104]. FT-ICR functions in a similar manner to ion traps but differs in that the ion trap is embedded in a magnetic field that makes the trapped ions resonate at their cyclotron frequency. The application of a secondary electric field at or near the cyclotron frequency of the trapped ions causes the ions to be excited into a larger orbit, which can then be measured as they pass the detectors, which are situated on opposite sides of the trap[103]. The ion-to-ion interaction in FT–ICR however, limits the range of possible measurements, which is dependent on the frequency of the oscillating ions. This added to the high cost of these systems are a major drawback for their use[105].

1.2.13 Orbitrap mass analyser

The Orbitrap mass analyser was invented by Makarov 1999; however, the first commercial systems were introduced to the market by Thermo Fisher Scientific in the year 2005[106–109]. In Orbitrap systems, ions are trapped via electrostatic trapping. Ions are injected into the trap (Orbitrap) by the C-trap, during analysis, which acts as a temporary storage trap of ions separated from the linear trap component of the system. The Orbitrap is made up of a barrel-like electrode on the outside and an inner spindle-like electrode. The injected ions travel both in a circular path around the inner electrode while also undergoing axial harmonic oscillations, the latter of which is dependent on the mass-to-charge (m/z) ratios of the ions. Ion detection occurs by means of an image current that is generated as the ions undergo lateral oscillations

along the axis of the inner electrode. The Fourier transformation of this image current gives the *m/z* ratios of the ions in the trap. Extremely low concentrations of ions (<1 ng/ml) can easily be measured due to the ability of the Orbitrap to detect extremely small variations in the image current. Orbital trapping relies on the axial rotations of the ions and is not related to their m/z ratios. Orbitraps have a large space-charge capacity at higher masses with large trapping volumes and high mass resolution which FT-ICR instruments lack, as well as the Paul trap[110].

1.3 Limitations of Analytical Assays, Comparison of HPLC and HPLC-MS/MS

HPLC alone is a less sensitive technique than HPLC-MS/MS, which can detect substances at much lower concentrations [111,112]. While HPLC provides good separation and identification, HPLC-MS/MS offers a higher degree of specificity by adding mass spectral data, which can help in distinguishing compounds with similar retention times[113]. HPLC-MS/MS allows for more accurate quantification, especially when working with complex samples, due to the mass data, which allows the distinction between analytes with similar masses or chemical properties[112,113].

Like with most analytical techniques, there are some limitations and challenges that are common to the utilisation of both HPLC and HPLC-MS/MS, such as sample preparation, especially when working with complex samples, which often require extensive preparation that can be time-consuming and may introduce variability[114–116]. Biological matrices such as blood or urine can sometimes interfere with the analysis and require specialised techniques to mitigate these effects while preparing samples for analysis.

HPLC-MS/MS is more expensive than HPLC and requires more highly specialized equipment and expertise when compared to HPLC alone. Both HPLC and HPLC-

MS/MS are invaluable tools for analysis, each with its own strengths. HPLC is effective and better suited for routine analysis, while HPLC-MS/MS is preferred for cases requiring highly sensitive, specific, and accurate measurements, especially for complex or trace-level analyses[115,117]. These techniques help analytical scientists detect and quantify drugs, toxins, and other substances of interest.

1.4 Illicit drugs of Interest Used in This study

Several examples of illicit drugs/classes of drugs of forensic importance were studied during this thesis, these include the amphetamines, benzodiazepines and pregabalin.

1.4.1 Benzodiazepines (BZDs)

Benzodiazepines (BZDs), which are often called "benzos," were first discovered in the mid-20th century and are one of the top-selling families of prescription drugs in the pharmaceutical industry but are prone to abuse and dependency.

BZDs are a class of psychoactive drugs with a wide range of physiological effects including sedative, anxiety-reducing (anxiolytic), muscle relaxing, sleep-inducing (hypnotic) and anticonvulsant properties[118]. A few of the BZDs commonly used in clinical practice are shown below (Figure 1.5).

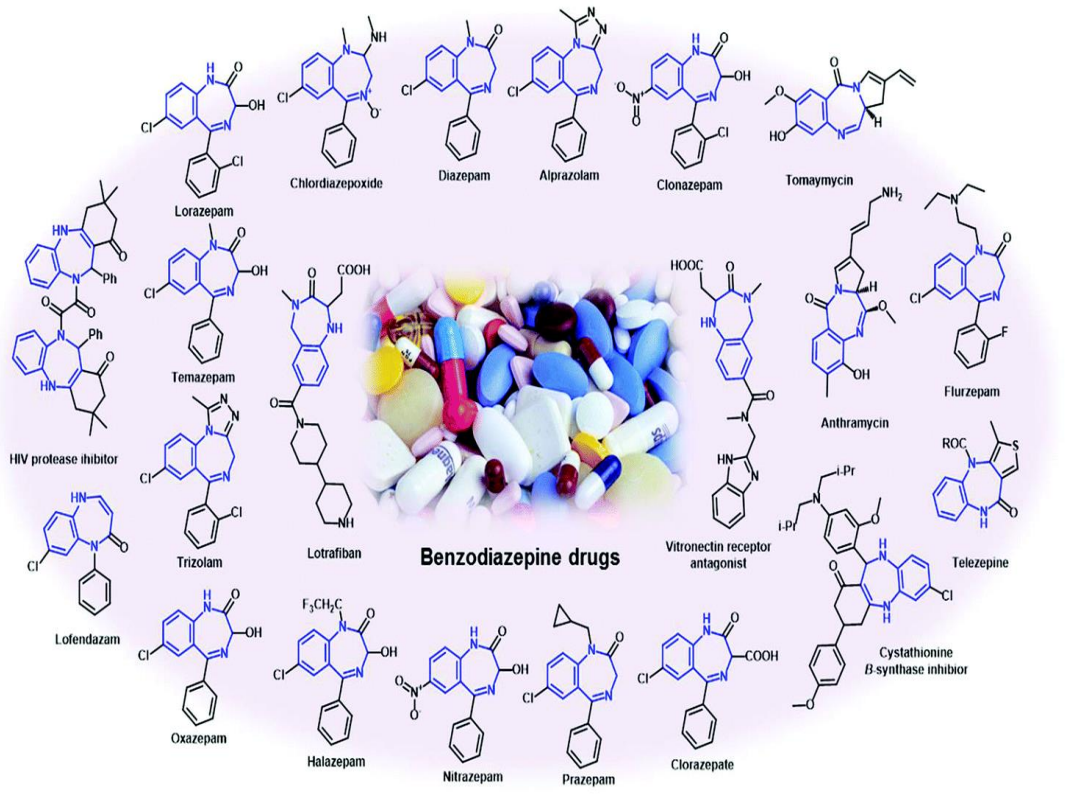


Figure 1. 5 Benzodiazepine-based clinically used drugs[119].

BZDs are commonly used with over 112 million prescriptions for different BZDs in the USA in 2008 which is an average of around one for every three residents[120]. The drug Xanax, commonly known as alprazolam, is one of the most widely prescribed BZDs on the market with over 48 million prescriptions dispensed in the USA in 2013[121].

BZDs, along with other non-benzodiazepine anxiolytic drugs, such as Zopiclone, Tandospirone and Buspirone, are some of the most often prescribed medications globally for use as anxiolytics, sedative hypnotics, anticonvulsants, and muscle relaxants are. These medicines, which are frequently used to induce intoxications, often lead to dependence in many patients. BZDs are the medications most often found to be linked with a wide range of crimes, including homicides, and violent

crimes, robberies, and sexual assaults, because of their sedative effect and their ability to cause amnesia in the victims, when used for reasons other than their intended therapeutic use [122–125].

BZDs are synthetic drugs whose pharmacological effects are mainly due to their ability to induce mild to severe central nervous system (CNS) depression because of the stimulation of the gamma-aminobutyric acid-A (GABAA) receptors in the brain[126]. Pharmaceuticals containing BZDs and benzodiazepine-related compounds are used for the treatment and management of anxiety, depression, sleep disorders, seizures, and for the induction of anaesthesia prior to surgery[127–130]. Short-term side effects such as drowsiness, dizziness, fatigue, headaches, disorientation, loss of appetite, and loss/decrease in muscular coordination may accompany the use of BZDs even when used as prescribed. In terms of onset of pharmacological effects, diazepam is rapid acting, alprazolam is intermediate acting, and clonazepam is slow acting[131]. Adverse short-term side effects including memory loss may however be more severe with increase in dose. Available evidence tends to suggest that any declines in cognitive function may continue even after cessation of use based on a meta-analysis study of reduced cognitive function following long-term BZD use[132]. When BZDs are taken together with other CNS depressants such as alcohol or opioids, it may result in severe and even fatal adverse effects[133,134]. A study in the USA in 2014, found that BZDs were the second-most prevalent class of drugs prescribed to drug dependent individuals after prescription opioids[135]. Due to their sedative effects which might make victims more compliant, BZDs are commonly utilised in drug-facilitated criminal activity such as robbery and sexual crimes and are also implicated in drug-impaired driving[125,136]. The recommended cut-off levels for prescription

benzodiazepines (PBZDs) for blood, urine, and oral fluid testing in cases of alcohol/drug-impaired driving were outlined in detail in a report by Logan et al[137]. As a result of their use in various forms of criminality including robberies and sexual assaults to either incapacitate or make victims more pliable/compliant, as well as their capacity to cause amnesia, there is an urgent requirement for the development of tools to detect the presence of these types of chemicals in relation to criminality and criminal behaviour[124,138]. Several BZD metabolites have significant pharmacological activities themselves which are comparable to those of the parent medicines such as *N*-desmethylclobazam (DMCLB) which is a metabolite of clobazam (CLB) and *N*-desmethyldiazepam a metabolite of diazepam[139,140]. However, because they are now among the most frequently prescribed medications, their risk of addiction and abuse is increased especially as they are frequently used in combination with other substances in in cases of drug-related fatalities[141]. To help reduce the incidence of drug related fatalities, prescription monitoring programs, (PMPs/PDMPs), which utilize BZDs as model medications for therapeutic drug monitoring, proactively collect and analyse data about controlled drugs of interest[142,143]. Clinical toxicology is becoming increasingly interested in screening for drugs and harmful substances in blood, urine, and stomach material because of its critical function in forensic case investigation.[144]. When assessing chemicals at low concentrations from biological samples, HPLC-MS/MS is often used and frequently ensures high levels of selectivity and sensitivity. Several HPLC-MS/MS techniques have been reported for the detection of pharmaceutical agents and hazardous substances from biological fluids including human blood, urine, whole blood, plasma, and serum [145,146].

1.4.1.1 Analysis and determination of BZDs (Liquid Chromatography)

Methods for liquid chromatography are those in which a solvent is used as the mobile phase. We differentiate between liquid chromatography (LC), which is the original method of chromatography, and more recent techniques like high pressure liquid chromatography, also known as high-performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC), thin-layer chromatography (TLC), and high performance thin-layer chromatography (HPTLC)[147]. Both qualitative and quantitative examination of various chemicals and combinations can be done using HPLC procedures. The foundation of qualitative analysis is separation and identification, which confirms the identity of the compounds, complicated mixtures, or pure substance under examination[148]. These methods are widely employed in a variety of fields, including toxicological analysis, forensic medicine, doping control, agriculture, the food and pharmaceutical industries[147–149].

Benzodiazepine compounds have been extensively analysed using high-performance liquid chromatography (HPLC), mostly from biological samples like urine, hair, plasma, serum, whole blood, nails, gastric fluids, tissue fragments, and oral secretion fluids, as well as in pharmaceutical preparations, i.e., tablets capsules and injection solutions[149–160]. HPLC methods, when properly sampled, also enable the identification and measurement of BZDs in pharmaceutical complexes and other medical compounds[161]. Additionally, it is employed in investigations about the stability of benzodiazepine derivatives in certain formulations, and the detection of impurities in pharmaceuticals containing BZDs, and enantiomer separation[157,162,163]. HPLC methods also permit the detection of metabolites such glucuronides from biological fluids[148,164]. For the study of benzodiazepines in biological materials, authors

frequently use a variety of extraction techniques, such as solid phase extraction (SPE) ,[165,166] utilizing Oasis HLB columns,[167] BondElut Certify,[168] Varian Bond Elut,[169] or columns modified with cyanopropyl groups,[170] liquid – liquid extraction (LLE) using different solvents,[154,171] solid phase microextraction (SPME),[164] SPME utilizing alkyl-diol-silica coated wall (ADS),[172] or molecularly imprinted polymer solid phase extraction (MISPE)[173]. From a practical point of view, reverse phase HPLC (RP-HPLC) is more frequently utilized than, normal phase HPLC (NP-HPLC)[174–176]. Typical RP columns include C18 and C8 columns[177,178]. In addition, there are also some reports involving the use of columns with an internal diameter of 1 mm and columns modified with hydrophobic groups[183,184]. There have also been reports about columns with polymer-coated stationary phases such as polyvinyl alcohol, or phenyl groups, cyano groups, or β-cyclodextrin[179–182]. In other examples, the use of silica-coated monolithic columns that ensure low pressure on the column and hence improve separation efficiency have also been reported. Separation of test compounds were performed utilizing isocratic conditions or gradients with different eluents as mobile phase, UV detection at wavelengths of 215 nm, 220 nm, 226 nm, 228 nm, 230 nm, 235 nm, 240 nm, 242 nm, 250 nm, 254 nm, 255 nm. Furthermore diode-array detectors (DAD) were reported to be useful as electrochemical detectors for benzodiazepine analysis[183,184]. There have also been several reports about the use of columns with polymer-coated stationary phases such as polyvinyl alcohol, or phenyl groups, cyano groups, or β-cyclodextrin.[179–182] In another example, the use of silica-coated monolithic columns that ensures low pressure on the column and hence improves separation efficiency was reported. Separation of test compounds was performed utilizing isocratic conditions or gradients with different eluents as mobile phase, UV

detection at wavelengths of 215 nm, 220 nm, 226 nm, 228 nm, 230 nm, 235 nm, 240 nm, 242 nm, 250 nm, 254 nm, 255 nm. Furthermore diode-array detector (DAD) was said to be useful as an electrochemical detector for benzodiazepine analysis[185].

The use of the combination of high-performance liquid chromatography and mass spectrometry (HPLC-MS) have been extensively reported for use in benzodiazepine analysis so also high-performance liquid chromatography tandem mass spectrometry (HPLC-MS-MS) while the use of HPLC-DAD-MS has also been proposed[149,159,164,186,187].

Ionisation methods used in benzodiazepine analysis include:

• Electrospray ionisation in positive mode (ESI +),[160,164,174] and electrospray ionisation in negative mode (ESI -)[151].

• Atmospheric pressure ionisation combined with electrospray ionisation (API-ESI)[167].

• Chemical and atmospheric pressure ionisation (APCI)[149].

• Thermospray ionisation (TSP)[188].

• Sound spray ionisation (SSI)[186].

• Triple quadrupole mass analyzers are frequently used in MS analysis[182] .

Additionally, ion trap mass analyzers and single quadrupoles have also been employed[189].

Due to potential issues, tandem mass spectrometry with thermospray ionisation was proposed for the analysis of thermolabile BZDs. Except for ketazolam, which completely decomposed into diazepam when analysis was performed under these specific conditions, this technique is however suitable for most BZDs[190]. Diazepam contamination in dietary supplements and herbal treatments has also been discovered

using HPLC-MS-MS. In this report diazepam and other adulterants including Sildenafil and Promethazine were successfully and reliably detected from herbal preparations. The samples which contained diazepam were advertised as being helpful to combat dysphoria and insomnia[191]. Ionisation matrix effects are one of the drawbacks of the HPLC-MS technology, as well as variations in peak retention times shapes brought about by the presence of co-eluting substances that can also affect the ionisation of analytes by increasing or decreasing their ionisation have been noted[192]. Micellar liquid chromatography (MLC) has been used, among other methods, to identify benzodiazepine compounds in pharmaceutical preparations[193]. The anticonvulsant medications of chlordiazepoxide and diazepam, which come in capsules, pills, tablets, infusions, drops, and suppositories, were determined using MLC. Bentazepam, Halazepam, Oxazepam, Pinazepam, and Tetrazepam concentrations in pills and capsules were also estimated using MLC[185]. Utilizing a carbon-fiber veil electrode (CFVE) in combination with a glassy carbon electrode, Honeychurch *et al*. were able to determine the presence of flunitrazepam and nitrazepam using high-performance liquid chromatography dual electrode detection (LC DED) in the reductive-reductive mode[194]. It was also able to identify BZDs from liquid samples using a straightforward and practical approach with a detection limit of 20 ng mL[195]. Drug analysis techniques using electrochemical technologies are continually being improved.

In order to determine BZDs, Ultra-Performance Liquid Chromatography (UPLC) may also be used. This tool can be used for pharmaceutical screening, tranquillizer identification, and antidepressant drug determination. The concentration, therapeutic or hazardous dose of BZDs in a blood sample can be determined using this method[196].

Pedersen et al. used a precise UPLC-TOF-MS screening methodology to seek out illegal substances and medications from blood samples. From nearly a thousand blood samples obtained from traffic case victims, they were able to identify the most often prescribed BZDs. This approach is particularly useful in forensic toxicology because the limit of identification (LOI) in whole blood samples obtained using this methodology ranged from 0.001-0.1 mg kg^{-1}. In cases where brain death was suspected, the BZDs level was determined using a UHPLC-MS/MS method (BDD)[197].

1.4.1.2 Analysis and Determination of BZDs (Gas Chromatography)

Additionally, BZD compounds have been detected from blood or urine samples, which were analysed using Gas chromatography utilizing extraction solvents, for example, *n*-butyl chloride or a chloroform-isopropanol (9:1) mixture. To transform BDZs into a variety of volatile derivatives that may be studied in the gaseous phase, a derivatization method is often necessary and is used to improve ionisation for more precise analysis[197,198].Derivatization was not always required, as in the case of oxazepam, which degrades thermally during chromatographic injection and was identified by the byproducts of that decomposition [198].

1.4.2 Amphetamines

Amphetamines such as Amphetamine, Methylenedioxyamphetamine (MDA), Methylenedioxymethamphetamine (MDMA), Methamphetamine, and dextroamphetamine are a class of chemical compounds, phenethylamines, that act on the central nervous system as stimulants.

Amphetamine was first discovered in 1887 by the Romanian chemist Lazăr Edeleanu but were not used as drugs until the 1920's[199–201].

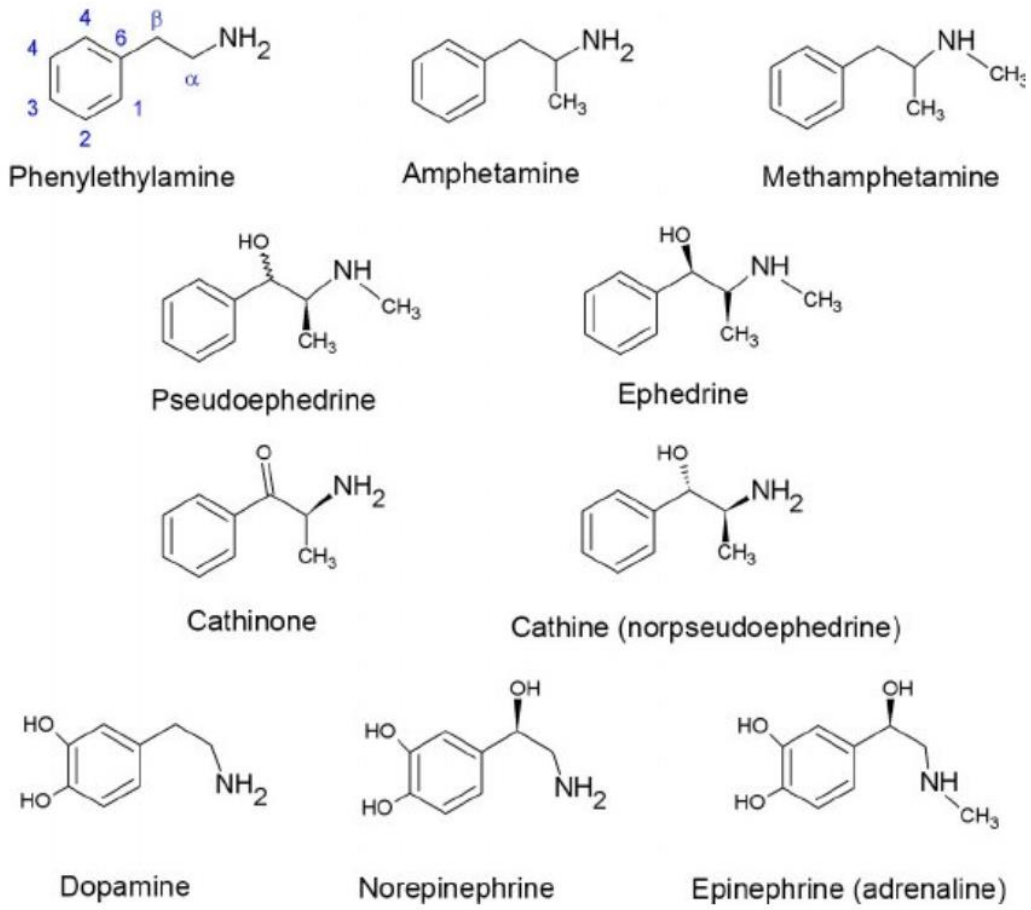


Figure1.6 Chemical structures of β -phenylethylamine (numbered), amphetamine, and methamphetamine. Also depicted are the natural amphetamines pseudoephedrine, ephedrine, cathinone, and cathine (norpseudoephedrine). The neurotransmitters dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline) are based on the same phenylethylamine structure202

Amphetamine derivatives are some of the most abused drugs[203]. They act by increasing dopamine concentration in terminal neurons resulting in effects which include a perception of increased energy, euphoria, increased concentration and mental alertness, and a perception of increased self-confidence and strength[204]. These compounds have been widely used to manage a range of medical conditions such as attention deficit hyperactivity disorder (ADHD), narcolepsy, obesity, and binge eating

disorder [200,201,205,206]. However, it has also been known for quite some time that they can induce neurotoxicity amongst other adverse health effects such as ischemic and haemorrhagic strokes, cardiac arrhythmia with cardioembolism, cerebral vasculitis, vasoconstriction, and acute hypertension associated with their use[203,207,208]. This and the potential for misuse raises important questions about the responsible prescription of amphetamines.

1.4.3 Analysis of Amphetamines The recommended methods for the identification and analysis amphetamines have been reported in a publication by the United Nations and are classified onto qualitative or quantitative methods of analysis[209,210]. Qualitative analytical methods are presumptive tests which provide for rapid screening for the presence or absence of the class of the substance of interest to quickly screen out negative samples. These include colour tests, which although being a simple and quick test, provide low accuracy with a high ratio of false test results[209]. Anion tests and microcrystal tests [209,211]. Several techniques can be utilised to provide quantitative results including TLC, which is rapid, sensitive and inexpensive but is not accepted in several countries [209,212]. Other methods include Gas chromatography with tandem flame ionisation detector (GC-FID)[209,213,214], GS-MS, which is the gold standard and one of the most widely used techniques capable of providing highly specific spectroscopic information about the components of a complex mixture without the need for prior separation [209,215,216]. HPLC is also a commonly used technique with similar technical features as GC-MS, such as similar sample preparation methods, analysis times, and the cost of instrumentation[209,212,217]. Fourier transform infrared (FTIR) spectroscopy is mainly used for qualitative analysis and is typically coupled to a GC [209,218,219], although it can be used as a stand-alone technique

by employing attenuated total reflectance FTIR [209,220]. Raman spectroscopy is also used but it is hindered by adulterants and cutting agents, which might interfere with readings due to their intrinsic fluorescence, which can result in high background fluorescence [209,221]. Techniques such as immunoassays , which allow for high specificity, are however, hindered by the high costs of equipment and reagents, and the highly trained personnel required for their operation [209,222–224]. Similarly, techniques such as capillary electrophoresis (CE) and nuclear magnetic resonance (NMR) , which have also been used for the detection and analysis of amphetamine type substances Amphetamine-Type Stimulants also require expensive equipment and highly skilled operatives [209,225–227].

Methamphetamine exists as two enantiomers D-and L-methamphetamine. D-methamphetamine has been found to produce a stronger stimulant effect therefore it has a higher potential for abuse. GC analysis, of methamphetamine often requires a derivatisation step which is not required for HPLC or Supercritical fluid chromatography analysis does not require this addition step[228]. A previous study found that MS detection was preferred over to DAD due to its lower limits of detection and specificity [228,229].

1.4.3 The Opium Alkaloids

The Opium poppy Papaver somniferum L. is believed to have originated about 5000 BC in the Sumer region of Mesopotamia, located in modern day Iraq and Kuwait, in the fertile area between the Euphrates and Tigris rivers[230–232]. A lot has been written about the opium poppy over the years covering not just its cultivation, medicinal/recreational uses, and its chemistry but also its trade, the politics and wars that have been fought because of opium[230,231,233–243]. Currently, the main legitimate

uses of the opium poppy are in the pharmaceutical industry as a source of alkaloids for medical applications and in the food industry as a source of poppy seeds used in food preparation[231,244]. Two species from the poppy (Papaveraceae) family, Papaver somniferum L. and *Paperver setigerum* D.C. (poppy of Troy or dwarf bread seed poppy) are the main medicinal plants of interest which contain the alkaloids morphine, codeine, noscapine (narcotine), thebaine and papaverine [231,245–247]. *Papaver somniferum* L. is believed to contain more of these alkaloids by weight compared to *Paperver setigerum* [247,248].

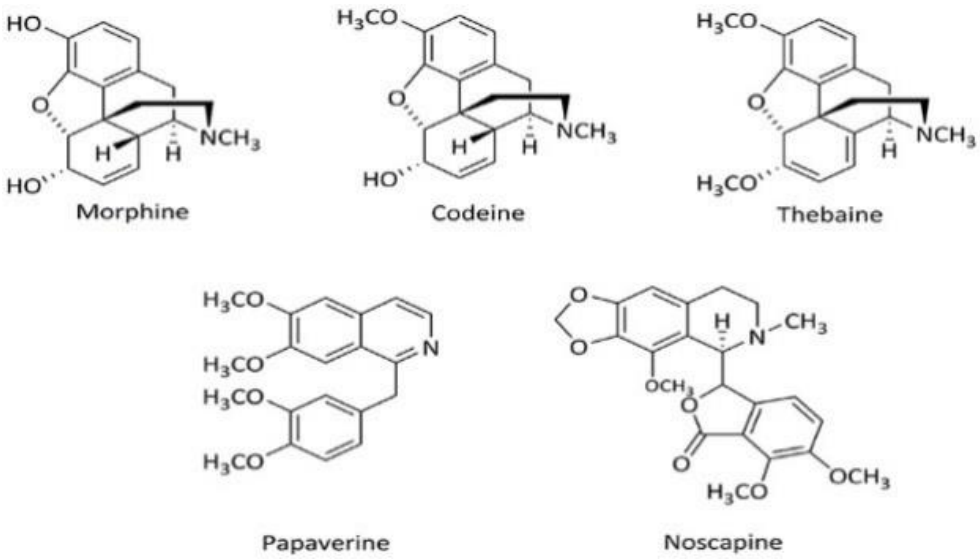


Figure 1. 7 Chemical structures of the main opium alkaloids.[231]

Although there have been reports of the detection of thebaine from both *Papaver orientale* q] *and Papaver bracteatum* Lindl., no evidence has been presented of the biosynthetic interconversion of thebaine to codeine and morphine in these species[231,249].

Wholly synthetic opioid drugs which mimic the effect of opioids have also been synthesised, such as fentanyl and its analogues, which are an order of magnitude more potent than both heroin and morphine and methadone[250–252].

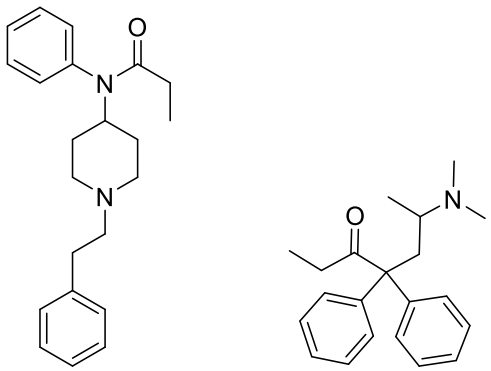


Figure 1. 8 Structures of Fentanyl and Methadone.

Although these drugs mimic the physiological effects produced by opium and bind to the same opioid receptors in the body, they are not derived from opium or any related alkaloids and have chemical structures that are unrelated to the opium alkaloids.

1.4.3.1 Analysis of Opioids

Common methodologies such as liquid-liquid extraction and solid-phase extraction have all been reportedly applied to the extraction of opioids from biological specimens such as plasma, blood, hair and urine[253,254].

Non-chromatographic methods are rare for the detection of opiates from blood, plasma or serum. are rare. There are, however, some reports for the use of immunoassays (IA) but any positive results need to be confirmed by an independent secondary technique at least as sensitive as the screening test to provide confidence in the result[255–262].

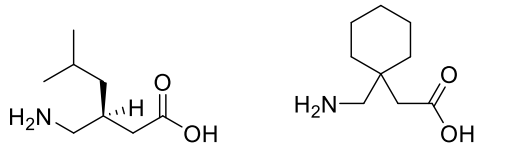
Chromatography and related techniques, including LC-MS/LC-MSMS, UPLC-MSMS, GC-MS have been extensively reported for the qualitative or quantitative determination of opioids but are generally considered expensive due to the high cost of equipment required and the highly skilled expert operators required for their efficient operation[253,263,264].

Numerous reports exist in the literature for the detection of opioids utilising immunoassays, HPLC coupled with fluorescence, electrochemical or UV detection, HPLC/UPLC- MS/MS and GC-MS [263–265]. A detailed review of techniques for the detection of opioids can be found in these excellent reports [255,266,267].

1.4.4 Pregabalin

The drug Pregabalin (Lyrica) is a gamma-aminobutyric acid analogue, widely used as a treatment for diabetic neuropathy, neuropathic pain, partial-onset seizures, anxiety disorders and other conditions [268]. The original research that led to its discovery was conducted at Northwestern University in the USA in 1990s, funded by public research grants. It was initially licensed to Parke-Davis and subsequently bought by Pfizer for further development and received FDA approval in 2004 [269]. In 2023, the market size for pregabalin was valued at $1.6b and set to rise to an estimated $ 2.2b. This implies that the drug has generated a significant amount of revenue for Northwestern University.

Pregabalin bears a close structural relationship to the antiepileptic drug gabapentin as it shows in (Figure 1.9), and both drugs have similar sites of action, the alpha2-delta (alpha2-delta) protein, which is an auxiliary subunit of voltage-gated calcium ion channels[270].



Pregabalin (Lyrica) Gabapentin (Neurontin)

Figure 1. 9 Structures of Pregabalin (Lyrica) and Gabapentin (Neurontin).

Studies have shown that pregabalin binds to presynaptic voltage-gated calcium channels (VGCC) at the α-2-δ subunit in the central nervous system [268,271–273]. This decreases the influx of calcium into neurons and the release of excitatory neurotransmitters. This decreases the influx of calcium into neurons and the release of excitatory neurotransmitters which is believed to be responsible for the analgesic and anticonvulsant effects of pregabalin. Pregabalin does not modify cyclooxygenase activity neither does it have any known activity at serotonin or opiate sodium channels, or receptors [270].

1.4.4.1 Analysis of Pregabalin Chromatographic Techniques

Several analytical methods have been reported in the literature for the analysis of pregabalin since 2001[274]. Techniques such as LC-MS/LC-MSMS, GC-MS, etc. have been reported for qualitative or quantitative determination of pregabalin and related compounds like gabapentin and etoricoxib[274,275]. According to the United States Pharmacopeia (USP) liquid chromatography is prescribed for the analysis of pregabalin[275]. Despite its pharmacopeial status, several non-compendial analytical methods have also developed. These include spectroscopic, chromatographic and hyphenated methods such as UV-Vis spectrophotometry, TLC, NMR, capillary electrophoresis, HPTLC, HPLC GC, HPLC-MS and GC-MS from a number of sample sources including hair, urine, blood, capsule formulations, tablet formulations,

bulk formulations, and plasma[274–282]. Several spectrophotometric methods were reported in a study of pregabalin by Shah et al, of which fifteen were UV-Vis, three were spectrofluorometric and one was a spectrometric method of luminescence[274,275] . As pregabalin is an aliphatic compound without a UV chromophore, derivatisation with reagents such as 1,2-naphthoquinone-4-sulphonate (NQS), 2,4-dinitrofluorobenzene (DNFB), 7-chloro-4-nitrobenzofurazon (NBD-Cl), 2,4-dinitrophenyl hydrazine (DNP) and 3-methyl-2-benzthiazolinone hydrochloride (MBTH) were used enhance the analysis[274,275].

1.4.4.2 Analysis of Pregabalin Hyphenated Chromatographic Techniques

Hyphenated techniques which combine various advanced chromatographic techniques with spectral techniques such as GC-MS, LC/ HPLC/UHPLC-MS/MS, or ESI-MS/MS enantioselective HPLC etc, have all been applied to the analysis of pregabalin due to their enhanced capabilities and detection abilities. These modern techniques combine the power of liquid chromatography's capabilities for the physical separation of the components of mixtures with the mass analysis capabilities of mass spectrometry. HPLC and UHPLC are analytical tools commonly used in analytical chemistry is for their enhanced abilities to separate the components of complex mixtures. The main differences between both techniques are that the stationary phases used for UHPLC columns have much smaller particle sizes than HPLC columns and they operate at a much higher pressures around 1000 bar compared to the 250 bar for HPLC[274,275,279].

1.5 The aim of the project

The aim of this thesis is to develop, optimize, and validate fast, robust, and reliable analytical methods for the identification, detection, and quantification of selected narcotics and illicit drugs, including benzodiazepines and their metabolites, commonly encountered in forensic and post-mortem toxicology in Kuwait. The study employs HPLC, short-column HPLC, HPLC–UV, HPLC–MS, GC, and GC–MS techniques for the analysis of standard laboratory solutions and urine samples, with the objective of evaluating the suitability of these methods for routine forensic and toxicological applications while overcoming challenges related to the physicochemical properties of the analytes.

Chapter 2 HPLC method development and validation for most used benzodiazepine compounds in Kuwait

2.1 Introduction

Benzodiazepines (BZDs) are some of the most detected substances encountered during investigations of drug-facilitated crimes (DFC), such as sexual assaults and robberies, due to their sedative effects as well as their ability to induce amnesia.[1,2] After the cannabinoids, benzodiazepines such as diazepam (DZ) and midazolam (MZ) are the second most common class of substances found[3]. Benzodiazepines are known prescription sedatives[4]. Benzodiazepines were also found in most cases to be used in combination with other illicit substances[3,5]. The combination of morphine and benzodiazepines is known to increase the risk of death due to overdose.[6,7] This is because both of these types of drugs can lead to sedation and the suppression of breathing[8,9]. An increased worldwide trend of benzodiazepine abuse has been reported by several authors from almost all continents of the globe and was highlighted in a 2019 systematic review by Votaw et al.[10]

It is therefore increasingly important from a pharmacological, toxicological, and clinical point of view to have rapid and reliable screening tests available capable of the analysis of numerous compounds of interest, such as BDZs, in a short time. These procedures often involve innovative and eco–friendly extraction and purification techniques. It is, however, often necessary to carry out preliminary sample preparation steps such as protein precipitation (plasma or whole blood). Globally, Alprazolam, sold under the brand name Xanax, appears to be the predominant drug of choice; however, diazepam (DZ), sold under the brand name Valium, was the BDZ almost exclusively found in Kuwait [3]. There is also a higher percentage of victims in Kuwait

when compared to figures reported in other studies worldwide[11]. In two studies on overdose deaths done on thousands of subjects in the United States of America and Sweden, the percentage of study subjects whose death certificates indicated a drug-related death that were found to have BDZs in their bloodstream were 16.1% and 19% respectively[12]. However, in Kuwait the percentage of similar deaths is 43%[11]. This might indicate that there may be easier access to these substances in Kuwait. Forensic analysis and investigation capabilities are vital in criminal and civil cases, for national security, environmental protection, and public safety. The manufacture and trafficking of illicit drugs, including the counterfeiting of legitimate pharmaceuticals, is a new emerging challenge faced by organized crime and terrorist groups[13]. This also underlines the vital requirement for sensitive and selective rapid identification protocols for illicit drugs in the field[14]. Presently, practical analytical technologies are needed to enable the "in-field" screening and analysis of evidence to provide fast, accurate, scientific information to support the forensic investigations[15]. Forensic analytical chemistry is defined as the discipline applied to crime scene analysis and to law, which is one of the areas of analytical chemistry. The nature of a sample and the use of its analytical chemical information play important roles in selecting and executing the appropriate chemical analysis technique. This part of forensic sciences deals with the characterization and quantification of chemical substances at trace levels. Most forensic samples are complex mixtures for which analysis generally requires separation prior to identification of chemical species. Thus, the principal tools of the forensic chemist are the instruments of analytical chemistry, with emphasis mainly on chromatographic techniques.

2.2 Validation of stability-indicating assay.

A stability-indicating assay is an essential analytical method that is used in the pharmaceutical and other chemical industries for the determination of the stability and integrity of a drug or chemical compound over a period of time. These types of assays are designed to detect and quantify any changes in the test substances' chemical composition, purity, and potency due to degradation over time and under various storage conditions[16]. They are specific and highly sensitive tests capable of detecting and quantifying degradation products, impurities, and changes in the active ingredient, even at very low con centrations.

A stability-indicating assay method has been defined as a "Validated quantitative analytical method that can detect the change with time in the chemical, physical or microbiological properties of the drug substance and drug products are specific so that the content of active ingredients and degradation products can be accurately measured without interference"[17].

Stability-indicating assays are used to assess the changes in the chemical, microbial and physical properties of all substances used in the formulation of a drug[18,19]. Since the chemical compounds used in drug formulations degrade to different extents over time and with exposure to different conditions, their degradation when exposed to blood components should be studied to help understand their performance under these conditions. Application of a stability-indicating assay is also essential in determining the behavior of the drug components[20]. The active ingredients in medicines are metabolised and excreted over a period. The metabolic process is essential to avoid

side effects caused by the buildup of toxins in the body. For example, after application of a drug to treat seizures, the drug should be cleared from the blood system after a given period.

The stability-indicating assay is essential for pharmacists to ensure the safety, efficacy, and quality of drug products for the duration of their shelf life[21]. Some drugs will degrade after being exposed to certain conditions[16]. Stability-indicating testing during manufacture and subsequent storage is essential and impacts several aspects of the manufacturing process, for example is used to determine the ideal storage conditions for drugs, the optimum formulation, and the ideal materials for packaging the drug to prolong its shelf life[22–24].

The development of a stability-indicating assay is applied to the identification and separation of various impurities from drugs[25]. Some drug impurities are formed during the synthesis of drugs. The manufacturing process involves several processes that can also increase the impurities in medicines. The assessment method helps determine the number of impurities in drugs and develop effective ways to remove them. The presence of impurities in medications can lead to off-target side effects, and the application of the assessment method plays a crucial role in minimizing giving patients peace of mind[26]. Through the application of the process, the right parameters for the manufacture of medications are set.

2.2.1 Development of Validated Stability-Indicating Assays.

The stability-indicating assay process was developed as a way of evaluating of the effect of exposing drugs to different stress conditions [27]. Drugs are exposed to base and acid hydrolysis processes, amongst others. Other processes that can be applied during the drug assessment process include oxidation of the ingredients used in the

drug. The photodegradation procedures affect drugs in different stages of their lifespan. Drugs are exposed to these steps to determine how individual ingredients in the drugs react to the conditions. Application of thermal degradation is one of the common processes that is applied to assess the stability of drugs. The development of the stability-indicating assay aims at addressing issues such as:

2.2.2 The Importance of Validated Stability-Indicating Assay.

Stability-indicating assay comes with several benefits. Its application in the drug manufacturing industry leads to high-quality drug production. Professionals apply it in the drug development stages to determine the reaction of different drug ingredients when exposed to different conditions[28]. The application of the process makes it possible to know what steps should be followed during the drug manufacturing process.

2.2.3 Differentiation of Degradation Products Related to the Drugs.

The drug formulation process involves the combination of various chemicals and excipients to make the drug under specific conditions. Using the process, experts learn the difference between drug degradation products and other products related to the drugs. Reaction between components of the drug can lead to degradation; therefore, the interactions of various components are tested to identify which components are most suitable and economical to formulate the drug. The application of the process makes it easy to establish the degradation process. Users of the process understand the degradation of the drug and other substances that react with the drug in the body to bring about specific outcomes. The process is beneficial in the drug manufacturing process.

2.2.4 Explaining the Structure of Substance Degradation.

The application of a stability-indicating assay aims at elucidating the structure of degradation products. There is a wide range of products that undergo different processes in the degradation process[29]. The process helps determine the degradation of the products. Some products will degrade under a wide range of conditions. This process can be used to explain how drug products behave when they are subjected to different conditions. The procedure helps manufacturers determine the right procedures to follow as they formulate the drugs and determine how the final product should be stored and handled. This process is highly effective in making the drugs more stable and extending their shelf life.

2.2.5 Determining the Intrinsic Stability of Drug Substances.

The process helps determine the intrinsic stability of the substances used in making the drugs. Some ingredients will degrade fast when subjected to certain conditions[30]. Knowing these conditions is essential to coming up with practical steps that will be applied to ensure the best outcome for the drug in development. Drug formulation utilises different steps. Application of the process clarifies the stability of various formulations. Knowing the stability of a drug is essential when coming up with the optimal measures for addressing the wide range of issues that can affect the drug.

2.2.6 Understanding Chemical Properties of Drugs.

Drugs come with different chemical properties[31]. It is essential to understand the chemical properties of drugs and then come up with appropriate ways to apply them. Through research, drug manufacturers understand different chemical properties. The process exposes the drugs to various conditions under which the chemical properties

are monitored. It is a clear way to determine the applications of the drugs. Users of the method can rely on it to learn about a wide range of issues related to the drug. Pharmacists get to know the correct procedures required to preserve the drugs or make them more effective after they assess the chemical properties. Exposing drugs to different conditions will make them react. The procedure is essential in determining the best procedures to be applied while establishing the chemical processes involved in the manufacture of the drug.

2.2.7 Production of a Degradation Profile.

Drug formulation companies rely on the process to develop degradation profiles that reflect the procedures to be followed when the drug is put into use[32]. Drugs undergo several tests to determine their degradation paths. Using this process simplifies the understanding of the degradation process and helps to determine the ideal conditions to get the optimum outcomes.

2.2.8 Solving Stability-Related Problems in Drugs

Drugs that treat specific ailments are required to achieve a specific stability standard. Through the application of the stability assay process, drug manufacturers establish proper procedures to ensure the stability of a given drug. These are practical steps that work towards making drugs more stable. Users can ultimately count on the manufacturer's information for the correct information regarding the storage or use of the drug to achieve the desired effects. Stability problems can make a drug less effective. The application of the processes works towards solving the stability issues. Changes can be made to the formulation of a drug to make it more stable. The information regarding the necessary changes required in the drug formulation can be obtained after it has been exposed to a stability test because stability tests reveal how

the drug will work when exposed to different conditions. It is a great way to ensure that the drugs developed will work in solving different health complications.

2.2.9 Providing Insights into Degradation Pathways.

The process offers useful insights into the degradation pathways of products. Understanding the degradation pathway is essential for coming up with measures to ensure that the drug is used appropriately. Drugs are developed to treat a wide range of issues. Through research, the process of ensuring that drugs are put to the correct use is simplified. The process is relied on to understand how the medication will work under different applications. The degradation pathways can also be studied to determine what ingredients can be added to the drug substance to make it more stable when applied under different conditions. There are many tests that have been developed to assess the stability of the drug. They are effective processes that can be applied to achieve the best results when working on various drug formulations.

2.2.10 Showing the Chemical Behavior of the Molecules.

The chemicals and excipients used in manufacturing a drug can potentially have different effects on the patients. There is a need to study the chemical behavior of these substances in the drug formulation. The chemical substances will begin to degrade after exposure to various environmental conditions. A clear strategy should therefore be developed to address the issue of chemical degradation. The combination of different chemical substances in a drug formulation can result in different effects [33,34]. Research is essential to discovering the correct measures to ensure the performance of drug formulations is as desired. Several drugs have been developed to address a wide range of issues by developing measures to analyse chemical degradation. The study of chemical interactions within the drug is essential for

addressing several issues that can come up during drug formulation. There is a need to come up with the right chemical formulations to make the drug more reliable. The introduction of certain chemicals can lead to better drug performance.

2.3 Aim of study

The aim of the studies in this chapter is to develop and validate a method to detect the drugs, alprazolam, clonazepam, flunitrazepam, bromazepam and diazepam, and their metabolites that are commonly encountered in post-mortem toxicology, and to apply the developed methodology to the analysis of urine samples. This data will then be used to evaluate the feasibility of this method used in the routine analysis of urine samples. These compounds were chosen since they are commonly detected during forensic investigations in Kuwait as it shows in (Figure 2,1)

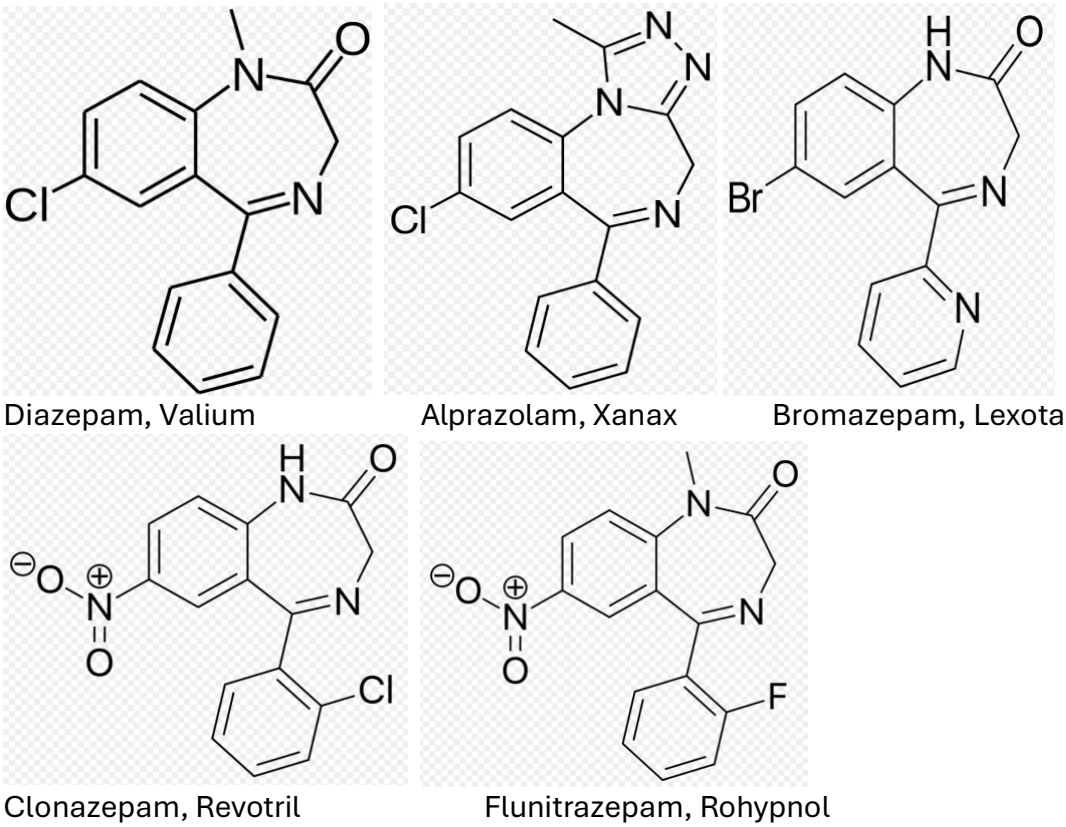


Figure 2. 1 Five BDZs most detected during forensic investigations in Kuwait.

2.4 Materials and Methods

2.4.1 Solvents and chemicals.

The solvents used for the studies were High-performance liquid chromatography (HPLC) grade. Acetonitrile (ACN) was purchased from either Fisher Scientific (Loughborough, UK), or Merck Life Science UK Limited, The Old Brickyard, New Rd, Gillingham, Dorset, United Kingdom. HPLC grade water was obtained from a Purite Select Ondeo system (Purite Limited, UK), or prepared using a MilliQ filter purchased from Millipore, Watford, UK. Syringe membrane filters (13 mm) were purchased from Kinesis Scientific Expert, Cambridgeshire, UK. Nylon solvent filters (0.45 um) were used for solvent filtration and Water. AnalaR-grade formic acid (98%) was obtained from BDH Merck (Poole, UK). Solvents, (Methanol, Water), Chemicals (Ammonium Formate,) and authentic standard stock solutions (Alprazolam, Bromazepam, Clonazepam, Diazepam and Flunitrazepam), Sigmatrix Urine Diluent, were obtained from Merck Life Science UK Limited, The Old Brickyard, New Road, Gillingham, Dorset, United Kingdom. Sigmatrix Urine Diluent was obtained from Sigma -Aldrich UK Limited, The Old Brickyard, New Road, Gillingham, Dorset, United Kingdom.

2.4.2 Chromatographic analysis.

HPLC analysis was performed on a Nexera LC-2400C HPLC (Shimadzu Corporation, Japan), fitted with a C18 ec, 250 x 4.6 mm, 3 µm column.

Analysis was performed using the Lab Solutions version 5,117 software, from Shimadzu.

The HPLC was set up with freshly prepared mobile phase and rinse/wash solutions required for the analysis installed on the appropriate lines. The autosampler needle and sample syringe were then flushed with the syringe wash solution (Methanol: Water, 1:1). The drain valve was opened, and the system was then initially flushed with mobile phase B (100%) followed by mobile phase A (100%) at a flow rate of 1.0 ml/min for 5 minutes for both mobile phases. The drain valve was then closed and the desired HPLC column was installed and conditioned with 50% of mobile phase B at a flow rate of 0.3 ml/min for 5 min, then slowly brought to the appropriate starting gradient and flow rate for each run over a further 10 minutes. Chromatographic separations were performed with a C18 250 x 4.6 mm, 3 µm column utilising isocratic elution over 26 minutes as shown below (Table 2.1) using the appropriate mobile phases and flow rates. While on the instrument, samples were kept in a vial tray, which was set to a constant temperature of 4˚C to avoid any degradation of samples.

2.4.2.1 Long Colum Validation HPLC Method Settings.

Mobile Phase A : 10 mM ammonium formte in water

Mobile Phase B : Methanol

Column : NUCLEODUR, C18 ec, 250 x 4.6 mm, 3µm (Length, ID and particle size)

Flow rate : 0.8 mL/min

Detector : UV and PDA

Detection Wavelength: 254 nm (200-400 nm scan for PDA)

Injection volume : 10 µL

Column Oven Temp : 50°C

Run time : 26 minutes (isocratic elution)

Diluent : Methanol

Table 2. 1 Gradient Composition for HPLC

	Time (mins)	% MP B Composition
1	0.01	5
2	26.0	5

2.5 Stability Indicating Assay of Benzodiazepines

A stability indicating HPLC method was performed on the 5 most used BDZs in Kuwait to investigate their stability or degradation in HPLC solvents (methanol) under several storage conditions chosen to mimic the conditions which forensic samples might be exposed to in Kuwait. The test was run at four temperatures over a four-week period.

2.6 Experimental

2.6.1 Standard solutions.

Standard solutions for the study were prepared as follows:

2.6.1.1 Preparation of individual standard stock solution: (1000 µg/mL)

10 mg each of the benzodiazepines (Alprazolam, Bromazepam, Clonazepam, Diazepam and Flunitrazepam) were accurately weighed and transferred individually into separate 10 mL volumetric flasks. Methanol (5 mL) was then added to each of the volumetric flasks to dissolve the samples by gentle swirling. The volume was then made up to the mark with methanol.

2.6.1.2 Preparation of individual intermediate standard stock solution: (100 µg/mL)

An aliquot (1 mL) of from each of the Alprazolam, Bromazepam, Clonazepam, Diazepam and Flunitrazepam standard stock solutions were transferred individually *via* pipette to a separate 10 mL volumetric flask. Methanol (5 mL) was added to each volumetric flask and mixed well by swirling. The volume was then made up to the mark with methanol. of the 5 BZDs were then prepared for each test temperature.

The experiment was run over 4 weeks at 4 different storage temperatures.

2.6.2 Analysis

The analysis was done using Lab Solutions version 5,117 software, from Shimadzu. The first run for time 0 (t_0) was run on the same day the samples were prepared. All the samples were stored in the HPLC vials at the 4 different storage temperatures respectively (4 °C, 25 °C, 37 °C, 50 °C).

The second run was carried out after one week and then all the samples were again stored at their respective storage temperatures for another week as the third run was after 2 weeks. The samples were again returned to storage for another two weeks and finally the last run was done in week 4.

2.6.3 Long Column Validation.

2.6.3.1 Preparation of calibration curves:

A series of mixed standard calibration solutions were prepared for 1, 2, 5, 10, 20, 40, 80 and 100 µg/mL concentrations using methanol as a diluent. Areas under the curves (AUCs) were plotted for the corresponding concentrations and regression equations computed using Microsoft excel 365.

2.6.3.2 System Suitability Criteria:

The system is deemed to be suitable if the peaks at 10 µg/mL calibration solution meet the following criteria[35].

- The Tailing factor for Alprazolam, Bromazepam, Clonazepam, Diazepam and Flunitrazepam does not exceed 2.0.
- The Resolution between Clonazepam and Alprazolam should be more than 1.5.
- The Resolution between Alprazolam and Flunitrazepam should be more than 1.5.

2.7 Results and discussion.

The results from the experiments conducted in this study are presented below.

2.7.1 Method development and optimization.

Table 2. 2 Results of HPLC Development

Name of the Component	Retention Time (min)	Peak Area	Tailing Factor	Capacity Factor (K')	Resolution	Number of theoretical plates
Bromazepam	8.596	658320	0.998	1.762	--	34375
Clonazepam	9.712	1023399	0.985	2.121	2.232	55698
Alprazolam	10.569	759213	1.099	2.396	1.586	64042
Flunitrazepam	13.580	769313	1.007	3.364	4.660	60794
Diazepam	23.755	955380	1.107	6.633	10.322	80151

The chromatogram obtained for the BDZ samples showed well resolved baseline separated, sharp and distinct peaks with good response levels for the chosen chromatographic conditions with a run time of 26 minutes isocratic (figure 2.8).

2.7.1.1 Results of HPLC Validation:

The method was developed and validated utilising ICH guideline Q2(R1)[36,37], and the results are presented below.

Table 2. 3 Specificity

Name of the Component	Peak Area	Retention Time (min)	Peak Purity (3-point Similarity)
Methanol Blank Solution			
Bromazepam	Not Detected	Not Detected	Not Applicable
Clonazepam			
Alprazolam			
Flunitrazepam			
Diazepam			
Individual Solution at 10 µg/mL			
Bromazepam	38068	8.581	0.9990
Clonazepam	76831	9.696	0.9998
Alprazolam	57593	10.543	0.9999
Flunitrazepam	41572	13.559	0.9998
Diazepam	47477	23.784	0.9999
Mix Standard Solution at 10 µg/mL			
Bromazepam	38009	8.596	0.9999
Clonazepam	83449	9.720	0.9999
Alprazolam	52196	10.580	0.9999
Flunitrazepam	50326	13.597	0.9999
Diazepam	56165	23.779	0.9999

Standard samples of each of the BDZs were analysed first individually and their peak areas, peak retention times and purities recorded. The samples were then combined into a single mixed standard solution which was then analysed and again the peak areas, retention times and purities were recoded again. Comparisons between the results obtained for each of the individual runs was comparable to those obtained for the mixed standard sample runs.

Specificity of the method was assessed by the analysis of laboratory prepared BDZ standard solutions individually and as mixtures at 10 µg/mL concentrations. The

precision of the protocol was tested over a range of concentrations, (1, 2, 5, 10, 20, 40, 80 and 100 µg/mL), for each BDZ in triplicates.

Table 2. 4 Precision (repeatability)

Name of the Component	Concetration (µg/mL)	% RSD
Bromazepam	20	0.50
Clonazepam	20	1.61
Alprazolam	20	0.44
Flunitrazepam	20	0.85
Diazepam	20	1.21

Table 2. 5 Intermediate Precision

Name of the Component	% RSD
Bromazepam	0.77
Clonazepam	0.97
Alprazolam	1.33
Flunitrazepam	1.04
Diazepam	1.08

Repeatability and intermediate precision were assessed at several concentrations of standard BDZ solutions also in triplicate. The %RSD were found to be under 2 .

Table 2. 6 LOQ and LOD.

Name of the Component	LOQ (2 µg/mL)		LOD (0.6 µg/mL)	
	% RSD (n=6)	Mean S/N (n=6)	% RSD (n=6)	Mean S/N (n=3)
Bromazepam	1.93	917.26	1.85	329.53
Clonazepam	1.14	1509.9	1.96	457.73
Alprazolam	0.93	1113.06	1.88	350.86
Flunitrazepam	1.15	701.53	1.60	205.66
Diazepam	1.17	500.86	0.98	188.73

The chromatographs showed a very clear separation between the BDZs of interest with limits of quantifications (LOQ (2 µg/mL)) and limits of detection (LOD (0.6 µg/mL)) respectively.

Table 2. 7 Linearity

Name of the Component	Correlation Coefficient (r^2)
Bromazepam	0.9993
Clonazepam	0.9995
Alprazolam	0.9992
Flunitrazepam	0.9995
Diazepam	0.9991

The linearity of the protocol was tested over a range of concentrations, (1, 2, 5, 10, 20, 40, 80 and 100 µg/mL), for each BDZ in triplicates. The r^2 values were found to be ≥ 0.99 which implies that the method can be deemed to be linear as it showed in following graphs (Figures 2.2-2.6).

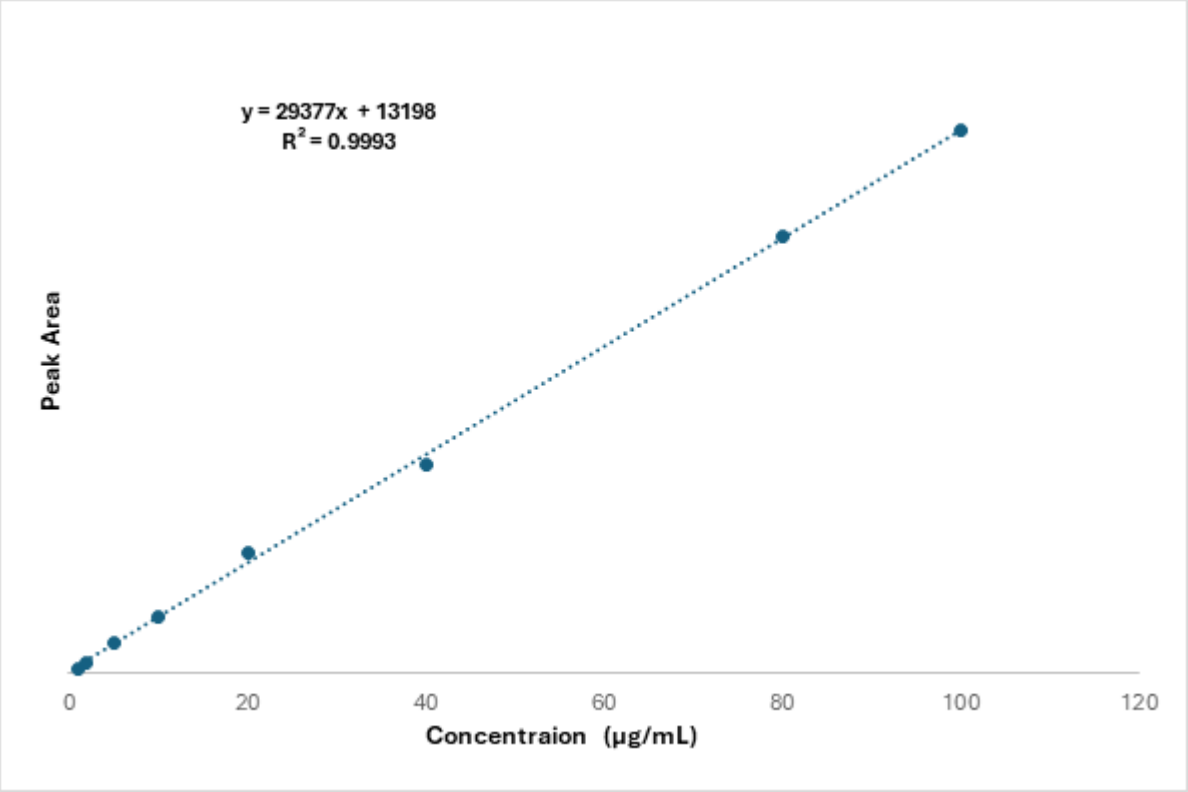


Figure 2. 2 Calibration curve for Bromazepam.

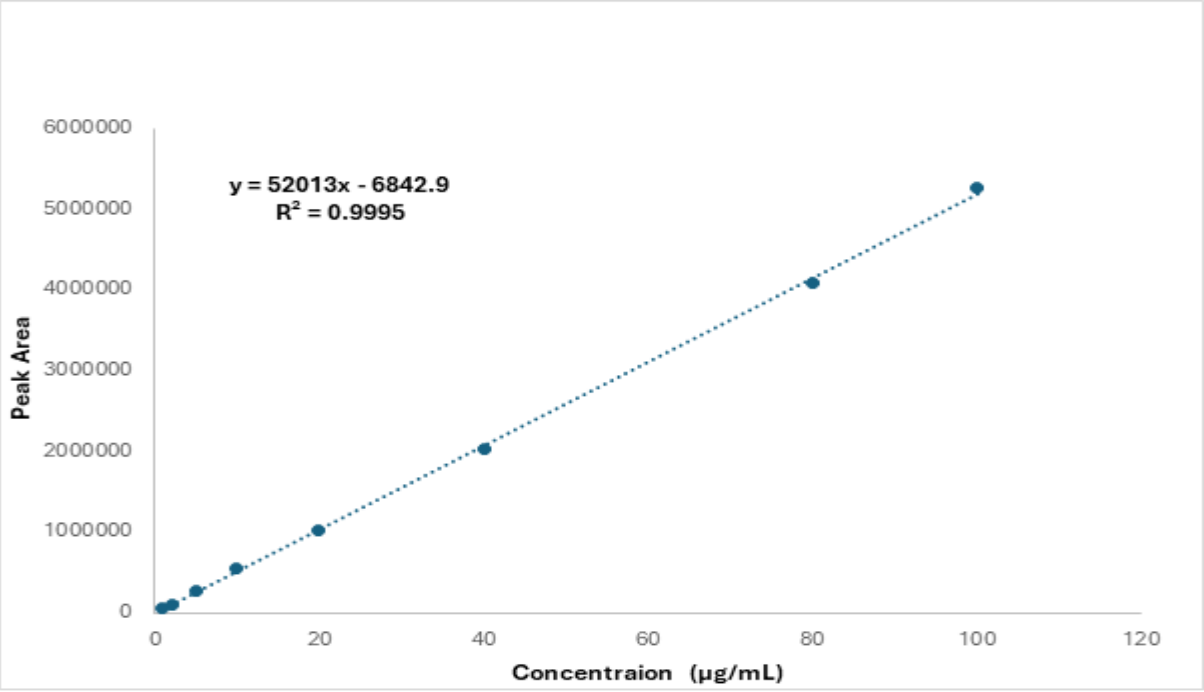


Figure 2. 3 Calibration curve for Clonazepam.

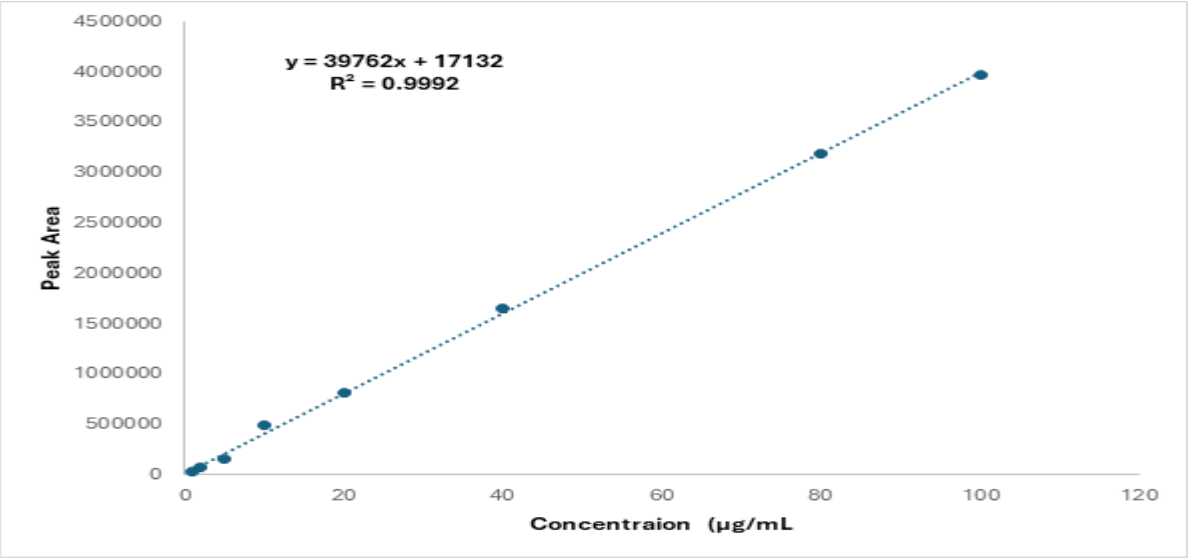


Figure 2. 4 Calibration curve for Alprazolam.

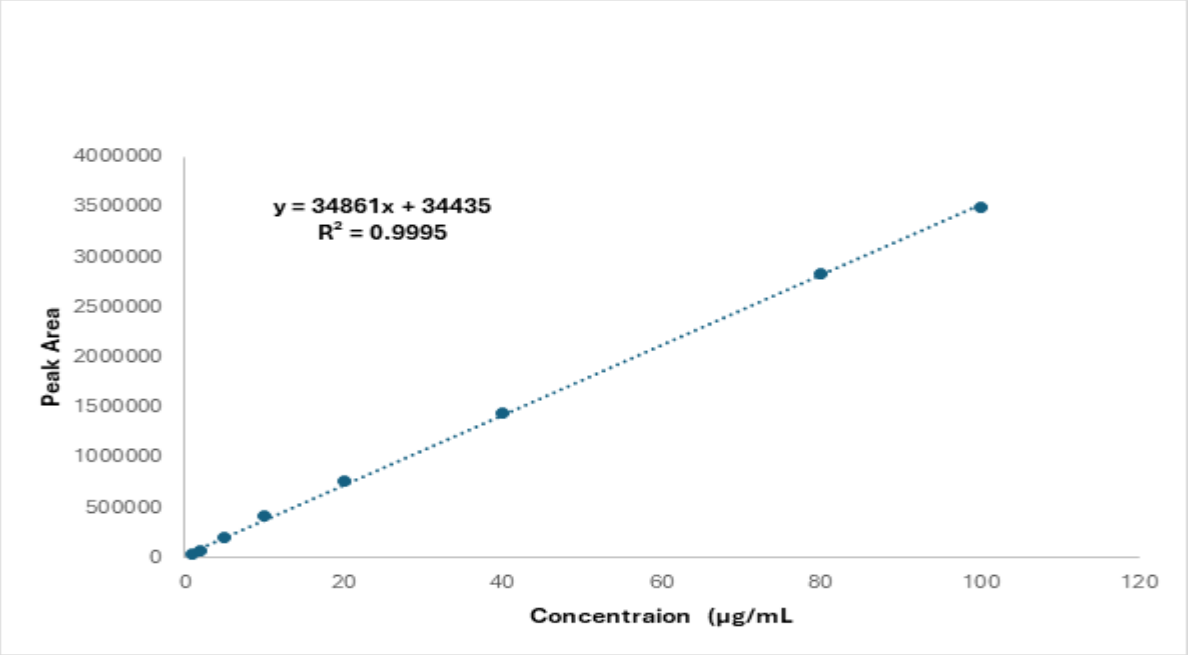


Figure 2. 5 Calibration curve for Flunitrazepam**.**

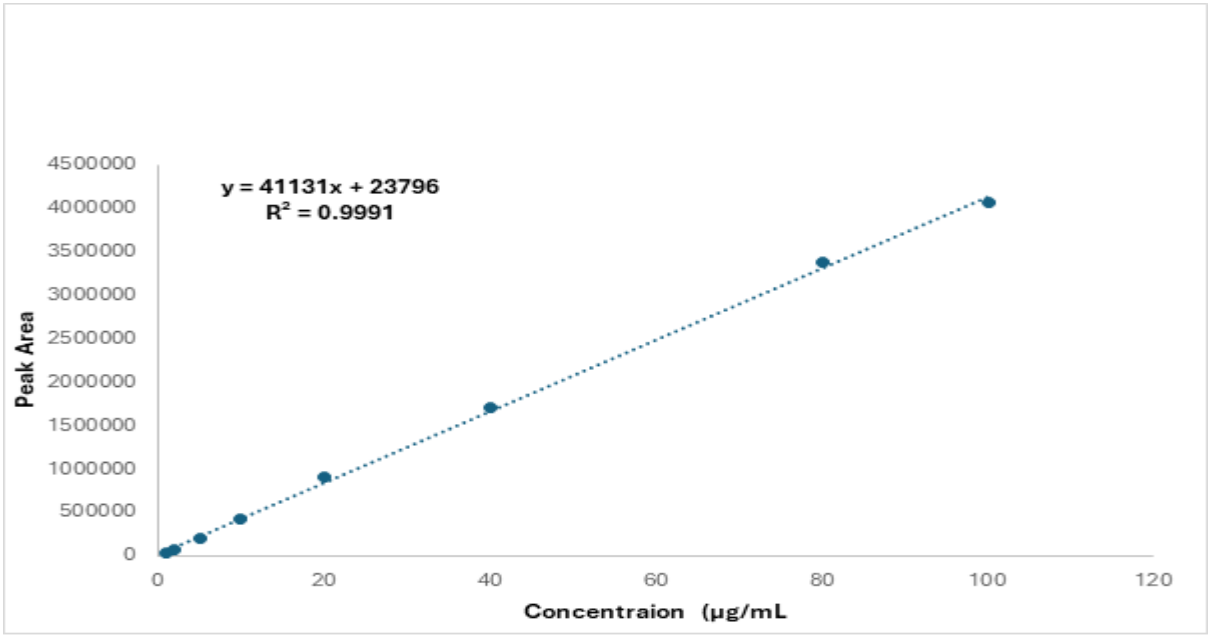


Figure 2. 6 Calibration curve for Diazepam.

Table 2. 8 Robustness

Name of the Component	%RSD					
	Flow Rate (ml/min)		Col Temp (°C)		Wavelength (nm)	
	0.75	0.85	48	52	252	256
Bromazepam	1.84	1.78	1.87	1,86	1.76	1.27
Clonazepam	1.88	1.35	1.81	1.17	0.89	1.27
Alprazolam	1.84	1.94	0.97	1.62	1.57	1.12
Flunitrazepam	1.51	1.91	1.60	1.46	1,62	1.17
Diazepam	1.82	1.61	1.87	0.88	1.69	1.33

The robustness of the protocol was assessed by the reliability of the analysis after small changes had been made to the experimental conditions. The parameters changed included oven temperature (±2°C), formic acid concentration (±1 mM) and flow rate (±0.1 mL/min) over several concentrations of standard BDZ solutions in triplicate changing only one parameter at a time.

Table 2. 9 System Suitability

Name of the Component	Tailing Factor	Resolution
Bromazepam	1.100	--
Clonazepam	1.038	10.18
Alprazolam	1.145	2.017
Flunitrazepam	1.053	2.328
Diazepam	1.127	8.375

Chromatograms from HPLC:

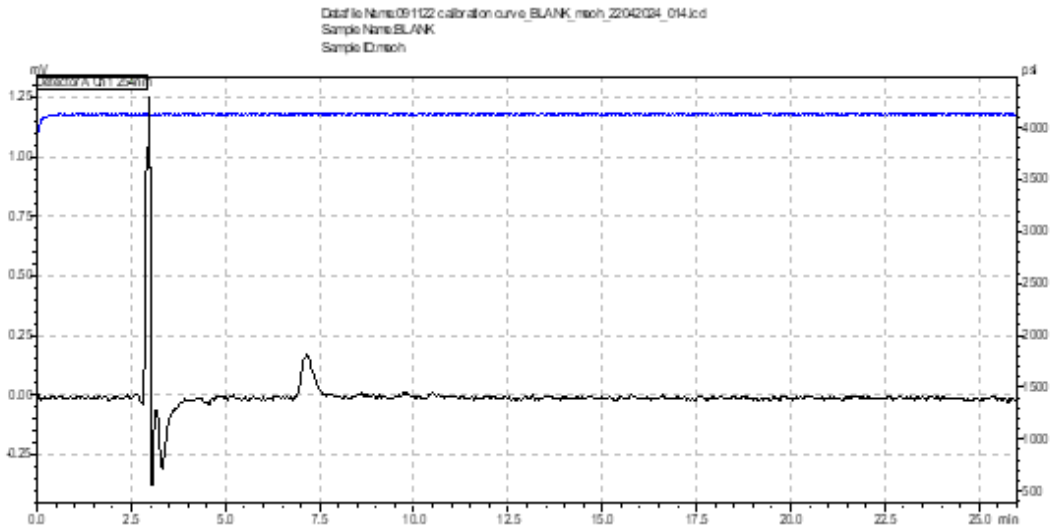


Figure 2. 7 Chromatogram for Methanol Blank.

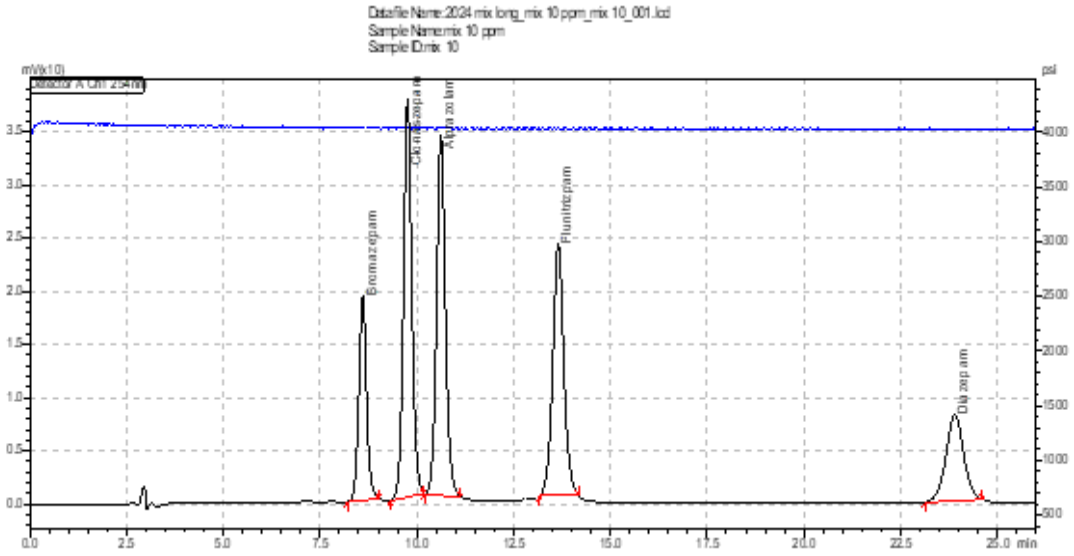


Figure 2. 8 Chromatogram for Mix Standard Solution at 10 µg/mL.

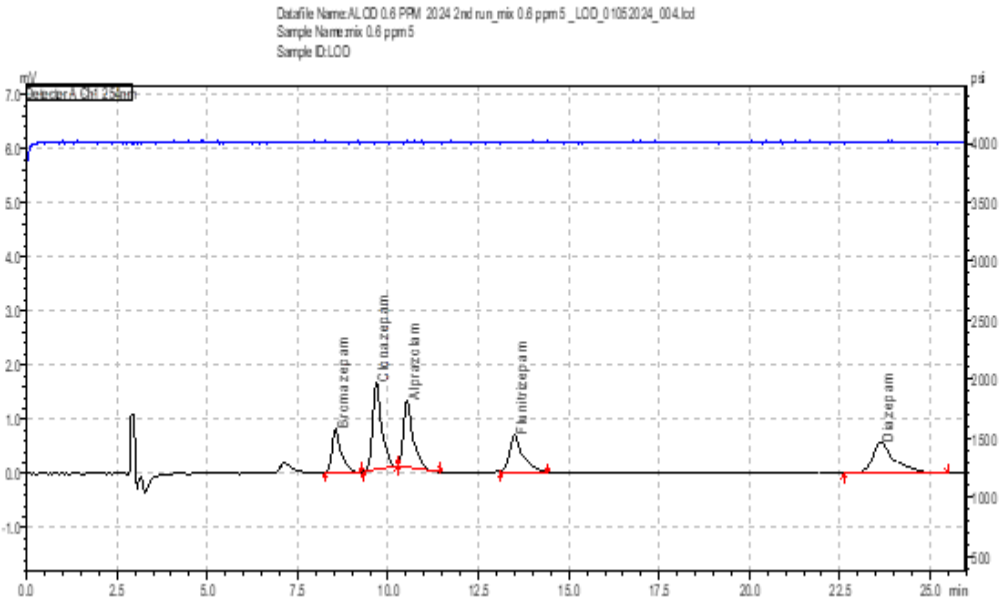


Figure 2. 9 Chromatogram for LOD.

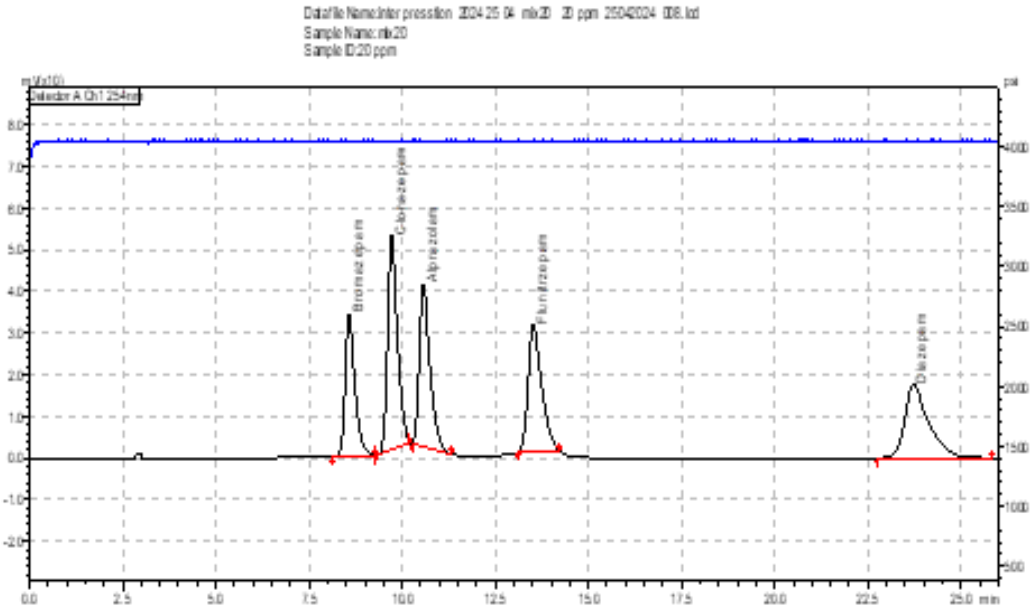


Figure 2. 10 Chromatograph for LOQ.

Following the development and validation of the method on the long (25cm) column, the method was applied to the conduction of a stability study on samples of the BDZs selected for the study. The aim of the study was to determine the ideal storage conditions for the samples once prepared and the duration for which samples could be kept before use before they are rendered unsuitable for analysis due to degradation.

Time zero (t_0) analysis runs were carried out for individual BZD samples on the day the samples were prepared for: Bromazepam, Clonazepam, Alprazolam, Flunitrazepam and Diazepam. The samples were then stored at 4 different temperatures (4, 25, 37 and 50°C) respectively for the duration of the study and rerun at set intervals as shown in table below (Table 2.10)

Table 2. 10 Sample run table for stability assay during 4 weeks for four different temperature (4°C , 25°C , 37°C and 50 °C).

Week 4	Week 2	Week 1	Temperatures °C
Last Run after storage	2nd Run after storage	1st Run after storage	4 °C
Last Run after storage	2nd Run after storage	1st Run after storage	25 °C
Last Run after storage	2nd Run after storage	1st Run after storage	37 °C
Last Run after storage	2nd Run after storage	1st Run after storage	50 °C

Overlays of representative runs from the HPLC analysis of the five BDZs for t_0 and t_4 are shown in the figure below (Figure 2.11).

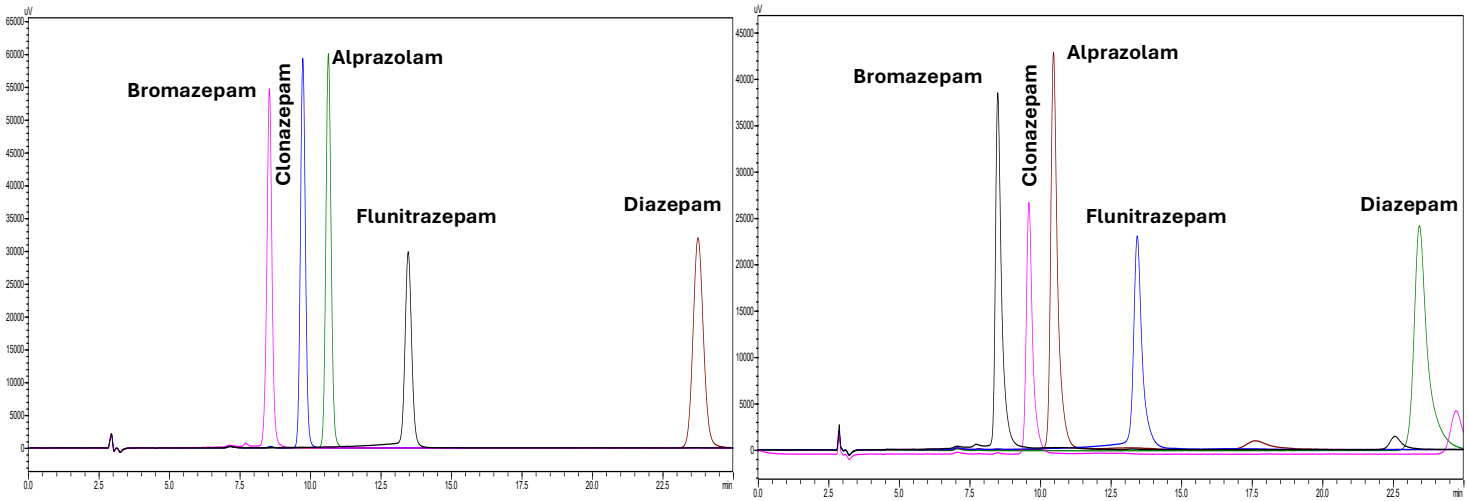


Figure 2. 11 Representative overlays of HPLC analysis for t_0 (left) and t_4 (right) 50°C experiment.

From the chromatographs, some very minor peaks can be seen to begin to appear in the samples at t_4 suggesting the samples begin to degrade after a few weeks of storage but are generally stable under the test conditions.

The average peak areas calculated for the peaks of each BDZ sample for the different time and temperature points are presented in table below (Table 2.11).

From the table, the peak areas for each BDZ generally show a slight decrease, less than 10% per week, which is greatest for all samples between t_2 and t_4. The greatest decrease in peak area was seen for Clonazepam in the 50°C experiment, 37%. This is probably due to the molecular structures of the BDZs. Clonazepam contains a nitro group which might be responsible for its faster metabolism because nitro groups can react with methanol to give *N*-methylated amines. This would indicate that the samples although relatively stable over the test period had begun to undergo some slight decomposition which appears to increase with temperature. This would suggest that samples are best stored under refrigeration if they are to be kept for extended periods of time and are best used within 3 weeks of preparation to avoid complications due to degradation of the samples. Considering the daily temperature range in Kuwait it would also be important to ensure samples are refrigerated as soon as possible after collection. Based on the method used, BDZs can begin to degrade when exposed to temperatures approaching 50°C, which is within the range commonly reached in Kuwait. Therefore, any samples or trace substances collected from a crime scene must be carefully handled and stored to avoid heat-related degradation from the time of collection until they reach the laboratory for analysis. Suspected drugs or powder should be collected while wearing gloves, placed in clean, properly labelled evidence

packaging, stored in an ice box, and transported under a documented chain of custody to preserve sample integrity prior to laboratory examination.

Table 2. 11 Summary of average peak areas for the various timepoints and temperatures.

Temp.(°C)	week	Peak Area for analyte				
4°C		Bromazepam	Clonazepam	Flunitrazepam	Alprazolam	Diazepam
	0	691642	755901	782140	476056	820621
	1	689856	732964	770534	469329	814482
	2	647912	738481	755812	464504	802586
	4	636696	695017	737848	462014	802005
	%loss	7.9	8.0	5.6	2.9	2.2
25°C	week	Bromazepam	Clonazepam	Flunitrazepam	Alprazolam	Diazepam
	0	699638	705901	762242	500799	819113
	1	687530	726769	757847	492646	812402
	2	677915	735191	737848	462014	795413
	4	636696	681129	733017	459532	767401
	%loss	8.9	3.5	3.8	8.2	6.3
37°C	week	Bromazepam	Clonazepam	Flunitrazepam	Alprazolam	Diazepam
	0	768919	705901	765510	492696	826170
	1	758269	718013	764182	476097	820905
	2	713541	705752	746065	462014	795413
	4	696696	659078	707848	458082	779962
	%loss	9.3	6.6	7.5	7.0	5.5

50°C	week	Bromazepam	Clonazepam	Flunitrazepam	Alprazolam	Diazepam
	0	666696	705901	754182	516513	841583
	1	642625	672879	737848	514660	817486
	2	633820	593390	727913	492056	795413
	4	601039	445632	670669	462014	794309
	%loss	9.8	36.8	11.0	10.5	5.6

Overlays of the linear representations of the peak areas from the HPLC runs for the 5 BDZs is presented below (Figure 12). This shows the fluctuation in the peak areas for each BDZ over the experiment duration and temperature range.

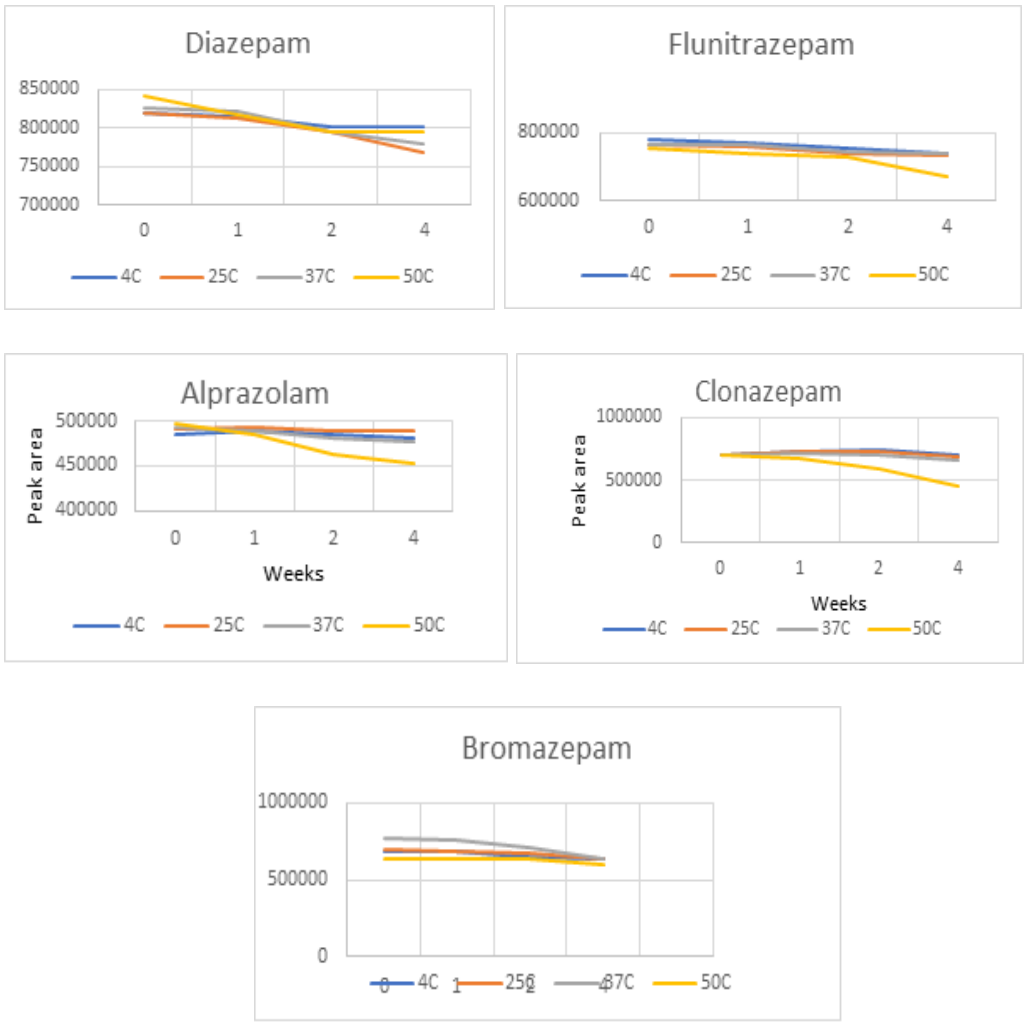


Figure 2. 12 Overlays of linear representations of peak areas from the HPLC under different temperatures.

2.7.1.2 Synthetic Urine samples

The detection of BDZ from biological samples can be challenging due to the complexity of the matrix in which the sample is in. Therefore, the protocol must be robust enough to overcome these challenges to limit interference from other components sample to the minimum.

The protocols for analysis of the BDZ samples were validated according to ICH guidelines. This guideline is also known as Q2(R1) Validation of Analytical Procedures.

2.7.1.3 Long Column HPLC Recovery from Synthesised Urine Samples

Samples for analysis were extracted using the liquid-liquid extraction method from synthesised urine samples made using Sigmatrix Urine Diluent obtained from Sigma -Aldrich and Sodium bicarbonate 1.5 M and PH 9.5 as buffer. 1 mL TERUMO syringes without needles with LLG-Syringe filters SPHEROS, PTFE, 0.22 µm Ø 13 mm, white, were used for liquid handling and filtration of the samples prior to analysis. Samples for the different concentrations were made up as shown in the table below (Table 12).

Table 2. 12 Synthesised urine samples used for HPLC recovery (Sample preparation and its concentration at different level).

	Urine	Sodium bicarbonate PH 9.5	Mix of 5 BDZs	MeOH
Control	200 µl	100 µl	No standard added	700 µl
1mg/ml	200 µl	100 µl	10 µl of 100 ppm	650 µl
2 mg/ml	200 µl	100 µl	20 µl of 100 ppm	600 µl
20 mg/ml	200 µl	100 µl	20 µl of 1000 ppm	600 µl
100 mg/ml	200 µl	100 µl	100 µl of 1000 ppm	200 µl

The appropriate amount of sample was carefully weighed into separate Eppendorf tubes respectively to which the urine diluent and buffer were then added and vortexed for 30 seconds. MeOH was then added, and the tubes were once again vortexed for 30 seconds then placed on a shaker for 10 minutes. The tubes were then centrifuged for 15 minutes at 130 RPM and the supernatant filtered off and transferred to a sample vail as analysed. Each sample was repeated three times. The summary of the results is presented below (Table2.13).

Table 2. 13 Results for recovery from urine samples.

Sample Name	Recovery level (concentration in mg/mL)							
	1 mg/mL		2 mg/mL		20 mg/mL		100 mg/mL	
	%RSD	%Rec	%RSD	%Rec	%RSD	%Rec	%RSD	%Rec
Bromazepam	5.637	101.634	6.056	88.857	4.627	94.928	2.445	90.361
Clonazepam	9.916	98.659	2.428	83.527	2.735	94.868	2.524	89.849
Alprazolam	3.849	100.417	4.552	97.511	2.967	95.102	2.184	105.676
Flunitrazepam	3.969	97.756	5.479	94.319	4.540	104.662	5.638	99.619
Diazepam	3.234	101.513	2.210	93.383	1.990	97.436	6.580	94.118

The percentage recovery is crucial for accurate quantification and validation of an analytical method. From the table the percentage recovery for each of the BDZ samples across the concentration range fell with the acceptable range, ± 20 % at every concentration for all samples, which is an indication that the method accurate, reproducible and therefore well suited for the analysis of BDZs from biological samples such as urine[38].

2.8 Conclusion

In this chapter, a method for the analysis of BDZs was developed and validated. The method was then used to conduct a stability-indicating assay on 5 representative BDZs selected because they are the most encountered BDZs in Kuwait. The results of the stability assay indicated that the samples were best stored in the refrigerator and used within 3 weeks of preparation because some degradation of the sample was detected during the analysis of the samples, 4 weeks after the preparation of the samples (t4 samples). The method was also utilised for a recovery study for BDZ samples extracted from sanitised urea. The recoveries obtained across all samples and concentrations fell within the acceptable range, indication that the method would be suitable for the analysis of urine samples for BDZs. Decided to optimise the run further to increase the throughput by reducing the run time. Switching to a shorter column could potentially reduce the run time by half.

Overall, the developed method demonstrated robustness, reliability, and suitability for routine BDZ analysis. Its successful application to stability and recovery studies highlights its potential use in forensic and clinical settings. From a forensic perspective, qualitative identification of BDZs is often of greater importance than quantitative determination, particularly in jurisdictions such as Kuwait where regulatory and medico-legal guidelines prioritise the presence or absence of the drug rather than its concentration. While pharmacokinetic data provide insight into drug disposition, pharmacodynamic effects are more directly relevant to impairment assessment. Consequently, qualitative analysis is sufficient to meet current forensic requirements in Kuwait. Nevertheless, the method retains the capability for quantitative application should future regulatory needs evolve.

Chapter 3 HPLC Short Column and LC-MS/MS method development and validation for most used benzodiazepine compounds in Kuwait

3.1 Introduction

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a powerful tool routinely applied to the identification of the components of samples in forensic investigations. Highly accurate mass analyser instrumentation is now widely available and direct methods for drug and metabolite analysis continue to replace traditional immunoassay methods.[1,2] The systematic toxicological analysis approach for dealing with drug-related analysis consists of two steps: primary drug screening using immunoassay techniques and confirmation using hyphenated chromatography techniques coupled with mass spectrometry identification and quantification.[3,4]

Following on from the study in the preceding chapter (Chapter 2) which discussed the successful development and validation a twenty-six-minute method for the detection, quantification and identification of the five most detected BZDs in Kuwait, it was which would double the throughput of the protocol. Shorter columns are also used as standard on HPLC-MS systems which would make a short column method suitable for use on both HPLC systems and benefit from the increased sensitivity afforded by HPLC-MS systems. In addition to the higher throughput and increased sensitivity, shorter run times require less solvents so are cheaper and more environmentally friendly[5]. HPLC-MS also offers the added advantage that it can provide approximate untargeted sample content identification based on the masses of the components of the sample as well as the retention times without the need for the use of standards[6].

3.2 Aim of study

The aim of this work in this chapter is to develop and validate short column HPLC and HPLC-MS methods suitable for the detection and quantification of five BDZ drugs, alprazolam, clonazepam, flunitrazepam, bromazepam and diazepam, and their metabolites that are commonly encountered in post-mortem toxicology, and to apply the developed methodology to the analysis of urine samples. This data will then be used to evaluate the feasibility of this method being used in the routine analysis of urine samples. These compounds were chosen since they are commonly detected during forensic investigations in Kuwait[7].

3.3 Materials and Methods

3.3.1 Solvents and chemicals

The solvents used for the studies were High-performance liquid chromatography (HPLC) grade. Acetonitrile (ACN) was purchased from either Fisher Scientific (Loughborough, UK), or Merck Life Science UK Limited**,** The Old Brickyard, New Rd, Gillingham, Dorset, United Kingdom. HPLC grade water was obtained from a Purite Select Ondeo system (Purite Limited, UK), or prepared using a MilliQ filter purchased from Millipore, Watford, UK. Syringe membrane filters (13 mm) were purchased from kinesis scientific expert, Cambridgeshire, UK. Nylon solvent filters (0.45 um) were used for solvent filtration and Water. AnalaR-grade formic acid (98%) was obtained from BDH Merck (Poole, UK). Chemicals, Formic acid and Sodium bicarbonate AR grade, and authentic standard stock solutions, Alprazolam, Bromazepam, Clonazepam, Clonazepam-D4, Diazepam and Flunitrazepam, HPLC grade methanol were obtained from Merck Life Science UK Limited, The Old Brickyard, New Road, Gillingham, Dorset, SP84XT, United Kingdom. Sigmatrix

Urine Diluent was obtained from Sigma -Aldrich UK Limited**,** The Old Brickyard, New Road, Gillingham, Dorset, United Kingdom.

3.3.2 Chromatographic analysis

HPLC (UV) analysis was performed on a Nexera LC-2400C HPLC (Shimadzu Corporation, Japan), fitted with either a Phenomenex C18 column (00F-4435-E0) 150 x 4.6 mm, 3µm (Length, ID and particle size. Analysis was performed using the Lab Solutions software, from Shimadzu.

The HPLC (UV) was set up with freshly prepared mobile phase and rinse/wash solutions required for the analysis installed on the appropriate lines. The autosampler needle and sample syringe were then flushed with the syringe wash solution (Methanol: Water, 1:1). The drain valve was opened, and the system was then initially flushed with mobile phases B (100%) followed by mobile phase A (100%) at a flow rate of 1.0 ml/min for 5 minutes for both mobile phases. The drain valve was then closed and the desired HPLC column was installed and conditioned with 50% of mobile phase B at a flow rate of 0.3 ml/min for 5 min then slowly brought to the appropriate starting gradient and flow rate for each run over a further 10 minutes. The operating pump pressure was continuously monitored to ensure that it did not exceed the maximum operating pressure for the machine. Chromatographic separations were performed on an ACE 3 C18 column from Merck, 150 x 4.6 mm, 3µm (Length, ID and particle size) utilising a linear gradient elution over 15 minutes, as shown below (Table 2.1) using the appropriate mobile phases and flow rates. While on the instrument, samples were kept in a vial tray which was set to a constant temperature of 4˚C to avoid any degradation of samples.

3.3.2.1 Analytical method for Short Column HPLC (UV):

Mobile Phase A : 0.1% v/v formic acid in 5% acetonitrile

Mobile Phase B : 0.1% v/v formic acid in 95% acetonitrile

Column : ACE 3, C18, 150 x 4.6 mm, 3µm (Length, ID and particle size)

Flow rate : 0.6 mL/min

Detector : UV and PDA

Detection Wavelength : 254 nm (200-400 nm scan for PDA)

Injection volume : 10 µL

Column Oven Temperature : 50 °C

Run time : 15 minutes (gradient elution)

Diluent : Methanol

Table 3. 1 Gradient Composition

Time in Min	% MP B Composition
0.01	30
1.00	30
10.00	100
12.00	100
12.10	30
15.00	30

3.3.3 LC-MS Analysis

LC-MS Analysis was conducted using a Shimadzu, LC-2040C, Nexera-i LCMS-8050 with a tandem Triple Quadrupole (TQ-8050) mass spectrometer utilizing Electrospray ionization (TQ-ESI) Mass Spectrometer (Shimadzu Corporation, Japan), operated in

ESI positive mode and multiple reactions monitoring (MRM) scan mode with analysis carried out using Lab Solutions software from Shimadzu.

3.3.3.1 LC-MS Setup

The mobile phase solutions for were freshly prepared with appropriate buffers and stored at room temperature for up to 48 h. LC-MS setup was performed by equilibration of the system with the appropriate column and mobile phase for the desired analysis. The quality of the data obtained from these experiments were ascertained utilising standard sample solutions to assess parameters such as peak width, height, retention time, and chromatographic resolution because the accuracy of the deductions that can be made from any study is dependent on the quality of data acquired by the instrument. The system was deemed to be suitable for use if the relative standard deviations (RSDs) of these parameters were no more than 20% from those expected for each of the standards. The HPLC system was checked for leaks if the retention times obtained at the beginning and at the end of a given sequence was shifted by more than 0.3 min. The MS accuracy was tested using standard calibration samples. Peaks from the calibrants were cross checked to ensure that mass deviations were within 5 ppm. The electrospray ionisation (ESI) interface was operated in positive mode with interface temperature of 300°C and desolvation temperature of 526°C.

3.3.4 LCMS Method Settings

Mobile Phase A : 0.1% v/v FA in water (pH 2.5)
Mobile Phase B : 0.1% v/v Acetonitrile
Column : ACE 3, C18, 150 x 4.6 mm, 3µm (Length, ID and particle size)

Flow rate : 1 mL/min

Detector : UV

Detection Wavelength : 254 nm for UV

Injection volume : 20 µL

Column Oven Temperature : 25 °C

Run time : 15 minutes (gradient elution)

Diluent : Methanol

Table 3. 2 Gradient Composition

	Time (mins)	**% MP B Composition**
1	0.01	30
2	1.00	30
3	10.00	100
4	12.00	100
5	12.10	30
6	15.00	30

3.3.5 Triple Quadrupole MS parameters:

Mode used: Positive.

MRM : Alprazolam (Precursor m/z: 309.10, Product m/z: 281.15, 205.15, CE: -26.0, -45.0)

Bromazepam (Precursor *m/z*: 317.95, Product m/z: 209.05, 182.05, CE: -28.0, -33.0)

Clonazepam (Precursor *m/z*: 316.05, Product m/z: 270.10, 214.00, CE: -25.0, -37.0)

Diazepam (Precursor *m/z*: 285.10, Product m/z: 193.15, 154.10, CE: -30.0, -27.0)

Flunitrazepam (Precursor m/z: 314.10, Product m/z: 268.10, 239.15, CE: -25.0, -36.0)

Interface	: Electrospray Ionisation
Interface Temperature	: 300 °C
Desolvation Temperature	: 526 °C
Heating Gas Flow	: 10 L/min
Drying Gas Flow	: 10 L/min

The MRM was optimized using the optimization settings of the Lab solution software.

3.4 Experimental.

3.4.1 Standard solutions.

Standard solutions for the study were prepared as follows:

3.4.1.1 Preparation of individual standard stock solution: (1000 µg/mL)

10 mg each of the BZDs Alprazolam, Bromazepam, Clonazepam, Diazepam and Flunitrazepam was accurately weighed and transferred individually into a separate 10 mL volumetric flask respectively. Methanol (5 mL) was then added to each of the volumetric flasks to dissolve the samples by gentle swirling. The volume was then made up to the mark with methanol.

3.4.1.2 Preparation of individual intermediate standard stock solution: (100 µg/mL)

An aliquot (1 mL) of from each of the Alprazolam, Bromazepam, Clonazepam, Diazepam and Flunitrazepam standard stock solutions were respectively transferred

individually *via* pipette to a separate 10 mL volumetric flask. Methanol (5 mL) was added to each volumetric flask and mixed well by swirling. The volume was then made up to the mark with methanol. Triplicate mixtures of the 5 BZDs were then prepared for each test temperature.

3.4.1.3 Preparation of calibration curves:

The standard solutions were further diluted with methanol to achieve the final concentrations of 2, 5, 10, 50, 100, 200 and 300 ng/mL concentrations using methanol as the diluent in each case. Areas under the curves AUCs were plotted for the corresponding concentrations and regression equations computed.

3.4.1.4 System Suitability Criteria:

The system is suitable if, the Correlation coefficient (r^2) is more than 0.99 for all peaks of interest from calibration curve[8]

The analysis was done by using Lab Solutions software, from Shimadzu.

3.5 Results and discussion

3.5.1 HPLC (UV) Method development and optimization

The results obtained from the analysis of the samples are summarised below (Table 3.4)

Table 3. 3 Results of HPLC Development.

Name of the Component	Retention Time (min)	Peak Area	Tailing Factor	Capacity Factor (K')	Resolution	Number of theoretical plates
Bromazepam	6.097	366922	0.998	1.439	--	34375
Clonazepam	7.467	400443	0.985	1.987	10.643	55698
Alprazolam	7.731	259006	1.099	2.092	2.127	64042
Flunitrazepam	8.036	504656	1.007	2.215	2.429	60794
Diazepam	9.178	534876	1.107	2.671	8.799	80151

3.5.2 Results of HPLC Validation:

These protocols for analysis of the BDZ samples were validated according to ICH guidelines.[9] These guidelines are also known as Q2(R1) Validation of Analytical Procedures.

Table 3. 4 Specificity.

Name of the Component	Peak Area	Retention Time (min)	Peak Purity (3-point Similarity)
Methanol Blank Solution			
Bromazepam	Not Detected	Not Detected3	Not Applicable
Clonazepam			
Alprazolam			
Flunitrazepam			
Diazepam			
Individual Solution at 10 µg/mL			
Bromazepam	362436	6.235	0.9990
Clonazepam	404622	7.573	0.9998
Alprazolam	249333	7.853	0.9999
Flunitrazepam	512671	8.148	0.9998
Diazepam	556312	9.298	0.9999
Mix Standard Solution at 10 µg/mL			
Bromazepam	385009	6.204	0.9999
Clonazepam	398449	7.549	0.9999
Alprazolam	252196	7.819	0.9999
Flunitrazepam	500326	8.120	0.9999
Diazepam	564165	9.264	0.9999

The specificity of the method was assessed by the analysis of laboratory prepared BDZ standard solutions individually and as mixtures at 10 µg/mL concentrations. The precision of the protocol was tested over a range of concentrations for each BDZ in triplicates.

Table 3. 5 Precision (repeatability).

Name of the Component	% RSD
Bromazepam	0.08
Clonazepam	0.13
Alprazolam	0.16
Flunitrazepam	0.11
Diazepam	0.09

Table 3. 6 Intermediate Precision.

Name of the Component	% RSD
Bromazepam	0.22
Clonazepam	0.16
Alprazolam	0.17
Flunitrazepam	0.21
Diazepam	1.73

Repeatability and intermediate precision were assessed at several concentrations of standard BDZ solutions also in triplicate. The robustness of the protocol was assessed by the reliability of the analysis after small changes had been made to the experimental conditions. The parameters changed included oven temperature (± 5 °C), formic acid concentration (±1 mM) and flow rate (±0.1 mL/min) over several concentrations of standard BDZ solutions in triplicate changing only one parameter at a time.

Table 3. 7 LOQ and LOD.

Name of the Component	LOQ (2 µg/mL)		LOD (0.6 µg/mL)
	% RSD (n=6)	Mean S/N (n=6)	Mean S/N (n=3)
Bromazepam	0.17	646.15	226.74
Clonazepam	0.23	855.39	232.97
Alprazolam	0.25	564.91	169.00
Flunitrazepam	0.21	1029.16	283.73
Diazepam	0.17	1064.96	387.57

The LOQ and LOD were determined utilising the visual evaluation approach. The limit of quantification is the minimum concentration at which analytes can be reliably quantified with acceptable accuracy (%Recovery should be within 80-120%) and precision (%RSD should be within 20%). The values obtained for each BDZ samples is displayed in the table above, (Table 3.7), with limits of quantifications (LOQ (2 µg/mL)) and limits of detection (LOD (0.6 µg/mL)) were respectively.

Table 3. 8 Linearity.

Name of the Component	Correlation Coefficient (r^2)
Bromazepam	0.9995
Clonazepam	0.9998
Alprazolam	0.9994
Flunitrazepam	0.9996
Diazepam	0.9991

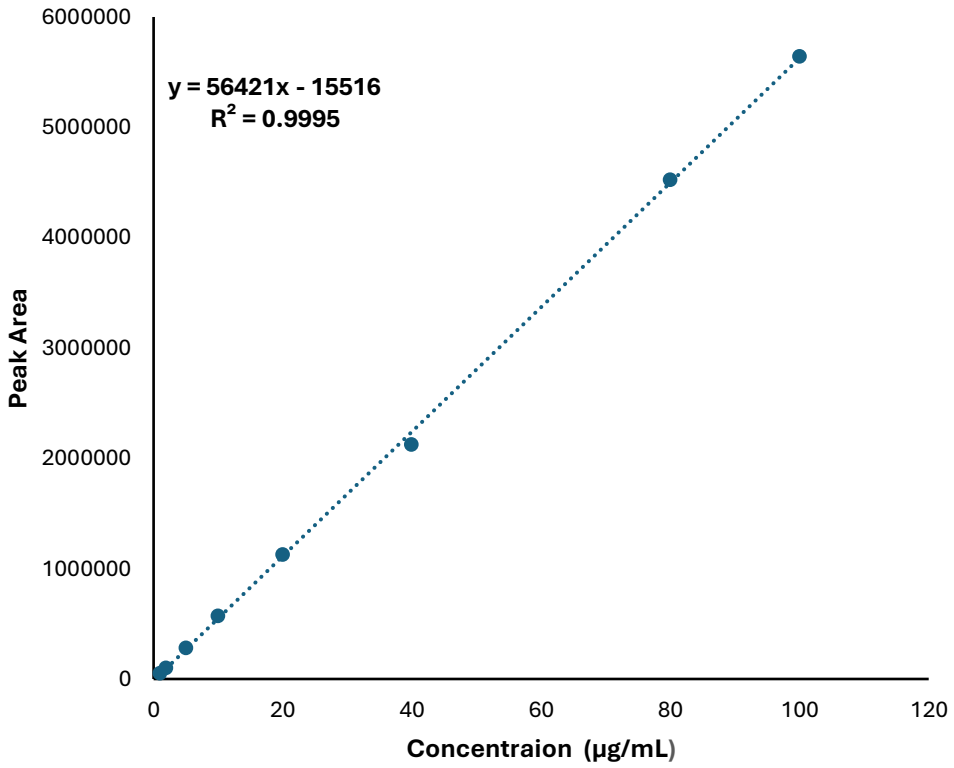


Figure3. 1 Calibration curve for Bromazepam

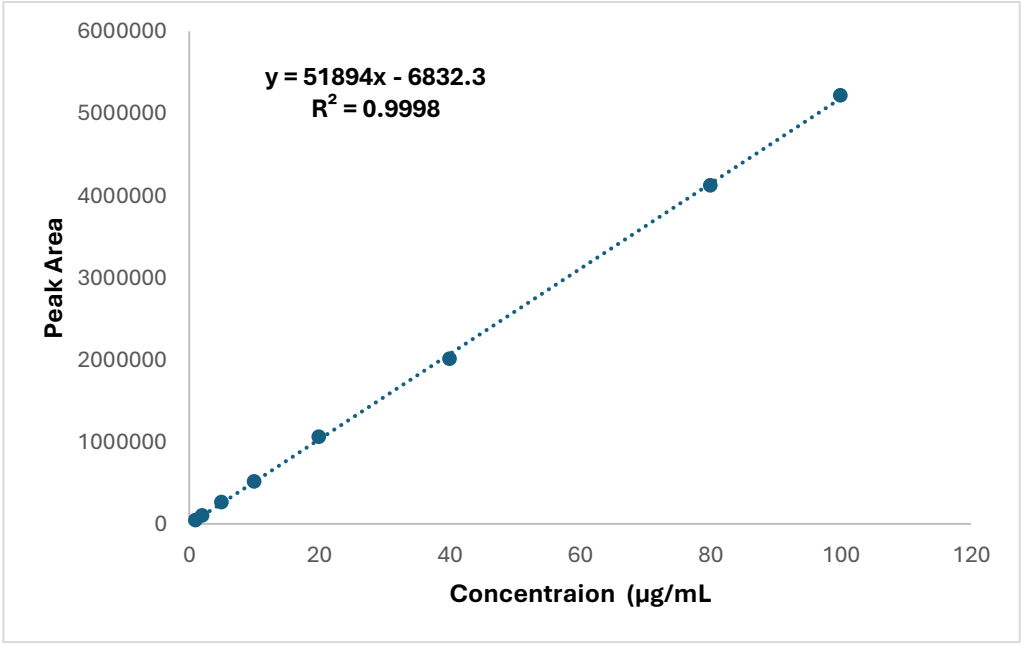


Figure3. 2 Calibration curve for Clonazepam

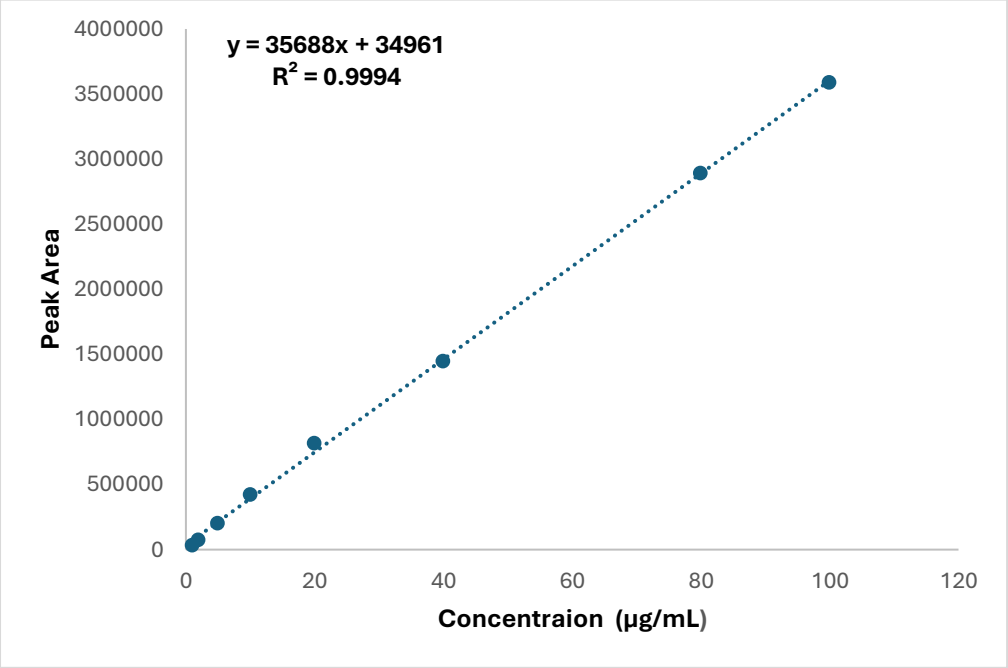


Figure3. 3 Calibration curve for Alprazolam

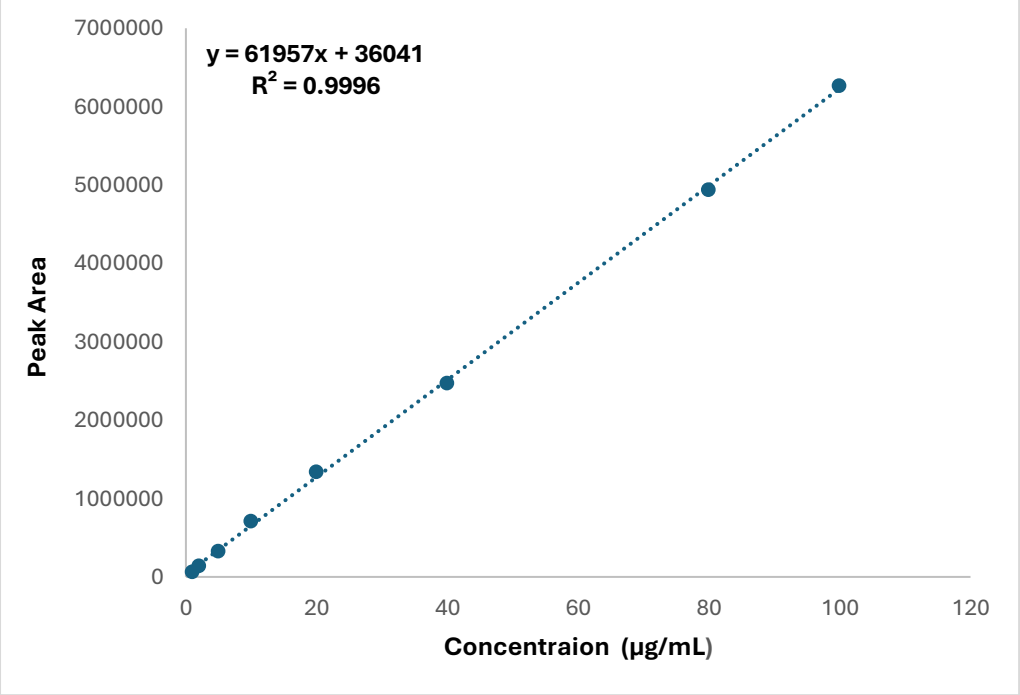


Figure3. 4 Calibration curve for Flunitrazepam

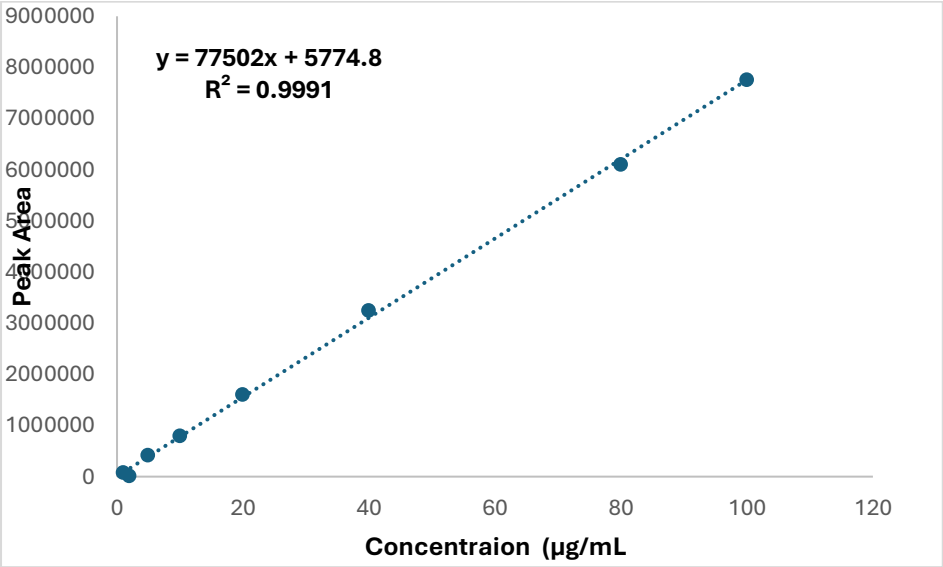


Figure3. 5 Calibration curve for Diazepam

Linearity was evaluated over a series of concentrations of the samples, and a calibration curve was plotted to determine the mean peak area ratio of standard versus its concentration. When the r^2 value is ≥ 0.99 the method is deemed to be linear which was observed for all analytes.

Table 3. 9 Robustness.

Name of the Component	%RSD					
	Flow Rate (ml/min)		Col Temp (°C)		Wavelength (nm)	
	0.55	0.65	48	52	252	256
Bromazepam	0.30	0.32	0.42	0.14	0.37	0.14
Clonazepam	0.89	0.74	1.22	0.19	0.33	0.18
Alprazolam	1.08	1.02	1.67	0.24	0.32	0.14
Flunitrazepam	0.29	0.10	0.23	0.23	0.33	0.16
Diazepam	0.19	0.15	0.21	0.12	0.33	0.16

Table 3. 10 System Suitability.

Name of the Component	Tailing Factor	Resolution
Bromazepam	1.100	--
Clonazepam	1.038	10.18
Alprazolam	1.145	2.017
Flunitrazepam	1.053	2.328
Diazepam	1.127	8.375

3.5.2.1 Chromatograms from HPLC:

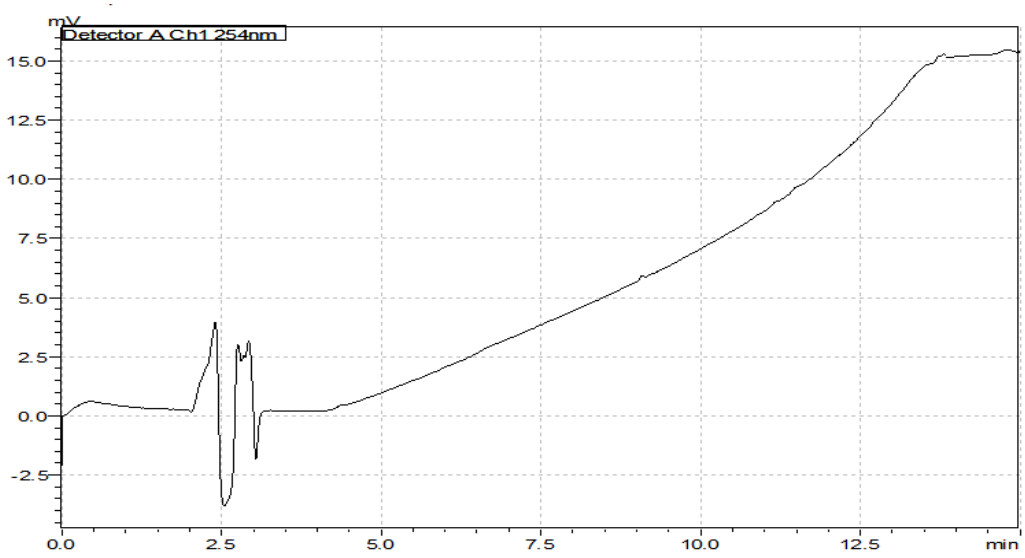


Figure3. 6 Chromatogram for Methanol Blank.

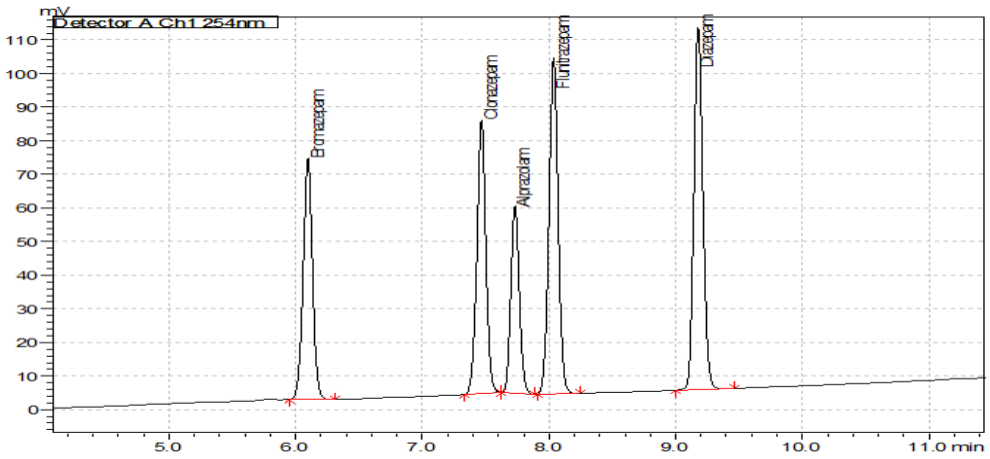


Figure3. 7 Chromatogram for Mix Standard Solution at 10µg/mL.

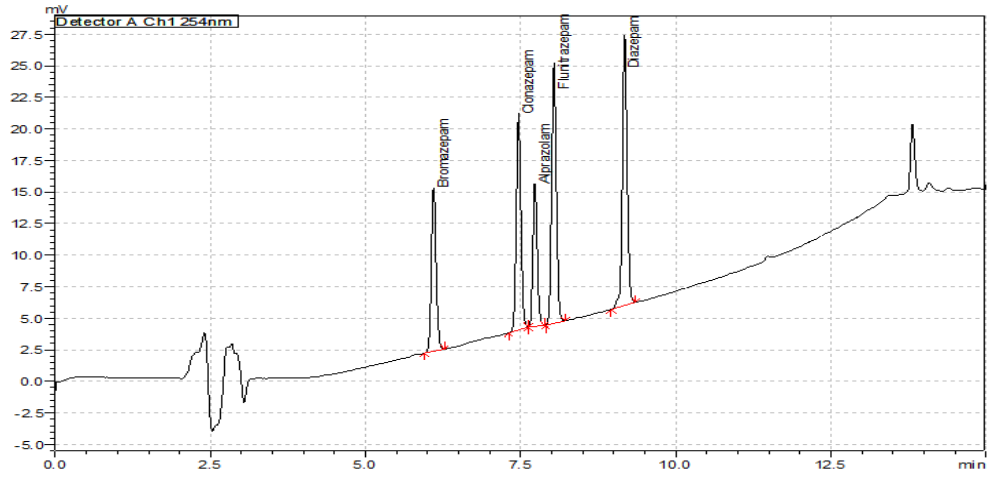


Figure3. 8 Chromatogram for LOQ.

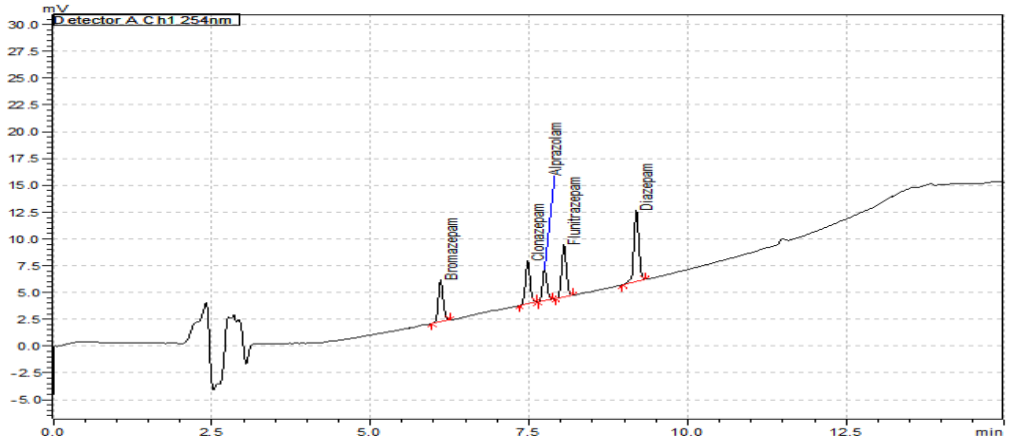


Figure3. 9 Chromatogram for LOD.

The chromatographs show noticeably clear separation between the BDZs of interest.

As LCMS is more sensitive than HPLC (UV), validation was repeated on the LCMS at lower concentrations (2, 5, 10, 50, 100, 200, 300, and 500 ng/mL). The results are presented below.

3.5.3 Results of LCMS Validation:

The protocols use MRM mode in the MS settings to detect only ions from the target analyte, minimizing interference from other sample components.

Table 3. 11 Specificity

Name of the Component	Peak Area	Retention Time (min)
Methanol Blank Solution		
Bromazepam	Not Detected	Not Detected
Clonazepam-D4		
Clonazepam		
Alprazolam		
Flunitrazepam		
Diazepam	Not Detected	
Individual Solution at 100 ng/Ml		
Bromazepam	10320388	6.172
Clonazepam-D4	79968903	7.520
Clonazepam	18346351	7.558
Alprazolam	7125895	7.818
Flunitrazepam	23375553	8.120
Diazepam	16025983	9.247

The method was deemed to be selective for the quantification of the BDZ samples because no interference was observed at the retention times for each of the BDZ samples and the internal standard, bromazepam, clonazepam-d4, clonazepam, alprazolam, flunitrazepam and diazepam in the Sigmatrix urine diluent when

compared to the blank. All results were found to be within the acceptable limit of not more than 20% of the LOQ in the blank Sigmatrix urine diluent for the samples.

Table 3. 12 Precision (repeatability).

Name of the Component	% RSD
Bromazepam	0.82
Clonazepam	1.34
Alprazolam	1.00
Flunitrazepam	0.95
Diazepam	0.82

Table 3. 13 Intermediate Precision.

Name of the Component	% RSD
Bromazepam	1.85
Clonazepam	3.05
Alprazolam	2.38
Flunitrazepam	2.43
Diazepam	1.21

Table 3. 14 LOQ and LOD

Name of the Component	LOQ (6 ng/mL)	LOD (2 ng/mL)
	% RSD	% RSD
Bromazepam	2.27	1.85
Clonazepam	1.20	3.05
Alprazolam	1.98	2.38
Flunitrazepam	1.504	2.43
Diazepam	0.122	1.21

The LOQ (n=6) was determined to be 6.0 ng/mL and the LOD (n=3) to be 2.0 ng/mL. The %RSD for all samples was within the acceptable range (no more than 20.0%).

Based on the results it was concluded that LOQ along with LOD was established at 6.0 ng/mL and 2.0 ng/mL respectively.

Table 3. 15 Linearity.

Name of the Component	Correlation Coefficient (r^2)
Bromazepam	0.9995
Clonazepam	0.9995
Alprazolam	0.9993
Flunitrazepam	0.9996
Diazepam	0.9997

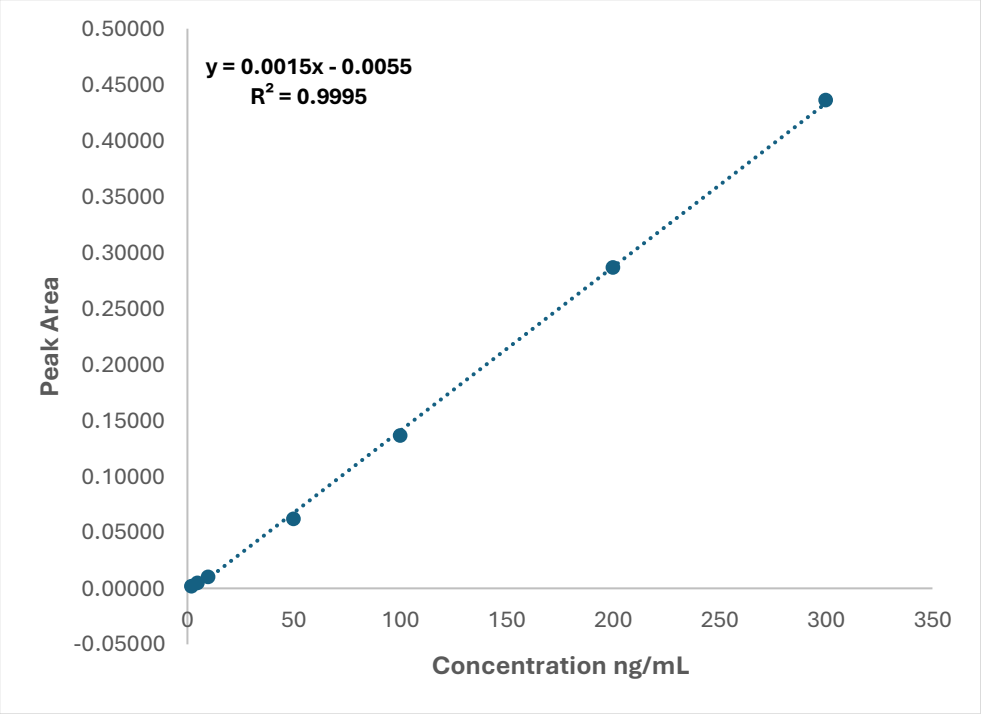


Figure3. 10 Calibration curve for Bromazepam

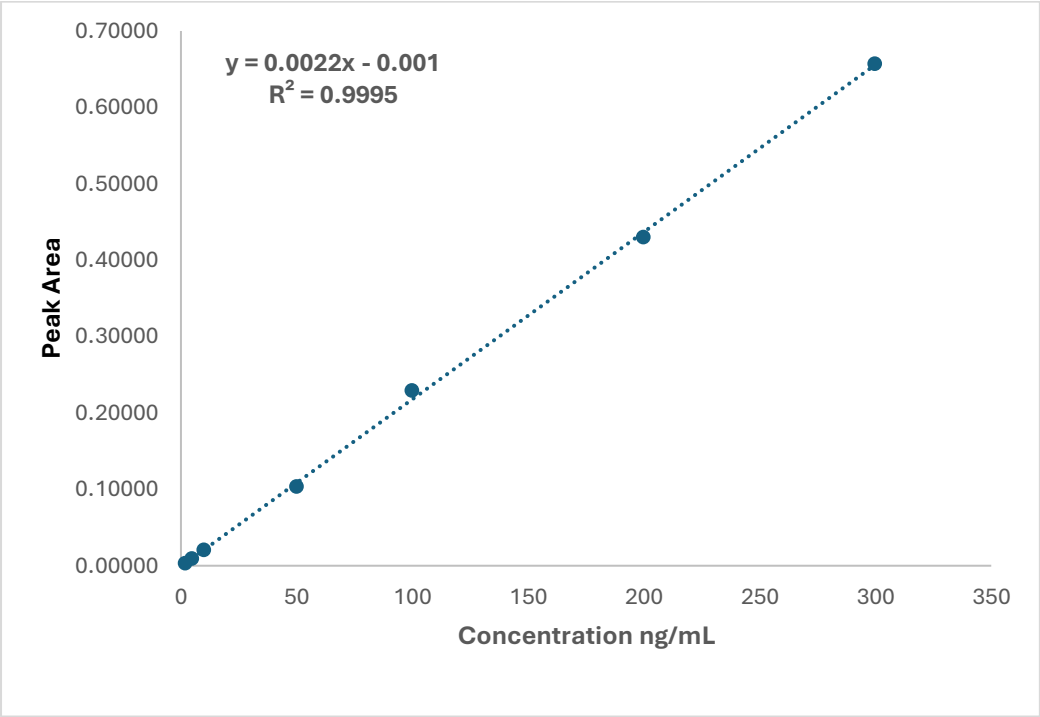


Figure3. 11 Calibration curve for Clonazepam

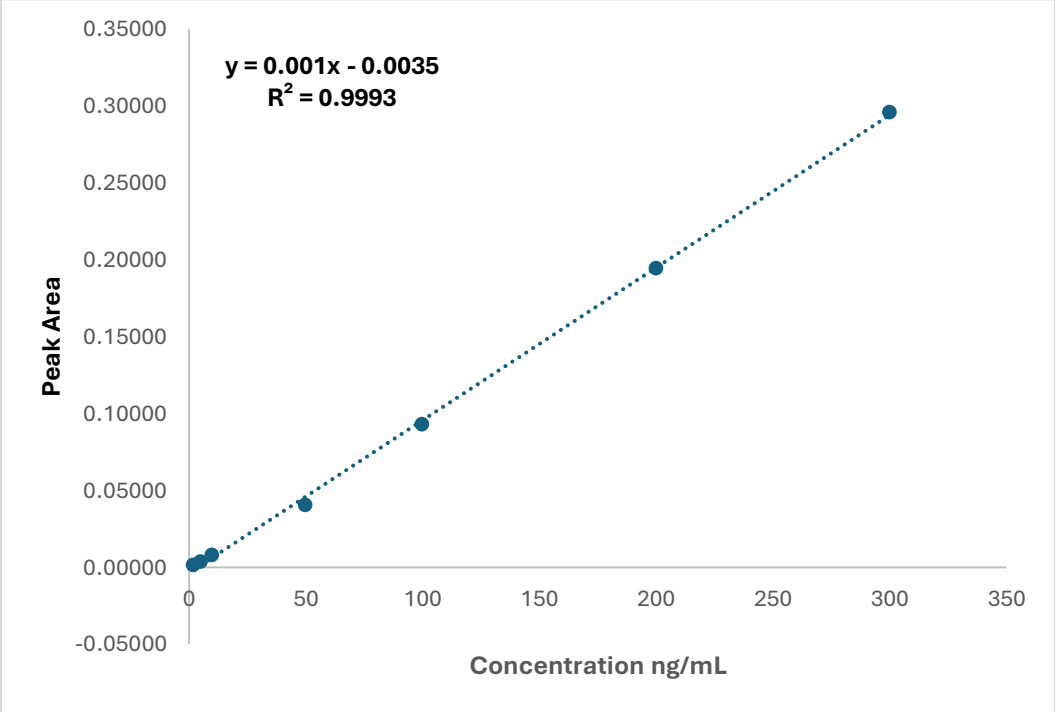


Figure3. 12 Calibration curve for Alprazolam

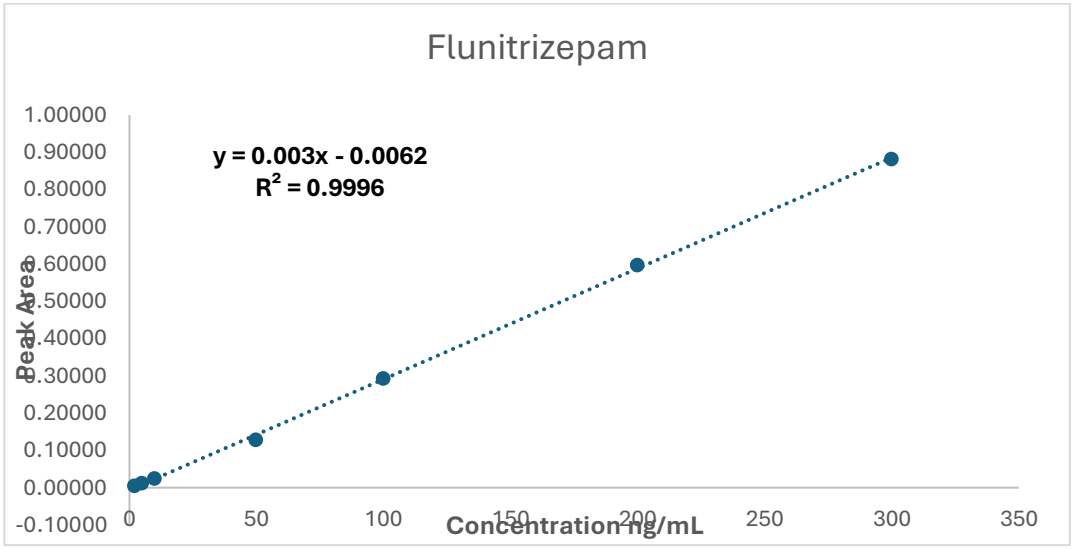


Figure3. 13 Calibration curve for Flunitrazepam

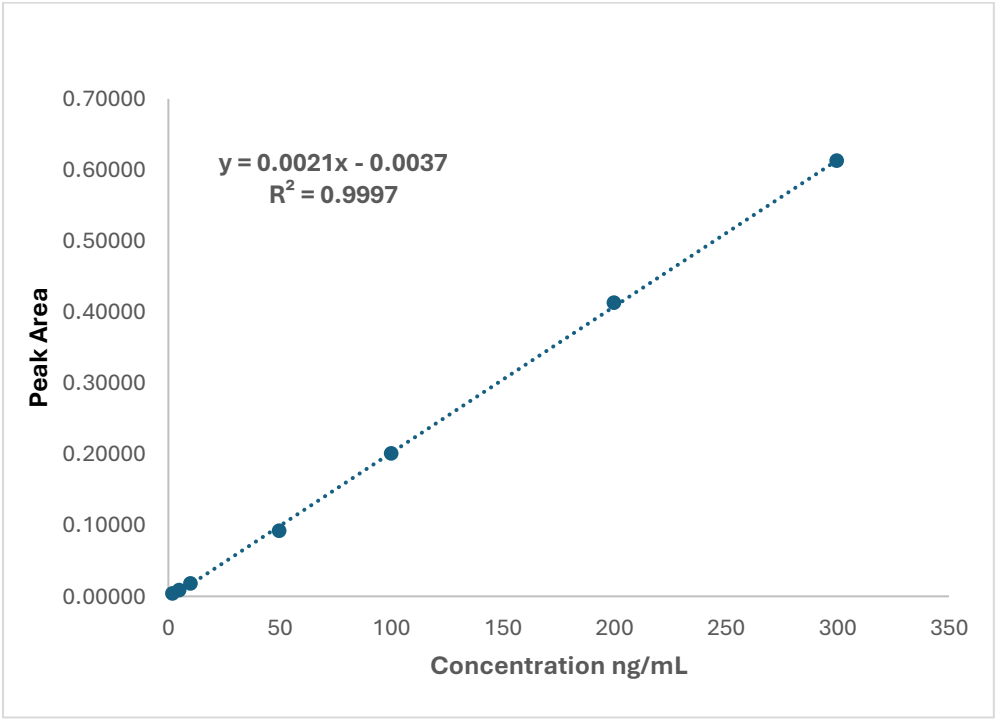


Figure3. 14 Calibration curve for Diazepam

The linearity was determined at n=3 for each level over the range of concentrations (2.0–300.0 ng/mL) for bromazepam, clonazepam, alprazolam, flunitrazepam,

diazepam using clonazepam-d4 as internal standard at a concentration of 100.0 µg/mL. A linear fit was observed for the calibration curve when the mean peak area ratio of standards was plotted against its concentration. The r^2 values obtained in all cases was found to lie well within the limit of $r^2 \geq 0.99$, therefore it is considered as linear over the calibration range.

Table 3. 16 Robustness.

Name of the Component	%RSD			
	Flow Rate (ml/min)		Col Temp (°C)	
	0.55	**0.65**	**48**	**52**
Bromazepam	1.30	1.36	1.86	2.42
Clonazepam	1.88	1.79	2.35	1.66
Alprazolam	1.44	1.75	2.28	1.77
Flunitrazepam	1.27	1.21	2.27	1.98
Diazepam	1.69	1.60	1.91	1.27

Table 3. 17 System Suitability.

Name of the Component	Correlation Coefficient (r^2)
Bromazepam	0.9995
Clonazepam	0.9995
Alprazolam	0.9993
Flunitrazepam	0.9996
Diazepam	0.9997

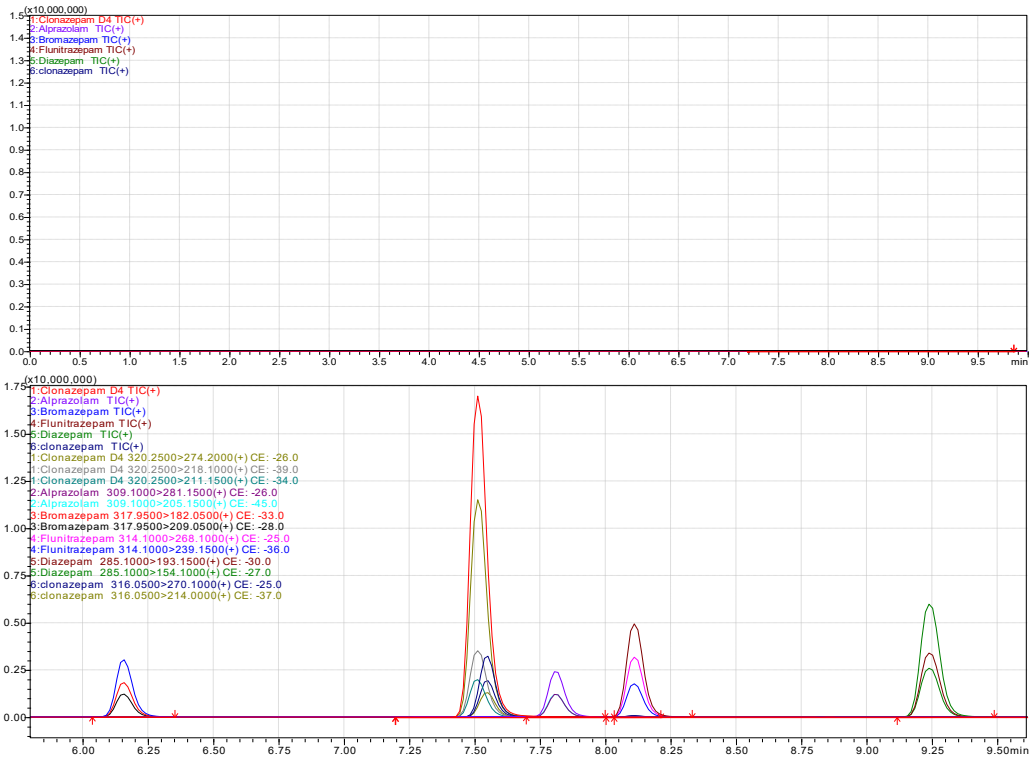


Figure3. 15 MeOH Blank (top), MIX STD 100 ng/mL (bottom).

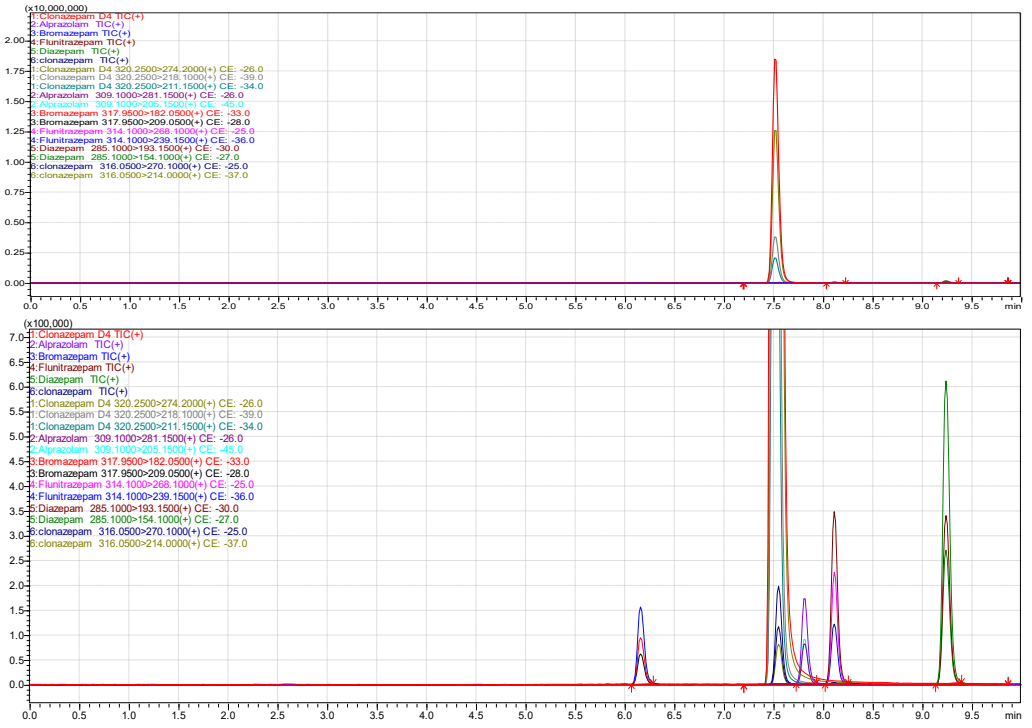


Figure3. 16 LOD 2ng/ml full view (top) zoom (Bottom).

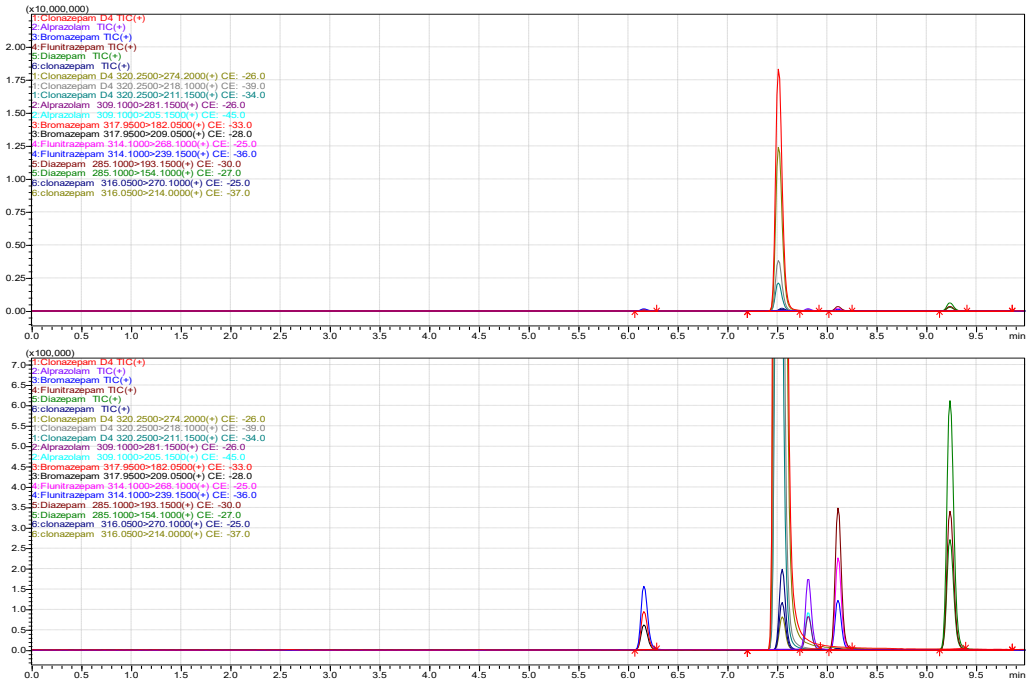


Figure3. 17 LOQ 6 ng/mL full view (top) zoom (bottom).

Clear baseline separation was observed between the BDZs test samples although the peaks for the samples were more closely bunched together and eluted over a shorter period of time using this method compared to the isocratic method developed in Chapter 2, (15 minutes v 26 minutes), with the limits of quantification at 6 ng/mL and detection at 2 ng/mL in this study.

The protocol must once again be robust enough to overcome the challenges associated with the detection of BDZ from biological samples due to the complexity of the matrix in which the sample is in therefore, for the successful analysis of biological samples, proper sample preparation is essential to separate the target analytes from the rest of the sample matrix. A recovery assay was therefore conducted to determine the extraction of the test sample from synthesized urine made using (Sigmatrix Urine Diluent) ordered from Sigma-Aldrich.

3.5.4 Recovery for HPLC

Samples were extracted using a liquid-liquid extraction protocol from synthesised urine samples made using Sigmatrix Urine Diluent obtained from Sigma -Aldrich and Sodium bicarbonate 1.5 M (PH 9.5) as buffer for the method used in the study (used in chapter 2). 1 mL TERUMO syringes without needles with LLG-Syringe filters SPHEROS, PTFE, 0.22 µm Ø 13 mm, white was used for liquid handling and filtration of the samples before analysis. Samples for the different concentrations were made up as shown in the table below for three times (Table 3.18).

Table 3. 18 Synthesized urine samples used for HPLC recovery (Sample preparation and its concentration at different level).

Concentration (mg/mL)	Urine	Sodium bicarbonate PH 9.5	Mix of 5 BENO	MeOH
Control	200 µl	100 µl	No standard added	700 µl
1mg/ml	200 µl	100 µl	10 µl of 100 ppm	650 µl
2 mg/ml	200 µl	100 µl	20 µl of 100 ppm	600 µl
20 mg/ml	200 µl	100 µl	20 µl of 1000 ppm	600 µl
100 mg/ml	200 µl	100 µl	100 µl of 1000 ppm	200 µl

The appropriate amount of sample was carefully weighed into separate Eppendorf tubes to which urine diluent and buffer were then added and vortexed for 30 seconds. MeOH was then added, and the tubes were then vortexed again for 30 seconds then placed on a shaker for 10 minutes. The tubes were then centrifuged for 15 minutes at 130 RPM and the supernatant filtered off and transferred to a sample vail as analysed. The summary of the results is presented below (Table 3.19).

Table 3. 19 Sample recovery results for HPLC validation.

Sample Name	Concentration (mg/mL)							
	1 mg/mL		2 mg/mL		20 mg/mL		100 mg/mL	
	%RSD	%Rec	%RSD	%Rec	%RSD	%Rec	%RSD	%Rec
Bromazepam	4.962	92.446	2.806	106.000	4.687	110.000	10.195	108.000
Clonazepam	4.672	85.363	1.735	94.896	0.019	100.925	1.835	101.125
Alprazolam	3.190	113.042	3.713	99.523	0.792	109.726	0.463	96.390
Flunitrazepam	6.737	82.306	6.016	98.273	2.598	89.063	0.826	83.924
Diazepam	8.183	98.538	5.628	97.501	1.110	103.343	1.882	103.300

3.5.5 Recovery for LCMS

A similar extraction protocol was used here as for the HPLC study above but with one modification. Clonazepam -D4 100 µg/mL in 1 mL Methanol obtained from Merck was used as internal standard (Table 3.20). Samples were prepared for three times individually.

Table 3. 20 Sample makeup for HPLC-MS recovery.

Concentration (ng/mL)	Urine	Sodium bicarbonate PH 9.5	Internal standard (I.S)	Mix of 5 BENO	MeOH
Control	200 µl	100 µl	Not added	Not added	700 µl
Control + (I.S)	200 µl	100 µl	25 µl	Not added	675 µl
2ng/ml	200 µl	100 µl	25 µl	20 µl of 0.1 ppm	655 µl
6ng/ml	200 µl	100 µl	25 µl	60 µl of 0.1 ppm	615 µl
100ng/ml	200 µl	100 µl	25 µl	10 µl of 10 ppm	665 µl
200ng/ml	200 µl	100 µl	25 µl	20 µl of 10 ppm	655 µl

The summary of the results is presented below (Table 3.21).

Table 3. 21 Sample recovery results for HPLC-MS validation.

Sample Name	Concentration (ng/mL)							
	2 ng/mL		6 ng/mL		100 ng/mL		200 ng/mL	
	%RSD	%Rec	%RSD	%Rec	%RSD	%Rec	%RSD	%Rec
Bromazepam	3.372	118.188	3.338	100.870	1.322	112.253	3.425	107.795
Clonazepam	6.676	111.044	5.252	104.750	1.422	94.961	1.363	93.734
Alprazolam	4.193	98.156	3.545	96.520	1.387	105.240	2.400	103.905
Flunitrazepam	9.459	100.249	6.529	107.440	6.825	82.356	0.821	86.120
Diazepam	7.562	104.655	6.277	98.591	2.305	105.282	2.149	105.407

In both studies, the sample recovery percentages were excellent, above 80% and below 120% for all concentrations, indicating that in both studies the extraction protocol and analysis methods are suitable for use with real human urine samples are suitable for the analysis of BZDs from urine samples[10].

3.5.6 Application to Urine Samples from Kuwait.

The optimised LCMS method was used to analyse urine samples collected from individuals in Kuwait suspected of drug use. The urine samples were received from the toxicology department of the general department of criminal evidence in Kuwait. rine was collected in sterile containers, aliquoted into screw-cap cryovials, frozen at −20 °C, and transported to the UK using triple packaging in insulated containers with dry ice to preserve sample integrity prior to analysis. A total of 48 urine samples collected from individuals under investigation for suspected drug abuse violations in 2022 were analysed in this study.

3.5.6.1 Declarations

Ethics approval and consent to participate

Ethical approval for the collection and analysis of the samples has been granted by the Ministry of Justice and Ministry of Interior Ethical Committee. Permission to use samples and data was obtained from the General Department of Criminal Evidence, Ministry of Interior.

Table 3. 22 Kuwait urine samples 5 Benzodiazepines

Ser	Sample no	Bromazepam	Clonazepam	Alprazolam	Fluntrizepam	Diazepam
		Concentration (ng/mL)				
1	sam46	5.474845681	1.225145854	6.06972381	-	2.17094975
2	sam47	5.488592447	2.242702258	3.93264726	-	3.94599353
3	sam48	4.943587257	0.464981775	4.52532057	-	2.28702387
4	sam49	7.251550396	-	5.7366481	-	2.51024332
5	sam50	5.021476216	0.62275878	5.3685604	-	2.06240053
6	sam 51	5.26955791	0.685750536	5.25199502	-	3.36626809
7	sam52	5.058254472	0.853199365	4.56585405		2.81308914
8	sam53	5.320460601	1.078158211	12.5619382	2.27985678	2.71649212
9	sam54	5.02512584	1.13831931	4.73156681		2.4004615
10	sam55	-	-	-	-	-
11	sam56	5.014152755	1.245011063	4.45690395	3.69550505	6.32656021
12	sam57	5.080518488	0.572539925	5.14893198	-	4.91936095
13	sam58	5.364581748	0.902567734	5.58564902	-	1.92692984
14	sam59	9.729948557	2.640923235	4.55940453	-	1.442678
15	sam60	5.018289874	1.039559002	5.33406209	-	1.99268262
16	sam61	5.15445425	0.985180847	5.56709069	-	1.9981417
17	sam62	6.266682788	0.985976561	4.93186973	-	3.40565977
18	sam63	6.10289242	1.201033594	6.25372572	-	2.43840602
19	sam64	4.954514333	9.165042818	4.63335557	5.45243868	13.622754
20	sam65	6.090283249	10.07875185	5.91304053	6.00088538	12.7134586
21	sam66	5.287761799	0.578650666	4.95977943	-	9.85225126
22	sam67	6.717325561	0.956642598	4.52803856	-	2.23463811
23	sam68	-	-	-	-	-
24	sam 69	5.784751644	1.206630576	6.04323612	-	12.3480508

25	sam 70	5.198501951	1.987736159	6.43980818	3.01395084	-
26	sam71	4.944103436	-	5.20733315	-	14.0140866
27	sam 72	6.929310586	0.725080811	5.99353168	2.94294096	3.27990604
28	sam 73	6.037095747	1.958470519	2.26168665	-	5.88135812
29	sam74	4.926940095	0.59097887	4.49886481	-	5.22069532
30	sam75	5.144737459	1.281035475	2.54733396	-	7.73725867
31	sam 76	5.188513607	0.639485219	4.37706555	-	8.41444333
32	sam77	5.260628097	1.207913174	233.7426	-	3.14505542
34	sam 78	5.024785608	0.587264606	4.72090042	2.59889733	2.30151952
35	sam79	5.148431014	0.547914265	5.21954687	-	1.9524503
36	sam 80	5.263714409	0.848881602	5.05447473	-	2.02279241
37	sam 81	5.143718637	0.751870923	6.18304255	-	2.13175723
38	sam82	4.949702358	0.72748192	99.796657	-	2.4242033
39	sam 83	4.97113644	0.609715607	5.98170375	-	2.21135285
40	sam 84	5.186444003	0.521288637	6.2792338	-	2.17580757
41	sam85	4.933658081	1.125869808	2.25145663	-	2.73989072
42	sam86	5.248128344	0.81194539	1.87352941	-	2.79062741
43	sam 87	-	-	-	-	-
44	sam 88	5.0336191	8.326140417	4.67159204	-	4.06633923
45	sam 89	8.27214832	2.233784527	4.7494235	-	11.3568204
	sam 90	5.012529142	-	5.05457859	-	10.4125472
	sam 91	5.038274972	0.783754317	4.51232584	-	5.62423689
	sam 92	4.910068524	0.794402842	178.995605	-	2.49106679

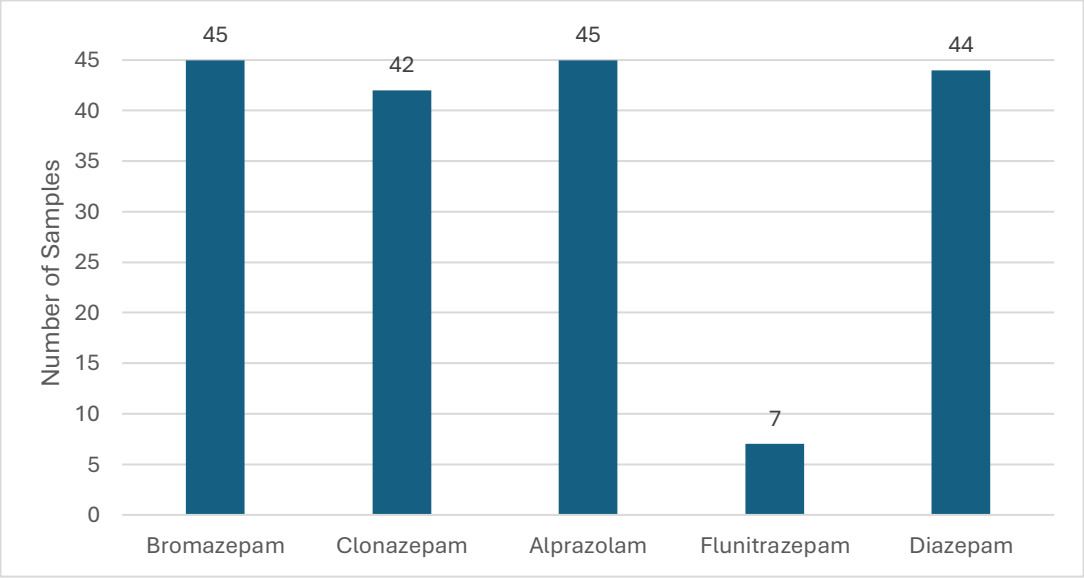


Figure3. 18 Analysis of urine samples from Kuwait showing the occurrence of each BDZ and combinations.

3.6 Discussion

The positive drug identification rate was 93.7%. Only 3 of the samples were found to contain none of the drugs being tested for.

In all other samples a mixture of BDZs were detected. Bromazepam and Alprazolam were the most detected BDZs closely followed by Diazepam and Clonazepam. This shows that illicit drug users tend to take a cocktail of drugs probably depending on availability rather than stick to a favourite or preferred drug. Flunitrazepam however was only identified in 7 samples (14.5%). This might be due to tighter restrictions on Flunitrazepam, sold under the brand name Rohypnol, which has been associated with date rapes and is under more stringent controls in some jurisdictions such as the USA where it has been made illegal to manufacture sell use or be in procession of the drug [11,12]. Flunitrazepam is also associated with date rapes due its ability to induce short term memory loss, dizziness confusion and loss of consciousness which might make it less popular among self-administering users in the Middle East[12–14]. The low detection rate might also be due to the low dose at which Flunitrazepam is often taken at and its poor stability even when samples are stored at low temparatures[1516]. The high rate of positive test results tends to imply that the developed protocol would be suitable for the routine screening of urine samples for determination of illicit use of the BDZs Alprazolam, Bromazepam, Clonazepam, Diazepam and Flunitrazepam in forensic laboratories.

3.7 Conclusion

A fast accurate and reliable protocol for the detection of BZDs by both HPLC and HPLC-MS have been successfully developed and validated according to ICH guidelines. The protocol was applied to the screening of urine 48 urine samples obtained in Kuwait by law enforcement officers from individuals under investigation for suspected drug abuse violations in 2022 for the presence of BZDs. The method successfully detected BDZs from 93.7% of the samples including the presence of Flunitrazepam, sold under the brand name Rohypnol, which is often difficult to detect from urine samples especially after 72 hours of administration, in 14.5% of the samples[16]. This would tend to indicate that the developed and validated protocol would be suitable for use for the routine analysis of human urine samples in forensic laboratories in Kuwait for the determination of Alprazolam, Bromazepam, Clonazepam, Diazepam and Flunitrazepam specifically and can be adapted to widen its scope for application to cover a wider range of BDZs.

Chapter 4 Development and Validation of Stability-Indicating Assay Method for a Novel Oxazolidinone (PH-192) with Anticonvulsant Activity by Using UHPLC-QToF-MS

As travel into the UK was restricted during the Covid19 lockdown, a series of HPLC experiments were conducted at Kuwait University. These experiments (the development and validation of an HPLC method to analysis a novel oxazolidinone) were published as a paper in Molecules entitled Development and Validation of Stability-Indicating Assay Method for a Novel Oxazolidinone (PH-192) with Anticonvulsant Activity by Using UHPLC-QToF-MS[1].

The text in the paper has been re-written to meet the thesis textual requirements and is reported in this chapter as the text below.

4.1 Introduction.

The study was based on a novel oxazolidinone derivative PH192 which was found to demonstrate anticonvulsant activity in vivo in rats and mice. People suffering from seizures often face numerous challenges because many of the available drugs are ineffective in controlling seizures.[1,2] During seizures, the brains nerve cells act inappropriately, sending a burst of uncontrolled electrical activity altering the sensory motor resulting in temporary abnormalities in muscular control, behavior, sensations or state of awareness.

4.2 Seizure Disorders.

The conditions arise when nerve cells spontaneously fire action potentials inappropriately 1,3. This spontaneous triggering results in alterations in the sensory, motor, and the psychological function of the affected individual. This inappropriate neuronal firing often results in convulsions and recurrent episodes of seizures resulting in a syndrome referred to as epilepsy3. Frequent seizures lead to several side

effects that affect the lifestyles of sufferers. Patients who suffer from seizures are unable to enjoy everyday life and need to take medication to keep the seizures under control4,5.

4.2.1 Treatment of Seizure Disorders.

Seizures can be fatal if they are not managed well6. Several classes of anticonvulsant agents are available for clinical use with patients7–9. A wide range of drugs with diverse chemical structures and pharmacophores have been introduced to the market to control the seizures10,11. Anti-seizure medications, also referred to as anticonvulsants or antiepileptic drugs (AEDs), are used clinically to manage and control seizures in sufferers of epilepsy or other seizure disorders. These drugs work by stabilizing the electrical activity in the brain, reducing the occurrence of abnormal and or excessive neuronal firing which results in seizures10 Various factors, including the type of seizures experienced, the individual's age, overall health, and potential side effects affect the choice of medication prescribed.

Some commonly prescribed anti-seizure medications include11:

Phenytoin (Dilantin), which works by blocking sodium channels in the brain, preventing excessive electrical activity12, Carbamazepine (Tegretol), which also acts on sodium channels and is effective in treating partial seizures and generalised tonic-clonic seizures13. Lamotrigine (Lamictal) is another drug that modulates sodium channels and is prescribed for partial seizures and generalised seizures in conditions like Lennox-Gastaut syndrome14. Oxcarbazepine (Trileptal), which similarly to carbamazepine, modulates sodium channels and is used for partial seizures13.

Valproic acid (Depakote), acts by enhancing the effect of the neurotransmitter GABA, which inhibits excessive neuronal activity. It is used for various seizure types13.

Clobazam (Onfi), also enhances the effects of GABA and is used for certain types of seizures, often as an adjunctive therapy15.

Levetiracetam (Keppra) whose exact mechanism of action is not well understood, but it is believed to affect synaptic vesicle protein and neuronal excitability. It is used for various seizure types16.

Topiramate (Topamax), which works by blocking sodium channels and enhancing the activity of GABA17. Topiramate is used for a range of seizures and is also prescribed for migraine prevention18.

Gabapentin (Neurontin) also has an unclear mechanism of action but is believed to affect calcium channels19. It is used for partial seizures and neuropathic pain.

It is important for individuals taking anti-seizure medications to adhere to their healthcare provider's instructions carefully, as proper dosage and regular monitoring is crucial for effective seizure control. Additionally, healthcare providers may need to adjust medications or try different combinations to achieve optimal seizure management with few side effects.

People who suffer from seizures can experience diminished social support, stress on family and cognitive development20,21. A lot of research has been performed to introduce drugs that can address the different issues that seizure suffers face including the issue of stigmatization. Patients with epilepsy find it hard to concentrate on their everyday lives22. Additionally, they often experience low employment levels23.

The learning process for people who have epilepsy is full of challenges. Ranging from low school attendance to issues in relationships24. Social interactions can be an issue when people are faced with seizures. Introducing the right treatment protocols is

therefore essential. The introduction of new and more effective treatments will hopefully help in addressing some of these issues.

4.2.2 Managing Therapy-Resistant Epilepsy.

Therapy-resistant epilepsy is also known as drug-resistant or refractory epilepsy and refers to a condition in which sufferers do not adequately respond to standard anti-seizure medications25,26. In therapy-resistant epilepsy, seizures persist despite the use of multiple appropriate antiepileptic drug combinations. The inability to control seizures with medication poses is extremely challenging for both the patients and healthcare providers. It can have a profound impact on their quality of life, cognitive function, as well as the mental health of patients.

Several factors contribute to therapy resistance in epilepsy. These often include the underlying causes of epilepsy, the specific type of seizures, genetic factors, and individual variations in drug metabolism. Identification of the cause and understanding the mechanisms of therapy resistance are essential for developing targeted treatment approaches27,28.

Research into novel therapeutic strategies, including new drugs and other advanced technologies, continues to provide effective solutions for individuals with therapy-resistant epilepsy29,30.

There are several therapeutic approaches routinely employed for the treatment of seizures. These approaches, however, tend to be less effective when dealing with a wide range of complex issues. For example, some patients do not respond optimally to many of the treatments. This highlights a need for new measures that overcome these shortcomings and work towards making treatments more reliable.

The study was therefore conducted to investigate the common physicochemical stability properties of a new anti-epilepsy drug, PH-192, to help shed some light on its duration of action and access its suitability as a potential new treatment for seizures. Analysis of the ADME process is necessary to understand the drug's stability and suitability to address some of the various side effects related to seizures. The in-vitro stability-indicating assay in plasma and other conditions can help unlock different dose strategies or suggest more stable analogues which are resistant to degradation and so might be effective alternatives that can be applied in the treatment of epilepsy.

4.3 Novel Triazolyl-oxazolidinone Derivative, PH-192.

The novel drug candidate, PH-192, was identified as a potential anticonvulsant agent that can be applied to control seizures1,2. There was however a need for further assessment to investigate its stability.

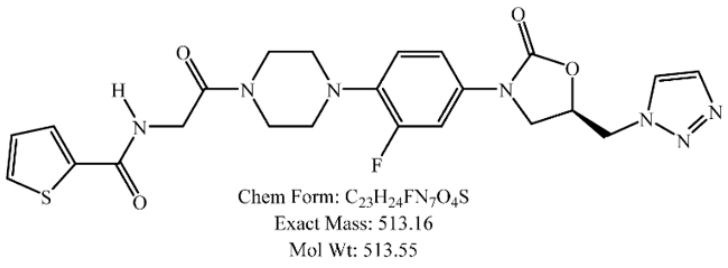


Figure 4. 1 Structure of PH-192[1,2].

Through a stability-indicating assay, different aspects of the drug can be assessed to determine the optimal way it can be used as a treatment. Stability-indicating assays are used to understand the way a potential drug product may degrade over time which

can help formulation scientists select excipients, packaging, and storage conditions to minimise degradation to optimise the shelf life, potency and quality of the drug31. The drug has a chemical composition comparable with phenytoin which is utilised for managing electrically induced seizures. The study began with tests on rodents.

The oxazolidinone-containing antibacterial agents, linezolid (1) and tedizolid (2) (Figure 4.2), have both been approved for use in humans based on their demonstrated clinical efficacy and safety against Gram-positive bacteria1,32–35.

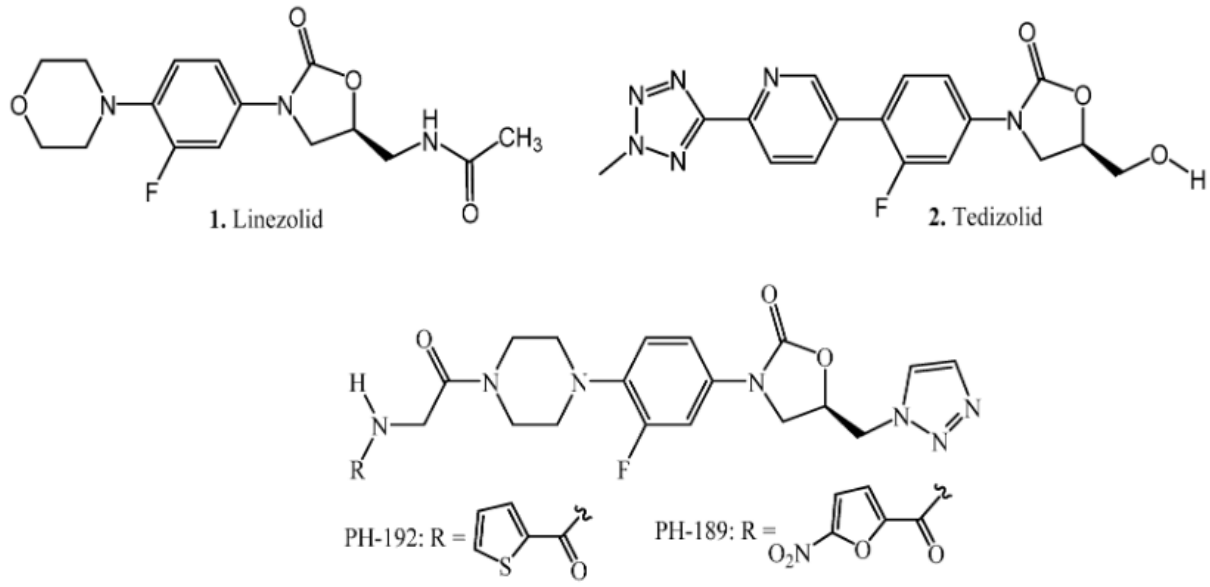


Figure 4. 2 Chemical structures of oxazolidinones with antibacterial and anticonvulsant properties[36].

Previous reports regarding the neuronal responses of oxazolidinone derivatives led to their assessment establishing if they could potentially be repurposed for use as anticonvulsant agents1,2,37,38. Following structural modifications and in-vivo screening of the anticonvulsant activity of the selected compounds in rodents, using both electrically and chemically induced models of seizures, it was highlighted that

some of the compounds protected rats and mice in both electrically and chemically induced models of seizures with no observable anticonvulsant side effects[1]. However, PH-192, which was adjudged to be the most efficacious and safest of these compounds, protected the animals only for 30 min[37]. PH-192 exhibited dose-dependent protection from seizures induced by using a 6 Hz stimulation protocol with estimated ED50's of 34.5 mg/kg and 90 mg/kg in mice and rats, respectively[1,39]. Pre-treatment of the test subjects for 30 min with 100 mg/kg of PH-192 protected 75% (mice) and 66.6% (rats), of the rodents, respectively, against 6 Hz-induced seizures. Additionally, a 30 min intraperitoneal (IP) pre-treatment of rats with 100 mg/kg PH-192 protected 80% of them from a pentylenetetrazole (PTZ) injection-induced seizure. This level of protection is comparable to that obtained for the reference antiepileptic drug phenytoin (40 mg/kg), which is used in the clinic for treating seizure disorders[37]. Despite its efficacy and safety profile, the short duration of action (30 min) for PH-192 raised some significant pharmacokinetic questions about this compound. Hence, it was decided to conduct an in-vitro stability-indicating assay in plasma, and under acidic, and basic conditions, with the aim of shedding some light on the physicochemical stability of PH-192 before conducting detailed pharmacokinetic behavior and brain distribution studies.

4.3.1 Investigating Stability-Indicating Assay of Ph-192 in Plasma and Other Solutions.

The investigation of PH-192 involved exposing it to different conditions. Its degradation process in human plasma was first studied. The IP application of the new substance for the treatment of seizures would bring it into contact with blood and would involve a reaction with blood plasma. This test is aimed at the determination

of the potential reactions of the drug in the blood. Drug-free human plasma was obtained from a blood bank in Kuwait. Samples of PH-192 were mixed with aliquots of plasma and incubated at 37 °C for 90 minutes before extraction and analysis LC-MS.

Substances react differently at different pH levels; therefore, respective samples of PH-192 were exposed to acidic and basic media to study the drug's stability. Stability to oxidation plays a very important role in determining the outcomes for potential novel drug products[40]. There was therefore a need to come up with an assessment of the compound under oxidative conditions.

4.4 Results and Discussion.

4.4.1 Materials and Methods Used in the Studies[1].

The study utilised drug-free human plasma obtained from the Kuwait blood bank. HPLC-grade solvents, acetonitrile, and other chemicals were of analytical grade and obtained from Sigma Aldrich, Dorset, UK. In-house HPLC-grade water was prepared using a MilliQ filter purchased from Millipore, Watford, UK. Syringe membrane filters (13 mm) were purchased from Kinesis Scientific Expert, (Cambridgeshire, UK). Nylon solvent filters (0.45 um) were purchased from Kinesis Scientific Expert, (Cambridgeshire, UK) were used for filtration of both solvents and water.

4.4.2 Solutions.

Stock standard solutions of PH-192 and the internal standard (PH-189) were prepared by dissolving 10 mg of each in 100 mL of a water: acetonitrile (75:25 v/v) mixture separately to give stock solutions with final concentrations of 0.1 mg/mL. Working solutions of both were then prepared by diluting the stock solutions with the water: acetonitrile (75:25 v/v) mixture to obtain final concentrations of 500 μg/mL.

4.4.3 Human Plasma Extraction Procedure.

Plasma samples of PH-192 were prepared by adding PH-192(10 μg/ml) to aliquots of human plasma (200 μL) to give mixtures with final concentrations of 210 μg/mL. PH-192 was extracted by transferring 210 μL of human plasma to an Eppendorf tube, followed by the addition of acetonitrile (ACN, 770 μL), which already contained the internal standard PH-189 (20 μL). The samples were then vortexed and centrifuged at 8000 rpm (10 min). The supernatant was then collected and analysed by LC-MS. Stability of the spiked plasma samples was maintained at the temperature of 37 °C to mimic the human body temperature.

4.5 Instrumentation.

4.5.1 Ultra Pressure Liquid Chromatography.

UPLC analysis for the analysis and method validation was conducted utilising Isocratic elution on a Waters Acquity UHPLC system with a quaternary Solvent Manager (H-Class), with a UV detector, fitted with an ACE C18 (50 × 3.0 mm, 3 μm) analytical column. Data processing and reporting was performed using the Empower® software. The mobile phases used were filtered and degassed water and acetonitrile containing 0.1% formic acid as an additive in a 75:25 *v*/*v* ratio and a flow rate of 0.2 mL/min. The column oven temperature was set to 30 °C and the sample volume injected was 10 μL. UV analysis was done at a wavelength of 254 nm.

4.5.2 Liquid Chromatography tandem Mass Spectrometry

LCMS analysis was conducted on a Waters® Xevo G2-S QToF coupled to a Waters® Acquity UPLC system equipped with binary Solvent Manager (I-Class) in ESI mode. The operating parameters (positive ion mode) were as follows:

Sheath gas and auxiliary flow rates: 30 and 5 (arbitrary units), respectively.

Capillary voltage: 3.5 V

Sampling cone voltage 55 V

Source temperature: 110 °C.

Desolvation temperatures: 450 °C.

Collision energies: - 2: 10 eV, 3: 15 eV, 4: 20 eV.

4.5.3 Calibration Procedure for Mass Spectrometry.

Calibration of mass spec was done using the standard internal calibration procedure with leucine enkephalin (*m*/*z* = 556.2771) as the reference compound which was introduced to the ionisation source simultaneously with the PH-192 sample. The error obtained was within 1 ppm of the detected charge to mass ratio (*m*/*z)* for leucine enkephalin (556.2772). Sodium formate (0.5 µM) was used as buffer (pH 4.5) in the standard solutions for the calibration of the machine and to assess the accuracy and resolution.

4.6 Method Validation.

The validation of all methods was performed in accordance with the International Council for Harmonisation (ICH) guidelines (Q1A R2)41,42.

4.6.1 Calibration curves.

To make up the calibration curves, accurately measured aliquots of the sample PH-192 were transferred from the stock solution (0.1 mg/mL) into a series of volumetric flasks (10 mL) and filled to the correct volume mark with the mobile phase. The calibration samples consisted of five concentrations of PH-192 (1–80 µg/mL) which were independently injected onto the system at a flow rate of 0.2 mL/min. The peak areas of each of the drug peaks was recorded against its concentration. The linearity

of the curves was constructed, and regression equations computed using Microsoft excel 365

4.6.2 Accuracy and Precision.

The accuracy of the results was verified by calculating the accuracy (%) of three replicates of three different concentrations covering the linearity. The concentrations were calculated from the corresponding regression equations. The precision of the UHPLC-UV method for the combination was assessed using two separate sets of PH-192 samples within the concentration range of the calibration curve. The precision of the UHPLC-UV method for PH-192 was estimated to be using three different concentrations of pure samples of PH-192 (1, 20, and 80 µg/mL) within the linear range. All samples were analysed in triplicate, in a single day on three consecutive days respectively, to determine the intra-day repeatability and inter-day precision (intermediate precision) of the proposed method, respectively. A set (n = 3) was prepared at room temperature (22 °C), whereas five other sets (n = 3) were prepared and stored at 4 °C for mixtures dissolved in mobile phase samples for three days. The relative standard deviation percentage (%RSD) was used to calculate the intra- and inter-assay precision.

4.6.3 Extraction Recovery and Matrix Effect.

Samples of standards containing 1, 20, 40, and 80 µg/mL of PH-192 were prepared in duplicate. One set was prepared using human plasma while the other set was prepared with the mobile phase. The standards in plasma were mixed with 20 µg/mL of internal standard and extracted as mentioned previously, while the standards in mobile phase analysed directly after mixing with internal standard (non-extracted samples). The

recoveries from the extractions were calculated based on the slopes of the standard curves of PH-192 in the plasma and the mobile phase. Absolute recoveries of PH-192 and internal standard were also indicated by comparing the absolute values of the peak areas of PH-192 and internal standard in both the extracted and non-extracted samples.

4.6.4 Evaluation of PH 192 Extraction and stability in Human Plasma.

Samples of PH192 containing (20 μg/mL) plus internal standard (20 μg/mL) were spiked into 160 μL human plasma (final concentration 0.1 μg/mL) and incubated in an oven for 90 min at 37 °C. The PH-192 and internal standard were then extracted from plasma by liquid-liquid extraction then the samples were filtered prior to analysis using syringe membrane filters (13 mm) kinesis®.

4.6.5 Limit of Detection (LOD) and Limit of Quantification (LOQ).

Stock solutions of PH-192 were prepared in a range of concentrations between 1–100 μg/mL. The LOD and LOQ for PH-192 was then determined at signal-to-noise ratios of 3:1 and 10:1, respectively.

4.6.6 Forced Degradation Studies.

Stability tests were conducted on PH-192, to indicate the best dosage form design and simulate the actual conditions under which the dosage form would be stored.

4.6.7 Acidic Degradation.

PH-192 (2 mg) was weighed into a 4 mL vial. Two millilitres of 1 N HCl was then added and the resulting mixture incubated at 90 °C for 90 min. The mixture was allowed to cool down for 15 min, before analysis by LC-MS.

4.6.8 Basic Degradation.

PH-192 (2 mg) was weighed into a 4 mL vial followed by 2 mL of aqueous 1 N NaOH solution to simulate basic stress conditions. The sample was incubated at 90 °C for 90 min, then allowed to cool down for 15 min prior to analysis by LC-MS.

4.6.9 Oxidative Degradation.

PH-192 (2 mg) was placed in a 4 mL vial and subjected to oxidative conditions by the addition of 2 mL of 1 N H_2O_2 . The sample was then incubated at 90 °C for 90 min, then allowed to cool down for 15 min, prior to analysis by LC-MS.

4.7 HPLC Data.

Acceptable resolution and peak shapes (symmetry factor) were obtained using water with acetonitrile (75:25 v/v) as mobile phase with 0.1% v/v formic acid as additive (Figure 1). A flow rate of 0.2 mL/min was chosen for to aid resolution and speed of separation.

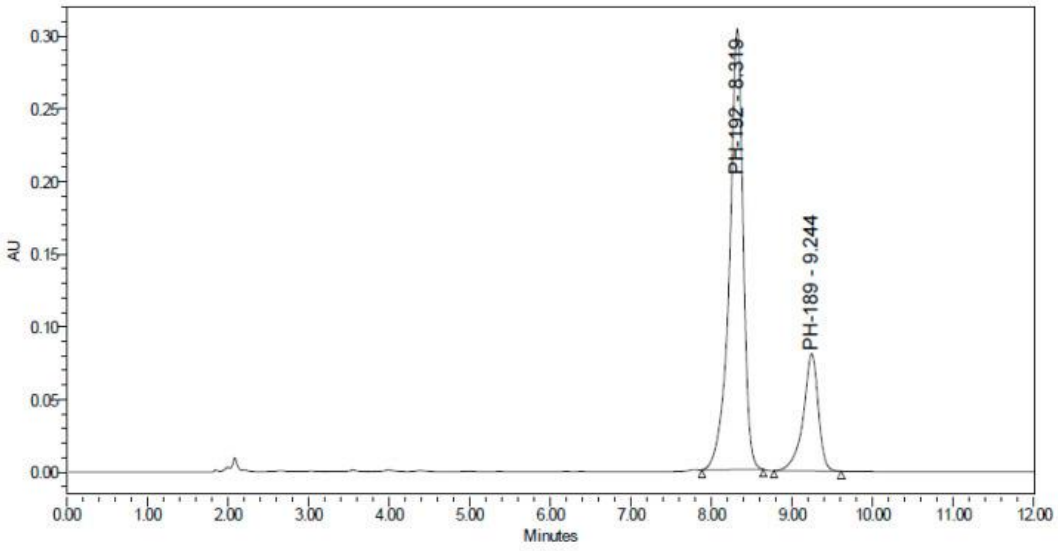


Figure 4. 3 UHPLC-UV chromatogram of 40 µg/mL of PH-192 and 20 µg/mL of PH-189 as an internal standard.

4.7.1 Method Validation

Linearity and Sensitivity

Linearity was achieved by plotting the respective peak areas against the concentrations for PH-192 in the range between 1–80 µg/mL with correlation coefficients (r) ≥0.999. The calibration curve was produced in triplicate, with the obtained slopes and correlation coefficients showing high consistency. This demonstrated the reliability of the standard curve over the chosen concentration range for the study, as shown in the table below (Table 4.1). The LOQ was found to be 1 µg/mL for PH-192 (RSD% = 5.8%). The LOD for PH-192 was similarly found to be 0.33 µg/mL, using 10 µL injections of sample also shown in the table below (Table 4.1).

Table 4. 1 Validation parameters of the UPLC-UV method.

Parameters	PH-192
Range µg/mL	1–80 µg/mL
Regression Equation	$y = 0.0867x - 0.0403$
Correlation coefficient (r)	0.9998
LOQ (µg/mL)	1
LOD (µg/mL)	0.33
Intra-assay precision [a]	5.8
Inter-assay precision [a]	7.4
Accuracy [b]	96.66%

[a] expressed as the RSD. [b] expressed as [mean % deviation = mean calculated concentration/nominal concentration].

4.7.1.1 Precision and Accuracy.

The results were expressed as accurate percentages (%) of PH-192 in the samples. The overall results for PH-192 are reproduced in the table below (Table 4.2). This is representation of the accuracy of the proposed UHPLC-UV method. The values of the %RSD for intra-day and inter-day variations are also reported in the table below (Table 4.2). In both cases, the % RSD values were found to be within the acceptable 2% limit, indicating the precision and repeatability of the developed method.

Table 4. 2 Intra and inter- assay precision and accuracy data for PH-192 determination in bulk powder using UPLC-UV.

Precision	PH-192 Concentration µg/mL	Mean ± SD (*n* = 3) Observed/µg/mL	Precision [a] (%)	Accuracy [b] (%)
Intra-Assay Precision and Accuracy Data for PH-192 Determination in Mobile Phase Using UPLC-UV.	1	0.966 ± 0.056	5.8	96.66
	20	19.91 ± 0.158	0.8	99.55
	80	79.96 ± 0.165	0.2	99.96
Inter-Assay Precision and Accuracy Data for PH-192 Determination in Mobile Phase Using UPLC-UV.	1	0.943 ± 0.070	7.4	94.33
	20	19.77 ± 0.305	1.5	98.85
	80	79.27 ± 0.409	0.5	99.08

[a] expressed as the RSD. [b] expressed as [mean % deviation = mean calculated concentration/nominal concentration ×100].

4.7.1.2 Evaluation of PH-192 Extraction and Stability in Human Plasma.

The efficiency of the liquid-liquid extraction method for PH-192 was assessed by calculating the recovery percentages for the extractions. The extraction recovery was estimated from the peak areas of the PH-192 samples in both plasma and mobile phase. The extraction method was found to be capable of recovering 94.38% of the PH-192 sample from human plasma. PH-192 demonstrated good stability in human plasma for 90 min at a temperature of 37 °C with no degradation products detected. The quantity of PH-192 recovered was calculated from the calibration curve equation and was found to be equivalent to 9.44 µg/mL from the 10 µg/mL of PH-192, which was spiked into the human plasma.

4.7.1.3 Stability Study.

A sample of PH-192 was then subjected to degradation study under basic conditions by incubating the sample in 1 N NaOH at 90 °C for 90 min. This resulted in the formation of three degradation products with the major degradation product at approximately 5.9 minutes as indicated by UHPLC-UV. A reproduction of the chromatograph is shown in the figure below (Figure 4.4).

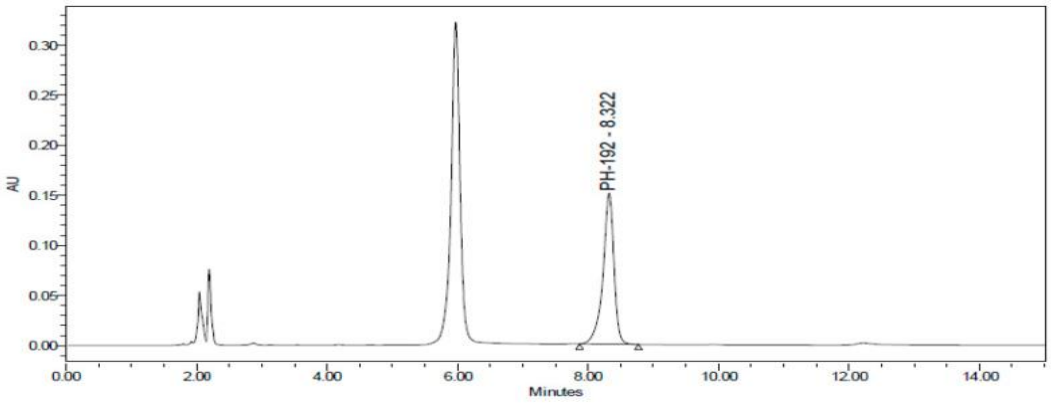


Figure 4. 4 UHPLC-UV chromatogram for the basic degradation products of PH-192.

This degradation product was identified and confirmed with the aid of LC-QToF-MS, as shown in the figure below (Figure 5). This major degradation product was identified as being the result of hydrolysis at the piperazine amide bond and decarboxylation of the oxazolidinone ring on PH-192.

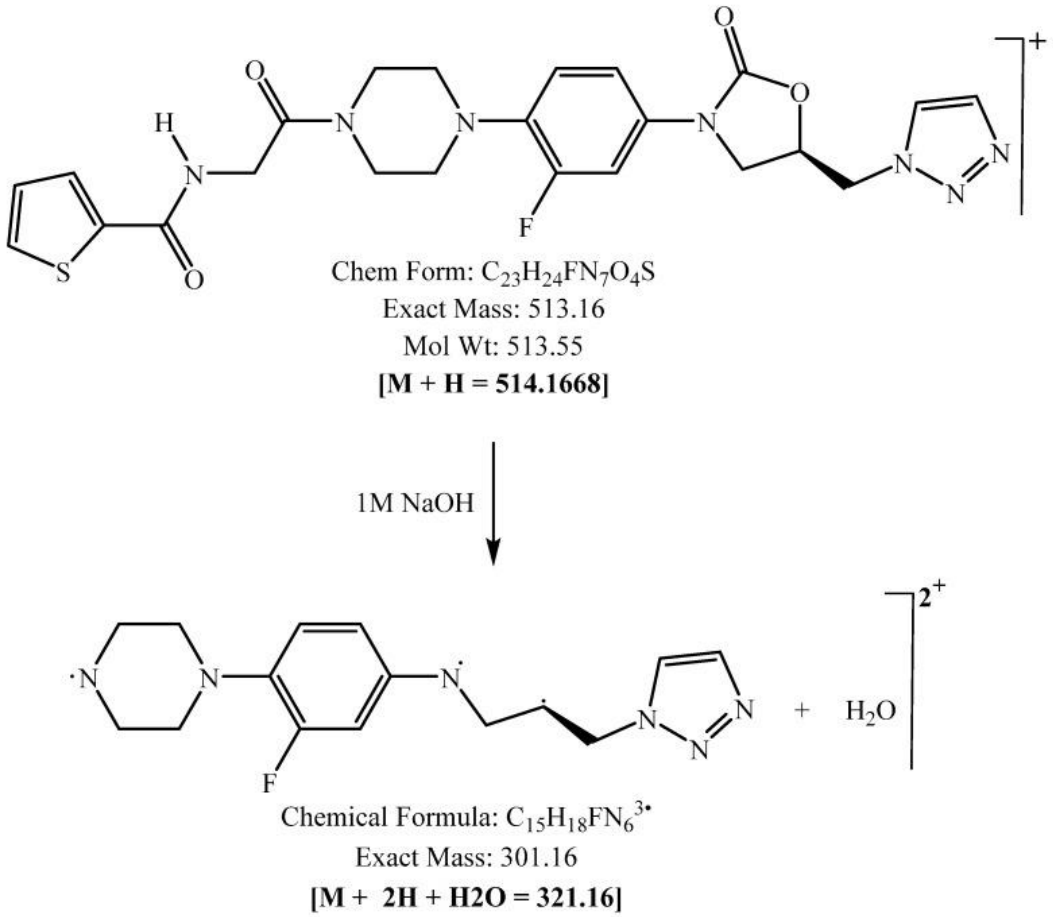


Figure 4. 5 Degradation product of PH-192 after adding 1 N of NaOH.

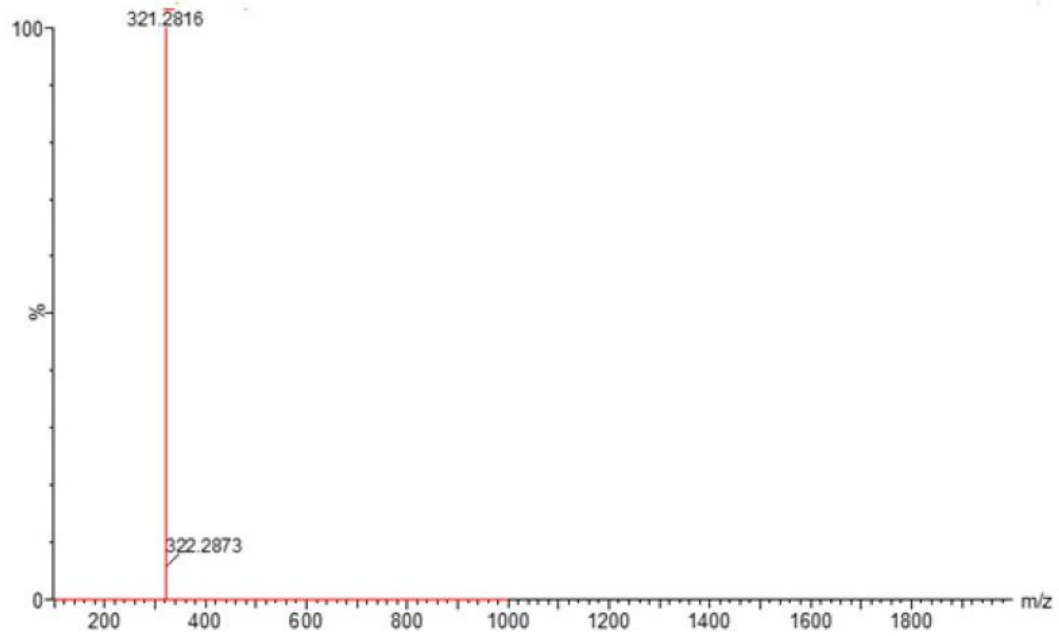


Figure 4. 6 LC-QToF-MS analysis of PH-192 post-exposure to basic degradation at a retention time of 5.9 minutes).

A sample of PH-192 was subjected to further degradation studies by exposure to oxidising conditions by incubation in 1 N H_2O_2 at 90 °C for 90 min and assessed for the formation of degradation products. Four degradation products were observed by UHPLC-UV with the major degradation product eluting at 1.8 min as highlighted in the figure below (Figure 4.7).

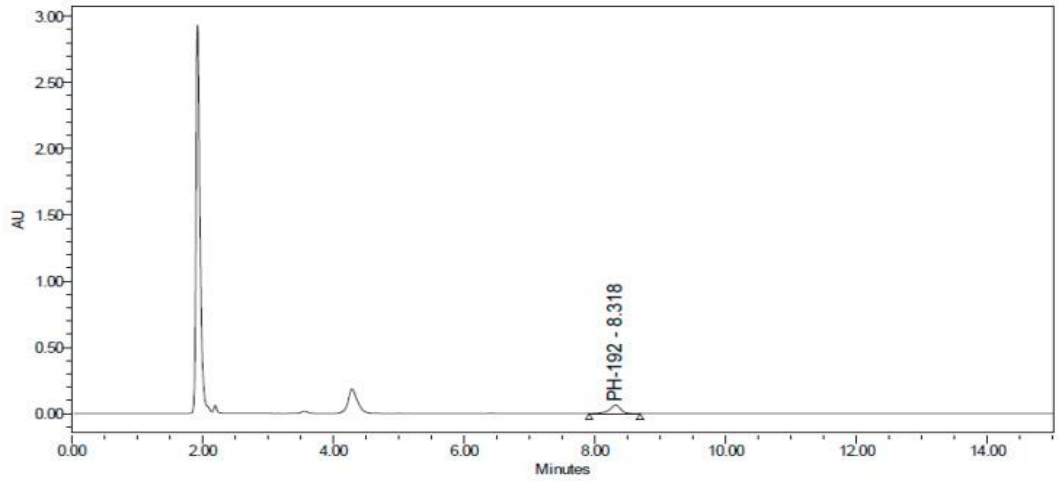


Figure 4. 7 UHPLC-UV chromatogram for the oxidative degradation products of PH-192 at retention time 1.8 minutes.

Mass identification of this major degradation product by LC-QToF is shown in the figure below (Figure 4.8), revealed that it was the result of oxidation on the piperazine ring resulting in the formation of the corresponding alcohol.

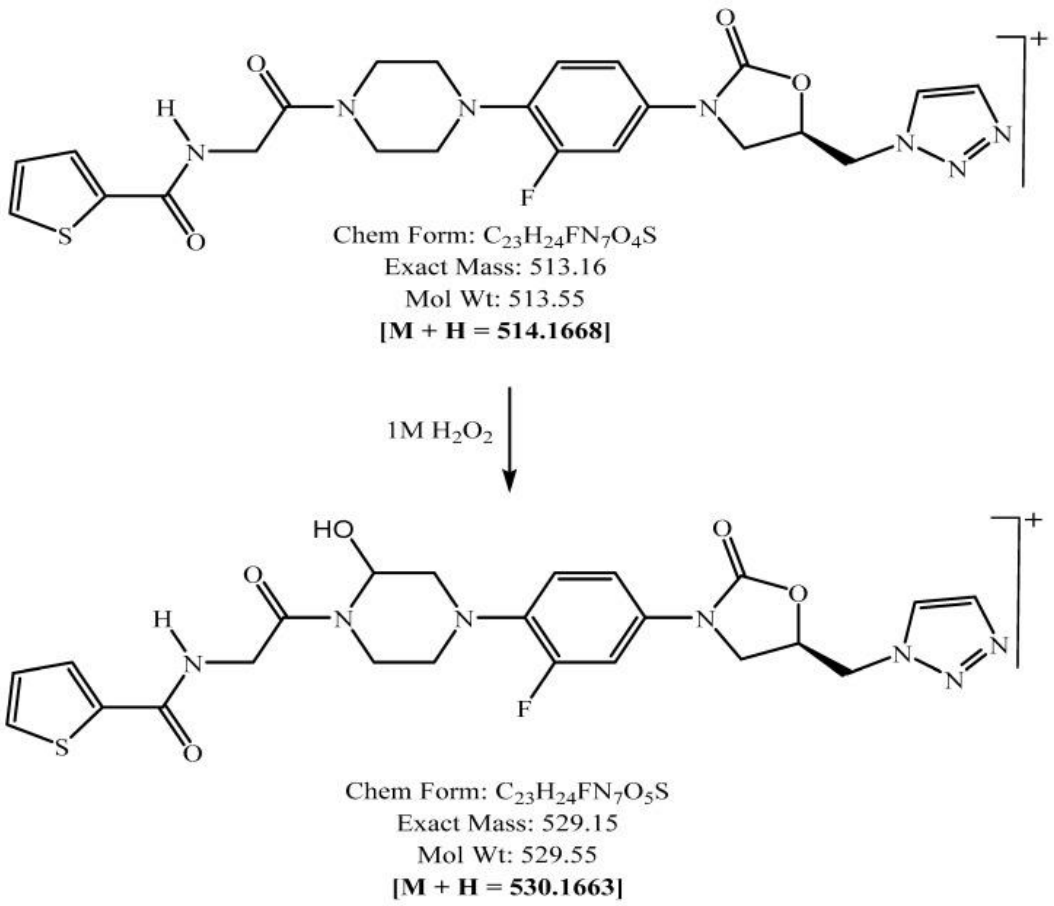


Figure 4. 8 Degradation products of PH-192 after adding 1 N of H_2O_2.

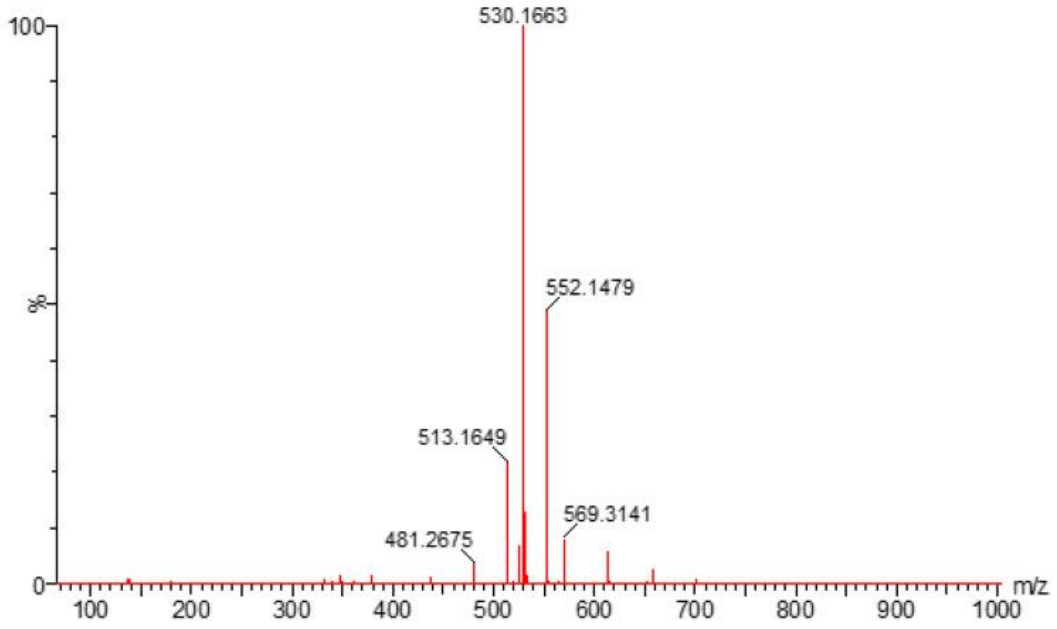


Figure 4. 9 LC-QToF-MS analysis of PH-192 post-exposure to oxidation degradation at a retention time of 1.8 minutes.

Finally, PH-192 was found to be stable when subjected further degradation studies by exposure to acidic conditions by incubation in 1 N HCl at 90 °C for 90 min with no significant degradation products found.

4.8 Conclusions

4.8.1 Stable in Acidic Stress Conditions

It was established from the study that the novel drug candidate was stable under acidic stress conditions[43].

4.8.2 Alkaline (NaOH) and Oxidative (H_2O_2) Solutions Test

The drug was found to be unstable under oxidative conditions in the hydrogen peroxide solution test. The test was crucial because it gives an indication of possible metabolic products and routes. Some active pharmaceutical ingredients tend to be deactivated when they undergo oxidation, which appears to be the case with PH-192 as it is almost entirely converted to the oxidised product under the test conditions.

However, more work is needed to synthesize and evaluate the oxidised by-product for its efficacy. When exposed to the alkaline conditions, PH-192 appeared to decompose with the cleavage at the piperazine ring resulting in the loss of the Thiophene containing side chain which appears to be important for its activity[1]. It will be informative to subject PH-192 to further metabolic studies to understand if the loss of anticonvulsant activity after 30 minutes which was observed is due to metabolism of the drug. This might point towards alternative analogues which can overcome this metabolic issue.

4.8.3 Stable in human plasma for 90 minutes at 37 °C.

PH-192 was found to be stable in human plasma for up to 90 minutes when maintained at 37 °C. This study was carried out to determine how the drug coped when exposed to human plasma under the test conditions.

Chapter 5 Bioanalytical Method Development and Validation of Amphetamine, Methamphetamine, Morphine, Codeine, Diazepam and Pregabalin by High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS) technique

5.1 Introduction

Kuwait, a Middle Eastern Arabian gulf country and home to 4.4 million people, is also struggling to overcome its own illicit drug problem [18]. The Global Organized Crime Index 2023 also reports that synthetic drug seizures have significantly increased in Kuwait, with prescription-only medications like methamphetamine, tramadol, and pregabalin being the main substances smuggled into the nation[19].

Anecdotal evidence indicates that the dramatic increase in reported cases of drug abuse has also resulted in increased pressure on local resources, stretching key administrative services such as policing and forensic services to breaking point[20,21]. The abuse of illicit drugs such as amphetamine, methamphetamine, morphine, codeine, diazepam and pregabalin is widespread and they are often taken as mixtures or cocktails by users[2,22,23]. These illicit substances have very different physicochemical properties such as partition coefficient (Log P), polarity and maximum Ultraviolet Visible absorbance (UV_{max}) values, which all serve to complicate the development of a single analysis method suitable for the rapid identification and quantification of all these drug components from biological samples[24].

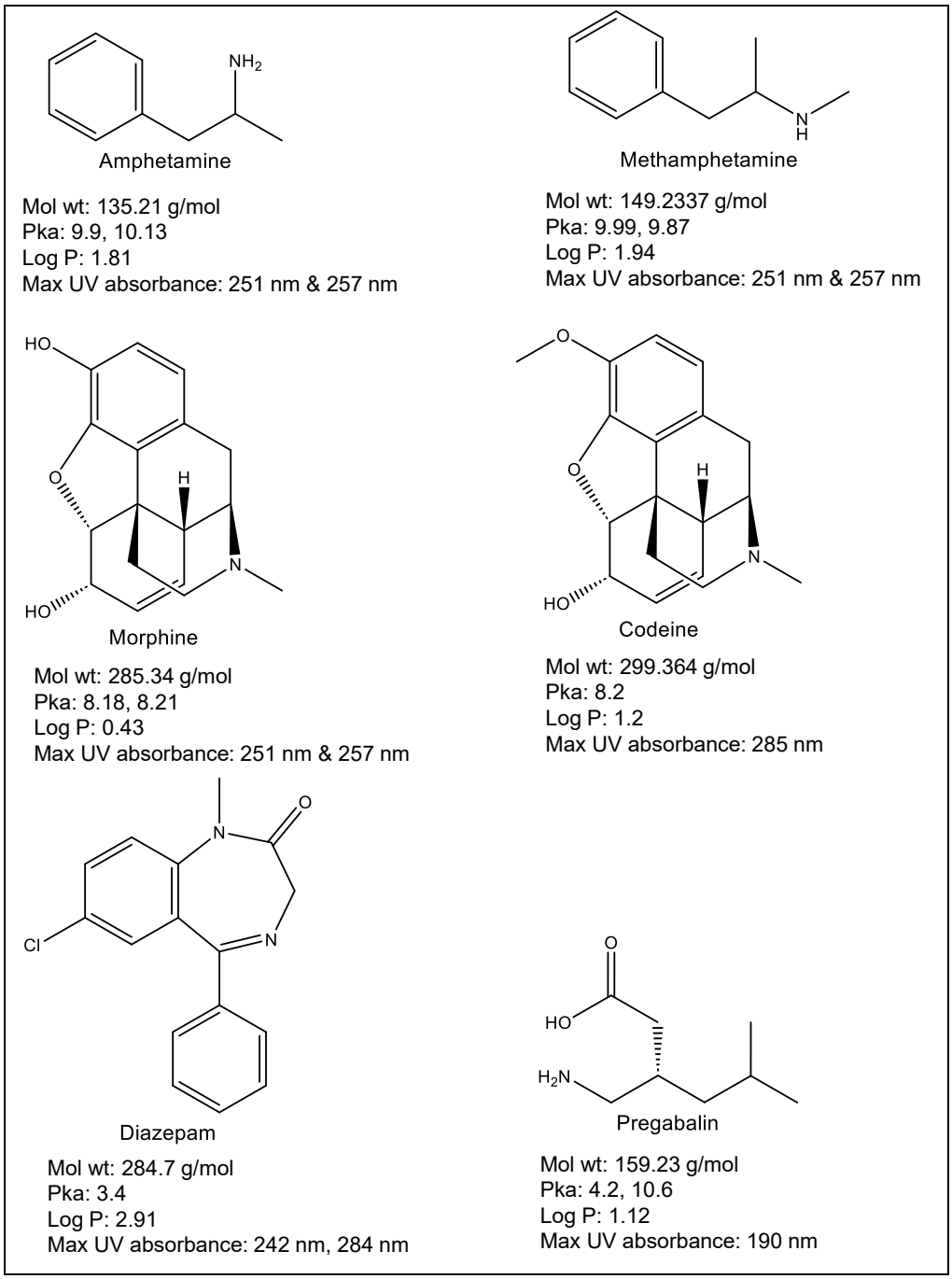


Figure 5.1 Chemical structures and Physicochemical properties of Amphetamine, Methamphetamine, Morphine, Codeine, Diazepam and Pregabalin [26].

5.2 Aim of the study

The studies in this chapter were to develop a fast, simple and robust analytical protocol for the identification and quantification of most illicit drugs in Kuwait such as amphetamine, methamphetamine, morphine, codeine, diazepam and pregabalin using HPLC-UV and GC-MS techniques. Hyphenated techniques were chosen to overcome any difficulties posed by the physiochemical properties of the test samples.

5.3 Materials & Methods.

5.3.1 Materials.

HPLC grade solvents, acetonitrile, methanol, ethanol and all other chemicals (Morphine, Codeine, Diazepam, Amphetamine, Pregabalin and Methamphetamine) of analytical grade were obtained from Sigma Aldrich, Dorset, UK. In-house HPLC grade water was prepared using a MilliQ filter purchased from Millipore, Watford, UK.

5.3.2 Instruments and Column.

HPLC-UV

Column	: LC Column, C18.
Dimensions	: 150 x 4.6 mm, 5µm (Length, ID & particle size)
Make	: Phenomenex
Serial No.	: H17-100640
Batch No.	: 5520-0186
Part No.	: 00F-4435-E0
HPLC Make and Model	: Shimadzu, LC-2040C, Nexera-i
Column	: SH-I-5MS

Dimensions : 30 cm x 0.25 mm, 0.25µm (Length, ID and particle size)

Make : Shimadzu

Serial No. : 12152774

Batch No. : 22291

Part No. : 221-75940-30

GC-MS

Make and Model : Shimadzu, Nexis GC-2030 Gas Chromatograph.

: GCMS-TQ8040 NX Gas Chromatograph Mass Spectrometer.

5.3.3 Methods

5.3.3.1 Analytical Method for HPLC-UV

Mobile Phase A : 0.1% v/v TFA in water (pH 2.5)

Mobile Phase B : Acetonitrile

Column : Phenomenex, 150 x 4.6 mm, 5µm (Length, ID and particle size)

Flow rate : 1 mL/min

Detector : UV

Detection Wavelength : 254 nm for UV

Injection volume : 20 µL

Column Oven Temp : 25°C

Run time : 35 minutes (gradient elution)

Diluent : Methanol

Table 5. 1 Gradient Composition for HPLC

	Time (mins)	% MP B Composition
1	0.01	5
2	2.10	5
3	25.00	50
4	27.00	95
5	30.00	95
6	32.00	5

5.3.3.2 Preparation of calibration series.

Preparation of individual standard stock solution: (1000 ppm)

Standard samples of Morphine, Codeine, Diazepam, Amphetamine and Methamphetamine were made up by accurately weighing (10 mg) of each standard respectively and transferring into separate 10 mL volumetric flasks. Methanol (5 mL) was added to each of the volumetric flasks and swirled gently to facilitate dissolution then the volume adjusted up-to the mark with methanol.

5.3.3.3 Preparation of HPLC calibration level.

A series of standard calibration solutions were prepared for the concentration range 1-250µg/mL using methanol as the diluent.

System Suitability Criteria [44]:

The system is deemed to be suitable if the peaks for Diazepam are at 10 PPM, 100 PPM for codeine, and 150 PPM for Morphine, Amphetamine and Methamphetamine. The system is also deemed to be suitable if the calibration solution (100% level) meets the following criteria.

1. The Tailing factor for all peaks should not be more than 2.0.
2. The Capacity factor (k') for all peaks should be more than 1.0.
3. The Resolution between Codeine and Amphetamine should be greater than 1.5.
4. The Resolution between Amphetamine and Methamphetamine should be greater than 1.5.

5.3.3.4 Analytical Method for GC-MS.

Parameters for GC

Temperature	: 280°C
Injection Mode	: Split
Sampling Time	: 1 min
Carrier Gas	: Helium
Flow Control Mode	: Linear Velocity
Pressure	: 107.2 kPa
Total Flow	: 18.1 mL/min
Column Flow	: 1.37 mL/min

Purge Flow : 3.0 mL/min

Split Ratio : 10.0

Column : SH-1-5MS, 30.0 m x 0.25 mm ID, 0.25 µm (film thickness)

Column Temperature : 110°C

Equilibration Time : 0.5 min

Temperature Gradient

Table 5. 2 GC temperature gradient program

	Rate	Temperature	Hold Time
1	-	110.0	2.00
2	5.00	230.0	2.00
3	2.50	260.0	2.00
4	50.00	320.0	2.00

Parameters for MS

Ion Source Temp : 250°C

Interface Temp : 300°C

Solvent Cut Time : 1.0 min

Detector Voltage : 0.1 kV

GC Program Time: : 45.20 min

Table 5. 3 GC results for the six compound samples

	Compound Name	Start Time (min)	End Time (min)	Acquired Mode	Event Time (sec)	Ch1 m/z	Ch2 m/z	Ch3 m/z	Ch4 m/z
1	Amphetamine	2.50	4.00	Q3 SIM	0.300	135.00	91.00	65.00	120.00
2	Methamphetamine	4.00	7.25	Q3 SIM	0.300	149.00	134.00	91.00	58.00
3	Pregabalin	7.25	11.00	Q3 SIM	0.300	141.00	111.00	126.00	84.00
4	Codeine	11.00	30.00	Q3 SIM	0.300	299.00	229.00	162.00	214.00
5	Morphine	30.00	30.50	Q3 SIM	0.300	285.00	268.00	215.00	162.00
6	Diazepam	30.50	44.00	Q3 SIM	0.300	284.00	256.00	221.00	241.00

5.3.3.5 Preparation of calibration series.

Preparation of individual standard stock solution: (1000 PPM)

10 mg samples each of Morphine, Codeine, Pregabalin, Diazepam, Amphetamine and Methamphetamine were accurately weighed and transferred into 10 mL standard volumetric flasks respectively, and 5 mL of ethanol to each volumetric flask and dissolved it by gentle swirling. Then volume was made up-to the mark with ethanol.

Preparation of GC calibration level:

A series of mixed standard calibration solutions were prepared for a concentration range of 1 - 250 µg/mL using ethanol as a diluent.

System Suitability Criteria [44]:

The system is deemed to be suitable if the peaks for Diazepam and Codeine are at 25 PPM, at 50 PPM for Morphine, Amphetamine, Methamphetamine and 100 PPM for Pregabalin.

The system is also deemed to be suitable if the calibration solution (100% level) meets the following requirements.

1. The Tailing factor for all peaks should be no more than 2.0.
2. The Resolution between Amphetamine and Methamphetamine should be greater than 1.5.
3. The Resolution between Morphine and Diazepam should be greater than 1.5.

5.4 Results

5.4.1 Development of Methods for HPLC-UV and GC-MS

Development of the HPLC-UV and GC-MS methods were carried out utilising the existing literature regarding the structure and properties of the test compounds, nature of the solvents and stationary phase, the composition of the solvents and several trials.

5.4.2 HPLC Results

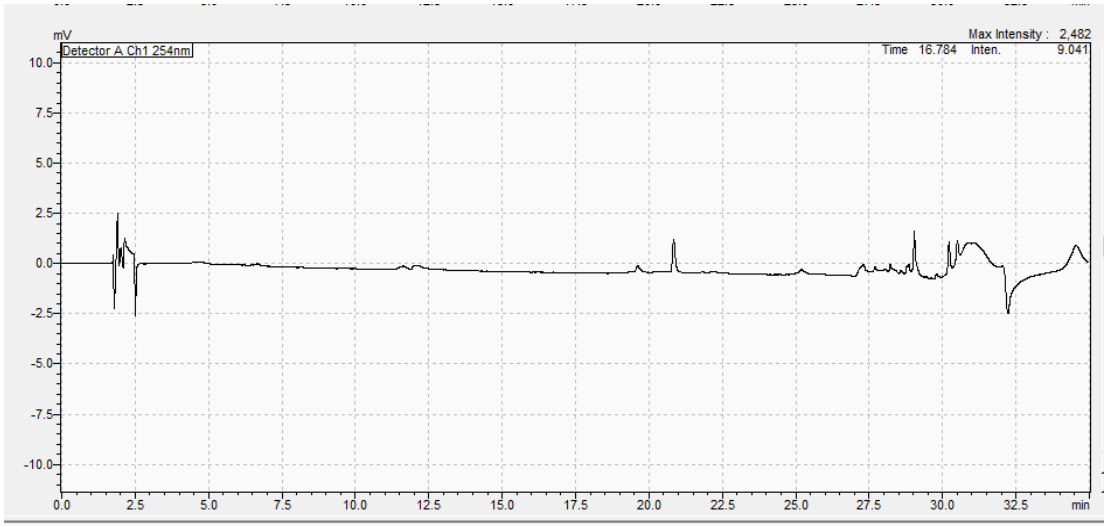


Figure 5.4 HPLC-UV for Blank (methanol) chromatogram

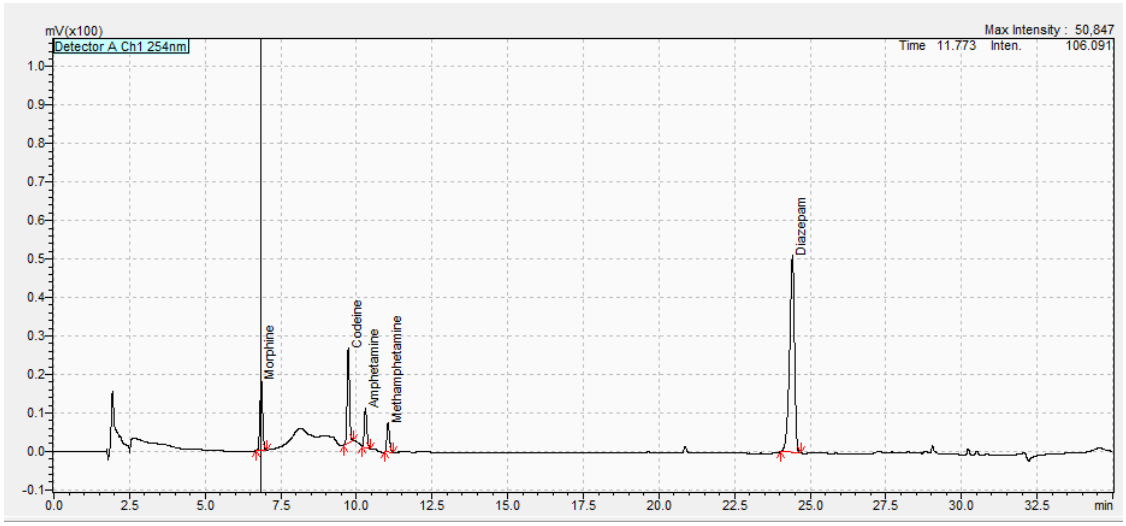


Figure 5.3 HPLC-UV for mixed Standard Solution at 100% level chromatogram

Table 5. 4 Result of Mix Standard solution at 100% level

Name of the Component	Retention Time (min)	Peak Area	Tailing Factor	Capacity Factor (K')	Resolution	Number of theoretical plates
Morphine	6.750	135779	0.944	2.472	--	24051
Codeine	9.642	170281	1.082	3.960	17.851	65440
Amphetamine	10.202	82454	1.324	4.248	3.501	57624
Methamphetamine	10.954	62861	1.240	4.635	4.461	67943
Diazepam	24.377	810170	0.981	11.539	66.269	167759

Basic information about the samples was derived from their structures, such as identifying the type of functional groups present, the nature of the compound, its physicochemical properties, such as lipophilicity, dissociation constant, and molecular weight. This information aids with things like mobile phase and column selection considering factors such as column length, particle size, and internal diameter, as well as determining the suitable wavelength required for the analysis [45,46]. These as well as ICH Q14 guidelines for analytical procedure development were also referred to for method development [44,47]. Drugs such as Amphetamine, Methamphetamine and Morphine are weakly basic, Codeine and Diazepam are amphoteric and neutral, while Pregabalin is acidic based on the functional groups they contain. All the compounds possess a UV chromophore in their structure except for Pregabalin. Therefore, it would

be unsuitable for method development that relies on UV absorption as the sole detection method. Pregabalin was therefore excluded from the HPLC-UV method.

Considering the polarity of the samples, reversed-phase chromatography was deemed to be better suited for the separation of samples. Trials were conducted using acidic mobile phase (0.1% v/v formic acid and 0.1% v/v trifluoroacetic acid (TFA)) with columns packed either with C8 octyl carbon chain and C18 octadecyl carbon chain stationary phase. As the samples are either basic and/or lipophilic in nature, they will either be partially or completely ionized therefore spending less time on the stationary phase. The C8 or C18 carbon chains reduce the polarity of the silanol groups of silica, creating a hydrophobic environment suitable for lipophilic molecules to partition well between the mobile phase and the stationary phase [48,49].

Using 0.1% formic acid in water as mobile phase A and ACN as mobile phase B, separation was achieved but the tailing factor for the Amphetamine and Methamphetamine peaks did not meet the criteria of less than 2. Therefore, mobile phase A was substituted with 0.1% TFA in water with a gradient flow rate of 1.0 mL/min and mobile phase B remained as ACN, affording well-separated peaks with good resolution and better peak shapes.

All samples, except Pregabalin, gave satisfactory UV response at 254 nm.

Considering all these factors, a suitable method was developed for the analysis of the UV active samples and the data obtained from the method gave satisfactory results with good peak shapes, acceptable tailing factors,

resolutions, capacity factor and theoretical plates, which was deemed suitable to proceed for validation.

5.4.3 GC-MS Results

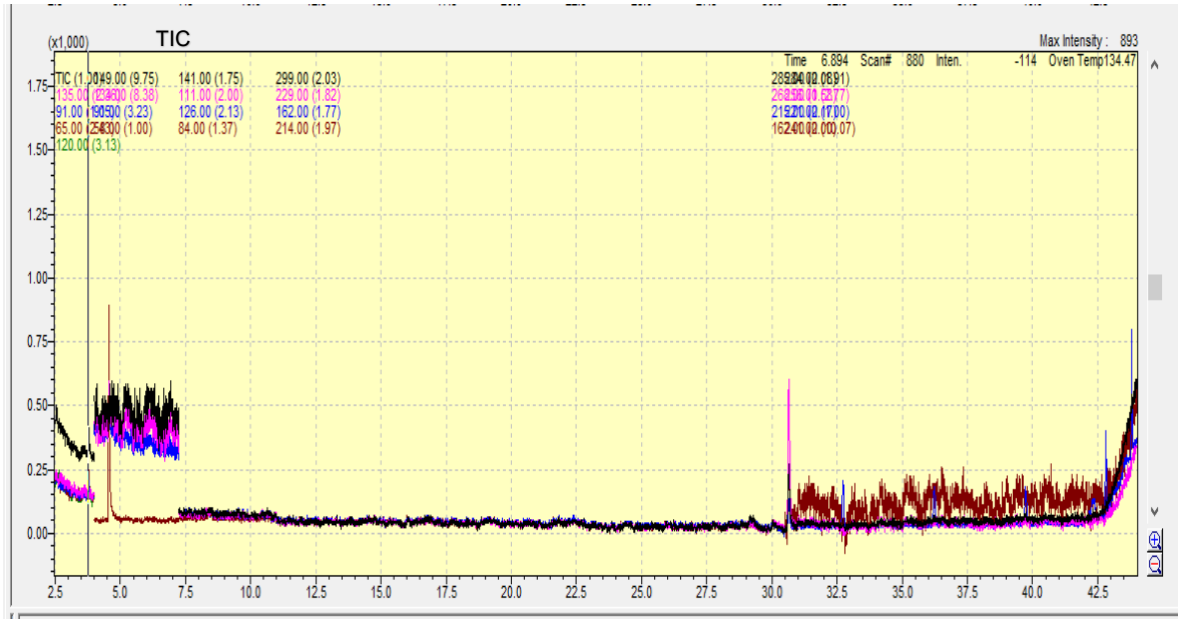


Figure 5.4 GC-MS Blank (ethanol) chromatogram

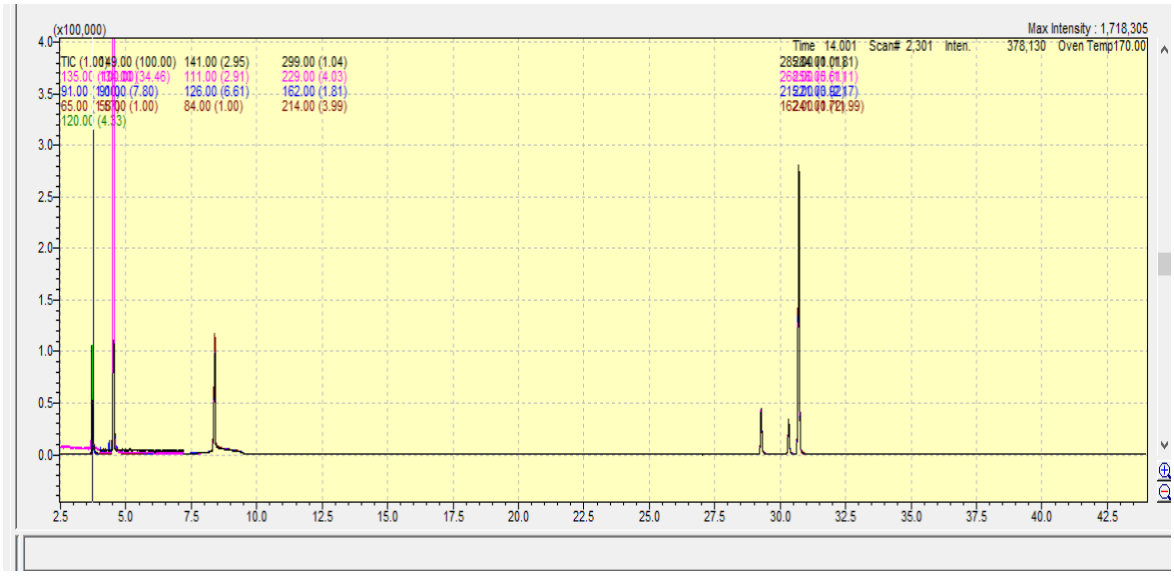


Figure 5.5 GC-MS Mix Standard Solution at 100% level chromatogram

Table 5. 5 Result of Mix Standard solution at 100% level

Name of the Component	Retention Time (min)	Peak Area	m/z
Amphetamine	3.731	608546	91.00
Methamphetamine	4.533	5612508	58.00
Pregabalin	8.399	349701	84.00
Codeine	29.275	159128	299.00
Morphine	30.327	125754	285.00
Diazepam	30.712	943019	256.00

Similarly to the HPLC-UV method above the physicochemical properties of the samples were taken into consideration along with ICH Q14 guidelines for analytical method development [44].

While a UV chromophore is required for detection in the HPLC-UV method, there is no such requirement for GC-MS as the mass spectrometer is the detector in this technique. The primary aim for the switch to the GC-MS was to develop a method suitable for the identification and quantification of all the samples without the requirement for derivatization to aid detection by MS. The initial literature study encompassed the functionalities and molecular weights of all the compounds [50]. Most of the compounds were non-polar, therefore, the SH-I-5MS basic column was selected for the analysis.

Methanol was initially used as a solvent and the development study started by scanning each drug individually to determine its peak intensity response to adjust the concentrations of each of the samples in the mixed standard solution.

The samples were then run again individually in selective-ion monitoring mode (SIM) to identify the fragments of each drug.

After the fragments were confirmed from SIM, a method was developed on the GC-MS to separate all the samples using the temperature gradient program with a column flow rate of 1.37 mL/min on the GC with inputs for exact fragments on the mass spectrometer in the method to capture only the specific fragments associated with the compounds.

The initial development showed a distorted peak shape for pregabalin, with morphine and diazepam fragments co-eluting. Ethanol was then substituted as the diluent in place of methanol to resolve the issues with the peak shape and the gradient temperature was optimised to achieve a better resolution between morphine and diazepam.

5.5 Validation Results for HPLC and GC-MS

Validation was performed as per ICH Guidelines Q2(R2).

5.5.1 HPLC Validation Results

5.5.1.1 Specificity and Selectivity:

Evaluation of interference from closely eluting peaks was done by retention time from the individual and mixed standard solutions and compared with blank solutions.

Table 5. 6 Results of Specificity and Selectivity

Name of the Component	Peak Area	Retention Time (min)	Acceptance Criteria [51,52]
Methanol Blank Solution			No interference shall be observed at the retention time of analyte peak from blank or any other contaminant.
Morphine	Not Detected	Not Detected	
Codeine			
Amphetamine			
Methamphetamine			
Diazepam			
Individual Solution at 100 % level			
Morphine	107761	6.670	
Codeine	123327	9.578	
Amphetamine	65385	10.107	
Methamphetamine	36574	10.865	
Diazepam	823456	24.436	
Mix Standard Solution at 100 % level			
Morphine	135779	6.750	
Codeine	170281	9.642	
Amphetamine	82454	10.202	
Methamphetamine	62861	10.954	
Diazepam	810170	24.377	

5.5.1.2 **Linearity and Range**

A series of mix standard solutions was prepared for a range of 1 – 250 µg/mL and analysed 3 times at each concentration. The correlation coefficient (r^2) was determined by plotting mean peak area on 'Y axis' against the concentration in µg/mL on 'X axis', which was then used to establish the linear range of method.

Table 5. 7 Results of Linearity and Range

Name of the Component	Correlation Coefficient (r^2)	Acceptance Criteria [51]
Morphine	0.9908	Correlation coefficient (r^2) should not be less than 0.99
Codeine	0.9925	
Amphetamine	0.9927	
Methamphetamine	0.9911	
Diazepam	0.9990	

Calculations: Linearity calculation for morphine

1. Below observed mean peak areas for linear increment in morphine concentration.

Table 5. 8 Mean peak areas for linear increment in morphine concentration

Concentration in PPM	Peak area response
50	47648
100	85682
150	123942
200	176555
250	233078

2. Concentration vs Peak area response scatter graph was plotted to check the linearity.

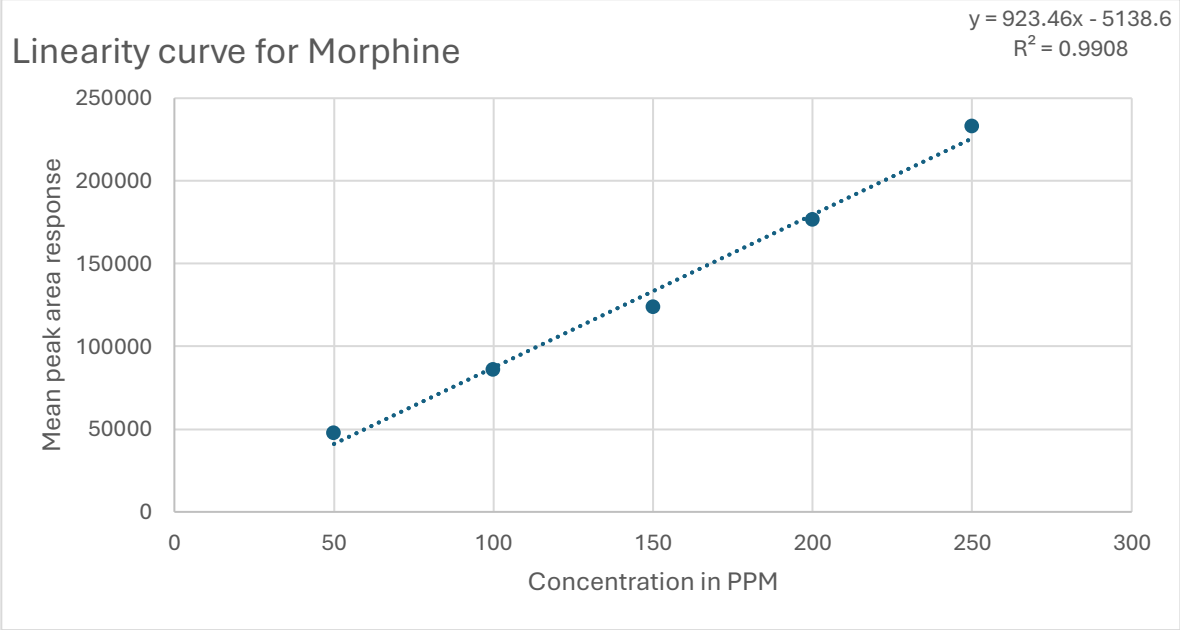


Figure 5.6 Linearity curve for morphine

5.5.1.3 **Precision (Repeatability)**

The reproducibility was evaluated by analysing mix standard solution at 100% level (n=6) and its %RSD was determined.

Table 5. 9 Results of Repeatability

Name of the Component	%RSD	Acceptance Criteria [51]
Morphine	0.734	The %RSD should not be more than15.0%.
Codeine	0.929	
Amphetamine	0.805	
Methamphetamine	0.859	
Diazepam	0.613	

5.5.1.4 Intermediate Precision:

The reproducibility was evaluated by analysing mix standard solution at 100% level for six times at different day and system and its %RSD was determined.

Table 5. 10 Results of Intermediate Precision

Name of the Component	%RSD	Acceptance Criteria [51]
Morphine	0.749	The %RSD should not be more than15.0%.
Codeine	1.345	
Amphetamine	2.750	
Methamphetamine	0.594	
Diazepam	0.763	

5.5.1.5 **Accuracy:**

Accuracy was evaluated utilising the data collected for linearity and predicted concentrations were calculated through linear regression. The data generated was then compared with actual concentration for % accuracy.

Table 5. 11 Results of %Accuracy

Name of Compound	%Accuracy	
	Mean	%RSD
Morphine	101.5	7.88
Codeine	103.6	16.73
Amphetamine	100.8	5.12
Methamphetamine	101.1	7.66
Diazepam	92.3	17.84
Acceptance Criteria [51]	The mean % Accuracy should be within 80.0-120.0%,	

5.5.1.6 **Robustness:**

The robustness of the protocol was evaluated by variations to the flow rate (± 0.5mL/min), column temperature (± 2°C), and wavelength (± 2 nm). Also, by injecting mix standard solutions at 100% level (150 PPM each for Morphine, Amphetamine, Methamphetamine, 100 PPM for Codeine and 10 PPM for Diazepam) for 6 repeats and the %RSD was determined

Table 5. 12 Results of Robustness

Name of the Component	%RSD						Acceptance Criteria [51]
	Flow Rate (ml/min)		Col Temp (°C)		Wavelength		
	0.95	**1.05**	**23**	**27**	**252**	**256**	
Morphine	2.37	0.31	0.05	0.84	0.67	0.88	The %RSD should not be more than15.0%.
Codeine	2.21	0.88	0.48	1.03	1.30	6.61	
Amphetamine	5.45	0.92	3.55	2.09	1.26	5.30	
Methamphetamine	4.28	0.17	0.48	0.40	0.70	4.46	
Diazepam	2.64	0.11	0.40	0.41	0.40	2.25	

5.5.1.7 **Limit of Detection (LOD) and Limit of Quantification (LOQ):**

LOD: The lowest concentration of an analyte that can be detected at an Signal-to-noise (S/N) ratio of 3:1. LOD = 3.3 × SD / S

SD = Standard Deviation

S = Slope

LOQ: The lowest concentration of an analyte which can be quantitatively determined at an Signal-to-noise (S/N) ratio of 10:1. LOD = 10 × SD / S

Table 5. 13 Calculated Values of LOD and LOQ

Name of the Component	LOD	LOQ
	ng/mL	ng/mL
Morphine	0.3	0.9
Codeine	0.4	1.1
Amphetamine	0.4	1.2
Methamphetamine	0.7	2.2
Diazepam	0.0074	0.023

The validation process was guided by ICH Guidelines Q2(R2), according to ICH Guidelines Q2(R2). A validation study is designed to provide sufficient evidence that the analytical procedure meets its objectives. These objectives are described with a suitable set of performance characteristics and related performance criteria, which can vary depending on the intended purpose of the analytical procedure and the specific technology selected and capable of producing acceptable results consistently over different time intervals or days and across different locations [52].

The results obtained were deemed to be satisfactory and are discussed below.

No peaks were observed at the retention times of sample peaks in blank methanol; hence it was considered that analyte peaks had no interference. All the sample peaks were clear, distinct sharp well resolved and were confirmed by their retention time by injecting individual and mix standard sample solutions.

The data represented a linear correlation between the mean peak area and concentration in µg/mL over the selected linearity range, the Correlation coefficient (r^2) obtained for all the samples were found to be greater than 0.99 which is an indication that the method is linear over the range.

The repeatability test and intermediate precision tests were carried out on different days. The %RSD obtained (n=6) was less than 15.0% which indicates that method is reliable, reproducible and precise.

Accuracy was determined through the generated linearity data using a calibration curve. The concentration was recalculated using the peak areas and predicted concentrations were compared with the actual concentrations. The % accuracy obtained for all the samples at each level were well within the limit of 80.0-120.0% and % RSD with the exception of Codeine and Diazepam were well within 15.0% respectively, overall, the method was deemed to be precise and accurate.

Diazepam and codeine had one outlier value which was different while calculating the mean for accuracy for lower concentrations i.e. 1 PPM for diazepam and 25 PPM for codeine this could be due to errors while dealing with such low quantities during preparation of the sample solutions and dilution or the sensitivity of the method at such low concentrations.

The robustness of the method was verified by changing various chromatographic parameters such as flow rate, column temperature and wavelength and observed by performing minor deliberate variations. From the results it was identified that the %RSD of all the samples were well within the

limit of not more than 15.0%, which implies that the method is robust and not impacted by slight variations.

5.5.2 GC-MS Validation Results

5.5.2.1 Specificity and Selectivity:

Evaluation for the interference from closely eluting peaks was done from retention time from individual and mix standard solutions were compared with blank solutions.

Table 5. 14 Results of Specificity and Selectivity

Name of the Component	Peak Area	Retention Time (min)	Acceptance Criteria [51,52]
Ethanol Blank Solution			No interference shall be observed at the retention time of analyte peak from blank or any other contaminant.
Amphetamine	Not Detected	Not Detected	
Methamphetamine			
Pregabalin			
Codeine			
Morphine			
Diazepam			
Individual Solution at 100 % level			
Amphetamine	381241	3.703	
Methamphetamine	3641777	4.574	
Pregabalin	106481	8.335	

Codeine	139663	29.238	
Morphine	105966	30.272	
Diazepam	270149	30.668	
Mix Standard Solution at 100 % level			
Amphetamine	407340	3.719	
Methamphetamine	4236648	4.512	
Pregabalin	157144	8.358	
Codeine	206597	29.260	
Morphine	160835	30.307	
Diazepam	509706	30.689	

5.5.2.2 **Linearity and Range:**

A series of mix standard solution were prepared for a range of 1 – 200 µg/mL, analysed (n=3) at each concentration. The correlation coefficient (r^2) was determined by plotting mean peak area on 'Y axis' against the concentration in µg/mL on 'X axis' which was used to establish the linear range of method.

Table 5. 15 Results of Linearity and Range

Name of the Component	**Correlation Coefficient (r^2)**	**Acceptance Criteria** [51]
Amphetamine	0.9957	Correlation coefficient (r^2) should not be less than 0.99
Methamphetamine	0.9905	
Pregabalin	0.9385	
Codeine	0.9752	
Morphine	0.9929	
Diazepam	0.9987	

5.5.2.3 Precision (Repeatability):

The reproducibility was evaluated by analysing mix standard solutions at 100% level for six repeats and the %RSD determined.

Table 5. 16 Results of Repeatability

Name of the Component	%RSD	Acceptance Criteria [51]
Amphetamine	4.822	The %RSD should not be more than15.0%.
Methamphetamine	5.462	
Pregabalin	8.229	
Codeine	14.931	
Morphine	23.653	
Diazepam	6.292	

5.5.2.4 Accuracy:

The accuracy of the protocol was evaluated through data collected for linearity and predicted concentrations were calculated through linear regression. The data generated was then compared with actual concentrations for % accuracy.

Table 5. 17 Results of %Accuracy

Name of Compound	%Accuracy	
	Mean	%RSD
Amphetamine	103.5	14.2
Methamphetamine	103.6	16.0
Pregabalin	108.0	27.7
Codeine	110.8	30.7
Morphine	102.4	15.3
Diazepam	105.9	17.5
Acceptance Criteria [51]	The mean % Accuracy should be within 80.0-120.0%,	

ICH guideline Q2(R2) validation for analytical procedures were followed for validating the method and the obtained results are discussed below.

No peaks were observed at retention times of the sample peaks in the blank ethanol; therefore, it was concluded that the sample peaks had no interference. All the analyte peaks were clear, distinct, sharp, well resolved and were confirmed by their retention times by the injection of individual and mixed standard solutions.

The data displayed a linear correlation between the mean peak areas and concentrations in µg/mL over the selected linearity range. The Correlation coefficient (r^2) obtained for all the samples was found to be greater than 0.99 except for codeine and pregabalin which indicated that the method could still benefit from optimisation, or this might be due to errors in dilution or variability in the injections.

Repeatability tests were carried out and the %RSD obtained for (n=6) was less than 15.0%, except for morphine, whose % RSD was more than 20%.

Accuracy was determined by the generated linearity data using calibration curves. The concentrations were recalculated using the peak areas and predicted concentrations were compared with the actual concentrations. The % accuracy obtained for all the samples at each concentration was well within the limits of 80.0-120.0%, however, the % RSDs did not meet the standard for any of the samples.

The results obtained from the validation of the GC method suggest that either the method is not yet optimised, there were some problems due to human error while preparing the GC samples, or some instrument malfunction, i.e. injection error occurred, which still needs to be ironed out. However, due to instrument issues and time constraints, some parameters, such as LOD, LOQ and repetitions to resolve the questions raised by some of the erratic results obtained to identify the exact issues

with the method were not carried out. Due to time constraints, I was unable to complete the full validation of the method or obtain the desired results. The limited time available in the lab prevented me from running the necessary tests and repetitions to resolve the inconsistencies in the data. This issue, however, has been identified as a priority for future work. With more time, I would be able to re-optimize the method, address the instrument-related challenges, and conduct further experiments to achieve more reliable and accurate results. I am confident that with additional time, the full validation process can be completed, and the remaining challenges can be effectively resolved.

Conclusion

The study successfully developed and validated an HPLC-UV method for analysing a range of pharmaceutical compounds often abused for illicit use. A promising GC-MS method for analysing a range of broader range of pharmaceutical compounds, which would enable the analysis on both UV active and samples and samples which do not contain a UV chromophore, still requires a bit more work to complete its validation.

Overall, while the HPLC method achieved satisfactory outcomes, while the GC-MS method requires additional work.

Chapter 6 Conclusion and Future Work

6.1. Conclusion

A robust and validated method for the analysis of benzodiazepines (BDZs) was developed and applied in a stability-indicating assay for five representative BDZs—alprazolam, bromazepam, clonazepam, diazepam, and flunitrazepam—chosen for their prevalence in the Kuwaiti market. The results of the stability assay indicated that samples should be stored under refrigerated conditions and used within three weeks of preparation, as degradation was detected in some cases during analysis.

A fast, accurate, and reliable protocol for the detection of BDZs using both HPLC and HPLC-MS was also successfully developed and validated in accordance with ICH guidelines. This method was applied to the analysis of 48 urine samples obtained in Kuwait in 2022 by law enforcement from individuals under investigation for suspected drug abuse. BDZs were detected in 93.7% of the samples, including the detection of flunitrazepam, demonstrating the method's suitability for routine forensic urine screening in Kuwait.

Additionally, a sensitive and specific HPLC-UV method was developed to separate six commonly abused drugs in Kuwait: morphine (MOR), amphetamine (AMP), methamphetamine (MAMP), codeine (COD), diazepam (DZP), and pregabalin (PGB). However, due to the absence of a UV chromophore in pregabalin, it was excluded from the HPLC-UV method. To address this limitation, A complementary GC–MS method was developed and partially validated, achieving successful separation of all six drugs, including pregabalin. While the method showed promising performance, full validation was not completed, and certain parameters, such as LOD and LOQ, did not meet ICH guideline requirements. Further optimization and repeated validation are therefore required to fully comply

with ICH guidelines and ensure acceptance for routine forensic and toxicological applications.

During the COVID-19 lockdown and the resulting travel restrictions, additional research activities were conducted at Kuwait University, focusing on the development and validation of an HPLC method for a novel oxazolidinone compound (PH-192) with anticonvulsant activity. This work culminated in a publication in *Molecules* titled:

"Development and Validation of Stability-Indicating Assay Method for a Novel Oxazolidinone (PH-192) with Anticonvulsant Activity by Using UHPLC-QToF-MS."

The investigation of PH-192 involved subjecting it to various conditions, with a primary focus on its degradation process in human plasma. As part of its potential application for the treatment of seizures, PH-192 would interact with blood plasma upon administration via injection. This test was designed to evaluate how the drug would react when exposed to blood. Human plasma, free of any drugs, was sourced from a blood bank in Kuwait. PH-192 samples were mixed with aliquots of this plasma and incubated at 37°C for 90 minutes, after which extraction and analysis were performed using LC-MS. The results showed that PH-192 remained stable in human plasma for up to 90 minutes at 37°C, providing insight into its stability under conditions that mimic the human body.

6.2. Future Work

The developed LC-MS/MS methods for the screening of illicit drugs will be extended to include the analysis of target substances in various biological matrices such as blood, urine, saliva, and hair. These extended methods will be employed to analyse

samples collected in Kuwait, aiming to provide insights into the trends and evolution of illicit drug abuse within the country.

The optimized and validated methods will further be utilized for the analysis of benzodiazepines from various sources in top of the urine sample . Real blood samples obtained from volunteers will be analysed to assess the performance and reliability of the method in practical scenarios.

Future work will also focus on the validation of a GC-MS method for the detection and quantification including amphetamine, methamphetamine, morphine, codeine, diazepam, and pregabalin Due to instrument-related challenges and time constraints, critical validation parameters—such as the determination of the limit of detection (LOD), limit of quantification (LOQ), and repeatability studies—were not completed. These steps are essential for identifying and resolving the erratic results observed during the initial validation phase. Future work should focus on addressing these limitations to ensure the robustness and reliability of the GC method. Additionally, the method will be adapted to detect other emerging illicit drugs found in the Kuwaiti market. Enhancements to the method will include procedures for blood sample extraction to improve its applicability for forensic and clinical toxicology.

Appendices

Articles

1. Naser F. Al-Tannak [1], Oludotun A. Phillips [1], **Husein Kamal** [1] and Ahmed Hemdan[2], *Development and Validation of Stability-Indicating Assay Method for a Novel Oxazolidinone (PH-192) with Anticonvulsant Activity by Using UHPLC-QToF-MS*,

Molecules 2022, 27(3), 1090

2. **Husein Kamal** [1], Varun Gandhi [1], Lina Akil [1], Naser Al-Tannak[2], Nicholas Rattray [1] and Ibrahim Khadra [1],

Development and validation of an LC-MS/MS method for the simultaneous determination of Alprazolam, Bromazepam, Clonazepam, Diazepam and Flunitrazepam in human urine and its application to samples from suspected drug abusers.

Molecules-3763522

Conferences

1. Strathclyde Institute of Pharmacy & Biomedical Sciences (SIPBS) Research Day, February 2023**. (Poster presentation)**

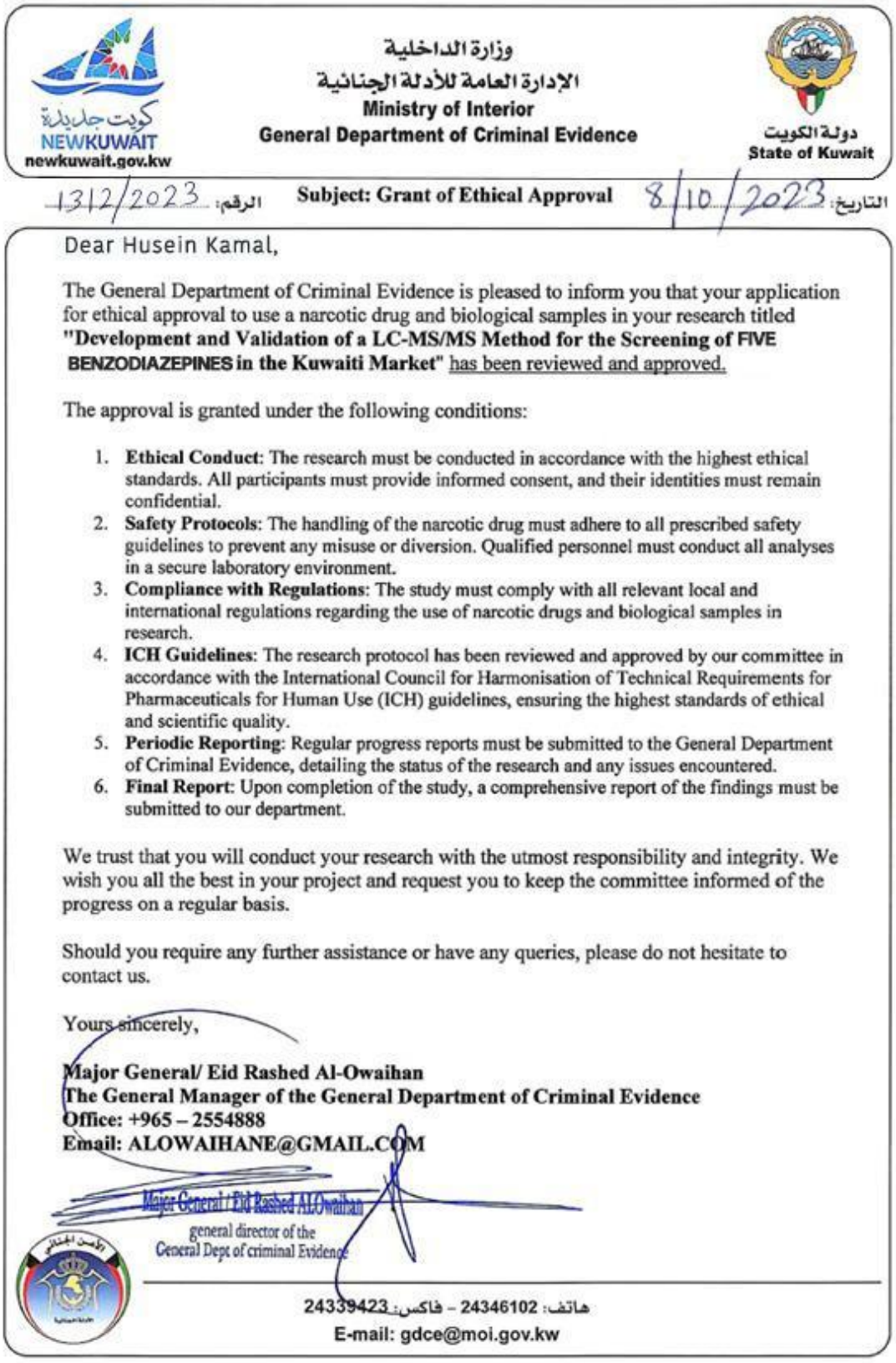
   Husein Kamal, Lina Akil, Nicholas Rattray, Ibrahim Khadra. IDENTIFICATION AND QUALIFICATION OF SOME BENZODIAZEPINES COMPOUND IN WHOLE BLOOD SAMPLE IN CRIME SCENE USING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

2. INTERNATIONAL CONFERENCE ON FORENSIC ANALYTICAL CHEMISTRY, CHEMOMETRICS AND STATISTICS (ICFACCS-24**,** ALEXANDRIA, EGYPT, January 2024 **. (Poster presentation)**

   Husein Kamal, Lina Akil, Nicholas Rattray, Ibrahim Khadra. IDENTIFICATION AND QUALIFICATION OF 6 BENZODIAZEPINES FROM HUMAN URINE SAMPLE VIA HIGH PRESSURE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

3. Participated in the SIPBS outreach activity “SIPBS Open Doors Day – Glasgow Science Festival 2024” June 2024

Ethical Approval for Chapter 2



وزارة الداخلية
الإدارة العامة للأدلة الجنائية
Ministry of Interior
General Department of Criminal Evidence

كويت جديدة
NEWKUWAIT
newkuwait.gov.kw

دولة الكويت
State of Kuwait

الرقم: 1312/2023 Subject: Grant of Ethical Approval التاريخ: 8/10/2023

Dear Husein Kamal,

The General Department of Criminal Evidence is pleased to inform you that your application for ethical approval to use a narcotic drug and biological samples in your research titled **"Development and Validation of a LC-MS/MS Method for the Screening of FIVE BENZODIAZEPINES in the Kuwaiti Market"** has been reviewed and approved.

The approval is granted under the following conditions:

1. **Ethical Conduct**: The research must be conducted in accordance with the highest ethical standards. All participants must provide informed consent, and their identities must remain confidential.
2. **Safety Protocols**: The handling of the narcotic drug must adhere to all prescribed safety guidelines to prevent any misuse or diversion. Qualified personnel must conduct all analyses in a secure laboratory environment.
3. **Compliance with Regulations**: The study must comply with all relevant local and international regulations regarding the use of narcotic drugs and biological samples in research.
4. **ICH Guidelines**: The research protocol has been reviewed and approved by our committee in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, ensuring the highest standards of ethical and scientific quality.
5. **Periodic Reporting**: Regular progress reports must be submitted to the General Department of Criminal Evidence, detailing the status of the research and any issues encountered.
6. **Final Report**: Upon completion of the study, a comprehensive report of the findings must be submitted to our department.

We trust that you will conduct your research with the utmost responsibility and integrity. We wish you all the best in your project and request you to keep the committee informed of the progress on a regular basis.

Should you require any further assistance or have any queries, please do not hesitate to contact us.

Yours sincerely,

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