University of Glasgow

STABILITY OF DRUGS AND PESTICIDES OF FORENSIC TOXICOLOGICAL INTEREST AND THEIR METABOLITES IN BIOLOGICAL SAMPLES

Thesis submitted in Accordance with Requirements of the University of Glasgow for the Degree of Doctor of Philosophy

by

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Table of Contents

List of Tables

List of Figures

Summary

Loss of analyte from biological samples during the post-mortem interval or during storage has potentially serious implications in forensic toxicology and represents a challenge for the forensic toxicologist, especially in the interpretation of case results. The initial aim of the studies in this thesis was to evaluate the stability of some important drugs and compounds in blood under different storage conditions in order to optimize the preservation of these compounds. A second aim was to evaluate a new method of stabilizing these compounds in blood by storing them as dried blood spots on filter paper. The third aim was to investigate methods by which corrections could be made for analyte losses based on quantification of their degradation products, which would serve as markers of the former presence of the compounds even if they were no longer detectable.

The background to toxicology and its classification systems is reviewed along with the most common areas of application, including forensic toxicology. Details are given of the most commonly-used matrices and of current problems facing forensic toxicologists, particularly the problem of analyte instability. The literature concerning stability of drugs and pesticides in biological samples are reviewed and discussed as well as methods applied to enhance and stabilize analytes for long storage periods. Background is provided on methodologies used in the work reported in this thesis, including extraction techniques and instrumental analysis by LC-MS/MS and GC/MS. Also, because of its importance in forensic toxicology at present validation procedures and requirements are also discussed.

An initial study was made of drug stability during storage in blood samples for 1 year under conventional laboratory conditions using selected drugs from the benzodiazepine group, alprazolam, lorazepam, oxazepam and estazolam. Blank blood containing these drugs at low and high concentrations was stored in tubes at -20° C, 4°C and room temperature. Half of the tubes contained fluoride-oxalate preservative. Blood samples were analysed on the first (day zero), second and fourth days, and after one week, two weeks, one month, two months, three months, six months and one year using a method which was developed and validated for this study based on solid phase extraction (SPE) and

liquid chromatography-tandem mass spectrometry (LC-MS/MS). Alprazolam and estazolam were stable at -20° C and 4° C, but decreased by almost 10% at room temperature (RT) at both concentrations. Lorazepam and oxazepam were stable at -20°C but were poorly stable at 4° C and decreased by 100% at RT by the end of the 1 year period. Sodium fluoride stabilised the drugs by approximately 13% compared to unpreserved samples. The long-term stability of alprazolam and estazolam is attributed to the presence of the trizolo ring in their structures which makes the compounds more resistant to hydrolysis, the most prominent degradation reaction affecting benzodiazepines.

A similar study was performed on the stability of morphine-3- and 6-glucuronide and codeine-6-glucuronide in blood and urine under the same storage conditions. These compounds were stable at -20° C, losing less than 7% but losses were higher at 4° C, up to 18% in blood and 28% in urine, and at room temperature up to 54% in blood and 78% in urine after 1 year. Sodium fluoride did not have a significant effect (<10% increase in stability).

An investigation was carried out on stabilisation of hydrolytically-labile benzodiazepines and cocaine in blood during storage as dried blood spots (DBS) on filter paper. An analytical method was developed and validated for this study based on SPE and LC-MS/MS analysis. The drugs selected were flunitrazepam, temazepam, oxazepam, lorazepam, nitrazepam, diazepam and cocaine. Blood spots (100 µl blood) on Guthrie card 903 containing the drugs at 1000 ng/ml were dried overnight at RT. Spots were cut out and extracted with buffer (pH 6), which was analysed with the validated method. DBS were stored in duplicate at RT, 4°C and -20°C for up to one year. Degradation of the drugs in DBS in all storage conditions was less than for the corresponding liquid blood samples stored under similar conditions. More than 80% of each analyte could be recovered from DBS after one month while 15 % cocaine and 74 % of the benzodiazepines were recovered after 1 year under all conditions.

The degradation of diazepam, temazepam, chlorodiazepoxide and oxazepam by hydrolysis was studied over a 1 month period under conditions designed to accelerate the reaction (80 °C, pH 2 and 12) and the hydrolysis products 2-methylamino 5-chlorobenzophenone (MACB) and 2-amino 5-chlorobenzophenone (ACB) were analysed by a method based on SPE and LC-MS/MS which was

developed and validated for this study. MACB and ACB in whole blood and urine were evaluated as indicators of the original drug concentrations. Blank blood and urine containing these compounds at 1000 ng/ml stored at high temperature (80°C) and under acidic (pH 2) and basic (pH 12) conditions at room temperature for one month. The samples were analyzed in duplicate at days 1, 2, 4, 7, 14 and 30. MACB and ACB were the main hydrolysis products and their concentrations increased as degradation of the drugs proceeded. They could be detected when the starting materials had completely disappeared. However, MACB and ACB were found to be further degraded under some of the conditions used and a further study was made of the conversion of MACB to ACB. It was concluded that the drugs studied were more sensitive to alkaline pH than to acidic pH or high temperature and that MACB and ACB can be used to confirm the original presence of these drugs in samples, especially when they have decomposed due to poor or prolonged storage conditions.

A final study was made of organophosphates (OPs) and their dialkylphosphate (DAP) hydrolysis products. A new method was developed and validated for analysis of OPs and DAPs in blood samples based on SPE and GCMS after derivatization with N-*tert*-butyldimethylsilyl-N-methyltrifluroacetamide. The influence of sodium fluoride preservative and storage as DBS on filter paper on the stability of OPs in blood was assessed over a 3 day period at RT. With preservative, DAPs concentrations increased as degradation of the OPs proceeded and they could be detected when the parent compounds had completely disappeared. OPs in DBS showed good stability in comparison to liquid blood samples containing NAF and the parent compounds were detected at the end of the observation period.

It was concluded that careful attention should be given to the storage of samples to avoid loss of analyte and erroneous interpretation of results. DBS could be an effective and inexpensive way of increasing analyte retention but routine use of preservatives without evaluation of their effects is discouraged, as these may accelerate loss of analyte.

Abbreviations

AB	2-Aminobenzophenone
ACB	2-Amino-5-chlorobenzophenone
ADB	2-Amino-2`, 5-dichlorobenzophenone
AEME	Anhydroecgonine Methyl Ester
ANB	2-Amino-5-nitrobenzophenone
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Ionization
BBB	Blood Brain Barrier
BZE	Benzoylecgonine
C6G	Codeine-6-glucuronide
CI	Cemichal Ionization
CNS	Central Nervous System
DAP	Dialkylphosphate
DBS	Dried Blood Spot
DEP	Diethylphosphate
DETP	Diethylthiophosphate
DFSA	Drug Facilitated Sexual Assault
DI	Deionized Water
DMP	Dimethylphosphate
DNA	Deoxyribonuclic Acid
EI	Electron Impact
EME	Anhydroecgonine Methyl Ester

ESI	Electrospray Ionization
HPLC	High Pressure Liquid Chromatography
GABA	Gamma-aminobutyric Acid
GC	Gas Chromatography
GCMS	Gas Chromatography Mass spectrometry
LC	Liquid Chromatography
LCMSMS	Liquid Chromatography Tandem Spectrometry
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LLOQ	Lower Limit of Quantitation
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
MACB	2-Methyl-5-chlorobenzophenone
MBSTFA	N-*tert*-Butyl-Dimethylsilyl-N-Methyl-Trifluoracetamide
MS	Mass Spectrometry
NaF	Sodium Flouride
NaN_3	Sodium Azide
NP	Normal Phase
OP	Organophosphate
P	Propability Value
pH	Negative log of the hydrogen ion concentration
Pka	pH at which 50% of anlyte is ionized and 50% is non-ionized

R^2	Linear Correlation Coefficient
RF	Radiofrequency
RP	Reversed Phase
RSD	Relative Standard Deviation
RT	Room Temperature
SAX	Strong Anion Exchange
SCX	Strong Cation Exchnage
SD	Standard Deviation
SIM	Selected ion monitorng
SOD	Sodium Metabisulfite
SPE	Solid phas Extraction
TBDMCS	Tert-Butyl-Dimethylchlorosilane

1 Introduction

1.1.1 Toxicology

Figure 1-1: Ebers papyrus

Toxicology is the study of the effects of poisons on living organisms. A poison or toxicant is any substance that causes illness or death when taken into the body.[1] Toxicology has a long history. The following are some of the prominent events in history. Even primitive man who was aware of the toxicity of plants and animals used poisons as a weapon, for suicide and for political assassination. The Ebers papyrus shown in Figure 1-1, written in 1500 BC, is one of the oldest preserved medical documents anywhere. It contains information about aconite, which was used by the Chinese as an arrow poison. Later, Socrates was condemned to commit suicide by drinking conium (hemlock), which was the poison of the Greeks, while Hindus used opium as both a poison and an antidote.[2]

The Romanian king Mithridates was so afraid of poisoning that he ingested a mixture of more than 36 antidotes in order to protect himself against it. However, his plan backfired when he was unable to commit suicide after being captured by his enemies because of the powerful antidotes he used.[1,2]

Paracelsus (1493-1541) was the first to discover the relationship between a dose and the body's reaction to it, which is now known as the dose-response relationship and most of pharmacology, toxicology and therapeutics are based on this. Paracelsus said that "all substances are poisons; there is none which is not

a poison. The right dose differentiates a poison from a remedy." We now understand that any compound when taken in a large enough dosage may cause a harmful effect. Even water, which is known as a very safe compound and the single largest constituent of the body, can cause a lethal electrolyte imbalance if a person swallows enough of it. However, small amounts of toxic substances such as cyanide and arsenic when ingested from food or inhaled from the air will not cause fatal toxicity.[1]

There are many types of toxic substances, which are illustrated in Figure 1-2. Toxicology can be categorized in three branches: environmental, clinical and forensic. Environmental toxicology is concerned with the harmful effect of industrial chemicals released into the atmosphere. Clinical toxicology is concerned with the adverse effects of drugs that are given intentionally for therapeutic purposes. Details about forensic toxicology are provided in the next section.

1.1.2. Forensic toxicology: an introduction

Forensic toxicology is the use of analytical chemistry, pharmacology, and toxicology to aid the medicolegal investigation of the effects of a poison. Orfila (1787-1853) is considered to be the first toxicologist who used chemical analysis of autopsy specimens to prove the presence of a poison. Therefore the forensic toxicologist should improve and develop methods to determine toxic compounds, decide which specimens are suitable for analysis, apply these methods to the samples and interpret the results obtained. Forensic toxicology can be divided into three subfields, which are discussed in the following sections.[3,4]

1.1.2.1. Post-mortem forensic toxicology

Many forensic toxicology cases are investigations of deaths by poisoning. Many of these cases deal with children who accidentally ingested a poisonous substance that was not safely stored, such as household materials, drugs, pesticides and detergents. Sometimes adults are also accidentally poisoned when a toxic substance is mislabelled or when a worker is exposed to a toxic substance due to lack of workplace safety. Concurrent use of two or more drugs may lead to drug

interactions when one drug elevates the concentration of another beyond the therapeutic level.[3]

Death can also result if a person ingests an illicit or controlled substance while seeking euphoria. People who use drugs in this way build up a tolerance to it, so they increase the dose to reach the desired effect, and death can occur as a result of toxicity.[5]

In addition, a person who is depressed or has another psychological disorder may commit suicide by ingesting a toxic substance, traditionally poisons such as cyanide, arsenic, or carbon monoxide. Recent research shows that most suicides today use prescribed medicine in high doses or a mixture of prescribed drugs.[5]

Death due to the ingestion of poison administered by another person is called homicide, and it is rarely seen by forensic toxicologists today; however, it has been reported in post-mortem cases.[3]

The forensic toxicologist plays a very important role in post-mortem cases confirming the presence or absence of a toxic compound and selecting a suitable specimen in which to quantify the amount of poison in order to interpret the cause of death.[3,4]

1.1.2.2. Human performance toxicology

This field is related to psychology and is concerned with drugs that can alter human behaviour by stimulating or inhibiting performance. Most toxicologists in this field are interested in drugs that impair performance including stimulants, because chronic use of stimulants results in deficiency of motor and cognitive performance. Such impairment will lead to the inhibition of a person's ability to perform skills requiring full attention such as operating machinery in industry or driving a vehicle. Besides illicit drugs such as opiates, cannabis and hallucinogens, legal drugs like antidepressants and benzodiazepines that exert an inhibitory effect on the central nervous system (CNS) have been reported in previous studies as the cause of automobile accidents.[6,7] Moreover, researchers have reported that benzodiazepines are used by rapists to facilitate sexual assault, especially when the victim takes them with alcohol, which enhances their effect.[8] Alcohol is the most common compound impairing human

performance, and its use is reported in connection with many driving accidents. Therefore many countries have prohibited its use by drivers and those operating machines.[4]

1.1.2.3. Forensic drug testing

Drug abuse has increased as a result of social or personal problems such as feelings of failure in education, on the job, or in family life, which may lead to depression and dysphoria and cause a person to seek euphoria by using drugs. Other factors include the availability of free time, ease of obtaining drugs, as well as curiosity to learn more and experience new feelings.[9] Since drug abuse may affect the combat readiness of the armed forces, cause accidents in the industrial and transportation sectors, and are used as performance enhancements in sports, drug testing is increasingly used in the military, criminal justice, public sector, and sports.[4,10]

1.2 Drugs and poisons – trends

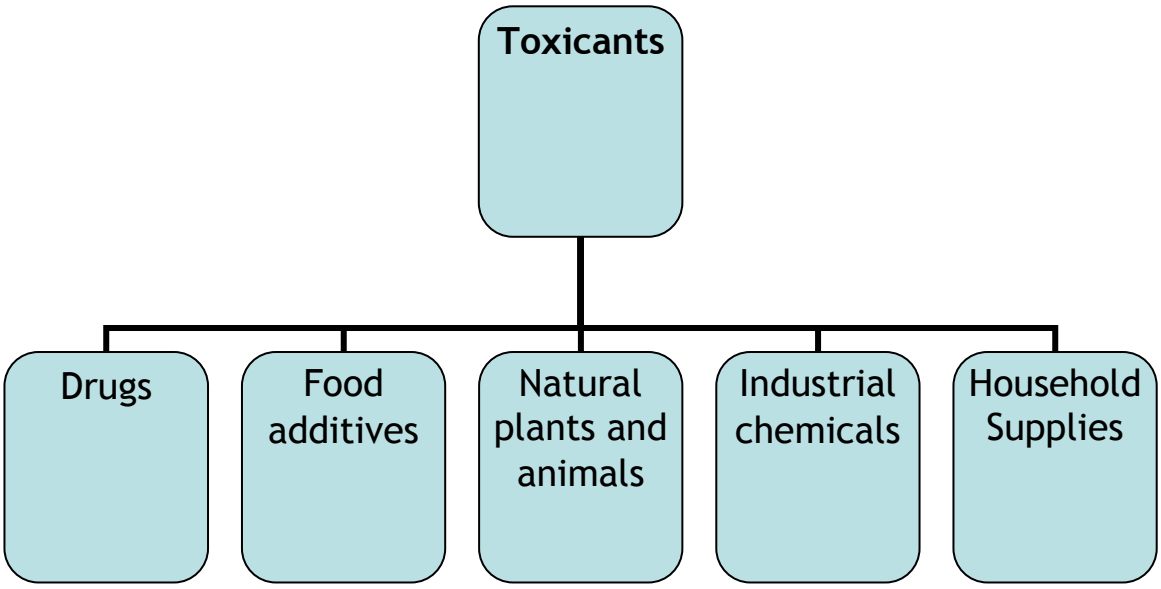


Figure 1-2: Poison classification[1]

Drugs are chemical substances that alter bodily functions by interacting with a biological target.[11] Toxicity of drugs may occur as a result of overdose, drug interactions, and genetic problems.[2] Even over-the-counter (OTC) drugs are not

safe when used inappropriately. For example, the most commonly used drug worldwide is paracetamol, an analgesic. A high dose of paracetamol taken accidentally or in order to commit suicide will cause hepatotoxicity (liver damage).[12] Thalidomide can interfere with the development of a foetus or cause birth defects if it is taken during pregnancy as a sedative or to treat morning sickness.[13,14] Genetic factors also play a role in drug toxicity, especially for those having glucose-6-phosphate degydrogenase deficiency.[15] Anti-malarial drugs such as primaquine will induce haemolytic anaemia in those who take them. In addition, poor metabolism of the antihypertensive drug debrisoquine may cause severe hypotension.[16]

Drug abuse has increased for the reasons mentioned earlier. Despite their harmful effects, alcohol and nicotine are still accepted in most societies and are legal in most countries. The following are descriptions of the psychoactive drugs most commonly abused.

Stimulant drugs such as cocaine and amphetamines improve the mood, leading to pleasant feelings (euphoria) and give one a sense of increased energy and enhanced physical performance. Stimulant drugs are highly addictive. Khat, widely used in some African and Middle Eastern countries, has the same effect as amphetamines.[4]

Depressant drugs such as alcohol, barbiturates, chloral hydrate, antipsychotic agents and benzodiazepines cause sedation, drowsiness, and euphoria.[4]

Hallucinogens include lysergic acid diethylamide (LSD), phencyclidine (PCP), and mescaline. This group of drugs has the ability to induce unpleasant effects like panic, fright, and hallucinations.[4]

Opioids such as heroin, morphine, pethidine, and codeine are often misused because they produce an intense sense of euphoria. [4]

Cannabis, more often called marijuana in the United States of America, refers to several variations of Cannabis sativa L. that contain the psychoactive drug delta-9-tetrahydrocannbinol (THC). Cannabis has both a depressant and a hallucinogenic effect. [4]

Food toxicity may occur because of additives added intentionally or as a result of contamination. The additives usually include flavouring agents, sweeteners, colouring agents, stabilizers, and preservatives. For example, tartrazine (E102) is a dye added to juice, foods, and pharmaceutical preparations to give them an orange colour. The toxic effects attributed to tartrazine include hyperkinetic behaviour in children, skin rash (urticria), and asthma in people who are hypersensitive to this material.[17] The sweetening agent saccharin was banned from United States because of its carcinogenic effect.[1,18] *Clostridium botulinum* is a bacterium that produces the toxin ***botulin***, the causative agent in botulism, which is a toxic syndrome characterized by weakness and difficulty breathing that can lead to death.[19] Furthermore, aflatoxins are any of the various related mycotoxins produced by a species of *Aspergillus,* commonly *A. flavus,* found as a contaminant in mouldy grains and meals, as in rice and peanut meal, and suspected of causing liver cancer in humans and other animals.[20] Oil mixtures containing rapeseed oil denatured with aniline were clearly linked by epidemiological and analytic chemical studies to the toxic oil syndrome that hit Spain in the spring and summer of 1981, in which 20,000 people became acutely ill, 12,000 were hospitalized, and more than 350 died in the first year of the epidemic.[21]

Pesticides are compounds used to kill certain organisms. They are used in herbicides, fungicides, insecticides, and rodenticides. Improper use of agricultural sprays containing these compounds or ingestion of food contaminated with pesticides can cause acute toxicity. The most common toxic pesticide is dichloro-diphenyl-trichlor-ethane (DDT) used to control the malarial mosquito, which also causes a toxic reaction in humans. In addition, organophosphates and paraquat were reported to cause toxic effects in humans.[22]

Industrial chemicals are also very threatening to humans. For example, chronic exposure to cadmium causes kidney damage while acute toxicity causes testicular damage.[23] Vinyl chloride used to manufacture plastics causes liver trauma.[24] Prolonged exposure to asbestos causes bronchial carcinoma (lung cancer).[25]

Some natural products are toxic to humans, too. For example, plant products such as pyrrolizidine alkaloid, pennyroyal oil, ricin, bracken, and fluoroacetate cause liver injury, abortion, cell damage, cancer, and heart failure, respectively.[26-30] Snake venom causes a lowering of arterial blood pressure, haemolytic anaemia, and a rapid pulse.[31]

Carbon monoxide from car exhaust and other sources combines with haemoglobin in red blood cells leading to respiratory failure, brain damage, and cardiac arrhythmia.[32] Ingestion of ethylene glycol, an ingredient in antifreeze, can lead to acidosis, as well as kidney and brain damage.[33] Cyanide causes cardiac and respiratory failure. In addition, chronic use of alcohol can lead to liver injury such as liver cirrhosis.[34] Glue sniffing is very common among teenagers: toluene, the most common solvent available in glue, causes narcosis.[35]

1.3 Pharmacology

Pharmacology is the study of drug effects on the functions of the body systems. The mutual interactions of drugs with the body are divided into two classes: *pharamcodynamics* and *pharmacokinetics*. Drugs are administered orally or parenterally through a non-gastrointestinal route such as intravenous, intramuscular, subcutaneous injection, topically, inhalation and sublingual. Forensic toxicologists should have knowledge about drug interactions with living humans and their biotransformation in order to assess toxicity and to give a reliable interpretation.[11]

1.3.1 Pharmacodynamics

Pharmacodynamics is the study of the effects of drugs on the body, including their mechanisms of action, medical uses and side effects. Most drugs produce their effects by activation or inactivation of receptors which normally respond to endogenous chemicals such as neurotransmitters or hormones, while some drugs are inhibitors for certain transport process or enzymes.[11]

1.3.2 Pharmacokinetics

Pharmacokinetics is the study of the effect of the body on the drug including absorption, distribution, metabolism, and excretion (ADME). Once a drug is administered, it must cross a membrane to be absorbed and enter the bloodstream. A membrane is composed of a strongly hydrophobic bilayered lipid, so drugs that are capable of dissolving in lipids are more easily absorbed. There are many factors that affect drug absorption such as the pK_a of the drug, the pH of its environment and its stability in the stomach acid. Drug absorption depends on the ionization form of drug, either acidic or basic. The Henderson-Hasselbach equations are used to determine how much of a drug will be absorbed (Equation 1-1 to 1-3).[5,11]

$pH = pK_a$ - log unionized/ionized Equation 1-1

For acids: $pH = pK_a + \log$ [ionized]/ [unionized] Equation 1-2

For bases: $pH = pK_a + \log$ [unionized]/ [ionized] Equation 1-3

For example, aspirin is an acidic drug and in the stomach (pH 2) will be unionized and lipophilic therefore absorbed readily, whereas basic drugs become more ionized and hydrophilic in the stomach and are not absorbed. By contrast basic drugs in the small intestine (pH8) become unionized and are absorbed easily.

When a drug enters the bloodstream, its distribution into the tissues of each organ is influenced by blood flow to the organs. Distribution to highly perfusion organs such as the brain, kidney and liver is faster than distribution to adipose tissue. Lipid-soluble substances have the ability to penetrate capillary and cell membranes such as the blood-brain barrier (BBB). Ionization effect affects not only the penetration of the drug through membranes but also the distribution between aqueous compartments, for instance urinary alkalinisation accelerates excretion of weak acids and retards weak bases whereas urinary acidification increases excretion of weak bases and reduces excretion of weak acids. Therefore, it is very important in cases of drug toxicity to accelerate the elimination of the drug from the body. One way to do this is through the alkalinisation of plasma by the administration of sodium bicarbonate, which

leads to the extraction of a weak acid drug from CNS to plasma. On the other hand, acidification of plasma by administering an acetazolamide will inhibit its extraction. Consequently, the drug will concentrate in CNS, and its neurotoxicity will be increased. In addition, urine alkalinisation through administering bicarbonate accelerates the elimination of aspirin in cases of aspirin toxicity.[5,11]

Metabolism or biotransformation is used to convert lipophilic compounds to hydrophilic compounds that can be excreted readily in urine. Therefore, the main function of metabolism is to detoxify drugs and xenobiotics (foreign compounds). This reaction mainly occurs in the liver and occurs in two phases described below.[36]

Phase I or functionalization is the preparation of the drug for *Phase II* by the addition of one ore more new functional groups to the compound such as -OH, -NH2, -COOH and –SH. The *Phase I* metabolic reaction can be oxidation involving cytochrome P_{450} enzymes, hydrolysis, hydration, oxidation by other enzymes, and reduction as shown in figure 1-3.

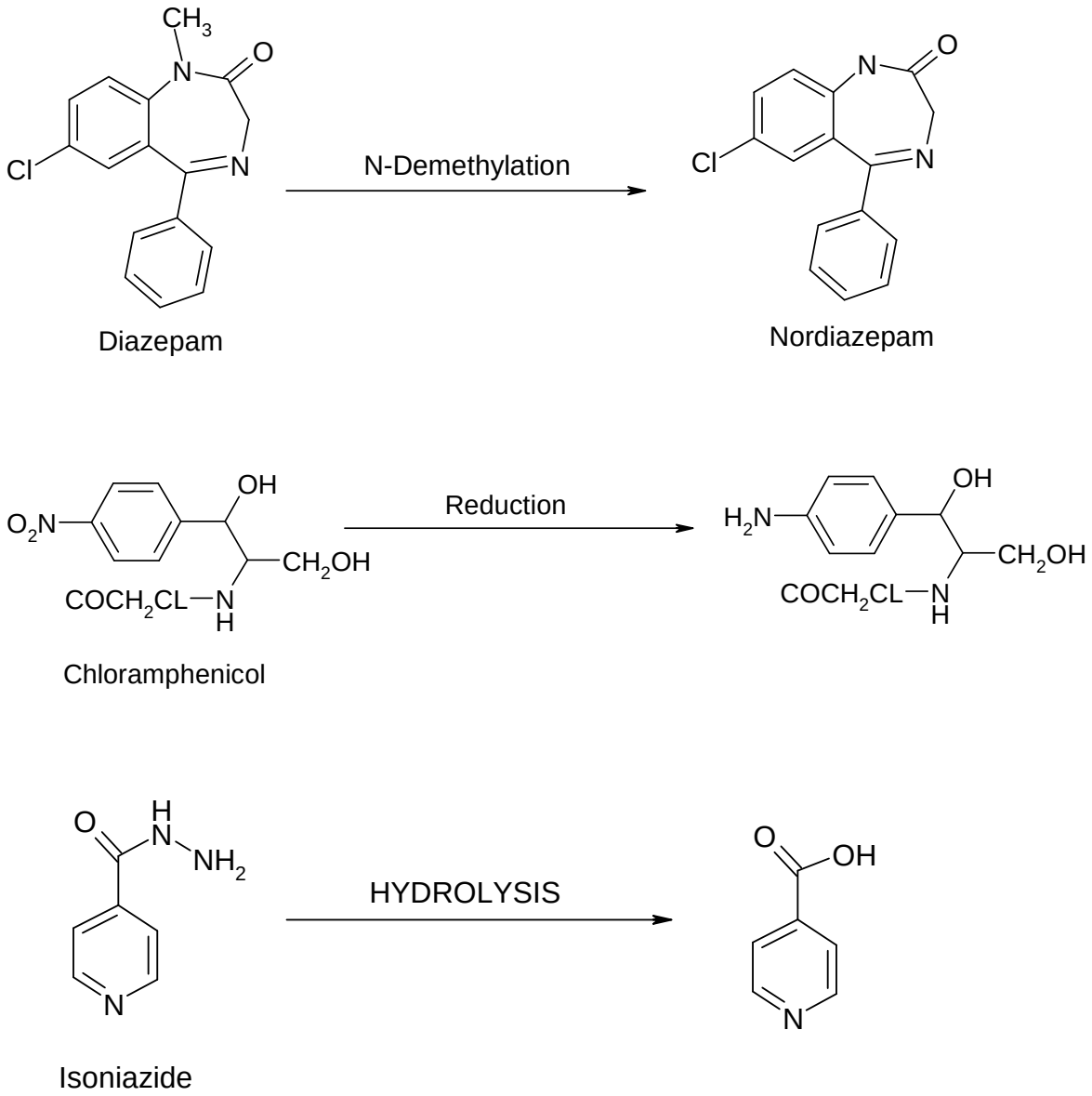
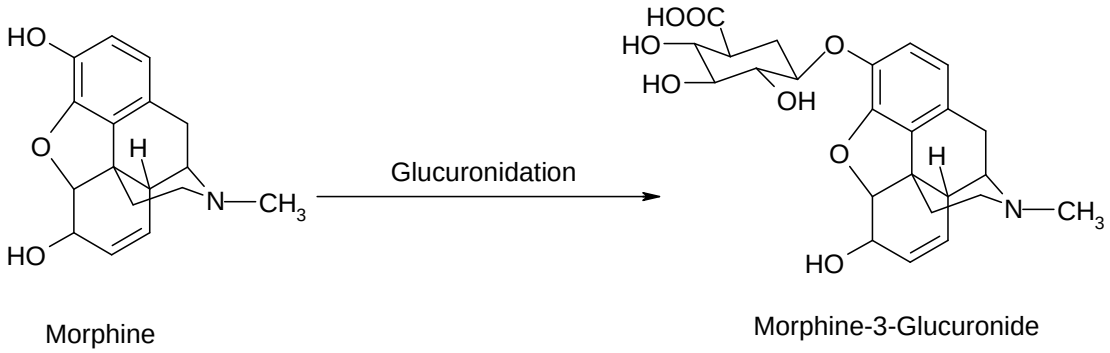


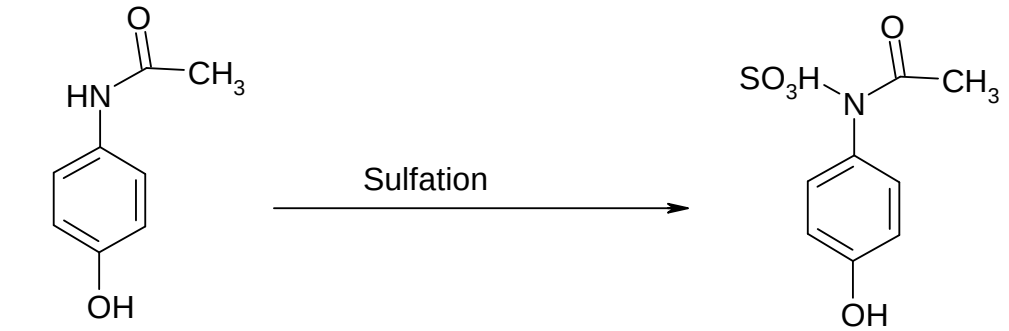
Figure 1-3: Phase I reactions

Phase II metabolism or conjugation is accomplished through the addition of an endogenous compound such as sugar, sulphate, or methyl to the functional group(s) added in *Phase I* as shown in figure *1-4*. The most common reaction in Phase II is glucuronidation due to an abundance of UDP-glucuronic acid, which is a part of the glycogen synthesis in the body and is found in all tissues. The enzyme involved in this reaction is UDP-glucuronosyltransferase, which is located in the cytosol.

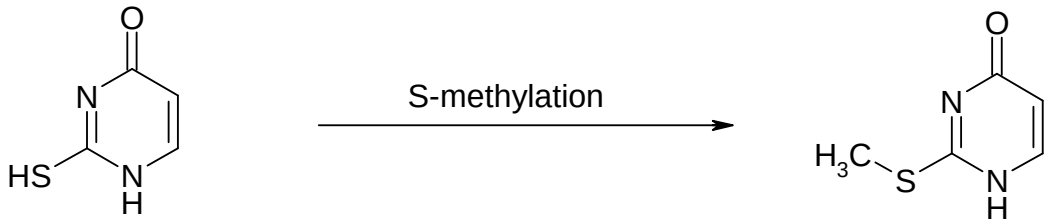


Morphine

Morphine-3-Glucuronide



Paracetamol



Thiouracil

Figure 1-4: Phase II reactions

In *Phase I,* cytochrome P_{450} enzymes catalyze the oxidation reactions of thousands of different drugs and chemicals with different structures. The catalysis also includes N-, O- and S-dealkylation. Most interactions between drugs and other substances such as another drug, food and chemicals occur by inducing or inhibiting the enzymes. For example, some drugs such as phenobarbitone, rifambicin, ethanol, and carbamazepine induce this enzyme and thereby decrease the concentration of a drug metabolized by this enzyme.

However, the most important of the interactions that play a major role in drug toxicity is a drug inhibitor because inhibition will lead to an increase in the concentration of drugs metabolized by the enzyme, causing toxicity. For instance, the antacid drug cimetidine is an enzyme inhibitor when taken with phenytoin, and warfarin will increase their toxicity. The concurrent use of the antibiotic drug erythromycin, which is an enzyme inhibitor, with narrow therapeutic index drugs such as theophylline and digoxin will lead to toxic effects of these drugs. Furthermore, grapefruit is an enzyme inhibitor and should be avoided when taking drugs.[36-38]

Toxicologists also need to be aware of genetic factors that are related to variations in responses to drugs (polymorphism). The study of this phenomenon is called *pharmacogenetics*. For instance, there is an individual variation between people in CYP2D6 (subtype of CYP450) leading to extensive or poor metabolism of the antihypertensive drug deprisquine.[16,36,39]

1.4. Samples used

Generally, ingestion or intake of a drug or poison is followed by absorption, distribution, metabolism, and excretion, so the compound can be detected in different matrices. The choice of the specimen depends on the type of case and compound being investigated. Therefore, the forensic toxicologist should carefully select the proper matrix for each specific case. The most common matrices are blood, urine, and hair.[4,11]

Blood is the complex fluid that transports the oxygen and nutrients to the cells and carries carbon dioxide and waste from the cells to be eliminated from the body. Blood plays a major role in the regulation of body temperature by transporting hormones within the body. It consists of plasma (55% by volume) and a cellular portion (45% by volume). Serum is a clear liquid exudate from clotted blood. In forensic toxicology, blood is considered to be the most useful biological fluid because of its advantages over other matrices. In other words, it can reflect the concentration of a poison at the time of death or time of the sample collection, and blood cannot be adulterated. However, many analytes cannot be detected in blood after a relatively short period of time, especially

drugs and other substances having short plasma half life. Furthermore, clotting of blood makes analysis difficult. Also, blood is an invasive matrix compared to the other matrices. The pH of blood is slightly alkaline and usually ranges between 7.2 and 7.4.[3,4]

Urine is 95% water, and the other 5% is made up of sugar and acids, urea, and creatinine. Urine is produced continuously by the kidneys to eliminate waste and metabolites from the body. Urine pH can range from 4 to 8 depending on diet and drugs ingested. Unlike blood, urine is essentially free of protein and lipids, so it can be analyzed directly by immunoassay. Urine is the specimen of choice in workplace drug abuse testing, traffic safety and sports doping because it contains drugs in higher concentrations and for longer periods of time compared to blood. The disadvantage of using this matrix is that drug concentrations are not correlated with the pharmacological effects of the drugs, so there is no indication of the concentration of the drug at the time of death or sample collection. Moreover, it is easy to adulterate a urine sample, which is often contaminated by bacteria.[4,40,41]

Hair is extensively used in drug screening, and it is considered to be the ideal matrix because it has many advantages over other specimens. For instance, it provides information about chronic use of a drug, it can be collected in a non-invasive manner, it is very stable, it is not affected by storage conditions and it is difficult to adulterate. However, there are a few problems with using hair. The uptake of a drug into hair depends on the colour of the hair, for example, dark hair has more affinity than blond hair, and the concentrations of some drugs detected in hair may be affected by the use of hair products.[42,43]

Oral fluid consists of saliva plus other components in the oral cavity. It is a non-invasive matrix in which concentrations of drugs may be correlated with those found in blood. Oral fluid is often used to screen recent use of a drug in cases where a person is suspected of driving under the influence of drugs. However, drugs which are highly protein-bound in blood have low concentrations in oral fluid.[44]

Other specimens such as bone, heart, bile, liver and gastric contents are collected at autopsy and are useful substances in case of extreme putrefaction.[4]

1.5 Forensic toxicology problems

There are many problems facing forensic toxicologists, including finding reliable methods to detect unknown compounds in small volumes of sample and distinguishing structurally similar compounds.

Decomposition of drugs during the post-mortem interval or during storage in the laboratory has been reported in previous studies, which may give errors in the interpretation of results.[45-50,55]

Post-mortem drug concentrations change due to continuing drug metabolism after death, chemical decomposition, redistribution of the drug from organs, or as a result of putrefaction. Bacteria enter the oral cavity and then penetrate the gastrointestinal wall five hours after death, leading to putrefaction and drug decomposition. The products originating from putrefaction as a result of enzymatic and bacterial activity include indole, phenylacetic acid, butyric acid, pyridine, tryptamine, and tyramine, all of which will interfere with drug analysis. The most likely bacteria to affect post-mortem drug metabolism are clostridium perfringens, Bacteroides fragilis, and Escherichia coli, which contain oxygen-sensitive nitroreductase enzymes that are able to reduce nitroaromatic compounds. The other bacteria involved in post-mortem drug metabolism are bacillus spp, proteus miribalis, staphylococcus aureus, streptococcus, and faecalis.[41,45-50]

In addition to contamination during the post-mortem period, contamination can be caused by poor sampling. An average person has between 100 to 100,000 microbes per square centimetre of the skin depending on the time of day and on the hygiene of the individual who is the subject of analysis, especially if he or she has been exercising and there is sweat on the skin, which contains bacteria that may contaminate the sample. Furthermore, extensive clotting of blood after death or when no anticoagulant is added to the sample can make the analysis difficult because blood will clot in ten minutes after it is placed in a tube.[45-51]

In addition, chemical adulteration of samples, especially urine, can lead to wrong interpretation of results due to decomposition of the analyte.[40]

1.6 Aims

The aims of the component studies described in this thesis were:

To investigate the stability over one year of four drugs from the benzodiazepine group in whole blood at low and high concentrations under different storage and temperature conditions, with and without additives. The study also intended to investigate the stability of three opiate glucouronides in blood and urine stored for one year under different conditions.

To develop a new method for the analysis of hydrolytically labile drugs such as benzodiazepines and cocaine in dried blood spots stored on filter paper and assess their degradation during a storage period of one year under different conditions.

To develop a procedure for correction of drug losses after decomposition of hydrolytically labile benzodiazepines during storage under different conditions in blood and urine by quantification of the products of degradation.

To study the stability of organophosphates when stored in blood for three days at room temperature after the addition of sodium fluoride as preservative and also to assess the loss of analytes resulting from addition of sodium fluoride by detection of their degradation products. Furthermore, to assess the stabilization of these compounds by storing them as dried blood spots on filter paper as an alternative method to protect them from decomposition.

2 Stability of Drugs and Poisons: Literature Review

2.1 Introduction

Skopp et al.[52] define stability as the "capability of a sample material to retain the initial value of a measured quantity for a defined period within specific limits when stored under defined conditions." Shah et al.[53] define stability as "the chemical stability of an analyte in a given matrix under specific conditions for a given time interval." Waterman et al.[54] refer to stability as the "ability to withstand loss of chemical due to decomposition" while for pharmaceuticals stability is related to the "storage time allowed before any degradation in the dosage form," which is expressed in time as shelf life.[54]

Drug concentration changes are observed in forensic cases. In the postmortem period a decrease in drug concentration can occur during the time between death and toxicological analysis of autopsy specimens as a result of drug redistribution,[247,248]ongoing drug metabolism by enzymes or bacteria and chemical degradation.[3-6], Drug concentration in collection tubes was influenced by storage conditions during the storage time in the laboratory or during transportation. The temperature plays a major role in the drug stability and additives and preservatives usually have not been added. Very often, a period of several days occurs between sampling, screening, and quantification. In general all biological samples are stored in the laboratory until the final confirmatory analysis is carried out. The final confirmation may not actually be required until the case reaches court for legal process, and it may be carried out after a period of time. Within the United Kingdom, autopsy specimens frequently become court exhibits and are retained in the court premises prior to the commencement of the proceedings, often for periods of weeks or months, and often at room temperature. Any difference in the results between initial and subsequent analyses may well be the basis for arguments and disagreements over interpretation of the results by the medico-legal community, and more reanalysis may be requested. Therefore, knowledge of drug stability under different storage conditions is necessary for toxicologists to interpret case results reliably.

2.2 Drugs

Drug loss during the post-mortem interval has been reported in previous studies. For example, Fumio and Yoshiaki investigated the tissue distribution of nitrazepam and its metabolite 7-aminonitrazepam in one case of nitrazepam toxicity.[48] An elderly woman was found dead during winter in a shallow ditch. The ambient temperature was 2—8 °C and no putrefaction was noted. Different concentrations of nitrazepam and its metabolite 7-aminonitrazepam were found at different sites. High concentrations of both analytes were found in the brain, whereas low concentrations were observed in the liver. In addition, largely different concentrations in the veins and arteries were noted; these changes in the concentration were attributed to dilution by water that entered in the circulation through the lung, and by bacterial metabolism, which reduced nitrazepam to 7-aminonitrazepam.

Robertson and Drummer investigated the stability of nitrobenzodiazepines, in particular nitrazepam, clonazepam and flunitrazepam, in blood and water.[46] The result of this study showed that nitrazepam and clonazepam were stable in sterile fresh blood containing 1% sodium fluoride/potassium oxalate preservative when stored at 22 °C over 28 days while flunitrazepam lost approximately 25% of its original concentration under the same conditions. All three drugs lost approximately 29–51% after 9 h when stored at 37 °C. 7-Aminonitrazepam was stable at 4 °C but unstable at 22 °C and 37 °C; also, it declined by 25% after 28 days whereas 7-aminoclonazepam was stable at all three temperatures. 7-aminoflunitrazepam lost approximately 10% at all storage temperatures. No degradation was found for the drugs and corresponding metabolites in water under the same storage conditions. In the absence of preservatives, 25—50% of parent drugs were lost in blood stored at 22 °C after 10 days. All parent drugs were completely converted to their metabolites over 8 h at 22 °C in bacterially contaminated postmortem blood.

In conclusion, all three parent drugs were found to be stable in blood stored at −20 °C over 24 months and 10 months at 4 °C, whereas their 7-amino metabolites were unstable at −20 °C after 2 months and 1 month at 4 °C.

Furthermore, postmortem nitrobenzodiazepines metabolism by bacteria has been investigated by Robertson and Drummer in blood at 4 °C, 22 °C, and 37 °C for 21 days.[45] Flunitrazepam, nitrazepam, and clonazepam were found to be metabolized completely to their 7-aminometabolites in the presence of eight species of enteric bacteria. Activity of bacteria with respect to bioconversion was observed at 22 °C and 37 °C but not at 4 °C except for clostridium perfringens. The rate of bioconversion increased as the temperature increased. However, addition of fluoride oxalate at a concentration of 0.7 % (w/v) was found to reduce the bioconversion loss of these drugs from 100% to 13%, whereas 1% (w/v) of fluoride oxalate reduced the loss to 4%. A higher concentration of fluoride oxalate, 2% w/v, did not further inhibit metabolism. The bioconversion activity is different from one species of bacteria to another. By contrast, no bioconversion was observed in sterile buffer after 21 days and less than 21 % of nitrobenzodiazepines were converted in sterile fresh whole blood.

Stability of drugs *in vitro* has been reported in previous studies. For example El Mahjob et al.[56] found clonazepam, flunitrazepam, midazolam, and oxazepam stable for 1 year when stored at −80 °C while the losses for all compounds when stored at −20 °C were 5% and 20% for high and low concentrations, respectively. In the refrigerator at 4 °C, the decreases in concentration were more than 50% for high concentration and more than 90% for low concentration. At room temperature the losses for all drugs were 100% and 70% for low and high concentrations, respectively.

Alhadidi and Oliver studied stability of temazepam in blood and in aqueous solution.[57] They found that temazepam was stable in blood when stored in the freezer at -20 °C for 1 year while in the refrigerator at 4 °C and at a room temperature of 20 °C significant decreases in concentration were noted after 3 months. The amount of temazepam recovered after 1 year ranged from 47% to 82% regardless of storage temperature. Temazepam in aqueous solution was found to be stable for 6 months regardless of the storage temperature and for 1 year in the freezer; in the refrigerator and at room temperature the recovery after 1 year ranged between 42% and 58% regardless of storage temperature and the drug loss was less in blood than in water.

Moreover, the same authors have reported on the stability of morphine and buprenorphine in whole blood.[60] Both drugs were stored at −20 °C, 4 °C, and 25

°C, and were found to be very stable. For storage periods of up to 6 months, 85% of morphine and 77% of buprenorphine were recovered. After 1 year more than 70% of the original concentrations were detected regardless of storage temperature, particularly when blood samples were stored in silanized glass vessels. In another study Alhadidi and Battah[59] found that the anticholinergic agent trihexyphenidyl (THP) was stable for 6 months in blood and urine at 4 °C and -20 °C; however, at 25 °C THP was only stable for 3 months and 4 months in blood and urine, respectively. Chlorpromazine was found to be stable in blood for 10 months at −20 °C, 8 months at 4 °C and for 2 months at 25 °C, as reported by Alhadidi et al.[60]

Levine et al.[55] studied the postmortem stability of benzodiazepines in blood and tissue for several months — in particular diazepam, chlordiazepoxide, flurazepam and their desalkyl metabolites. Diazepam was found to be very stable at room temperature and under refrigeration over a 5-months period. Flurazepam and N-1-desalkylflurazepam were found to be stable under both storage conditions despite the slight decrease observed, which was less than 20%. Chlorodiazepoxide and norchlordiazepoxide were unstable at the same storage temperatures. Neither chlorodiazepoxide nor norchlordiazepoxide could be detected after 18 days at room temperature from the spiking date. Addition of preservative and anticoagulant had little effect on the stability of norchlordiazepoxide at room temperature compared to chlordiazepoxide; the preservative partially protected chlorodiazepoxide from decomposition compared to the unpreserved sample. The results obtained were the same in blood and tissue.

Lin et al.[61] investigated the effect of storage temperature on the stability of morphine and codeine in urine samples stored at freezer, refrigerator, and room temperatures over 11 months. They showed that total morphine and total codeine concentrations decreased approximately up to 31% in the freezer and 39% in the refrigerator, whereas free morphine and free codeine were only noted to have slight increases in all samples. In contrast, a large decrease of concentration of these drugs was observed when stored at room temperature.

Baselt et al.[62] reported a long-term stability study of blood containing cocaine, benzoylecgonine and ethanol, stored in glass collection tubes containing a

combination of preservative and anticoagulant at 4 °C for 1 year. The study was done to determine if minor improvements could prolong the long-term stability of drugs. The results of this study showed 100% loss of cocaine when the sample was stored in a collection tube containing sodium fluoride and potassium oxalate. However, substitution of oxalic acid instead of potassium oxalate reduced the loss to 76% from the day zero concentration while less improvement was noted in the concentration of benzoylecgonine and ethanol. Moreover, cocaine loss was after 1 year reduced to 60% after the addition to the sample of 10 mg echothiophate, a known cholinesterase inhibitor, while no enhancements were noted for the stability of benzoylecognine and ethanol, which were found to be stable and not affected over the time in all preserved tubes, compared to cocaine.

McCurdy et al.[63] investigated the stability of cocaine, benzoylecognine (BZE), and 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in whole blood stored in four different types of tube. Three of these contained different additives: sodium fluoride, EDTA and heparin, respectively. The fourth tube did not contain any additives. The samples were stored for 1 month at refrigerator and room temperatures. BZE and THC-COOH were found to be stable under all storage conditions whereas cocaine was unstable and losses were noted at both temperatures. Also, no improvement was noted in the sample containing additives.

Holmgren et al.[64] investigated the stability of 46 drugs in blood at −20 °C for 1 year and the influence of preservative on drug stability when added to samples of vitreous humor. In this study, concentrations of ethanol, desmethylmianserin, 7-amino-nitrazepam, THC, and zopiclone in blood were found to have decreased after 1 year, whereas ketobemidone and thioridazine were found to have increased in concentration. The study also showed a significant beneficial effect of potassium fluoride on the stability of ethanol and zopiclone in vitreous humor, which were found to decrease during storage in the absence of potassium fluoride. The rest of drugs were found to be stable under the same conditions.

The stability of the ring-substituted amphetamines 3,4-methylenedioxy-amphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA) have been examined by Clauwaert et

al.[65] for 21 weeks at −20 °C, 4 °C, and 20 °C in blood, urine, water and serum. They found these compounds to be stable for the whole investigation period at high and low concentrations, in water and urine, and at all storage temperatures. A similar result was noted for storage in serum for 17 weeks and in whole blood for 5 weeks under all storage conditions. This study showed that the drugs are stable up to 21 weeks when stored at −20 °C in all matrices.

Zaitsu et al.[66] studied the long-term stability of various drugs and metabolites for 5 months at 25 °C, 4 °C, and -20 °C; the study also investigated the effect of addition of sodium azide ($NaN_{3)}$ as preservative and the use of an aseptic urine collection kit. In urine which was slightly contaminated by bacteria, amphetamine and methamphetamine were found to be stable for 5 months under all storage conditions whereas more than 32% of methamphetamine and 44% of amphetamine were lost in severely contaminated urine. However, the losses of both analytes were completely prevented by either use of NaN3 or the aseptic collection kit. Estazolam was found to be stable under all conditions with or without preservative or aseptic collection kit whereas nitrazepam disappeared completely within 14 days in severely contaminated urine at room temperature. However this loss was completely inhibited by either NaN_3 or the aseptic collection kit. Both nitrazepam and 7-aminoflunitrazepam (7AF) were found to be stable at −20 °C for 5 months. Further addition of NaN3 alone had a moderate effect on 7AF stability at the other two temperatures, while addition of NaN_3 plus filtration sterilization was found to be most effective. Cocaine (COC) and 6-acetylmorphine (6AM) were found to be stable at 4 °C and −20 °C for 5 months in all storage conditions while both of them were completely lost after 1 month in severely contaminated urine at 25 °C and after 75 days in slightly contaminated urine under the same conditions without any preservative treatment. Addition of NaN_3 accelerated the decomposition of COC and 6AM whereas the decreases were completely inhibited after using the aseptic urine collection kit.

Skopp et al.[67] performed a study on approximately 13 benzodiazepines and their metabolites in blood and plasma for storage periods of up to 8 months at 4 °C. All compounds were found to decrease by at least 60% over the full period of study in both matrices; flunitrazepam and norflunitrazepam were less stable

than the other analytes followed by prazepam and lorazepam. The stability of the compounds was better in blood than in plasma.

Skopp et al.[68] also reported a study on the stability of morphine, morphine-3-glucuronide (M3G), and morphine-6-glucuronide (M6G) in fresh blood, plasma, and postmortem blood in glass vials at −20 °C, 4 °C, and 20 °C for 6 months. Morphine and its glucuronide metabolites were found to be stable in both blood and plasma at −20 °C and 4 °C. At room temperature M3G was found to be stable in both matrices while morphine was stable in blood and decreased by 23% in plasma by the end of study. M6G was lost from blood and plasma, approximately 23% and 14%, respectively at room temperature. In postmortem samples all compounds were found to be stable at −20 °C. Morphine was stable at 4 °C whereas M6G and M3G decreased by 10% and 40%, respectively. At 20 °C, the morphine concentration increased to 134% of the initial concentration whereas its metabolites decreased.

Lutfi studied the stability of three benzodiazepines and four tricyclic antidepressant drugs in blood for up to 1 year.[69] The stability results showed that diazepam was stable at −20 °C while at 4 °C and 25 °C losses occurred of 20% and 46%, respectively. Desmethyldiazepam decreased by 43%, 53%, and 70% and temazepam by 30%, 38%, and 44% at −20 °C, 4 °C, and 25 °C, respectively. Amitriptyline was found to be stable at −20 °C and 4 °C while at 25 °C it was stable up to 5 months but had decreased by 42% after 12 months. Nortriptyline showed poor stability, with decreases in concentration of 44%, 54%, and 73% at −20 °C, 4 °C and 25 °C, respectively. Imipramine was stable for 1 year at −20 °C and for 7 months at 4 °C; however, at 25 °C it showed poor stability and decreased by more than 50% after 1 year.

Cocaine, benzoylecgonine, nonconjugated morphine, codeine, methamphetamine and phencyclidine (PCP) stability in blood were studied by Giorgi and Meeker for 5 years at ambient temperature.[70] The specimens were collected in gray–top container tubes containing sodium fluoride and potassium oxalate. These blood samples were collected from live individuals and stored at room temperature. Cocaine and its metabolites showed poor stability. Cocaine was not detected after reanalysis while BZE was not detected after 2 years. Morphine had decreased by 61.8% at the end of study whereas codeine was fairly

stable during the study. Methamphetamine significantly decreased after 1 year of storage. PCP was measured at only 69.4% of the original concentration at the end of study.

Isenschmid et al.[71] noted that cocaine was stable in blood for 150 days if preservative (2% NaF) or organophosphate was added to the sample adjusted to pH 5 and stored at 4 °C or less. Unpreserved cocaine was hydrolysed to ecgonine methyl ester, while the addition of pseudocholinesterase (Pch E) inhibitor without reduction in pH caused cocaine to hydrolyse into benzoylecgonine. Furthermore, cocaine is hydrolysed to BE in a phosphate buffer. Cocaine was found to be more stable in unpreserved postmortem blood samples than blank blood because of the lower pH of the former. Also ecgonine methyl ester was found to be less stable than benzoylecgonine in unpreserved blood.

The stability of the muscle relaxant pancuronium in blood has been studied by Kala and Lechowics.[72] Pancuronium was stable for 7 months and 3 months when stored at −20 °C and room temperature, respectively.

Dugan and Bogema studied the stability of amphetamine, methamphetamine, morphine, codeine, cocaine, phencyclidine, and 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid in urine at −20 °C for 12 months.[73] The concentrations of these drugs did not change except for cocaine, which decreased by 37% of its original concentration after a 12-month period.

The stability of the digitalis drugs digoxin and digitoxin in serum at 4 °C has been investigated by Datta.[74] Both drugs were found to be stable for 3 days. In addition digoxin stability in saliva was studied by Lori et al.[245], which was found to be stable for 2 weeks at room temperature.

Forsdahl and Gmeiner measured the loss of salbutamol in urine at two different concentrations: this ranged between 2—4% at −18 °C and 8—12% at 4 °C after 2 months.[75]

Celma et al.[76] studied short-term stability of paracetamol and chlorpheniramine in plasma. Both drugs were found to be stable for 1 day in the autosampler at

room temperature. Also, both drugs were resistant to three freezing and thawing cycles over a 3 month storage period at −20 °C.

The stability of the antiretroviral non-nucleoside reverse transcriptase inhibitor, nevirapine (NVP, used for treatment and prevention of HIV) was investigated by Bennetto et al.[77] in whole blood and serum in the refrigerator, at room temperature and in an incubator at 37 °C. Nevirapine was found to be stable for 1 day at all storage conditions at both high and low concentrations.

Barnada et al.[78] studied calcium channel antagonist (CCA) photodegradation in serum samples during preparation for analysis by LC-MS/MS. The degradation of solutions containing nifedipine or nisoldipine in a clean glass vial was 96% after 2 h when exposed to laboratory light, whereas the degradation in plasma was 25% after 2 h for both drugs. All compounds were found to be stable in plasma for 2 months when stored at −20 °C and for 24 h at room temperature.

The stabilities of the antipsychotic drug promazine and its metabolite desmethylpromazine were investigated by Oliver and Stephen in plasma and urine for 64 days at −20 °C.[79] Both analytes were found to be stable during the whole observation period.

Skopp et al.[80] reported on the short-term stability of THCCOOH glucuronide (THC-COOHglu) in plasma for 10 days at −20 °C, 4 °C, 20 °C, and 40 °C. The results indicated that THC-COOHglu was stable only when stored at −20 °C and that the rate of decrease in THC-COOHglu concentration increased with temperature.

Skopp et al.[81] studied the stability of the hallucinogenic agent lysergic acid diethylamide (LSD) and its metabolites N-desmethyl–LSD and 2-oxo-3-hydroxy-LSD in urine for 3 days. All analytes were found to be stable at −20 °C. At 4 °C and 22 °C, only LSD was stable and at 40 °C all analytes were unstable.

The influence of cosmetic treatments such as bleaching and perming formulas on the stability of opiates in hair was examined by Putsch and Skopp.[82] About 2−18% of starting opiate concentration could be detected after bleaching and about 20−30% after perming. Morphine was found to be more sensitive to oxidative

destruction followed by codeine and dihydrocodeine. In another report Yegles et al.[83] studied the effect of bleaching on the stability of benzodiazepines and opiates in hair. Concentrations of diazepam, nordiazepam, and 7-aminoflunitrazepam decreased by 39.7%, 67.7%, and 61.8%, respectively in comparison to unbleached hair. Furthermore, significant decreases occurred in the concentrations of codeine, 6-acetylmorphine and morphine amounting to 57.5%, 88.6%, and 67.4%, respectively. Cocaine and benzoylecgonine concentrations decreased by 24.6% and 36.4%, respectively.[84]

Skopp et al.[85] investigated the *in vitro* stability of cocaine (COC) in whole blood and plasma preserved with 0.25% potassium fluoride (KF) for 15 days at 4 °C and 20 °C and for 3 days at 40 °C. The study also included the stability of benzoylecgonine (BZE), ecgonine methyl ester (EME) and ecgonine (ECG) stored in plasma at 4 °C and 20 °C. Cocaine continuously decreased whereas its metabolites appeared after storage for 1 day at 4 °C and 20 °C. At 40 °C, COC was detected for only 2 days. The sum of all hydrolysis products comprised 82% of the initial COC concentration at 40 °C. The degradation of cocaine in whole blood was less than in plasma. Both BZE and EME were found to be unstable and were hydrolysed to ECG, which was found to be the major hydrolysis product for COC, BZE, and EME. ECG was found to be stable at all storage temperatures and could be detected even if samples were stored under unfavorable conditions, so could be used to correct for loss of the parent drug.

The stability data of different classes of drugs in biological samples has been reviewed recently by Peters and Maurer.[86]

2.3 Pesticides

2.3.1 Organophosphates

Ageda et al.[87] studied the stability of 14 organophosphorous insecticides in fresh blood at 37 °C, 25 °C, and 4 °C for 1 day. The study showed that concentrations decreased as the temperature increased. All compounds except malathion, trichlorfon, and fenitrothion were found to be stable at 4 °C. At room temperature all compounds were found to be unstable except thiometon,

isoxathion, diazinon, and sulprofos. Sulprofos is the only compound that was found stable at 37 °C.

Moriya et al.[88] investigated the effect of preservative (NaF) on the stability of organophosphates in blood for 3 days. Instead of preserving the compounds from degradation, sodium fluoride was found to accelerate the chemical degradation of the compounds. Dichlorvos completely disappeared within 15 min of addition of NaF (2% W/V) whereas chlorpyrifos decreased by 95% after 3 days. A similar observation was reported by Asri[99] and this loss was attributed to alkaline hydrolysis after the addition of NaF. Therefore, Moriya et al.[88] recommended that NaF preservatives should be avoided for samples containing organophosphorous compounds.

2.4 Stabilisation of samples

Degradation mechanisms may occur as a result of three main reactions: reduction, oxidation and hydrolysis. Therefore, stabilisation of samples can be achieved by inhibiting these reactions from occurring as well as inhibiting clotting and enzymatic and bacterial activity, in order to keep compounds stable in the sample for a long period of time. Since stability is an essential issue in forensic toxicology, the influence of the environmental and storage conditions on compound stability in different matrices should be understood in order to select a suitable method to preserve compounds against degradation as well as to optimize the best storage conditions.

Some methods that have been used to stabilize analytes are listed below.

2.4.1 Additives

Blood clotting occurs very quickly, 4—8 min after placing blood in the collection tube. This makes sample analysis very difficult. Calcium plays a major role in this process. Chemical substances that prevent the clot formation process are called anticoagulants and include potassium oxalate, citrate, ethylenediaminetetraacetic acid (EDTA) and heparin. Usually oxalate is

combined with preservatives such as sodium fluoride (NaF), which is considered a weak anticoagulant. Fluoride/oxalate works by precipitating calcium while citrate and EDTA bind calcium to stop the clotting process from occurring. Heparin works by preventing conversion of prothrombin to thrombin, thereby preventing clot formation.[51]

Sodium fluoride is an antienzymatic agent that inhibits the activity of enzymes, such as cholinesterase, and bacteria, thus inhibiting the reduction of nitroaromatic compounds by bacterial metabolism and enhancing the stability of drugs. Addition of a cholinesterase inhibitor such as echothiophate as well as fluoride/oxalate to samples containing cocaine reduced the loss of cocaine by 60% compared to the loss after adding only fluoride/oxalate to the sample.[45]

Brogan et al.[89] studied the stability of cocaine in blood samples stored at room temperature for 2 days in 5 different tubes containing no additives, 0.25% NaF, 0.5% NaF, 1% NaF and 0.25% NaF/K respectively. Cocaine was unstable in containers not containing any additives while equal enhancement of stability was observed in containers containing 0.25% and 0.5% NaF and increased when 1% of NaF tube was used. The greatest inhibition of cocaine degradation was achieved when the gray-top vacutainer® containing 0.25% NaF/K was used and stored at room temperature or at 4 °C.

Addition of sodium azide (NaN_3) as preservative showed enhancement of drug stability in urine. An aseptic urine collection kit showed greater enhancement of drug stability than NaN_3.[66]

In most studies additives showed enhancement of the drug stability when added to the sample whereas some studies showed little effect. However, additives should be carefully selected for such cases and analytes. For example, adding EDTA to samples containing morphine showed an increase in the morphine concentration by 4.8% compared to heparin anticoagulant.[90] Furthermore, NaF accelerated the chemical hydrolysis of dichlorvos and chlorpyrifos when added to blood samples containing these compounds. Thus, it should be avoided in samples containing organophosphates.[88] NaN_3 has also been found to accelerate the degradation of cocaine and 6-acetylmorphine when added to urine samples.[66]

2.4.2 Filter paper

Preserving compounds in dried blood spots on filter paper has been advocated in previous studies. For example, the antibacterial drugs enrofloxacin (EFX) and ciprofloxacin (CFX) were found to be stable for 4 weeks when stored in dried blood spots at −20 °C, 4 °C and room temperature.[7] Tacrolimus, an immunosuppressant drug, was present in blood samples collected from patients and stored on filter paper for 1 month at 4 °C. It was found stable under these storage conditions.[91] Furthermore, tacrolimus was found to be stable when stored at room temperature, 37 °C, 70 °C and −20 °C for 9, 7, 1, and 8 days, respectively.[92]

Skopp[93] has used filter paper to assess the degradation of some drugs. For example, ester type drugs, amphetamines, diazepam and its metabolites were found to be stable in dried blood spots for several days even when stored under a high temperature of 40 °C. Furthermore, cocaine is known to be very unstable and to degrade rapidly in blood samples.[94] However, when stored in filter paper it was stable at −20 °C and at room temperature for 17 days.

Metformin is used for type 2 diabetic patients: its stability was assessed for 2 months in blood spots stored on filter paper at −70 °C and was found to be stable for the entire observation period. [95]

The stabilities of drugs in urine samples stored in filter paper have been investigated at −20 °C, 4 °C and room temperature for 12 weeks compared to aliquots of urine stored at −20 °C. Benzoylecgonine, morphine and PCP were found to be stable at all storage temperatures in both dried and frozen aliquot samples for the whole study period. Amphetamine was found to be stable in dried stains at all storage temperatures whereas 16% of its original concentration was lost from aliquots of urine stored at −20 °C after 12 weeks. Furthermore, THC-COOH was found to be stable in dried spots at −20 °C and 4 °C for 12 weeks while at room temperature it was stable for only 4 weeks. In frozen urine aliquot samples, THC-COOH was found to be stable for 12 weeks.[96]

Stabilities of the antimalarial drugs chloroquine (CQ) and proguanil (PG) and their metabolites monodesethylchloroquine (MDCQ) and cycloguanil (CG) respectively were evaluated at −20 °C, 4 °C, 20 °C, and 50 °C for 1, 5, and 20

days. All compounds were found to be stable for 1 day at all storage conditions. CG was not affected by temperature and was found to be stable for 20 days at all storage conditions. CQ decrease by more than 50% at 20 °C and 50 °C after 5 and 20 days of storage. Significant decreases of 30% and 80% in PG concentration were observed after 5 or 20 days at 50 °C respectively. However, MDCQ decreased at 20 °C after 5 and 20 days and completely disappeared after 5 or 20 days of storage at 50 °C.[97]

2.4.3 Freezing

Usually samples after collection are kept at ambient temperature during transportation; in the laboratory reception or during registration then the samples will be stored at 4 °C for a few days until screening and confirmation are performed. After that samples will be stored at negative temperatures (freezer) for several months or years for reanalysis and legal purpose requirements. Most studies showed that as temperatures increase the stability of drugs decreased. Freezing enhances stability if drugs are stored at −20 °C or lower, such as −70 °C or −80 °C. Consequently the rate of degradation reactions will be minimized at low temperatures. Therefore, it is recommended by most studies that samples are kept in the freezer after collection, even during transportation from the place of collection.[61-81]

2.4.4 Acidification

Acidification of samples showed enhancement of the stability of some drugs, for example, substitution of the anticoagulant potassium oxalate by oxalic acid minimized the decrease in cocaine concentration from 100% to 76% loss after 1 year of storage.[62]

Hashimoto and Moriya[98] investigated the postmortem stability of cocaine and cocaethylene in blood and tissues of humans and rabbits. They found that cocaine is less stable than cocaethylene in postmortem samples, and brain and muscle were considered the specimens of choice to detect cocaine and cocaethylene. In acidic conditions cocaine was stable and did not decompose. Moreover a study by Gupta showed that cocaine was stable in aqueous solution below pH 4 at 24 °C for 45 days.[246]

Asri[99] found that acidification of solutions containing malathion in deionized water with preservatives such as EDTA/K and Heparin/Lithium lowered the pH to (5-6) and 7 respectively and reduced the degradation of the compound whereas addition of NaF was found to increase the pH value to basic (around 8) consequently accelerated the degradation of the compound.

2.4.5 Antioxidants

Addition of an antioxidant to serum samples enhanced the stability of drugs sensitive to oxidation reactions. The new-generation neuroleptic drug olanzapine was stabilised by adding 0.25% ascorbic acid as antioxidant during the extraction procedure and storage period. Samples not containing ascorbic acid lost 16% of the olanzapine compared to those with the antioxidant. Storage of samples not containing antioxidant for 1-2 weeks showed decreases in olanzapine concentration of 13-45%, respectively. At room temperature, about 40% was lost after 24 h compared to samples containing ascorbic acid. Different concentrations of ascorbic acid, 0.125%, 0.25% and 0.5% showed the same result: no differences were noted between them.[100]

Ueyama et al.[101] investigated the effects of three antioxidants in preventing oxidation of dialkylthiophosphates to their corresponding dialkylphosphates. About 10 mg/ml of ascorbic acid, sodium metabisulfite (SOD) or pyrogallol were added individually to human urine spiked with dimethylthiophosphate (DMTP) and diethylthiophosphate (DETP). Without antioxidant, dimethylphosphate (DMP) and diethylphosphate (DEP) were produced in 25% yield. In contrast, addition of any of the antioxidants to the sample prevented production of DMP and DEP effectively (although pyrogallol was less effective). Ascorbic acid showed an interference peak in the subsequent analysis and SOD was selected for use, which prevents the unwanted production of DMP and DEP to < 1%.

The stability studies of drugs and pesticides are summarized in Table 2-1.

Table 2-1: Summary of drugs and pesticides stability

Author	Compound	Matrix	Conditions	Results	Comments
Robertson and Drummer[46]	Nitrazepam	Blood	-20 °C, 24 months 4 °C, 10 months 22 °C, 28 days	Stable	With 1% (w/v) NAF
	Clonazepam	Blood	-20 °C, 24 months 4 °C, 10 months 22 °C, 28 days	Stable	
	Flunitrazepam	Blood	-20 °C, 24 months 4 °C, 10 months	Stable	
			22 °C, 28 days	25% decrease	
	7-aminonitrazepam	Blood	-20 °C, 2 months 4 °C, 1 months	Stable	
			22 °C, 28 days	25% decrease	
	7-aminoclonazepam	Blood	-20 °C, 2 months 4 °C, 1 months 22 °C, 28 days	Stable	
	7-aminflunitrazepam	Blood	-20 °C, 2 months 4 °C, 1 months	Stable	
			22 °C, 28 days	10% decrease	

El Mahjoub et al.[56]	Clonazepam	Blood	-80 °C, 1 year	Stable	No additives
			-20 °C, 1 year	20% and 5% decrease for LC and HC, respectively	
			4 °C, 1 year	90% and 50% decrease for LC and HC, respectively	
			22 °C, 1 year	100% and 90% decrease for LC and HC, respectively	
	Flunitrazepam	Blood	-80 °C, 1 year	Stable	
			-20 °C, 1 year	20% and 5% decrease for LC and HC, respectively	
			4 °C, 1 year	90% and 50% decrease for LC and HC, respectively	
			22 °C, 1 year	100% and 90% decrease for LC and HC, respectively	
	Midazolam	Blood	-80 °C, 1 year	Stable	
			-20 °C, 1 year	20% and 5% decrease for LC and HC, respectively	
			4 °C, 1 year	90% and 50% decrease for LC and HC, respectively	
			22 °C, 1 year	100% and 90% decrease for LC and HC, respectively	

	Oxazepam	Blood	-80 °C, 1 year	Stable	
			-20 °C, 1 year	20% and 5% decrease at LC and HC, respectively	
			4 °C, 1 year	90% and 50% decrease at LC and HC, respectively	
			22 °C, 1 year	100% and 90% decrease at LC and HC, respectively	
Alhadidi and Oliver[57]	Temazepam	Blood	-20 °C, 1 year	Stable	No additives
			4 °C, 1 year	18% decrease	
			25 °C, 1 year	53% decrease	
Alhadidi and Oliver[58]	Morphine	Blood	-20 °C, 1 year	Stable	No additives
			4 °C, 1 year	30% decrease	
			25 °C, 1 year	30% decrease	
	Buprenorphine	Blood	-20 °C, 1 year	Stable	No additives
			4 °C, 1 year	30% decrease	
			25 °C, 1 year	30% decrease	
Alhadidi and Battah[59]	Trihexyphenidyl	Blood	-20 °C, 6 months	Stable	No additives
			4 °C, 6 months		
			25 °C, 3 months		
		Urine	-20 °C, 6 months		
			4 °C, 6 months		
			25 °C, 4 months		
Alhadidi et al.[60]	Chlorpromazine	Blood	-20 °C, 10 months	Stable	No additives
			4 °C, 8 months		
			25 °C, 2 months		

Levine et al.[55]	Diazepam	Blood	4 °C, 5 months	Stable	With NAF
			25 °C, 5 months		
	Nitrazepam	Blood	4 °C, 5 months	< 20% decrease	
			25 °C, 5 months	> 50% decrease	
	Flurazepam	Blood	4 °C, 5 months	< 20% decrease	
			25 °C, 5 months		
	N-1-desalkylflurazepam	Blood	4 °C, 5 months	< 20% decrease	
			25 °C, 5 months		
	Chlordiazepoxide	Blood	4 °C, 5 months	> 50% decrease	
			25 °C, 5 months	100% decrease	
	Norchlordiazepoxide	Blood	4 °C, 5 months	< 20% decrease	
			25 °C, 5 months	100% decrease	
Lin et al.[61]	Morphine	Blood	-20 °C, 11 months	5.3-31% decrease	No additives
			4 °C, 11 months	5.4-39% decrease	
			25 °C, 11 months	1-100% decrease	
	Codeine	Blood	-20 °C, 11 months	14-25% decrease	
			4 °C, 11 months	12-22% decrease	
			25 °C, 11 months	9.5-66% decrease	
Baselt et al.[62]	Cocaine	Blood	4 °C, 1 year	100% decrease	1 % (w/v) NAF/K Oxalate
				76% decrease	1 % (w/v) NAF/Oxalic Acid
				60% decrease	0.1% (w/v) echothiophate
	Benzoylecgonine Ethanol	Blood	4 °C, 1 year	Stable	1 % (w/v) NAF/K Oxalate
					1 % (w/v) NAF/Oxalic Acid
					0.1% (w/v) echothiophate

McCurdy et al.[63]	Cocaine	Blood	4 °C, 1 months	> 50% decrease	No improvement in any of the 4 tubes (NAF, EDTA, Heparin and no additives)
			25 °C, 1 months		
	Benzoylecgonine THC-COOH	Blood	4 °C, 1 months	Stable	
			25 °C, 1 months		
Clauwaert et al.[65]	Amphetamine MDA MDMA MDEA	Urine and water	-20 °C, 21 weeks	Stable	No additives
			4 °C, 21 weeks		
			20 °C, 21 weeks		
		Blood	-20 °C, 21 weeks		
			4 °C, 21 weeks		
			20 °C, 21 weeks		
		Serum	-20 °C, 21 weeks		
			4 °C, 21 weeks		
			20 °C, 21 weeks		
Zaitsu et al.[66]	Estazolam	Urine	-20 ° C, 5 months	Stable	No improvement with NaN_3 and aseptic collection kit
			4 °C, 5 months		
			25 °C, 5 months		
	Amphetamine	Urine	-20 °C, 5 months	Stable in sterile sample but unstable in severely contaminated sample	Stability enhanced with NaN_3 and aseptic collection kit
			4 °C, 5 months		
			25 °C, 5 months		
	Methamphetamine	Urine	-20 °C, 5 months		
			4 °C, 5 months		
			25 °C, 5 months		
	Nitrazepam	Urine	-20 °C, 5 months	Stable	
			4 °C, 5 months		
			25 °C, 5 months	100% decrease	

	7-aminoflunitrazepam	Urine	-20 °C, 5 months	Stable	
			4 °C, 5 months		
			25 °C, 5 months	100% decrease	
Zaitsu et al.[66]	Cocaine	Urine	-20 °C, 5 months	Stable	Stability enhanced with aseptic collection kit but degradation increased when NaN_3 used
			4 °C, 5 months		
			25 °C, 5 months	100% decrease	
	6-acetylmorphine	Urine	-20 °C, 5 months	Stable	
			4 °C, 5 months		
			25 °C, 5 months	100% decrease	
Skopp et al.[67]	Diazepam Midazolam Prazepam Flurazepam Hydroxyethylflurazepam Lorazepam Nordazepam Flunitrazepam Norflunitrzepam Desalkylflurazepam Clorazepate Clobazam Bromazepam	Blood and plasma	4 °C, 8 months	> 60% decrease	No additives

Skopp et al.[68]	Morphine	Blood	-20 ° C, 6 months	Stable	No additives
			4 °C, 6 months		
			25 °C, 6 months		
		Plasma	-20 ° C, 6 months	Stable	
			4 °C, 6 months		
			25 °C, 6 months	23% decrease	
	M3G	Blood	-20 ° C, 6 months	Stable	
			4 °C, 6 months		
			25 °C, 6 months		
		Plasma	-20 ° C, 6 months		
			4 °C, 6 months		
			25 °C, 6 months		
	M6G	Blood	-20 ° C, 6 months	Stable	
			4 °C, 6 months		
			25 °C, 6 months	23% decrease	
		Plasma	-20 ° C, 6 months	Stable	
			4 °C, 6 months		
			25 °C, 6 months	14% decrease	

Lutfi[69]	Diazepam	Blood	-20 °C, 1 year	Stable	No additives
			4 °C, 1 year	20% decrease	
			25 °C, 1 year	46% decrease	
	Desmethyldiazepam	Blood	-20 °C, 1 year	43% decrease	
			4 °C, 1 year	53% decrease	
			25 °C, 1 year	70% decrease	
	Temazepam	Blood	-20 °C, 1 year	30% decrease	
			4 °C, 1 year	38% decrease	
			25 °C, 1 year	44% decrease	
	Amitriptyline	Blood	-20 °C, 1 year	Stable	
			4 °C, 1 year	Stable	
			25 °C, 1 year	42% decrease	
	Nortriptyline	Blood	-20 °C, 1 year	44% decrease	
			4 °C, 1 year	54% decrease	
			25 °C, 1 year	73% decrease	
	Imipramine	Blood	-20 °C, 1 year	Stable	
			4 °C, 1 year	Stable	
			25 °C, 1 year	50% decrease	
Giorgi and Meeker[70]	Cocaine	Blood	25 °C, 5 year	100% decrease	No additives
	Benzoylecgonine	Blood	25 °C, 5 year	100% decrease	
	Morphine	Blood	25 °C, 5 year	62% decrease	
	Codeine	Blood	25 °C, 5 year	Stable	
	Methamphetamine	Blood	25 °C, 5 year	100% decrease	
	Phencyclidine	Blood	25 °C, 5 year	69.4% decrease	

Kala and Lechowics.[72]	Pancuronium	Blood	-20 °C, 7 months 25 °C, 3 months	Stable	No additives
Dugan and Bogema[73]	Amphetamine	Urine	-20 °C, 1 year	Stable	No additives
	Methamphetamine				
	Morphine				
	Codeine				
	Phencyclidine				
	THC-COOH				
	Cocaine	Urine	-20 °C, 1 year	37% decrease	
Datta.[245]	Digoxin	Saliva	25 °C, 2 weeks	Stable	No additives
Lori et al.[74]	Digoxin Digitoxin	Serum	4 °C, 3 days	Stable	No additives
Forsdahl and Gmeiner[75]	Salbutamol	Urine	-18 °C, 2 months	4% decrease	No additives
			4 °C, 2 months	12% decrease	
Celma et al.[76]	Paracetamol	Plasma	-20 °C, 3 freezing and thawing cycles for 3 months	Stable	No additives
	Chlorpheniramine		25 °C, 1 day in autosampler		
Bennetto et al.[77]	Nevirapine	Blood and serum	4 °C, 1 day	Stable	No additives
			25 °C, 1 day		
			37 °C (incubator), 1 day		
Barnada et al.[78]	Nifedipine Nisoldipine	Plasma	-20 °C, 2 months 25 °C, 1 day	Stable	Protected from laboratory light
Oliver and Stephen[79]	Promazine Desmethylpromazine	Plasma and urine	-20 °C, 2 months	Stable	No additives

Skopp et al.[80]	THC-COOH-glucuronide	Plasma	-20 °C, 10 days	Stable	No additives
			4 °C, 10 days	Degradation increased when temperature increased	
			20 °C, 10 days		
			40 °C, 10 days		
Skopp et al.[81]	LSD	Urine	-20 °C, 3 days	Stable	No additives
			4 °C, 3 days		
			22 °C, 3 days		
			40 °C, 3 days	Unstable	
	N-desmethyl-LSD 2-oxo-hydroxy-LSD	Urine	-20 °C, 3 days	Stable	
			4 °C, 3 days		
			22 °C, 3 days	Unstable	
			40 °C, 3 days		
Yegles et al.[83]	Diazepam	Hair	Room temperature	39.7% decrease	Bleached
	Nordiazepam			67.7% decrease	
	7-Aminoflunitrazepam			61.8% decrease	
	Codeine			57.5% decrease	
	6-acetylmorphine			88.6% decrease	
	Morphine			67.4% decrease	
	Cocaine			24.6% decrease	
	Benzoylecgonine			88.6% decrease	
Moriya et al.[88]	Dichlorvos	Blood	22 °C, 3 days	100% decrease within15 minutes	NAF (2% W/V)
	Chlorpyrifos			95% decrease after 3 days	

Posyniak et al.[91]	Enrolfloxacin	Dried blood spot	-20 °C, 4 weeks	Stable	Stored in filter paper
			4 °C, 4 weeks		
	Ciprofloxacin		22 °C, 4 weeks		
Hoogtanders et al.[92]	Tacrolimus	Dried blood spot	4 °C, 1 month	Stable	Stored in filter paper
Skopp[94]	Amphetamine Diazepam	Dried blood spot	40 °C, 17 days	Stable	Stored in filter paper
Skopp[93]	Cocaine	Dried blood spot	-20 °C, 17 days	Stable	Stored in filter paper
AbuRuz et al.[95]	Metformin	Dried blood spot	-70 °C, 2 month	Stable	Stored in filter paper
Dubey et al.[96]	Benzoylecgonine Morphine Phencyclidine THC-COOH	Dried urine spot	-20 °C, 12 weeks	Stable	Stored in filter paper
			4 °C, 12 weeks		
			25 °C, 12 weeks		
		Aliquot of urine	-20 °C, 12 weeks		
	Amphetamine	Dried urine spot	-20 °C, 12 weeks	Stable	
			4 °C, 12 weeks		
			25 °C, 12 weeks		
		Aliquot of urine	-20 °C, 12 weeks	16% decrease	

Lejeune et al.[97]	Chloroquine	Dried blood spot	-20 °C, 20 days	Stable	Stored in filter paper
			4 °C, 20 days		
			25 °C, 20 days	>50% decrease	
			50 °C, 20 days		
	Proguanil	Dried blood spot	-20 °C, 20 days	Stable	
			4 °C, 20 days		
			25 °C, 20 days		
			50 °C, 20 days	80% decrease	
	Monodesethylchloroquine	Dried blood spot	-20 °C, 20 days	Stable	
			4 °C, 20 days		
			25 °C, 20 days	100% decrease	
			50 °C, 20 days		
	Cycloguanil	Dried blood spot	-20 °C, 20 days	Stable	
			4 °C, 20 days		
			25 °C, 20 days		
			50 °C, 20 days		
Gupta[247]	Cocaine	Aqueous solution	24 °C, 45 days	Stable	pH < 4
Olesen et al.[100]	Olanzapine	Serum	4 °C, 2 weeks	Stable	0.25% ascorbic acid
				45% decrease	No antioxidant
			25 °C, 1 day	Stable	0.25% ascorbic acid
				40% decrease	No antioxidant
Ueyama et al.[101]	Dimethylthiophosphate (DMTP)	Urine	During the extraction	Conversion to DMP and DEP reduced to < 1%	1%(w/v) of SOD, ascorbic acid or pyrogallol
	Diethylthiophosphate (DETP)				

Skopp et al.[85]	Cocaine	Blood	40 °C, 15 days	100% decrease while degradation product increased	0.25%(w/v) NAF
Fumio and Yoshiaki[48]	Nitrazepam	Postmortem blood	2-8 °C	Different concentration of nitrazepam and 7-amino-nitrazepam at different sites of nitrazepam case sample toxicity	The change in the concentration is attributed to bacterial contamination or dilution by water that entered in the circulation through the lung
Robertson and Drummer[45]	Flunitrazepam Nitrazepam Clonazepam	Postmortem blood	22 °C, 21 days	100% converted to 7-aminometabolites	No additives
				13% converted to 7-aminometabolites	0.7%(w/v) NAF
				4% converted to 7-aminometabolites	1%(w/v) NAF
				4% converted to 7-aminometabolites	2%(w/v) NAF

Ageda et al.[87]	Dichlorvos	Blood	4 °C, 1 day	100% decrease	No additives
			25 °C, 1 day		
			37 °C, 1 day		
	Malathion	Blood	4 °C, 1 day	44% decrease	
			25 °C, 1 day	100% decrease	
			37 °C, 1 day		
	Trichlorfon	Blood	4 °C, 1 day	14% decrease	
			25 °C, 1 day	100% decrease	
			37 °C, 1 day		
	Phenthoate	Blood	4 °C, 1 day	18% decrease	
			25 °C, 1 day	61% decrease	
			37 °C, 1 day	86% decrease	
	Fenitrothion	Blood	4 °C, 1 day	15% decrease	
			25 °C, 1 day	28% decrease	
			37 °C, 1 day	82% decrease	
	Cyanophos	Blood	4 °C, 1 day	Stable	
			25 °C, 1 day	36% decrease	
			37 °C, 1 day	74% decrease	
	Methidathion	Blood	4 °C, 1 day	5% decrease	
			25 °C, 1 day	21% decrease	
			37 °C, 1 day	63% decrease	
	Dimethoate	Blood	4 °C, 1 day	7% decrease	
			25 °C, 1 day	17% decrease	
			37 °C, 1 day	43% decrease	
	Thiometon	Blood	4 °C, 1 day	Stable	
			25 °C, 1 day	8% decrease	
			37 °C, 1 day	38% decrease	
	Isoxathion	Blood	4 °C, 1 day	8% decrease	
			25 °C, 1 day	9% decrease	
			37 °C, 1 day	35% decrease	

Ageda et al.[87]	EPN	Blood	4 °C, 1 day	Stable	No additives
			25 °C, 1 day	11% decrease	
			37 °C, 1 day	28% decrease	
	Acephate	Blood	4 °C, 1 day	Stable	
			25 °C, 1 day	12% decrease	
			37 °C, 1 day	22% decrease	
	Diazinon	Blood	4 °C, 1 day	Stable	
			25 °C, 1 day	6% decrease	
			37 °C, 1 day	21% decrease	
	Sulprofos	Blood	4 °C, 1 day	Stable	
			25 °C, 1 day		
			37 °C, 1 day		

3 General Experimental Procedures Used in this Project

3.1 Extraction

The main role of extraction is to separate the analytes from their matrix and preferably also remove as many interferences from the sample as possible, such as protein and other biological components, before analysis. However, some difficulties normally occur in forensic toxicology in determining compounds in blood samples, such as haemolysis of the samples and putrefaction of a postmortem sample, as described earlier. Some samples may be contaminated by components of the container such as the rubber septa, which may make contact with the sample during collection and storage. The samples may also have solidified and coagulated, especially if no additives were used. Furthermore, the amount of the sample collected may be small or have very low drug concentrations. Therefore, using an efficient, first-rate technique for the extraction of samples is necessary in order to obtain a high recovery of the analytes of interest from biological samples.[102]

The most common extraction techniques are liquid liquid extraction (LLE) and solid phase extraction (SPE). However, SPE posses advantages over LLE, as discussed in detail in the next section and solid phase extraction was used in the studies in this thesis.

3.1.1 Solid phase extraction

Solid phase extraction, first reported in 1974, is a relatively new technique and has become rapidly established in the clinical, biochemical, industrial, environmental, and pharmaceutical fields because of its powerful ability to provide clean extracts of dirty samples, such as those containing protein, salts, and resins in high amounts.[102]

SPE has largely replaced LLE because of its advantages over LLE; for example, a lower sample quantity (100 µL or less) can be used in SPE. In addition, the small volume of solvents required in SPE is less than those used in LLE, making SPE

more economical. Moreover, the sorbents used in SPE are cheap and can be discarded after use. LLE is also more time consuming than SPE, which can provide higher recovery, selectivity, and reproducibility in comparison with LLE, and its procedure can be easily automated.

The SPE extraction procedure is achieved through five steps, usually carried out with a vacuum work station. The first is *conditioning* of the column to solvate the functional groups of the sorbent in order to interact with and retain the analytes of interest. This also removes any fine particles and dust from the sorbent.

The next step is the *loading of the sample* at a low flow rate without vacuum application to allow enough time for the target analyte to be retained in the column by interaction with the sorbent functional groups. However the sample should be pretreated with a suitable buffer before this step and addition of a suitable internal standard (IS), mixing then centrifuging.

The third step is *washing the column* with appropriate solvents to remove unwanted matrix interferences retained in the sorbent without removing the analytes of interest. Then, *drying of the column* is achieved by applying full vacuum to remove as much residual aqueous solvent as possible, that may be immiscible with the elution solvent.

Finally, it is necessary to *elute the analytes* of interest by using a small volume of solvent to disrupt all retention mechanisms operating between the sorbent functional groups and the analytes.

Compounds may be retained on SPE sorbents by different mechanisms, depending on the functional groups bonded to the sorbent backbone (usually silica but also organic polymers). The most common SPE columns are described below.

3.1.1.1 Hydrophobic SPE columns

The sorbent backbone is bonded with hydrocarbon chains, such as C8 and C18. A non-polar analyte binds to the sorbent by hydrophobic interaction caused by Van Der Wall forces. Disruption is achieved by applying a suitable organic solvent to

displace the analytes from the column. The C18 column, for example the commercial product Bond Elut®, is the most common and widely used hydrophobic column because of its ability to retain a wide range of organic compounds by non-selective interactions. However, its disadvantage is that because it is non-selective, it also retains non-polar interferences from the matrix.

3.1.1.2 Hydrophilic SPE columns

This column is composed of a silica or polymer backbone bonded with carbon chains containing polar functional groups such as amines, hydroxyls and carbonyls. The analyte is retained in the sorbent as a result of polar interactions, including hydrogen bonding, π-π interactions, or dipole-dipole interactions. Polar analytes are often extracted from non-polar matrices such as oils with polar columns which need very strongly polar solvents to elute the analyte. The Oasis® column is an example of a hydrophilic column using a polymeric backbone.

3.1.1.3 Ion exchange SPE columns

This sorbent is composed of a silica or polymeric backbone bonded with carbon chains terminated with negatively or positively charged functional groups. Analytes are retained by ionic bonding between the charged sorbent and the oppositely charged analytes. There are two types of ion exchange: cationic and anionic.

In a cationic ion exchange column, the sorbent is substituted with negatively charged groups, such as benzenesulfonic acid, propylsulfonic acid or a carboxylic acid, whic ion-pair with positively charged analytes (bases, usually amines) to form strong bonds. In elution, the analyte is carried out by using a basic solvent to neutralize and release the analyte from the cationic ion exchange sorbent.

In contrast, the sorbent in an anionic ion exchange column carries positively charged substituents, such as primary, secondary or quaternary amines, aminopropyl or diethylamino groups, which interact with negatively charged analytes (acids) through strong ionic bonds. These interactions are disrupted by

using an acidic solvent to neutralize analytes that are to be eluted from the column.

3.1.1.4 Mixed mode SPE columns

The sorbent contains both hydrophobic and ionic functional groups which provide two retention mechanisms. This type of column provides a clean extract because of its ability to retain a wide range of compounds with high selectivity without any interferences. Thus, it is widely used in forensic toxicology. For example, Bond Elut Certify® and CLEAN SCREEN® SZDAU020 contain hydrophobic C8 groups and a strong cationic exchanger (benzosulfonic acid), while Bond Elut Certify II® contains hydrophobic C8 and a strong anionic exchange (quaternary amine).[102]

3.2 Derivatization

The presence of a polar functional group in an analyte such as a hydroxy or amino groups may cause a polar interaction between the analyte and the analytical column stationary phase or polar residues in the injector and column which have accumulated from previous analyses, especially in gas chromatography and gas chromatography/mass spectrometry, leading to peak tailing and poor detection of the compound. Therefore, the derivatization process is used to modify the chemical properties of a compound by chemically altering the polar active sites on the molecule, producing a thermally stable, low polarity and more volatile compound, thereby improving selectivity and detectability of the analyte prior to analysis.

Derivatization is classified into three main groups: silylation, acylation and alkylation. Silylation is the most common and widely used derivatisation reaction in forensic toxicology and was used in this thesis for silylation of dialkylphosphate (DAP) compounds.[102,103]

3.2.1 Silylation

Silylation is the introduction of a silyl group, usually the trimethylsilyl group, $Si(CH_3)_3$, into the analyte by substitution of the active hydrogen in the analyte

with the silyl group, thus reducing the polarity of the compound and decreasing hydrogen bonding.

There are a large number of silylation reagents available commercially. In this thesis, N-*tert*-butyl-dimethylsilyl-N-methyl-trifluoracetamide (MTBSTFA) containing 1% t-butyldimethylchlorosilane (TBDMCS) was used as described in a previous study that used this derivatizing agent for DAP compounds. MTBSTFA reacts with carboxyls, hydroxyls thiols, and primary and secondary amines in a short time and at room temperature and the solution of analyte in the reagent can be injected directly into the gas chromatograph. Temperature and catalysts such as trimethylchlorosilane (TMCS) and TBDMCS are often used to increase the rate of reaction.[103]

3.3 Gas Chromatography/Mass Spectrometry (GC/MS)

Gas chromatography (GC) is a separation technique used to separate volatile compounds from the sample by differential migration through a column containing the stationary phase.The samples should be injected into the flowing mobile phase rapidly. The injection port is connected to the top of the column which should be hot to vaporize the sample before enter to the column. There are different injection methods and the most common techniques are split and splitless injection mode. Split injection is less sensitive than splitless because a small fraction of the injected sample enters the column while the rest vents to the atmosphere via a control valve. Transportation of analytes through the column to the detector is performed by an inert gaseous mobile phase, usually helium or nitrogen. [104]

Analytical columns are coiled to allow them to be housed within a fan-assisted thermostatically controlled oven. Currently, GC uses mostly open tubular capillary columns made from fused silica, distinguished by their internal diameter, for example narrow bore columns have internal diameters of approximately 0.25 mm and megabore columns have a diameter of 0.5 mm. Columns also vary according to their length, from 10-100 m, stationary phase composition, from non-polar to polar, without or with microparticulate support (SCOT columns) and stationary phase layer thickness, from 0.1-several microns. Packed GC columns are still used for particular applications, such as analysis of gases, especially in an industrial environment. [104]

After the analytes are separated by the column, they are eluted into the detector to be registered and produce the gas chromatogram. The most important detector that is widely used in forensic toxicology is the mass spectrometer and the combined technique is termed gas chromatography/ mass spectrometry (GC/MS). [104]

GC/MS is used to identify and quantify analytes. The GC effluent from a capillary column is conducted directly into the ion source of the mass spectrometer, which is composed of an interface, an ion source, a mass analyzer and a detector. The interface is maintained at a high temperature to ensure that the sample components remain in the gas phase. The ion source is maintained under

vacuum and molecules eluting from the GC column are ionized before passing into the mass analyser. The most common ionization methods are electron impact ionization (EI, hard ionization) and chemical ionization (CI, soft ionization). In EI, molecules are bombarded with high energy electrons (70 electron volts, eV), which is higher than the ionization potential of most molecules and enough to break internal bonds within the molecule to create a charged ion, the "molecular ion" and fragment ions. After that, the ions are separated according to their mass/charge ratios, which is achieved with a mass analyzer such as the time of flight, ion trap and quadrupole analyzers. The quadrupole is the most common analyzer because it is small in size, inexpensive, has a lower operating voltage and offers ease of computer control. [104]

When ions enter the detector a significant amount of energy is tranferred to its surface. That energy is used to generate a more easily detected species, usually electrons or photons that can be detected by an electron or photo-multiplier. The multiplier can enormously amplify the signals produced by the ions. The amplified signal is converted into digital form with a high frequency analogue-to-digital converter, then relayed to the computer data acquisition system.[104]

The dynamic combination of GC and MS techniques results in a powerful separation and structural identification technique and has been used to analyse a wide range of compounds.[105-109]

3.4 Liquid chromatography - tandem mass spectrometry (LC/MS/MS)

In this thesis, LC-MS/MS was used to analyze the compounds discussed in Chapters 4-7. The instrument contains three main components: the HPLC system, the interface and the mass spectrometer.

Liquid chromatography (LC) is a separation technique using a liquid mobile phase to separate analytes by differential migration through a column containing a microparticulate stationary phase. The LC system can be normal phase (NP) or reversed phase (RP). In NP, the stationary phase is more polar than the organic solvent and systems of this type are used for the analysis of non-polar compounds. However, NP is avoided in most laboratories because it generally involves mobile phases containing hazardous solvents such as hexane and dichloromethane. In RP, the stationary phase is less polar than the mobile phase and this type of system is suitable for analysis of polar compounds and is commonly used in forensic toxicology. The most common non-polar silica bonded stationary phase is C_{18}, which has been used to separate many drugs.[112]

Transportation of the analyte in a RP system is achieved by the mobile phase, which usually contains an aqueous buffer and an organic solvent, such as acetonitrile or methanol. Selection of the mobile phase components is very important; for example, organic solvents such as acetonitrile and methanol are preferred because they are less hazardous than the solvents used in NP systems. The buffer used must be volatile, especially in LC/MS/MS, such as ammonium formate, ammonium acetate, acetic acid, and ammonia because a non volatile buffer such as phosphate buffer will precipitate in the ion source, leading to clogging and degradation of instrument performance. Further, high buffer concentrations should be avoided in order to prevent saturation of the surface of droplets formed in the LC-MS interface, consequently inhibiting the formation of the gaseous form of analytes and leading to a low instrument response. In addition, it is very important to adjust the pH of the mobile phase in order to ionize analytes of interest, either basic or acid, because the addition of a small amount of volatile acid to the mobile phase will ionize basic analytes while volatile bases will ionize acidic compounds.[110,112]

Before the analyte is introduced into the mass analyzer in the mass spectrometer component, it must be ionized and the solvent from the LC eluent must be eliminated in order to introduce the analyte in ionized gaseous form. This process is achieved in the interface. The most common interfaces are atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), which is the most common ionization mode in analytical toxicology. In ESI, a high voltage (3-5 Kv) is applied to the ESI needle to evaporate the solvent into charged droplets, which exit the ESI probe. When the solvent evaporates, the droplet size decreases, ions come closer together and repell each other. When the repulsion overcomes the cohesive forces of surface tension, a series of columbic explosions results, which splits the droplet into microdroplets as a result of these explosions until the gas phase ions are produced. The ions move through the ion optic assembly to the mass analyzer.[111,112]

The most common mass analyzer is an ion trap, which is known as the quadrupole ion trap mass analyzer. It consists of a ring "doughnut" electrode, to which an oscillating radiofrequency (RF) field is applied, and two hemispherical electrodes (endcaps) which are placed above and below the ring electrode. The end caps have small holes in the center allowing ions to pass in and out of the cavity. The ions that enter the cavity will be trapped in the ring electrode until RF is applied. As the frequency increases, trapped ions are selectively ejected out through the exit lens into the ion detection system. [111,112]

In the detection system, selected ions will strike the surface of a conversion dynode to produce one or more secondary particles. Then the secondary particles are focused by the curved surface of the conversion dynode and accelerated into the electron multiplier. If the secondary particles have sufficient energy, the electron multiplier cathode will eject electrons, which will strike the inner surface of the cathode to produce more electrons, thus the cascade of electrons produced results in a measurable current. The current will be converted to a voltage by an electrometer circuit and recorded by the MS data system. [111,112]

LC-MS/MS has been used to analyse a wide variety of compounds.[113-125]

3.5 Method validation

Method validation is the application of experimental procedures in order to ensure that an analytical method is reliable and fit for its intended purpose. Validation of a method used in drug analysis is very important because an unreliable method may lead to false positive or negative results or to underestimating or overestimating the concentration of an analyte, resulting in a false interpretation. An incorrect interpretation may lead to the wrong treatment of the patient or unjustified legal consequences for a defendant in court. It is therefore very important to use validated methods in forensic toxicology in order to give accurate results which can form the basis of correct interpretations.

Different guidelines have been published for method validation.[126-133] The validation parameters used in this study were as follows:

3.5.1 Linearity

The linearity of a method, or calibration model, investigates the relationship between the concentration of the analyte in the sample and the detector response. This is achieved by analyzing the six sets of samples spiked with different concentrations of the analyte and plotting the resulting responses (y) versus the corresponding concentration (x). The degree of linearity is provided by the value of the correlation coefficient (r^2). The method is considered acceptable if the linear correlation coefficient (r^2) exceeds 0.99.

3.5.2 Limit of Detection and Lower Limit of Quantitation

3.5.2.1 Limit of detection (LOD)

The LOD is defined as the lowest concentration of an analyte that can be detected and that can be differentiated from background noise. The LOD is measured by analysing samples spiked with decreasing concentrations of the analyte. LODs were calculated using Equations 3-1 and 3-2.

$$Y_{LOD} = Y_B + 3S_B \qquad \text{Equation 3-1}$$

$$LOD = (Y_{LOD}-Y_B)/m \quad \text{Equation 3-2}$$

Where Y_B is the intercept, S_B is the standard error of the regression line, and m is the gradient.

3.5.2.2 Lower limit of quantitation (LLOQ)

LLOQ is defined as the lowest concentration of an analyte that can be quantitated with the required precision and accuracy. The LLOQ is measured by analyzing samples spiked with decreasing concentrations of the analyte. LLOQs were calculated using Equations 3-3 and 3-4.

$$Y_{LLOQ} = Y_B+10S_B \quad \text{Equation 3-3}$$

$$LLOQ = (Y_{LLOQ}-Y_B)/m \quad \text{Equation 3-4}$$

Where Y_B is the intercept, S_B is the standard error of the regression line, and m is the gradient.

3.5.3 Recovery

Recovery is the percentage of analyte extracted from a sample and is used to measure the efficiency of the extraction method. Recovery of analytes was determined at three concentrations - low, medium and high - using Equation 3-5.

Recovery (%) = (peak area ratio of extracted standard/peak area ratio of unextracted standard) x 100 Equation 3-5

3.5.4 Precision

Precision is the closeness of agreement between a series of tests results obtained under a set of prescribed conditions. In this thesis, precision was measured intraday and interday, and was calculated as the coefficient of the variation expressed as a percentage (CV %) or as the percent relative standard deviation (RSD %).

3.5.4.1 Intraday precision

Standards were prepared at three concentrations - low, medium and high - and five replicates at each concentration were extracted. Each extract was analysed and the relative standard deviations at each concentration were calculated as the RSD %. An RSD % less than 20 % is considered to be acceptable.[126]

3.5.4.2 Interday precision

Five sets of standards at three different concentrations - low, medium and high - were extracted over five different days. The RSD% was calculated for each concentration. An RSD % less than 20 % is considered to be acceptable. [126]

3.6 Statistical evaluation of stability of substances during storage

In this project, a statistical evaluation was made of the stability of drugs and other substances during storage based on the coefficient of variation of the method, obtained from measurement of the first day concentration (n=10 or n=5). A substance was considered to be significantly unstable ($p < 0.05$) if its concentration at the time selected was less than the value obtained from Equation 3-6.[59,134]

Concentration threshold = $C° - (1.96 \times CV\ \%)$ Equation 3-6

Where

$C°$ =initial concentration

CV %= coefficient variation of initial concentration

Student's t value for any sample in a series was calculated using Equation 3-7 and the *p* value was calculated in Excel® using the one tail t-test.

t value = $(C° - C_n)/ (CV\ \%)$ Equation 3-7

Where $C°$ is the initial concentration and C_n is the concentration of the significant loss point.

4 Stability of Benzodiazepines in Whole Blood under Different Storage Conditions

4.1 Introduction

Benzodiazepines, one of the most common drug classes, act on the central nervous system as CNS depressants. Chlordiazepoxide, the first drug in this group, was discovered in 1955 and marketed in 1960 after a clinical application trial, which revealed different clinical effects such as its hypnotic, sedative, and anticonvulsant activities. Since that time, benzodiazepines have been widely prescribed and are used to treat insomnia and anxiety and as muscle relaxants. Benzodiazepines are classified according to structural features into N_1-substituted-1,4-benzodiazepines, diazolobenzodiazepines, triazolobenzo-diazepines and nitrobenzodiazepines subtypes. Alternatively they are classified according to pharmacokinetic half life into short- and long-acting benzodiazepine subtypes, which means that benzodiazepines with a half life less than 24 hours are considered to be short-acting and those with a half life greater than 24 hours are considered to be long-acting. Short-acting benzodiazepines are used to treat insomnia, whereas long-acting benzodiazepines are used to treat anxiety-related symptoms such as panic attack or social phobia. Many benzodiazepine drugs are controlled by the United Nations Convention on Psychotropic drugs (1971). These drugs are frequently abused by illicit users in combination with other compounds such as opiates, antidepressants, and alcohol. [135,136]

Benzodiazepines are often present in the blood of drivers involved in road accidents because they impair a driver's attention and cognition.[137,142]
Benzodiazepines are also capable of causing drug dependency and tolerance after regular use which may take weeks or months to develop.[135,136]
Since benzodiazepines impair human performance and attention, some of them, such as flunitrazepam and alprazolam, are used by rapists to facilitate sexual assaults, Drug Facilitated Sexual Assault (DFSA).[143,144]

4.2 Benzodiazepines included in the present study

Stability studies were carried out on four benzodiazepines which are described below and whose structures are given in Figure 4-1.

Alprazolam is a triazolobenzodiazepine used therapeutically to treat panic disorder, panic attack, depression, agoraphobia, and generalized anxiety.
Alprazolam has a therapeutic level in plasma ranging between 0.005 and 0.05 mg/L, whereas a toxic concentration ranges between 0.1 mg and 0.4 mg/L, with 2.1 mg/l being reported as the highest postmortem blood concentration. The side effects of alprazolam usually include sedation, drowsiness, confusion, aggression, nightmares, tachycardia, and palpitation. Alprazolam is metabolized by oxidation followed by conjugation to produce active metabolites α-hydroxyalprazolam, 4-hydroxyalprazolam, and α, 4-dihydroxyalprazolam.[145]

Lorazepam is a 3–hydroxy-benzodiazepine. It is administered orally as an anxiolytic, tranquilizer, muscle relaxant, anticonvulsant, and hypnotic drug.
Lorazepam is rapidly absorbed from the digestive tract and its therapeutic plasma concentration reaches 0.018 mg/L after 2 hours while toxic concentrations in the blood range between 0.28-1.0 mg/L. Lorazepam is rapidly conjugated with glucuronic acid to produce an inactive metabolite that accumulates in plasma, achieving concentrations that exceed those of its parent[145].

Oxazepam is a 3-hydroxybenzodiazepine used clinically as an anxiolytic and hypnotic. The maximum therapeutic serum concentration has been reported as 0.31 mg/L after 1.5 hours. Toxicity has been reported in 14 drivers arrested for impaired ability who had an average blood concentration of 2.4 mg/L. Moreover, two cases of oxazepam poisoning have been reported, with blood concentrations found at 4.4 and 6.1 mg/L. Oxazepam is rapidly conjugated with glucuronic acid to produce an inactive metabolite. [145]

The triazolobenzodiazepine estazolam is widely used as a sedative and hypnotic drug. Its molecular structure is similar to that of alprazolam and triazolam. Maximum plasma therapeutic concentrations of estazolam after an oral dose of 1 mg ranged between 42-70 μg/L, while the blood concentration of estazolam in an overdose case was 1250 μg/L two days after a suicide attempt. Moreover, a

postmortem case has been reported involving overdose with estazolam and alcohol which had a blood concentration of estazolam of 480 µg/L. In addition, estazolam undergoes oxidative metabolism to produce 1-oxoestazolam and 4-hydroxyestazolam. Both of these metabolites have some pharmacological activity. [145]

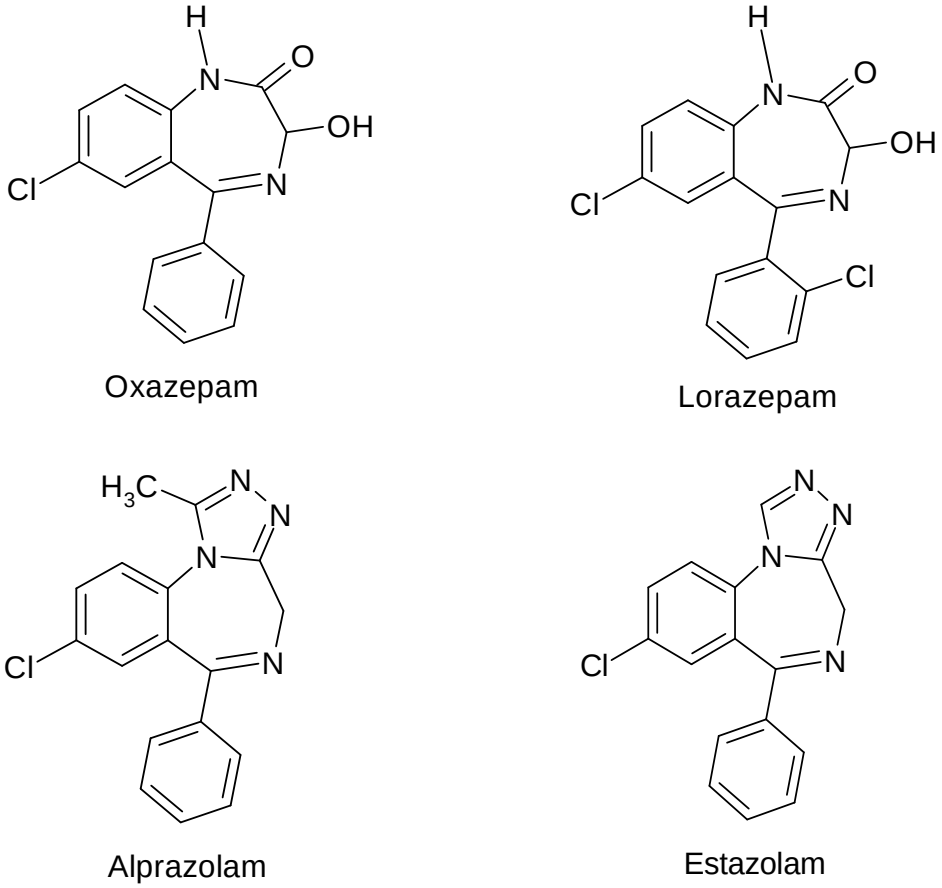


Figure 4-1: Chemical structures of benzodiazepines included in this study.

4.3 Aims

The aim of this study was to determine the stability of selected benzodiazepines under a range of storage conditions which represent those likely to be encountered in routine forensic toxicology investigations in different parts of the world. The drugs selected for this work have not previously been studied with respect to their long-term stability during storage. The effect of preservatives on drug stability was also studied using sodium fluoride and potassium oxalate as preservative and anticoagulant respectively, which are the most common

additives used in forensic toxicology and which have been found to affect the rate of hydrolysis of some types of analyte, such as organophosphate pesticicides (See Chapter 2 and Chapter 8).

Alprazolam, lorazepam, oxazepam and estazolam stabilities at low and high concentrations in blood were investigated in preserved and unpreserved tubes in freezer, refrigerator and at room temperature for one year.

4.4 Experimental Section

4.4.1 Chemical and material:

Alprazolam, lorazepam, oxazepam, and estazolam were purchased from LGC-Promochem (Middlesex, UK) as 1 mg/ml solutions in methanol. Alprazolam-d5, lorazepam-d4, oxazepam-d5 and estazolam-d5 were purchased from LGC-Promochem (Middlesex, UK) as 100 µg/ml solutions in methanol. Acetonitrile, methanol, cyclohexane, ethyl acetate, ammonium hydroxide, formic acid and potassium dihydrogen phosphate were obtained from BDH (Poole, UK). Ammonium formate, anhydrous disodium orthophosphate and sodium dihydrogen orthophosphate were purchased from Sigma-Aldrich (Dorset, UK). Clean screen® ZSDAU020 cartridges were purchased from United Chemical Technology UK. Tubes containing sodium oxalate were obtained from Tek Lab Ltd.

4.4.2 Solutions:

4.4.2.1 Preparation of 0.1M phosphate buffer pH 6.0:

1 litre of solution was prepared by dissolving 1.7 g of anhydrous disodium orthophosphate and 12.14 g of sodium dihydrogen orthophosphate monohydrate in 800 ml of deionized water. The pH was adjusted to 6 with 1 M potassium hydroxide and the solution made up to volume with deionized water.

4.4.2.2 Preparation of 3mM ammonium formate and 0.001% formic acid

This mobile phase was prepared by dissolving 0.189 g of ammonium formate in 800 ml deionized water followed by addition of 10 µl of concentrated formic acid and making up to one litre with deionized water.

4.4.2.3 Standard solutions of analytes

Working standards of unlabelled and labelled drugs were prepared by diluting 250 µl from each drug stock solution in 25 ml methanol to obtain a concentration of 1 µg/ml. Stock solutions of standards and internal standards were stored at -20 ºC.

4.4.3 Preparation of blood samples:

Packed human red blood cells, which had passed their usable date, were obtained from the Scottish National Blood Transfusion Service and were re-suspended in an equal volume of isotonic saline to give a total volume of 800 ml. The original blood collection used ethylene diamine tetra acetic acid (EDTA) as anticoagulant. It was screened to check whether it contained benzodiazepines of interest in this study, and the result was negative.

The blank blood was divided into two portions (400 ml each). One portion was spiked with a low concentration of four benzodiazepines drugs and mixed on the roller for one hour to give a final concentration of 50 ng/ml. The second portion of blank blood was spiked with a high concentration of drugs and mixed for one hour to produce a final concentration of 400 ng/ml. Ten aliquots of volume 1 mL from each portion were analyzed and found to be homogeneous (RSD% < 14 for all analytes as shown in Tables 4-6 to 4-13 as day zero concentrations), and the mean for each analyte was considered to be the day zero concentration. Subsequently, aliquots of blood (volume 2.5 mL) were pipetted into 300 x 2.5 ml tubes, half of which contained 2 mg sodium fluoride and 3 mg potassium oxalate per ml in 2.5 ml container while the others contained no additives. These prepared samples were stored as real case samples in the 300 containers.

4.4.4 Sample storage and time for analysis

The prepared samples were divided into three groups and were then stored at three different temperatures (-20 ºC, 4 ºC, and room temperature) as shown in Figure 4-2. These storage temperatures were selected because they are frequently-used laboratory storage temperatures. The storage temperatures were monitored daily using permanently mounted digital thermometers with a resolution of 0.1 C°at the start of the study and subsequently at weekly intervals during the period of study. Temperatures were stable within a range of approximately 1 C ° (freezer and refrigerator) or 5 C° (room temperature, average temperature 20°C). The times selected for analysis were at day 1 (day zero), 2, 4, 7, 14, 30, 60, 90, 180, and 365.

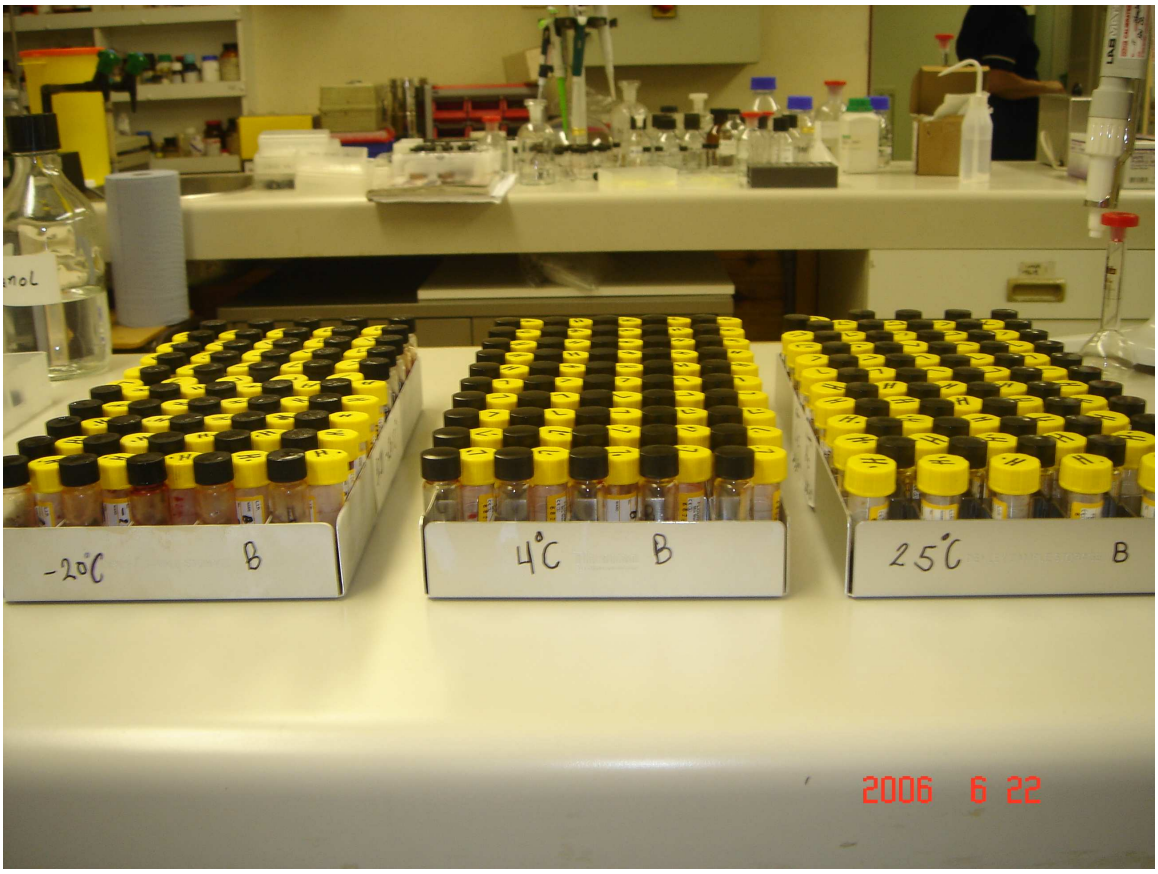


Figure 4-2: Prepared samples were stored under different conditions

4.4.5 Analytical method

The analytical method used in this study was based on a procedure published in the literature for analysis of benzodiazepines in oral fluid, in which a mixed mode sorbent was used for extraction followed by LC-MS/MS analysis.[125] Since the method was to be applied to blood rather than oral fluid, it required to be fully validated for its intended purpose before it was used in the stability study as summarized in Table 4-1. Details of the final method are given below.

Table 4-1: Validation of analytical method

Parameter	Samples analysed	Calculation of parameter and acceptance criteria
Linearity	Benzodiazepines standards were spiked into blood to produce two concentration ranges, 2-200 ng/ml and 25-800 ng/ml. The first set contained standards at concentrations of 2, 5, 10, 25, 50, 100, 200ng/ml and the second set contained standards at 25, 50, 100, 200, 400 and 800 ng/ml. 100 ng/ml of internal standards were added to each calibrator then these were extracted by SPE and analysed by LC-MS-MS.	Ratio of peak areas of standards/internal standards 100 ng/ml plotted versus the spiked analyte concentrations and linear correlation coefficient (r^2) calculated. The linearity of analytes were acceptable if $r^2 > 0.99$
LOD	Blank blood was spiked with 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 2 and 5 ng/ml to produce regression lines used to calculate LOD and LLOQ. Then extracted by SPE and analysed by LC-MS-MS.	LOD statistically calculated as the intercept of the calibration graph plus three times the standard error of the regression line as shown in the equation 3-1 and 3-2 in chapter 3.
LLOQ		LLOQ statistically calculated as the intercept of the calibration graph plus ten times the standard error of the regression line as shown in the equation 3-3 and 3-4 in chapter 3.

Recovery	Blank blood was spiked with analyte at three concentrations (5, 50 and 100 ng/ml). Each concentration was extracted five times without addition of internal standard. Unextracted standards (n=2) at each concentration were also prepared without internal standard present and kept in fridge until the extraction achieved. Before drying the sample under nitrogen stream, 100 ng/ml of internal standard was added to extracted and unextracted samples.	The % of recovery at each concentration was calculated using the equation 3-5 in which mean peak area ratio of extracted samples divided by mean peak area ratio of unextracted samples.(peak area ratio is peak area of product ion/ peak area of internal standard).
Intraday precision	Standards were prepared at three concentrations - 5, 50 and 100 ng/ml - and five replicates at each concentration were extracted.	Each extract was analysed and the relative standard deviations at each concentration were calculated as the RSD %. An RSD % less than 20 % is considered to be acceptable.
Interday precision	Five sets of standards at 5, 50 and 100 ng/ml were extracted over five different days.	The RSD% was calculated for each concentration. An RSD % less than 20 % is considered to be acceptable.

Each day samples were analysed, two replicates were taken for analysis from the storage racks for each of the different storage conditions, extracted within the day and left to run on the LC-MS/MS instrument overnight. If the analysis could not be completed within 24 hours due to instrumental problems, extracts were kept in the deep freeze at -20°C until they could be analysed. A calibration curve was prepared for each set of samples over part of the linear range, 25-800 ng/ml, which was considered sufficient for the analysis of the stored blood samples cotaining high and low drug concentrations. Benzodiazepine standards were spiked into blood to produce concentrations of 25, 50, 100, 200, 400, 800 ng/ml and internal standards were added at concentrations of 100 ng/ml to each calibrator solution before extraction by SPE and analysis by LC-MS/MS. The

calibration models for all analytes were linear with R^2 > 0.99 for all drugs (0.994, 0.996, 0.996 and 0998 for alprazplam, lorazepam, oxazepam and estazolam respectively).

4.4.5.1 Extraction of blood samples

Prior to extraction, 1 ml from each blood sample was mixed with 3.5ml of phosphate buffer (pH6) and 100 µl of internal standard followed by centrifugation for 10 minutes at 2500 RPM.

The solid phase columns were conditioned with 3ml of methanol, 3ml deionized water, and 2 ml of phosphate buffer (pH6), and then the supernatants of the samples were poured into the cartridges with no vacuum applied and allowed to pass through the absorbent under gravity flow. The columns were then washed with 2ml deionized water followed by 2ml phosphate buffer (pH6): acetonitrile (80:20 v/v) then dried for 1 minute under full vacuum. After that, 2 ml of cyclohexane was passed through the columns, and they were dried for 1 minute. The final step in the column washing was the addition of 2 ml of deionized water followed by drying for 5 minutes.

Elution of analytes was achieved using 3 ml of ethyl acetate: ammonia (98:2 v/v). The extracts were dried under a gentle stream of nitrogen at 40 °C, and then the residues were reconstituted in 80µl of initial HPLC mobile phase (3mM ammonium formate and 0.001% formic acid: acetonitrile, 75:25 v/v), of which 20µl was injected into the LC-MS/MS instrument.

4.4.5.2 LC-MS/MS analysis

LC-MS/MS analysis was performed using a Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San Jose, CA, USA) equipped with a surveyor HPLC system. Chromatographic separation was achieved using a Gemini C18 column (150 mm x 2 mm ID, 5µm particle size) fitted with a guard column (4 mm x 2 mm, 5µm) with the same column packing. The mobile phase was a mixture of 3mM aqueous ammonium formate solution containing 0.001% formic acid and acetonitrile starting at 75:25 v:v and changing to 20:80 v:v between 15 and 17 minutes. It

was then held at 10:90 v:v between 17 and 18.5 minutes then reset to the staring composition between 18.5 and 25 minutes.

The optimum mass spectrometric tuning parameters for analytes are shown in Table 4-2. Internal standard data were acquired in selected ion monitoring (SIM) mode and analytes were identified on the basis of their full MS-MS spectra. Two product ions were monitored. The underlined ions in Table 4-2 were used as the quantification ions because they were the most intense ions produced.

Table 4-2: Optimum tuning parameters.

Drug	Precursor ion (m/z)	Monitored ions* (m/z)	Collision energy (ev)
Alprazolam	309	281, 274	41
Lorazepam	321	303, 275	28
Oxazepam	287	269, 241	26
Estazolam	295	267, 269	40
Internal standards			
Alprazolam-d5	314	314	Not used
Lorazepam-d4	325	325	Not used
Oxazepam-d5	292	292	Not used
Estazolam-d5	300	300	Not used

- Underlined ion was used as the quantification ion. For internal standards, only the pseudomolecular ions were monitored and no collision energies were specified.

4.5 Results

4.5.1 Validation results

All validation parameters mentioned in Chapter 3 were investigated to validate the method before it was used in the stability study. The method was found to be satisfactory, as demonstrated in Tables 4-3, 4-4 and 4-5 and suitable for use in the stability study.

4.5.1.1 Linearty

The calibration models for all analytes were linear over the range 2-200 ng/ml with R^2 >0.99 for all drugs as shown in Table 4-3.

4.5.1.2 LOD and LLOQ

As shown in Table 4-3 the LOD and LLOQ for all benzodiazepines ranged from 0.29-1.13 ng/ml and 0.98-3.8 ng/ml respectively.

Table 4-3 : Linearity, LOD, and LLOQ of drugs in 1 ml of blood

Drug	Linearity r^2 (2-200 ng/ml)	LOD (ng/ml)	LLOQ (ng/ml)
Alprazolam	0.999	1.13	3.80
Lorazepam	0.994	0.76	2.50
Oxazepam	0.995	0.53	1.78
Estazolam	0.998	0.29	0.98

4.5.1.3 Recoveries

The recoveries for all analytes at concentrations of 5, 50 and 100 ng/ml are given in Table 4-4 and ranged from 82-106 %.

Table 4-4: Mean recoveries of analytes.

Drug	Mean Recovery% (RSD%, n=5)		
	5ng/ml	50ng/ml	100ng/ml
Alprazolam	84 (13)	83 (10)	88 (4)
Lorazepam	106 (15)	83 (6)	83 (4)
Oxazepam	82 (12)	98 (12)	86 (11)
Estazolam	92 (12)	89 (10)	96 (6)

4.5.1.4 Inter-day and intra-day precision

The inter-day and intra-day precisions were all acceptable according to SOFT guidelines, being less than ± 20%.[126] The values of % RSD were lower at higher concentrations and the intraday precision was lower than interday precision for most analytes. Results are shown in Table 4-5.

Table 4-5: Interday and intraday precision results.

Drug	Interday mean (RSD%, n=5)			Intraday mean (RSD%, n=5)		
	5ng/ml	50ng/ml	100ng/ml	5ng/ml	50ng/ml	100ng/ml
Alprazolam	4 (17)	51 (14)	104 (6)	5 (14)	46 (3)	90 (3)
Lorazepam	5 (19)	51 (11)	106 (6)	4 (16)	50 (8)	103 (4)
Oxazepam	7 (18)	45 (4)	98 (4)	5 (10)	47 (5)	99 (4)
Estazolam	4 (12)	51 (8)	107 (2)	5 (10)	46 (4)	96 (2)

4.5.2 Stability results

4.5.2.1 Alprazolam

In the freezer (-20 °C), alprazolam is apparently stable for one year at both low and high concentrations, in preserved and unpreserved samples, and no significant loss was noted ($P > 0.05$).

In the refrigerator (4 °C), alprazolam was also found to be stable regardless of the concentration and the addition of a preservative ($P > 0.05$).

At room temperature, alprazolam was found to be stable for 6 months, but losses of 9% and 6% from the initial concentration for unpreserved and preserved samples, respectively were found after 1 year at low concentration. At high concentration, alprazolam was found to be stable for 6 months, but losses were observed after 1 year of 9% and 7% for unpreserved and preserved samples, respectively. Alprazolam concentrations versus time for the different storage conditions are shown in Table 4.6-4.7 and Figures 4.3-4.4. The losses observed were not statistically significant for low concentration whereas at high concentration it was significant after one year.

Table 4-6: Stability of alprazolam 50 ng/ml under different storage conditions.

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	49 (6)	49 (6)	49 (6)	49 (6)	49 (6)	49 (6)
2	51 (2.8)	50 (1.4)	50 (1.5)	49 (1.6)	48 (0.7)	48 (1.5)
4	50 (1.4)	50 (2.7)	49 (4.5)	48 (6.0)	49 (0.7)	49 (0.8)
7	50 (1.4)	50 (1.4)	49 (4.4)	49 (1.5)	49 (0.7)	48 (1.0)
14	50 (0.7)	50 (1.4)	50 (1.4)	50 (1.2)	50 (1.8)	50 (2.1)
30	50 (1.4)	50 (2.8)	50 (4.3)	51 (2.8)	51 (2.8)	51 (3.6)
60	50 (1.4)	50 (1.4)	50 (4.5)	49 (1.6)	49 (0.7)	50 (2.2)
90	50 (1.4)	49 (2.9)	50 (1.4)	50 (2.9)	50 (1.8)	49 (0.9)
180	49 (1.4)	49 (1.5)	49 (4.6)	48 (3.0)	48 (0.8)	48 (1.3)
365	49 (1.3)	48 (3.0)	49 (2.9)	48 (4.5)	47 (1.9)	46 (3.6)

P*: preserved, N-P*: unpreserved. (Concentration threshold=37)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

Table 4-7: Stability of alprazolam 400 ng/ml under different storage conditions.

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	397 (14)	397 (14)	397 (14)	397 (14)	397 (14)	397 (14)
2	394 (0.9)	399 (0.2)	391 (1.8)	376 (3.7)	396 (0.2)	394 (0.5)
4	399 (0.4)	398 (0.2)	390 (0.2)	390 (2.6)	398 (0.2)	396 (0.2)
7	398 (0.2)	396 (0.3)	372 (3.5)	380 (0.7)	397 (1.1)	398 (0.3)
14	400 (0.2)	399 (0.2)	399 (1.4)	408 (5.8)	390 (1.3)	392 (0.9)
30	400 (0.2)	397 (0.1)	408 (3.0)	399 (4.2)	399 (0.4)	391 (1.1)
60	399 (0.9)	399 (0.2)	408 (3.1)	398 (4.0)	399 (0.2)	392 (0.9)
90	399 (0.7)	398 (0.9)	396 (0.9)	394 (3.3)	395 (0.4)	391 (1.0)
180	397 (0.3)	396 (0.4)	394 (0.5)	393 (3.1)	392 (0.8)	392 (0.9)
365	397 (0.4)	397 (0.2)	392 (0.5)	392 (2.9)	372 (0.4)	364 (0.6)

P*: preserved, N-P*: unpreserved. (Concentration threshold=369)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

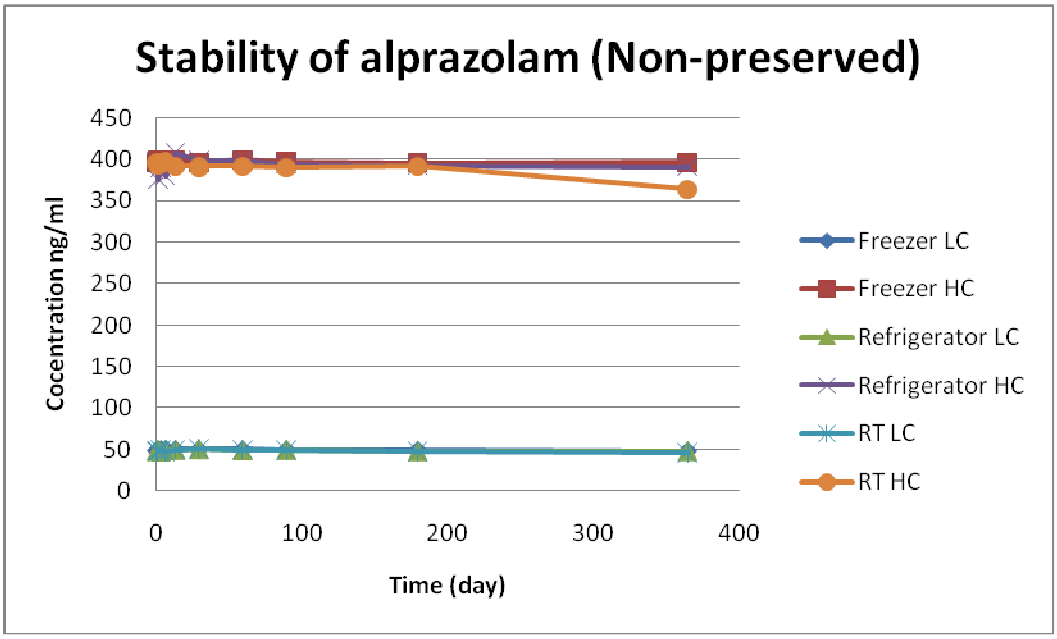


Figure 4-3: Stability of unpreserved alprazolam at varying storage temperature. LC: Low concentration, HC: High concentration and RT: Room temperature

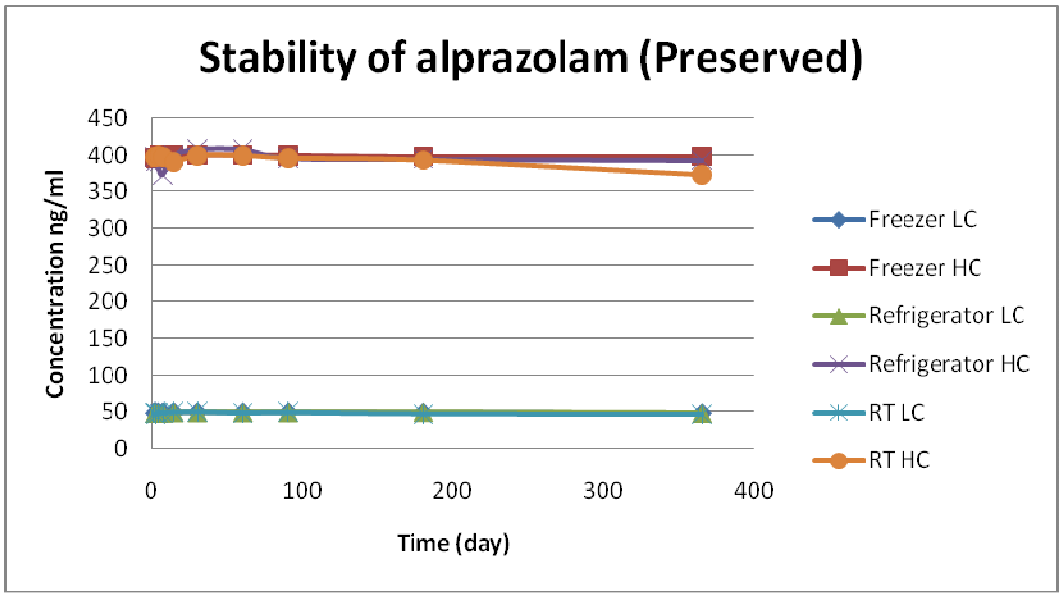


Figure 4-4: Stability of preserved alprazolam at varying storage temperature. LC: Low concentration, HC: High concentration and RT: Room temperature

4.5.2.2 Lorazepam

In the freezer (-20 °C), lorazepam was found to be stable for 6 months at low and high concentrations for both unpreserved and preserved samples, but after that, a loss of lorazepam was noted at low concentration. The loss from the original concentration was 19 % and 9 % for unpreserved and preserved samples, respectively. A decrease in lorazepam at high concentration was observed after one year of storage. The losses were 5% and 3% for unpreserved and preserved samples, respectively.

In the refrigerator (4 °C), lorazepam at low concentration was found to be stable for 3 month. A loss in the concentrations of lorazepam was observed after six months of storage, and the losses were 30% and 26% for unpreserved and preserved samples, respectively. After one year, the losses in the concentrations were 50% and 42% for unpreserved and preserved samples, respectively. However, at high concentrations, lorazepam was found to be stable for 2 months in both samples, and a loss was observed after 3 months. The losses were 7% and 5 % for unpreserved and preserved samples, respectively. After 1 year, the losses from the initial concentration were approximately 28% and 15% for unpreserved and preserved samples, respectively.

At room temperature, lorazepam in unpreserved, low concentration samples were found to be stable for only 4 days, while a significant loss of 27 % from the original concentration was observed after 1 week ($P < 0.05$). After 3 months, only 10 % of lorazepam could be detected, and it was completely lost after 6 months. The lorazepam at a low concentration with preservative was found to be stable for only 4 days and a significant decrease in concentration was observed after 1 week. The loss was approximately 25% with a p value < 0.05, and a continuous loss in the concentration was observed to proceed during the storage time. After three months, only 13% of the lorazepam could be detected, and it completely disappeared after 6 months. Lorazepam at high concentrations was stable for 2 days and 4 days in unpreserved and preserved samples repectively. The concentrations decreased by 5% after 4 days and 1 week in the unpreserved and preserved samples respectively ($P < 0.05$), and approximately 4% could be detected for both samples after 6 months. After 1 year, lorazepam

could not be detected in either storage condition. Stability data are given in Table 4.8-4.9 and are shown in Figures 4.5-4.6.

Table 4-8: Stability of lorazepam 50 ng/ml under different storage conditions

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	48 (10.0)	48 (10.0)	48 (10.0)	48 (10.0)	48 (10.0)	48 (10.0)
2	50 (1.4)	51 (2.1)	50 (2.9)	50 (4.3)	49 (1.1)	49 1.0)
4	51 (2.7)	50 (1.4)	50 (1.4)	49 (0.3)	43 (9.2)	43 (10.0)
7	51 (0.7)	50 (3.1)	47 (1.5)	47 (2.2)	36 (8.6)	35 (11.2)
14	51 (1.4)	49 (4.3)	48 (3.6)	46 (6.7)	36 (9.5)	32 (3.3)
30	49 (1.4)	49 (4.6)	47 (6.5)	45 (9.6)	27 (5.4)	24 (7.9)
60	50 (2.8)	49 (0.4)	44 (7.6)	42 (4.9)	17 (7.9)	10 (8.5)
90	49 (1.4)	49 (2.2)	42 (5.2)	40 (14.3)	5 (9.4)	5 (14.6)
180	47 (3.9)	47 (3.5)	35 (3.9)	37 (5.9)	0 (0.0)	0 (0.0)
365	46 (4.6)	41 (8.9)	29 (7.7)	25 (10.5)	0 (0.0)	0 (0.0)

P*: preserved, N-P: unpreserved. (Concentration threshold=39)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

Table 4-9: Stability of lorazepam 400 ng/ml under different storage conditions.

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	396 (6.0)	396 (6.0)	396 (6.0)	396 (6.0)	396 (6.0)	396 (6.0)
2	396 (0.4)	396 (0.5)	392 (0.2)	388 (2.2)	398 (0.2)	399 (0.3)
4	404 (0.9)	404 (1.1)	402 (2.1)	396 (1.1)	389 (2.3)	380 (2.7)
7	404 (1.1)	400 (0.7)	394 (4.1)	396 (2.9)	384 (4.9)	372 (4.7)
14	404 (0.7)	404 (0.9)	396 (0.5)	396 (2.1)	332 (3.4)	324 (2.6)
30	404 (0.7)	398 (0.2)	396 (2.2)	396 (0.5)	296 (4.2)	296 (3.7)
60	398 (0.2)	396 (0.5)	392 (2.8)	392 (1.3)	252 (5.3)	236 (8.6)
90	399 (0.2)	396 (0.3)	382 (1.2)	374 (0.1)	144 (7.4)	132 (6.1)
180	393 (0.7)	392 (0.8)	377 (2.5)	360 (1.4)	17 (6.2)	14 (9.9)
365	388 (2.4)	380 (0.5)	340 (3.8)	288 (1.5)	0 (0.0)	0 (0.0)

P*: preserved, N-P: unpreserved. (Concentration threshold=384)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

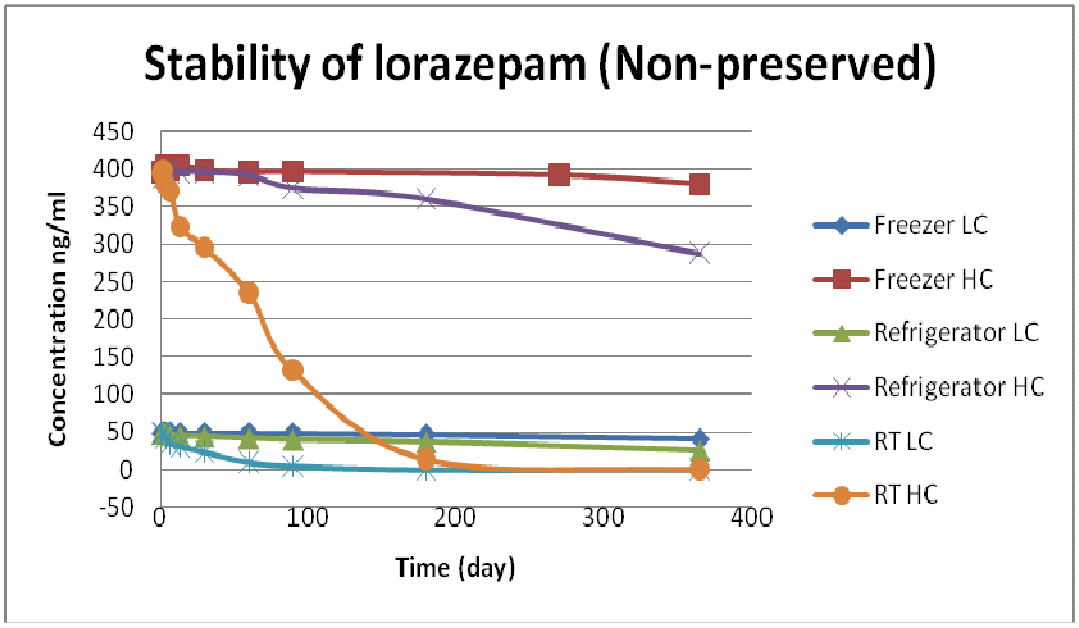


Figure 4-5: Stability of unpreserved lorazepam at varying storage temperature. LC: Low concentration, HC: High concentration and RT: Room temperature.

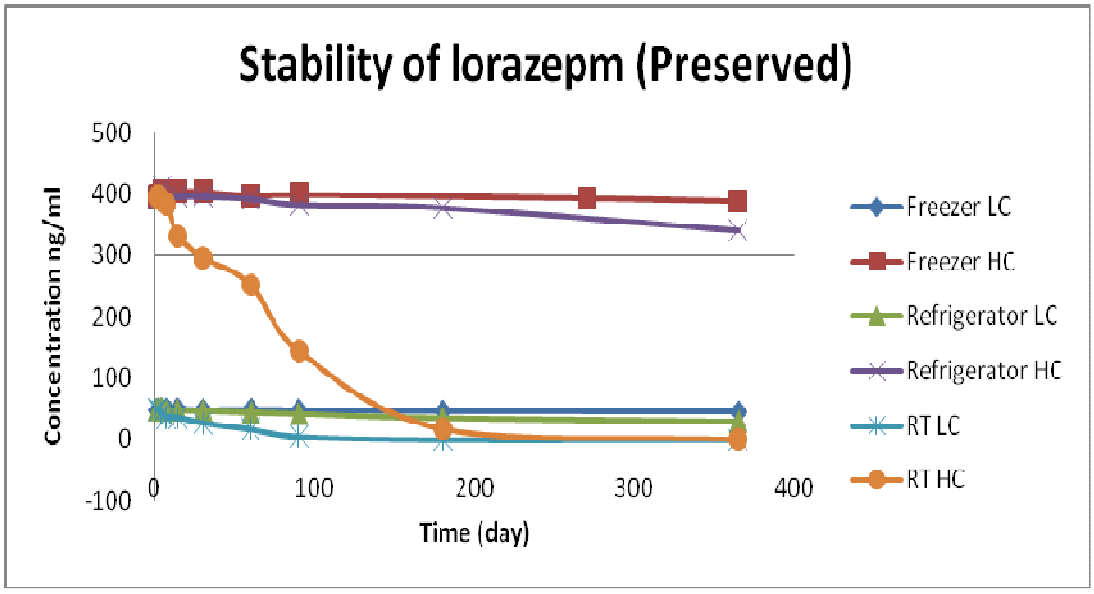


Figure 4-6: Stability of preserved lorazepam at varying storage temperature. LC: Low concentration, HC: High concentration and RT: Room temperature.

4.5.2.3 Oxazepam

Oxazepam at low concentrations in both unpreserved and preserved samples was found to be stable in the freezer (20 °C) for 12 months. Oxazepam concentrations decreased by approximately 5% in both conditions. On the other hand, oxazepam at high concentration was found to be stable for 6 months in both conditions while a significant loss was observed after one year. There was a 19% and 12% loss in the concentrations in unpreserved and preserved samples, respectively ($P < 0.05$).

In the refrigerator (4 °C) oxazepam at low concentration in both conditions was found to be stable for 6 month but it was not stable after 12 months. After 12 months, oxazepam decreased by 31% and 28% for unpreserved and preserved samples, respectively ($P < 0.05$). Oxazepam at a high concentration was stable for 2 months, and a significant loss was observed under both conditions after 3 months. The decrease in the concentration was 9% and 7% for unpreserved and preserved samples, respectively ($P < 0.05$). The decrease in the concentrations reached 26% and 23% for unpreserved and preserved samples, respectively, after 12 months.

At room temperature, oxazepam at a low concentration was found to be stable for 2 week under both conditions. The loss in the concentration was significant after 1 month. The loss was approximately 55% ($P < 0.05$) under both conditions. Further decreases in the concentrations proceeded dramatically. Approximately 8% of the original concentration could be recovered after 3 months of storage under both conditions and it disappeared completely after 6 months. Oxazepam at a high concentration was stable for only 2 days under both conditions while a significant decrease was observed after 4 days. The loss was approximately 10% and 9% for unpreserved and preserved samples, respectively ($P < 0.05$) under both conditions. Approximately 5% of the initial concentration could be detected after 6 months in both unpreserved and preserved samples; however, no concentration could be detected after 1 year in either sample. Stability data are given in Table 4.10-4.11 and are shown in Figures 4.7-4.8.

Table 4-10: Stability of oxazepam 50 ng/ml under different conditions.

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	49 (8.0)	49 (8.0)	49 (8.0)	49 (8.0)	49 (8.0)	49 (8.0)
2	50 (1.4)	50 (1.8)	50 (2.9)	49 (1.5)	44 (4.6)	44 (6.1)
4	50 (1.9)	50 (2.8)	50 (1.4)	49 (4.5)	44 (7.6)	43 (9.2)
7	49 (0.4)	50 (0.3)	49 (2.9)	49 (0.7)	39 (3.5)	38 (5.4)
14	50 (0.3)	50 (2.8)	49 (1.4)	49 (2.9)	35 (8.6)	34 (4.3)
30	50 (4.1)	50 (2.8)	49 (2.8)	47 (5.7)	28 (8.0)	24 (9.4)
60	50 (4.2)	49 (0.3)	46 (4.5)	46 (5.9)	23 (13.5)	18 (12.9)
90	49 (1.4)	49 (1.8)	45 (7.4)	45 (6.0)	5 (12.9)	4 (9.4)
180	48 (1.4)	48 (1.5)	43 (3.2)	41 (4.9)	0 (0.0)	0 (0.0)
365	48 (1.5)	48 (0.1)	36 (3.8)	35 (7.6)	0 (0.0)	0 (0.0

P*: preserved, N-P: unpreserved. (Concentration threshold=33)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements.

Table 4-11: Stability of oxazepam 400 ng/ml under different storage conditions.

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	396 (10.0)	396 (10.0)	396 (10.0)	396 (10.0)	396 (10.0)	396 (10.0)
2	396 (0.4)	400 (0.2)	396 (0.9)	396 (3.7)	392 (0.8)	392 (0.9)
4	397 (0.4)	396 (0.3)	396 (1.1)	392 (0.4)	364 (0.8)	360 (1.2)
7	396 (0.4)	396 (0.1)	388 (2.9)	388 (1.5)	344 (0.6)	344 (0.8)
14	396 (0.7)	396 (0.8)	388 (1.9)	386 (3.9)	336 (0.8)	308 (0.9)
30	396 (0.7)	392 (0.9)	384 (4.3)	384 (2.7)	272 (1.8)	252 (2.5)
60	395 (0.7)	394 (0.8)	384 (4.3)	377 (4.0)	200 (3.1)	192 (2.5)
90	393 (1.0)	392 (1.1)	372 (0.8)	364 (1.9)	97 (5.3)	78 (10.9)
180	388 (1.6)	385 (1.9)	357 (1.4)	344 (1.8)	21 (15.0)	20 (12.8)
365	352 (0.9)	324 (0.7)	308 (3.1)	296 (3.4)	0 (0.0)	0 (0.0)

P*: preserved, N-P: unpreserved. (Concentration threshold=376)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

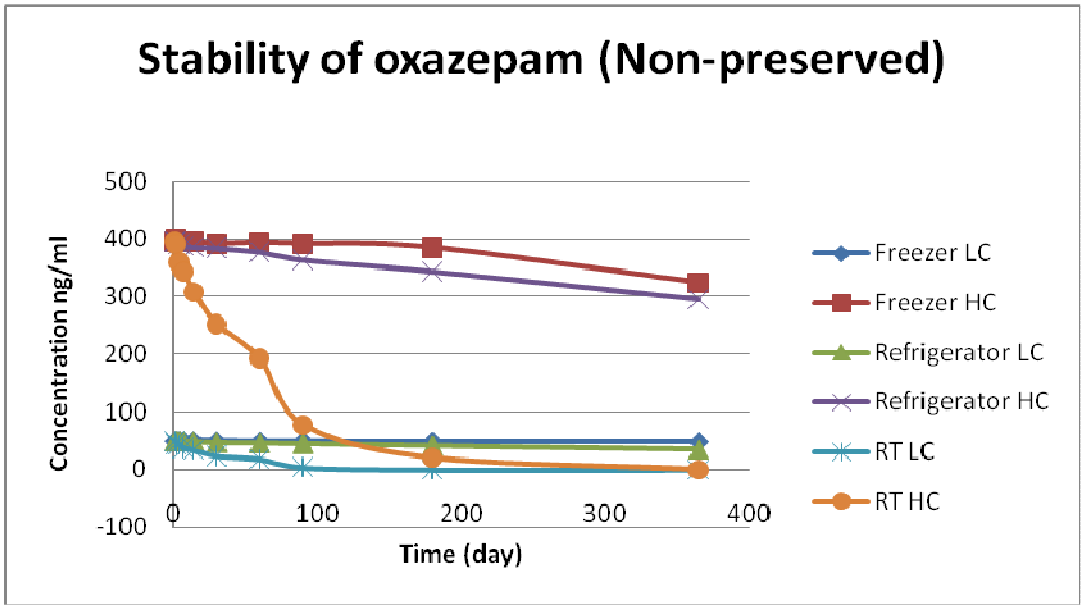


Figure 4-7: Stability of unpreserved oxazepam at varying storage temperature. LC: Low concentration, HC: High concentration and RT: Room temperature.

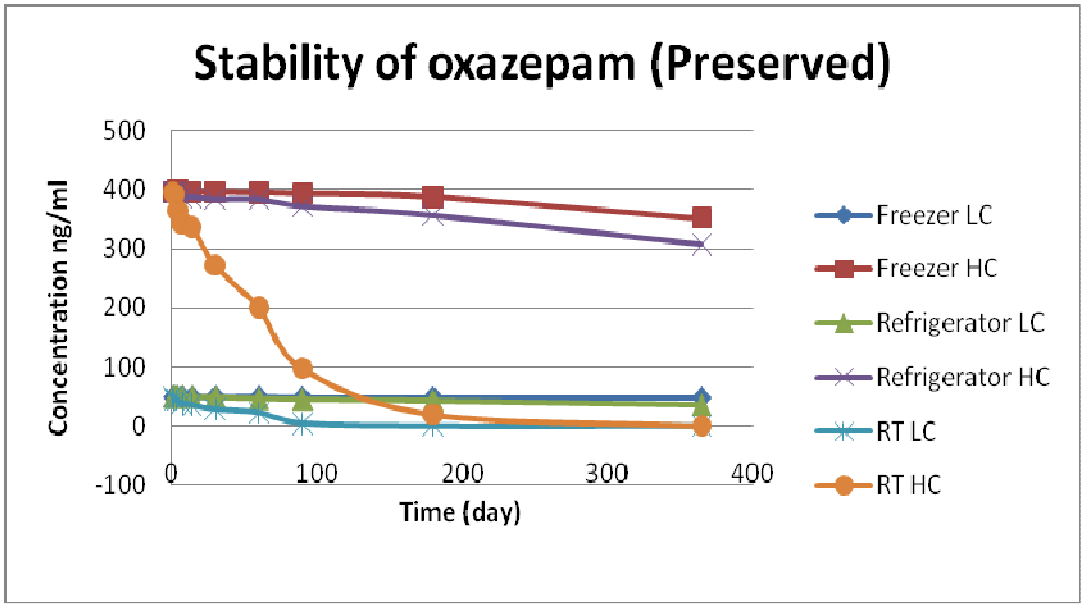


Figure 4-8: Stability of preserved oxazepam at varying storage temperature. LC: Low concentration, HC: High concentration and RT: Room temperature.

4.5.2.4 Estazolam

In the freezer (-20 °C), estazolam at low and high concentrations was found to be stable for one year in both unpreserved and preserved samples and no significant loss was observed ($P > 0.05$).

In the refrigerator (4 °C), there was no significant decrease in the low and high concentrations in the preserved and unpreserved samples. The loss was less than 2% of the original concentration ($P > 0.05$).

At room temperature, no significant loss was observed for 12 months of storage at low and high concentrations under both conditions. However, after 1 year, estazolam concentration was decreased at low concentration by 9% and 8% in unpreserved and preserved samples, respectively. At high concentrations, it decreased by 4% and 3% for unpreserved and preserved samples, respectively ($P > 0.05$). Stability data are given in Table 4.12-4.13 and are shown in Figures 4.9-4.10.

Table 4-12: Stability of estazolam 50 ng/ml under different storage conditions.

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	49 (10.0)	49 (10.0)	49 (10.0)	49 (10.0)	49 (10.0)	49 (10.0)
2	50 (2.8)	49 (0.4)	49 (1.4)	49 (1.5)	50 (1.7)	49 (0.7)
4	50 (1.8)	50 (0.7)	50 (1.0)	49 (2.9)	49 (0.7)	49 (0.3)
7	50 (0.4)	50 (0.7)	50 (1.1)	49 (1.5)	49 (0.7)	48 (0.4)
14	50 (0.2)	49 (2.1)	49 (0.7)	49 (2.9)	48 (1.8)	48 (2.3)
30	50 (1.4)	49 (4.2)	49 (2.8)	48 (3.6)	49 (2.5)	48 (3.6)
60	49 (0.1)	49 (0.3)	48 (0.7)	48 (2.9)	48 (0.7)	49 (2.2)
90	49 (0.7)	49 (1.4)	49 (0.9)	49 (1.5)	50 (0.3)	49 (0.7)
180	50 (0.4)	49 (0.1)	49 (1.4)	49 (1.5)	49 (0.6)	49 (0.7)
365	49 (1.0)	49 (1.3)	49 (2.8)	48 (4.3)	46 (1.8)	46 (0.7)

P*: preserved, N-P*: unpreserved. (Concentration threshold=39)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

Table 4-13: Stability of estazolam 400 ng/ml under different storage conditions.

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	391 (7)	391 (7)	391 (7)	391 (7)	391 (7)	391 (7)
2	398 (0.7)	400 (0.2)	395 (0.7)	400 (1.9)	397 (0.1)	396 (0.2)
4	400 (0.2)	400 (0.4)	400 (1.7)	396 (1.1)	399 (0.2)	396 (0.2)
7	396 (0.3)	392 (0.7)	398 (4.8)	396 (2.9)	398 (0.2)	394 (0.7)
14	396 (0.7)	392 (1.3)	394 (0.8)	395 (2.3)	398 (1.4)	394 (0.3)
30	392 (1.4)	392 (0.9)	392 (2.8)	394 (0.9)	395 (0.7)	392 (0.2)
60	395 (0.8)	392 (1.3)	392 (2.8)	393 (1.0)	394 (0.8)	392 (0.3)
90	395 (0.6)	392 (1.1)	394 (0.4)	392 (0.3)	395 (0.2)	391 (0.7)
180	392 (0.9)	391 (0.8)	392 (0.4)	390 (0.5)	394 (0.3)	390 (0.4)
365	389 (1.2)	390 (1.3)	391 (0.1)	390 (0.4)	388 (2.9)	384 (3.8)

P*: preserved, N-P*: unpreserved. (Concentration threshold=377)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

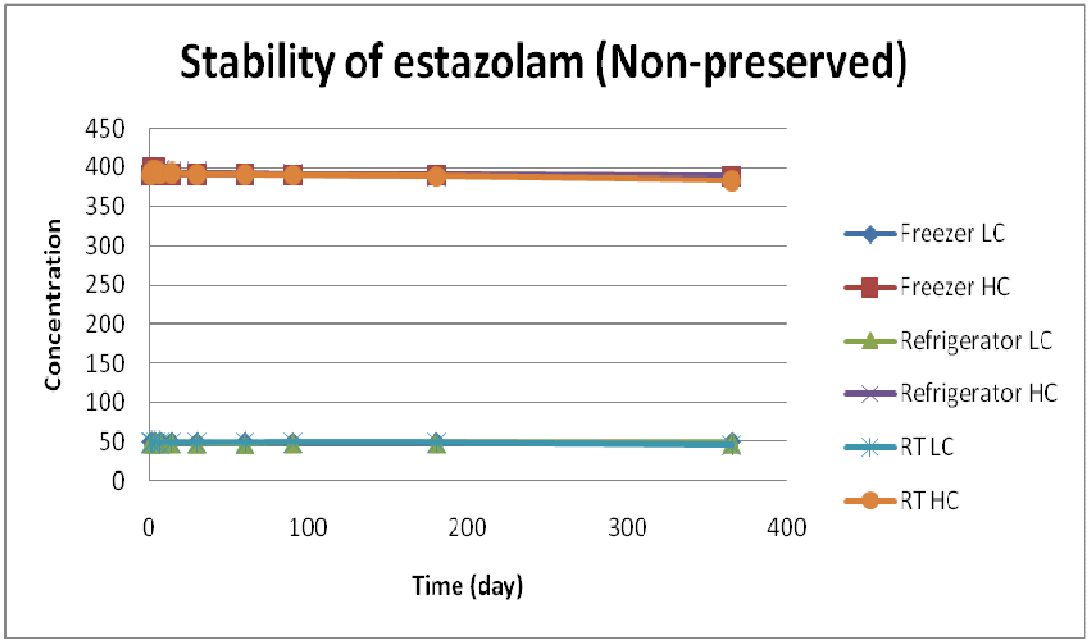


Figure 4-9: Stability of unpreserved estazolam at varying storage temperature. LC: Low concentration, HC: High concentration and RT: Room temperature.

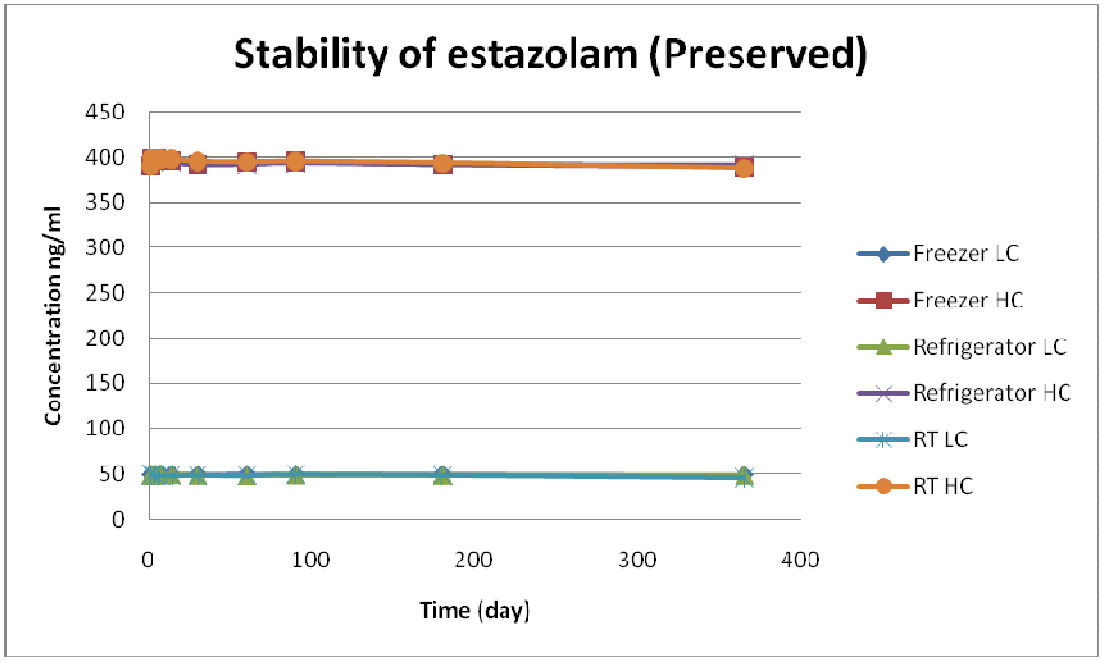


Figure 4-10: Stability of preserved estazolam at varying storage temperature. LC: Low concentration, HC: High concentration and RT: Room temperature.

4.5.3 Summary of benzodiazepine stability in blood samples

The stability of alprazolam, estazolam, lorazepam, and oxazepam in blood samples stored for one year under different storage conditions is summarized in Table 4-14.

Table 4-14: Summary of benzodiazepine stability in blood under varied storage conditions

Drug	Storage conditions	Results	Comment
Alprazolam	-20 °C, 1 year 4 °C, 1 year	Stable	• Less than 13% enhancement in stability when NAF (2% W/V) used • As temperature increased, stability decreased • Alprazolam and estazolam are more stable than lorazeam and oxazepam • Higher concentration of drugs can be detected for long time compared with low concentration
	25 °C, 1 year	Less than 9% decrease at HC	
Estazolam	-20 °C, 1 year	Stable	
	4 °C, 1 year		
	25 °C, 1 year		
Lorazepam	-20 °C, 1 year	5% decrease at HC	
	4 °C, 1 year	50% decrease at LC and 28% decrease at HC	
	25 °C, 1 year	100% decrease at LC after 6 months and 100% decrease at HC after 1 year	
Oxazepam	-20 °C, 1 year	19% decrease at HC	
	4 °C, 1 year	31% decrease at LC and 26% decrease at HC	
	25 °C, 1 year	100% decrease at LC after 6 months and 100% decrease at HC after 1 year	

4.6 Discussion

This study was undertaken to investigate the stability of four benzodiazepines that are widely prescribed in Saudi Arabia, and for which no information about their stability is available in the literature, although oxazepam has been investigated in whole blood without the addition of a preservative.[56] In this study, the influence of temperature, concentration, and the addition of an anticoagulant and a preservative were examined to discover whether they had an effect on the stability of these compounds in blood over a period of one year. In forensic toxicology, a study of stability should comply with actual forensic cases and conditions. Therefore, the blood was prepared at low and high concentrations. The low concentration represented the therapeutic levels that would be found in drug monitoring in hospital or encountered in drug abusers accused of driving under the influence of drugs. The high concentration samples were similar to those encountered in a drug overdose case. Since authentic samples are routinely opened several times, in this study the spiked blood was distributed in small tubes, and each tube was used one time only to avoid any contamination that may occur after opening, consequently affecting the drug concentration by contamination with bacteria which may be in the air. The estimation of stability in this study is based on the results obtained in equation 6 in Chapter 3 and the *p* value. If the loss in the concentration at the time selected during the study is more than 1.96 of the coefficient variation and the *p* value is < 0.05, then the decrease in the drug concentration is considered significant.[59,134]

The study indicates that the concentrations of the 4 benzodiazepines are influenced by storage conditions. The storage temperature plays a major role in drug stability. As the temperature increased, drug concentrations were observed to decrease. All compounds showed good stability in the freezer. However, as clearly demonstrated in this study, a decrease in the high concentration can be expected after 12 months of storage for lorazepam and oxazepam in the freezer while alprazolam and estazolam were found to be stable for all periods of the study when stored in the freezer. In the refrigerator, which is the most common and feasible storage temperature used in laboratories and the place used to keep samples before analysis, a loss in lorazepam and oxazepam concentrations can be expected after 6 months for low concentrations and after 3 months for

high concentrations under both conditions whereas alprazolam and estazolam were found to be stable for one year, and no significant losses were observed. Contrary to what happened in the freezer, high losses in the concentrations were observed for all drugs including alprazolam and estazolam at room temperature. Lorazepam and oxazepam showed poor stability at room temperature. The concentration of oxazepam decreased significantly after 2 weeks and 4 days at low and high concentration, respectively, in both conditions, while the level of lorazepam decreased significantly from the original concentration after 1 week and 4 days at low and high concentrations, respectively.

Degradation of benzodiazepines has been reported in previous studies, and this was attributed to hydrolysis reactions because most benzodiazepines contain an amide bond in their structure and are therefore prone to undergoing a hydrolysis reaction leading to cleavage of the amide. Two drugs in this study have amide bonds: lorazepam and oxazepam. For this reason, lorazepam and oxazepam exhibited poor stability compared to alprazolam and estazolam, which belong to the triazolobenzodiazpines and do not have an amide group in their structures. Instead, alprazolam and estazolam contain triazolo ring, which seems to make the drugs resistant to the hydrolysis reaction. It seems to enhance the stability of these compounds by protecting the compounds from decomposition when the biological samples are in storage.

Hydrolysis of compounds may be chemical or enzymatic. The blood used in this study consisted of red blood cells suspended in isotonic saline and therefore did not contain plasma esterases. However, enzyme activity due to bacterial contamination would have been possible. In this situation, the reaction seemed to be chemical hydrolysis because enzymatic activity is reported to continue after sampling in unpreserved samples compared to those that are preserved, this enzyme activity being related to esterase, which affects ester-containing compounds but is not known to be involved in benzodiazepine breakdown. Moreover, the enzymatic effect of bacteria, which are reported to have degraded benzodiazepines such as nitrobenzodiazepines, is to convert nitrobenzodiazepines into corresponding aminobenzodiazepine metabolites. However, the nitro group was not present in the compounds investigated in this study; this effect can be excluded in this case, even if there was contamination involved, especially in unpreserved samples. Sodium fluoride (NaF) is an

antienzymatic preservative that showed little effect in this study because there was no clear role for this preservative to minimize or protect the compounds from degradation or maybe because the concentration of NaF in the tubes used to preserve the drugs in this study is small (0.2% w/v) while in the previous studies increase of NaF concentration showed reduction in the drugs degradation as mentioned in Chapter 2.[62] NaF also seemed to have no effect in accelerating the hydrolysis reaction compared to unpreserved samples as has been reported in ester-containing compounds such as organophosphates which decompose quickly after the addition of sodium fluoride.[88] Sodium fluoride is therefore safe if the analytes in the sample are benzodiazepines.

Something similar was reported by Levine et al.[55] (1983) regarding the effect of additives on the stability of benzodiazepines. Additives had a small effect on drug stability under all conditions, and the enhancement in the stability was estimated to be less than 10% compared to the unpreserved samples.[3] This small effect may have been a result of anticoagulant activity, which was not noticeable in the unpreserved samples compared to preserved samples, especially those at room temperature. Elmahjob et al.[56] found the same results as those obtained in this study, namely, that oxazepam is not detected at all at room temperature after 1 year at both low and high concentrations. Moreover, high concentrations can be detected for a long time in comparison to low concentrations, and this should be considered in the interpretation of either therapeutic or toxic concentration samples.

4.7 Conclusions

The possibility of a decrease in benzodiazepine concentration should be considered, even if samples are stored at -20 ºC. Therefore, the analysis should be performed as soon as possible. In addition, samples should be stored at -20 ºC or lower to keep the drug stable for as long as possible. Furthermore, the addition of a preservative to the sample could enhance the stability without a possible loss of concentration by NaF, as reportedly happened with organophosphourous compounds. Anticoagulant should be added to the blood sample to prevent it from coagulating, an effect that could increase at a high temperature.

The drugs containing an ester or an amide group are prone to degradation by hydrolysis and this should be carefully considered when they are being transported or stored and during the interpretation of results. Optimal storage conditions to stop or minimize the degradation of the drug are necessary to keep the drug stable for a long time. Further determination of degradation products as alternatives could also help the forensic toxicologist to confirm the presence of the parent drug in case the drug decomposes partially or completely as observed in the case of oxazepam and lorazepam after one year.

5 Stability of Opiate Glucuronides in Blood and Urine

5.1 Introduction

Opium, which means juice in Greek, is the name given to milky exudates obtained from the immature fruits of the plant *papaver somniferum*. The milky exudate contains several pharmacologically active compounds, including morphine and codeine. Members of the group of natural, semi synthetic or synthetic alkaloids are called opoids. Morphine and codeine are naturally occurring compounds known as opiates, while examples of semi-synthetic and synthetic compounds are oxycodone, fentanyl, buprenorphine, methadone, and tramadol. Opioids exert their effect by binding receptors at several sites in the CNS.[135,136]

There are four major types of opioid receptors located throughout the body: μ (mu), κ (kappa), δ (delta), and σ (sigma). An analgesic effect is produced by opioids due to the interaction between the opoid and specific receptors at the terminal nerve ending, which blocks the release of neurotransmitters or pain stimuli. Interaction with the μ receptor results in analgesia, euphoria, miosis, and bradycardia, while binding to the κ receptor produces analgesia, miosis, diueresis, and sedation. The δ receptors are the site of binding of most endogenous opioid peptides (endorphins), which produce analgesia, dysphoria, delusions, and hallucinations. Binding to the σ receptor may produce central excitation, resulting in tachycardia, hallucinations, and hypertension. Opioids may be used therapeutically as postoperative analgesics after surgery, and for myocardial infarction (MI), trauma, and burns. Additionally, opioids are indicated for chronic pain, such as the pain associated with cancer. Opioids are also used as anti-tussives and anti-diarrhoeals, as well as antidotes - naloxone, is used for treatment of opioid intoxication and is an extremely potent opioid receptor antagonist. The main advantage of using opioids clinically is that they can produce an analgesic effect without loss of consciousness, unlike anaesthesia. However, the analgesia is accompanied by euphoria and sedation. Furthermore, respiratory depression may result from opioids, which is

considered a major disadvantage and side effect that can cause death in intoxication cases.[135,136]

Morphine was the first compound discovered in opium, in 1803, when the German pharmacist Feredrik Serturner succeeded in isolating an analgesic compound, which he named morphine. Since that time, morphine has become an important treatment for severe pain and diarrhoea. Parenteral morphine is rapidly absorbed, while extensive first pass hepatic metabolism results in low bioavailability when morphine is taken orally. Therefore, the analgesic effect of morphine is greater when given parenterally rather than orally. [135,136]

The analgesic effect of morphine following oral administration in cancer patients is obtained at a mean serum concentration of 18 ng/ml. The major metabolic pathway for morphine is the conjugation with glucuronic acid on the free phenolic hydroxyl or alkanol groups to produce morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), respectively. M3G is a highly water-soluble metabolite and the predominant metabolite. M3G has a low affinity to opioid receptors; thus, no opioid reaction will be produced, but it seems to produce the side effects of morphine. On the other hand, M6G is a pharmacologically active metabolite, which is an analgesic twice as potent as morphine. Since morphine is the active metabolite of heroin and codeine, the presence of free morphine or its metabolites may also indicate heroin or codeine use. Following administration of a 15mg oral dose of morphine every 6 hours for 5 days, cancer patients have steady state plasma concentrations of morphine, M3G, and M6G of 0.014 mg/L, 0.515 mg/L and 0.077 mg/L, respectively. [135,136,145]

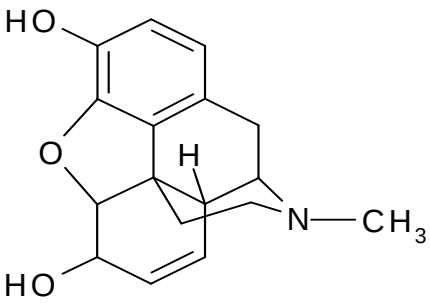
Codeine is naturally occurring and can be obtained from opium or synthesised commercially by 3-O-methylation of morphine. Codeine is less potent than morphine and is used clinically in the treatment of mild to moderate pain and as an anti-tussive. Some over-the-counter (OTC) remedies include low doses of codeine in combination with non-opioid analgesics, such as paracetamol and aspirin. Oral and parenteral doses of codeine are well absorbed. Codeine is metabolised via O-demethylation to morphine and via N-demethylation to norcodeine, and conjugated to codeine-6-glucuronide (C6G), which has a similar pharmacological activity to codeine. Morphine has a 200 times greater affinity to the μ receptor than codeine and its pharmacological effect depends on the

formation of morphine. Cytochrome P450 (CYP) 2D6 catalyses the biotransformation of codeine to morphine. Around 7% of Caucasians and 50% of Chinese are deficient in this enzyme and do not experience the analgesic effect of codeine compared to those who have this enzyme. [135,136,146]

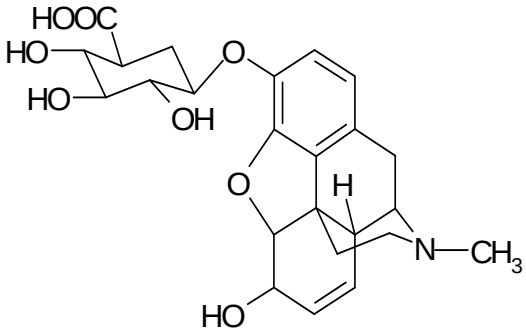
Peak concentrations following a 50 mg oral dose of codeine range between 0.05 and 0.15 mg/L after 1 hour. The amounts of codeine and metabolites remaining in urine 24 hours after a 22 mg oral dose were as follows: free codeine (5-17%), codeine-6-glucuronide (C6G) and free codeine (32-46%), conjugated norcodeine and free morphine (10-21%), and conjugated morphine (5-13%). The composition of the metabolite in the urine from codeine ingestion after 3 days is similar to that following morphine and heroin use. However, the presence of C6G and norcodeine in the urine is indicative of codeine use, not morphine or heroin use.[145]

The determination of a parent drug and its glucuronide metabolite is very important to interpretations in clinical and forensic toxicology. Estimation of metabolite/drug ratios can be used to assess the time interval after drug intake. Additionally, metabolite detection can be used to differentiate between chronic and recent drug use. Glucuronides are usually determined by cleavage of the glucuronide with an enzyme such as β-glucuronidase to release the parent compound, which is subsequently quantified. However, there are limitations with this method. The enzymatic hydrolysis may be inhibited competitively by matrix components such as phosphate, resulting in incomplete reaction. Also, conjugates may be cleaved more readily at some positions than other, for example, morphine-3-glucuronide is more easily cleaved than morphine-6-glucuronide. In addition, different rates of hydrolysis of conjugates are obtained for many drugs depending on the enzyme preparation used. Furthermore, enzymatic reactions are time-consuming. Direct detection of the intact metabolites is very important to overcome the limitations involved in the enzymatic cleavage method. The opiate glucuronides have been detected directly by different methods listed in the literature.[146-155]

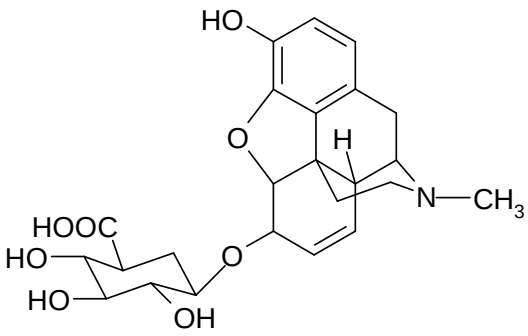
Morphine, codeine, M3G, M6G and C6G chemical structures are shown in Figure 5-1.



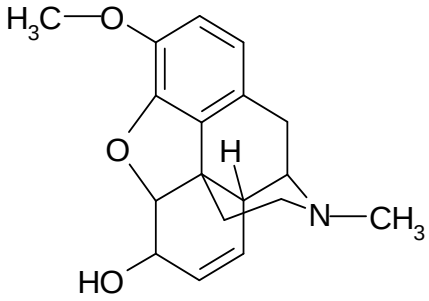
Morphine



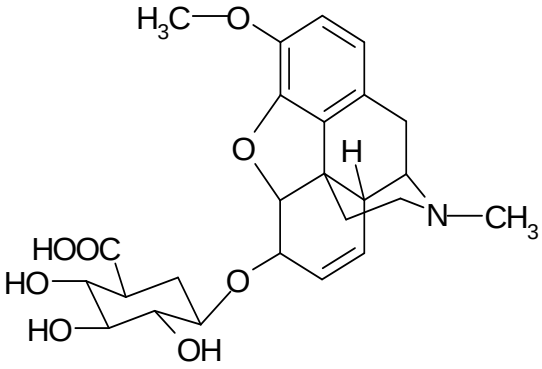
Morphine-3-glucuronide



Morphine-6-glucuronide



Codeine



Codeine-3-glucuronide

Figure 5-1: Chemical structures of morphine and codeine and their glucuronide metabolites.

5.2 Aims

The aim of this study was to determine the stability of M3G, M6G and C6G in blood and urine samples with and without additives when stored in the freezer, in the refrigerator and at room temperature for 1 year.

5.3 Experimental Section

5.3.1 Chemical and material:

Morphine-3-glucuronide (M3G), Morphine-6-glucuronide (M6G), Morphine-d_6 (MOR-d_6), Codeine-6-glucuronide (C6G), and Codeine-6-glucuronide-d3 (C6G-d_3) were purchased from Lipomed (Arleshide, Switzerland) as 1mg/ml solutions in methanol. Bond Elut LRC-C18 cartridges were purchased from Varian (Palo Alto, CA, USA). Ammonium carbonate, formic acid and ammonium hydroxide were purchased from BDH (Poole, UK). Methanol and acetonitrile were also obtained from BDH (Poole, UK). Ammonium formate was obtained from Acros Organic (Morris Plains, NJ, USA).

5.3.2 Solutions:

5.3.2.1 Preparation of 0.01M ammonium carbonate pH 9.3

0.94 g of ammonium carbonate was weighed into a 1 litre volumetric flask. Approximately 800 ml of deionized water were added and the pH was adjusted to 9.3 with ammonium hydroxide. Then, the solution was made up to volume with deionized water.

5.3.2.2 Preparation of 10mM ammonium formate and 0.001% formic acid

The mobile phase was prepared by dissolving 0.631 g of ammonium formate in 800 ml de-ionised water in a 1 L volumetric flask followed by the addition of 10 µl of concentrated formic acid. The volume was then made up to the mark with deionized water.

5.3.2.3 Standard solutions of analytes

Working standards of unlabelled and labelled drugs were prepared by diluting 250 µl from each drug stock solution in 25 ml methanol to obtain a concentration of 1 µg/ml. Stock solutions of standards and internal standards were stored in a freezer at -20ºC.

5.3.3 Preparation of blood and urine samples:

Time-expired packed red blood cells were obtained from a blood bank (Scottish Transfusion Service, Glasgow Western Infirmary) and were re-suspended in equal volume of isotonic saline (0.1M sodium chloride solution) before use to give a final volume of 400 ml. They were screened to check whether they contained the opiate glucuronide, of interest in this study, and the result was negative.

The blank blood was spiked with M-3G, M-6G, and C-6G to give final concentration of 100 ng/ml of each analyte and was then mixed on a roller for 1 hour to ensure that all compounds were distributed equally in the blood (RSD% < 13.7 for all analytes as shown in Tables 5-2, 5-4 and 5-6 at day zero concentrations). This concentration was selected according to the average concentrations of these analytes found in the case samples reported by Bogus et al. (1997).[148] Five samples from the prepared blood were analysed and found to be homogeneous, and the mean concentration of each analyte was considered to be the day zero concentration.

Blank urine obtained from a healthy volunteer was spiked with the same compounds as added to the blood to produce a final concentration of 100 ng/ml of each analyte in a total volume of 400 ml. The urine was mixed on the roller to

ensure all compounds were distributed equally. Five samples from the prepared urine were analysed and found to be homogeneous (RSD% < 9.8 for all analytes as shown in Tables 5-3, 5-5 and 5-7 at day zero concentrations), while the mean concentration obtained for each compound was considered to be the initial concentration.

The prepared blood sample was distributed into 150 tubes, half of them preserved (fluoride/oxalate) and the other half unpreserved containers. The prepared urine was distributed into tubes in a similar manner to the blood samples. Each day samples were analysed, two replicates were taken for analysis from the storage racks for each of the different storage conditions, extracted within the day and left to run on the LC-MS/MS instrument overnight. If the analysis could not be completed within 24 hours due to instrumental problems, extracts were kept in the deep freeze at -20°C until they could be analysed. A calibration curve was prepared for each set of samples over the range of 5-200 ng/ml.

Opiate glucuronide standards were spiked into blood or urine to produce concentrations of 5, 10, 25, 50, 100, 200 ng/ml and 100 ng/ml of internal standards were added into each concetrations then these were extracted by SPE and analysis by LC-MS-MS.

5.3.4 Sample storage and times of analysis

The prepared samples were divided into three groups and were then stored at three different temperatures (-20 ºC, 4 ºC, and room temperature). These storage temperatures were selected because they are frequently-used laboratory storage temperatures. The storage temperatures were monitored daily using permanently mounted digital thermometers with a resolution of 0.1 C°at the start of the study and subsequently at weekly intervals during the period of study. Temperatures were stable within a range of approximately 1 C ° (freezer and refrigerator) or 5 C° (room temperature, average temperature 20°C). The times selected for analysis were at day 1 (day zero), 2, 4, 7, 14, 30, 60, 90, 180, and 365.

5.3.5 Analysis

The method used for extraction and analysis was based on a published method for extraction of opiates and their glucuronides from blood.[147] The same method was subsequently validated for the same compounds in urine.[249]

Prior to extraction, 1ml from each sample was added to 3ml of 0.01M ammonium carbonate (pH 9.3). Next, 100 μl (100 ng/ml) of working internal standard solution (1 μg/ml), containing deuterated analogues of the target analytes, was added, then the mixture was vortex mixed and centrifuged for 10 minutes at 2500 rpm.

Bond Elut C18 SPE columns were conditioned with 3ml methanol, 3ml deionized water, and 3ml of 0.01M ammonium carbonate (pH 9.3), and then the supernatant of each sample was applied to the column, after which the SPE cartridges were washed twice with 3ml 0.01 ammonium carbonate (pH 9.3) and dried for 10 minutes. Elution of the retained analytes was achieved using 3ml methanol. The eluate was evaporated to dryness under a gentle stream of nitrogen at 50 °C. The residue was reconstituted in 150 μl of the initial mobile phase (97:3 v/v), of which 20 μl was injected into the LC-MS/MS instrument.

5.3.6 Instrumentation

The method used for analysis by liquid chromatography-tandem mass spectrometry was based on a published method.[147]

LC-MS/MS analysis was performed using a Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San Jose, CA, USA) equipped with a surveyor HPLC system. Chromatographic separation was achieved using a Synergy Polar RP column (150mm x 2mm ID, 4μm particle size) fitted with a guard column (4mm x 2mm, Phenomenex, Torrance) with the same column packing. The mobile phase was a mixture of (A) 10mM aqueous ammonium formate solution containing 0.001% formic acid and (B) acetonitrile. The gradient started at 97% of solution A for 3 minutes, decreasing to 84.5% at 8 minutes, to 74% at 13 minutes, and to 20% at 26 minutes; 5% of solution A was maintained for 3 minutes before resetting to

the starting composition between 29 and 36 minutes. The parameters selected for these compounds are shown in Table 5-1. Inernal standard data was aquired in selected ion monitoring (SIM) mode and analytes were identified on the basis of their full MS-MS spectra. One product ion was monitored and used as the quantification ion because it was the most intense ion produced. In the published method M3G-d3 and M6G-d3 coeluted with some analytes and were replaced with MOR-d6. As a result, MOR-d6 was used for M3G and M6G quantification while C6G-d3 was used for C6G quantification.[147]

Table 5-1: Optimum tuning parameters

Drug	Precursor ion (m/z)	Monitored ion* (m/z)	Collision energy (eV)
Morphine-3-G	462	286	29
Morphine-6-G	462	286	29
Codeine-6-G	476	300	30
Internal standards			
Morphine-d6	292	292	Not used
Codeine-6-G-d3	479	479	Not used

- Underlined ion was used as the quantification ion. For internal standards, only the pseudomolecular ions were monitored and no collision energies were specified.

M3G and M6G had the same precursor ion and product ion, which are produced by the same collision energy. However, they had different retention times. The M3G retention time was 3.42 min, while the M6G retention time was 6.25 min. The C6G retention time was 11.09 min.

5.4 Stability results

5.4.1 Stability of M3G in blood and urine

In the freezer (-20 °C), M3G was found to be stable in blood for 1 year. Small changes in the concentration were oberved after 1 year of storage in which approximately 3% and 5% of the compound was lost in preserved and unpreserved samples, respectively ($0.1 > p > 0.05$). In urine, M3G was also found to be stable for 1 year in preserved and unpreserved samples. A statistically insignificant loss in preserved samples was observed at month 12, in which M3G decreased by approximately 5% of the initial concentration, while in unpreserved samples, the compound had decreased by 7% at month 12 ($0.1 > p > 0.05$ for both points). After one year, more than 92% of the compound was recovered from the blood and urine in both preserved and unpreserved samples.

In the refrigerator (4 °C), M3G was found to be stable for 6 months in both preserved and unpreserved blood samples. Significant losses of 14% and 18% were observed after 1 year for preserved and unpreserved samples, respectively ($p < 0.05$). M3G was found to be stable for 3 months in preserved and unpreserved urine, whereas after 6 months, M3G had decreased by 13% and 17% in preserved and unpreserved samples, respectively ($p < 0.05$). More than 82% and 72% of the compound was recovered after 1 year from blood and urine samples, respectively.

At room temperature, M3G was found to be stable for 2 months in preserved and for 1 month in unpreserved blood samples. In the preserved blood sample the compound had significantly decreased by 14% from day zero concentration, while approximately 14% was lost in the unpreserved sample after 2 monts of storage ($p < 0.05$ for both samples). In urine M3G was found to be stable for 2 weeks for both conditions. Thereafter, the concentrations in preserved and unpreserved urine decreased by 14% and 16%, respectively by the end of the first month of storage ($p < 0.05$). After one year, more than 46% of M3G was recovered from blood samples, and more than 22% was recovered from urine samples in both conditions. Stability data are given in Tables 5-2 and 5-3.

Table 5-2: M3G stability in blood at 100 ng/ml

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	98 (6.0)	98 (6.0)	98 (6.0)	98 (6.0)	98 (6.0)	98 (6.0)
2	99 (0.7)	102 (2.8)	96 (1.5)	95 (2.2)	102 (2.8)	90 (6.0)
4	100 (2.1)	97 (1.5)	96 (0.7)	97 (2.9)	98 (2.2)	93 (3.1)
7	101 (3.4)	99 (5.2)	95 (5.0)	98 (1.5)	95 (1.4)	94 (0.7)
14	98 (5.2)	97 (2.9)	102 (4.9)	96 (1.4)	97 (2.1)	95 (4.9)
30	97 (4.5)	97 (2.2)	98 (2.1)	95 (5.7)	94 (3.0)	92 (1.5)
60	98 (4.8)	101 (1.9)	99 (3.7)	98 (4.8)	87 (1.9)	84 (3.8)
90	98 (1.9)	95 (4.1)	96 (1.0)	92 (3.7)	84 (3.2)	81 (2.5)
180	97 (0.7)	98 (5.7)	89 (8.1)	88 (5.9)	73 (7.8)	68 (6.1)
365	95 (3.2)	93 (1.8)	84 (6.4)	80 (7.3)	55 (5.1)	46 (3.9)

P*: preserved, N-P*: unpreserved. (Concentration threshold=86)

Day zero concentration is mean of 5 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

Table 5-3: M3G stability in urine at 100 ng/ml

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	99 (5.0)	99 (5.0)	99 (5.0)	99 (5.0)	99 (5.0)	99 (5.0)
2	102 (2.1)	99 (2.2)	99 (1.4)	98 (0.7)	102 (2.1)	100 (0.7)
4	100 (3.4)	99 (1.4)	99 (2.1)	99 (0.8)	99 (0.7)	99 (2.2)
7	102 (0.7)	101 (2.1)	102 (1.3)	98 (1.4)	98 (2.9)	99 (5.2)
14	103 (2.8)	104 (4.9)	104 (4.9)	100 (2.8)	94 (7.1)	92 (7.3)
30	99 (5.9)	97 (3.7)	97 (2.2)	96 (2.4)	85 (2.8)	83 (4.9)
60	98 (5.2)	98 (2.2)	94 (0.8)	93 (1.5)	71 (4.8)	66 (7.9)
90	99 (2.9)	97 (2.3)	93 (5.5)	91 (7.4)	62 (2.2)	57 (6.5)
180	95 (0.7)	94 (1.5)	86 (6.9)	82 (8.2)	44 (2.3)	32 (9.2)
365	94 (1.4)	92 (1.5)	78 (6.6)	71 (9.5)	30 (9.4)	22 (12.8)

P*: preserved, N-P*: unpreserved. (Concentration threshold=89)

Day zero concentration is mean of 5 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

5.4.2 Stability of M6G in blood and urine

In the freezer (-20 °C), M6G was found to be stable for 1 year in both blood and urine and no significant loss was observed during this period ($0.1 > p > 0.05$). More than 96% of analyte was recovered from the blood and urine after 1 year of storage.

In the refrigerator (4 °C), M6G in the blood sample was found to be stable for 1 year, with losses less than 15% of the original concentrations ($0.1 > p > 0.05$). After 1 year, more than 87% of the original concentration of M6G was recovered from the preserved and unpreserved blood samples. The preserved and unpreserved urine samples were stable up to 3 months and 2 months respectively. A significant loss was observed in the preserved sample after 6 months, which decreased by approximately 10% ($p < 0.05$) from the initial concentration, while the unpreserved sample decreased by approximately 12% ($p < 0.05$) after 3 months of storage. More than 76% of the compound was recovered after 1 year from urine samples in both conditions.

At room temperature, M6G was found to be less stable than in the freezer and refrigerator. M6G was found to be stable for 2 months in blood and two weeks in the urine. Three months after day zero, approximately 16% of M6G had been lost in the preserved and unpreserved blood samples ($p < 0.05$), while after 1 month approximately 14% and 16% had been lost from the urine preserved and unpreserved samples, respectively ($p < 0.05$). After 1 year of storage, approximately 64% and 57% was recovered from the preserved and unpreserved blood samples, respectively, while approximately 37% and 30% were recovered from the preserved and unpreserved urine samples, respectively. Stability data are given in Tables 5-4 and 5-5.

Table 5-4: M6G stability in blood at 100 ng/ml

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	98 (7.0)	98 (7.0)	98 (7.0)	98 (7.0)	98 (7.0)	98 (7.0)
2	99 (0.7)	101 (2.1)	96 (1.5)	97 (3.7)	103 (3.5)	95 (2.2)
4	100 (0.7)	99 (4.4)	98 (0.7)	97 (5.3)	98 (0.7)	95 (2.1)
7	99 (1.5)	98 (3.8)	97 (3.7)	98 (1.5)	97 (2.9)	98 (1.4)
14	99 (2.9)	98 (2.2)	98 (1.4)	96 (2.2)	101 (1.4)	100 (3.6)
30	98 (2.2)	99 (5.2)	100 (3.6)	99 (5.9)	99 (5.9)	95 (3.8)
60	101 (1.7)	99 (5.1)	97 (5.3)	97 (3.6)	88 (3.8)	89 (1.7)
90	99 (1.0)	100 (3.5)	97 (0.3)	95 (0.4)	83 (1.6)	82 (3.3)
180	100 (3.5)	98 (2.2)	94 (4.6)	93 (4.1)	76 (1.4)	75 (6.7)
365	97 (8.0)	96 (2.3)	87 (4.1)	85 (2.3)	63 (9.1)	56 (3.7)

P*: preserved, N-P*: unpreserved. (Concentration threshold=84)

Day zero concentration is mean of 5 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

Table 5-5: M6G stability in urine at 100 ng/ml

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	97 (5.0)	97 (5.0)	97 (5.0)	97 (5.0)	97 (5.0)	97 (5.0)
2	98 (0.7)	97 (1.5)	97 (2.9)	99 (5.2)	98 (0.7)	97 (1.4)
4	100 (1.4)	99 (1.4)	99 (2.9)	99 (1.5)	99 (0.7)	98 (0.9)
7	99 (0.7)	98 (0.8)	100 (1.4)	98 (2.2)	101 (2.1)	96 (0.8)
14	101 (0.9)	100 (1.5)	103 (2.1)	99 (0.7)	97 (0.6)	94 (2.2)
30	97 (2.1)	96 (2.2)	93 (5.1)	92 (4.5)	83 (4.1)	81 (5.1)
60	98 (1.2)	97 (1.4)	91 (1.5)	89 (2.3)	77 (5.7)	68 (3.4)
90	98 (1.3)	96 (2.1)	88 (2.4)	85 (3.3)	68 (4.0)	59 (4.6)
180	96 (0.6)	97 (2.3)	87 (0.8)	81 (3.4)	57 (7.6)	48 (9.4)
365	95 (2.4)	94 (0.8)	81 (5.1)	74 (6.4)	36 (5.7)	29 (9.1)

P*: preserved, N-P*: unpreserved. (Concentration threshold=87)

Day zero concentration is mean of 5 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

5.4.3 Stability of C6G in blood and urine

In the freezer (-20 °C), C6G was apparently stable in the blood and urine samples for 12 months. Losses were less than 7% ($p > 0.05$) in the concentrations in both matrix conditions after 1 year and more than 93% of the compound could be detected after 1 year in both matrices.

In the refrigerator (4 °C), the compound was found to be stable for 12 months in blood and 2 months in urine samples, preserved and unpreserved, while a significant loss was observed after 3 months in urine sample. In the urine samples, C6G decreased by 8% and 10% in the preserved and unpreserved, respectively, after 3 months. At the 1-year interval following storage, more than 91% and 89% of analyte was recovered from preserved and unpreserved blood samples, respectively, while more than 86% and 75% of analyte was recovered from the preserved and unpreserved urine samples, respectively.

At room temperature, the compound was found to be stable for 3 and 2 months in the preserved and unpreserved blood samples respectively, while beyond this time a significant loss of 13% and 16% was estimated in the preserved and unpreserved samples, respectively, ($p < 0.05$). After 1 year, approximately 75% and 70% of the analyte was recovered from preserved unpreserved samples of blood, respectively. In the urine samples, the compound was found to be stable for 2 weeks in both preserved and unpreserved samples. In preserved urine, C6G decreased by 15.5% ($p < 0.05$) after 1 month of storage, while approximately 45% of the compound was detected after 1 year of storage. In unpreserved urine, C6G decreased by 18% after 1 month, while only 38% of the initial concentration was recovered after 1 year. Stability data are shown in Table 5-6 and 5-7.

Table 5-6: C6G stability in blood at 100 ng/ml

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	99 (6.0)	99 (6.0)	99 (6.0)	99 (6.0)	99 (6.0)	99 (6.0)
2	100 (3.5)	98 (2.2)	99 (3.7)	98 (2.5)	98 (4.4)	98 (3.7)
4	101 (3.3)	99 (1.4)	98 (2.8)	100 (1.7)	99 (0.7)	97 (1.4)
7	99 (0.7)	97 (3.0)	102 (0.6)	99 (0.8)	98 (0.5)	99 (0.9)
14	98 (2.1)	97 (3.6)	101 (2.8)	98 (2.2)	99 (1.3)	99 (1.5)
30	101 (2.7)	102 (1.4)	100 (3.6)	99 (4.4)	101 (1.4)	97 (5.3)
60	99 (2.6)	99 (1.7)	98 (0.8)	98 (1.9)	91 (2.3)	90 (2.4)
90	98 (5.6)	98 (2.8)	98 (3.8)	97 (6.4)	89 (4.9)	83 (4.7)
180	99 (5.2)	98 (6.5)	95 (8.1)	94 (5.5)	86 (3.3)	79 (2.1)
365	96 (5.0)	93 (1.3)	91 (3.1)	89 (2.8)	75 (5.5)	70 (3.6)

P*: preserved, N-P*: unpreserved. (Concentration threshold=88)

Day zero concentration is mean of 5 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

Table 5-7: C6G stability in urine at 100 ng/ml

Time (day)	Storage condition					
	Freezer (-20 °C)		Refrigerator (4 °C)		Room temperature	
	P* (ng/ml)	N-P* (ng/ml)	P (ng/ml)	N-P (ng/ml)	P (ng/ml)	N-P (ng/ml)
Day zero#	97 (3.0)	97 (3.0)	97 (3.0)	97 (3.0)	97 (3.0)	97 (3.0)
2	99 (1.4)	100 (2.1)	102 (3.6)	97 (1.5)	100 (2.1)	99 (1.5)
4	97 (2.2)	98 (4.5)	99 (1.4)	99 (2.9)	98 (0.7)	97 (2.2)
7	99 (1.4)	96 (1.5)	97 (0.7)	98 (1.3)	99 (1.2)	96 (0.7)
14	98 (0.7)	98 (4.5)	98 (0.5)	95 (1.8)	97 (1.4)	95 (0.8)
30	97 (2.9)	96 (1.5)	96 (0.9)	94 (2.2)	82 (1.7)	80 (3.4)
60	108 (6.1)	98 (2.9)	94 (2.3)	93 (2.9)	80 (1.7)	73 (2.9)
90	95 (1.5)	97 (0.7)	89 (3.8)	87 (4.7)	69 (4.2)	60 (3.4)
180	96 (0.9)	96 (1.3)	87 (1.6)	83 (3.3)	50 (2.9)	46 (4.8)
365	94 (2.2)	93 (3.7)	86 (2.5)	75 (4.6)	44 (4.9)	37 (8.1)

P*: preserved, N-P*: unpreserved. (Concentration threshold=99)

Day zero concentration is mean of 5 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).

5.4.4 Summary of M3G, M6G, and C6G stability in blood and urine samples

The stability of M3G, M6G, and C6G in blood and urine samples under different storage conditions is summarized in Table 5-8.

Table 5-8: Summary of stability of M3G, M6G, and C6G in blood and urine samples for 1 year under different storage conditions

Compound	Storage conditions	Results		Comment
		Blood	Urine	
M3G	-20 °C, 1 year	Stable	Stable	• Compounds more stable in blood than in urine • NaF (0.2% w/v) enhances the stability by less than 10% • C6G more stable than M3G and M6G • M6G more stable than M3G
	4 °C, 1 year	18% decrease	28% decrease	
	25 °C, 1 year	53% decrease	78% decrease	
M6G	-20 °C, 1 year	Stable	Stable	
	4 °C, 1 year	Stable	24% decrease	
	25 °C, 1 year	43% decrease	70% decrease	
C6G	-20 °C, 1 year	Stable	Stable	
	4 °C, 1 year	Stable	22% decrease	
	25 °C, 1 year	29% decrease	62% decrease	

5.5 Discussion

This study demonstrated that decrease in the concentrations of these opiate metabolites must be expected during storage periods of up to 1 year. All compounds showed good stability when stored in the freezer during the whole observation period and no significant losses were noted. Higher temperatures significantly influenced the stability of M3G in blood samples, in which the compound steadily decreased after 2 months at room temperature and 1 year in the refrigerator, while in the urine sample it decreased after 1 month at room temperature and 6 months in the refrigerator. M6G in blood was very stable in refrigerator while in urine samples it was stable for 3 months and 2 months in preserved and unpreserved samples respectively. Similar findings were observed for M6G as for M3G at room temperature.

No significant decrease was observed in the concentrations of C6G in blood or urine after 1 year in the freezer while in the refrigerator changes were observed in urine samples after 3 months whereas C6G was found to be stable in blood for 1 year and no significant loss was observed. At room temperature, C6G was found to be stable in blood for 3 months and 2 months in preserved and unpreserved samples respectively, and was more stable than in urine, in which it was stable for only 2 weeks; beyond 2-4 weeks, significant losses were observed in both matrices in both conditions.

Decomposition of these metabolites is clearly dependent on the storage conditions. Although the C6G decay was similar to M3G and M6G at the beginning, the C6G recovered at the end of the study was more than that of M3G and M6G. This implies that the chemical structure of these compounds has diffeent degrees of thermal instability. The phenol moiety in M3G and M6G might undergo an oxidation reaction through a process involving the phenolic group in their structure. This reaction was reported for morphine, in which the oxidation process yielded pseudomorphine as a result of oxidation. However, this reaction did not occur for codeine because the phenolic group is alkylated. Therefore, samples containing morphine and its metabolite should be protected from light and stored at negative temperatures after collection from individuals.

Degradation by hydrolysis of the opiate glucuronides is not a predominant reaction, which was reported in post-mortem cases to yield free morphine. Meanwhile, the oxidation is a minor process that may be affected by light.[68] At the beginning, these compounds exhibited similar stability, but the samples may have been exposed to light during the opening and closing of the refrigerator, freezer, and cabinet doors at room temperature. Additionally, active oxygen may be scavenged by blood components such as haemoglobin.

Summing up, morphine and M3G and M6G calculated as morphine equivalents, provided that the degradation of the morphine metabolite is not a result of a hydrolysis reaction or other minor degradation processes, is attributed to oxidation degradation as reported previously.[68] In post-mortem samples bacteria may play a role in this reaction, which transmigrate through the gastrointestinal wall into the blood 5 hours after death occurred, as reported in the metabolism of nitrobenzodiazepines by intestinal bacteria. Furthermore, bacteria showed high β-glucuronidase activity in heart blood, which was reported by Skopp et al.[68] The presence of bacteria, common in post-mortem blood and urine, as well as in the unprotected skin of less hygienic individuals, might affect the stability of these compounds, leading to accelerated degradation reactions. Bacteria might also utilise the glucose moiety of glucuronide metabolites to produce alcohol. In addition, urine from unhealthy persons, such as those complaining of urinary tract infection (UTI), may contain a high bacteria count which can strongly influence the stability of these compounds. Bacterial contamination of samples and its effect on drug stability should be considered and minimized by using preservatives. In the current study, the role of bacteria in the decomposition is excluded because these matrices were collected from healthy volunteers and the prepared samples were transferred into tubes, which were opened once and not reopened for subsequent analyses. Additives showed enhancement of the stability of these metabolites but only to a small extent, as observed in the work reported in Chapter 4, and that may be due to the small concentration of NaF (0.2% w/v) in the tube. However, if samples are collected from real post-mortem cases, the effect of the additives on the stability of these compounds may be greater.

M3G was less stable than M6G, in agreement with the results reported by Skopp et al., while C6G was found to be the most stable among these compounds.[68]

In addition, the stability of these metabolites is not only influenced by temperature and additives but by the matrices, and the stability of these metabolites in blood was greater than in the urine, which might be the result of a lack of protective effects resulting from partitioning of the analytes into red blood cells. It has been reported that whole blood provides better protection against light exposure than plasma. Additionally, it was found that these metabolites are more stable in whole blood than in plasma, despite the fact that plasma contains protein, which may protect the compounds from degradation as a result of protein binding. The same finding was observed in this study although the blood matrix used did not contain plasma proteins. Blood contains haemoglobin which binds oxygen and this might have been expected to play a role in the degradation of compounds. However, the glucuronides were more stable in blood than in urine. The net effect may be the combined result of a protective effect due to partitioning of the metabolites into red blood cells as mentioned earlier and of the pH of the matrix. Blood pH is buffered by bicarbonate as well as by proteins, which in the present study could have been released by lysis of erythrocytes, whereas, as noted earlier, the urine pH may change significantly during storage. The pH of urine (pH 4-8) may accelerate the degradation process of compounds compared to that of blood (pH 7.2-7.4). Furthermore, at high temperatures and with time, urine pH may shift to alkaline as a result of the breakdown of urea to ammonia, and this could also accelerate the hydrolysis of these compounds.[156]

5.6 Conclusion

This study investigated the stability of M3G, M6G and C6G in blood and urine at three different storage temperatures, with and without additives, and demonstrated that a low storage temperature is the optimum for preserving these compounds. Ideally, analysis of samples should be achieved as soon as they are received. Samples should be stored in a freezer at -20 °C or lower in order to protect analytes for a long period, while additives should be added even though their effect was small in this study, as they may show greater effects with post-mortem samples which are known to contain bacteria that may accelerate the decomposition of compounds and limiting their detection. The addition of antioxidant substances, such as ascorbic acid or sodium metabisulfite, may enhance the stability of morphine metabolites against the oxidation process, and further study is suggested using antioxidants to preserve morphine metabolites. Storage conditions were not the only influence on the stability of compounds, but the nature of the biological matrix also had an effect such that compounds were more stable in blood than urine. Interpretation of results obtained from samples stored long-term should be carefully interpreted to avoid serious errors.

6 Stability of Cocaine and Benzodiazepines in Dried Blood Spots

6.1 Introduction

In the review given earlier (Section 2.4.2), the use of dried blood spots (DBS) is one of several approaches to stabilising drugs in blood specimens. The Guthrie card has been used for the collection of blood spots since 1960, when Dr Guthrie used blood collected on filter paper to measure phenylalanine in newborns.[157] Dried blood spots (DBS) have also been used in therapeutic drug monitoring and diagnostic screening as well as having been found suitable for drug analysis and for reducing the breakdown of drugs.[91-97,158-172] However, only a few studies in this area have been published and the present work considerd DBS as a method for stabilising hydrolytically-labile drugs, including cocaine and selected benzodiazepines.[91-97]

Cocaine is an active alkaloid obtained from the leaves of the plant *Erythroxylon coca*. Cocaine induces its effect via stimulation of release of the neurotransmitters dopamine, noradrenaline and serotonin in the peripheral organs and brain. Cocaine also stimulates the synthesis of these neurotransmitters by activation of the enzyme tyrosine hydroxylase. Further, cocaine prevents reuptake of released neurotransmitters into their respective neurones. Beside these effects cocaine inhibits release of the central nervous system (CNS) depressant Gamma-aminobutyric acid (GABA). Cocaine was used therapeutically as a local anaesthetic and is widely abused due to its stimulant properties and so it is frequently detected in forensic cases. Benzoylecgonine (BZE) is the main metabolite of cocaine in blood and urine which is produced by chemical and enzymatic hydrolysis of the methyl ester in cocaine. Other metabolites include norcocaine, ecgonine methyl ester (EME) and ecgonine. Smoking of cocaine produces anhydroecgonine methyl ester (AEME) by pyrolysis rather than metabolism while cocaethylene is a unique active metabolite of cocaine when cocaine is co-administered with alcohol. Peak plasma concentrations of cocaine were 104 to 424 ng/ml at 50 to 90 min after administration of cocaine hydrochloride in gelatine capsule to healthy male volunteers.[135,136]

Flunitrazepam (Rohypnol®) is a member of the 7-nitrobenzodiazepine class which has fluorine at position 2 of the phenol group and is used clinically as a hypnotic and anaesthetic induction agent. It is also implicated as drug of abuse in drug facilitated sexual assault (DFSA). Flunitrazepam is administered orally or intravenously. The metabolism of flunitrazepam is via N-demethylation, N-hydroxylation and glucuronidation, and reduction of the nitro group to the corresponding amine. The average peak plasma concentrations of flunitrazepam and 7-aminoflunitrazepam within 6-24 hours following a 2 mg oral dose were 0.5-3 μg/l and 50-500 μg/l respectively. Blood concentrations in cases of flunitrazepam toxicity ranged from 0.01-0.05 mg/l. [135,136,144,145]

Diazepam (Valium®) is the second benzodiazepine used clinically to treat anxiety, muscle spasm and convulsion, orally or parenterally. Diazepam undergoes N-demethylation to nordiazepam while both of them are hydroxylated to give temazepam and oxazepam respectively. The peak blood concentration after a 10 mg oral dose of diazepam is 0.148 mg/L at 1 hour. Serum diazepam concentrations in intoxicated patients reach up to 20 mg/L.[135,136,145]

Temazepam (Restoril®) is the 3-hydroxylated metabolite of diazepam and is used therapeutically as a hypnotic. An oral dose of temazepam (10 mg) leads to a peak plasma concentration in the range 0.205-0.43 mg/L within 15-90 minutes. Temazepam is metabolised and excreted in the urine as free and conjugated temazepam and free and conjugated oxazepam. Toxic doses of temazepam lead to blood temazepam concentrations in the range 0.9-14 mg/L. [135,136,145]

Nitrazepam (Mogadon®) is a 7-nitro–benzodiazepine which is used clinically as a hypnotic drug. The therapeutic plasma level following a 5 mg oral dose of nitrazepam is 0.035 mg/L after 2 hours while the blood concentration following toxic doses of nitrazepam (250 mg) are in the range 1.2-9 mg/L. Nitrazepam is reduced to an inactive metabolite, 7-aminonitrazepam. [135,136,145]

The chemical structures of cocaine and benzodiazepines used in this study are shown in Figures 6-1 and 6-2.

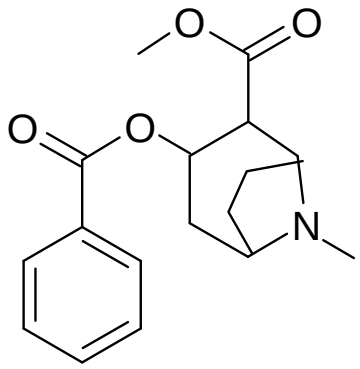
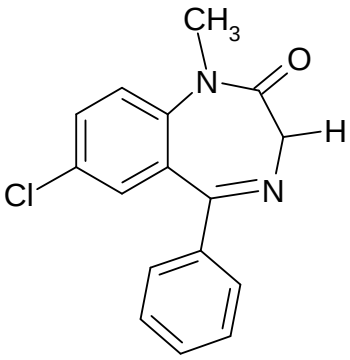
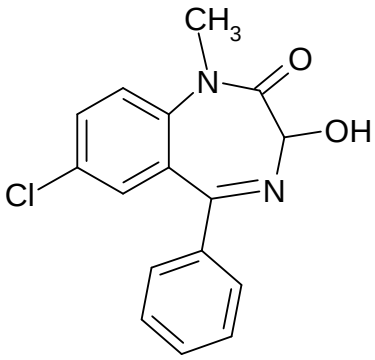


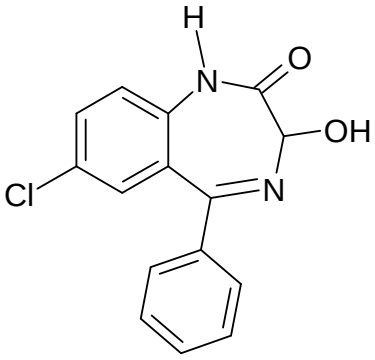
Figure 6-1: Chemical structure of cocaine



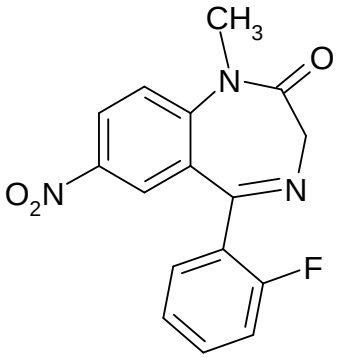
Diazepam



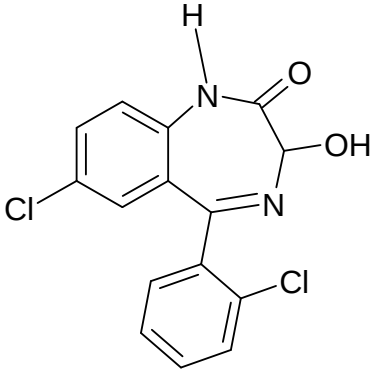
Temazepam



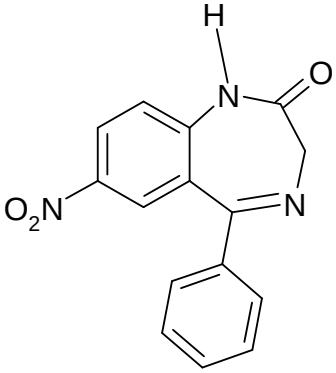
Oxazepam



Flunitrazepam



Lorazepam



Nitrazepam

Figure 6-2: Chemical structures of benzodiazepines used in this study.

The aim of this study was to investigate the stability of hydrolytically labile drugs in dried blood spots stored for 1 year in the freezer, the refrigerator and at room temperature.

6.2 Experimental Section

6.2.1 Chemical and material:

Flunitrazepam, temazepam, lorazepam, oxazepam, nitrazepam and diazepam and the corresponding deuterated internal standards (flunitrazepam-d7, temazepam-d5, oxazepam-d5, lorazepam-d4 and diazepam-d5) were purchased from LGC Promochem (Middlesex, UK) as 100µg/ml solutions in methanol. Cocaine and cocaine-d3 were purchased from LGC Promochem as 100 µg/ml solutions in acetonitrile. Stock solutions of standards and internal standards were stored at -20 ºC. Acetonitrile, methanol, cyclohexane, ethyl acetate, ammonium hydroxide, formic acid and potassium dihydrogen phosphate were obtained from BDH (Poole, UK). Ammonium formate was purchased from Sigma-Aldrich (Dorset, UK). Clean Screen ZSDAU020 cartridges were purchased from United Chemical Technology UK. Sheets of Guthrie card 903® specimen collection paper (210 x 297 mm) were purchased from Whatman UK.

6.2.2 Solutions:

6.2.2.1 Preparation of 0.1M *phosphate buffer pH 6.0*

1 litre of solution was prepared by dissolving 1.7 g of anhydrous disodium orthophosphate and 12.14 g of sodium dihydrogen orthophosphate monohydrate in 800 ml of deionized water. The pH was adjusted to pH 6 with 1 M potassium hydroxide and the solution made up to volume with deionized water.

6.2.2.2 Preparation of 3mM ammonium formate and 0.001% formic acid

This mobile phase was prepared by dissolving 0.189 g of ammonium formate in 800 ml deionized water followed by addition of 10 µl of concentrated formic acid and making up to one litre with deionized water.

6.2.2.3 Standard solutions of analytes

Working standards of unlabelled and labelled drugs were prepared by diluting 250 µl from each 100 µg/ml drug stock solution in 25 ml methanol to obtain a concentration of 1 µg/ml. Stock solutions of standards and internal standards were stored in the freezer at -20 ºC.

6.2.3 Preparation of DBS samples:

Packed human red blood cells which had passed their usable date were obtained from the Scottish National Blood Transfusion Service and were re-suspended in an equal volume of isotonic saline. The original blood collection used ethylene diamine tetra acetic acid (EDTA) as anticoagulant. The blood was spiked with drug compounds to produce a final concentration of 1000 ng/ml of each analyte (total volume was 10 ml) then mixed for one hour to ensure the homogeneity of all analytes in the blood. After that, 100 µl of the spiked blood was spotted on the filter paper to produce 100 spots. The spot diameter was equal to 13mm and each spot contained 100 ng of each analyte.

The spotted samples were allowed to dry overnight at ambient temperature in the fume hood. Ten samples were analysed on the first day to check on their homogeneity and treated as the first day concentrations (RSD% < 9 for analyte as shown in Tables 6-6 to 6-12 at day zero concentration). Then the prepared samples were divided into three groups and placed in sealed plastic bags to protect them from contamination and humidity and were then stored at three different temperatures (-20ºC, 4ºC and room temperature). These storage temperatures were selected in relation to frequently-used laboratory storage temperatures.The storage temperatures were monitored daily using permanently mounted digital thermometers with a resolution of 0.1 C°at the start of the study and subsequently at weekly intervals during the period of study.

Temperatures were stable within a range of approximately 1 C ° (freezer and refrigerator) or 5 C° (room temperature, average temperature 20°C). The times selected for analysis were at day 1 (day zero), 2, 4, 7, 14, 30, 60, 90, 180, and 365.

The bags remained sealed until they were opened for processing of the DBS. DBS were analysed in duplicate for each time and storage temperature.

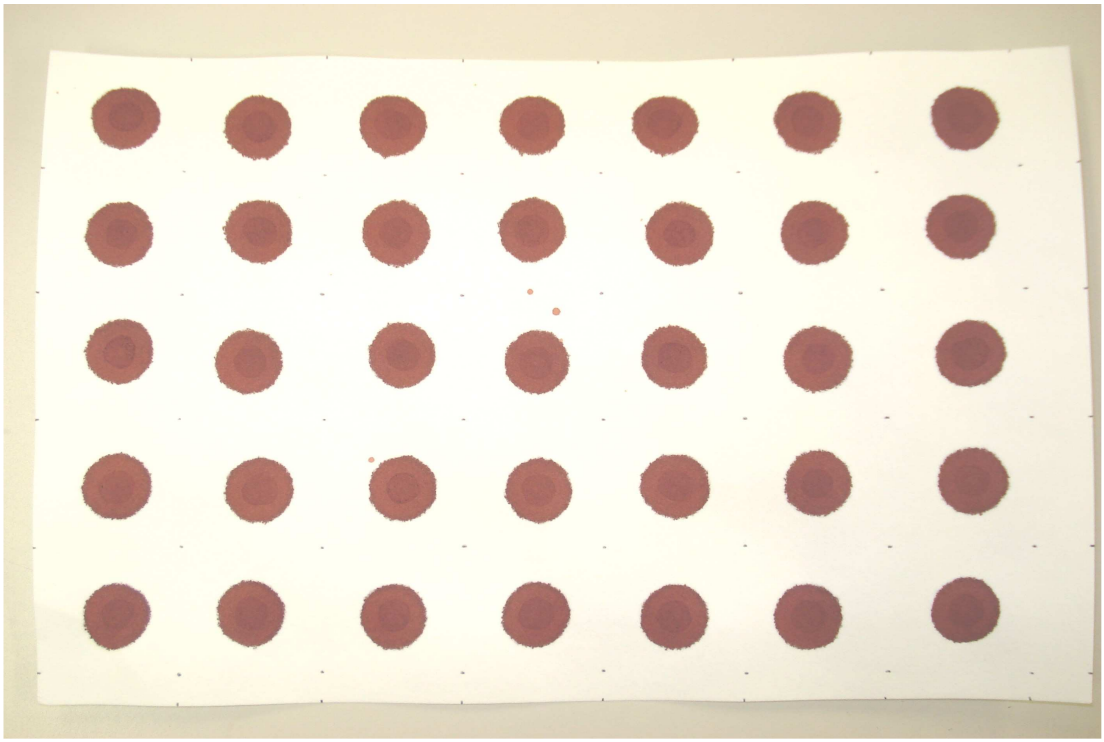


Figure 6-3: 100µl aliquots of spiked blood were spotted on sheets of paper 903® to produce 100 spots in total.

6.2.4 Extraction procedure

6.2.4.1 Optimisation of the extraction of analytes from DBS

An evaluation was carried out of four different methods of extracting the analytes from DBS on filter paper, based on three different solvents (methanol, ethyl acetate and acetonitrile) and phosphate buffer (pH6), to establish which would give the best recoveries of the analytes.

2ml blank blood was spiked with the analytes and was then spotted on the sheet of paper to give a final concentration of 50 ng of each analyte per spot. The

spots were allowed to dry overnight. The spots were cut from the sheet with scissors, transferred to extraction tubes and 3.5 ml extraction solutions were added. The tube was vortex-mixed and placed in an ultrasonic bath for one hour to extract the drugs from the paper. The paper was discarded then the extract was centrifuged at 2500 RPM for 10 minutes. Solid phase extraction columns were conditioned with 3ml of methanol, 3ml water and 2 ml phosphate buffer (pH6). After this, the sample extracts were passed through the cartridges with no vacuum applied. The columns were washed with 2ml water followed by 2ml phosphate buffer (pH6): acetonitrile (80:20 v:v). The final column washing step was the addition of 2 ml of water followed by drying for 5 minutes. Elution of analytes was achieved using 3 ml of ethyl acetate: ammonia (98:2 v:v).

Unextracted standards (50 ng of each analyte) were prepared in duplicate.
Before evaporation under a stream of nitrogen, 50ng of internal standard was added to all samples, and the samples were analysed by LC-MS/MS. Extracts of DBS and the unextracted standards were analysed by LC-MS/MS and recoveries were calculated using the equation 3-5. The mean recoveries for all analytes are shown in Table 6-1.

Table 6-1: Recovery of (50 ng/spot) drugs from DBS using three different solvents and buffer:

Drug	Mean recovery % (RSD %, n=4)			
	Methanol	Acetonitrile	Ethyl acetate	Buffer *
Cocaine	84 (1)	97 (20)	82 (7)	90 (13)
Flunitrazepam	86 (11)	71 (12)	57 (17)	93 (3)
Temazepam	94 (9)	68 (8)	76 (14)	86 (2)
Oxazepam	63 (2)	31 (10)	37 (17)	95 (12)
Lorazepam	64 (4)	49 (3)	59 (6)	83 (12)
Nitrazepam	65 (15)	53 (13)	54 (18)	99 (4)
Diazepam	98 (7)	68 (10)	78 (11)	100 (5)

* Phosphate Buffer pH 6

Phosphate buffer pH 6 showed the highest recovery compared to organic solvents (Table 6-1). Haemoglobin was also released from the spot when using buffer and consequently the buffer was used in the study to extract the drugs from DBS followed by SPE.

6.2.4.2 Final extraction procedure for DBS

DBS samples were removed from the paper with scissors and added to 3.5ml of phosphate buffer pH 6 containing 100µL of internal standard then vortex-mixed and placed in an ultrasonic bath for one hour to extract the drugs from the paper. The paper was discarded then the extract was centrifuged at 2500 RPM for 10 minutes.

Solid phase extraction columns were conditioned with 3ml of methanol, 3ml water and 2 ml phosphate buffer (pH6). After this, the sample extracts were passed through the cartridges with no vacuum applied. The columns were washed with 2ml water followed by 2ml phosphate buffer (pH6): acetonitrile (80:20 v:v). The final column washing step was the addition of 2 ml of water followed by drying for 5 minutes. Elution of analytes was achieved using 3 ml of ethyl acetate: ammonia (98:2 v:v). The extracts were dried under a gentle stream of nitrogen, then the residues were reconstituted in 100µl of HPLC mobile phase, of which 20µl was injected into the LC-MS/MS instrument. The analysis was carried out in duplicate for each storage temperature.

Each day samples were analysed, two replicates were taken for analysis from the storage racks for each of the different storage conditions, extracted within the day and left to run on the LC-MS/MS instrument overnight. If the analysis could not be completed within 24 hours due to instrumental problems, extracts were kept in the deep freeze at -20°C until they could be analysed. A calibration curve was prepared for each set of samples over the range of 5-200 ng/spot. Cocaine and benzodiazepines standards were spiked into blood to produce concentrations of 5, 10, 25, 50, 100, 200 ng/spot and 100 ng/spot of internal standards were added into each concentration, then these were extracted by SPE and analysed by LC-MS/MS.

6.2.5 Instrumentation

LC-MS/MS analysis was performed using a Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San Jose, CA, USA) equipped with a surveyor HPLC system. Chromatographic separation was achieved using a Gemini C18 column (150 mm x 2 mm ID, 5µm particle size) fitted with guard column (4 mm x 2 mm, 5µm) with the same column packing. The mobile phase was a mixture of 3mM aqueous ammonium formate solution containing 0.001% formic acid and acetonitrile starting at 65:35 v:v and changing to 10:90 v:v between 13 and 13.5 minutes. It was then held at 10:90 v: v between 13.5 and 16.5 minutes then reset to the staring composition between 16.5 and 20 minutes. Optimum tuning parameters selected for all analyte are shown in the Table 6-2. The m/z of the nitrazepam-d5 internal standard precursor ion is the same as the m/z of the oxazepam precursor ion (m/z=287) also both have the same t_R of 5.8 min resulting to undesirable coelution. Therefore, oxazepam-d5 was used as internal standard in nitrazepam quantitation. Internal standard data was acquired in selected ion monitoring (SIM) mode and analytes were identified on the basis of their full MS-MS spectra. Two product ions were monitored. The underlined ions in Table 6-2 were used as the quantification ions because they were the most intense ions produced.

Table 6-2: Optimum tuning parameters

Drug	Precursor ion (m/z)	Monitored ions* (m/z)	Collision energy (ev)
Cocaine	304	182, 150	32
Flunitrazepam	314	286, 268	40
Temazepam	301	283, 255	28
Oxazepam	287	269, 241	26
Lorazepam	321	303, 275	28
Nitrazepam	282	254, 236	41
Diazepam	285	257, 222	40
Internal standards			
Cocaine-d3	307	307	Not used
Flunitrazepam-d7	321	321	Not used
Temazepam-d5	306	306	Not used
Oxazepam-d5	292	292	Not used
Lorazepam-d4	325	325	Not used
Diazepam-d5	300	300	Not used

- Underlined ion was used as the quantification ion. For internal standards, only the pseudomolecular ions were monitored and no collision energies were specified.

6.3 Validation of analytical method

The validation parametrs applied in this study were similar to the parameters in Table 4-1 except that the low concentration in this method was 10 ng/spot.

6.4 Results

6.4.1 Method validation results

6.4.1.1 Linearity

The relationships between analyte concentrations and peak area ratios were linear over the range 5-200 ng/spot and the linear correlation coefficients (r^2) were better than 0.99 for all analytes as shown in Table 6-3.

6.4.1.2 LODs and LLOQs

LOD values for all analytes were 0.29-0.74 ng/spot, and LLOQ values were 0.99-2.46 ng/spot. The results are shown in Table 6-3.

Table 6-3: Linear correlation coefficients, LODs and LLOQs of drugs in DBS.

Drug	Linear correlation coefficient (r^2)	LOD (ng/spot)	LLOQ (ng/spot)
Cocaine	0.994	0.74	2.46
Flunitrazepam	0.999	0.47	1.58
Temazepam	0.996	0.32	1.08
Oxazepam	0.999	0.62	2.06
Lorazepam	0.996	0.32	1.10
Nitrazepam	0.998	0.54	1.80
Diazepam	0.996	0.29	0.99

6.4.1.3 Recoveries

The recoveries for all analytes were in the range 81-106% as shown in Table 6-4.

Table 6-4: Recoveries of analytes from DBSs

Drug	Mean Recovery% (RSD%, n=5)		
	10ng/spot	50ng/spot	100ng/spot
Cocaine	90 (12)	91 (16)	97 (11)
Flunitrazepam	89 (16)	93 (9)	103 (13)
Temazepam	89 (13)	93 (15)	94 (12)
Oxazepam	95 (17)	106 (12)	105 (5)
Lorazepam	81 (13)	88 (10)	86 (8)
Nitrazepam	90 (12)	99 (16)	100 (2)
Diazepam	83 (18)	97 (15)	99 (15)

6.4.1.4 Intraday and interday precision

As shown in Table 6-5 the method showed good precision and the relative standard deviations (RSD%) for intra- and inter-day precision were in the ranges 1.9-14.7 and 1.6-18.3 % respectively, which are considered to be acceptable.

Table 6-5: Inter-day and intra-day precision results.

Drug	Interday mean (RSD%, n=5)			Intraday mean (RSD%, n=5)		
	10ng/spot	50ng/spot	100ng/spot	10ng/spot	50ng/spot	100ng/spot
Cocaine	10 (6.7)	53 (6.7)	98 (9.7)	13 (12.8)	48 (8.0)	96 (5.4)
Flunitrazepam	12 (11.7)	47 (11.7)	98 (6.9)	10 (8.1)	48 (5.6)	95 (4.2)
Temazepam	10 (14.2)	49 (9.3)	95 (6.0)	11 (6.6)	44 (4.5)	92 (3.3)
Oxazepam	10 (14.1)	47 (12.7)	100 (7.3)	11 (5.9)	55 (14.7)	97 (2.8)
Lorazepam	10 (18.3)	51 (13.8)	107 (2.6)	10 (14.7)	57 (10.3)	100 (1.9)
Nitrazepam	13 (16.9)	48 (16.8)	99 (2.9)	14 (10.9)	46 (8.0)	93 (3.2)
Diazepam	9 (11.9)	55 (5.9)	103 (1.6)	13 (10.3)	51 (11.3)	99 (4.0)

6.4.2 Stability results

6.4.2.1 Cocaine

Cocaine in dried blood spots were found to be stable in the freezer and significant loss was not observed until after 2 months of storage, when there was a 17 % loss from the original concentration ($p < 0.05$). After 1 year approximately 60% of the cocaine was recovered from DBS.

In the refrigerator, cocaine was found to be stable for only 2 weeks. After 1 month a significant loss of 15% from the day zero concentration was noted ($p < 0.05$). Approximately 36% of the original cocaine could still be detected in DBS after 1 year.

At room temperature cocaine was found to be stable for 4 days before a significant decrease was observed after 1 week which was an 18% decrease from the initial concentration at day zero ($P < 0.05$). The remaining cocaine that could be recovered from DBS after 1 year of storage at room temperature was approximately 14% of the starting concentration. The stability data are shown in Table 6-6.

Table 6-6: Stability of cocaine in dried blood spots stored in filter paper

Time (day)	Storage condition		
	Freezer (-20 °C) (ng/spot)	Refrigerator (4 °C) (ng/spot)	Room temperature (ng/spot)
Day zero#	105 (8.0)	105 (8.0)	105 (8.0)
2	106 (8.2)	104 (11.5)	100 (2.8)
4	105 (2.9)	100 (3.5)	92 (3.6)
7	103 (2.0)	98 (5.6)	86 (4.6)
14	98 (3.2)	95 (4.3)	83 (1.4)
30	96 (10.4)	89 (9.4)	81 (7.4)
60	87 (10.1)	73 (1.0)	54 (10.5)
90	72 (10.9)	59 (8.7)	33 (2.4)
180	68 (4.2)	46 (8.3)	20 (4.2)
365	63 (1.5)	38 (12.8)	15 (11.0)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %). (Concentration threshold=90).

6.4.2.2 Flunitrazepam

Flunitrazepam was found to be stable in DBS when stored in the freezer and refrigerator for one year and no significant loss was noted during the complete observation period ($0.1 > p > 0.05$). The quantities of flunitrazepam recovered after one year in the freezer and refrigerator were 94% and 88% respectively.
Significant loss of flunitrazepam at room temperature was observed after 1 month of storage. Flunitrazepam decreased by 18% from the day zero concentration ($p < 0.05$) while approximately 76% of the compound was detected after 1 year. The stability results are shown in the table 6-7.

Table 6-7: Stability of flunitrazepam in dried blood spots stored in filter paper.

Time (day)	Storage condition		
	Freezer (-20 °C) (ng/spot)	Refrigerator (4 °C) (ng/spot)	Room temperature (ng/spot)
Day zero#	104 (7.0)	104 (7.0)	104 (7.0)
2	99 (3.3)	98 (1.3)	102 (0.7)
4	99 (4.6)	97 (1.0)	99 (0.6)
7	98 (1.2)	96 (1.4)	99 (3.4)
14	96 (1.4)	92 (6.6)	93 (2.3)
30	95 (2.2)	91 (3.9)	86 (3.0)
60	98 (10.4)	92 (13.7)	83 (2.6)
90	99 (8.9)	97 (1.1)	84 (4.6)
180	97 (0.3)	96 (0.7)	83 (0.8)
365	98 (14.3)	92 (6.8)	79 (7.9)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %). (Concentration threshold=90).

6.4.2.3 Temazepam

Temazepam was apparently stable in the freezer and refrigerator for 1 year of storage with no significant loss being observed ($0.1 > p > 0.05$). The quantities of temazepam recovered from DBS in the freezer and refrigerator after 1 year of storage were 96% and 89% respectively. At room temperature temazepam showed good stability for 6 months and a significant decrease of the drug concentration was observed after 1 year which was approximately 18% of starting concentration ($p < 0.05$). Approximately 82% of temazepam was recovered after 1 year of storage. Stability data are given in Table 6-8.

Table 6-8: Stability of temazepam in dried blood spots stored in filter paper

Time (day)	Storage condition		
	Freezer (-20 °C) (ng/spot)	Refrigerator (4 °C) (ng/spot)	Room temperature (ng/spot)
Day zero#	100 (8.0)	100 (8.0)	100 (8.0)
2	97 (2.6)	98 (3.8)	99 (9.5)
4	98 (1.8)	96 (3.9)	98 (2.5)
7	97 (4.5)	94 (3.3)	96 (2.2)
14	97 (3.8)	95 (2.7)	88 (10.4)
30	98 (4.5)	94 (4.8)	86 (7.1)
60	96 (6.9)	93 (8.9)	87 (1.3)
90	95 (3.4)	89 (2.1)	86 (5.4)
180	97 (1.4)	90 (4.9)	87 (0.2)
365	96 (6.8)	89 (4.8)	82 (7.1)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %). (Concentration threshold=84).

6.4.2.4 Oxazepam

Oxazepam was found to be stable in DBS for the whole observation period when stored in the freezer and refrigerator ($p > 0.05$) whereas a significant decrease occurred 3 months after storage at room temperature ($p < 0.05$), which was 20% from the day zero concentration. The amounts of drug recovered from DBS after 1 year of storage in the freezer, refrigerator and at room temperature were 90%, 85% and 75% respectively. Stability data are given in Table 6-9.

Table 6-9: Stability of oxazepam in dried blood spots stored in filter paper

Time (day)	Storage condition		
	Freezer (-20 °C) (ng/spot)	Refrigerator (4 °C) (ng/spot)	Room temperature (ng/spot)
Day zero#	102 (9.0)	102 (9.0)	102 (9.0)
2	101 (7.6)	98 (3.2)	98 (8.9)
4	99 (5.2)	98 (4.1)	98 (1.8)
7	99 (5.3)	97 (3.9)	97 (2.1)
14	98 (0.3)	95 (3.1)	93 (7.9)
30	97 (4.1)	94 (5.0)	89 (13.5)
60	97 (2.1)	92 (5.8)	85 (12.2)
90	95 (2.5)	88 (5.4)	82 (8.3)
180	93 (4.3)	89 (5.2)	79 (2.4)
365	92 (3.7)	87 (8.8)	77 (1.9)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %).(Concentration threshold=84).

6.4.2.5 Lorazepam

In the freezer and refrigerator lorazepam was found to be stable in DBS for 1 year ($p > 0.05$) and no significant loss of the drug concentration was noted during the observation period. The drug remaining after 1 year was 97% and 88% of the starting amount in the freezer and refrigerator respectively. At room temperature a significant decrease of 16% was observed after 1 year of storage ($p < 0.05$) and the quantity recovered was 84% of its initial concentration. Stability data are given in Table 6-10.

Table 6-10: Stability of lorazepam in dried blood spots stored in filter paper

Time (day)	Storage condition		
	Freezer (-20 °C) (ng/spot)	Refrigerator (4 °C) (ng/spot)	Room temperature (ng/spot)
Day zero#	101 (8.0)	101 (8.0)	101 (8.0)
2	101 (2.4)	99 (5.1)	97 (1.8)
4	99 (0.1)	99 (0.7)	98 (3.0)
7	100 (1.4)	99 (1.5)	98 (1.7)
14	99 (0.7)	95 (2.8)	93 (3.9)
30	98 (5.8)	94 (3.2)	90 (2.0)
60	99 (1.9)	97 (6.3)	93 (3.1)
90	103 (10.1)	101 (2.1)	98 (1.2)
180	98 (8.4)	92 (6.1)	90 (9.7)
365	97 (4.5)	88 (4.9)	84 (1.6)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %). (Concentration threshold=85).

6.4.2.6 Nitrazepam

Nitrazepam was also found to be stable in the freezer and refrigerator, and no significant decrease was observed in the concentration over a 1 year period (p > 0.05). The amounts of nitrazepam detected after 1 year were 93% and 84% from DBS stored in freezer and refrigerator respectively. However nitrazepam stored at room temperature showed a significant 18% decrease in concentration after 2 months (p < 0.05) while 74% was recovered from DBS after 1 year of storage at room temperature. Stability data are shown in Table 6-11.

Table 6-11: Stability of nitrazepam in dried blood spots stored in filter paper

Time (day)	Storage condition		
	Freezer (-20 °C) (ng/spot)	Refrigerator (4 °C) (ng/spot)	Room temperature (ng/spot)
Day zero#	102 (9.0)	102 (9.0)	102 (9.0)
2	102 (0.4)	101 (2.7)	101 (10.2)
4	100 (0.5)	99 (3.1)	97 (2.8)
7	100 (1.7)	97 (5.3)	96 (4.5)
14	98 (3.3)	96 (3.4)	91 (2.3)
30	98 (1.6)	96 (6.1)	85 (3.4)
60	97 (1.9)	94 (1.8)	84 (3.9)
90	96 (2.8)	92 (3.2)	81 (1.7)
180	95 (12.0)	89 (10.1)	78 (11.6)
365	95 (4.6)	86 (3.8)	74 (8.2)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %). (Concentration threshold=84).

6.4.2.7 Diazepam

Diazepam was found to be very stable in both freezer and refrigerator, with no significant change in the concentration being observed ($p > 0.05$). Approximately 95% and 87% of drug was recovered from DBS samples after 1 year period of storage in freezer and refrigerator respectively. At room temperature a significant decrease in concentration was observed at the end of study which was approximately 20 % of the initial concentration ($p < 0.05$). After 1 year of storage at room temperature 80% of diazepam could be detected in DBS samples. Stability data are given in Table 6-12.

Table 6-12: Stability of diazepam in dried blood spots stored in filter paper

Time (day)	Storage condition		
	Freezer (-20 °C) (ng/spot)	Refrigerator (4 °C) (ng/spot)	Room temperature (ng/spot)
Day zero#	100 (7.0)	100 (7.0)	100 (7.0)
2	100 (0.9)	98 (1.2)	98 (6.5)
4	99 (2.4)	98 (0.9)	96 (5.1)
7	99 (0.8)	98 (1.3)	96 (4.7)
14	98 (3.7)	98 (3.9)	96 (2.8)
30	98 (4.6)	96 (6.9)	88 (1.1)
60	99 (8.2)	97 (3.8)	90 (3.7)
90	96 (2.1)	94 (4.0)	89 (5.5)
180	98 (1.3)	93 (9.5)	86 (7.2)
365	95 (4.4)	87 (12.2)	80 (2.3)

Day zero concentration is mean of 10 measurements (RSD %). All other days concentration is average of two replicate measurements (RSD %). (Concentration threshold=85).

6.4.2.8 Summary of stability of cocaine and benzodiazepines in dried blood spot (DBS) for 1 year under different storage temperatures

The stability of cocaine and benzodiazepines in DBS for 1 year under different storage conditions is summarized in Table 6-13.

Table 6-13: Summary of the stability of cocaine and benzodiazepines in DBS stored under different storage conditions for 1 year

Drug	Storage conditions	Results	Comment
Cocaine	-20 °C, 1 year	40% decrease	• All drugs can be detected after 1 year regardless of storage temperature • As temperature increased, stability decreased • Benzodiazepines more stable than cocaine • More than 15% of cocaine and 74% benzodiazepines can be recovered after 1 year regardless of the storage temperature
	4 °C, 1 year	64% decrease	
	25 °C, 1 year	86% decrease	
Flunitrazepam	-20 °C, 1 year	Stable	
	4 °C, 1 year		
	25 °C, 1 year	24% decrease	
Temazepam	-20 °C, 1 year	Stable	
	4 °C, 1 year		
	25 °C, 1 year	18% decrease	
Oxazepam	-20 °C, 1 year	Stable	
	4 °C, 1 year		
	25 °C, 1 year	25% decrease	
Lorazepam	-20 °C, 1 year	Stable	
	4 °C, 1 year		
	25 °C, 1 year	16% decrease	
Nitrazepam	-20 °C, 1 year	Stable	
	4 °C, 1 year		
	25 °C, 1 year	27% decrease	
Diazepam	-20 °C, 1 year	Stable	
	4 °C, 1 year		
	25 °C, 1 year	20% decrease	

6.5 Discussion

As mentioned previously, dried blood spots stored on paper, such as the Guthrie card, have been used since 1960 for therapeutic drug monitoring, diagnostic screening and to reduce drug decomposition.[157,91-97,158-172] The residual drug amounts in DBS for a 1-year period for the present study are shown in Figure 6-4, which compare favourably with stabilities in liquid aliquot samples.

The stability of benzodiazepines under different storage conditions has been studied and published reports indicate that they are of low stability when stored at ambient temperature or higher. Most benzodiazepines other than triazolobenzodiazepines contain an amide group, which facilitates hydrolysis reactions to produce aminobenzophenone derivatives, whereas the presence of a triazolo ring in the structure makes the compounds more resistant to hydrolysis. Hydrolysis is a chemical reaction that uses water to split an amide or ester bond.[173] In the benzodiazepine stability study described in Chapter 4, oxazepam in liquid aliquot samples was found to be stable in the freezer for 1 year and six months at low and high concentration respectively whereas in DBS it was very stable for one year. In the refrigerator it was stable for 1 year and 2 month at low and high concentration respectively while in DBS it was stable for 1 year without any significant loss and the drug remaining in DBS was more than in low and high concentration aliquot samples. After one year of storage at room temperature oxazepam completely disappeared from liquid samples while approximately 75% was recovered from DBS.

Lorazepam aliquot samples containing low and high concentrations of the drug were stable for 1 year and 6 months respectively when stored in freezer and for 3 months and 2 months in the refrigerator while in DBS it was stable for 1 year without any significant loss observed. At room temperature lorazepam degradation is slower than in liquid samples. After 1 year lorazepam had completely disappeared from liquid samples. However, more than 84% of lorazepam was recovered from DBS samples stored at room temperature. These results prove the value and advantages of filter paper in preserving benzodiazepines, which was superior than the usual preservatives for liquid samples, which showed little effect on stability, as described in Chapter 4 and shown in table 6-14. Previous studies of diazepam and temazepam found that

these drugs are not stable when stored at 4 °C and 25 °C. However, DBS showed enhanced stability of these drugs at the same storage temperatures. Nitrobenzodiazepine compounds such as flunitrazepam and nitrazepam showed poor stability in liquid samples as a result of reduction and hydrolysis degradation reactions whereas in DBS reasonable amounts of the compounds were detected after 1 year even when stored at room temperature.[46]

Cocaine is a widely abused CNS stimulant frequently detected in forensic cases. Cocaine is known to be unstable in blood samples and is rapidly degraded by enzymatic and chemical hydrolysis. The addition of a preservative with cholinesterase inhibitor activity can decrease enzymatic hydrolysis but does not stop chemical degradation from proceeding. Breakdown of cocaine in biological samples is attributed to the presence of the ester linkages in the structure. In the refrigerator, cocaine disappeared completely after 1 year of storage in tubes containing sodium fluoride and potassium oxalate. Acidification of the sample by substituting potassium oxalate with oxalic acid enhanced the stability by 24%. Further addition of cholinesterase inhibitor to the sample improved the stability by 40%. However, in the current study the loss of cocaine at the same storage temperature was reduced to approximately 60% of the initial concentration similar to effect of the addition of 10 mg echothiophate, a known cholinesterase inhibitor, to the sample. Addition of EDTA, heparin and sodium fluoride to samples containing cocaine showed no improvement.[62] In this study more than 77% of cocaine was recovered from DBS after one month of storage at three storage temperatures, which is in agreement with the results reported by Skopp et al.[93] who found that more than 75% could be detected after 17 days of storage in filter paper. In DBS cocaine can be detected after 1 year of storage at all three storage temperatures.

The low stability of cocaine compared to benzodiazepines is because it has two ester groups in its structure, making the compound more susceptible to degradation by hydrolysis. Also, compounds containing an ester are more readily decomposed by hydrolysis compared to those containing an amide group, because oxygen is more electronegative than nitrogen, which accelerates the hydrolysis of the compound.

Dehydration of the samples on the filter paper minimized the hydrolysis of drugs which are liable to this type of degradation and so the stability of the drugs in DBS was enhanced compared to liquid blood samples. DBS technique can be used to quantify the drugs from blood spot even if the volume of blood is unknown. The influence of storage temperature on the stability of drugs in DBS, as shown in Figure 6-4, was much less than when the drugs are stored in whole blood specimens.

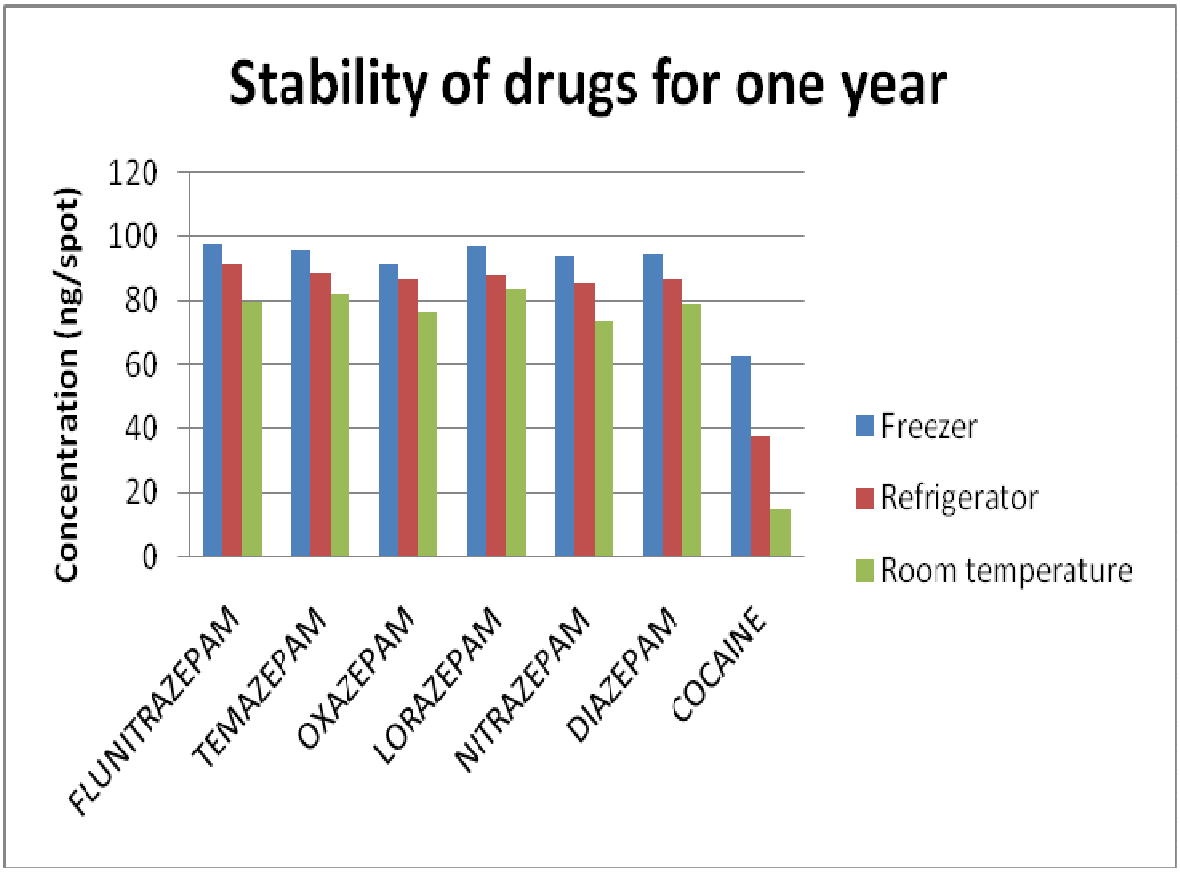


Figure 6-4: Stability of benzodiazepines and cocaine in DBS for one year. Initial concentrations were 100 ng/spot.

Table 6-14: Comparison of stability of lorazepam and oxazepam in liquid blood and DBS for 1 year under different storage temperatures

Drug	Storage conditions	Results	
		Liquid blood sample	DBS
Lorazepam	-20 °C, 1 year	5% decrease at HC	Stable
	4 °C, 1 year	50% and 28% decrease at LC and HC, respectively	Stable
	25 °C, 1 year	100% decrease after 6 months and 100% decrease at HC after 1 year	16% decrease
Oxazepam	-20 °C, 1 year	19% decrease at HC	Stable
	4 °C, 1 year	31% and 26% decrease at LC and HC, respectively	Stable
	25 °C, 1 year	100% decrease at LC after 6 months and 100% decrease at HC after 1 year	25% decrease

LC: Low concentration, HC: High concentration.

6.6 Conclusion

A validated method has been developed which can be used for the quantitative analysis of drugs in small volumes of blood contained in dry blood spots. The DBS technique provides a suitable procedure for the storage and analysis of samples in forensic toxicology because they are easy to handle, transport and to store in the laboratory, even in the absence of refrigeration, which can be a problem in some countries. It permits the analysis of a small volume of sample and as a result frequent collection of blood samples from donors is possible. In addition, the test analytes chosen for this study were drugs which are known to be rapidly degraded in biological samples and these were preserved when stored on paper, instead of adding the usual preservatives and anticoagulants. For some drugs the addition of preservatives is not recommended as they may accelerate the degradation of these drugs, for example organophosphorus pesticides, while anticoagulants are not recommended for other drugs as they can increase their concentrations, for example morphine. The DBS technique is therefore recommended as an additional procedure to be used in conjunction with conventional methods for preserving unstable drugs from decomposition and for avoiding potential errors in interpretation of analytical results resulting from the addition or absence of preservatives and anticoagulants.

The water present in blood samples plays a very important role in hydrolysis reactions as an active reagent which cleaves drug molecules. The use of filter paper to store samples resulted in enhancement of drug stability due to the dehydration of the sample on the paper and consequent minimalisation of enzymatic and chemical hydrolysis of the drugs.

7 Correction for Loss of Benzodiazepines During Storage

7.1 Introduction: degradation of drugs

Degradation refers to the chemical breakdown of a parent compound or starting material to produce new molecules. Chemical degradation is different from metabolism, which takes place during life, but may take place at the same time as metabolism. Equally, post-mortem changes can also be caused if samples are contaminated by microbiological oragnisms. Chemical degradation of drugs in a sample may occur as a result of three main types of reaction: reduction, oxidation, and hydrolysis. Since benzodiazepines contain an amide group in their structures, it is susceptible to decomposition through a hydrolysis reaction, which is considered to be the most common degradation process in this group. Degradation of benzodiazepines in aqueous media yields benzophenone derivatives and glycine. Many authors have studied the degradation of selected compounds.

For benzodiazepines, Nudelman and Waisbaun[174,175] studied the acidic degradation of diazepam in an aqueous methanol solution, which produced new products through hydrolytic cleavage of the benzodiazepinone ring, such as 2-methylamine-5-chlorobenzophenone and glycine. Furthermore, they also studied the kinetics of the reaction of 2-amino-5-chlorobenzophenone with hydrochloric acid in aqueous methanol and found that different products were obtained at different acid concentrations.

Cabrera et al.[176] investigated the mechanisms of alprazolam and diazepam hydrolysis. They found that diazepam is highly sensitive to hydrolysis and that the ring can be opened by cleavage of the amide C-N bond. In contrast, alprazolam showed high stability against acid and base hydrolysis. This stability of alprazolam was attributed to the triazole ring, which seems to inhibit the opening of the ring by hydrolysis.

Identification of degradation products has been reported in previous studies. For example, diazepam and temazepam produce 2-methylamino-5-

chlorobenzophenone (MACB), while chlordiazepoxide and oxazepam produce 2-amino-5-chlorobenzophenone (ACB). The nitrazepam hydrolysis product is 2-amino-5-nitobenzophenone (ANB), while hydrolysis of prazepam will produce N-cyclopropylmethyl-2-amino-5-chlorobenzophenone (CMCB). Flunitrazepam degradation produces 2'-fluoro-2-methylamino-5-nitobenzophenone (MNFB), while bromazepam and lorazepam degradation produce (2-amino-5-bromophenyl)(2-pyridyl) methanone (ABP) and 2-amino-2',5-dichlorobenzophenone (ADB), respectively.[177,188]

Breton et al.[189] have reported instability of avizafone (pro-drug of diazepam), which degraded into unexpected new compounds—in particular diazepam, carbostyril, MACB, and ACB. These benzodiazepine degradation products were identified by chemical ionisation mass spectrometry.

Cocaine is known to be rapidly degraded enzymatically and chemically by hydrolysis reactions. Detection of degradation product in biological samples has been reported previously by Skop et al.[85], who reported a new method to quantify cocaine and its degradation products, BE, EME, and ecgoine. Ecgonine was found to be the final degradation product of cocaine and its metabolites.

Sulphonamides are antimicrobial agents used in agriculture, animal husbandry, and human medicine to treat many kinds of infection by bacteria and other microorganisms. Sulfamethoxazole is one of the most common sulphonamides. Sulfamethoxazole and its degradation product N^4-acetylsulfamethoxazole were detected in effluent samples collected from wastewater treatment plants (WWTPs) and in final effluent samples from five different WWTPs in the UK. Chloramine-T (N-sodium-N-chloro-p-toluenesulfonamide) used in drug treatment of different diseases of swine and poultry was found to degrade into p-toluenesulfonamide and o-toluenesulfonamide in wastewater and these were detected in WWTPs and in groundwater below a former sewage farm.[190]

The natural (17β-estradiol, E2) and synthetic (17α-ethinylestradiol, EE2) estrogens are among the most potent endocrine-disrupting chemicals found in the environment. The biological treatment of these compounds in WWTPs in order to reduce estrogenicity of the effluent prior to discharge into the environment is very important. Different methods were used in the treatment,

which produced different degradation products, and was observed in different WWTPs that used different treatments to remove estrogen. Identification of these products was reported; however, knowledge about their estrogenicity is very important in order to optimize the method that can remove estroginicity efficiently before discharging it to the environment.[191]

Therefore, it is important both in therapeutics and in toxicology to detect the degradation products of drugs and other compounds. In forensic toxicology, it is important to detect the decomposition products as markers of drugs and poisons especially unstable substances, which can indicate the earlier presence of the drugs in cases in which samples were not properly stored or else were collected a long time after death.

The aims of this study were to identify the degradation products of some benzodiazepine drugs and to develop a system for correcting for loss of benzodiazepines during storage by analysing their degradation products in blood and urine using SPE and LC-MS/MS. An additional aim was to assess the degradation of benzodiazepines when stored under unfavourable conditions for 1 month. Initially, a method for the analysis of benzodiazepines and their degradation products in blood and urine needed to be developed and optimised prior to full validation of the method.

7.2 Experimental Section

7.2.1 Chemicals and Materials

Diazepam, temazepam, oxazepam, and chlordiazepoxide and their corresponding deuterated internal standards (diazepam-d5, temazepam-d5, and oxazepam-d5) were purchased from LGC-Promochem (Middlesex, UK) as 100 µg/ml solutions in methanol. Aminobenzophenone (AB), 2-amino-5-chloro aminobenzophenone (ACB), 2-methylamino-5-chloro aminobenzophenone (MACB) ammonium formate, ammonium acetate, and β-glucuronidase crude solution were obtained from Sigma-Aldrich (Dorset, UK). Stock solutions of standards and internal standards were stored at -20 ºC. Acetonitrile, methanol, cyclohexane, ethyl acetate, ammonium hydroxide, formic acid, isopropanol, dichloromethane, and potassium dihydrogen phosphate were obtained from BDH (Poole, UK). Clean Screen ZSDAU020 cartridges were purchased from United Chemical Technology UK.

7.2.1 Solutions

7.2.1.1 Preparation of 0.1 M Phosphate Buffer pH 6.0

1 litre of solution was prepared by dissolving 1.7 g of anhydrous disodium orthophosphate and 12.14 g of sodium dihydrogen orthophosphate monohydrate in 800 ml of deionised water. The pH was adjusted to 6 with 1 M potassium hydroxide and the solution made up to volume with deionised water.

7.2.1.2 Preparation of 0.1 M Acetate Buffer pH 4.5

2.93 g of sodium acetate trihydrate was weighed into 400 ml of deionised water in a 500 ml volumetric flask, and then 1.62 ml of glacial acetic acid was added to the mixture. The pH was adjusted to 4.5 with 0.1 M acetic acid. The solution was made up to volume with deionised water.

7.2.1.3 Preparation of 3mM Ammonium Formate and 0.001%, 0.002%, 0.004% and 0.01% Formic Acid Solution

These mobile phases were prepared by dissolving 0.189 g of ammonium formate in 5 x 1 L volumetric flasks filled with 800 ml deionized water followed by the addition of 10, 20, 40, or 100 µl of concentrated formic acid respectively and making up to one litre with deionized water.

7.2.1.4 Preparation of 5mM Ammonium Acetate

38.54 g of ammonium acetate was weighed out into a 500 ml volumetric flask and made up to volume with deionised water to prepare 1 M solution of ammonium acetate. 5 ml of this solution was added to a 1 litre volumetric flask and 800 ml of deionised water was added. Ammonia was used to adjust the pH to 4.7 and made up to volume with deionised water, resulting in 5mM of solution.

7.2.1.5 Standard Solutions of Analytes

Working standards of unlabelled and labelled drugs were prepared by diluting 250 µl from each drug stock solution in 25ml methanol to obtain a concentration of 1 µg/ml. Stock solutions of standards and internal standards were stored in a freezer at -20 ºC.

7.3 Optimisation of Analytical Method for Diazepam, Temazepam, and MACB

7.3.1 Optimization of Mobile Phase

7.3.1.1 Optimum tuning parameters for diazepam, temazepam and MACB

In order to obtain good mass spectrometry conditions, solutions of standards 1 µg/ml were introduced into the mass spectrometry interface using a syringe pump at 5 µl/minutes. The capillary temperature, sheath and auxiliary gas flow rates and collision energy were optimized for each analyte. The compounds were analysed with MS parameters slected in table 7-1. Inernal standard data was aquired in selected ion monitoring (SIM) mode and analytes were identified on the basis of their full MS-MS spectra. Two product ions were monitored. The underlined ions in Table 7-1 were used as the quantification ions because they were the most intense ions produced.

Table 7-1: Optimum tuning parameters for diazepam, temazepam and MACB.

Drug	Precursor ion (m/z)	Monitored ions* (m/z)	Collision energy (ev)
Diazepam	285	257, 222	40
Temazepam	301	283, 255	28
MACB	246	228, 168	28
Internal standards			
Diazepam-d5	300	300	Not used
Temazepam-d5	306	306	Not used

- Underlined ion was used as the quantification ion. For internal standards, only the pseudomolecular ions were monitored and no collision energies were specified.

7.3.1.2 Comparison of 5mM Ammonium Acetate and 3mM Ammonium Formate Mobile Phases

In a previous study, Breton et al. used 5mM ammonium acetate and trifluroacetic acid as the mobile phase to analyse avizafone and its degradation products including diazepam and MACB; diazepam was well detected while MACB showed poor detection .[189] 3mm ammonium formate was used as the mobile phase for analysing benzodiazepines in many published papers.[120,125] Therefore, this experiment was devised to investigate the ionization of these compounds in LC-MS/MS by comparing 5 mM ammonium acetate and 3mm ammonium formtate as mobile phases. An unextracted standard was used, composed of 100 µl of working solution and 100 µl of internal standard mixture, which was evaporated under a stream of nitrogen and reconstituted in 200 µl of mobile phase: 20 µl was injected 5 times. An average peak area was calculated for each analyte under each condition. The results are shown in Table 7-2.

Table 7-2: Comparison of effect of 5mM ammonium acetate and 3mM ammonium formate on analyte response.

Compound	Average peak area	
	5mM ammonium acetate	3mM ammonium formate
Diazepam	81,436,949	504,035,879
Diazepam-d5	147,335,965	2,616,514,447
Temazepam	111,024,296	848,728,838
Temazepam-d5	156,902,245	2,779,937,122
MACB	3,352,629	24,533,361

It is clear from the results demonstrated in Table 7-2 that 3mM ammonium formate gives a high response for diazepam, temazepam, MACB and internal standards compared to 5mM ammonium acetate; thus, it was used as the mobile phase in this study. Further investigation using different concentrations of formic acid in addition to 3mM ammonium formate may improve the response of the analytes.

7.3.1.3 Comparison between 0.001%, 0.002%, 0.004% and 0.01% of Formic Acid in 3mM Ammonium Formate Solution

Formic acid was added routinely to the mobile phase at different concentrations, especially if the analyte was basic; therefore, this experiment was carried out to investigate whether increasing the formic acid concentration could increase the response for these analytes and to identify the best concentration to give maximum sensitivity for analysis of the analytes. Four mobile phases were prepared containing different concentrations of formic acid. 0.001%, 0.002%, 0.004%, and 0.01% in 3mM ammonium formate solution. An unextracted standard was used, composed of 100 µl of working solution, and 100 µl of internal standard mixture were blown down under nitrogen. Then it was reconstituted in 200 µl of mobile phase, and 20 µl was injected 5 times. An average peak area was calculated for each analyte under each condition as shown in Table 7-3. A previous study reported that an increase in the response of the analyte was obtained after the addition of formic acid to the mobile phase; however, adding high concentrations of formic acid resulted in a decrease in the response. Therefore, the concentrations investigated in this experiment ranged from 0.001% to 0.01% of formic acid.

Table 7-3: Effect of formic acid on analyte response

Compound	Average peak area			
	3mM ammonium formate			
	0.001% F.A	0.002% F.A	0.004% F.A	0.01% F.A
Diazepam	590,778,121	698,968,355	819,240,270	767,030,169
Diazepam-d5	2,761,448,341	2,804,888,014	2,887,214,387	2,788,373,228
Temazepam	1,164,515,006	1,390,582,615	1,408,164,021	1,429,463,318
Temazepam-d5	3,018,607,830	3,152,031,037	3,339,199,932	3,304,103,181
MACB	66,000,325	94,113,152	147,862,546	120,441,274

As shown in Table 7-3, the addition of 0.004% formic acid to 3mM ammonium formate solution gave a higher response for MACB, diazepam, and dueterated standards while 0.01% of formic acid was better for temazepam only. However, its response with 0.004% of formic acid was still good and better than a low concentration of formic acid. Therefore, a mobile phase containing 3mM ammonium formate and 0.004% as buffer was used to analyse these compounds by LC-MS/MS.

7.3.2 Optimisation of Extraction Method

The extraction method used to extract benzodiazepines described in Chapter 4 and as summarized in Table 7-4 was used here to extract diazepam and temazepam and their degradation product MACB from blood.[125]

Table 7-4: SPE method for extraction of benzodiazepines from blood

Step	Solvent
Conditioning	3ml methanol, 3ml DI water, 2ml phosphate buffer pH 6
Washing	2ml DI water, 2ml phosphate buffer pH6/acetonitrile (80:20 v/v), 2ml cyclohexane then dry for 5 minutes, 2ml DI water then dry for 5 minutes
Elution	3ml ethyl acetate/NH_4OH (98:2)

An unextracted standard containing 50 ng of working solution was prepared in duplicate. An extract of 50 ng of these compounds from blood was also prepared in duplicate. After extraction and before evaopration, 50 ng of internal standard mixture was added to the extracted and unextracted standards to assess the recovery obtained using this method. The results are shown in Table 7-5.

Table 7-5: Average recoveries (%) of diazepam, temazepam, and MACB using SPE procedure

Compound	Average Recovery% (n=2)
Diazepam	90
Temazepam	88
MACB	16

As shown in Table 7-5, this method showed good recovery for diazepam and temazepam, but MACB recovery was not acceptable. It may be that the compound was lost during the washing step, was not attracted to the sorbent, or did not elute efficiently from the sorbent; thus, a series of studies were undertaken to modify this method in order to enhance the recovery of this compound.

7.3.2.1 Monitoring of Extraction Wash

The effects of cyclohexane and acetonitrile on the recovery were investigated in this method. Cyclohexane was used in the washing step to remove the interferences from the cartridge; instead of discarding the cyclohexane solvent, 50 ng of standard was extracted and the cyclohexane was collected and analysed to determine whether analyte was lost in this step. The chromatogram in Figure 7-1 shows that MACB was eluted by cyclohexane, whereas there was no elution of diazepam and temazepam.

Recoveries without cyclohexane and without cyclohexane and acetonitrile in the washing step were consequently investigated. Extracted and unextracted 50 ng standards of these compounds were prepared in duplicate. Before blow down, 50 ng of internal standard mixture were added to the samples before analysis.

Table 7-6: Average recoveries (%) of diazepam, temazepam, and MACB after modifying washing step

Compound	Recovery % (n=2)	
	No cyclohexane	No cyclohexane and acetonitrile
Diazepam	96	91
Temazepam	81	83
MACB	36	47

It is clear from Table 7-6 that after excluding cyclohexane and acetonitrile solvents from the washing step, the recovery of MACB increased with little effect on the diazepam and temazepam recoveries, so these solvents were excluded in the following experiments.

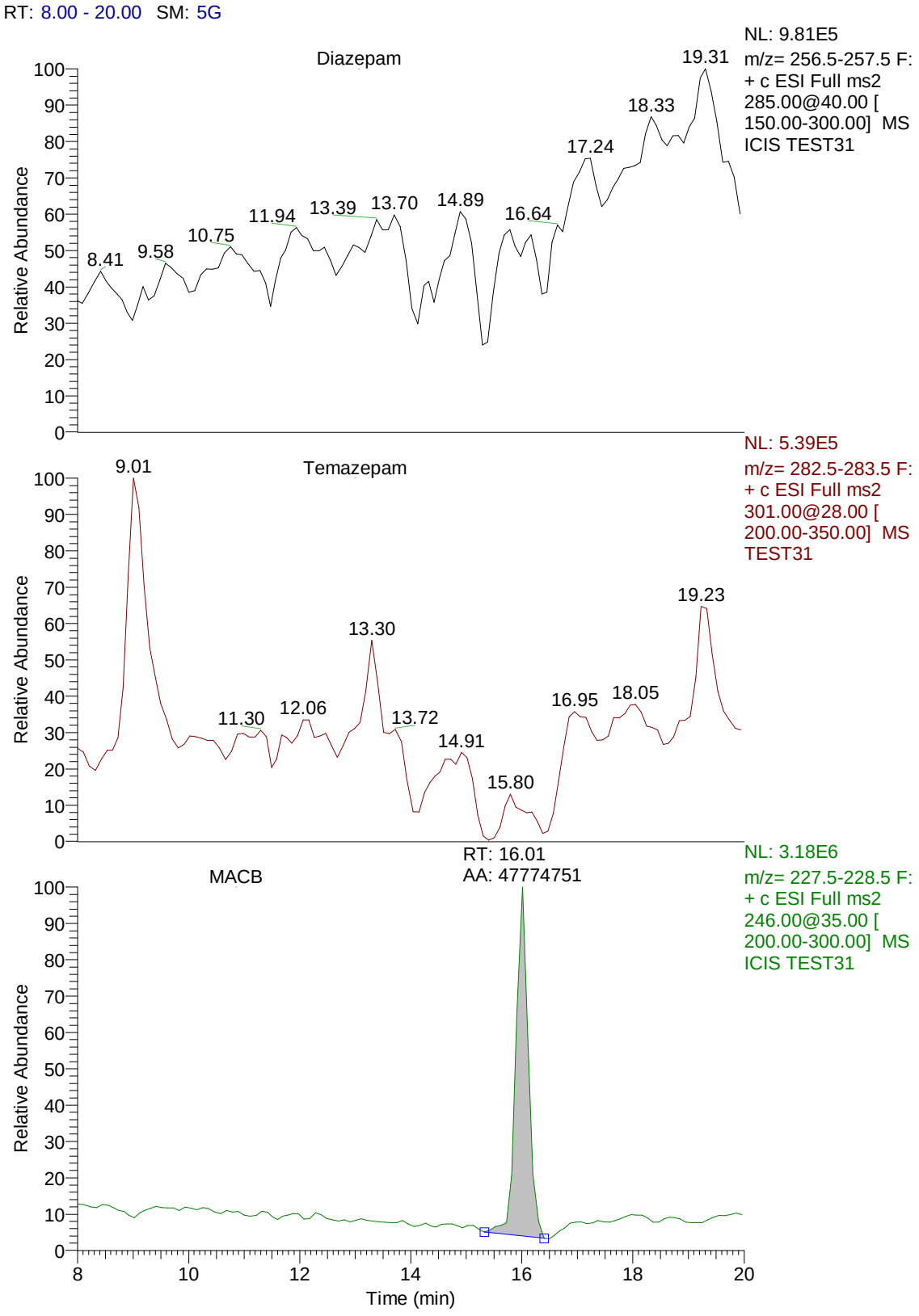


Figure 7-1: Analysis of cyclohexane wash by LC-MS/MS

7.3.2.2 Comparison of pH 6 and 4.5 as a Buffer

The use of a pH lower than pH 6 was also investigated to examine which buffer pH could provide high recovery. An standard mixture containing 50 ng of all analytes in blood was extracted in duplicate. Unextracted 50 ng standards of these compounds were also prepared in duplicate. Before evaporation under a stream of nitrogen, 50 ng of internal standard mixture was added to all prepared samples, extracted and unextracted. The results are shown in Table 7-7 below.

Table 7-7 : Average recoveries (%) of diazepam, temazepam, and MACB using buffers of different pH.

Compound	Average Recovery % (n=2)	
	pH 6	pH 4.5
Diazepam	92	94
Temazepam	87	90
MACB	45	59

Using the lower pH showed an improvement in the recoveries of all compounds. This may be explained because, as the pH of the buffer decreased, the ionization of the analytes increased, leading to enhanced ionic interactions between the analytes and the SPE sorbent. It was decided to use an acetate buffer with pH 4.5 in the subsequent experiment.

7.3.2.3 Comparison of Elution Solvents

Selection of elution solvent may have an influence on the recovery of analyte. Sometimes a change from one elution solvent to another shows an enhancement; Ethyl acetate-Ammonium hydroxide ETAc-NH_4OH (98:2 v/v) and Methylene chloride-Isopropanol-Ammonium hydroxide CH_2Cl_2-IPA-NH_4OH (78:20:2 v/v/v) were compared in this experiment to investigate which solvent gave the best recoveries. Unextracted and extracted standards containing 50 ng of these compounds were prepared in duplicate. Before evaporation under a stream of

nitrogen, 50 ng of internal standard was added to all samples, and the samples were analysed by LC-MS/MS. The results are shown below in Table 7-8.

Table 7-8: Average recoveries (%) of diazepam, temazepam, and MACB using different elution solvents

Compound	Mean Recovery % (n=2)	
	CH_2Cl_2-IPA-NH_4OH (78:20:2 v/v/v)	ETAc-NH_4OH (98:2 v/v)
Diazepam	103	95
Temazepam	107	92
MACB	84	62

Diazepam and temazepam recoveries remained relatively unaffected or slightly higher, while MACB showed improvement in the recovery when CH_2Cl_2-IPA-NH_4OH (78:20:2 v/v/v) was used as elution solvent compared to ETAc-NH_4OH (98:2 v/v). The energy of elution and the pH (12) of both elution solvents are the same; however, the difference in the recovery of MACB may be attributed to solubility differences of MACB in these elution solvents. Therefore, it was decided to use CH_2Cl_2-IPA-NH_4OH (78:20:2 v/v/v) to elute the compounds in SPE. The final procedures for the SPE method are listed in Table 7-15.

7.3.3 Validation of Analytical Method

The validation parameters applied in this study were similar to the parameters in Table 4-1 except that the low concentration in this method was 10 ng/ml. The results are shown in tables 7.9-7.14.

7.4 Validation Results

7.4.1 Validation Results in Blood

7.4.1.1 Linearity

The relationships between analyte concentrations and peak area ratios were linear over the range 5-200 ng/ml, and the linear correlation coefficients (r^2) were better than 0.99 for all analytes as shown in Table 7-9.

7.4.1.2 LOD and LLOQ

LOD values for all analytes were 0.10-2.30 ng/ml, and LLOQ values were 0.40-7.50 ng/ml. The results are shown in Table 7-9.

Table 7-9: Linearcorrelation coefficients, LODs, and LLOQs of drugs in blood

Analyte	Linear correlation coefficients (r^2)	LOD (ng/ml)	LLOQ (ng/ml)
Diazepam	0.999	0.20	0.70
Temazepam	0.994	0.10	0.40
MACB	0.998	2.00	6.80
Chlordiazepoxide	0.998	0.50	1.70
Oxazepam	0.999	0.46	1.50
ACB	0.992	2.30	7.50
AB	0.993	2.10	6.95

7.4.1.3 Recoveries

The recoveries for all analytes were in the range 70-102% as shown in Table 7-10.

Table 7-10: Recoveries of analytes from blood

Analyte	Mean Recovery, % (RSD%, n=5)		
	10ng/ml	50ng/ml	100ng/ml
Diazepam	102 (18)	101 (9)	94 (8)
Temazepam	84 (19)	90 (5)	93 (1)
MACB	86 (14)	90 (9)	96 (8)
Chlordiazepoxide	93 (10)	91 (7)	95 (5)
Oxazepam	92 (18)	97 (13)	94 (9)
ACB	83 (8)	84 (16)	92 (11)
AB	70 (13)	82 (11)	84 (6)

7.4.1.4 Intraday and Interday Precision

The method showed good precision, and the relative standard deviations (RSD %) for the intraday and interday precision were in the range of 1-18 and 2-18% respectively, which are considered to be acceptable. The results are shown in Table 7-11.

7-11: Interday and intraday precision results in blood

Analyte	Interday mean (RSD%, n=5)			Intraday mean (RSD%, n=5)		
	10ng/ml	50ng/ml	100ng/ml	10ng/ml	50ng/ml	100ng/ml
Diazepam	11 (17)	54 (5)	100 (6)	8 (10)	55 (6)	111 (6)
Temazepam	11 (15)	53 (5)	104 (4)	11 (4)	51 (6)	93 (5)
MACB	9.6 (18)	48 (7)	99 (8)	8 (18)	46 (17)	93 (14)
Chlordiazepoxide	9 (16)	52 (9)	103 (2)	9.8 (5)	50 (4)	102 (2)
Oxazepam	13 (17)	46 (3)	101 (2)	9 (13)	49 (4)	99 (3)
ACB	12 (18)	49 (6)	100 (5)	12 (5)	50 (4)	101 (1)
AB	12 (18)	53 (8)	97 (10)	13 (5)	51 (6)	97 (3)

7.4.2 Validation Results in Urine

7.4.2.1 Linearity

The relationships between analyte concentrations and peak area ratios were linear over the range 5-200 ng/ml, and the linear correlation coefficients (r^2) were better than 0.99 for all analytes as shown in Table 7-12.

7.4.2.2 LOD and LLOQ

LOD values for all analytes were 0.20-2.70 ng/ml, and LLOQ values were 0.78-9.10 ng/ml. The results are shown in Table 7-12.

Table 7-12: Linearity, LOD and LLOQ of drugs in urine

Analyte	Linearity r^2	LOD (ng/ml)	LLOQ (ng/ml)
Diazepam	0.996	0.20	0.78
Temazepam	0.998	0.30	1.00
MACB	0.99	1.35	4.50
Chlordiazepoxide	0.991	0.49	1.66
Oxazepam	0.99	0.29	0.99
ACB	0.994	2.70	9.10
AB	0.994	1.88	6.25

7.4.2.3 Recoveries

The recoveries for all analytes were in the range 74-103% as shown in Table 7-13.

Table 7-13: Recovery of analytes from urine

Analyte	Mean Recovery% (RSD%, n=5)		
	10ng/ml	50ng/ml	100ng/ml
Diazepam	91(15)	103 (6)	96 (8)
Temazepam	88(7)	89 (12)	103 (10)
MACB	84 (16)	86 (8)	97 (7)
Chlordiazepoxide	86 (15)	87(9)	95 (6)
Oxazepam	94(16)	95 (14)	95 (13)
ACB	78 (17)	94 (4)	96 (6)
AB	74(16)	90 (1)	91 (3)

7.4.2.4 Intraday and Interday Precision

The method showed good precision, and the relative standard deviations (RSD %) for the intraday and interday precision were in the range of 1-15 and 2-17% respectively, which are considered to be acceptable. The results are shown in Table 7-14.

7-14: Interday and intraday precision results in urine

Analyte	Interday mean (RSD%, n=5)			Intraday mean (RSD%, n=5)		
	10ng/ml	50ng/ml	100ng/ml	10ng/ml	50ng/ml	100ng/ml
Diazepam	10 (10)	54 (8)	97 (3)	11 (5)	50 (5)	99 (2)
Temazepam	9 (13)	54 (7)	100 (5)	10 (5)	49 (6)	101 (4)
MACB	12 (15)	45 (6)	100 (2)	13 (14)	51 (5)	101 (2)
Chlordiazepoxide	11 (14)	49 (11)	91 (4)	11 (6)	46 (6)	104 (4)
Oxazepam	11 (16)	44 (9)	97 (5)	12 (15)	47 (7)	96 (2)
ACB	10 (17)	45 (16)	94 (2)	13 (9)	48 (10)	95 (5)
AB	10 (13)	53 (11)	101 (10)	11 (10)	52 (8)	100 (1)

7.5 Degradation Study: Diazepam and Temazepam

The aim of this study was to measure the rate of degradation of diazepam and temazepam in stored blood and urine samples and the rate of formation of their hydrolysis product MACB (2-methylamino-5-chlorobenzophenone). With these results an assessment would then be made of a method for correcting for losses based on the concentration of MACB. With this objective in mind, conditions of storage were selected in order to result in degradation of the drugs in a short timescale.

7.5.1 Introduction

7.5.2 Stability Study in Blood and Urine

Packed red blood cells were obtained from the Scottish National Blood Transfusion Service and were suspended in an equal volume of isotonic saline. This was used as blank blood. Blank blood (120 ml) was divided into two 60 ml portions, A and B, which were spiked with diazepam and temazepam respectively to give final concentrations of 1000 ng/ml. Each portion was divided into three aliquots of 20 ml and the drugs were subjected to accelerated decomposition for one month under three different storage conditions. The first condition was at high temperature in the oven at 80 °C, the second was in acidic conditions (pH 2) and the third was in alkaline conditions (pH 12). The times selected for analysis were at days 1 (day zero), 2, 4, 7, 14, and 30.

A similar study was carried out using drug-free urine obtained from a healthy volunteer.

7.5.3 Extraction Procedure

Prior to extraction, 1ml of blood or urine was added to 3.5 ml of acetate buffer pH 4.5, and then 100 μl of internal standard mixture was added. This was vortex mixed for 2 minutes then centrifuged for 5 minutes at 2500 rpm. The supernatant was loaded onto conditioned CleanScreen® ZSDAU020 cartridges. The SPE procedure used is listed in Table 7-15.

Table 7-15: SPE extraction procedure

Step	Solvent
Conditioning	3ml methanol, 3ml DI water, 2ml acetate buffer pH 4.5
Washing	2ml DI water, 2ml acetate buffer, 2ml DI water
Dry for 5 minutes	
Elution	3ml CH_2Cl_2-IPA-NH_4OH (78:20:2 v/v/v)

For urine case samples in section 7.8.2, the same SPE method was used; however, before that, 1ml of urine sample was added to a tube containing 2ml of acetate buffer pH 4.5, 100 μl of internal standard mixture, and 10 μl of β-glucuronidase solution, mixed vortex then incubated for 3 hours in an oven at 40 °C in order to cleave the glucuronic acid moiety from drugs to detect the parent compounds.

7.5.4 Instrumentation

LC-MS/MS analysis was performed using a Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San Jose, CA, USA) equipped with a Surveyor HPLC system. Chromatographic separation was achieved using a Gemini™ C18 column (150 mm x 2 mm ID, 5μm particle size) fitted with a guard column (4 mm x 2 mm) with the same packing material. The mobile phase was a mixture of 3mM ammonium formate solution containing 0.004% formic acid and acetonitrile starting at 65:35 v:v and changing to 10:90 v:v between 13 and 13.5 minutes. It was then held at 10:90 v:v between 13.5 and 16.5 minutes and then reset to the staring composition between 16.5 and 20 minutes.

7.5.5 Results of Diazepam and Temazepam Degradation

7.5.5.1 Diazepam

Diazepam started to decompose from the first day of incubation in the oven at 80 °C, while MACB started to be detected after 4 days of degradation. After 1 month, 30% of the original concentration of diazepam had been lost, whereas MACB increased from 17 to 98 ng/ml between day 4 and the end of the study, corresponding to 1.7% to 10% of the original amount of diazepam. In urine under the same conditions, diazepam degraded faster than in blood and completely disappeared after one month, whereas MACB gradually increased after 4 days of incubation and still was detected at 192 ng/ml (20% of the original amount of diazepam) after one month of observation.

At pH 2, diazepam decreased gradually, while MACB appeared after one week of storage. After 1 month, 25% of the initial concentration of diazepam was lost, while MACB reached 91 ng/ml (9% of the original amount of diazepam). For urine under the same conditions, diazepam continuously decreased from the first day until it was not detected after one month, whereas MACB appeared in day 2 and increased to reach 254 ng/ml (27% of the original amount of diazepam) after one month.

Under basic conditions at pH 12, diazepam decomposed in blood more quickly than in the other two conditions and lost approximately 50% of its initial concentration by day 30, whereas MACB increased from day 2 through day 30 to reach 167 ng/ml (17% of the original amount of diazepam). In urine, diazepam was not detected after 2 weeks, while MACB was detected on day 2 and gradually increased, then decreased after 1 week from 217 ng/ml to 95 ng/ml (23% and 10% of the original amount of diazepam, respectively).

These results are shown graphically in Figures 7-2 to 7-7, which also shows graphs of the sum of diazepam and MACB during the 30-day period of the study.

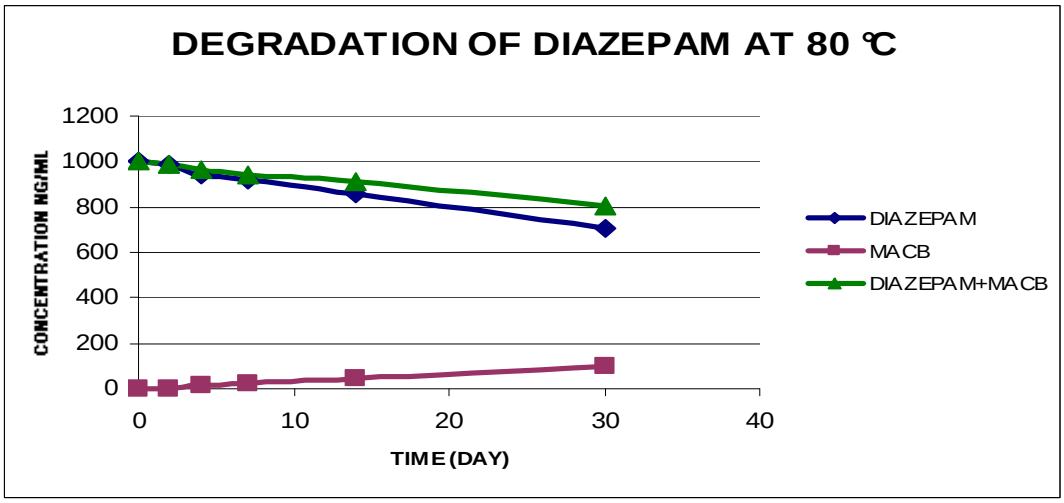


Figure 7-2: Degradation of diazepam in blood at 80 °C

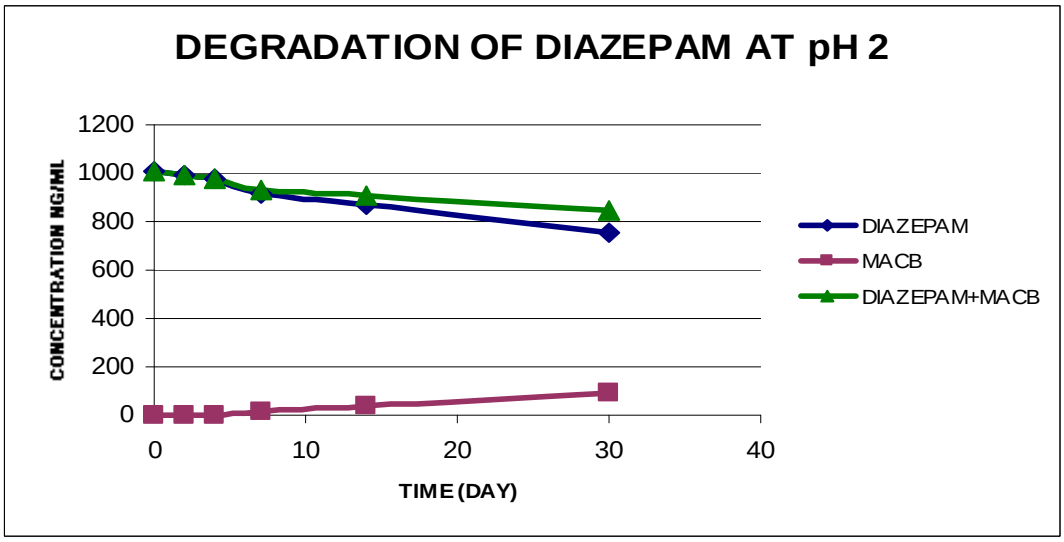


Figure 7-3: Degradation of diazepam in blood at pH 2

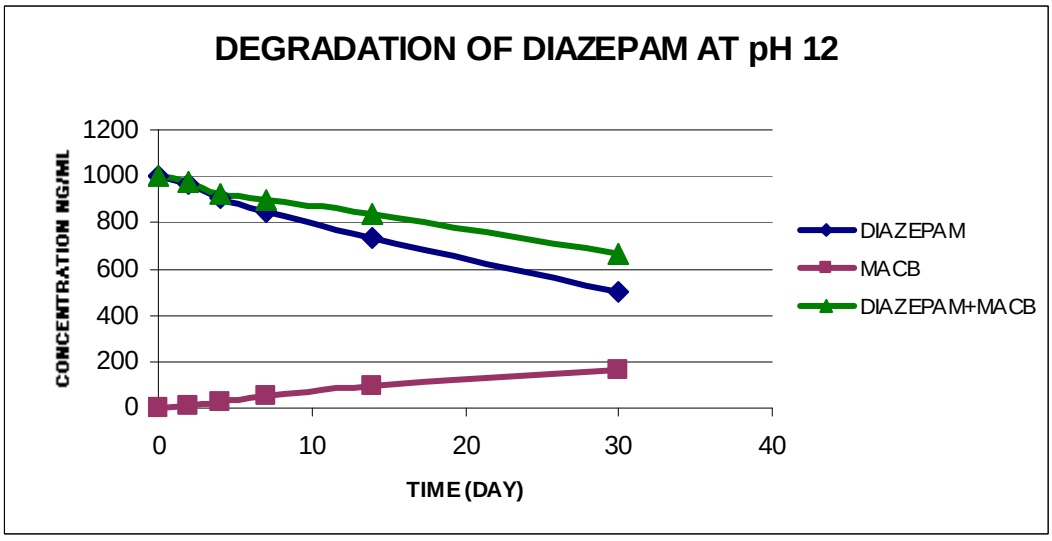


Figure 7-4: Degradation of diazepam in blood at pH 12

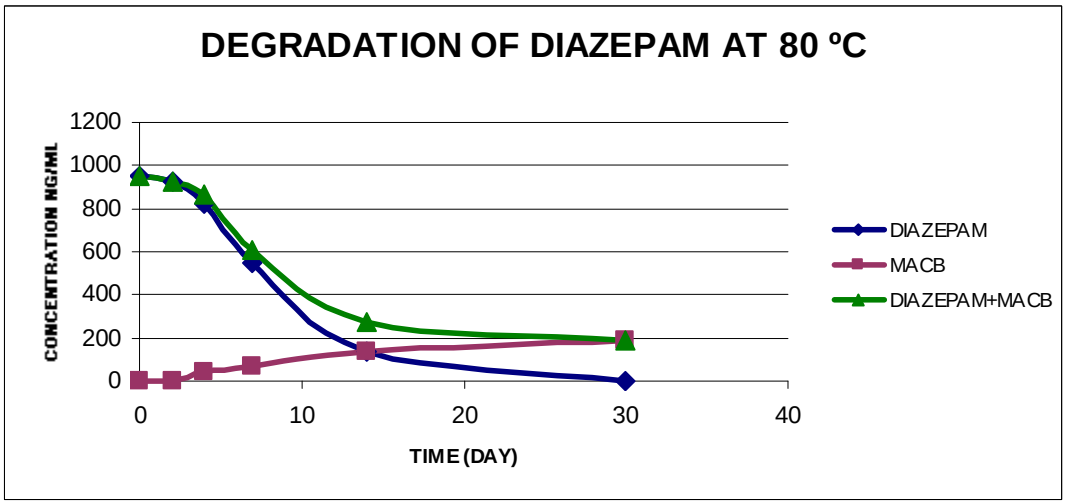


Figure 7-5: Degradation of diazepam in urine at 80 °C

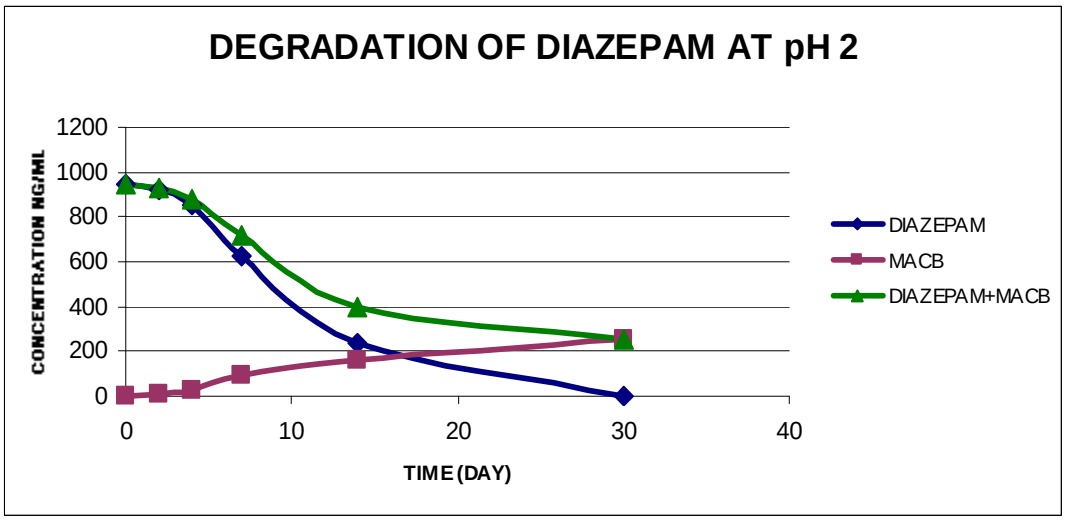


Figure 7-6: Degradation of diazepam in urine at pH 2

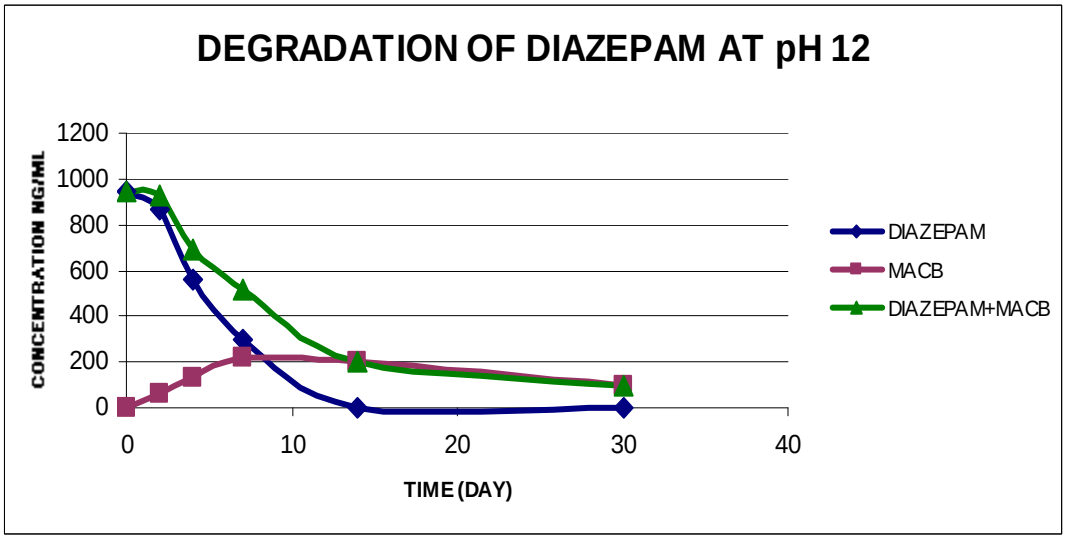


Figure 7-7: Degradation of diazepam in urine at pH 12

7.5.5.2 Temazepam

At 80 °C, the temazepam concentration decreased gradually from day zero until it was not detected after 1 month of incubation, while MACB was detected after 1 day and its concentration increased to 317 ng/ml (32% of the original amount of temazepam) after 2 weeks, then dropped to 149 ng/ml (15% of the original amount of temazepam) after 1 month. In urine, temazepam decomposed very quickly and was not detected after one month of incubation, while MCB appeared after 1 day and increased gradually to the highest concentration after 1 week at 193 ng/ml (21% of the original amount of temazepam), then dropped to 29 ng/ml (3.1% of the original amount of temazepam) after 2 weeks and was not detected after 1 month.

In acid conditions at pH 2, temazepam lost more than 75% of its original concentration in the blood, while MACB increased gradually from day 2 to day 30 to reach 272 ng/ml (27% of the original amount of temazepam). In the urine, it decomposed much more quickly than in blood and completely disappeared after one month, while MACB was detected on day 2 and continued to increase to 154 ngl/ml (17% of the original amount of temazepam), then dropped to 65 ng/ml (7% of the original amount of temazepam) after 2 weeks and disappeared after 1 month.

In basic conditions at pH 12, temazepam concentration decreased gradually and was not detected after 2 weeks, whereas MACB was detected after 1 day and reached its highest concentration at day 4; then it decreased gradually from 191 ngl/ml to 25 ng/ml after one month (from 19 to 3% of the original amount of temazepam). Temazepam in urine decreased more quickly and was not detected after 2 weeks, while MACB was detected after 1 day and increased to 274 ng/ml (29% of the original amount of temazepam) on day 4, then decreased gradually to 22 ngl/ml (2.4% of the original amount of temazepam) after 2 weeks and decomposed completely after 1 month. These results are shown graphically in Figures 7-8 to 7-13, which also shows graphs of the sum of temazepam and MACB during the 30-day period of the study.

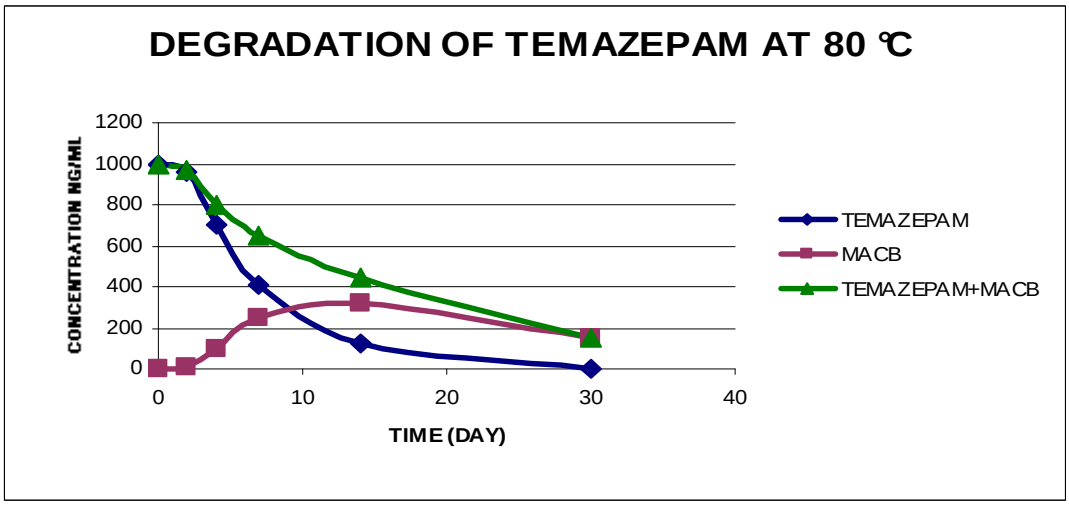


Figure 7-8: Degradation of temazepam in blood at 80 ℃

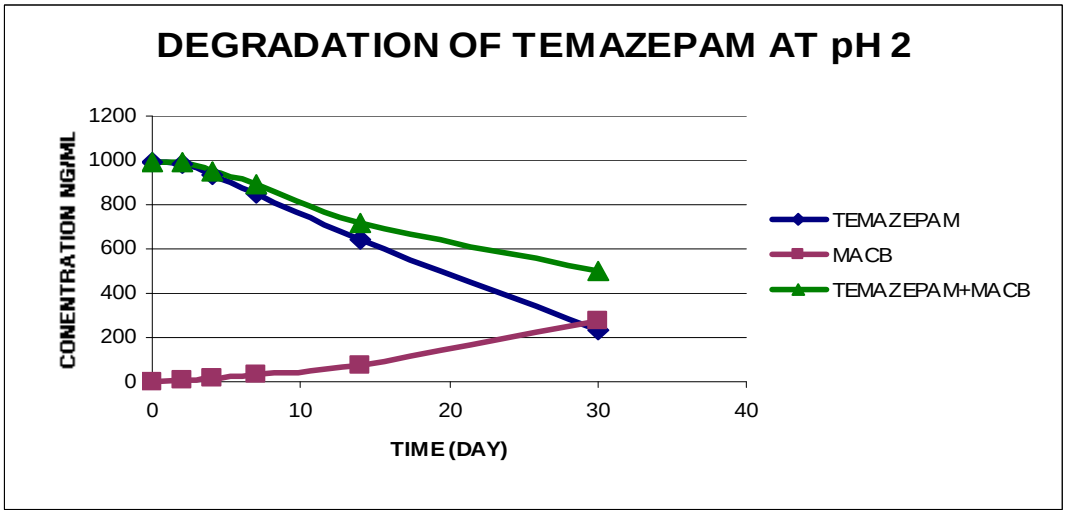


Figure 7-9: Degradation of temazepam in blood at pH 2

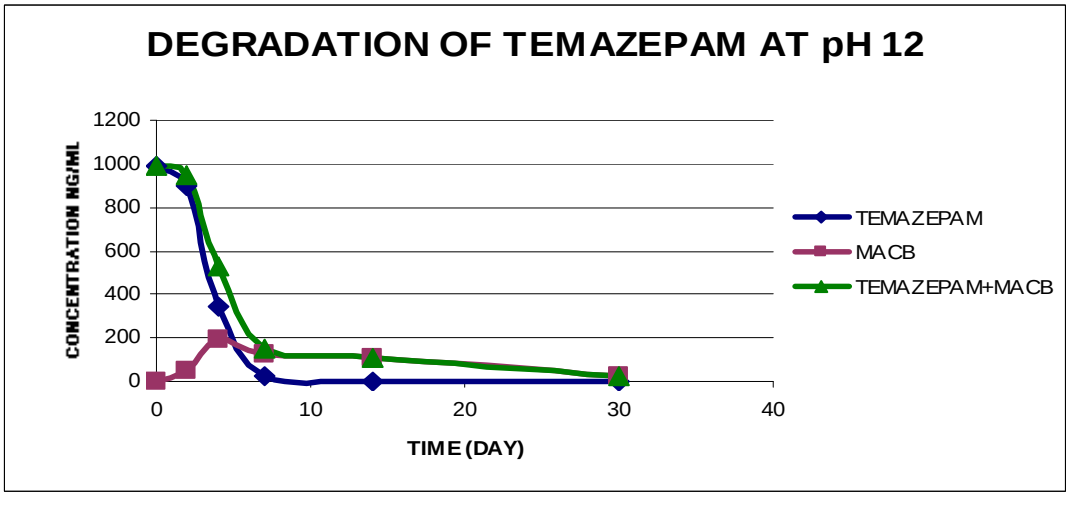


Figure 7-10: Degradation of temazepam in blood at pH 12

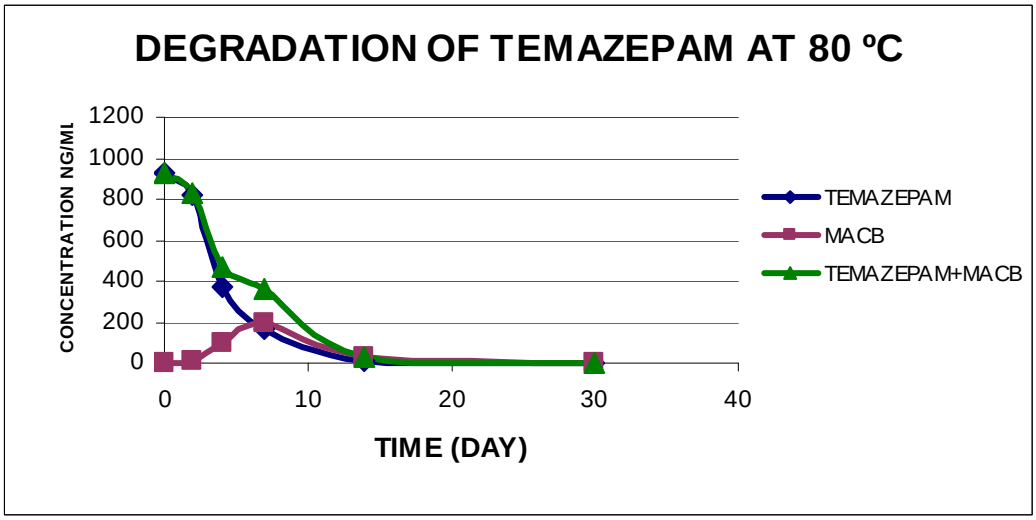


Figure 7-11: Degradation of temazepam in urine at 80 °C

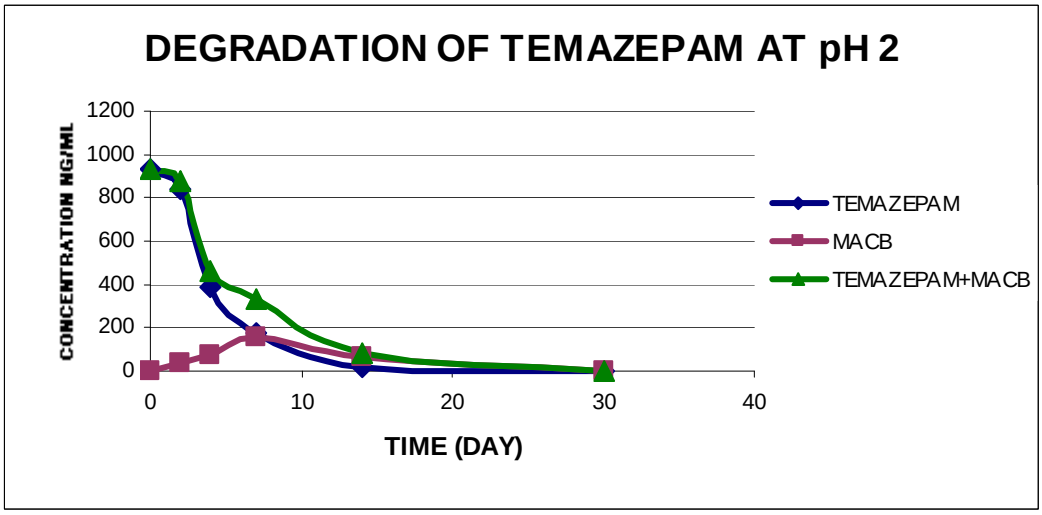


Figure 7-12: Degradation of temazepam in urine at pH 2

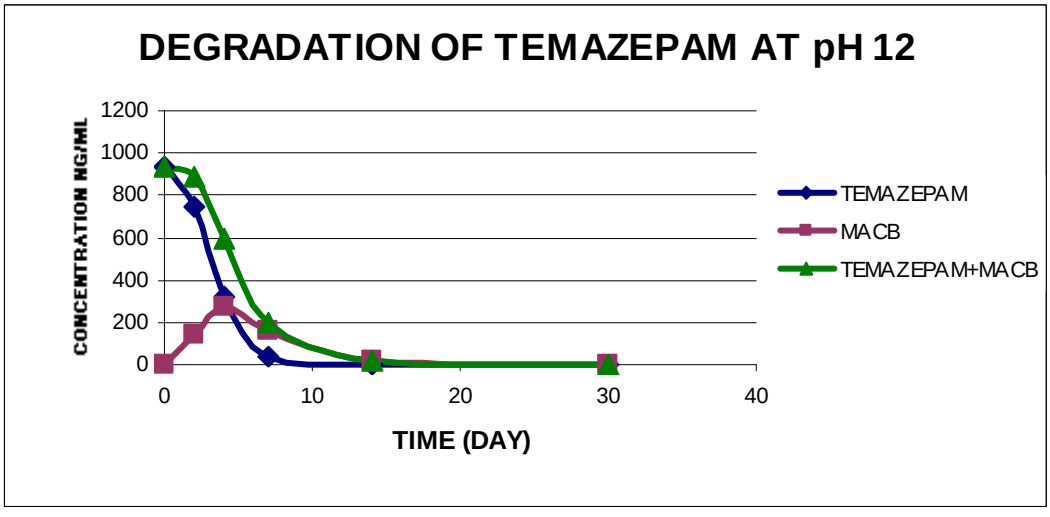


Figure 7-13: Degradation of temazepam in urine at pH 12

7.6 Degradation Study: Chlordiazepoxide and Oxazepam

7.6.1 Introduction

The aim of this study was to measure the rate of degradation of chlordiazepoxide and oxazepam in stored blood and urine samples and the rate of formation of their hydrolysis product ACB (2-amino-5-chlorobenzophenone). As for diazepam and temazepam, these results would allow an assessment to be made of a method for correcting for losses based on the concentration of ACB and conditions of storage were selected in order to result in degradation of the drugs in a short timescale.

7.6.2 Stability Study in Blood and Urine

Packed red blood cells were obtained from the Scottish National Blood Transfusion Service and were suspended in an equal volume of isotonic saline. This was used as blank blood. Blank blood (120 ml) was divided into two 60 ml portions, A and B, which were spiked with chlordiazepoxide and oxazepam respectively to give final concentrations of 1000 ng/ml. Each portion was divided into three aliquots of 20 ml and the drugs were subjected to accelerated decomposition for one month under three different storage conditions. The first condition was at high temperature in the oven at 80 °C, the second was in acidic conditions (pH 2) and the third was in alkaline conditions (pH 12). The times selected for analysis were at days 1 (day zero), 2, 4, 7, 14, and 30.

A similar study was carried out using drug-free urine obtained from a healthy volunteer.

7.6.3 Analytical method

Extraction and analysis were performed using the method for diazepam and temazepam (Section 7.4). Mass spectrometric conditions are given in Table 7-16. Internal standard data was aquired in selected ion monitoring (SIM) mode and analytes were identified on the basis of their full MS-MS spectra. Two

product ions were monitored. The underlined ions in Table 7-16 were used as the quantification ions because they were the most intense ions produced. It was not financially viable to use chlordiazepoxide-d5 therefore oxazepam-d5 was selected for chlordiazepoxide quantitation since its retention time was close to that of chlordiazepoxide (5.1 min and 5.95 min, respectively).

Table 7-16: Optimum tuning parameter for chlordiazepoxide, oxazepam and ACB

Drug	Precursor ion (m/z)	Monitored ions* (m/z)	Collision energy (ev)
Chlordiazepoxide	300	283,241	30
Oxazepam	287	269,241	26
ACB	232	154, 197	36
Internal standard			
Oxazepam-d5	292	292	Not used

- Underlined ion was used as the quantification ion. For internal standards, only the pseudomolecular ions were monitored and no collision energies were specified.

7.6.4 Results of Chlordiazepoxide and Oxazepam Degradation Study

7.6.4.1 Chlordiazepoxide

In the oven at 80 °C, the chlordiazepoxide concentration steadily decreased to reach 38% of its initial concentration at the end of the study. During this time, ACB appeared after 2 weeks and increased further by 1 month to 124 ng/ml (12% of the original amount of chlordiazepoxide). In urine, chlordiazepoxide decreased very quickly and was not detected after one month, while ACB was produced after 4 days, the concentration continued to increase until 2 weeks, then dropped to 140 ng/ml (13% of the original amount of chlordiazepoxide) by the end of the incubation period.

In the acidified blood sample (pH 2), chlordiazepoxide decreased gradually to reach 49% of its original concentration, while the ACB concentration increased between day 4 and day 30 from 29 nmol/ml to 97 ng/ml (3% to 10% of the original amount of chlordiazepoxide, respectively). In urine, chlordiazepoxide was less stable than in blood and decomposed very quickly, with approximately 7% of the starting concentration remaining after 1 month, while the ACB concentration increased from 57 ng/ml to 284 ng/ml (5% and 27% of the original amount of chlordiazepoxide, respectively) between day 4 and day 30.

In the basified blood sample (pH 12), chlordiazepoxide decomposed very quickly, losing half of its original concentration after 24 hours, and was not detected after 4 days, while ACB was detected after 1 day and decreased gradually from 118 ng/ml on day 2 to 81 ng/ml on day 7 and to zero ng/ml on day 14. In urine, more than 50% of the original chlordiazepoxide concentration decreased after 1 day, while ACB started to appear after 1 day and dropped from 125 to 73 ng/ml between days 2 and 4 (12% and 7% of the original amount of chlordiazepoxide, respectively). After this time, neither the parent drug nor its degradation product was detected. These results are shown graphically in Figures 7-14 to 7-19, which also shows graphs of the sum of chlordiazepoxide and ACB during the 30-day period of the study.

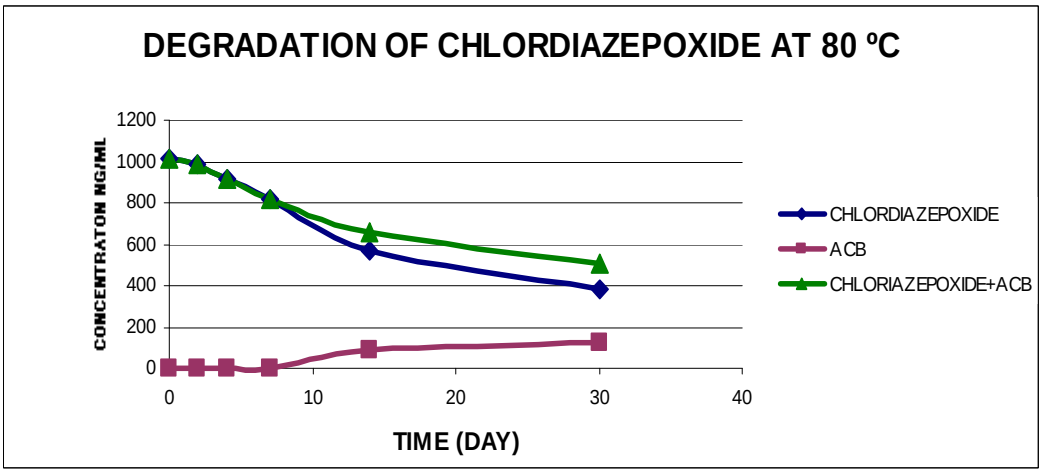


Figure 7-14: Degradation of chlordiazepoxide in blood at 80 °C

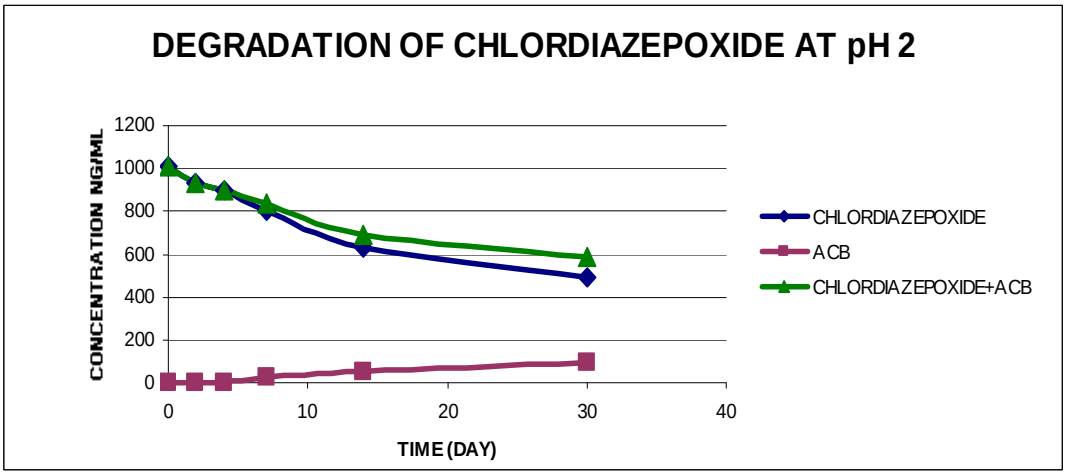


Figure 7-15: Degradation of chlordiazepoxide in blood at pH 2

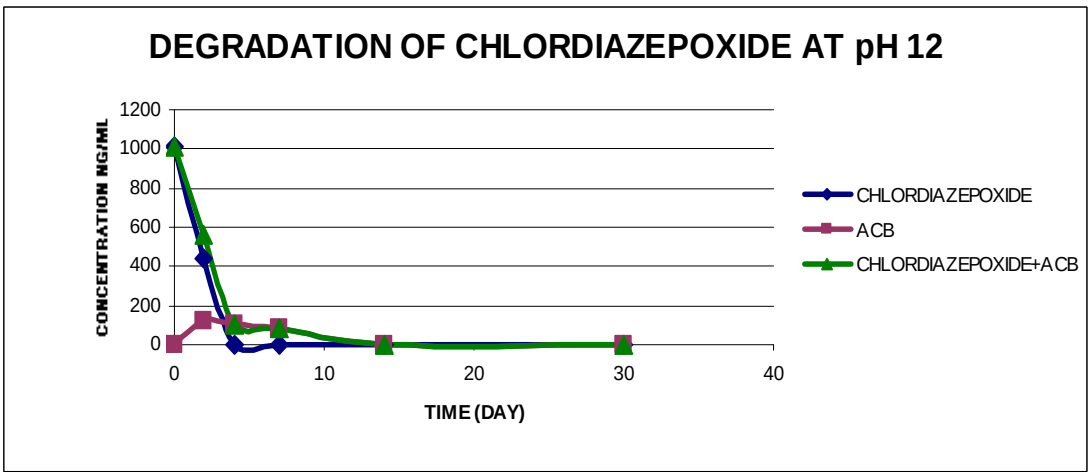


Figure 7-16: Degradation of chlordiazepoxide in blood at pH 12

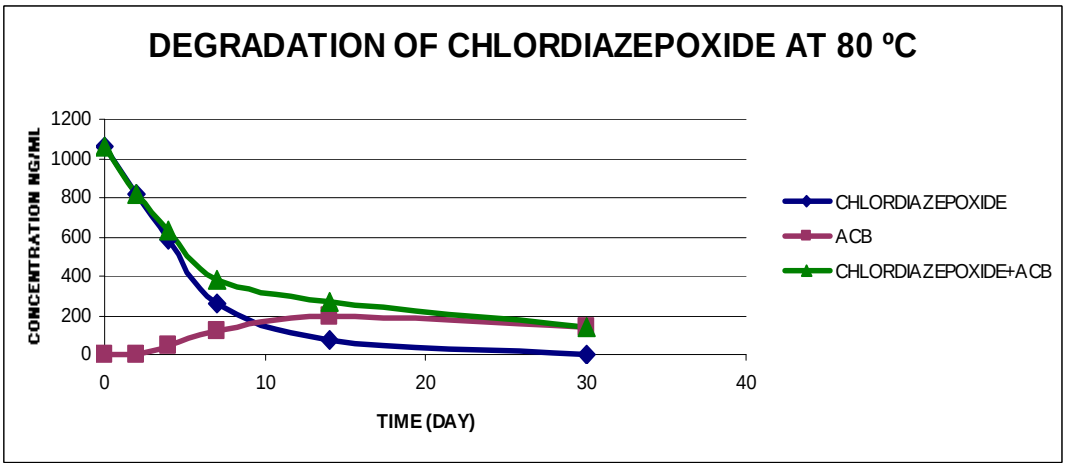


Figure 7-17: Degradation of chlordiazepoxide in urine at 80 °C

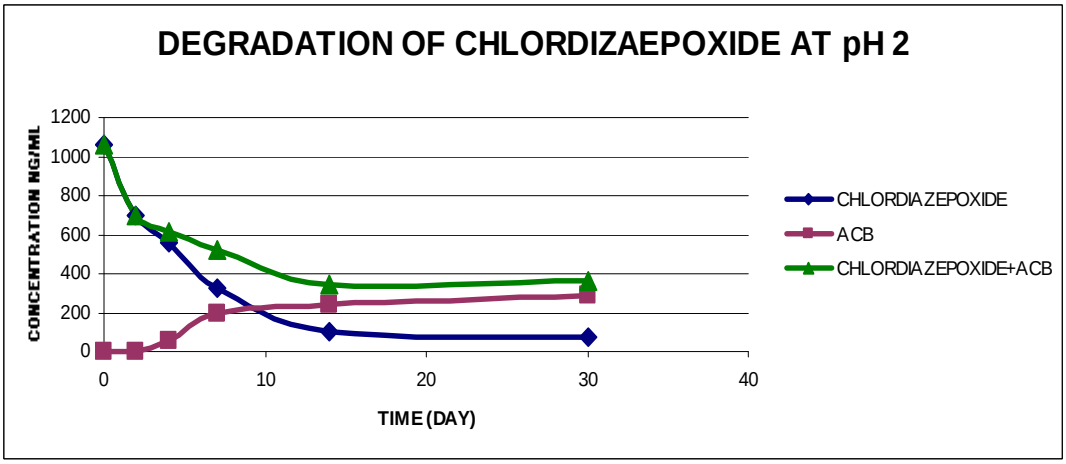


Figure 7-18: Degradation of chlordiazepoxide in urine at pH 2

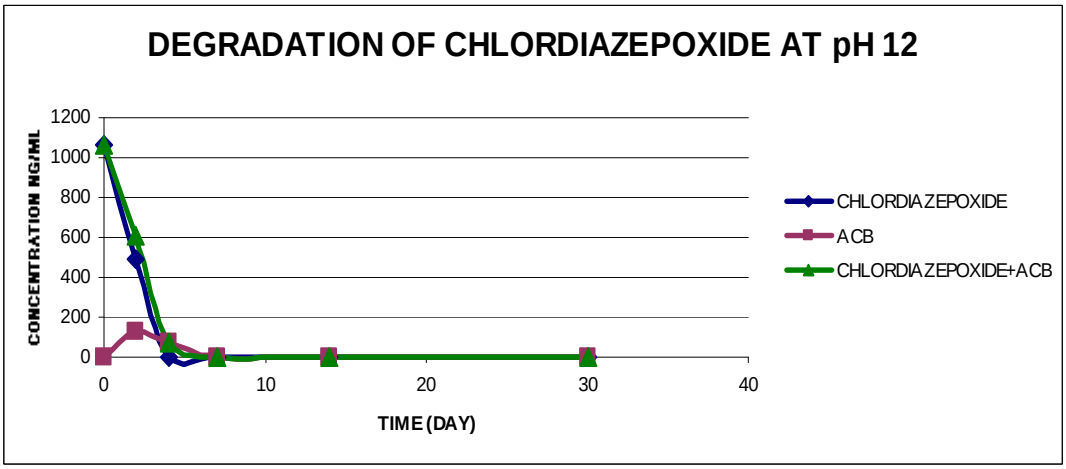


Figure 7-19: Degradation of chlordiazepoxide in urine at pH 12

7.6.4.2 Oxazepam

At 80 °C, oxazepam decreased gradually from 100% at day zero to 2% at week 2 and was not detected after one month, while ACB increased gradually from day 2 to week 2, then dropped to 340 ng/ml (34% of the original amount of oxazepam) at day 30. The same degradation pattern was observed in urine but faster, in which oxazepam decreased from day 2 to day 7 to 17% of the original concentration, while ACB increased from 204 ng/ml on day 2 to 310 ng/ml at week 2 (19.8% and 30% of the original amount of oxazepam, respectively), then dropped to 241 ng/ml (23.5% of the original amount of oxazepam) on day 30.

In acidified conditions at pH 2, oxazepam lost 88% of the original concentration after 2 weeks and was not detected after 1 month, while ACB was detected on day 2, which increased when oxazepam decreased. In urine, oxazepam lost approximately 95% of the initial concentration after 2 weeks and was not detected after this time, while ACB was observed to increase from day 2 to week 2, then dropped to 311 ng/ml (30.3% of the original amount of oxazepam) when oxazepam was completely lost.

In basified conditions at pH 12, approximately 50% of the original concentration of oxazepam was detected after 1 day and was not detected after 4 days, while ACB was detected after 1 day and dropped from 250 ng/ml to 143 ng/ml at day 4 and 1 month, respectively (25% and 14% of the original amount of oxazepam, respectively). In urine, more than 50% of the initial concentration was lost after 1 day and completely disappeared after 4 days, while ACB rose from 94 ng/ml on day 2 to 247 ng/ml on day 4, then dropped to 105 ng/ml after 1 month. These results are shown graphically in Figures 7-20 to 7-25, which also shows graphs of the sum of oxazepam and ACB during the 30-day period of the study.

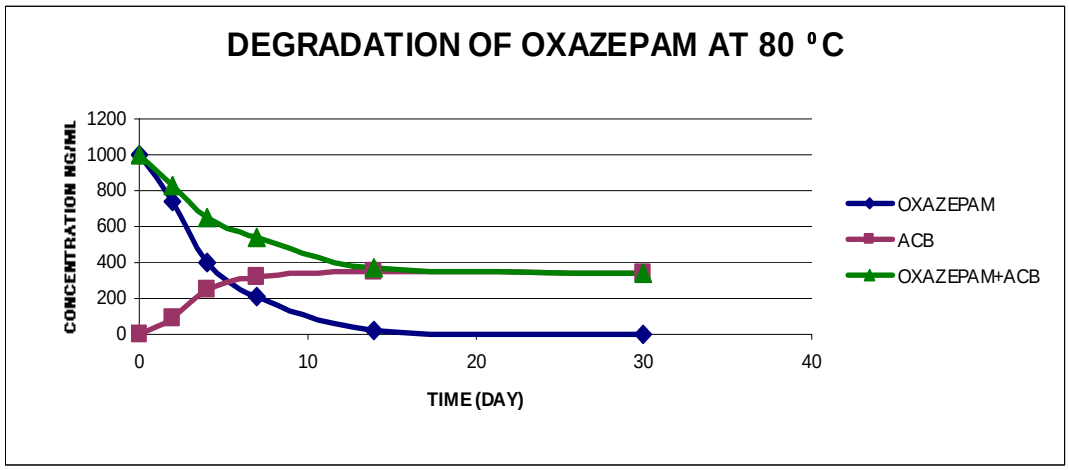


Figure 7-20: Degradation of oxazepam in blood at 80 °C

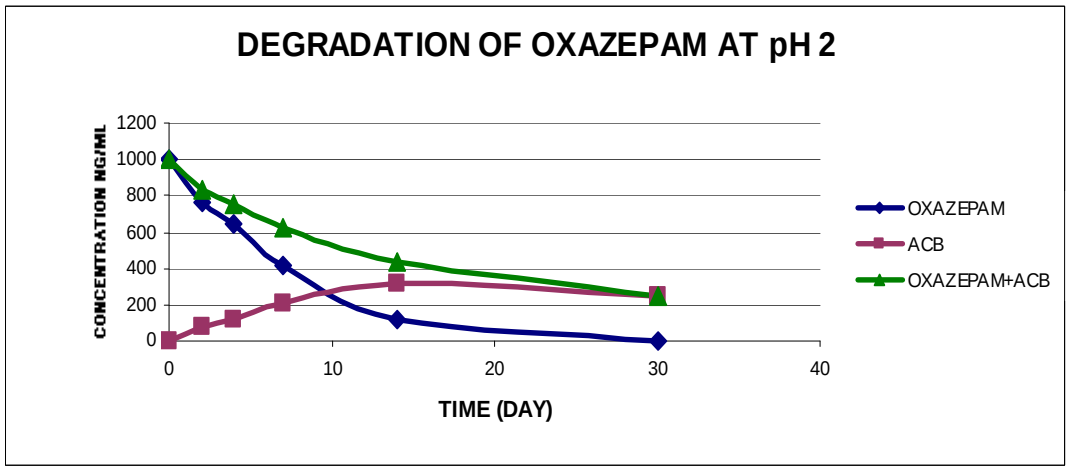


Figure 7-21: Degradation of oxazepam in blood at pH 2

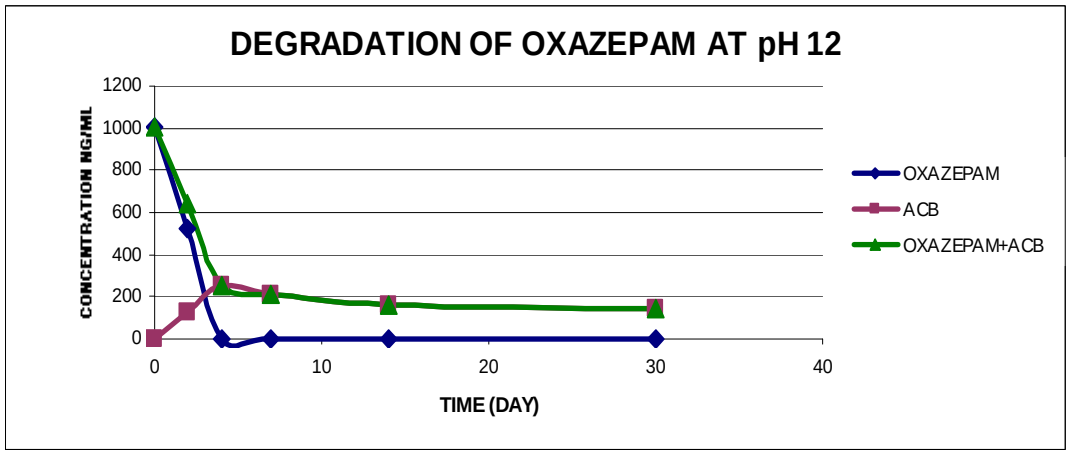


Figure 7-22: Degradation of oxazepam in blood at pH 12

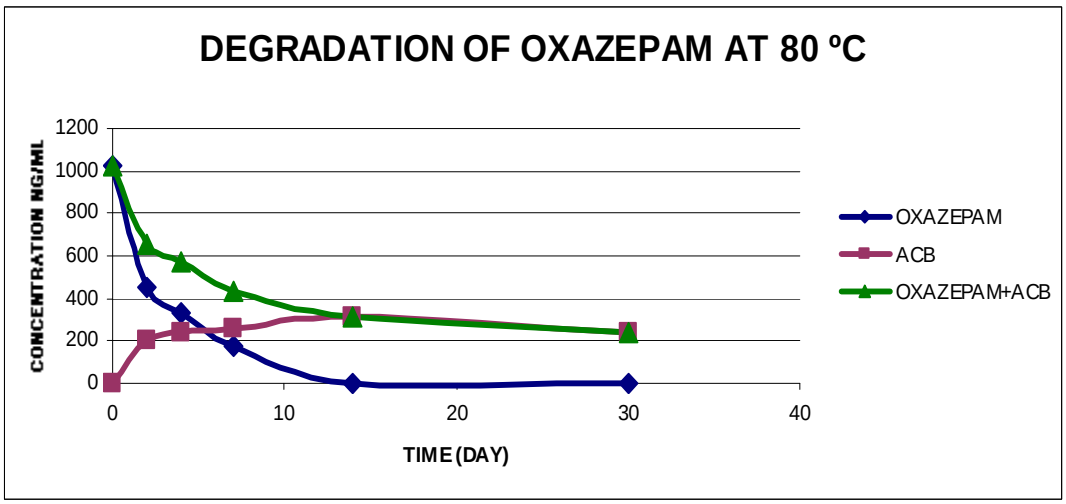


Figure 7-23: Degradation of oxazepam in urine at 80 °C

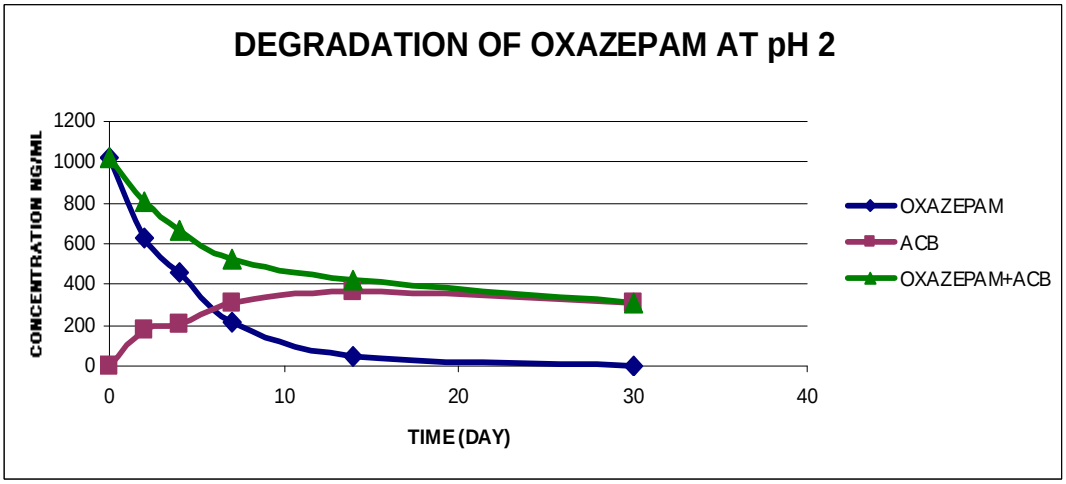


Figure 7-24: Degradation of oxazepam in urine at pH 2

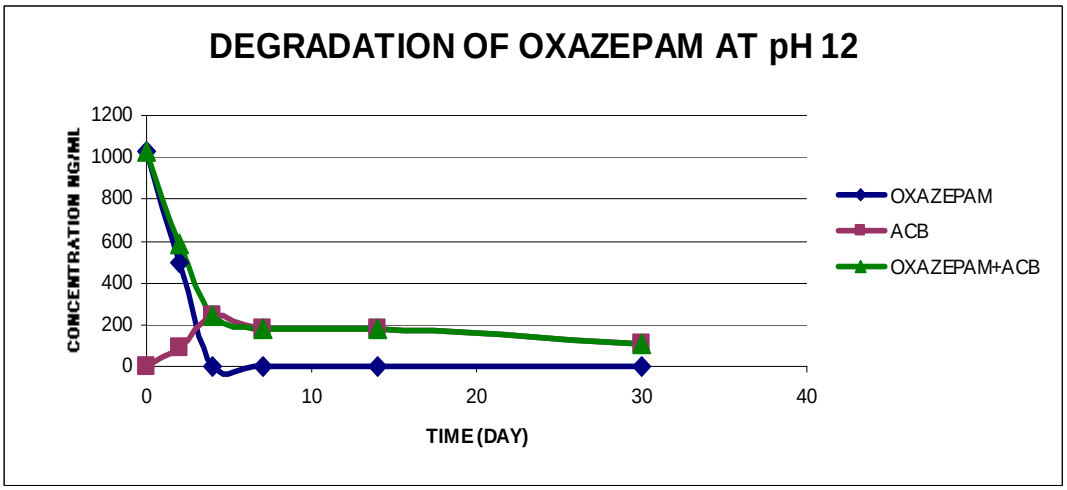


Figure 7-25: Degradation of oxazepam in urine at pH 12

7.7 Further Degradation of MACB to ACB and AB

7.7.1 Stability Study in Blood

The degradation of MACB and ACB in the previous studies suggested an additional experiment to investigate whether MACB can be degraded to ACB and ACB to aminobenzophenone (AB).

MACB was subjected to a process of rapid decomposition at high temperature to investigate and detect further degradation products, in particular ACB and the possible end product, AB.

Blank blood (20 ml) was spiked with MACB to give a final concentration of 1000 ng/ml.The prepared samples were submitted to accelerated decomposition for one month under high temperature in the oven at 80 °C. The times selected for analysis were at days 1 (day zero), 2, 4, 7, 14, and 30. The prepared sample was analysed in duplicate after spiking and designated as the day zero concentration. Aminobenzophenone was analysed with the MS parameters listed in table 7-17.

Table 7-17: Optimum tuning parameter for 2-aminobenzophenone (AB).

Drug	Precursor ion (m/z)	Monitored ions* (m/z)	Collision energy (ev)
AB	198	120,105	28

Underlined ion was used as the quantification ion.

7.7.2 Results of MACB Degradation

In blood at 80 °C, MACB degraded gradually and approximately 65% was lost from the day zero concentration by the end of study, whereas ACB increased gradually after 4 days of incubation to reach 91 ng/ml (9% of the original amount of MACB) and continued to increase to reach 203 ng/ml (21% of the original amount of MACB) at the end of study. AB was not detected during the observation period. This result is shown graphically in Figure 7-26.

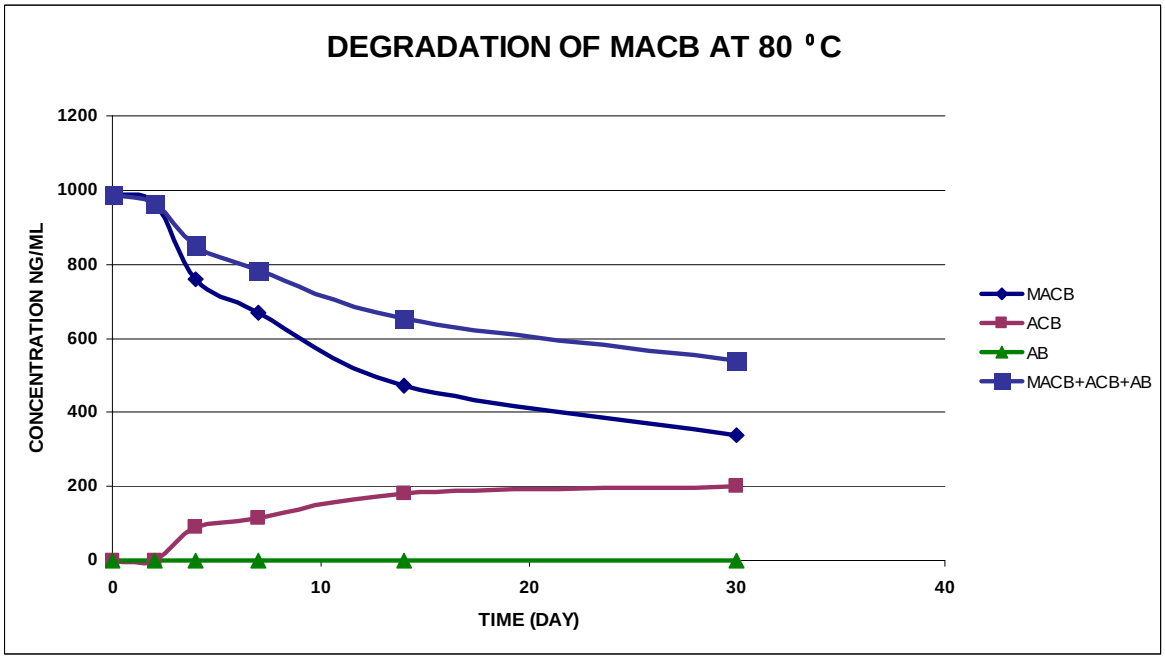


Figure 7-26: Degradation of MACB in blood at 80 °C

7.8 Effect of Cosmetics on Benzodiazepine Degradation

7.8.1 Introduction

Human natural hair colour is produced by a unique chemical pigment in the cortex of the hair called melanin. The production of this pigment is inherited and controlled by gene expression. The most common human hair colour is black or dark brown produced by a special pigment known as eumelanin; fair hair and red hair are produced by a specific melanin called phaeomelanin.[192]

Alteration of the natural hair colour using artificial or natural products is influenced by many personal, psychological, and practical aspects. The most common reason to change the colour is to cover grey hair. Artificial hair colour has been used since Egyptian times when kohl and henna were used as artificial pigments in cosmetics. Roman women used a mixture of natural products such as wood ash, unsliced lime and sodium bicarbonate, wild fruit, and alcohol to mimic their light-haired slaves. In Restoration times, alkali, salt, and sun were popular while potassium hydroxide was also fashionable for lightening hair.[192]

Para-phenylenediamine (PPD) was discovered in 1863 by the chemist Dr Hofmann, who reported the dyestuff properties of this substance. This discovery still is dominant; over 70% of permanent dyes in all products are derived from PPD.[192]

In 1931 Clairol produced their first home colour. Millions of women used this colouring product, which was considered a real breakthrough. Since that time the development continued for the next 20 years when Wella launched the first crème permanent dyes in 1953. Recently modern international manufacturers of hair dye products have established research and development organizations to produce safe, reliable, and qualified hair colour products. The modern hair dyes are classified into four basic categories: temporary, semi-permanent, permanent, and other dyes. [192]

Temporary colourants can easily be removed from the hair at the first shampooing. Semi-permanent dyes are not exposed to oxidation and not bound to hair protein. Therefore they can be washed out after four to six wash cycles.

Permanent products cannot be washed out; they are the most popular because of their extreme versatility. [192]

The essential ingredients in permanent hair dye are precursors such as PPD (for dark brown or black shades) and p-aminophenol (for light auburn), which is oxidized in the hair shaft by hydrogen peroxide to active intermediates. Within the hair shaft these intermediates react with the additional ingredients in the product called “colour couplers” such as resorcinol, 1-naphthol, and pyrogallol. Then the product of this coupled reaction is oxidized to the final coloured dye molecules. These reaction products have low water solubility, which makes the product wash-resistant. [192]

Other active ingredients are used in the colourant base such as ammonia, which is needed to activate the system when hair is treated with hydrogen peroxide. Without ammonia no bleaching is observed. The level of ammonia and peroxide will determine how much bleaching will be produced. More is added for blonde shades than for dark shades; however, a high level of ammonia irritates the scalp and makes the product smell bad. It also raises the pH of the product to about 10, which makes the hair swell. [192]

Hydrogen peroxide is also used to provide oxygen to fuel the reactions; no permanent colour occurs without this ingredient. Peroxide acts by oxidizing the dye species. The typical level is 3% to 4.5% on the head. [192]

Solvents such as dipropylene glycol, hexylene glycol, ethanol, and glycerin are used to solubilize the dye materials and improve viscosity. Surfactants/fatty alcohols are also added in the colourant base formula such as ceteareth-25 and steareth-2, which helps to remove oil and sebum from the hair surface. [192]

The presence of these compounds in hair may impact the instability of drugs in hair samples; it is reported that cosmetics influence the stability of some drugs.

Hair cosmetic treatment has been shown to influence the stability of opiates and benzodiazepines, as mentioned in Chapter 2 and reported in literature.[82-84,192-195] The current study was undertaken to investigate whether hair bleaching materials have an effect on the degradation of these drugs or if the observed instability is a result of the washing procedure, or hair washing with water.

7.8.2 Chemicals and Materials

Three commercial bleaching cosmetics with different brand names, Gerreni®, Loreal®, and Clairol® were purchased from a local pharmacy.

7.8.3 Experimental section

Each drug, diazepam, temazepam, oxazepam, and chlordiazepoxide was spiked with the bleaching materials in measuring cylinders to give a final concentration of 1000 ng/10μl to allow use of a small volume of bleaching material in order to avoid any blockage of SPE cartridges that might limit the extraction of the analytes of interest. Each drug was treated with three different bleaching agents. The prepared samples were mixed by vortexing for 5 minutes to dilute the drugs in the solution. Prepared samples (10 μl) were diluted with 3.5ml of an acetate buffer (pH 4.5) followed by the addition of 100 μl of internal standard, and centrifuging for 5 minutes at 2500 rpm. The SPE and analysis method were the same as those described earlier in this chapter. The prepared samples were analysed on the same day as spiking and one week after spiking to investigate the effect of the bleach on the drug and determine whether the degradation products could be detected, in the event that the drugs decomposed as a result of the action of bleaching material components.

7.8.4 Results

The drug concentration decreased for all compounds during the time of the investigation and with all cosmetic materials being used in this study. However, the degradation products that were previously detected in blood and urine were not detected in this study, except for a small amount of MACB that was detected after degradation of temazepam in with the presence of Gerreni® components, which had the most pronounced effect on degradation of all drugs.

7.8.4.1 Diazepam

More than 50% of the initial concentration of diazepam was lost when treated with Gerreni® and approximately 35% with Loreal®, while negligible amounts were lost when Clairol® was used. In all situations, MACB was not detected after one week of study. The results are shown in Table 7-18.

Table 7-18: Decomposition of diazepam by cosmetic materials

Bleaching material	Time			
	Day zero		1 week	
	Diazepam ng/10µl	MACB ng/10µl	Diazepam ng/10µl	MACB ng/10µl
Gerreni®	868	0	421	0
Loreal®	976	0	627	0
Clairol®	945	0	943	0

7.8.4.2 Temazepam

A very large proportion of temazepam was lost when added to Gerreni® after one week, while MACB was detected in small amounts, approximately 22ng /10µl. In the tube containing Loreal®, more than 40% of temazepam was lost, while in the Clairol® tube, less than 3% of temazepam was lost. In Loreal® and Clairol®, no MACB was detected. The results are shown in Table 7-19.

Table 7-19: Decomposition of temazepam by cosmetic materials

Bleaching material	Time			
	Day zero		1 week	
	Temazepam ng/10µl	MACB ng/10µl	Temazepam ng/10µl	MACB ng/10µl
Gerreni®	897	0	53	22
Loreal®	963	0	557	0
Clairol®	987	0	965	0

7.8.4.3 Chlordiazepoxide

As shown in Table 7-20, more than 62% of chlordiazpoxide was lost in the tube containing Gerreni® material, while ACB was not detected after one week. In Loreal® and Clairol® 19% and 5% of chlordiazepoxide were lost respectively, while ACB was not detected.

Table 7-20: Decomposition of chlordiazepoxide by cosmetic materials

Bleaching material	Time			
	Day zero		1 week	
	Chlordiazepoxide ng/10µl	ACB ng/10µl	Chlordiazepoxide ng/10µl	ACB ng/10µl
Gerreni®	902	0	338	0
Loreal®	913	0	743	0
Clairol®	934	0	890	0

7.8.4.4 Oxazepam

Oxazepam decreased by 89%, 32%, and 4% in Gerreni®, Loreal®, and Clairol® tubes respectively, while ACB was not detected in any of the tubes. The results are shown in Table 7-21.

Table 7-21: Decomposition of oxazepam by cosmetic materials

Bleaching material	Time			
	Day zero		1 week	
	Oxazepam ng/10µl	ACB ng/10µl	Oxazepam ng/10µl	ACB ng/10µl
Gerreni®	820	0	94	0
Loreal®	938	0	634	0
Clairol®	963	0	925	0

7.9 Case Samples

The established analytical method was applied to 12 blood and 7 urine samples from forensic cases, obtained from the Department of Forensic Medicine and Science, University of Glasgow. These samples had been kept in the deep freeze for up to 6 months after they were originally received and analysed. This study was intended to assess whether significant degradation of benzodiazepines had occurred during storage. The results are shown in Tables 7-22 and 7-23.

7.9.1 In Blood

Table 7-22: Benzodiazepines and their degradation products in blood case samples

SAMPLE NO	Diazepam mg/L		Temazepam mg/L		Oxazepam mg/L		Time Interval (month)	AB mg/L	ACB mg/L	MACB mg/L
	1^{st}*	2^{nd}*	1^{st}*	2^{nd}*	1^{st}*	2^{nd}*				
1	0.15	0.12	0.10	0.09	0.12	0.10	<1	ND	ND	ND
2	0.25	0.22	0.08	0.07	0.03	0.10	<1	ND	ND	ND
3	0.02	0.02	0.01	0.02	ND*	ND	1	ND	ND	ND
4	0.04	0.03	0.32	0.30	0.03	ND	1	ND	ND	ND
5	0.26	0.27	0.03	0.04	0.04	0.02	2	ND	ND	ND
6	0.12	0.12	0.01	0.02	0.03	0.02	2	ND	ND	ND
7	0.89	0.90	0.05	0.06	ND	ND	3	ND	ND	ND
8	1.30	1.27	0.22	0.21	0.26	0.23	3	ND	ND	ND
9	1.40	1.37	1.70	1.68	>1	1.53	4	ND	ND	ND
10	1.20	1.18	0.21	0.20	ND	ND	4	ND	ND	ND
11	0.20	0.19	0.06	0.05	ND	ND	4	ND	ND	ND
12	0.10	0.09	0.02	0.01	ND	ND	4	ND	ND	ND

* 1^{st}: The concenration of drug in the case sample at received time, 2^{nd}: The concentration of drug in case sample after period of time.

*ND: Not detected.

7.9.2 In Urine

Table 7-23: Benzodiazepines and their degradation products in urine case samples (not matched to the blood samples in Table 7-22)

SAMPLE NO	DIAZEPAM mg/L	TEMAZEPAM mg/L	OXAZEPAM mg/L	CHLORODIAZEPOXIDE mg/L	AB mg/L	ACB mg/L	MACB mg/L
1	0.030	>1	0.397	ND*	ND	0.111	0.072
2	0.148	>1	>1	ND	ND	0.424	0.097
3	0.484	>1	>1	ND	ND	0.903	>1
4	0.069	>1	0.288	ND	ND	0	0.321
5	>1	>1	>1	ND	ND	>1	>1
6	0.053	>1	>1	ND	ND	0.319	0.323
7	0	>1	0.337	ND	ND	0.121	ND

*ND: Not detected.

7.10 Summary of benzodiazepines degradation in blood and urine under different storage conditions

The degradation of diazepam, temazepam, chlordiazepoxide, and oxazepam in blood and urine samples for one month under different storage conditions is summarized in Table 7-24.

Table 7-24: Summary of degradation of diazepam, temazepam, chlodiazepoxide, and oxazepam in blood and urine samples under different storage conditions

Drug	Conditions	Results		Comment
		Blood	Urine	
Diazepam	80 °C, 1 month	30% decrease	100% decrease	• MACB concentration increased when concentration of diazepam and temazepam decreased • ACB concentration increased when chlordiazepoxide and oxazepam concentration decreased • MACB and ACB can be detected even if the parent compounds are undetectable • Degrades in urine faster than in blood • Degradation is dependent upon basic condition more than on the level of acidity and temperature conditions
	Acidified pH 2, 1 month	25% decrease	100% decrease	
	Basified pH 12, 1 month	50% decrease	100% decrease	
Temazepam	80 °C, 1 month	100% decrease	100% decrease	
	Acidified pH 2, 1 month	77% decrease	100% decrease	
	Basified pH 12, 1 month	100% decrease	100% decrease	
Chlordiazepoxide	80 °C, 1 month	62% decrease	100% decrease	
	Acidified pH 2, 1 month	51% decrease	93% decrease	
	Basified pH 12, 1 month	100% decrease	100% decrease	
Oxazepam	80 °C, 1 month	100% decrease	100% decrease	
	Acidified pH 2, 1 month	100% decrease	100% decrease	
	Basified pH 12, 1 month	100% decrease	100% decrease	

7.11 Discussion and Conclusions

Hydrolysis generally splits compounds into two molecules. Since benzodiazepines contain an amide group in their structure, they are susceptible to decomposition by hydrolysis reactions involving this functional group to produce carboxylic acids and amines. Benzodiazepine hydrolysis was extensively studied: degradation of benzodiazepines in aqueous media yielded benzophenone derivatives and glycine as degradation products; thus, in this study benzophenone derivatives were the major degradation products to be detected because the other product, glycine, may be produced in the sample from other sources: for example, glycine is used in medical and industrial applications as a sweetening agent, antacid agent, stabilizer, buffer and regulator, as a material in cosmetics, and as a supportive therapy for treatment of schizophrenia. Further, most proteins contain small quantities of glycine which in principle may be released by protein catabolism. Glycine was therefore excluded from this study, unlike the benzophenone derivatives, which are specific degradation products for benzodiazepine drugs. [199]

Temperature and pH significantly affected the rates of the hydrolysis reactions. Sometimes samples are kept for a period of time under unfavourable conditions, such as in high temperatures, according to the climate or during transportation at ambient temperature which may accelerate the hydrolysis reaction. This is a recognised problem in hot countries, especially in developing countries, which may not have the necessary resources to provide refrigeration during transport. The internal temperature of closed vehicles on a hot day can exceed 65 °C, and temperatures on exposed surfaces such as dashboards can reach 93 °C. The normal blood pH ranges from 7.2-7.4. Plasma pH shift was reported to increase to above pH 8 only a few hours after incubation at 37 °C. The effect of diet basicity or acidity on the blood pH is regulated in vivo by metabolic and renal mechanisms, whereas in vitro, this regulation system is not present, and it becomes more alkaline than the normal physiological pH a few hours after collection, as a result of CO_2 losses during storage. [196]

On the other hand, urine pH ranges between 4 and 8 depending on diet, medication intake and state of health of the sample donor. Urine also contains other components such as ammonia, phosphates, salts, and bicarbonate, which

may change the pH of the sample after collection, leading to difficulty in the quantification of pH-labile compounds after storage. Also, it was reported that an increase in temperature is directly correlated with an increase in the urine pH. This explains the observed difference in the degradation of all drugs involved in this study, which was faster in urine than in blood. Urine becomes alkaline immediately after a meal and then gradually becomes acidic between meals. A high-protein diet is associated with acidic urine, while a vegetarian diet produces more alkaline urine as a result of the formation of bicarbonate from fruits and vegetables. Male and female urethras are colonized with microorganisms, even if samples are collected with prior external cleansing of the genitalia, with further contamination from intestinal flora in females. Urine samples are as a result often bacterially contaminated. Individual health status and drug intake also affect the urine pH. Respiratory and metabolic alkalosis, prolonged vomiting and urinary tract infection are the most common causes of alkaline urine, while respiratory and metabolic acidosis, diabetes mellitus, starvation and severe diarrhoea are the most common causes of acidic urine. Some medications also change the urine pH. Clinically, in drug overdose, it is observed that the urine pH changes to accelerate the clearance of some drugs.[8] Urine contains urea, uric acid and non-protein nitrogenous compounds, and during the time of storage, the stability of these compounds is affected by bacterial contamination. For example, urea is split by bacterial urease into ammonia and carbon dioxide, which may yield a urinary pH>8. In vitro adulteration with some manufactured products containing very basic or very acidic components can destroy the drugs in the sample. SAMHSA guidelines have established that urine pH cut-offs <3 or >11 are indicate adulterated urine samples. At high temperatures, urine pH changes to reach >9, and some authors have attributed that to the loss of CO_2, while other authors argue that it is due to the production of ammonia. Therefore, all of these factors can accelerate the rate of hydrolysis reactions and lead to loss of the analyte of interest and, consequently, a false interpretation of case results. Under these expected conditions, the hydrolabile drugs were submitted to a range of non-ideal conditions in order to detect the degradation product specific for each analyte. The measured concentrations of degradation products can be used to correct for loss of the parent drugs and as indicators of drug intake in case in which the parent drug has completely decomposed under unfavourable conditions.[156]

The drug degradation reactions investigated in this study accelerated under all conditions. During decomposition of drugs under alkaline conditions, the hydrolysis of the compounds is initiated by nucleophilc attack of the hydroxide ion on the positively charged carbonyl carbon atom of the amide bond, leading to the production of a negatively charged intermediate compound that exhibited basic character; thus, it may induce the dissociation of the water molecule followed by protonation of the nitrogen atom. Finally, the C-N bond is broken, yielding a benzophenone product (Figure 7-27). Under acidic conditions, the hydrolysis reaction starts by protonation of the oxygen atom of the carbonyl group followed by nucleophilic attack by the hydroxide ion, protonation of the nitrogen atom and finally opening of the C-N bond. In both acidic and basic conditions, water assists this reaction, while in absence of acidic or basic catalysts, high temperature accelerates this reaction in the presence of water as observed in an oven at 80 °C.[198,199]

The degree of hydrolysis was different under different conditions. An excess amount of OH^- in basic conditions acts as a nucleophile, attacking the electrophilic carbonyl carbon, which explains the observed sensitivity of the drugs to degradation in basic conditions. Another reason explaining why drugs degraded faster in basic than in acidic conditions is that hydrolysis of drugs under acidic conditions may favour cleavage of the 4,5-imine bond, while in basic conditions hydrolysis favours cleavage of the 1,2 amide bond. However, in acidic conditions, the degradation product produced is benzophenone imine, ultimately hydrated to produce the benzophenone derivatives that were detected in this study under both conditions.[199]

Diazepam, temazepam, and oxazepam contain an amide group that is prone to decompose following the previous mechanism by either amide or imine bond cleavage under all conditions, and the main degradation products were MACB and ACB as found in this study (Figures 7-27 and 7-28).

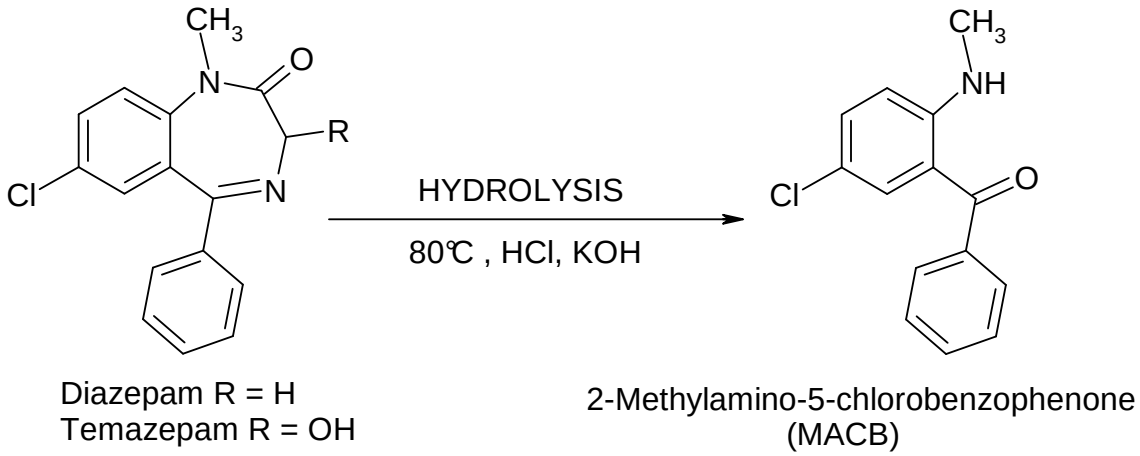


Figure 7-27: Hydrolysis of diazepam and temazepam

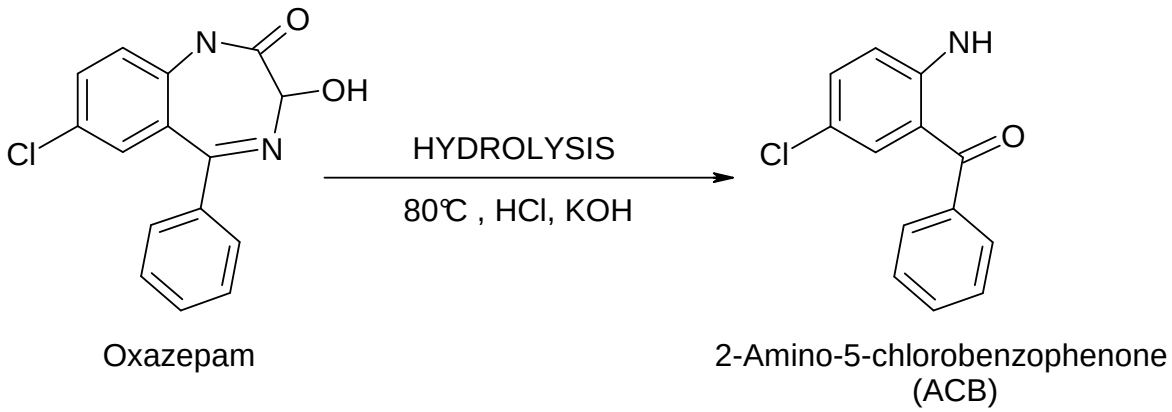


Figure 7-28: Hydrolysis of oxazepam

In contrast, chlordiazepoxide does not contain an amide group in its structure. However, some studies have reported that ACB was detected as a degradation product in cases of chlordiazepoxide degradation similar to what was observed in this study. The proposed degradation mechanism of chlordiazepoxide is hydrolysis first to an intermediate lactam (demoxepam), which is formed by hydrolytic cleavage of methylamino substituent at the 2-position of the parent compound, which decomposes further to nordiazepam, which is then hydrolyzed to produce ACB as a final degradation product. That is why the ACB produced from chlordiazepoxide degradation is less than that produced from oxazepam; therefore, demoxepen and nordiazepam can be quantified in addition to ACB in case of chlordiazepoxide decomposition (Figure 7-29).

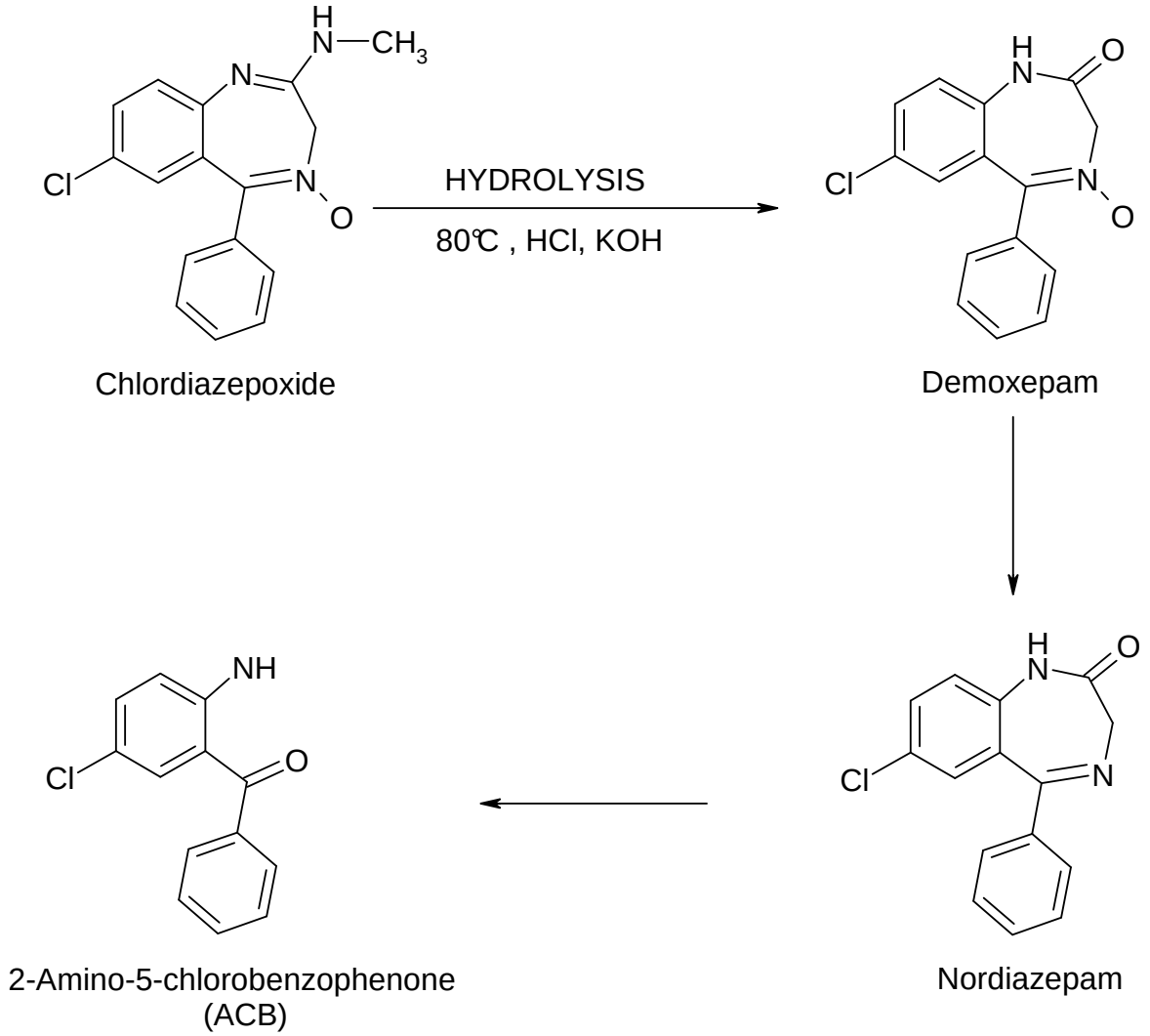


Figure 7-29: Hydrolysis of chlordiazepoxide

Temazepam and oxazepam were found to be less stable than diazepam, perhaps because of the presence of (OH) on position 3 of the diazepine ring that may exhibit electron withdrawing properties, thereby accelerating the degradation of these compounds.

MACB and ACB itself were found to be further degraded under some conditions created in this study. MACB was found to degrade further to ACB, and this result is similar to those of some previous studies, while AB was not detected as a final degradation product for these compounds as expected, perhaps because the bond energy between chlorine and carbon is higher than those between nitrogen and carbon thus another compound rather than AB may produced.

MACB and ACB were not detected in the case blood samples, perhaps because the samples were stored properly in the refrigerator and freezer, which

stabilized the drugs from degradation. However, MACB and ACB were detected in the urine samples, which may be attributed to urine pH, bacterial contamination, or the enzyme used to cleave the drug glucuronide.

Decomposition of benzodiazepines resulting from the presence of cosmetics was noted; however, hydrolysis is not the predominant degradation reaction under this condition, which is explained by the absence of MACB and ACB after degradation except for a small amount of MACB that was detected after hydrolysis of temazepam by Granini® bleaching. Also, this study indicates that the components of cosmetic materials are different in different brands, resulting in different effects on the stability of these compounds. In previous studies it was reported that oxidation of diazepam produced MACB.[200,201] However, they used HCL in the experiment, which may explain why MACB was produced, meaning that it was produced by a hydrolysis reaction rather than an oxidation process; it seems likely that a compound other than a benzophenone is produced by an oxidation process. Further study is suggested to investigate the degradation products that may be produced by oxidation.

In this study and under all conditions, the MACB and ACB concentration increased as degradation of the drugs proceeded and these products could be detected when the parent drugs had completely disappeared. Degradation of diazepam and temazepam in blood and urine under different conditions will produce MACB as the main hydrolysis product, while degradation of oxazepam and chlordiazepoxide will produce ACB as the main degradation hydrolysis product.

MACB and ACB can be reliably detected in whole blood and urine and can be used, at least in part, to correct for losses of parent drugs. They also serve as to indicate the original presence of these compounds in samples kept under poor or prolonged storage conditions if the case samples are suspected to contain benzodiazepines and the parent compound is not detected.

This study showed that temperature and pH have important implications for the decomposition of benzodiazepines. Extended transportation time and increased environmental temperature may affect the pH of the urine samples, which may exceed 9 within 2 days, destroying the drugs and giving false negative results. Therefore, degradation products are promising compounds as markers that can

indicate the former presence of specific drugs in cases in which the drug is lost during transport and storage.

8 Stability of Organophosphorus Pesticides During Storage in Preserved Blood and Dried Blood Spots

8.1 Introduction

Organophosphorus compounds (OPs) are highly toxic pesticides that contain esters of phosphoric acid attached through oxygen, sulphur and nitrogen atoms. OPs are used in agriculture as insecticides, acricides, and fungicides. OPs exert their effect by inhibiting acetylcholinesterase enzyme (AChE) activity by irreversible phosphorylation of the enzyme; thus, human exposure to these compounds causes nerve synapse toxicity as a result of the accumulation of acetylcholine in the synaptic cleft, leading to paralysis and possibly death by asphyxia.[9] It can be seen from Figure 8.1 that OPs usually have three organic substituents, including two methyl or ethyl substituents plus one other which is different between each OP. The unique substituent is termed the "leaving group" as it is this substituent which is displaced when AChE is phosphorylated.

OPs are rapidly metabolised in blood to yield dialkylphosphates (DAPs), which are excreted in urine within 6 to 24 hours. For example, the dichlorvous half-life in blood is 30 minutes, so detection of the parent compound after a period of time is difficult, whereas its metabolites can be found for a long time in urine. Phosphothionate undergoes oxidative desulfuration to produce its active phosphate analogue, which is a more powerful AChE inhibitor than the parent compound. For example, parathion is oxidised to paraoxon, which is more potent than parathion.[202]

Acute and chronic exposure to OPs has been reported in forensic cases.[203,204] In many parts of the developing world, pesticides account for most substance-related deaths because they are readily accessible in these agricultural communities. Assessment of exposure to OPs was achieved by measurement of the activity of AChE in erythrocytes. If the activity of this enzyme is less than 85% of the population mean value, then toxicity by OPs is suspected. However, inter-individual variation makes this assessment unsuitable for monitoring exposure to OPs and determination of exposure markers, such as OPs and their

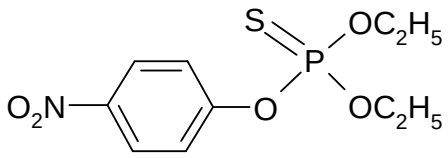
metabolites, is a more powerful approach and suitable parameter to evaluate OP toxicity.[203-205] In some medicolegal jurisdictions, the courts require unambiguous identification of the parent compound, which poses a problem for the toxicologist, and much effort has been expended on developing suitable analytical approaches.

Numerous methods have been reported for determination of intact OPs in biological fluids,[206-219] while other studies determined their DAPs as markers of occupational OP exposure.[220-236] Few methods have been reported for determination of both OPs and their DAP metabolites.[237-239] By looking at their molecular structure, we find that each OP compound will produce one of several different DAPs (see Figures 8.1 and 8.2). Dichlorvos degradation produces dimethylphosphate (DMP), while diazinon, chlorpyrifos, and parathion decomposes into diethylthiophosphate (DETP), and paraoxon decomposes to produce diethylphosphate (DEP). Since DAPs have high polarity and water solubility characteristics, they required suitable derivatisation before analysis. In previous studies, the derivatising agents used were diazoalkanes and pentafluorobenzyl bromide (PFBBr) to form volatile derivatives prior to analysis by GCMS. However, diazolkanes is highly toxic (carcinogenic) and readily explosive, while PFBBr is a potent lachrymator and its decomposition product may cause damage to the GC column.[226,233,235] Some studies used MTBSTFA + 1 % TDM to derivatize DAPs and this derivatising agent was used in this study for reasons of safety and efficacy.[229,239]

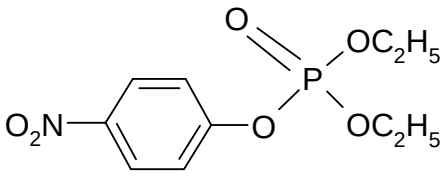
Little can be done with respect to preventing OPs from degrading in the post mortem interval but some consideration can be given to stabilisation of samples after collection at autopsy. However, Fumio et al. reported that the addition of the anti-enzymatic preservative sodium fluoride (NaF) to a blood sample containing OPs accelerated the degradation of dihlorvos and chlorpyrifos in the blood.[88]

The aim of this study was to analyse the DAPs for selected OP produced as a result of oxidation and hydrolysis as markers for the parent compounds. The OPs selected for this study were those of significance in forensic cases in Saudi Arabia. Additionally, an alternative approach was evaluated, to investigate whether filter paper could avoid or reduce the degradation rate of these

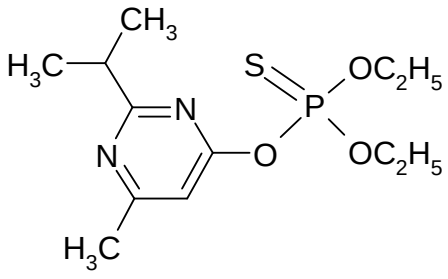
compounds in blood samples stored as DBS rather than by adding a preservative. These aims required the development of a new, practical, routine method to extract OPs and their DAP metabolites from blood samples by SPE, followed by analysis by GCMS after suitable derivatisation.



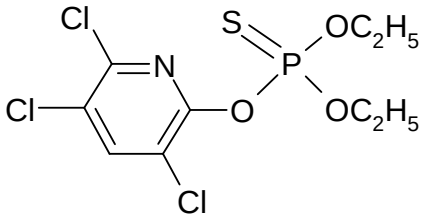
Parathion



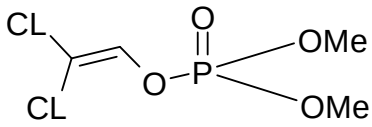
Paraoxon



Diazinon

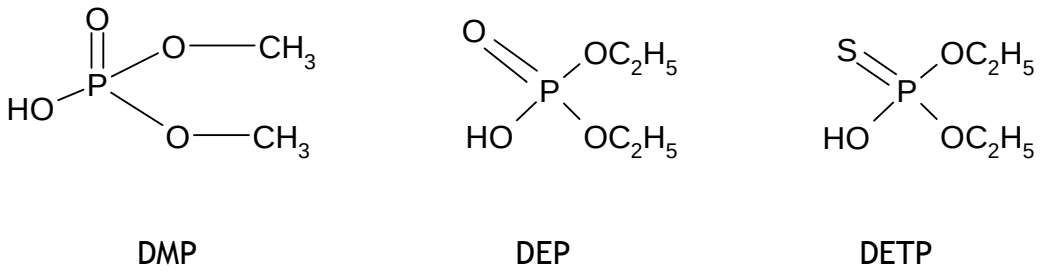


Chlorpyrifos



Dichlorvos

Figure 8-1: Chemical structures of organophosphates (OPs).



DMP DEP DETP

Figure 8-2: Chemical structures of dialkylphosphates (DAPs).

8.2 Experimental section

8.2.1 Chemicals and materials

Diazinon, chlorpyrifos, parathion, paraoxon, and dichlorvos were purchased from Sigma Aldrich (Dorset, UK) as 100μg/ml solutions in acetonitrile. Dimethylphosphate (DMP), diethylphosphate (DEP) and deithylthiophosphate (DETP) were also purchased from Sigma Aldrich and used to prepare standard solutions containing 100μg/ml in acetonitrile. Internal standard diazinon-d10 was purchased from Sigma Aldrich and prepared as a 100μg/ml solution in acetonitrile. Acetonitrile, methanol, cyclohexane, ethyl acetate, ammonium hydroxide, formic acid, isopropanol, dichloromethane and potassium dihydrogen phosphate were obtained from BDH (Poole, UK). Clean Screen ZSDAU020 cartridges were purchased from United Chemical Technology UK. Bond Elut LRC-C18 cartridges and Bond Elut Certify II cartridges were purchased from Varian (Palo Alto, CA). *tert*-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) containing 1 % *tert*-butyldimethylchlorosilane (TBDMCS) was purchased from Sigma Aldrich. Sheets of Guthrie card 903® specimen collection paper (210 x 297mm) were purchased from Whatman UK.

8.2.2 Solutions

8.2.2.1 Preparation of 0.04M phosphate buffer pH 2

Sodium dihydrogen orthophosphate (4.8 g) was dissolved in 800 ml DI water in a 1 L volumetric flask. The pH was adjusted to pH 2 with 1M hydrochloric acid. The volume was made up to the mark with DI water.

8.2.2.2 Preparation of 0.1M phosphate buffer pH 6.0

1 litre of solution was prepared by dissolving 1.7 g of anhydrous disodium orthophosphate and 12.14 g of sodium dihydrogen orthophosphate monohydrate in 800ml of DI water. The pH was adjusted to pH 6 with 1M potassium hydroxide and the solution made up to volume with DI water.

8.2.2.3 Preparation of 0.1M sodium acetate buffer pH 7 with 5% methanol

Sodium acetate trihydrate (6.8 g) was dissolved in 400 ml of DI water in a 500 ml volumetric flask. The pH was adjusted to pH 7 with 1M hydrochloric acid. The solution was made up to the volume with DI water then 25ml of the prepared solution was replaced with 25 ml of methanol.

8.2.2.4 Standard solutions of analytes

Working standards of unlabelled and labelled OPs and DAPs were prepared by diluting 100 µl from each OP stock solution in 10 ml acetonitrile to obtain a concentration of 1µg/ml. Stock solutions of standards and internal standard were stored at -20ºC.

8.2.3 Instrumentation (GC-MS)

A ThermoQuest GCMS instrument was used, fitted with an HP-5 capillary column (30m × 0.32mm × 0.25µm film thickness) and helium carrier gas. The column temperature was programmed from 100 °C to 300 °C at a rate of 12 °C /minute and held at 300 °C for 5 minutes. The temperature of the injector was 280 °C.

The split/splitless injection mode was used with a purge time of 40 s. The ionisation energy was 70eV in the EI+ mode.

8.2.4 Identification of retention times and fragmentation

Retention times and mass fragments for all compounds and their de-uterated standards were identified using unextracted standards after derivatision. 100 μl of standard working solutions of OPs, DAPs and internal standards (1 μg/ml) were evaporated to dryness under a stream of nitrogen at room temperature. For OPs, the residue was reconstituted in 50 μL of ethyl acetate; then 1 μl was injected directly into the GCMS, while DAP residues were derivatised in a mixture using 50 μl of ethyl acetate/MBSTFA+1% TDM (50:50 v/v) and placed in the heating block for 30 minutes. 1 μl was injected for analysis by GCMS (Table 8-1).

Table 8-1: Retention times and fragment ions of OPs, DAPs and internal standards.

Compound	Retention time (min.)	Fragment ions (m/z)
DMP	4.9	153, 183
DEP	6.06	155, 211
DETP	6.59	171, 227
Dichlorvos	3.94	109, 185
Chlorpyrifos	12.88	97, 197
Diazinon	10.94	137, 179
Parathion	12.91	97,139
Paraoxon	12.15	109, 149
Diazinon-D10	10.85	138, 153

The underlined ions were used for quantitation because they were the most intense ions obtained. Fragmentation of some compounds produced the same ions but this was acceptable if they had different retention times, for example, the ion at m/z 109 was produced from the fragmentation of dichlorvos and paraoxon but these OPs had different retention times. Diazinon-D10 was used as an internal standard.

8.2.5 Optimisation of derivatisation

Some authors reported that high temperature is required to derivatise DAPs which do not contain sulphur, while DAPs containing sulphur atoms must be derivatised at room temperature to avoid loss of sulphur by oxidation during this step.[229] Other authors proposed an intermediated temperature 40-50 °C to derivatise DAPs simultaneouslyand 40 °C was used for derivatisation in the next step.[239] OPs should not react with the derivatising reagent but an evaluation was made of their stability in the presence of the reagent.

Reconstitution of all compounds in the derivatising agent only gave poor detection for some OPs. Thus, OPs and DAPs were reconstituted in different solvent: derivatising agent ratios to optimise the response for all compounds. 100 μL of standard solutions of all compounds and internal standard, all at 1 μl/ml, were added to sealed vials in duplicate. Following evaporation, derivatisation was carried out at 40°C for 30 min using three different ratios of MTBSTFA +1%TBDMCS:ethyl acetate (EtOAc), which were 1:1 v/v, 2:3 v/v and 3:2 v/v. The reaction mixtures were subsequently analysed directly by GCMS and peak areas recorded. The results are shown in Table 8-2.

Table 8-2: Relative peak areas for OPs, DAPs and IS at different reagent compositions*.

COMPOUND	50 µl of (MTBSTFA+1%TBDMCS:EtOAc) at 40°C for 30 min		
	(1:1)	(2:3)	(3:2)
DMP	1.00	1.05	1.11
DEP	1.00	0.97	1.05
DETP	1.00	1.02	1.02
DICHLORVOS	1.00	1.12	0.51
CHLORPYRIFOS	1.00	1.34	1.39
DIAZINON	1.00	1.04	0.86
PARATHION	1.00	1.43	0.95
PARAOXON	1.00	1.02	0.96
Diazinon-D10	1.00	1.02	0.97

*Relative to the area obtained with the reagent with a 1:1 v/v ratio.

Table 8-2 demonstrates that the highest peak intensity for most analytes was produced with MBSTFA+1%TDM:EtOAc 2:3 v/v. Additionally, there was no significant enhancement in the sensitivity of DAPs when using more than 20 µl of the derivatising agent in the derivatising mixture, while OPs decreased when EtOAc was less than the derivatising agent, especially for dichlorvos. This ratio was selected for analyte reconstitution and derivatisation. In order to optimise the derivatisation reaction time for OPs and DAPs and enhance the response of DAPs without affecting OPs compounds, the derivatisation process was repeated in duplicate using three different reaction times at 30, 60, and 90 minutes. The results are presented in Table 8-3.

Table 8-3: Peak area at different incubation times

COMPOUND	Incubation time (minutes)		
	30 min.	60 min.	90 min.
DMP	1.00	1.18	1.14
DEP	1.00	1.11	1.10
DETP	1.00	0.96	0.94
DICHLORVOS	1.00	0.99	0.92
CHLORPYROFOS	1.00	0.82	0.79
DIAZINON	1.00	0.98	0.97
PARATHION	1.00	0.92	0.86
PARAOXON	1.00	1.05	1.01
Diazinon-D10	1.00	0.93	0.91

As shown in Table 8-3, the highest peak areas for DMP and DEP were obtained with a reaction time of 60 minutes, while for DETP at the optimum was 30 minutes. However, beyond 1 hour, all DAP peak areas decreased. Furthermore, the OP and diazinon-d10 areas decreased as the incubation time increased. The optimal reaction time for all compounds was 30 minutes and this was subsequently used for analyte derivatisation. Additionally, the antioxidant sodium metabisulfite (SOD) which was used to prevent conversion of DETP to DEP by oxidation, as described by Ueyama et al. was not added to the samples in this study to avoid introducing unwanted side-effects which might affect the stability of OPs, as, for example, resulted when NaF was added to samples containing these compounds.[88,101]

The final procedure adopted was as follows: the eluate obtained by SPE was evaporated to dryness at room temperature under a stream of nitrogen, the residue was derivatised using 50 μl of MTBSTFA+1%TBDMCS: EtOAc (2:3 v/v) in a sealed vial, which was vortexed and placed in a 40ºC heating block for 30 minutes; 1 μl was injected for analysis by GCMS.

8.2.6 Optimisation of extraction method

Most of the methods described in the literature for the extraction of OPs or DAPs were based on LLE, column clean-up, lyophilisation, azotropic distillation, and SPE using SAX columns.[219,222,224-226,232,234-236,240,241] However, because SPE has advantages over LLE, a new SPE method was developed to provide an efficient method to extract OPs and their alkyl phosphate metabolites which would be simple and robust enough for routine application. Three literature methods for the extraction of acidic compounds were selected to determine which could provide the highest recoveries for OPs and DAPs from blood samples. The procedures are listed in Table 8-4.[242-244]

Table 8-4: Procedures for three extraction methods used for comparison

Method	Extraction procedure
A[242]	Bond Elut C18 • 2ml methanol, 2ml 0.04M phosphate buffer pH2 • apply sample • 2ml 0.04M phosphate buffer pH2 • dry • elute with 3ml DCM/EtOAc (1:1 v/v)
B[243]	Bond Elut Certify® • 2ml methanol, 2ml distilled water, 2ml 0.1M phosphate buffer pH 6, • load sample • 2ml 0.1M phosphate buffer pH 6, 2ml 1M acetic acid • elute with 3ml DCM/EtOAc (4:1 v/v)
C[244]	Bond Elut Certify® II • 2ml methanol, 2ml 0.1M sodium acetate buffer pH 7 with 5% methanol • add sample • 1ml 0.1 M sodium acetate buffer pH 7 with 5% methanol

	• dry • elute with 3ml hexane/EtOAc (95:5 v/v)

Method evaluation was carried out by extracting 1ml blood containing 50 ng of each standard in duplicate. 50 ng of internal standard was added after extraction. An unextracted 50 ng standard containing 50ng of internal standard was also prepared in duplicate. The unextracted and extracted standards were derivatised and analysed by GCMS and recoveries were calculated by comparing the peak area ratios of the extracted and unextracted standards (Table 8-5).

Table 8-5: Mean recoveries for analytes, using three different methods

COMPOUND	Average recovery % (n=2)		
	Method A	Method B	Method C
DMP	43.6	17.1	59.5
DEP	53.8	8.8	65.8
DETP	42.4	18.8	64.3
DICHLORVOS	57.8	59.4	83.6
CHLORPYRIFOS	35.8	31.2	79.5
DIAZINON	31.5	16.7	87.6
PARATHION	26.7	14.2	79.6
PARAOXON	25.9	22.6	92.5

As shown in Table 8-5, the highest recoveries were obtained using method C and this method was used subsequently. However, the method was modified to improve the recoveries of DAP analytes, which showed the lowest recoveries obtained by this procedure.

8.2.6.1 Comparison of elution solvents

The sorbent in the Certify II column has strong anion (quaternary amine) exchange residues, which are always positively charged, while the analyte contains a negative charge at pH 7. Thus, the interaction will be between the negative charge of the analyte and the positive charge on the sorbent. Furthermore, the presence of a hydrophobic sorbent enables an extra mechanism of attraction of analytes to the cartridge. Since DAPs have a high polarity and acidity, these compounds required strong polar, acidified solvents to neutralise the acidic compounds and elute them.

The results in Table 8.5 show that DAPs have low recoveries compared to OPs and further investigation was conducted to improve this. The previous procedure was repeated using a different elution solvent containing ethyl acetate with a high concentration of formic acid (98:2 v/v). Each extraction was carried out in duplicate. The results are shown in Table 8-6.

Table 8-6: Average recoveries obtained for OPs and DAPs using different elution solvents

Compound	Average recovery % (n=2)	
	Hexane/EtOAc (95:5)	EtOAc:Formic Acid (98:2 v/v)
DMP	58.2	72.1
DEP	62.6	83.2
DETP	71.1	89.0
DICHLORVOS	81.8	91.7
CHLORPYRIFOS	86.4	83.9
DIAZINON	87.5	92.9
PARATHION	74.3	97.5
PARAOXON	82.7	102.3

As shown in Table 8-6, acidification of the elution solvent gave improved recoveries compared to hexane/EtOAc. This acidified elution solvent reduced

the ionisation of the acidic compounds, which allowed them to be eluted with the strong elution solvent. However, DMP showed a low recovery because it is a very strong acid with a pK_a around 2 which would need a more acidic elution solvent. The final procedure used to extract OPs and DAPs is listed in Table 8-7.

Table 8-7: Optimised extraction and derivatisation method for OPs and DAPs.

Step	Procedure
Sample preparation	• 1ml of blood added to 2ml of 0.1M sodium acetate buffer pH 7 with 5 % methanol • 100 μl of internal standard added • Vortex for 5 minutes • Centrifuge at 2500rpm for 5 minutes.
Conditioning	• 2ml methanol • 2ml 0.1 M sodium acetate buffer pH7 with 5% methanol • Add sample.
Washing	• 1ml 0.1 M sodium acetate buffer pH7 with 5% methanol
Elution	• 2ml EtOAc:Formic Acid (98:2 v/v)
Derivatisation	• Evaporate to dryness under stream of nitrogen • Vortex mix with 50μl of (2:3) MBSTFA+1%TDM:EtOAc in a sealed vial • Place in a 40 °C heating block for 30 minutes • 1μl injected for analysis by GCMS.

The optimised method summarised in table 8-7 was used in method validation and stability studies. However, for DBS samples the spots were cut from the sheet with scissors, transferred to extraction tubes and 3.5 ml extraction buffer were added. The tube was vortex-mixed and placed in an ultrasonic bath for one hour to extract the drugs from the paper. The paper was discarded then the extract was centrifuged at 2500 RPM for 5 minutes.

8.2.7 Method validation

The validation parameters applied in this study were similar to parameters in Table 4-1 except that the recovery and precision were determined at two concentrations, 50 ng and 100 ng/ml.

8.2.7.1 Linearity

The relationships between analyte concentrations and peak area ratios were linear over the range 10-400 ng/ml, and the linear correlation coefficients (r^2) were better than 0.99 for all analytes, as shown in Table 8-8.

8.2.7.2 LODs and LLOQs

LOD values for all analytes were 2.30-7.10 ng/ml blood and LLOQ values were 7.50-23.70 ng/ml blood. The results are shown in Table 8-8. The high LOD and LLOQ values obtained for analytes in this study compared to those obtained in the literature are attributed to the sensitivity of the GCMS instrument used in this experiment.

Table 8-8: Linear correlation coefficients, LODs and LLOQs of DAPs and OPs in blood.

Compound	Linear correlation coefficients (r^2)	LOD ng/ml	LLOQ ng/ml
DMP	0.991	5.80	19.40
DEP	0.993	4.60	15.30
DETP	0.992	5.40	18.00
DICHLORVOS	0.998	2.60	8.60
CHLOPYRIFOS	0.999	7.10	23.70
DIAZINON	0.994	2.30	7.50
PARATHION	0.992	5.20	17.40
PARAOXON	0.997	6.00	20.00

8.2.7.3 Recoveries

Recoveries of analytes were determined at two concentrations above the LLOQs (50 ng and 100 ng/ml, n=5). The recoveries for all analytes were in the range of 75-101%, as shown in Table 8-9.

Table 8-9: Recoveries of DAPs and OPs.

COMPOUND	MEAN RECOVERY % (RSD%, n=5)	
	50ng/ml	100ng/ml
DMP	75 (9)	78 (8)
DEP	85 (11)	82 (5)
DETP	81 (13)	86 (7)
DICHLORVOS	98 (14)	93 (11)
CHLOPYRIFOS	90 (7)	95 (5)
DIAZINON	97 (6)	101 (6)
PARATHION	90 (7)	95 (5)
PARAOXON	99 (13)	97 (5)

8.2.7.4 Intraday and interday precision

The precision obtained by this method was good, and the relative standard deviations (RSD %) for intra- and interday precisions were in the ranges 0.9-9.8 and 1.1-10.8%, respectively, which are considered acceptable, as shown in Table 8-10.

Table 8-10: Intraday and interday precision results.

Compound	Intraday mean (RSD%, n=5)		Interday mean (RSD%, n=5)	
	50ng/ml	100ng/ml	50ng/ml	100ng/ml
DMP	51(5.5)	103(1.6)	50(10.2)	104(2.8)
DEP	50(9.8)	90(2.6)	50(10.8)	98(3.1)
DETP	48(4.2)	99(1.3)	46(5.4)	96(4.5)
DICHLORVOS	50(4.5)	101(2.9)	50(5.5)	101(2.7)
CHLOPYRIFOS	49(5.6)	101(4.5)	48(5.9)	97(6.7)
DIAZINON	52(2.7)	101(1.3)	53(2.6)	101(1.1)
PARATHION	48(5.0)	100(3.9)	47 (7.2)	100(4.1)
PARAOXON	49(1.8)	100(0.9)	47(4.2)	99(1.7)

8.2.8 Stability study

8.2.8.1 Stabilisation study using NaF

It has previously been reported that the addition of NaF to blood samples accelerates the degradation of dichlorvos and chlorpyrofos. A similar experiment was done here to assess the degradation of five OPs and to quantify their degradation products (DAPs).

Blank blood (100 ml, pH 7.2) was divided into 5 x 20ml portions. Portions 1, 2, 3, 4, and 5 were spiked with dichlorvous, chlorpyrifos, diazinon, parathion, and paraoxon, respectively, to produce a final concentration of 1000 ng/ml. Each portion was vortex mixed for 30 minutes on roller, then extracted in duplicate, and these concentrations were designated as the time zero concentrations. The rest of the prepared samples were spiked with NaF to give a final concentration of 2% (w/v) for each portion, which were then stored at room temperature. The prepared samples were analysed after 5 and 15 minutes, and 3, 15, 24, 48, and 72 hours in duplicate. The pH value was measured after addition of NaF and before each analysis using a pH meter.

8.2.8.2 Stabilisation in DBS

Blank blood (10 ml) was divided into 5 x 2ml portions and each portion was spiked with a single OP, dichlorvous, chlorpyrifos, diazinon, parathion and paraxon at 1000 ng/ml. The prepared samples were vortex mixed for 30 minutes on roller then 100 μl of each spiked preparation was spotted on five separate sheets of paper to produce 20 spots, each containing 100 ng of the compound. The samples were analysed in duplicate at time zero, after 5 and 15 minutes, and 3, 15, 24, 48, and 72 hours. These results were compared with those of the previous experiment involving NaF. The papers holding the prepared samples were stored at room temperature in sealed plastic bags.

8.3 Results

8.3.1 Dichlorvos

Dichlorvos rapidly decomposed after the addition of NaF to the sample, and only a small amount of the compound was detected after 5 minutes, which was less than 3% of the original concentration. It then completely disappeared after 15 minutes. Its degradation product, DMP, was detected 5 minutes after the addition of NaF to the blood and then decomposed gradually to 260 ng/ml after 72 hours. The pH value of the sample dropped from pH 7.8 after addition of NaF to pH 6.5 at the end of the study. On the other hand, the degradation of dichlorvos in DBS was much slower than in the aliquots containing NaF, and approximately 75% of the parent compound could be detected after 72 hours. Very little DMP was detected after 24 hours of spotting, increasing gradually to 11ng/spot. The stability results are shown in Tables 8-11 and 8-12.

Table 8-11: Stability of dichlorvos in blood samples preserved with 2% (w/v) NaF

TIME	DICHLORVOS (RSD%, n=2)	DMP (RSD%, n=2)	Total	pH
	ng/ml	ng/ml	ng/ml	
Zero	983 (1.3)	0 (0.0)	983	7.8
5 MINS	30 (6.7)	426 (2.1)	456	7.5
15 MINS	0 (0.0)	395 (1.4)	395	7.4
3 HOURS	0 (0.0)	368 (2.5)	368	7.2
15 HOURS	0 (0.0)	336 (1.3)	336	7.1
24 HOURS	0 (0.0)	298 (2.2)	298	6.8
48 HOURS	0 (0.0)	281 (1.3)	281	6.7
72 HOURS	0 (0.0)	260 (2.8)	260	6.5

Table 8-12: Stability of dichlorvos in dried blood spots stored on filter paper

TIME	DICHLORVOS (RSD%, n=2)	DMP (RSD%, n=2)	Total
	ng/spot	ng/spot	ng/spot
Zero	101 (2.0)	0 (0.0)	101
5 MINS	101 (1.4)	0 (0.0)	101
15 MINS	102 (2.7)	0 (0.0)	102
3 HOURS	100 (1.3)	0 (0.0)	100
15 HOURS	98 (1.5)	0 (0.0)	98
24 HOURS	89 (2.2)	6 (12.8)	95
48 HOURS	81 (1.2)	9 (8.3)	90
72 HOURS	76 (1.3)	11 (6.1)	87

8.3.2 Chlorpyrifos

After the addition of NaF to the sample containing chlorpyrifos, the concentration of the compound decreased gradually and lost more than 62% of the day zero concentration. DETP appeared after 15 minutes and the concentration increased gradually during the observation period. The pH of the sample dropped from pH 7.6 at time of adding the NaF to pH 6.6 at the end of study.In DBS, the degradation of chlorpyrifos was very slow and the remaining content of the parent compound was more than 80% of the starting concentration, while DETP was detected in small concentrations after 72 hours. The stability results are shown in Tables 8-13 and 8-14.

Table 8-13: Stability of chlorpyrifos in blood samples preserved with 2% (w/v) NaF.

TIME	CHLORPYRIFOS (RSD%, n=2)	DETP (RSD%, n=2)	Total	pH
	ng/ml	ng/ml	ng/ml	
Zero	1009 (0.5)	0 (0.0)	1009	7.6
5 MINS	941 (0.4)	0 (0.0)	941	7.6
15 MINS	928 (1.5)	25 (3.1)	953	7.5
3 HOURS	821 (2.3)	82 (1.7)	903	7.3
15 HOURS	626 (2.3)	184 (1.1)	810	7.2
24 HOURS	517 (2.5)	216 (1.6)	733	6.9
48 HOURS	462 (2.7)	230 (2.4)	692	6.8
72 HOURS	381 (1.8)	264 (1.0)	645	6.6

Table 8-14: Stability of chlorpyrifos in dried blood spots stored on filter paper.

TIME	CHLORPYRIfOS (RSD%, n=2)	DETP (RSD%, n=2)	Total
	ng/spot	ng/spot	ng/spot
Zero	101 (0.6)	0 (0.0)	101
5 MINS	98 (1.5)	0 (0.0)	98
15 MINS	93 (0.8)	0 (0.0)	93
3 HOURS	96 (1.2)	0 (0.0)	96
15 HOURS	92 (0.8)	0 (0.0)	92
24 HOURS	91 (0.9)	4 (5.5)	95
48 HOURS	89 (1.2)	5 (6.7)	94
72 HOURS	87 (1.9)	6 (8.1)	93

8.3.3 Diazinon

The diazinon concentration decreased gradually after the addition of NaF to the sample and approximately 43% of the original concentration was detected after 72 hours, while DETP started to appear 3 hours after the start time and continuously increased throughout the observation time. The pH dropped from pH 7.6 to pH 6.8. In DBS, the diazinon concentration decreased very slowly, and approximately 81% of the original concentration was detected after 72 hours. A small amount of DETP was detected after 15 hours and increased to a small amount at the end of the study. The stability results are shown in Tables 8-15 and 8-16.

Table 8-15: Stability of diazinon in blood samples preserved with 2% (w/v) NaF.

TIME	DIAZINON (RSD%, n=2)	DETP (RSD%, n=2)	Total	pH
	ng/ml	ng/ml	ng/ml	
Zero	1002 (1.5)	0 (0.0)	1002	7.7
5 MINS	956 (0.9)	0 (0.0)	956	7.6
15 MINS	944 (0.4)	0 (0.0)	944	7.6
3 HOURS	845 (0.9)	47 (3.1)	892	7.2
15 HOURS	682 (0.8)	121 (3.9)	803	7.1
24 HOURS	594 (1.9)	163 (1.8)	757	6.9
48 HOURS	464 (1.5)	225 (1.9)	689	6.8
72 HOURS	431 (1.8)	238 (1.2)	669	6.8

Table 8-16: Stability of diazinon in dried blood spots stored on filter paper

TIME	DIAZINON (RSD%, n=2)	DETP (RSD%, n=2)	Total
	ng/spot	ng/spot	ng/spot
Zero	98 (0.6)	0 (0.0)	98
5 MINS	99 (1.1)	0 (0.0)	99
15 MINS	100 (0.4)	0 (0.0)	100
3 HOURS	93 (1.3)	0 (0.0)	93
15 HOURS	84 (0.9)	7 (9.4)	91
24 HOURS	82 (1.7)	8 (5.2)	90
48 HOURS	79 (1.3)	8 (3.9)	87
72 HOURS	80 (1.9)	8 (2.8)	88

8.3.4 Parathion

Degradation of parathion was observed after the addition of NaF to the blood samples and the remaining content after 72 hours was approximately 15% of the original concentration. The degradation product of parathion was detected after 5 minutes and increased gradually until the end of the experiment. During the degradation of parathion and the increase of DETP, the pH value of the sample dropped from pH 7.8 to pH 6.6 at the end of the study. In DBS, the parathion concentration decreased slowly and more than 82% of the parent compound could be detected at the end of study. The degradation product was detectedable after 15 hours. The stability results are shown in Tables 8-17 and 8-18.

Table **8-17: Stability of parathion in blood samples stored with 2% (w/v) NaF.**

TIME	PARATHION (RSD%, n=2)	DETP (RSD%, n=2)	Total	pH
	ng/ml	ng/ml	ng/ml	
Zero	1000 (0.6)	0 (0.0)	1000	7.8
5 MINS.	833 (0.3)	75 (4.9)	908	7.7
15 MINS.	727 (0.9)	112 (3.7)	839	7.5
3 HOURS	580 (1.8)	137 (2.1)	717	7.2
15 HOURS	414 (2.8)	217 (1.9)	631	7.1
24 HOURS	366 (3.4)	243 (1.2)	609	6.9
48 HOURS	245 (4.7)	290 (3.6)	535	6.8
72 HOURS	154 (4.9)	325 (2.3)	479	6.6

Table 8-18: Stability of parathion in dried blood spots stored on filter paper.

TIME	PARATHION (RSD%, n=2)	DETP (RSD%, n=2)	Total
	ng/spot	ng/spot	ng/spot
Zero	96 (0.2)	0 (0.0)	96
5 MINS.	93 (0.8)	0 (0.0)	93
15 MINS.	93 (0.6)	0 (0.0)	93
3 HOURS	90 (0.3)	0 (0.0)	90
15 HOURS	83 (0.9)	5 (3.4)	88
24 HOURS	82 (1.6)	6 (3.4)	88
48 HOURS	79 (2.2)	8 (4.6)	87
72 HOURS	79 (2.4)	8 (6.2)	87

8.3.5 Paraoxon

Paraoxon decomposed gradually 5 minutes after the addition of NaF and completely disappeared after 24 hours, while its degradation product DEP was detected after 5 minutes and increased gradually during the degradation of the parent compound. It then decreased gradually after 15 hours and continued to decrease until the end of the study. The sample pH value decreased from pH 7.9 after the addition of NaF to pH 6.9 at the end of the study. Paraoxon in DBS decreased slowly during the study and approximately 76% of the original concentration was recovered at the end. DEP was detected in a small amount after 24 hours and at the end of the study. The stability results are given in the table 8-19 and 8-20.

Table 8-19: Stability of paraoxon in blood sample preserved with 2% (w/v) NaF.

TIME	PARAOXON (RSD%, n=2)	DEP (RSD%, n=2)	Total	pH
	ng/ml	ng/ml	ng/ml	
Zero	999 (0.2)	0 (0.0)	999	7.9
5 MINS.	790 (0.1)	89 (3.2)	879	7.9
15 MINS.	625 (0.4)	115 (1.3)	740	7.7
3 HOURS	107 (3.4)	308 (1.2)	415	7.6
15 HOURS	56 (5.2)	315 (0.9)	371	7.5
24 HOURS	0 (0.0)	203 (1.7)	203	7.3
48 HOURS	0 (0.0)	191 (1.5)	191	7.1
72 HOURS	0 (0.0)	188 (3.1)	188	6.9

Table 8-20: Stability of paraoxon in dried blood spot stored on filter paper.

TIME	PARAOXON (RSD%, n=2)	DEP (RSD%, n=2)	Total
	ng/spot	ng/spot	ng/spot
Zero	102 (1.4)	0 (0.0)	102
5 MINS.	101 (2.1)	0 (0.0)	101
15 MINS.	101 (1.1)	0 (0.0)	101
3 HOURS	99 (0.2)	0 (0.0)	99
15 HOURS	97 (0.8)	0 (0.0)	97
24 HOURS	85 (0.7)	6 (10.8)	91
48 HOURS	83 (0.9)	7 (5.5)	90
72 HOURS	78 (1.8)	9 (6.5)	87

8.3.6 Summary of pesticides stability using different preservative methods

Summary of pesticides stability after 3 days using 2% (W/V) NAF or DBS as stabilizing method is shown in Table 8-21.

Table 8-21: Summary of pesticides stability in DBS or using 2% (W/V) NAF

Compound	Conditions	Results	Comment
Dichlorvos	2% (W/V) NAF, 3 days	100% decrease after 15 minutes	• DAP concentration increased as parent compound concentration decreased • Stability increased when using filter paper instead of NAF • NAF accelerated formation of degradation compounds • NAF must not be used as a preservative for OPs compounds • The extent of degradation is different from compound to compound and is related to the chemical structure
	DBS, 3 days	25% decrease	
Chlorpyrifos	2% (W/V) NAF, 3 days	62% decrease	
	DBS, 3 days	13% decrease	
Diazinon	2% (W/V) NAF, 3 days	57% decrease	
	DBS, 3 days	18% decrease	
Parathion	2% (W/V) NAF, 3 days	85% decrease	
	DBS, 3 days	18% decrease	
Paraoxon	2% (W/V) NAF, 3 days	100% decrease after 24 hours	
	DBS, 3 days	24% decrease	

8.4 Discussion

OPs were found to be unstable under different storage temperatures as reported previously.[87] The presence of ester functional groups in the OPs made them prone to hydrolysis reactions, which accelerated under alkaline or acidic conditions to produce acidic and phenolic compounds as degradation products.

Esterases metabolise OPs through hydrolysis to produce dialkylphosphates and a leaving group. These enzymes are divided into three groups: A, B, and C. The classification is based on their activity toward phosphate triesters. Esterase activity is known to continue a short time after death and some of these enzymes can hydrolyse OPs, such as A-esterase (paroxygenase), while others are inhibited by OPs, such as AChE. The addition of an anti-enzymatic compound is essential to preserve the OPs against the activity of these enzymes.[202]

NaF is the most common preservative, usually used in forensic toxicology to preserve the analyte from decomposition by enzymes or bacterial activity that may take place in the sample and may influence compound stability. Although NaF can inhibit the enzyme that hydrolyses OPs, its addition to the blood sample was found to accelerate the degradation of these compounds, as reported previously and shown again in this study.[88,99] The blood pH became more alkaline during hydrolysis of the OPs and the degradation can be attributed to an autocatalytic alkaline hydrolysis. Hydrolysis of OPs yields DAPs as major degradation products and their detection in a sample usually indicates OP exposure. However, these markers are common to several OPs and are not specific for a particular starting compound. They are generally analysed to assess OP exposure. According to the chemical structure of the compound, each OP is hydrolysed to yield a single DAP. For example, dichlorvos is hydrolysed to produce DMP, while chlorpyrifos, diazinon, and parathion are hydrolysed to produce DETP and paraoxon is hydrolysed to produce DEP. Therefore, the detection of DAP can indicate the presence of a specific OP. All compounds were sensitive to NaF and the pH increased after the addition of this preservative to the sample. The decomposition rates of the compounds were not the same because of differences in structure.

Dichlorvos and paraoxon were found to be less stable than chlorpyrifos, diazinon, and parathion. They also decomposed very quickly, which may be because both compounds are derivatives of orthophphoric acid whereas the other compounds are derivatives of thiophosphoric acid. Oxygen is more electronegative than sulphur and thiophosphates exhibit more resistance to hydrolysis than dichlorvos and paraoxon. Furthermore, diazinon showed greater stability than chlorpyrifos and parathion, possibly because there was no electron withdrawing substituent in the leaving group of diazinon, unlike chlorpyrifos, which has 3 chlorine atoms in its structure and parathion, which has a nitro group that could accelerate the hydrolysis process.

The role of NaF may not only be to increase the pH of the sample to alkaline, but also to supply fluoride anion to act as a nucleophile, which attacks the positively charged phosphorus atom, thereby accelerating the decomposition process.

The findings of the current study are in agreement with the results of Moriya et al. who reported that dichlorvos completely disappeared within 15 minutes of the addition of NaF to the blood sample while chlorpyrifos was found to be very unstable under these conditions.[88] In the present study, all compounds showed poor stability after the addition of NaF.

The drop in pH during the study is attributed to the acidity of DAPs, produced when the parent compound decomposed while the loss in the concentration was decreased in acidic pH. The parent OPs decomposed more rapidly than the DAP products, which may degrade further to monoalkyl phosphates and then to inorganic phosphate. The advantages of detection of DAPs in addition to the parent OPs, are that they can be detected even when the parent compounds have completely disappeared.

Storing OPs in DBS minimised the hydrolysis degradation of all compounds compared to the addition of NaF, and all compounds could be detected after 3 days. Furthermore, DAPs were detected in small levels. Therefore, the degradation of OPs at room temperature or during transportation at a high temperature should be considered and preserving these compounds on filter paper would help to minimise their degradation. A practical approach to the use of DBS would be to collect blood at autopsy as usual and spot an aliquot on

paper, allow it to dry and then seal in a plastic or paper bag, the latter being used for wet DNA samples.

The initial blood pH ranged from 7.2 to 7.4 and, as observed in this study, OPs were found to decompose continuously. Acidification of the sample with EDTA/K or heparin/lithium reduced the degradation of OPs, as reported by Asri.[99] Alos in this study and under acidic conditions, OPs were noted to decrease slower than in an alkaline pH. Furthermore, these additives are anticoagulants and don't inhibit esterase, which may be present in the blood sample. Therefore, dehydration of the sample in filter paper is a promising preservation method to minimise the enzymatic and chemical hydrolysis of these compounds.

The total amount of OPs and their DAP metabolites can be used to indicate the original OP concentration. Further studies seem to be necessary in order to assumption of the OP and its specific leaving group product (if they are available commercially), in addition to the specific DAP and its further degradation products in order to give the original OP concentration.

Decomposition of OP after death may result from the continuous activity of the esterase enzyme and the concentration of OP in a suspected poisoning case is lower than at the time of death. Detection of the degradation products may help forensic toxicologists estimate the concentration of OPs that may have caused the death.

8.5 Conclusions

In conclusion, NaF should not be added as a preservative to blood samples containing OPs as it may act as a catalyst for their hydrolysis. The rate of decomposition can be reduced when blood samples are stored in filter paper, which was found to be a promising alternative method to preserving OPs against degradation. The concentrations of degradation products for each OP can be added to those of the parent compounds to estimate the original concentration. Dehydration of the sample stored in paper, in addition to the assumption of degradation products to the parent compound, could help to preserve and indicate the original concentration of the parent compound at the time of sampling. Furthermore, storing the DBS samples in a cold place, such as the freezer, will enhance the stability, as described earlier in the previous chapters of this thesis.

9 Conclusions and Future Work

The possibility of a decrease in concentration of an analyte in biological specimens during storage should be considered, even if samples are stored at -20ºC. Under the same storage conditions different compounds exhibit different stabilities according to their chemical structures. Drugs containing ester or amide groups are prone to degradation by hydrolysis reactions, and this should be carefully considered when they are being transported or stored and during the interpretation of results. Although oxazepam, lorazepam, alprazolam and estazolam belong to the same drug class, they exhibit different stabilities that can be attributed to the presence of an amide group in oxazepam and lorazepam which make these compounds more sensitive to degradation by hydrolysis reactions, leading to a decrease in their concentration during storage, whereas alprazolam and estazolam exhibit more stability due to the presence of a triazolo ring instead of an amide group that make these compounds more resistant to this degradation process and more stable for long time periods. Addition of preservatives and anticoagulants to samples containing benzodiazepines showed little enhancement of stability without a possible loss of concentration by preservatives used NaF while anticoagulant should be added to blood samples to prevent them from coagulating, which makes sample analysis difficult. High and low concentrations of the same drug showed different stabilities: high concentrations can be detected for a long time in comparison to low concentrations, and this should be considered in the interpretation of samples with either therapeutic or toxic concentrations of drugs.

The opiate glucuronides M3G, M6G and C6G exhibited different stabilities under different storage conditions and in different matrices. These metabolites showed more stability when stored at negative temperatures however even then significant losses were observed and these could influence the interpretation. Samples should be stored in a freezer at -20 °C or less in order to preserve the compounds for a long period, while additives should be added even if they result in only a small improvement in stability as this may be greater in post-mortem samples, which are known to contain bacteria that may accelerate the decomposition of these compounds and limit their detection. C6G was more stable than M3G and M6G which can be explained on the basis of differences in their chemical structures. M3G and M6G may undergo both hydrolysis and

oxidation reactions because of the presence of a phenolic moiety in their structures while in C6G this group is alkylated, so further study is suggested on the use of antioxidant substances, such as ascorbic acid or sodium metabisulfite to examine their effects on the stability of morphine metabolites with respect to oxidation. Storage conditions were not the only factor influencing the stability of these metabolites, but the nature of the biological matrix also had an effect: they are more stable in blood than urine, which should be considered in interpretation of results.

An assessment was made of the degradation of diazepam, temazepam, chlordiazepoxide and oxazepam stored in blood and urine for one month at a high temperature of 80 °C under alkaline and acidic conditions. Under all conditions, the degradation of diazepam and temazepam in blood and urine produced MACB as the main hydrolysis product, while degradation of oxazepam and chlordiazepoxide produced ACB as the main hydrolysis product. The rate of degradation is accelerated most by alkaline conditions, then by high temperature then by acidic conditions. Similar to opiate glucuronides, the degradation of benzodiazepines in urine is faster than in blood. The pH of the matrix and lack of protective protein binding in urine may explain this. The degradation product MACB was found to be degraded further into ACB. A validated method was applied to case samples of blood and urine and the degradation products MACB and ACB were detected in all urine samples but not in blood. That may have been because these compounds were influenced by urine matrix or affected by enzymes used in the sample preparation step of the analysis to cleave the glucuronides. MACB and ACB concentrations increased as the degradation of the drugs proceeded and they could be detected when the parent drugs had completely disappeared. MACB and ACB can be reliably detected in whole blood and urine and can be used to correct for losses of parent drugs. They can also indicate the original presence of these compounds if case samples were suspected to have contained benzodiazepines and the parent compounds were not detected.

A new method was developed and validated to determine OPs and their degradation product DAPs by SPE followed by GCMS analysis after suitable derivatization. Addition of preservatives to the blood samples containing OPs elevated the pH value of the samples, thereby accelerating the degradation of

these compounds. However, during this degradation process DAP compounds were produced and these increased in concentration as the parent compounds decreased. Esters of orthophosphoric acid are degraded faster than those of thiophosphoric acid because the electronigativity of oxygen is higher than sulphur. Storage of blood samples containing OPs in filter paper showed enhancement of their stability compared to the use of preservatives and the compounds were detected for longer time periods. The DBS technique has been shown to be useful in reducing the enzymatic and chemical hydrolysis of these compounds.

For benzodiazepines and cocaine a validated method was developed which can be used for the quantitative analysis of drugs in small volumes of blood contained in dry blood spots. The DBS technique provides a suitable procedure for the storage and analysis of samples in forensic toxicology because they are easy to handle, transport and to store in the laboratory, even in the absence of refrigeration, which can be a problem in some countries. It permits the analysis of a small volume of sample and, as a result, frequent collection of blood samples from living donors is possible. In addition, the test analytes chosen for this study were drugs which are known to be rapidly degraded in biological samples and these were preserved when stored on paper, instead of adding the usual preservatives and anticoagulants. For some substances the addition of preservatives is not recommended as they may accelerate degradation, for example, organophosphorus pesticides, while anticoagulants are not recommended for other drugs as they can increase their concentrations, for example morphine. The DBS technique is therefore recommended as an additional procedure to be used in conjunction with conventional methods for preserving unstable drugs from decomposition and for avoiding potential errors in interpretation of analytical results resulting from the addition or absence of preservatives and anticoagulants.

The water present in blood samples plays a very important role in hydrolysis reactions as an active agent which cleaves drug molecules. The use of filter paper to store samples resulted in enhancement of drug stability due to the dehydration of the sample and consequent minimalisation of enzymatic and chemical hydrolysis of the drugs and hydrolycally labile compounds. Degradation of drugs in DBS at all storage conditions was less than for the corresponding liquid blood samples stored under similar conditions.

Interpretation of results obtained from samples stored long-term should be carefully interpreted to avoid serious errors. Ideally, analyses should be performed as soon as sample received and the samples should be stored at -20 °C or lower to keep the drug stable for a long time. The preservative should be carefully selected according to the analyte while NaF should not be added to samples containing OP compounds.

Extended transportation time and increased environmental temperature may affect the pH of the sample, for example the pH of urine samples may exceed pH 9 within 2 days, destroying drugs and giving false negative results. Degradation products are promising compounds as markers that can indicate the former presence of specific drugs in cases in which the parent drug has been lost due to poor storage conditions. Further preservation of the sample in filter paper can keep the drug stable for a longer period of time. Stabilization of drugs using filter paper in addition to quantification of degradation products can help in estimating the original concentrations of compounds at the time of sampling.

The DBS technique also needs to be validated and used for other groups of drugs to assess their stability using this technique in comparison to liquid samples. In some countries, including Saudi Arabia, urine samples are usually transported from distant places and that may take 1 or 2 days before they arrive in the central screening lab. Most of the samples are negative despite the suspected presence of drugs, and that may be as a result of poor storage conditions during transportation under high temperature. Stabilization of samples using the DBS technique may present an inexpensive, practical method to stabilize drugs of interest during transportation.

Further studies should be carried out to determine the degradation products of other benzodiazepines such as ANB, ADB and MNFB which produced from degradation of nitrazepam, lorazepam and flunitazepam respectively. Similarly, further work would permit the determination of degradation products of other OPs, in particular DAPs and their leaving groups.

10 References

1. Timbrell J. ***introduction to toxicology*****. 3 ed. taylor and francis; 2002.**

2. Klaassen C. ***Toxicology the basic science of poisons*****. 7 ed. Mcgraw-Hill; 2008.**

3. Eckert WG. ***introduction to forensic science*****. 2 ed. CRS Press; 1997.**

4. Levine B. ***principles of forensic toxicology*****. 2 ed. AACC press; 2003.**

5. Haddad LM, Shannon MW, Winchester JF. ***Clinical management of poisoning and drug overdose*****. 3 ed. Saunders Company; 1998.**

6. Bernhoft IM, Steentoft A, Johansen SS, Klitgaard NA, Larsen LB, Hansen LB. Drugs in injured drivers in Denmark. ***Forensic Science International*** **2005; 150(2-3):181-189.**

7. Skurtveit S, Abotnes B, Christophersen AS. Drugged drivers in Norway with benzodiazepine detections. ***Forensic Science International*** **2002; 125(1):75-82.**

8. Kintz P, Villain M, Cheze M, Pepin G. Identification of alprazolam in hair in two cases of drug-facilitated incidents. ***Forensic Science International*** **2005; 153(2-3):222-226.**

9. Eltahir K. ***Narcotics and mind-manifesting drus*****. 2002.**

10. Mottram D. ***Drugs in sport*****. 4 ed. taylor and francis group; 2005.**

11. Rang HP, Dale MM, Ritter JM, Moore PK. ***Pharmacology*****. 5 ed. Churchill Livingstone; 2003.**

12. Kaushal R, Dave KR, Katyare SS. Paracetamol hepatotoxicity and microsomal function. ***Environmental Toxicology and Pharmacology*** **1999; 7(1):67-74.**

13. Fleming FJ, Vytopil M, Chaitow J, Jones HR, Darras BT, Ryan MM. Thalidomide neuropathy in childhood. ***Neuromuscular Disorders*** **2005; 15(2):172-176.**

14. Melchert M, List A. The thalidomide saga. *International Journal of Biochemistry & Cell Biology* 2007; 39(7-8):1489-1499.

15. Barrett EL. Glucose-6-Phosphate Dehydrogenase Deficiency. A Brief Review. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1966; 60(2):267-&.

16. Eichelbaum M, Gross AS. The Genetic-Polymorphism of Debrisoquine Sparteine Metabolism - Clinical Aspects. *Pharmacology & Therapeutics* 1990; 46(3):377-394.

17. Elhkim MO, Heraud F, Bemrah N, Gauchard F, Lorino T, Lambre C et al. New considerations regarding the risk assessment on Tartrazine - An update toxicological assessment, intolerance reactions and maximum theoretical daily intake in France. *Regulatory Toxicology and Pharmacology* 2007; 47(3):308-316.

18. Roe FJC, Levy LS, Carter RL. Feeding Studies on Sodium Cyclamate, Saccharin and Sucrose for Carcinogenic and Tumour-Promoting Activity. *Food and Cosmetics Toxicology* 1970; 8(2):135-&.

19. Chou JH, Hwang PH, Malison MD. An Outbreak of Type-A Foodborne Botulism in Taiwan Due to Commercially Preserved Peanuts. *International Journal of Epidemiology* 1988; 17(4):899-902.

20. Abdulkadar AHW, Al Ali AA, Al Kildi AM, Al Jedah JH. Mycotoxins in food products available in Qatar. *Food Control* 2004; 15(7):543-548.

21. Aldridge WN. The Toxic Oil Syndrome (Tos, 1981) - from the Disease Towards A Toxicological Understanding of Its Chemical Etiology and Mechanism. *Toxicology Letters* 1992; 64-5:59-70.

22. Bayen S, Giusti P, Lee HK, Barlow PJ, Obard JP. Bioaccumulation of DDT pesticide in cultured Asian seabass following dietary exposure. *Journal of Toxicology and Environmental Health-Part A-Current Issues* 2005; 68(1):51-65.

23. Chater S, Douki T, Garrel C, Favier A, Sakly M, Abdelmelek H. Cadmium-induced oxidative stress and DNA damage in kidney of pregnant female rats. *Comptes Rendus Biologies* 2008; 331(6):426-432.

24. Huber H. Vinyl-Chloride Problem. *Chemie Ingenieur Technik* 1975; 47(19):803-807.

25. Nymark P, Wikman H, Hienonen-Kempas T, Anttila S. Molecular and genetic changes in asbestos-related lung cancer. *Cancer Letters* 2008; 265(1):1-15.

26. Cerutti VF, Peters RA. Observations Upon Effect of Fluoroacetate and Pyruvate Upon Isolated Atria from Rat Heart. *Biochemical Pharmacology* 1969; 18(9):2264-&.

27. Chojkier M. Hepatic sinusoidal-obstruction syndrome: toxicity of pyrrolizidine alkaloids. *Journal of Hepatology* 2003; 39(3):437-446.

28. Gordon WP, Forte AJ, Mcmurtry RJ, Gal J, Nelson SD. Hepatotoxicity and Pulmonary Toxicity of Pennyroyal Oil and Its Constituent Terpenes in the Mouse. *Toxicology and Applied Pharmacology* 1982; 65(3):413-424.

29. Rao PVL, Jayaraj R, Bhaskar ASB, Kumar O, Bhattacharya R, Saxena P et al. Mechanism of ricin-induced apoptosis in human cervical cancer cells. *Biochemical Pharmacology* 2005; 69(5):855-865.

30. Shahin M, Smith BL, Prakash AS. Bracken carcinogens in the human diet. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis* 1999; 443(1-2):69-79.

31. Peterson ME. Snake bite: Pit vipers. *Clinical Techniques in Small Animal Practice* 2006; 21(4):174-182.

32. Prockop LD, Chichkova RI. Carbon monoxide intoxication: An updated review. *Journal of the Neurological Sciences* 2007; 262(1-2):122-130.

33. Leth PM, Gregersen M. Ethylene glycol poisoning. *Forensic Science International* 2005; 155(2-3):179-184.

34. BALLANTY.B, Bright JE, Williams P. Post-Mortem Rate of Transformation of Cyanide. *Forensic Science* 1974; 3(1):71-76.

35. Ikeda N, Takahashi H, Umetsu K, Suzuki T. The Course of Respiration and Circulation in Toluene-Sniffing. *Forensic Science International* 1990; 44(2-3):151-158.

36. Gibson GG, Skett P. *Introduction to drug metabolism*. 3 ed. Nelson Thornes; 2001.

37. Fromm MF, Kroemer HK, Eichelbaum M. Impact of P450 genetic polymorphism on the first-pass extraction of cardiovascular and neuroactive drugs. *Advanced Drug Delivery Reviews* 1997; 27(2-3):171-199.

38. Girennavar B, Poulose SM, Jayaprakasha GK, Bhat NG, Patil BS. Furocoumarins from grapefruit juice and their effect on human CYP 3A4 and CYP1B1 isoenzymes. *Bioorganic & Medicinal Chemistry* 2006; 14(8):2606-2612.

39. Fromm MF, Kroemer HK, Eichelbaum M. Impact of P450 genetic polymorphism on the first-pass extraction of cardiovascular and neuroactive drugs. *Advanced Drug Delivery Reviews* 1997; 27(2-3):171-199.

40. Cassells NP, Craston DH. The effects of commonly used adulterants on the detection of spiked LSD by an enzyme immunoassay. *Science & Justice* 1998; 38(2):109-117.

41. Dumoulin GC, Paterson DG. Clinical Relevance of Postmortem Microbiologic Examination - A Review. *Human Pathology* 1985; 16(6):539-548.

42. Kintz P. Value of hair analysis in postmortem toxicology. *Forensic Science International* 2004; 142(2-3):127-134.

43. Wennig R. Potential problems with the interpretation of hair analysis results. *Forensic Science International* 2000; 107(1-3):5-12.

44. Skopp G, Potsch L. Perspiration versus saliva - basic aspects concerning their use in roadside drug testing. *International Journal of Legal Medicine* 1999; 112(4):213-221.

45. Robertson MD, Drummer OH. Postmortem Drug-Metabolism by Bacteria. *Journal of Forensic Sciences* 1995; 40(3):382-386.

46. Robertson MD, Drummer OH. Stability of nitrobenzodiazepines in postmortem blood. *Journal of Forensic Sciences* 1998; 43(1):5-8.

47. Leikin JB, Watson WA. Post-mortem toxicology: What the dead can and cannot tell us. *Journal of Toxicology-Clinical Toxicology* 2003; 41(1):47-56.

48. Moriya F, Hashimoto Y. Tissue distribution of nitrazepam and 7-aminonitrazepam in a case of nitrazepam intoxication. *Forensic Science International* 2003; 131(2-3):108-112.

49. Stevens HM. The Stability of Some Drugs and Poisons in Putrefying Human-Liver Tissues. *Journal of the Forensic Science Society* 1981; 21(2):146.

50. Tsunenari S, Yonemitsu K, Uchimura Y, Kanda M. The Influence of Putrefactive Changes on the Determination of Paraquat in Autopsy Materials. *Forensic Science International* 1981; 17(1):51-56.

51. Lynn B. *The Complete Textbook of Phlebotomy*. 2 ed. 2000.

52. Skopp G. Preanalytic aspects in postmortem toxicology. *Forensic Science International* 2004; 142(2-3):75-100.

53. Shah VP, Midha KK, Dighe S, Mcgilveray IJ, Skelly JP, Yacobi A et al. Analytical Methods Validation - Bioavailability, Bioequivalence, and Pharmacokinetic Studies. *Journal of Pharmaceutical Sciences* 1992; 81(3):309-312.

54. Waterman KC, Adami RC. Accelerated aging: Prediction of chemical stability of pharmaceuticals. *International Journal of Pharmaceutics* 2005; 293(1-2):101-125.

55. Levine B, Blanke RV, Valentour JC. Postmortem Stability of Benzodiazepines in Blood and Tissues. *Journal of Forensic Sciences* 1983; 28(1):102-115.

56. El Mahjoub A, Staub C. Stability of benzodiazepines in whole blood samples stored at varying temperatures. *Journal of Pharmaceutical and Biomedical Analysis* 2000; 23(6):1057-1063.

57. Alhadidi KA, Oliver JS. Stability of Temazepam in Blood. *Science & Justice* 1995; 35(2):105-108.

58. Hadidi KA, Oliver JS. Stability of morphine and buprenorphine in whole blood. *International Journal of Legal Medicine* 1998; 111(3):165-167.

59. Battah AH, Hadidi KA. Stability of trihexyphenidyl in stored blood and urine specimens. *International Journal of Legal Medicine* 1998; 111(3):111-114.

60. Alhadidi K. A Toxicological Study of the Effect of Putrefaction on the Analysis of Drugs of Forensic Interest in Biological Material Department of Forensic Medicine and Science, University of Glasgow; 1991.

61. Lin DL, Liu H, Chen CY. Storage-Temperature Effect on the Stability of Morphine and Codeine in Urine. *Journal of Analytical Toxicology* 1995; 19(5):275-280.

62. Baselt RC, Yoshikawa D, Chang J, Li J. Improved Long-Term Stability of Blood Cocaine in Evacuated Collection Tubes. *Journal of Forensic Sciences* 1993; 38(4):935-937.

63. Mccurdy HH, Callahan LS, Williams RD. Studies on the Stability and Detection of Cocaine, Benzoylecgonine, and 11-Nor-Delta-9-Tetrahydrocannabinol-9-Carboxylic Acid in Whole-Blood Using Abuscreen Radioimmunoassay. *Journal of Forensic Sciences* 1989; 34(4):858-870.

64. Holmgren P, Druid H, Holmgren A, Ahlner J. Stability of drugs in stored postmortem femoral blood and vitreous humor. *Journal of Forensic Sciences* 2004; 49(4):820-825.

65. Clauwaert KM, Van Bocxlaer JF, De Leenheer AP. Stability study of the designer drugs "MDA, MDMA and MDEX" in water, serum, whole blood, and urine under various storage temperatures. *Forensic Science International* 2001; 124(1):36-42.

66. Zaitsu K, Miki A, Katagi M, Tsuchillashi H. Long-term stability of various drugs and metabolites in urine, and preventive measures against their decomposition with special attention to filtration sterilization. *Forensic Science International* 2008; 174(2-3):189-196.

67. Skopp G, Potsch L, Konig I, Mattern R. A preliminary study on the stability of benzodiazepines in blood and plasma stored at 4 degrees C. *International Journal of Legal Medicine* 1998; 111(1):1-5.

68. Skopp G, Potsch L, Klingmann A, Mattern R. Stability of morphine, morphine-3-glucuronide, and morphine-6-glucuronide in fresh blood and plasma and postmortem blood samples. *Journal of Analytical Toxicology* 2001; 25(1):2-7.

69. Lutfi L. Stability of Drugs of Forensic Interest in Post Mortem Blood Department of Forensic Medicine and Science, University of Glasgow; 2000.

70. Giorgi S, Meeker J. A 5-Year stability study of common illicit drugs in blood. Journal of Analytical Toxicology 19, 392-398. 1995.
Ref Type: Journal (Full)

71. Isenschmid DS, Levine BS, Caplan YH. A Comprehensive Study of the Stability of Cocaine and Its Metabolites. *Journal of Analytical Toxicology* 1989; 13(5):250-256.

72. Kala M, Lechowicz W. Instability of pancuronium in postmortem blood and liver taken after a fatal intramuscular Pavulon injection. *Forensic Science International* 2004; 143(2-3):191-198.

73. Dugan S, Bogema S, Schwartz RW, Lappas NT. Stability of Drugs of Abuse in Urine Samples Stored at -20-Degrees-C. *Journal of Analytical Toxicology* 1994; 18(7):391-396.

74. Datta P. Stability of digoxin and digitoxin in specimens collected in blood collection tubes containing serum separator gels. *Clinical Biochemistry* 1998; 31(4):273-275.

75. Forsdahl G, Gmeiner G. Quantification and stability of salbutamol in human urine. *Journal of Separation Science* 2004; 27(1-2):110-114.

76. Celma C, Allue JA, Prunonosa J, Peraire C, Obach R. Simultaneous determination of paracetamol and chlorpheniramine in human plasma by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* 2000; 870(1-2):77-86.

77. Bennetto CJ, King JR, Turner ML, Stringer JSA, Acosta EP. Effects of concentration and temperature on the stability of Nevirapine in whole blood and serum. *Clinical Chemistry* 2004; 50(1):209-211.

78. Baranda AB, Alonso RM, Jimenez RM, Weinmann W. Instability of calcium channel antagonists during sample preparation for LC-MS-MS analysis of serum samples. *Forensic Science International* 2006; 156(1):23-34.

79. Hu OYP, Curry SH. Stability, Human-Blood Distribution and Rat-Tissue Localization of Promazine and Desmonomethylpromazine. *Biopharmaceutics & Drug Disposition* 1989; 10(6):537-548.

80. Skopp G, Potsch L, Mauden M, Richter B. Partition coefficient, blood to plasma ratio, protein binding and short-term stability of 11-nor-Delta(9)-carboxy tetrahydrocannabinol glucuronide. *Forensic Science International* 2002; 126(1):17-23.

81. Skopp G, Potsch L, Mattern R, Aderjan R. Short-term stability of lysergic acid diethylamide (LSD), N-desmethyl-LSD, and 2-oxo-3-hydroxy-LSD in urine, assessed by liquid chromatography-tandem mass spectrometry. *Clinical Chemistry* 2002; 48(9):1615-1618.

82. Potsch L, Skopp G. Stability of opiates in hair fibers after exposure to cosmetic treatment. *Forensic Science International* 1996; 81(2-3):95-102.

83. Yegles M, Marson Y, Wennig R. Influence of bleaching on stability of benzodiazepines in hair. *Forensic Science International* 2000; 107(1-3):87-92.

84. Skopp G, Potsch L, Moeller MR. On cosmetically treated hair - Aspects and pitfalls of interpretation. *Forensic Science International* 1997; 84(1-3):43-52.

85. Skopp G, Klingmann A, Potsch L, Mattern R. In vitro stability of cocaine in whole blood and plasma including ecgonine as a target analyte. *Therapeutic Drug Monitoring* 2001; 23(2):174-181.

86. Peters FT, Maurer HH. Bioanalytical method validation and its implications for forensic and clinical toxicology - A review. *Accreditation and Quality Assurance* 2002; 7(11):441-449.

87. Ageda S, Fuke C, Ihama Y, Miyazaki T. The stability of organophosphorus insecticides in fresh blood. Legal Medicine 8, 144-149. 2006.

88. Moriya F, Hashimoto Y, Kuo TL. Pitfalls when determining tissue distributions of organophosphorus chemicals: Sodium fluoride accelerates chemical degradation. *Journal of Analytical Toxicology* 1999; 23(3):210-215.

89. Brogan W, Kemp P, Bost R, Glamann D, Lange R, Hillis L. Collection and handling of clinical blood samples to assure the accurate measurement of cocaine concentration. Journal of Analytical Toxicology 16[3], 152-154. 1992.

90. Westerling D, Bengtsson HI, Thysell C, Hoglund P. The influence of preanalytical factors on concentrations of morphine and metabolites in patients receiving morphine. *Pharmacology & Toxicology* 1996; 78(2):82-85.

91. Posyniak A, Zmudzki J, Niedzielska J. Liquid chromatography analysis of enrofloxacin and ciprofloxacin in chicken blood spotted on filter-paper disks. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2002; 780(2):309-314.

92. Hoogtanders K, van der Heijden J, Christiaans M, Edelbroek P, van Hooff JP, Stolk LML. Therapeutic drug monitoring of tacrolimus with the dried blood spot method. *Journal of Pharmaceutical and Biomedical Analysis* 2007; 44(3):658-664.

93. Skopp G, Potsch L. Detection of cocaine in blood stains. Arch Kriminol 207[(3-4)], 81-88. 2001.

94. Skopp G. Blood Spot Analysis. 2007. Proceeding of The International Association of Forensic Toxicologists meeting. 2007.

95. AbuRuz S, Millership J, McElnay J. Dried blood spot liquid chromatography assay for therapeutic drug monitoring of metformin. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2006; 832(2):202-207.

96. Dubey IS, Caplan YH. The storage of forensic urine drug specimens as dry stains: Recovery and stability. *Journal of Forensic Sciences* 1996; 41(5):845-850.

97. Lejeune D, Souletie I, Houze S, Le Bricon T, Le Bras J, Gourmel B et al. Simultaneous determination of monodesethylchloroquine, chloroquine, cycloguanil and proguanil on dried blood spots by reverse-phase liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis* 2007; 43(3):1106-1115.

98. Moriya F, Hashimoto Y. The effect of postmortem interval on the concentrations of cocaine and cocaethylene in blood and tissues: An experiment using rats. *Journal of Forensic Sciences* 1996; 41(1):129-133.

99. Asri K. Solid Phase Extraction Techniques for the analysis of Pesticides and Drugs in Biological Specimens Department of Forensic Medicine and Science , University of Glasgow; 2001.

100. Olesen OV, Linnet K. Determination of olanzapine in serum by high-performance liquid chromatography using ultraviolet detection considering the easy oxidizability of the compound and the presence of other psychotropic drugs. *Journal of Chromatography B* 1998; 714(2):309-315.

101. Ueyama J, Saito I, Kamijima M, Nakajima T, Gotoh M, Suzuki T et al. Simultaneous determination of urinary dialkylphosphate metabolites of organophosphorus pesticides using gas chromatography-mass spectrometry. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2006; 832(1):58-66.

102. Telepchak M, August T, Chaney G. *Forensic and Clinical Applications of Solid Phase Extraction*. New Jersey: Human Press Inc.; 2004.

103. Blau K, Halket J. *Handbook of Derivatives for Chromatography*. Second ed. Sussex: John Wiley and Sons Ltd; 1993.

104. Kealey D, Haines P. *Analytical Chemistry*. Oxford: BIOS Scientific Publisher; 2002.

105. Begerow J, Jermann E, Keles T, Koch T, Dunemann L. Screening method for the determination of 28 volatile organic compounds in indoor and

outdoor air at environmental concentrations using dual-column capillary gas chromatography with tandem electron-capture-flame ionization detection. *Journal of Chromatography A* 1996; 749(1-2):181-191.

106. Bressolle F, BrometPetit M, Audran M. Validation of liquid chromatographic and gas chromatographic methods - Applications to pharmacokinetics. *Journal of Chromatography B-Biomedical Applications* 1996; 686(1):3-10.

107. Forrest M, Holding S, Howells D. The use of two-dimensional GC-MS for the identification and quantification of low molecular weight compounds from high performance elastomers. *Polymer Testing* 2006; 25(1):63-74.

108. Plzak Z, Polanska M, Suchanek M. Identification and Determination of Butyltin Compounds in Water by Ion-Trap Gas-Chromatography Mass-Spectrometry After Conversion to Methyl Or Hydride Derivatives. *Journal of Chromatography A* 1995; 699(1-2):241-252.

109. Strife RJ, Simms JR, Lacey MP. Combined Capillary Gas-Chromatography Ion Trap Mass-Spectrometry Quantitative Methods Using Labeled Or Unlabeled Internal Standards. *Journal of the American Society for Mass Spectrometry* 1990; 1(3):265-271.

110. Snyder L, Kirkland J, Glajch J. *Practical HPLC Method Development*. Second ed. USA: John Wiley and Sons, Inc.; 1997.

111. Thermo Finnigan LCQ Deca Hardware Manual. 2005.

112. Polettini A. *Applications of LC-MS in Toxicology*. London: Pharmaceutical Press; 2006.

113. Cheze M, Villain M, Pepin G. Determination of bromazepam, clonazepam and metabolites after a single intake in urine and hair by LC-MS/MS application to forensic cases of drug facilitated crimes. *Forensic Science International* 2004; 145(2-3):123-130.

114. Jeanville PM, Estape ES, Needham SR, Cole MJ. Rapid confirmation/quantitation of cocaine and benzoylecgonine in urine utilizing

high performance liquid chromatography and tandem mass spectrometry. *Journal of the American Society for Mass Spectrometry* 2000; 11(3):257-263.

115. Johansen SS, Jensen JL. Liquid chromatography-tandem mass spectrometry determination of LSD, ISO-LSD, and the main metabolite 2-oxo-3-hydroxy-LSD in forensic samples and application in a forensic case. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2005; 825(1):21-28.

116. Kintz P, Villain M, Concheiro M, Cirimele V. Screening and confirmatory method for benzodiazepines and hypnotics in oral fluid by LC-MS/MS. *Forensic Science International* 2005; 150(2-3):213-220.

117. Liang HR, Foltz RL, Meng M, Bennett P. Method development and validation for quantitative determination of methadone enantiomers in human plasma by liquid chromatography/tandem mass spectrometry. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2004; 806(2):191-198.

118. Maralikova B, Weinmann W. Confirmatory analysis for drugs of abuse in plasma and urine by high-performance liquid chromatography-tandem mass spectrometry with respect to criteria for compound identification. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2004; 811(1):21-30.

119. McClean S, O'Kane E, Hillis J, Smyth WF. Determination of 1,4-benzodiazepines and their metabolites by capillary electrophoresis and high-performance liquid chromatography using ultraviolet and electrospray ionisation mass spectrometry. *Journal of Chromatography A* 1999; 838(1-2):273-291.

120. Miller EI, Wylie FA, Oliver JS. Simultaneous detection and quantification of amphetamines, diazepam and its metabolites, cocaine and its metabolites, and opiates in hair by LC-ESI-MS-MS using a single extraction method. *Journal of Analytical Toxicology* 2008; 32(7):457-469.

121. Rook EJ, Hillebrand MJX, Rosing H, van Ree JM, Beijnen JH. The quantitative analysis of heroin, methadone and their metabolites and the simultaneous detection of cocaine, acetylcodeine and their metabolites in

human plasma by high-performance liquid chromatography coupled with tandem mass spectrometry. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2005; 824(1-2):213-221.

122. Weinmann W, Vogt S, Goerke R, Muller C, Bromberger A. Simultaneous determination of THC-COOH and THC-COOH-glucuronide in urine samples by LC/MS/MS. *Forensic Science International* 2000; 113(1-3):381-387.

123. Weng ND, Lee JW, Jiang XY, Wehling M, Hulse JD, Lin PP. Simultaneous assay of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human plasma using normal-phase liquid chromatography-tandem mass spectrometry with a silica column and an aqueous organic mobile phase. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 1999; 735(2):255-269.

124. Wood M, Laloup M, Fernandez MDR, Jenkins KM, Young MS, Ramaekers JG et al. Quantitative analysis of multiple illicit drugs in preserved oral fluid by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *Forensic Science International* 2005; 150(2-3):227-238.

125. Wylie FM, Torrance H, Anderson RA, Oliver JS. Drugs in oral fluid - Part I. Validation of an analytical procedure for licit and illicit drugs in oral fluid. *Forensic Science International* 2005; 150(2-3):191-198.

126. Forensic Laboratory Guidelines. 2006. SOFT/AAFS.

127. Bressolle F, BrometPetit M, Audran M. Validation of liquid chromatographic and gas chromatographic methods - Applications to pharmacokinetics. *Journal of Chromatography B-Biomedical Applications* 1996; 686(1):3-10.

128. Causon R. Validation of chromatographic methods in biomedical analysis - Viewpoint and discussion. *Journal of Chromatography B* 1997; 689(1):175-180.

129. Karnes HT, Shiu G, Shah VP. Validation of Bioanalytical Methods. *Pharmaceutical Research* 1991; 8(4):421-426.

130. Lindner W, Wainer IW. Requirements for initial assay validation and publication in J. Chromatography B. *Journal of Chromatography B* 1998; 707(1-2):1-2.

131. Peters FT, Maurer HH. Bioanalytical method validation and its implications for forensic and clinical toxicology - A review. *Accreditation and Quality Assurance* 2002; 7(11):441-449.

132. Peters FT, Drummer OH, Musshoff F. Validation of new methods. *Forensic Science International* 2007; 165(2-3):216-224.

133. Shah VP, Midha KK, Dighe S, Mcgilveray IJ, Skelly JP, Yacobi A et al. Analytical Methods Validation - Bioavailability, Bioequivalence, and Pharmacokinetic Studies. *Journal of Pharmaceutical Sciences* 1992; 81(3):309-312.

134. Swinscow T, Campbell M. *Statistics at Square One*. Tenth ed. London: BMJ Books Publishing Group; 2002.

135. Drummer O. *The Forensic Pharmacology of Drugs of Abused*. London: Arnold, a member of Hodder Headline Group; 2001.

136. Karch S. *Pharmacokinetics and Pharmacodynamics of Abused Drugs*. New York: CRC Press Taylor and Francis Group; 2008.

137. Longo MC, Hunter CE, Lokan RJ, White JM, White MA. The prevalence of alcohol, cannabinoids, benzodiazepines and stimulants amongst injured drivers and their role in driver culpability - Part I: the prevalence of drug use in drivers, and characteristics of the drug-positive group. *Accident Analysis and Prevention* 2000; 32(5):613-622.

138. Longo MC, Hunter CE, Lokan RJ, White JM, White MA. The prevalence of alcohol, cannabinoids, benzodiazepines and stimulants amongst injured drivers and their role in driver culpability - Part II: The relationship between drug prevalence and drug concentration, and driver culpability. *Accident Analysis and Prevention* 2000; 32(5):623-632.

139. Seymour A, Oliver JS. Role of drugs and alcohol in impaired drivers and fatally injured drivers in the Strathclyde police region of Scotland, 1995-1998. *Forensic Science International* 1999; 103(2):89-100.

140. Bramness JG, Skurtveit S, Morland J. Clinical impairment of benzodiazepines - relation between benzodiazepine concentrations and impairment in apprehended drivers. *Drug and Alcohol Dependence* 2002; 68(2):131-141.

141. Papadodima SA, Athanaselis SA, Stefanidou ME, Dona AA, Papoutsis I, Maravelias CP et al. Driving under the influence in Greece: A 7-year survey (1998-2004). *Forensic Science International* 2008; 174(2-3):157-160.

142. Smink BE, Ruiter B, Lusthof KJ, de Gier JJ, Uges DRA, Egberts ACG. Drug use and the severity of a traffic accident. *Accident Analysis and Prevention* 2005; 37(3):427-433.

143. Cheze M, Villain M, Pepin G. Determination of bromazepam, clonazepam and metabolites after a single intake in urine and hair by LC-MS/MS application to forensic cases of drug facilitated crimes. *Forensic Science International* 2004; 145(2-3):123-130.

144. Lebeau M, Mozayani A. *Drug-Facilitated Sexual Assault*. Barcelona: Academic Press; 2001.

145. Baselt R. *Disposition of Toxic Drugs and Chemicals in Man*. Sixth ed. California: Biomedical Publications; 2002.

146. Lotsch J, Skarke C, Schmidt H, Rohrbacher M, Hofmann U, Schwab M et al. Evidence for morphine-independent central nervous opioid effects after administration of codeine: Contribution of other codeine metabolites. *Clinical Pharmacology & Therapeutics* 2006; 79(1):35-48.

147. Al Asmari AI, Anderson RA. Method for quantification of opioids and their metabolites in autopsy blood by liquid chromatography-tandem mass spectrometry. *Journal of Analytical Toxicology* 2007; 31(7):394-408.

148. Bogusz MJ. Liquid chromatography-mass spectrometry as a routine method in forensic sciences: a proof of maturity. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2000; 748(1):3-19.

149. Glare PA, Walsh TD, Pippenger CE. A Simple, Rapid Method for the Simultaneous Determination of Morphine and Its Principal Metabolites in

Plasma Using High-Performance Liquid-Chromatography and Fluorometric Detection. *Therapeutic Drug Monitoring* 1991; 13(3):226-232.

150. Kaushik R, Levine B, LaCourse WR. A brief review: HPLC methods to directly detect drug glucuronides in biological matrices (Part I). *Analytica Chimica Acta* 2006; 556(2):255-266.

151. Mason JL, Ashmore SP, Aitkenhead AR. Simple Method for the Determination of Morphine and Its Active Glucuronide Metabolite in Human Plasma by High-Performance Liquid-Chromatography with Electrochemical Detection. *Journal of Chromatography-Biomedical Applications* 1991; 570(1):191-197.

152. Nishikawa M, Tsuchihashi H, Miki A, Katagi M, Schmitt C, Zimmer H et al. Determination of ethyl glucuronide, a minor metabolite of ethanol, in human serum by liquid chromatography electrospray ionization mass spectrometry. *Journal of Chromatography B* 1999; 726(1-2):105-110.

153. Rotshteyn Y, Weingarten B. A highly sensitive assay for the simultaneous determination of morphine, morphine-3-glucuronide, and morphine-6-glucuronide in human plasma by high-performance liquid chromatography with electrochemical and fluorescence detection. *Therapeutic Drug Monitoring* 1996; 18(2):179-188.

154. Svensson JO, Rane A, Sawe J, Sjoqvist F. Determination of Morphine, Morphine-3-Glucuronide and (Tentatively) Morphine-6-Glucuronide in Plasma and Urine Using Ion-Pair High-Performance Liquid-Chromatography. *Journal of Chromatography* 1982; 230(2):427-432.

155. Svensson JO. Determination of Morphine, Morphine-6-Glucuronide and Normorphine in Plasma and Urine with High-Performance Liquid-Chromatography and Electrochemical Detection. *Journal of Chromatography* 1986; 375(1):174-178.

156. Cook JD, Strauss KA, Caplan YH, Lodico CP, Bush DM. Urine pH: the effects of time and temperature after collection. *Journal of Analytical Toxicology* 2007; 31(8):486-496.

157. Guthrie R, Susi A. A Simple Phenylalanine Method for Detecting Phenylketonuria in Large Populations of Newborn Infants. *Pediatrics* 1963; 32(3):338-&.

158. AbuRuz S, Millership J, McElnay J. Dried blood spot liquid chromatography assay for therapeutic drug monitoring of metformin. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2006; 832(2):202-207.

159. Allard P, Cowell LD, Zytkovicz TH, Korson MS, Ampola MG. Determination of phenylalanine and tyrosine in dried blood specimens by ion-exchange chromatography using the Hitachi L-8800 analyzer. *Clinical Biochemistry* 2004; 37(10):857-862.

160. Barfield M, Spooner N, Lad R, Parry S, Fowles S. Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2008; 870(1):32-37.

161. Barns RJ, Bowling FG, Brown G, Clague AE, Thompson A. Carnitine in Dried Blood Spots - A Method Suitable for Neonatal Screening. *Clinica Chimica Acta* 1991; 197(1):27-34.

162. Beaudette P, Bateman KP. Discovery stage pharmacokinetics using dried blood spots. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2004; 809(1):153-158.

163. El Hajjar DF, Swanson KH, Landmark JD, Stickle DF. Validation of use of annular once-punched filter paper bloodspot samples for repeat lead testing. *Clinica Chimica Acta* 2007; 377(1-2):179-184.

164. Henderson LO, Powell MK, Hannon WH, Miller BB, Martin ML, Hanzlick RL et al. Radioimmunoassay Screening of Dried Blood Spot Materials for Benzoylecgonine. *Journal of Analytical Toxicology* 1993; 17(1):42-47.

165. Higashi T, Nishio T, Uchida S, Shimada K, Fukushi M, Maeda M. Simultaneous determination of 17 alpha-hydroxypregnenolone and 17 alpha-hydroxyprogesterone in dried blood spots from low birth weight infants using

LC-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis* 2008; 48(1):177-182.

166. Mercader S, Featherstone D, Bellini WJ. Comparison of available methods to elute serum from dried blood spot samples for measles serology. *Journal of Virological Methods* 2006; 137(1):140-149.

167. Minzi OMS, Massele AY, Gustafsson LL, Ericsson O. Simple and cost-effective liquid chromatographic method for determination of pyrimethamine in whole blood samples dried on filter paper. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2005; 814(1):179-183.

168. Oliveira EJ, Watson DG, Morton NS. A simple microanalytical technique for the determination of paracetamol and its main metabolites in blood spots. *Journal of Pharmaceutical and Biomedical Analysis* 2002; 29(5):803-809.

169. Schutz H, Gotta JC, Erdmann F, Risse M, Weiler G. Simultaneous screening and detection of drugs in small blood samples and bloodstains. *Forensic Science International* 2002; 126(3):191-196.

170. Sosnoff CS, Ann QH, Bernert JT, Powell MK, Miller BB, Henderson LO et al. Analysis of benzoylecgonine in dried blood spots by liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry. *Journal of Analytical Toxicology* 1996; 20(3):179-184.

171. Strasser S, Zink A, Kada G, Hinterdorfer P, Peschel O, Heckl WM et al. Age determination of blood spots in forensic medicine by force spectroscopy. *Forensic Science International* 2007; 170(1):8-14.

172. Tawa R, Matsunaga H, Fujimoto T. High-performance liquid chromatographic analysis of aminoglycoside antibiotics. *Journal of Chromatography A* 1998; 812(1-2):141-150.

173. Waterman KC, Adami RC, Alsante KM, Antipas AS, Arenson DR, Carrier R et al. Hydrolysis in pharmaceutical formulations. *Pharmaceutical Development and Technology* 2002; 7(2):113-146.

174. Nudelman N, Waisbaum R. Kinetic Study of the Reactions of 2-Amino-5-Chlorbenzophenone with HCL in MeOH-H_2O. Physical organic chemistry 10, 97-106. 1997.

175. Nudelman NS, Dewaisbaum RG. Isolation and Structure Elucidation of Novel Products of the Acidic Degradation of Diazepam. *Journal of Pharmaceutical Sciences* 1995; 84(2):208-211.

176. Cabrera CG, de Waisbaum RG, Nudelman NS. Kinetic and mechanistic studies on the hydrolysis and photodegradation of diazepam and alprazolam. *Journal of Physical Organic Chemistry* 2005; 18(2):156-161.

177. Archontaki HA, Gikas EE, Panderi IE, Ovezikoglou PM. Kinetics and mechanism of acidic hydrolysis of nordazepam studied by high-performance liquid chromatography and fourth-order derivative ultraviolet spectrophotometry. *International Journal of Pharmaceutics* 1998; 167(1-2):69-81.

178. Buret D, Breton D, Clair P, Lafosse M. Identification by CI-mass spectrometry of an unexpected benzodiazepine degradation product. *International Journal of Mass Spectrometry* 2006; 248(1-2):36-41.

179. Damjanovic T, Popovic G, Verbic S, Pfendt L. Study of acid hydrolysis of bromazepam. *Canadian Journal of Chemistry-Revue Canadienne de Chimie* 2004; 82(8):1260-1265.

180. Drummer OH. Methods for the measurement of benzodiazepines in biological samples. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 1998; 713(1):201-225.

181. Han WW, Yakatan GJ, Maness DD. Kinetics and Mechanisms of Hydrolysis of 1,4-Benzodiazepines .1. Chlordiazepoxide and Demoxepam. *Journal of Pharmaceutical Sciences* 1976; 65(8):1198-1204.

182. Han WW, Yakatan GJ, Maness DD. Kinetics and Mechanisms of Hydrolysis of 1,4-Benzodiazepines-3 - Nitrazepam. *Journal of Pharmaceutical Sciences* 1977; 66(6):795-798.

183. Han WW, Yakatan GJ, Maness DD. Kinetics and Mechanisms of Hydrolysis of 1,4-Benzodiazepines .2. Oxazepam and Diazepam. *Journal of Pharmaceutical Sciences* 1977; 66(4):573-577.

184. Hancu G, Gaspar A, Gyeresi A. Separation of 1,4-benzodiazepines by micellar elektrokinetic capillary chromatography. *Journal of Biochemical and Biophysical Methods* 2007; 69(3):251-259.

185. Moro ME, Novillofertrell J, Velazquez MM, Rodriguez LJ. Kinetics of the Acid-Hydrolysis of Diazepam, Bromazepam, and Flunitrazepam in Aqueous and Micellar Systems. *Journal of Pharmaceutical Sciences* 1991; 80(5):459-468.

186. Peel HW, Perrigo BJ. Toxicological Analysis of Benzodiazepine-Type Compounds in Postmortem Blood by Gas-Chromatography. *Journal of Analytical Toxicology* 1980; 4(3):105-113.

187. Sawada H, Hara A, Asano S, Matsumoto Y. Isolation and Identification of Benzodiazepine Drugs and Their Metabolites in Urine by Use of Amberlite Xad-2 Resin and Thin-Layer Chromatography. *Clinical Chemistry* 1976; 22(10):1596-1603.

188. Schuetz H. Tlc Data of Hydrolysis Products of 1,4-and 1,5-Benzodiazepines and Major Metabolites. *Journal of Analytical Toxicology* 1978; 2(4):147.

189. Breton D, Buret D, Mendes-Oustric AC, Chaimbault P, Lafosse M, Clair P. LC-UV and LC-MS evaluation of stress degradation behaviour of avizafone. *Journal of Pharmaceutical and Biomedical Analysis* 2006; 41(4):1274-1279.

190. Galan M, Cruz S, Barcelo D. Identification and determination of metabolites and degradation products of sulfonamide antibiotics. Trend in Analytical Chemistry. 2008.

191. Pitak J, Garcia E, Pitak M, Aga D. Identification of transformation products of 17 alpha-ethinylestradiol and 17 beta-estradiol by mass spectrometry and other intrumental techniques. Trend in Analytical Chemistry. 2008.

192. Gray J. *The World of Hair Colour A Scientfic Cmpanion*. London: Thomson Learning; 2005.

193. Potsch L, Skopp G, Becker J. Ultrastructural Alterations and Environmental Exposure Influence the Opiate Concentrations in Hair of Drug-Addicts. *International Journal of Legal Medicine* 1995; 107(6):301-305.

194. Wennig R. Potential problems with the interpretation of hair analysis results. *Forensic Science International* 2000; 107(1-3):5-12.

195. Yegles M, Marson Y, Wennig R. Influence of bleaching on stability of benzodiazepines in hair. *Forensic Science International* 2000; 107(1-3):87-92.

196. Fura A, Harper TW, Zhang HJ, Fung L, Shyu WC. Shift in pH of biological fluids during storage and processing: effect on bioanalysis. *Journal of Pharmaceutical and Biomedical Analysis* 2003; 32(3):513-522.

197. Olson K. *Poisoning and Drug Overdose*. Third ed. USA: Appleton and Lang; 1999.

198. Zahn D. On the role of water in amide hydrolysis. *European Journal of Organic Chemistry* 2004;(19):4020-4023.

199. Testa B, Mayer J. *Hydrolysis in Drug and Prodrug Metabolim*. Zurich: VHCA and WILEY-VCH; 2003.

200. Nanda N, Mayanna SM, Gowda NNM. Mechanism of oxidation of diazepam by Chloramine-B: A kinetic approach. *International Journal of Chemical Kinetics* 1998; 30(9):605-611.

201. Nanda N, Sheshadri BS, Mayanna SM. Mechanism of oxidation of diazepam by 1-chlorobenzotriazole in acidic medium. A kinetic approach. *Reaction Kinetics and Catalysis Letters* 1999; 67(1):35-41.

202. Gupta R. *Toxicology of Organophosphate and Carbamate Compounds*. California: Elsevier Academic Press; 2006.

203. Aprea C, Sciarra G, Orsi D, Boccalon P, Sartorelli P, Sartorelli E. Urinary excretion of alkylphosphates in the general population (Italy). *Science of the Total Environment* 1996; 177:37-41.

204. Nutley BP, Cocker J. Biological Monitoring of Workers Occupationally Exposed to Organophosphorus Pesticides. *Pesticide Science* 1993; 38(4):315-322.

205. Tsatsakis AM, Tzatzarakis MN, Tutudaki M. Pesticide levels in head hair samples of Cretan population as an indicator of present and past exposure. *Forensic Science International* 2008; 176(1):67-71.

206. Amini N, Crescenzi C. Feasibility of an on-line restricted access material/liquid chromatography/tandem mass spectrometry method in the rapid and sensitive determination of organophosphorus triesters in human blood plasma. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2003; 795(2):245-256.

207. Ballesteros E, Parrado MJ. Continuous solid-phase extraction and gas chromatographic determination of organophosphorus pesticides in natural an drinking waters. *Journal of Chromatography A* 2004; 1029(1-2):267-273.

208. Chouhan RS, Babu KV, Kumar MA, Neeta NS, Thakur MS, Rani BEA et al. Detection of methyl parathion using immuno-chemiluminescence based image analysis using charge coupled device. *Biosensors & Bioelectronics* 2006; 21(7):1264-1271.

209. Haib J, Hofer I, Renaud JM. Analysis of multiple pesticide residues in tobacco using pressurized liquid extraction, automated solid-phase extraction clean-up and gas chromatography-tandem mass spectrometry. *Journal of Chromatography A* 2003; 1020(2):173-187.

210. Hayama T, Yoshida H, Todoroki K, Nohta H, Yamaguchi M. Determination of polar organophosphorus pesticides in water samples by hydrophilic interaction liquid chromatography with tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 2008; 22(14):2203-2210.

211. Hernandez F, Serrano R, Pitarch E, Lopez FJ. Automated sample clean-up procedure for organophosphorus pesticides in several aquatic organisms using normal phase liquid chromatography. *Analytica Chimica Acta* 1998; 374(2-3):215-229.

212. Inoue S, Saito T, Mase H, Suzuki Y, Takazawa K, Yamamoto I et al. Rapid simultaneous determination for organophosphorus pesticides in human serum by LC-MS. *Journal of Pharmaceutical and Biomedical Analysis* 2007; 44(1):258-264.

213. Lacassie E, Dreyfuss MF, Gaulier JM, Marquet P, Daguet JL, Lachatre G. Multiresidue determination method for organophosphorus pesticides in serum and whole blood by gas chromatography-mass-selective detection. *Journal of Chromatography B* 2001; 759(1):109-116.

214. Lacassie E, Marquet P, Gaulier JM, Dreyfuss MF, Lachatre G. Sensitive and specific multiresidue methods for the determination of pesticides of various classes in clinical and forensic toxicology. *Forensic Science International* 2001; 121(1-2):116-125.

215. Liu JT, Suzuki O. Conditions of solid-phase extraction for the mixture of organophosphates and synthetic pyrethroids in human body fluids. *Forensic Science International* 1999; 99(2):159-161.

216. Mol HGJ, van Dam RCJ, Steijger OM. Determination of polar organophosphorus pesticides in vegetables and fruits using liquid chromatography with tandem mass spectrometry: selection of extraction solvent. *Journal of Chromatography A* 2003; 1015(1-2):119-127.

217. Stalikas CD, Pilidis GA. Development of a method for the simultaneous determination of phosphoric and amino acid group containing pesticides by gas chromatography with mass-selective detection - Optimization of the derivatization procedure using an experimental design approach. *Journal of Chromatography A* 2000; 872(1-2):215-225.

218. Tollback J, Tamburro D, Crescenzi C, Carlsson H. Air sampling with Empore solid phase extraction membranes and online single-channel desorption/liquid chromatography/mass spectrometry analysis: Determination of volatile and semi-volatile organophosphate esters. *Journal of Chromatography A* 2006; 1129(1):1-8.

219. Tsoukali H, Theodoridis G, Raikos N, Grigoratou I. Solid phase microextraction gas chromatographic analysis of organophosphorus pesticides

in biological samples. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2005; 822(1-2):194-200.

220. Black RM, Read RW. Analysis of degradation products of organophosphorus chemical warfare agents and related compounds by liquid chromatography mass spectrometry using electrospray and atmospheric pressure chemical ionisation. *Journal of Chromatography A* 1998; 794(1-2):233-244.

221. Blair D, Roderick HR. Improved Method for Determination of Urinary Dimethyl-Phosphate. *Journal of Agricultural and Food Chemistry* 1976; 24(6):1221-1223.

222. De Alwis GKH, Needham LL, Barr DB. Measurement of human urinary organophosphate pesticide metabolites by automated solid-phase extraction, post extraction derivatization, and gas chromatography-tandem mass spectrometry. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2006; 843(1):34-41.

223. Draper WM, Behniwal P, Wijekoon D. Direct determination of dialkyl phosphates by strong anion-exchange liquid chromatography/atmospheric pressure chemical ionization mass spectrometry using a quadrupole ion trap instrument. *Rapid Communications in Mass Spectrometry* 2008; 22(17):2613-2620.

224. Dulaurent S, Saint-Marcoux F, Marquet P, Lachatre G. Simultaneous determination of six dialkylphosphates in urine by liquid chromatography tandem mass spectrometry. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2006; 831(1-2):223-229.

225. Georgiou PP, Liapis KS, Miliadis GE, Siskos PA. Solid-phase extraction cleanup of tomato samples for the determination of pesticide residues by gas chromatography-electron capture detection. *International Journal of Environmental Analytical Chemistry* 2006; 86(1-2):69-76.

226. Hardt L, Angerer J. Determination of dialkyl phosphates in human urine using gas chromatography-mass spectrometry. *Journal of Analytical Toxicology* 2000; 24(8):678-684.

227. Hernandez F, Sancho JV, Pozo OJ. An estimation of the exposure to organophosphorus pesticides through the simultaneous determination of their main metabolites in urine by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2004; 808(2):229-239.

228. Imhof P, Fischer S, Kramer R, Smith JC. Density functional theory analysis of dimethylphosphate hydrolysis: effect of solvation and nucleophile variation. *Journal of Molecular Structure-Theochem* 2005; 713(1-3):1-5.

229. Kataoka M, Seto Y. Discriminative determination of alkyl methylphosphonates and methylphosphonate in blood plasma and urine by gas chromatography-mass spectrometry after tert.-butyldimethylsilylation. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2003; 795(1):123-132.

230. Kralj AB, Franko M, Trebse P. Photodegradation of organophosphorus insecticides - Investigations of products and their toxicity using gas chromatography-mass spectrometry and AChE-thermal lens spectrometric bioassay. *Chemosphere* 2007; 67(1):99-107.

231. Kupfermann N, Schmoldt A, Steinhart H. Rapid and sensitive quantitative analysis of alkyl phosphates in urine after organophosphate poisoning. *Journal of Analytical Toxicology* 2004; 28(4):242-248.

232. Lin WC, Kuei CH, Wu HC, Yang CC, Chang HY. Method for the determination of dialkyl phosphates in urine by strong anion exchange disk extraction and in-vial derivatization. *Journal of Analytical Toxicology* 2002; 26(3):176-180.

233. Lin WC, Hsieh CY, Chang HY. Improved analysis of dialkylphosphates in urine using strong anion exchange disk extraction and in-vial derivatization. *Journal of Separation Science* 2007; 30(9):1326-1333.

234. Miki A, Katagi M, Tsuchihashi H, Yamashita M. Determination of alkylmethylphosphonic acids, the main metabolites of organophosphorus nerve agents, in biofluids by gas chromatography mass spectrometry and liquid-liquid-solid-phase-transfer-catalyzed pentafluorobenzylation. *Journal of Analytical Toxicology* 1999; 23(2):86-93.

235. Oglobline AN, Elimelakh H, Tattam B, Geyer R, O'Donnell GE, Holder G. Negative ion chemical ionization GC/MS-MS analysis of dialkylphosphate metabolites of organophosphate pesticides in urine of non-occupationally exposed subjects. *Analyst* 2001; 126(7):1037-1041.

236. Tan BLL, Mohd MA. Analysis of selected pesticides and alkylphenols in human cord blood by gas chromatograph-mass spectrometer. *Talanta* 2003; 61(3):385-391.

237. Jager ME, Bourbon C, Levsen K. Analysis of pesticides and their degradation products in rainwater: A probe into their atmospheric degradation. *International Journal of Environmental Analytical Chemistry* 1998; 70(1-4):149-162.

238. Lima A, Vega L. Methyl-parathion and organophosphorous pesticide metabolites modify the activation status and interleukin-2 secretion of human peripheral blood mononuclear cells. *Toxicology Letters* 2005; 158(1):30-38.

239. Timchalk C, Busby A, Campbell JA, Needham LL, Barr DB. Comparative pharmacokinetics of the organophosphorus insecticide chlorpyrifos and its major metabolites diethylphosphate, diethylthiophosphate and 3,5,6-trichloro-2-pyridinol in the rat. *Toxicology* 2007; 237(1-3):145-157.

240. Ballesteros E, Parrado MJ. Continuous solid-phase extraction and gas chromatographic determination of organophosphorus pesticides in natural an drinking waters. *Journal of Chromatography A* 2004; 1029(1-2):267-273.

241. Hernandez F, Serrano R, Pitarch E, Lopez FJ. Automated sample clean-up procedure for organophosphorus pesticides in several aquatic organisms using normal phase liquid chromatography. *Analytica Chimica Acta* 1998; 374(2-3):215-229.

242. Tan BLL, Mohd MA. Analysis of selected pesticides and alkylphenols in human cord blood by gas chromatograph-mass spectrometer. *Talanta* 2003; 61(3):385-391.

243. Yu NH, Ho ENM, Tang FPW, Wan TSM, Wong ASY. Comprehensive screening of acidic and neutral drugs in equine plasma by liquid

chromatography-tandem mass spectrometry. *Journal of Chromatography A* 2008; 1189(1-2):426-434.

244. Wylie FM, Torrance H, Seymour A, Buttress S, Oliver JS. Drugs in oral fluid - Part II. Investigation of drugs in drivers. *Forensic Science International* 2005; 150(2-3):199-204.

245. Lori W, Michael V. Evaluation of the in vitro stability of digoxin in saliva. *Therapeutic Drug Monitoring* 1994; 16(2):221-223.

246. Gupta V. Stability of cocaine hydrolchloride solutions at various pH values as determined by high-pressure liquid chromatography. *International journal of pharmaceutics* 1982; 10:249-257.

247. Olaf HD. Postmortem toxicology of drugs of abuse. *Forensic Science International* 2004; 142:101-113.

248. Moriya F, Hashimoto Y. Redistribution of methamphetamine in the early postmortem period. *Journal of Analytical Toxicology* 2000; 23:69-70.

249. Al Asmari Al, Anderson RA. Determination of 27 opioids and their metabolites in urine by LC-MS/MS. *Proceeding of the international association of forensic toxicologists meeting, La Martinique, French west indies,* 2008.

11 Appendices:

11.1 Conference Papers and Journal Publication in Support of this Thesis.

1. Stability of benzodiazepines in whole blood under different storage conditions. Proceedings of the 9th Indo-Pacific Congress on Legal Medicine and Forensic Sciences, Colombo, Sri Lanka, 22-27 July, 2007.

2. Stability of benzodiazepines and cocaine in dried blood spots. Proceedings of the First Arab International Forensic Science & Forensic Medicine Conference, Riyadh, Saudi Arabia, 2007.

3. Correction for drug loss during storage: decomposition of diazepam and temazepam in whole blood under different storage conditions. Proceedings of the 46th Annual Conference of the International Association of Forensic Toxicologists, La Martinique, French West Indies, 2-8 June, 2008.

4. Correction for Drug Loss during Storage: Decomposition of Chlorodiazepoxide and Oxazepam in Whole Blood under Different Storage Conditions. Proceedings of the Society of Forensic Toxicologists Annual Meeting, Phoenix, Arizona, United States of America, 27-31 October, 2008.

5. Stability of benzodiazepines and cocaine in dried blood spots. Journal of Analytical Toxicology (2008); 32: 511-515.

Stability of alprazolam, estazolam, lorazepam and oxazepam in whole blood at different storage conditions

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This study has been undertaken to investigate the stability over one year of four drugs, all within the benzodiazepines class. For these tests, blank blood was spiked at low and high concentrations and stored in tubes containing no preservative or else containing fluoride-oxalate at -20° C, 4°C and room temperature. The times selected for analysis were, the first (day zero), second and fourth days, one week, two weeks, one month, two months, three months, six months and one year. Solid phase extraction was used for extracting the samples while the analysis was performed using liquid chromatography-tandem mass spectrometry. Alprazolam and estazolam were stable at -20° C and 4° C, however a decrease of almost 10% in the concentration was observed at room temperature for both drugs at low and high concentrations. Lorazepam and oxazepam were stable at -20°C. However, at 4° C lorazepam decreased by 50% and 28% for low and high concentrations respectively, while oxazepam decreased by 31% and 26% for low and high concentration respectively. At room temperature, both oxazepam and lorazepam decreased by 100% at low and high concentration. The addition of sodium fluoride enhanced the stability of the drugs by 10% in comparison with unpreserved samples. The long-term stability of alprazolam and estazolam is attributed to the presence of the trizolo ring in their structures, which makes the compounds more resistant to hydrolysis reactions, which of the most like degradation reactions affecting the stability of benzodiazepines drugs.

Key words: stability, benzodiazepines, storage condition, blood.

STABILITY OF BENZODIAZEPINES AND COCAINE IN BLOOD SPOTS STORED ON FILTER PAPER.

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Previous studies have shown that drug concentrations in blood can change during storage, especially at room temperature, but even labile drugs such as cocaine may be stable in dried blood spots (DBS). A new method has been developed for the analysis of hydrolytically-labile drugs in blood spots on filter paper in order to assess their degradation during a storage period of one month.

The drugs selected included flunitrazepam, temazepam, oxazepam, lorazepam, nitrazepam, diazepam and cocaine. A Guthrie card 903 was spotted with 100 µl of blood containing the drugs at concentrations of 1000ng/ml and left overnight to dry at room temperature. The filter paper was suspended in extraction buffer for 1 hour with ultrasonication. Drugs were then extracted from the buffer by solid phase extraction using Clean Screen® columns and analysed by liquid chromatography-tandem mass spectrometry. Method validation showed that all calibration curves were linear over the concentration range 5-200 ng/spot with correlation coefficients of 0.994-0.999. Inter-day and intra-day precisions at three concentrations (10, 50 and 100ng/spot) were 1.6-18.3% and 2.8-14.7% respectively. Limits of detection were 0.29-0.74 ng/spot while lower limits of quantitation were 0.99-2.46 ng/spot. Recoveries of all analytes were in the range 81-106%.

DBS were stored in duplicate at room temperature, 4°C and -20°C for up to one month. Degradation of the drugs in DBS at all storage conditions was less than for the corresponding liquid blood samples stored under similar conditions and more than 80% of each analyte could be recovered from the samples.

Key words: dried blood spots, filter paper, drug stability, blood analysis.

Correction for drug loss during storage: decomposition of diazepam and temazepam in whole blood under different storage conditions

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Introduction: Loss of analyte from biological samples during the post-mortem interval or during storage has potentially serious implications in forensic toxicology, and it would be of value to make corrections for these losses. This initial study evaluated one approach to this problem based on measurement of the main hydrolysis decomposition product of diazepam and temazepam, 2-methylamino 5-chlorobenzophenone (MACB). Previous studies have reported on the instability of diazepam and temazepam under different storage conditions. The aims of this study were to determine MACB in whole blood as an indicator of the extent of analyte degradation as well to investigate the effects of temperature and pH on the stability of diazepam and temazepam for one month.
Method: Blank blood was spiked with diazepam and temazepam to produce final concentrations of 1000 ng/ml of each. It is known from previous work that these drugs are stable for long periods of time, therefore, for the purposes of this study, degradation of the drugs was accelerated by storing aliquots of blood at high temperature (80°C) and under acidic (pH 2) and basic (pH 12) conditions at room temperature for one month. The samples were analyzed in duplicate at days 1, 2, 4, 7, 14 and 30. Samples were extracted by solid phase extraction and extracts were analysed by liquid chromatography-tandem mass spectrometry.
Results: Recoveries of all analytes were between 85-102%. The linear correlation coefficients for all three analytes were better than 0.99. Limits of detection and lower limits of quantitation were 0.1-2 ng/ml and 0.4-7 ng/ml respectively. Intra-day and inter-day precisions were found to be 4-17% and 4-18% respectively. After one month, the diazepam concentration at 80 °C, pH 2 and pH 12 had decreased from the original concentration by 30, 25 and 49 % respectively whereas temazepam decreased by 100%, 77% and 100% respectively. Under all sets of conditions the MACB concentration was observed to increase as degradation of the drugs proceeded and it could be detected when the parent drugs concentration were completely disappeared. However, MACB itself was found to be further degraded under some of the conditions used. Diazepam and temazepam are more sensitive to alkaline pH than to acidic pH or high temperature. Diazepam was noted to be more stable than temazepam.
Conclusion: Degradation of diazepam and temazepam in the blood under different conditions will produce MACB as the main hydrolysis product. MACB can be reliably detected in whole blood and can be used to correct for losses of diazepam and temazepam and to indicate the original diazepam concentration. MACB can also be used to confirm the presence of these drugs in samples, especially when the drug has decomposed due to poor or prolonged storage conditions.

KEYWORDS: benzodiazepines, degradation, whole blood, MACB.

Correction for Drug Loss During Storage: Decomposition of Chlorodiazepoxide and Oxazepam in Whole Blood Under Different Storage Conditions

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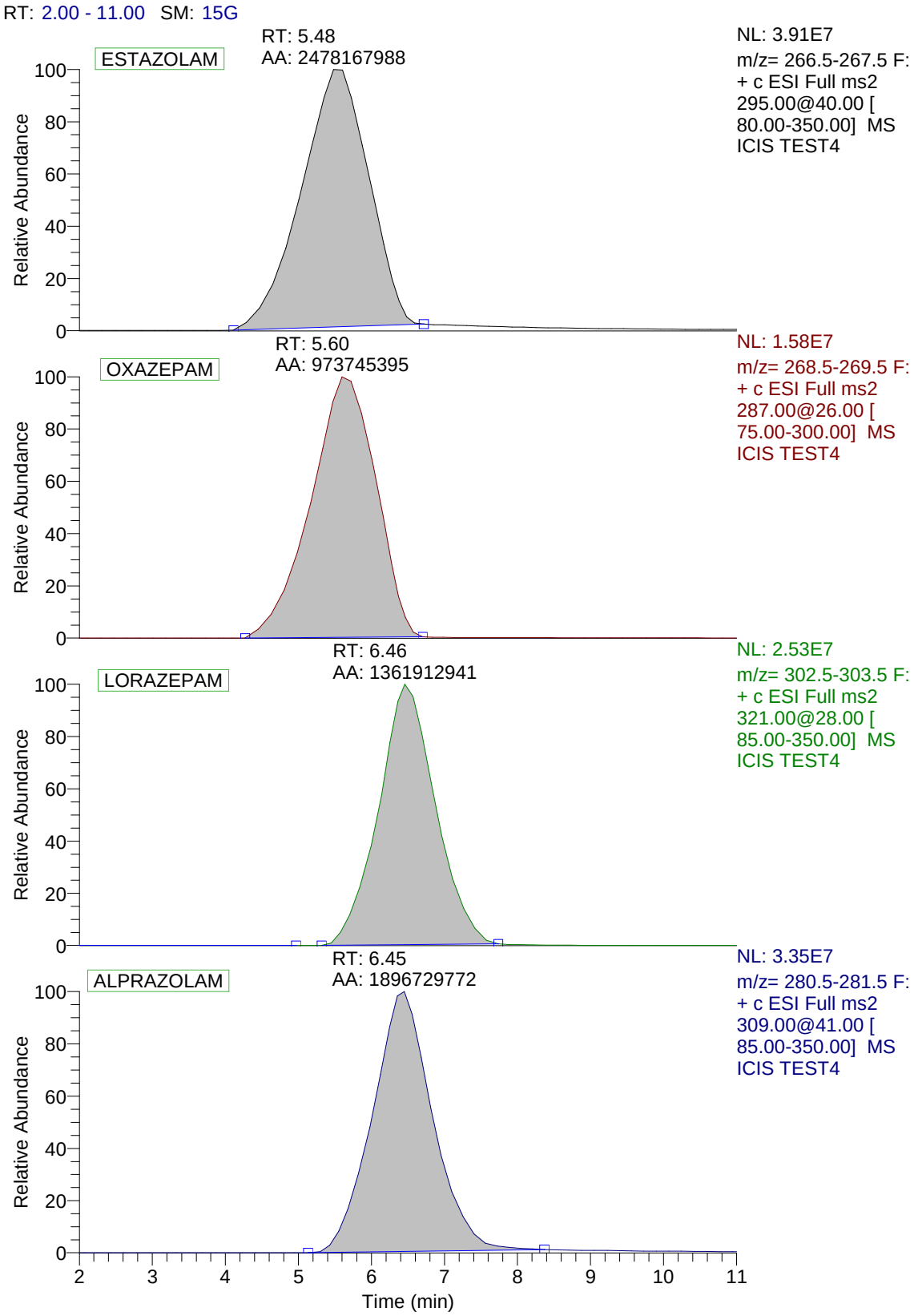
Introduction: Decomposition of analytes in biological samples during the post-mortem interval or during storage has been reported in the previous studies which leading to loss of the original concentration thus it would be of value to make corrections for these losses. This initial study evaluated one approach to this problem based on measurement of the main hydrolysis decomposition product of chlorodiazepoxide and oxazepam, 2-amino 5-chlorobenzophenone (ACB). Previous studies have reported on the instability of chlorodiazepoxide and oxazepam under different storage conditions. The aims of this study were to determine ACB in whole blood as an indicator of the extent of analyte degradation as well to investigate the effects of temperature and pH on the stability of chlorodiazepoxide and oxazepam for one month using the same method of our work in TIAFT2008 to correct the loss of diazepam and temazepam in whole blood.
Method: Blank blood was spiked with chlorodiazepoxide and oxazepam to produce final concentrations of 1000 ng/ml of each. It is known from previous work that these drugs are stable for more than one month, therefore, for the purposes of this study, degradation of the drugs was accelerated by storing aliquots of blood at high temperature (80°C) and under acidic (pH 2) and basic (pH 12) conditions at room temperature for one month. The samples were analyzed in duplicate at days 1, 2, 4, 7, 14 and 30. Samples were extracted by solid phase extraction and extracts were analysed by liquid chromatography-tandem mass spectrometry.
Results: Recoveries of all analytes were between 83-97%. The linear correlation coefficients for all three analytes were better than 0.99. Limits of detection and lower limits of quantitation were 0.46-2.3 ng/ml and 1.5-7.5 ng/ml respectively. Intra-day and inter-day precisions were found to be 1-13% and 2-18% respectively. After one month, the chlorodiazepoxide concentration at 80 °C, pH 2 and pH 12 had decreased from the original concentration by 62, 51 and 100 % respectively whereas oxazepam decreased by 100%, 99.7% and 100% respectively. Under all sets of conditions the ACB concentration was observed to increase as degradation of the drugs proceeded and it could be detected when the parent drugs concentration were completely disappeared. However, ACB itself was found to be further degraded under some of the conditions used. Chlorodiazepoxide and oxazepam are more sensitive to alkaline pH than to acidic pH or high temperature. Chlorodiazepoxide was noted to be more stable than oxazepam.
Conclusion: Degradation of chlorodiazepoxide and oxazepam in the blood under different conditions will produce ACB as the main hydrolysis product. ACB can be reliably detected in whole blood and can be used to correct for losses of chlorodiazepoxide and oxazepam and to indicate the original drug concentration. ACB can also be used to confirm the presence of these drugs in samples, especially when the drug has decomposed due to poor or prolonged storage conditions.

KEYWORDS: benzodiazepines, degradation, ACB.

12 Specimen Chromatograms



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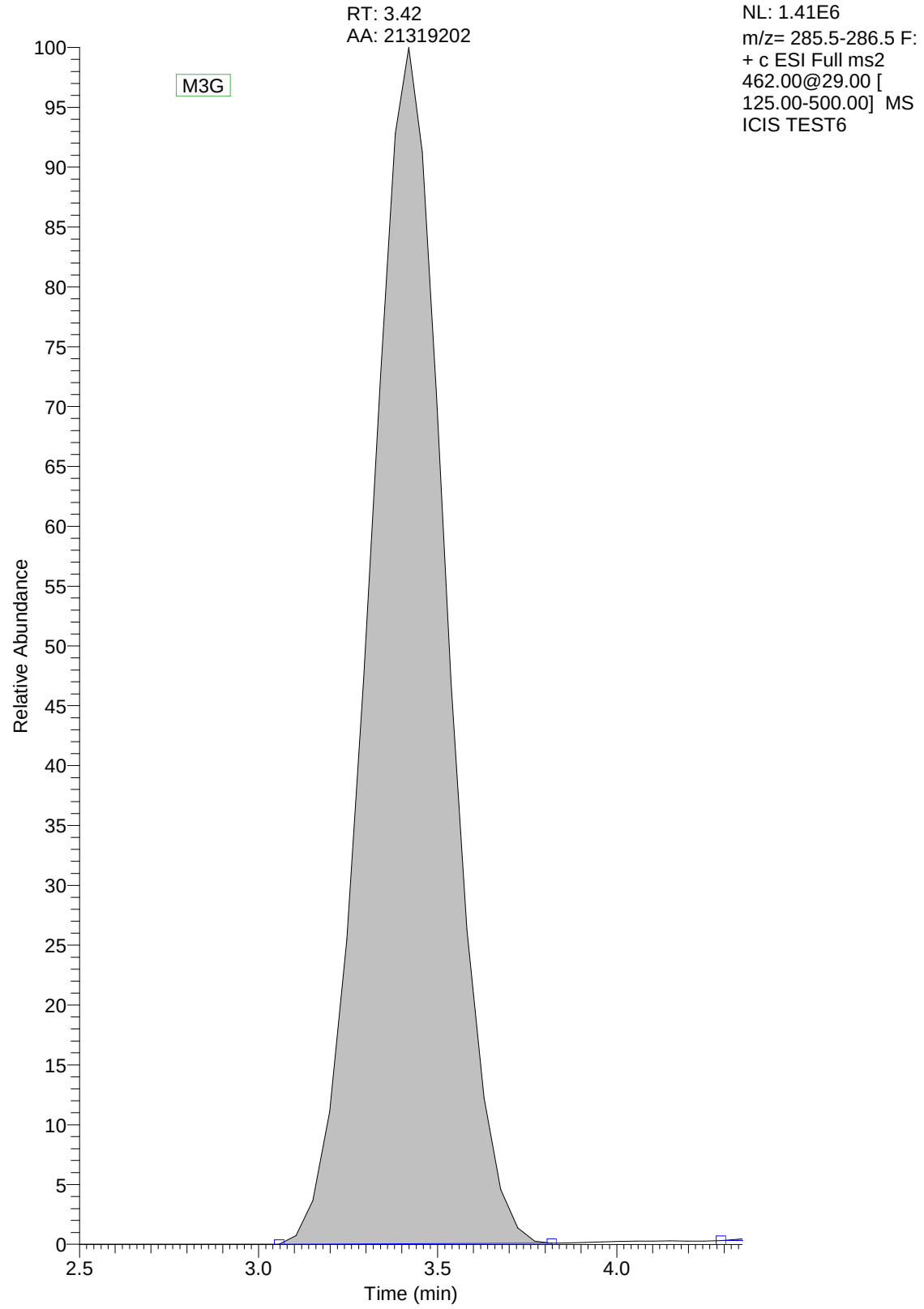

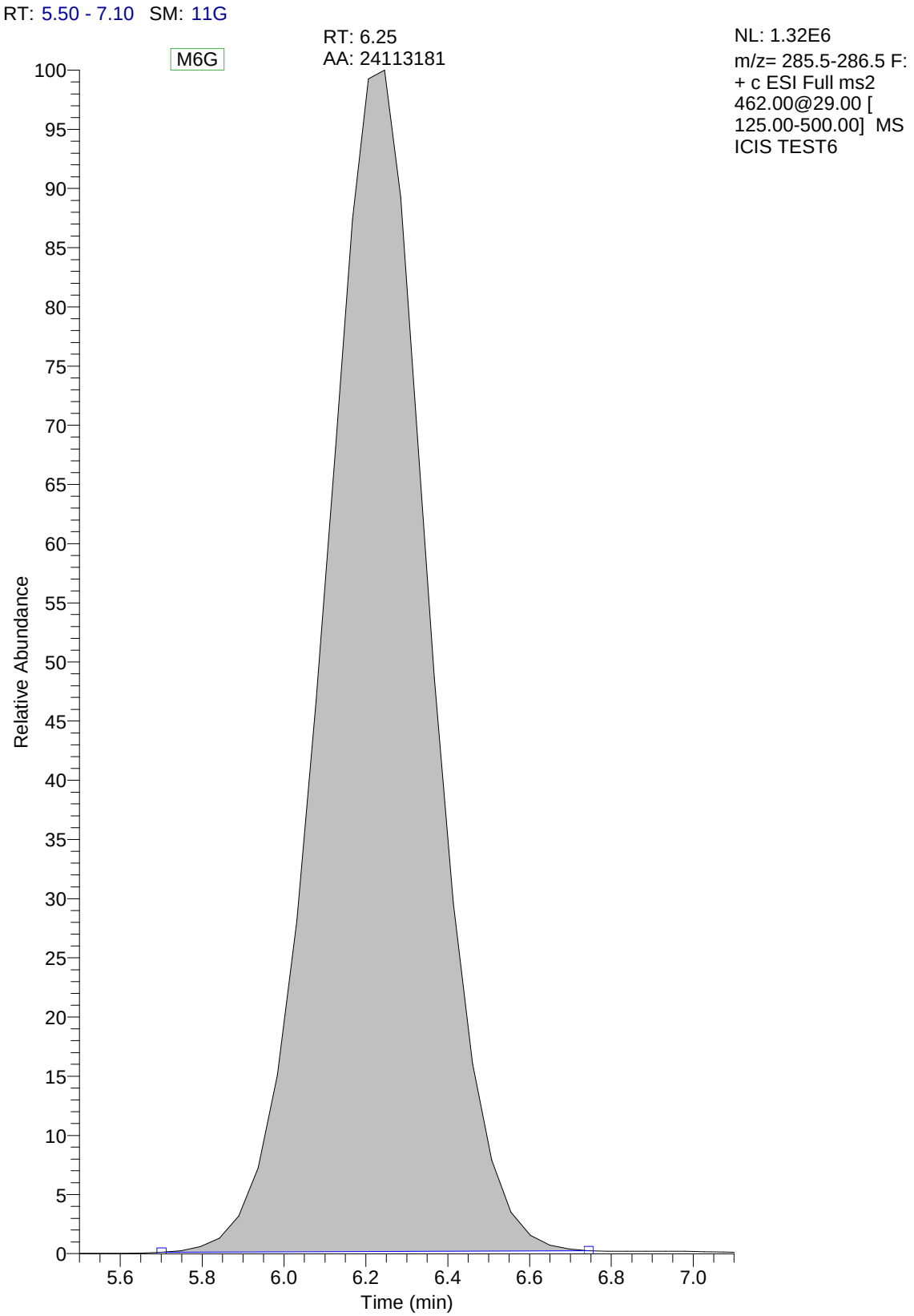
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M6G
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Relative Abundance
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90
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80
75
70
65
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55
50
45
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35
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20
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6.6
6.8
7.0

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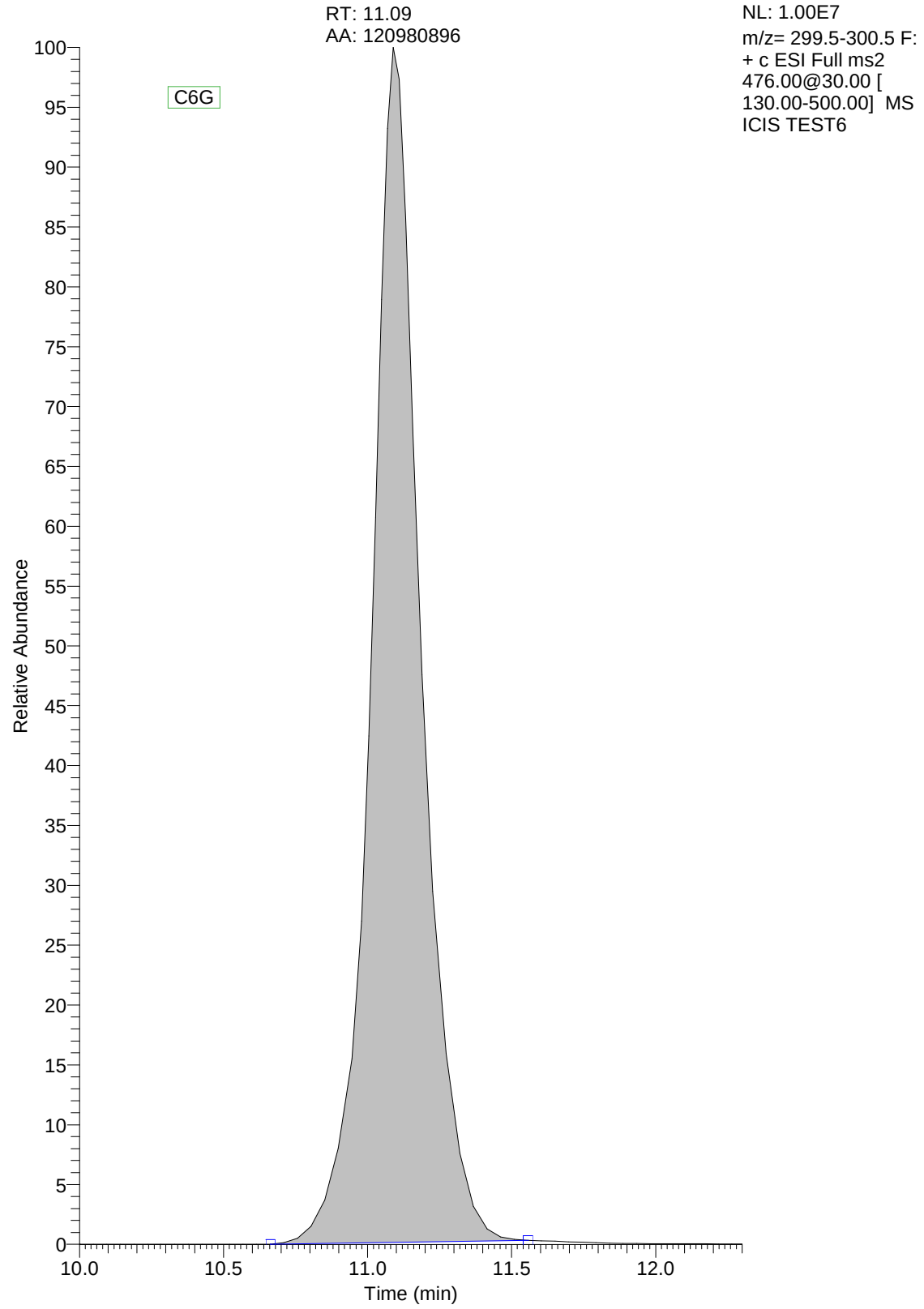

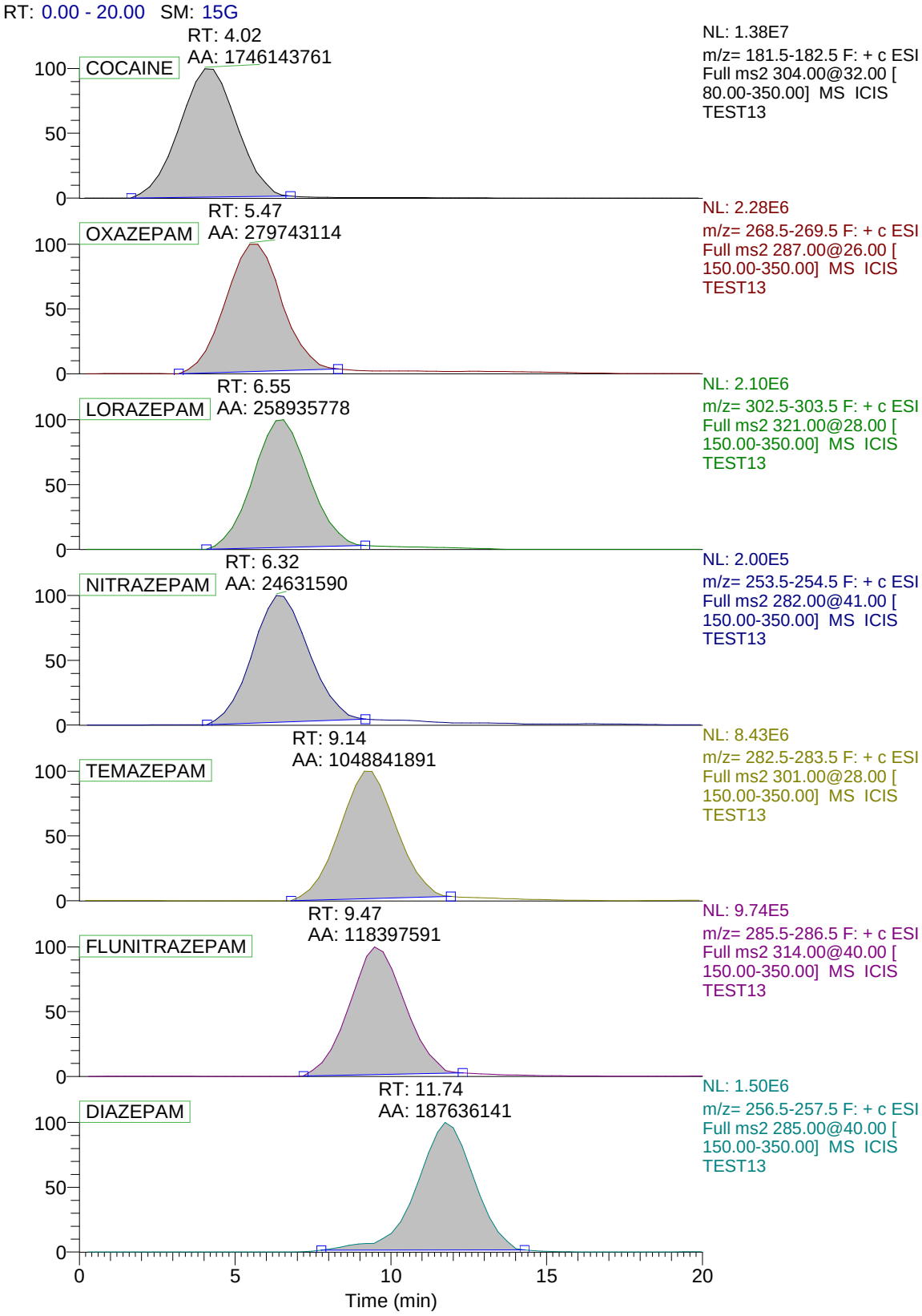
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TEST13
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0
5
10
15
20
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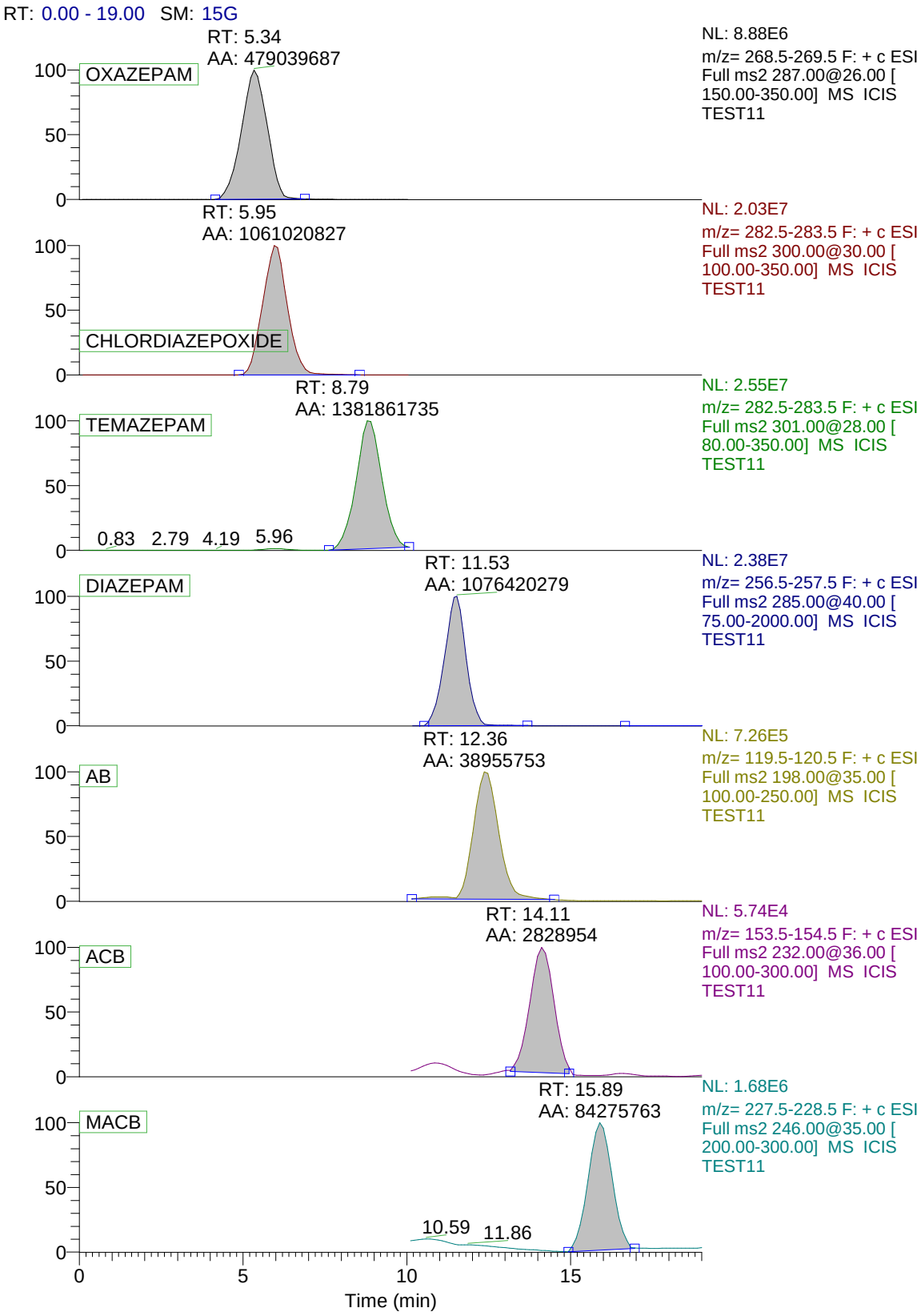
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TEST11
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TEST11
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AB
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ACB
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MACB
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0
0
5
10
15
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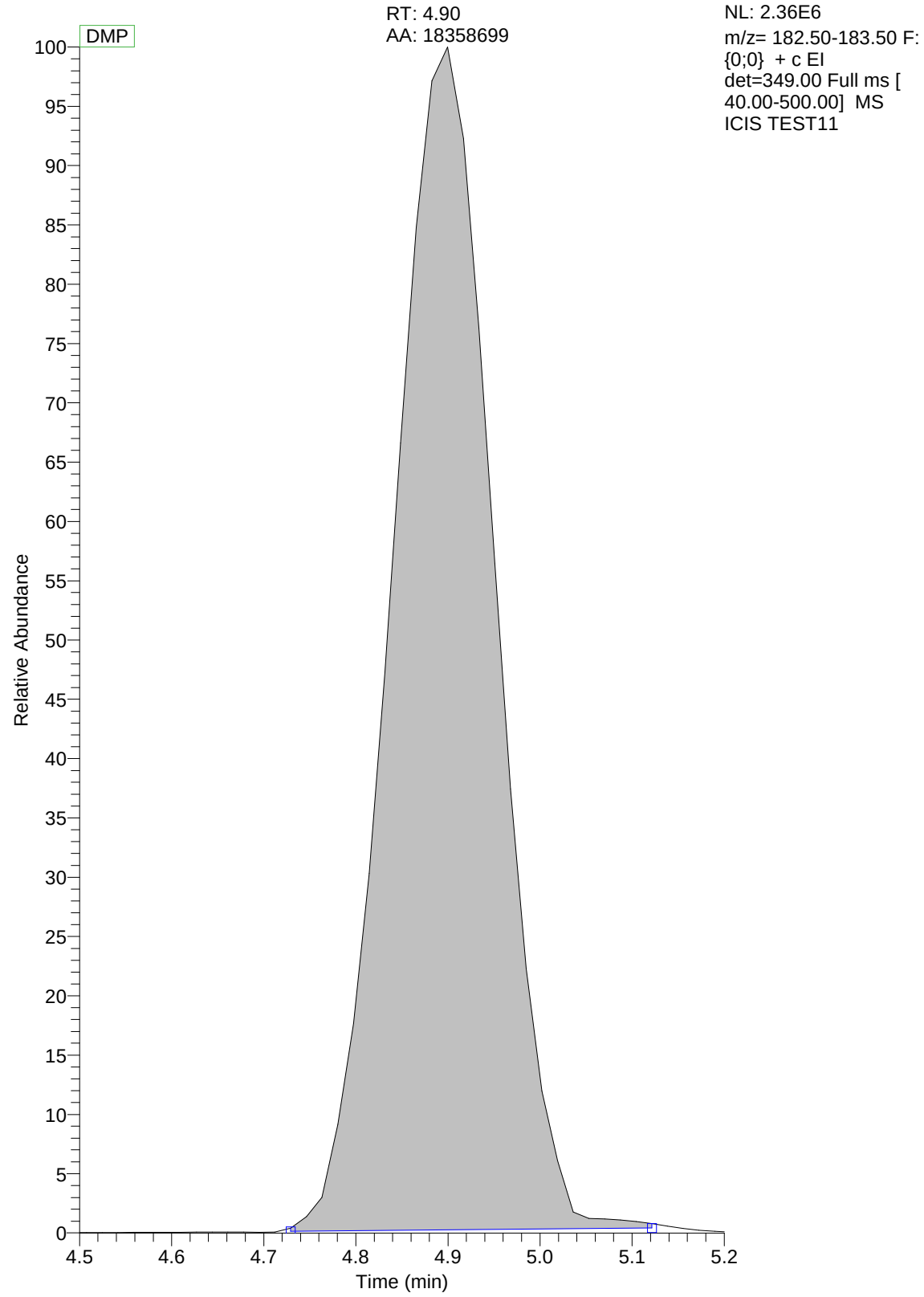

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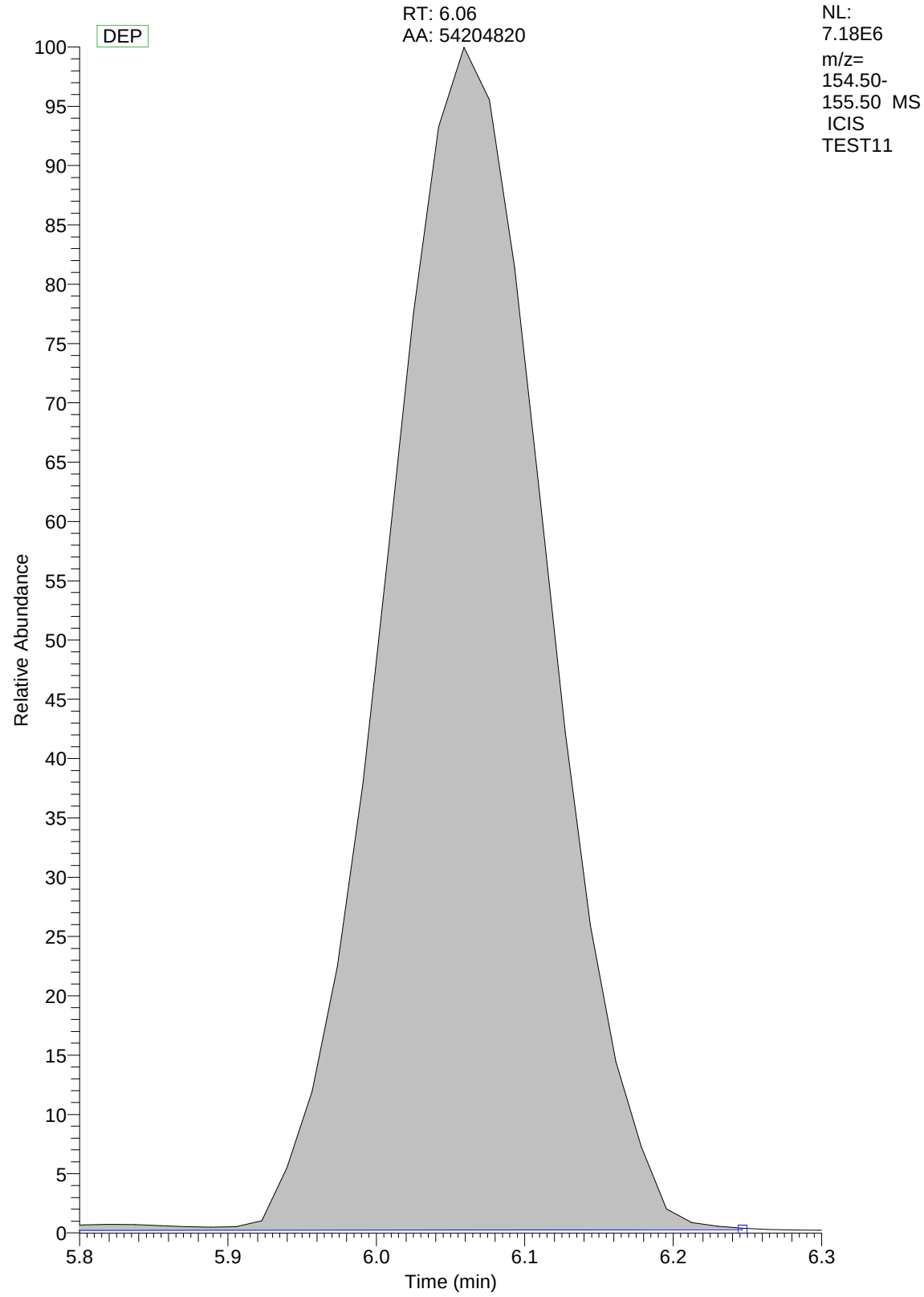

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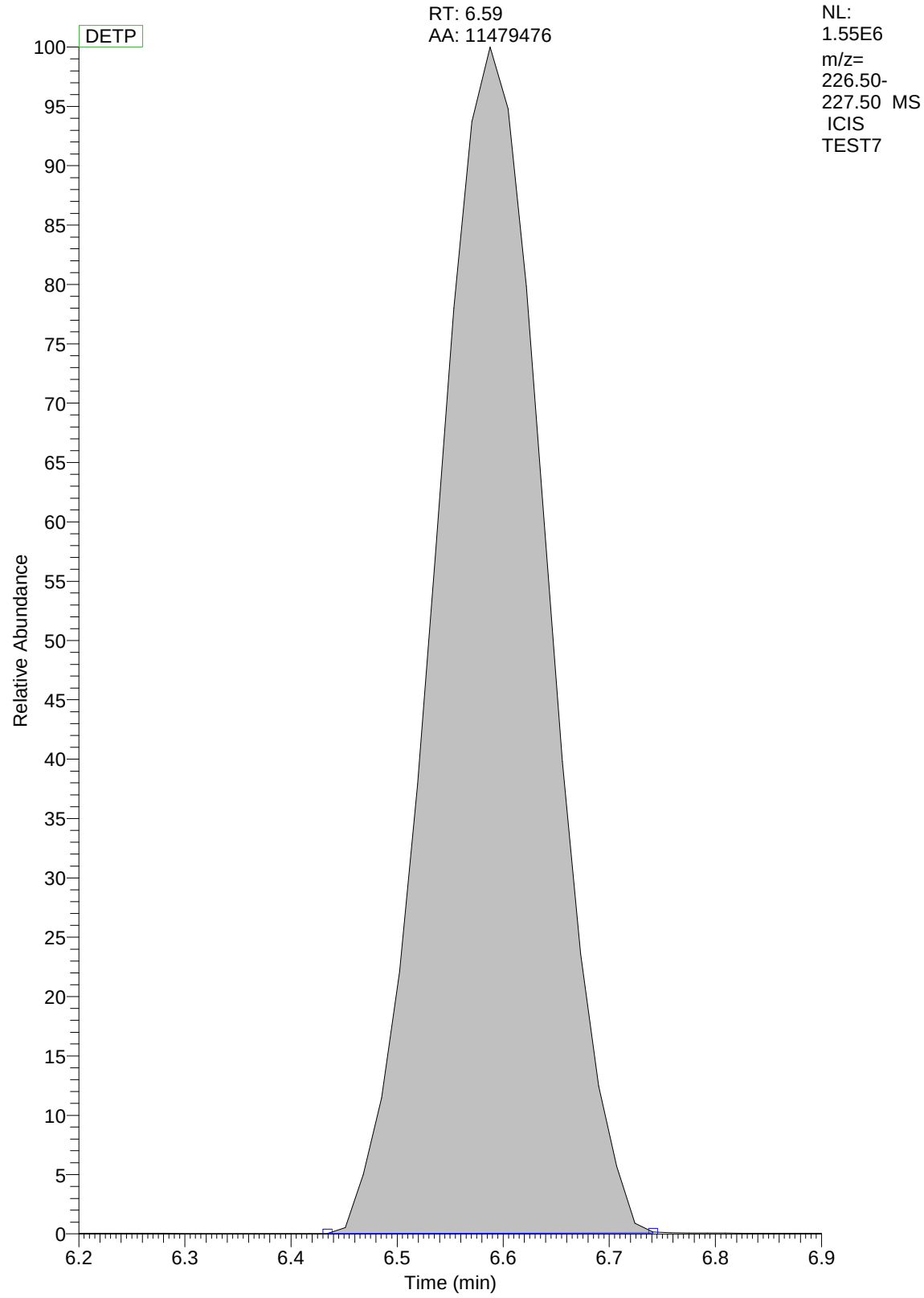

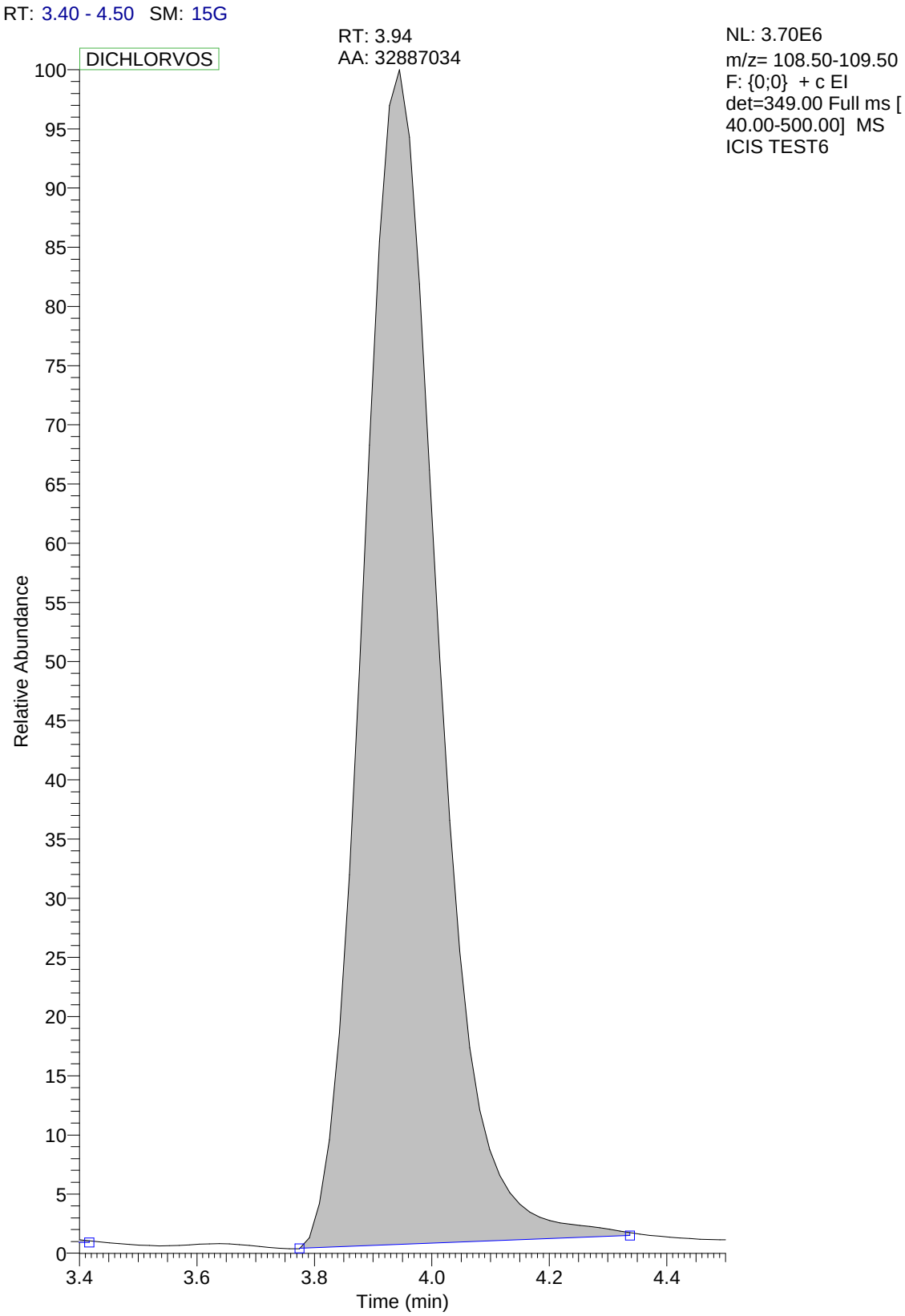
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ICIS TEST6
Relative Abundance
Time (min)
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95
90
85
80
75
70
65
60
55
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3.6
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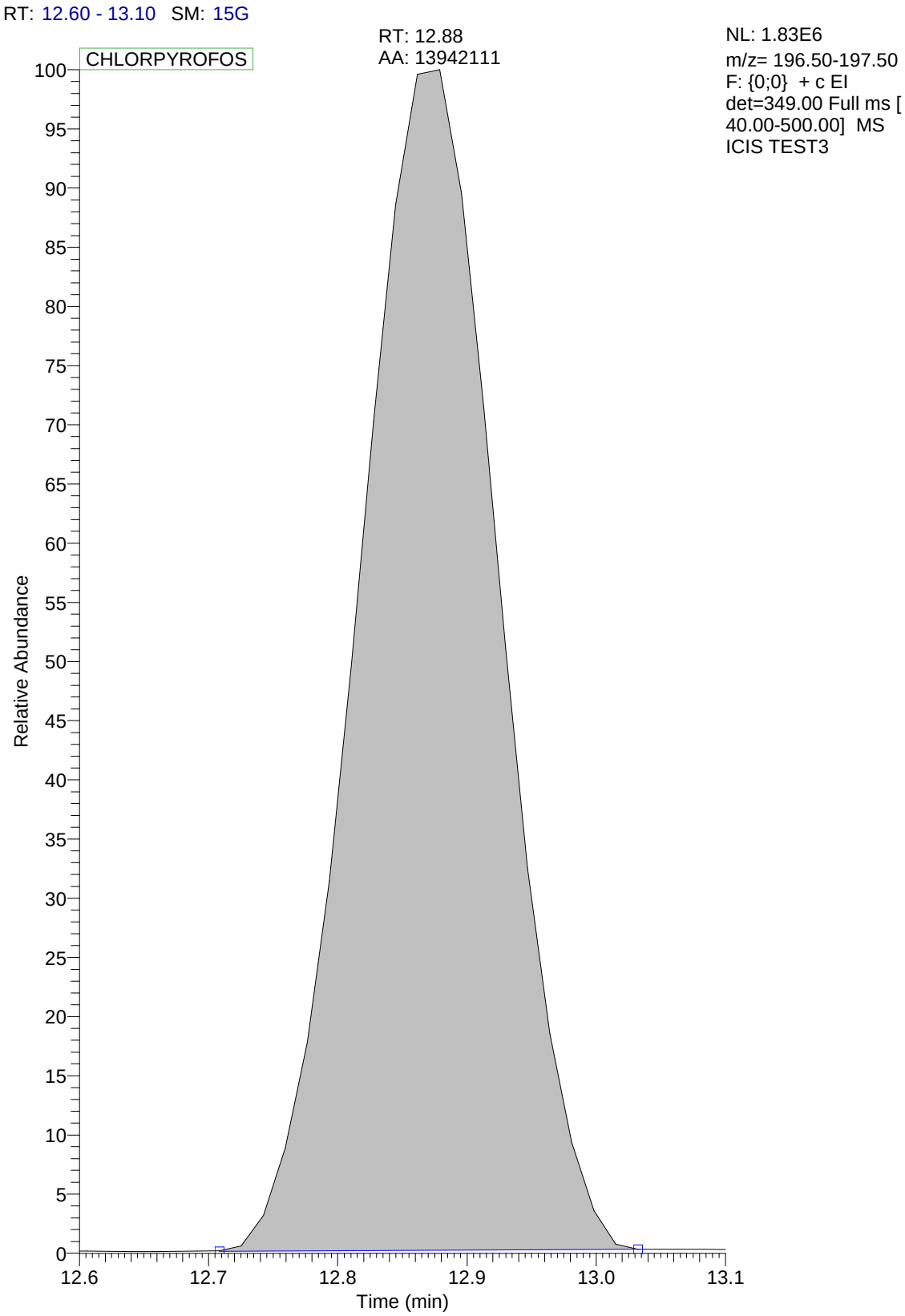
4.2
4.4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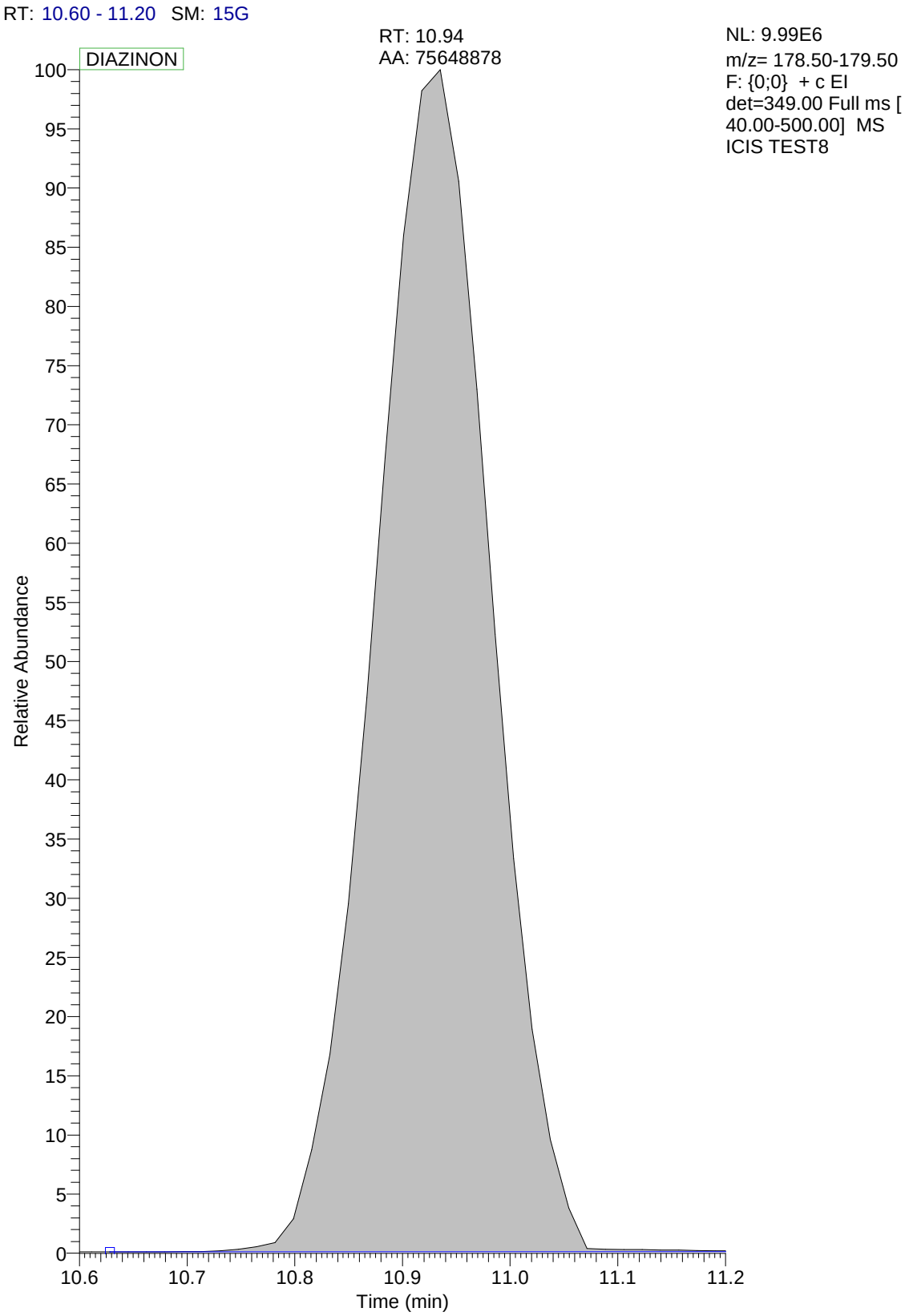
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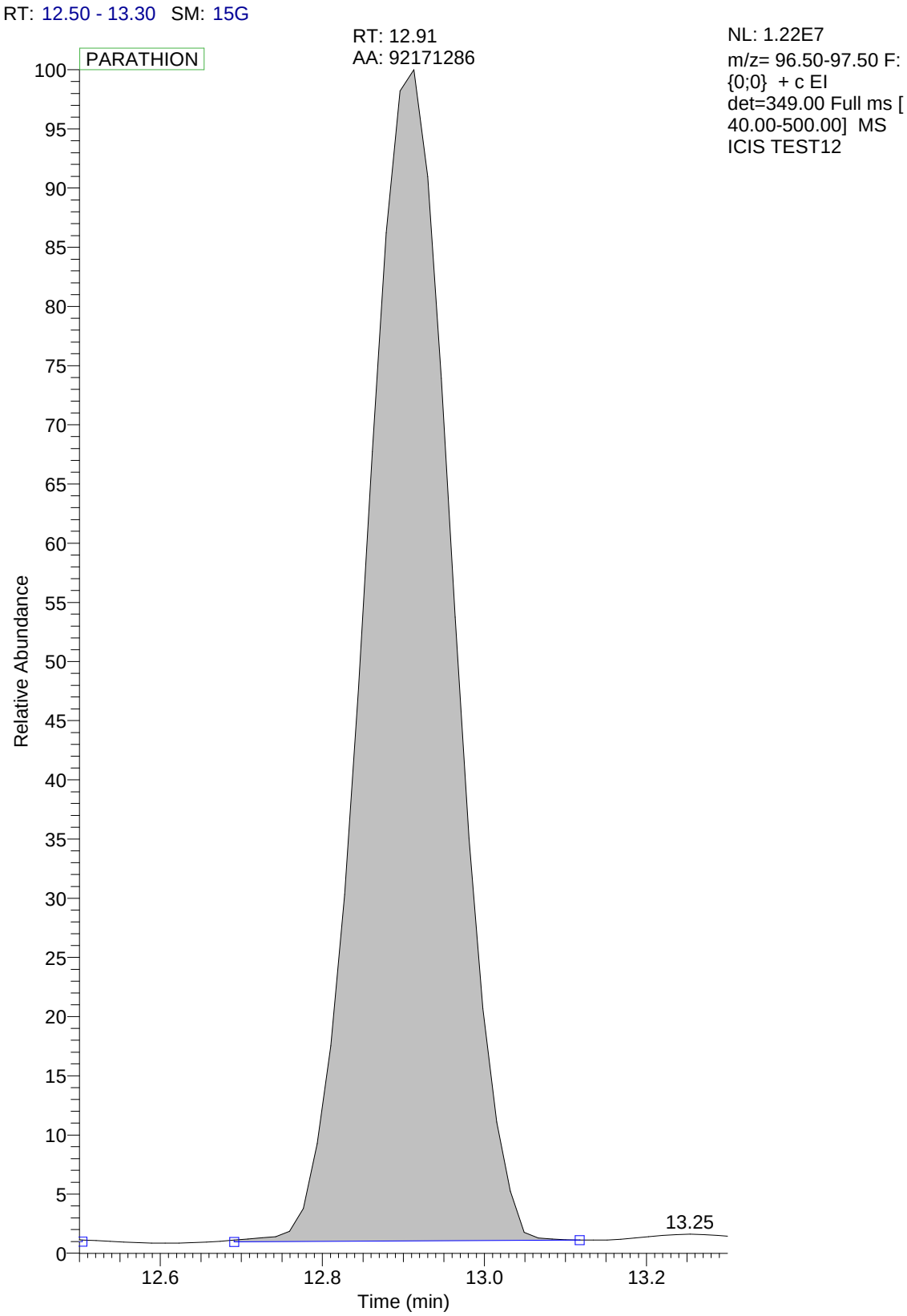
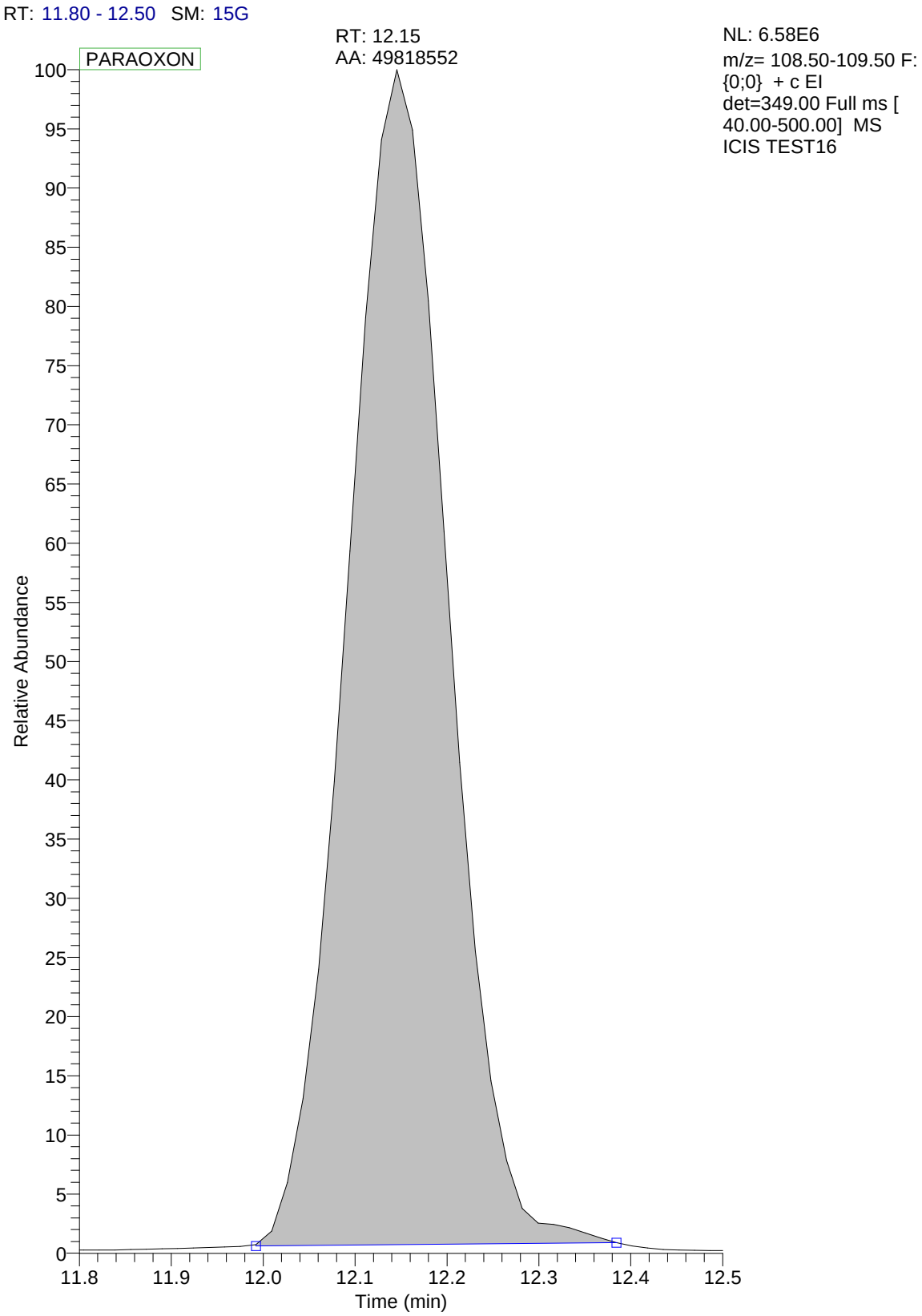
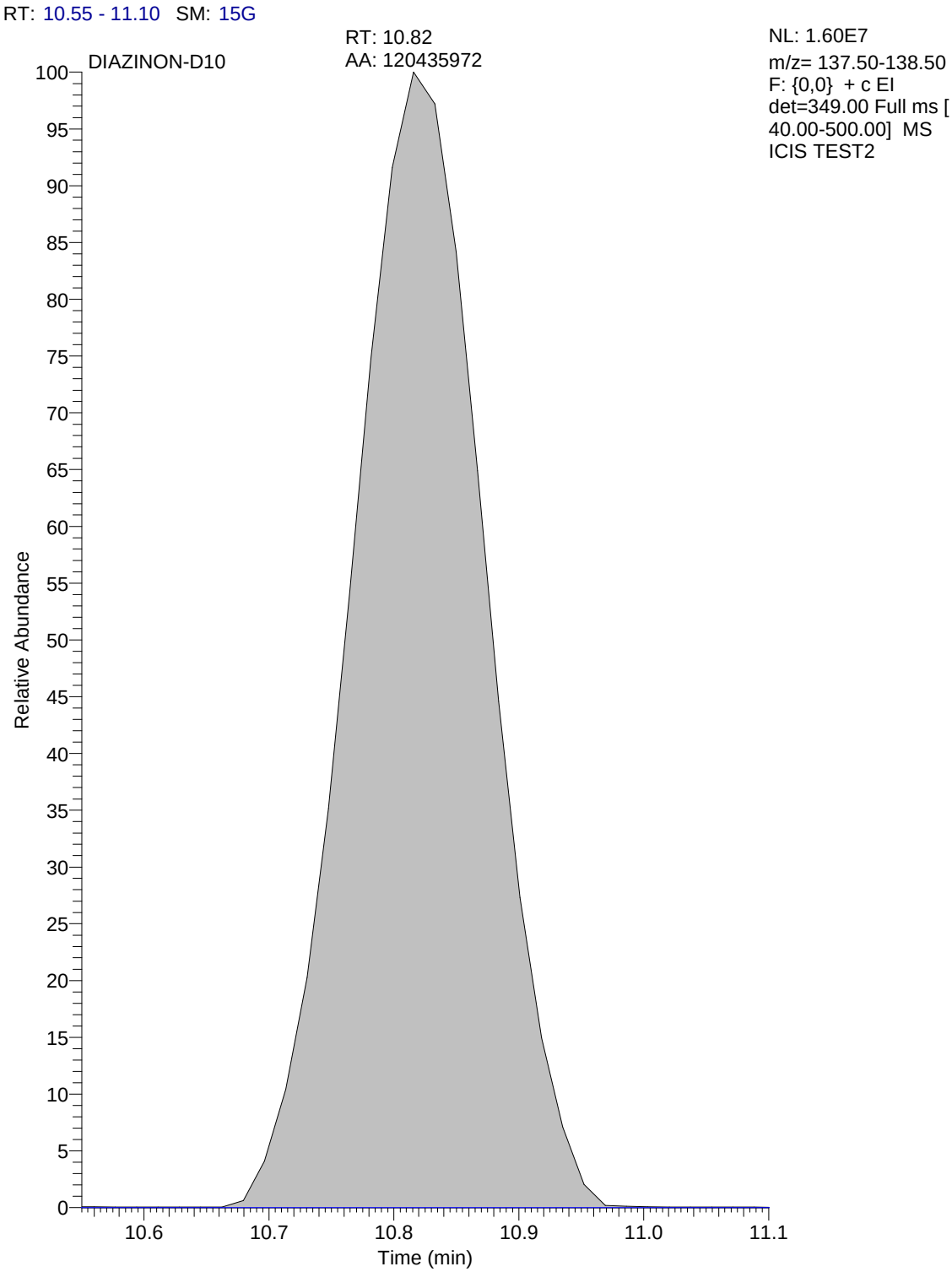
det=349.00 Full ms [
40.00-500.00] MS
ICIS TEST8
Relative Abundance
Time (min)
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
10.6
10.7
10.8
10.9
11.0
11.1
11.2


RT: 12.50 - 13.30 SM: 15G
PARATHION
RT: 12.91
AA: 92171286
NL: 1.22E7
m/z= 96.50-97.50 F: {0;0} + c EI det=349.00 Full ms [40.00-500.00] MS ICIS TEST12
Relative Abundance
Time (min)
13.25
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
12.6
12.8
13.0
13.2


RT: 11.80 - 12.50 SM: 15G
PARAOXON
RT: 12.15
AA: 49818552
NL: 6.58E6
m/z= 108.50-109.50 F: {0;0} + c EI det=349.00 Full ms [40.00-500.00] MS ICIS TEST16
Relative Abundance
Time (min)
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
11.8
11.9
12.0
12.1
12.2
12.3
12.4
12.5


RT: 10.55 - 11.10 SM: 15G
DIAZINON-D10
RT: 10.82
AA: 120435972
NL: 1.60E7
m/z= 137.50-138.50
F: {0,0} + c EI
det=349.00 Full ms [
40.00-500.00] MS
ICIS TEST2
Relative Abundance
Time (min)
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
10.6
10.7
10.8
10.9
11.0
11.1