In vitro studies of drug transformations: application to forensic toxicology

Thesis submitted in part fulfilment of the requirements of the University of Glasgow for the Degree of Doctor of Philosophy

By

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February 2016

Summary

The forensic toxicologist faces challenges in the detection of drugs and poisons in biological samples due to transformations which occur both during life and after death. For example, changes can result from drug metabolism during life or from the use of formalin solution for post mortem embalming purposes. The former requires the identification of drug metabolites and the latter the identification of chemical reaction products in order to know which substances had been administered. The work described in this thesis was aimed at providing ways of tackling these challenges and was divided into two parts. Part 1 investigated the use of *in vitro* drug metabolism by human liver microsomes (HLM) to obtain information on drug metabolites and Part 2 investigated the chemical reactions of drugs and a carbamate pesticide with formalin solution and formalin-blood.

The initial aim of part I was to develop an *in vitro* metabolism method using HLM, based on a literature review of previous studies of this type. MDMA was chosen as a model compound to develop the HLM method because its metabolism was known and standards of its metabolites were commercially available. In addition, a sensitive and selective method was developed for the identification and quantitation of hydrophilic phase I drug metabolites using LC/MS/MS with a conventional reverse-phase (C18) column. In order to obtain suitable retention factors for polar drug metabolites on this column, acetyl derivatives were evaluated for converting the metabolites to more lipophilic compounds and an optimal separation system was developed. Acetate derivatives were found to be stable in the HPLC mobile phase and to provide good chromatographic separation of the target analytes.

In vitro metabolism of MDMA and, subsequently, of other drugs involved incubation of 4 µg drug substance in pH 7.4 buffer with an NADPH generating system (NGS) at 37^{o}C for 90 min with addition of more NGS after 30 min. The reaction was stopped at 90 min by the addition of acetonitrile before extraction of the metabolites.

Acetate derivatives of MDMA metabolites were identified by LC/MS/MS using multiple reaction monitoring (MRM). Three phase I metabolites (both major and

minor metabolites) of MDMA were detected in HLM samples. 3,4-dihydroxy-methamphetamine and 4-hydroxy-3-methoxymethamphetamine were found to be major metabolites of MDMA whereas 3,4-methylenedioxyamphetamine was found to be a minor metabolite. Subsequently, ten MDMA positive urines were analysed to compare the metabolite patterns with those produced by HLM. An LC/MS method for MDMA and its metabolites in urine samples was developed and validated. The method demonstrated good linearity, accuracy and precision and insignificant matrix effects, with limits of quantitation of 0.025 µg/ml. Moreover, derivatives of MDMA and its metabolites were quantified in all 10 positive human urine samples. The urine metabolite pattern was found to be similar to that from HLM.

The second aim of Part 1 was to use the HLM system to study the metabolism of some new psychoactive substances, whose misuse worldwide has necessitated the development of analytical methods for these drugs in biological specimens. Methylone and butylone were selected as representative cathinones and *para*-methoxyamphetamine (PMA) was chosen as a representative ring-substituted amphetamine, because of the involvement of these drugs in recent drug-related deaths, because of a relative lack of information on their metabolism, and because reference standards of their metabolites were not commercially available.

An LC/MS/MS method for the analysis of methylone, butylone, PMA and their metabolites was developed. Three phase I metabolites of methylone and butylone were detected in HLM samples. Ketone reduction to *β*-OH metabolites and demethylenation to dihydroxy-metabolites were found to be major phase I metabolic pathways of butylone and methylone whereas *N*-demethylation to nor-methylone and nor-butylone were found to be minor pathways. Also, demethylation to *para*-hydroxyamphetamine was found to be a major phase I metabolic pathway of PMA whereas *β*-hydroxylation to *β*-OH-PMA was found to be a minor pathway.

Formaldehyde is used for embalming, to reduce decomposition and preserve cadavers, especially in tropical countries such as Thailand. Drugs present in the body can be exposed to formaldehyde resulting in decreasing concentrations of the original compounds and production of new substances. The aim of part II of the study was to evaluate the *in vitro* reactions of formaldehyde with selected

drug groups including amphetamines (amphetamine, methamphetamine and MDMA), benzodiazepines (alprazolam and diazepam), opiates (morphine, hydromorphone, codeine and hydrocodone) and with a carbamate insecticide (carbosulfan). The study would identify degradation products to serve as markers for the parent compounds when these were no longer detectable.

Drugs standards were spiked in 10% formalin solution and 10% formalin blood. Water and whole blood without formalin were used for controls. Samples were analysed by LC/MS/MS at different times from the start, over periods of up to 30 days.

Amphetamine, methamphetamine and MDMA were found to rapidly convert to methamphetamine, DMA and MDDMA respectively, in both formalin solution and formalin blood, confirming the Eschweiler-Clarke reaction between amine-containing compounds and formaldehyde.

Alprazolam was found to be unstable whereas diazepam was found to be stable in both formalin solution and water. Both were found to hydrolyse in formalin solution and to give open-ring alprazolam and open-ring diazepam. Other alprazolam conversion products attached to paraformaldehyde were detected in both formalin solution and formalin blood.

Morphine and codeine were found to be more stable than hydromorphone and hydrocodone in formalin solution. Conversion products of hydromorphone and hydrocodone attached to paraformaldehyde were tentatively identified in formalin solution. Moreover, hydrocodone and hydromorphone rapidly decreased within 24 h in formalin blood and could not be detected after 7 days.

Carbosulfan was found to be unstable in formalin solution and was rapidly hydrolysed within 24 h, whereas in water it was stable up to 48 h. Carbofuran was the major degradation product, plus smaller amounts of other products, 3-ketocarbofuran and 3-hydrocarbofuran. By contrast, carbosulfan slowly hydrolysed in formalin-blood and was still detected after 15 days.

It was concluded that HLM provide a useful tool for human drug metabolism studies when ethical considerations preclude their controlled administration to humans. The use of chemical derivatisation for hydrophilic compounds such as polar drug metabolites for analysis by LC/MS/MS with a conventional C18 column

is effective and inexpensive, and suitable for routine use in the identification and quantitation of drugs and their metabolites. The detection of parent drugs and their metabolites or conversion and decomposition products is potentially very useful for the interpretation of cases in forensic toxicology, especially when the original compounds cannot be observed.

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Ethical Approvals for this Thesis

The human biological specimens used in the work described in this thesis are listed below along with details of applicable ethical approval requirements.

Blank urine specimens used in method development and validation studies	Existing blank urine samples from a previous research project, obtained with informed consent and appropriately stored in Forensic Medicine and Science, were used. The chair of the University Medical Research Ethics Committee, Dr Jesse Dawson, Institute of Cardiovascular and Medical Sciences, University of Glasgow confirmed that approval would not be needed for these samples.
Post mortem urine samples	Three post mortem urine samples known to be positive for MDMA were analysed and reported as part of the medico-legal investigation of the three cases. Ethical approval and consent are not required if the analytical results are reported to the Procurator Fiscal.
Clinical urine samples	Human urine samples which were known to be positive for MDMA were obtained from Glasgow Royal Infirmary for routine toxicology service analysis and the results of the analyses were reported as part of the case investigation. The Infirmary Ethics Committee advised Dr Hazel Torrance that ethical approval was not required as the work formed part of medical systems development.

Acknowledgments

I would firstly like to thank Dr Robert A. Anderson for his help, support and guidance as supervisor and encouragement during my laboratory experimental work and in writing my thesis. I will miss him for his kindness and care during these three years under his supervision.

I would also like to thank Dr Hazel Torrance, my second supervisor, and all toxicologists and staff in Forensic Medicine and Science who gave me support and advice during my studies. Also I would like to thank all students in the section for giving me a good time.

I would also like to thank my whole family who has always supported me and gave me strength in my study and in my life.

Finally I would like to thank the Royal Thai Government for supporting me financially during my studies.

Abbreviations

2C-B	2,5-Dimethoxy-4-bromophenethylamine
3-HMMA	3-Hydroxy-4-methoxymethamphetamine
4-HMC	4-Hydroxy-3-methoxycathinone
4-HMMA	4-Hydroxy-3-methoxymethamphetamine
A/D	Analog/Digital
AA	Acetic anhydride
ACh	Acetylcholine
AChE	Acetylcholinesterase
amu	Atomic mass unit
API	Atmospheric pressure ionisation
ATS	Amphetamine-type stimulants
AUC	Area under the curve
bk-BDB	Nor-butylone
bk-MBDB	Butylone
CE	Collision energy
CID	Collision-induced dissociation
C_{max}	Maximum concentration
CNS	Central nervous system
COMT	Catechol-*O*-methyltransferase
CYPs	Cytochromes
Da	Dalton
DC	Direct-current
DMA	*N,N*-dimethylamphetamine
DOM	4-Methyl-2,5-dimethoxyamphetamine
ECD	Electron capture detector
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact ionisation
EIC	Extracted-ion chromatogram
ELISA	Enzyme-linked immunosorbent assay
EMCDDA	European Monitoring Centre on Drugs and Drug Addiction
ESI	Electrospray ionisation
FID	Flame ionisation detector
FMOs	Flavin-containing monooxygenases

FPD	Flame photometric detector
FV	Fragmentor voltage
G6P	D-glucose 6-phosphate dipotassium salt hydrate
G6PD	Glucose-6-phosphate dehydrogenase
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
GC-NICI-MS	GC/MS with negative ion chemical ionisation
GHB	Gamma-hydroxybutyrate
GLC	Gas-liquid chromatography
Gluc	Glucuronide
GST	Glutathione S-transferase
HFBA	Heptafluorobutyric acid anhydride
HHMA	3,4-Dihydroxymethamphetamine
HHMC	3,4-Dihydroxymethcathinone
HILIC	Hydrophilic interaction chromatography
HLM	Human liver microsomes
HMA	4-Hydroxy-3-methoxyamphetamine
HMC	3,4-Methylenedioxycathinone
HMMA	Hydroxymethoxymethamphetamine
HMMC	4-Hydroxy-3-methoxymethcathinone
HPLC	High-performance liquid chromatography
HPLC/DAD	High-performance liquid chromatography/diode array detection
HPLC/ED	High-performance liquid chromatography/electrochemical detection
IPs	Identification points
IS	Internal standard
k'	Retention factors
LC	Liquid Chromatography
LC/MS	Liquid chromatography/mass spectrometry
LC/MS/MS	Liquid chromatography/tandem mass spectrometry
LLOQ	Lower limit of quantitation
LOD	Limit of detection
LSD	Lysergic acid diethylamide

m/z	Mass-to-charge ratio
MBTFA	*N*-Methyl-bis(trifluoroacetamide)
MDA	3,4-Methylenedioxyamphetamine
MDC	3,4-Methylenedioxycathinone
MDDMA	3,4-Methylenedioxydimethylamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MDMA-d_5	3,4-Methylenedioxymethamphetamine-d_5
MDPV	3,4-Methylenedioxypyrovalerone
ME	Matrix effect
MRM	Multiple reaction monitoring
MS	Mass spectrometer
MSTFA	*N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide
NADP	Nicotinamide adenine dinucleotide phosphate
NAT	*N*-Acetyl transferase
NGS	NADPH generating system
NIDA	National Institute on Drug Abuse
NPD	Nitrogen-phosphorus detector
NPS	New psychoactive substances
PE	Process efficiency
PFPA	Pentafluoropropionic acid anhydride
PHA	*para*-Hydroxyamphetamine/4-Hydroxyamphetamine
PMA	*para*-Methoxyamphetamine
QC	Control samples/quality control
QqQ	Triple quadrupole mass analyser
R^2	Correlation coefficient
RE	Recovery
RF	Radio-frequency/Response factors
RPC	Reverse-phase chromatography
RSD	Relative standard deviation
S-HFBPCl	*S*-Heptafluoro-butyrylprolyl chloride
SIM	Selected ion monitoring
SNS	Sympathetic nervous system
SPE	Solid phase extraction
SRM	Selected reaction monitoring
ST	Sulphotransferase

Sul	Sulphate
TCD	Thermal conductivity detector
TFA	Trifluoroacetic acid anhydride
TMS	Trimethylsily
t_R	Retention time
UDPGA	Uridine diphosphate glucuronic acid
UGTs	Uridine diphosphoglucuronosyl transferases
UN	United Nations
UNODC	United Nations Office on Drugs and Crime
β-NADP	*β*-Nicotinamide adenine dinucleotide phosphate hydrate

Chapter 1 General introduction

1.1 Forensic toxicology

Toxicology is the study of how toxic substances (toxins) or poisons interact with living organisms resulting in harmful effects or death after exposure. All substances can be poisons depending on the size of the dose. Drugs are used for treatment within therapeutic levels. At higher doses, drugs may cause harm to the human body or death at lethal doses. There are many subdivisions of toxicology such as medical toxicology, clinical toxicology, environmental toxicology, forensic toxicology and others[1,2].

Forensic toxicology is involved with the identification, detection and interpretation of the presence of drugs and poisons for medico-legal purposes. Two main purposes of forensic toxicology are to assist in death investigations and in drug of abuse testing in the living. Forensic toxicology also includes drug facilitated crimes, road safety, workplace drug testing and the development of analytical methods for identification and confirmation of drugs and poisons. Studies on pharmacokinetics (absorption, distribution, metabolism, and excretion) are also important for toxicologists to understanding the likely effects of drugs and for interpretation of reported results[3,4].

The most common substances involved in forensic analysis are amphetamine-type stimulants, benzodiazepines, barbiturates, cocaine alkaloids, opioids such as heroin, morphine and codeine and cannabis products. However, other substances can also be targeted such as ketamine, gamma-hydroxybutyrate (GHB), lysergic acid diethylamide (LSD), pesticides and new psychoactive substances whose misuse continues to increase.

Samples for forensic toxicology analysis are biological fluids such as blood and urine. However, other biological samples such as vitreous humour, bile, stomach contents, liver, kidney and other organ tissues, hair and oral fluid are also required for toxicology analysis in some cases. The major work of the forensic toxicologist in post mortem cases involves the identification and quantitation of

drugs or poisons which are present in the samples and which may be relevant to the cause of death.

The results obtained from forensic toxicological analysis are important to the investigation of crimes or accidents. Analytical methods with high selectivity, sensitivity and rapidity are required. The most common analytical methods are immunoassay, which is used as a screening method, and chromatography-based methods which are used as confirmatory methods. To interpret the analytical results, many parameters need to be considered such as post mortem changes, post mortem redistribution and drug biotransformation which will convert drugs to other forms (metabolites)[3].

1.2 Drug transformations

Chemical transformation is defined as the conversion of a substrate to a product. Drugs are transformed both inside and outside the body. Inside the body, drugs are processed via the chemical and biochemical modifications made by a living organism, so-called “biotransformation”. Lipophilic compounds are converted to hydrophilic compounds, so-called “metabolites”, and excreted from the body mainly via the urinary system. Not only for formation of metabolites, biotransformation also includes drug interactions, bioactivation, substrate inhibition, enzyme induction and carcinogenesis. Basic reactions for biotransformation are functionalisation and synthetic conjugation reactions[5].

Outside the body, drugs are transformed through chemical reactions usually resulting in a reduction in drug concentrations. Drugs can form degradation products which may be toxic substances via both chemical and physical reactions such as photo-degradation. The expiration date is one parameter that expresses the degradation of drugs[6]. Chemical reactions will occur when drugs or compounds are exposed to environmental conditions such as heat, radiation, humidity and acidic or basic solvents. The most common reactions are hydrolysis, oxidation and reduction. In the analytical process, chemical transformations are sometimes used deliberately by the toxicologist, for example, chemical derivatisation is used to transform a target substance to a product with desirable properties.

Forensic toxicologists have been able to respond to the challenge of detecting drug transformation. Analytical methods in the forensic laboratory should be able to detect drug metabolites or decomposition products which affect the concentrations of the original substances measured in samples. It is necessary to quantitate parent drug, metabolites and decomposition products to obtain information on the drug concentrations before the transformation(s) for the interpretation of reported results.

1.3 General aims

The general aims of this study were to identify *in vitro* transformation reactions related to forensic toxicology including the identification of drug metabolites using human liver microsomes and the identification of products of the reaction between formaldehyde with various drugs and poisons. Specific aims were:

- To identify MDMA metabolites using *in vitro* metabolism with human liver microsomes and analysis by LC/MS/MS.
- To identify methylone, butylone and PMA metabolites using *in vitro* metabolism with human liver microsomes and analysis by LC/MS/MS.
- To identify conversion products of the reactions between formaldehyde and amphetamine, methamphetamine, MDMA, alprazolam, diazepam, codeine, hydrocodone, morphine, hydromorphone and carbosulfan.

Chapter 2 Metabolism studies

2.1 Introduction

In recent years, a large number of new drugs from new drug classes such as synthetic cathinones or synthetic cannabinoids have appeared on the illicit drug market. Previous studies have shown that some active metabolites can contribute a toxic effect greater than that of their parent drugs, such as the metabolites of MDMA[7,8]. Knowledge of the metabolism of new drugs is important in clinical and forensic toxicology for developing an analytical method. However, current knowledge on the human metabolism of new drugs is very limited. This chapter will review basic concepts of drug metabolism relevant to the studies reported subsequently.

2.2 Drug metabolism

Drugs are introduced into the body via many routes such as oral, dermal or intravascular. After a drug has entered the body, it is eliminated either as unchanged drug or as metabolism products, including both active and inactive metabolites. Drugs are removed from the body by biological processes called “metabolism” or “bio-transformation” which occur in two steps, phase I and phase II. Metabolites are usually removed from the human body faster than the parent drug due to their chemical structures which are commonly more water-soluble than their parent drugs and more readily excreted by the kidney which is the major route of elimination of most drugs[9-11]. Biotransformation occurs in many human organs such as the liver, kidneys, lungs and intestines. However, most biotransformation occurs in the liver.

Phase I drug metabolism typically involves oxidation, reduction, and hydrolysis of xenobiotics to generate more polar derivatives such as alcohols, phenols or carboxylic acid compounds whereas phase II metabolism is responsible for conjugation reactions such as glucuronidation, sulphation or addition of amino acids such as glycine. Typically phase I oxidation is involved mainly with hepatic cytochromes P450 (CYPs) that utilize NADPH and oxygen in their catalytic cycle.

CYPs play an important role in the metabolism of drugs, steroids and biogenic amines and can also transform nontoxic chemicals into toxic or carcinogenic products. Other metabolic enzymes are alcohol dehydrogenases, aldehyde dehydrogenases or flavin-containing monooxygenases (FMOs). Some of the metabolites produced by phase I reactions can undergo excretion, but many others require additional transformation by phase II metabolism. Enzymes that are involved in phase II metabolism include uridine diphosphoglucuronosyl transferases (UGTs) which are generally bound to the endoplasmic reticulum, with liver a major, but not the only, location, glutathione S-transferases (GSTs) which are located mainly in the cytoplasm but are also present in the nucleus, mitochondria and peroxisomes, and *N*-acetyl transferases (NATs), and sulphotransferases (STs) which are located in the cytosol in a range of tissues including the liver[12-15].

However, there are some disadvantages of drug metabolism. Some metabolites are still pharmacologically active and toxic after the parent drug structure has been modified, such as morphine-6-glucuronide, and some are more toxic than their parents, such as the neurotoxic metabolites of MDMA[16-18]. Therefore, the relationship between drug biotransformation and toxicity needs to be evaluated. There are multiple methods used in the investigation of drug metabolism such as *in vitro* and *in vivo* methods.

2.3 *In vitro* metabolism studies

There are many different *in vitro* methods or models used to study drug metabolism. *In vitro* data can be used to predict the metabolic pathway of each drug or toxic substance in humans. Because metabolism of drugs and toxic compounds occurs mainly in the liver, the majority of *in vitro* metabolism studies are derived from liver tissue, which contains drug metabolising enzymes. There are many liver preparations used for *in vitro* metabolism studies such as liver homogenates, isolated hepatocytes and sub cellular liver fractions (S9 fraction or microsomes) that constitute a different level of complexity and capability[14,19]. However, results obtained from *in vitro* experiments need to be confirmed by *in vivo* studies. This review focuses on phase I metabolism studies using human liver microsomes.

2.3.1 Human liver microsomes (HLM)

Human liver microsomes (HLM) are amongst the most popular *in vitro* metabolism study methods due to their price and method of use. Microsomes are subcellular fractions derived from the endoplasmic reticulum of hepatic cells. They are prepared and isolated from other cellular components by homogenisation of liver followed by differential centrifugation at 10,000 g and 100,000 g[20]. HLM contain only the membrane-bound metabolising enzymes such as CYPs, FMOs and UGTs which can produce partial metabolic profiles.

To evaluate phase I metabolism using HLM, a cofactor such as nicotinamide adenine dinucleotide phosphate (NADP) needs to be added. HLM can also be applied to glucuronidation studies (phase II) by the addition of uridine diphosphate glucuronic acid (UDPGA). Pooled HLM is a mixture of microsomes from different donors and is recommended to reduce the inter-individual variations in the activity of HLM[13].

The general method for the determination of drug metabolites using HLM is incubation of HLM with drugs in buffer and NADPH generating system, which usually consists of *β*-NADP and glucose-6-phosphate dehydrogenase. These are incubated at 37^{o}C for an appropriate time (40, 60, 90 min, etc.) depending on the target drugs. To stop the reaction, a stop reagent is added to the incubation mixture to precipitate or denature the proteins, including the enzymes.

2.3.2 S9 fractions

S9 liver fractions have been used to study both phase I and phase II drug metabolism since the 1970s. The S9 fraction contains both microsomal and cytosolic fractions which offer a more complete metabolic profile than HLM. S9 fractions also need to be applied with an NADPH generating system. However, the concentrations of metabolites produced by S9 fractions are lower because they contain lower enzyme activities compared to microsomes[13].

2.4 *In vivo* metabolism studies

In vivo animal or human studies have been used in drug development for the determination of pharmacokinetic properties and identification of metabolites. It is the most useful tool to obtain full information on drug biotransformation, especially an *in vivo* human model. However, *in vivo* models have some limitations such as the low amount of drugs that can ethically be administered to animals or humans, potentially resulting in a low concentration of drug metabolites in plasma or urine. Moreover, some intermediate metabolites may not be observed with these models. *In vivo* biotransformation studies have been applied in both animal and human models[19,21].

2.4.1 Animal models

Animal models give information for understanding the role of drug-metabolising enzymes in the organism. However, animal studies cannot entirely predict all metabolism pathways that occur in humans due to species differences. Common animals used for *in vivo* biotransformation studies are small rodents, guinea pigs and rabbits. However, animal studies remain a legal requirement in most countries[22,23].

2.4.2 Human model

The human model involves drug biotransformation experiments with human volunteers. The use of human volunteers for these studies provides full drug metabolic profiles. However, bio-variability amongst volunteers such as age or ethnicity needs to be identified. There are multiple parameters that need to be considered with *in vivo* human studies such as concentration of drug, single or multiple dose administration and route of administration. Moreover, ethical issues and the risk to volunteers participating in clinical studies should also be considered[22,23].

Chapter 3 Derivatisation

Derivatisation is a chemical reaction of an analyte which transforms it into a product with desirable properties, a so-called "derivative", by adding an extra group to the molecule. Derivatisation can be applied in chromatography techniques in both off-line mode and automatic mode and can be done before (pre-derivatisation) or after the separation step (post-column derivatisation)[24]. However, only pre-derivatisation is mentioned in this chapter.

In forensic toxicology, GC/MS, which is suitable for volatile and temperature-stable compounds, is a gold standard method for identification and quantitation of drugs and poisons. However, many drugs or poisons are compounds whose structures contain polar functional groups (hydroxyls, ketones, carboxylic acids and amines), especially metabolites of drugs which are often of extremely high polar character. It is necessary to convert some analytes chemically to another form to make them appropriate for GC/MS analysis. Derivatisation is used in GC to increase the volatility and thermal stability of an analyte. Furthermore, it can also improve the gas chromatographic properties of a compound resulting in a more symmetrical peak shape and give more characteristic fragments in mass spectra for identification[25-27].

For LC/MS, derivatisation is often applied in metabolomic profiling, identification and quantitation of drug metabolites or polar compounds such as aliphatic amines, carboxylic acids and alcohols which are difficult to detect at low concentrations to make them suitable for reverse-phase HPLC. Derivatisation is used in LC/MS to increase the capacity factors of hydrophilic compounds, improve selectivity, improve stability and enhance sensitivity by increasing the chargeability and hydrophobicity of the analytes[28-30].

To perform derivatisation, analytes must have at least one functional group in their structure with an active hydrogen, such as –COOH, –OH, –NHR and $–NH_2$, and the derivative should have adequate stability. Most common derivatisation reactions involve alkylation, silylation and acylation[24,31].

3.1 Acylation: acetylation

Acylation is a common derivatisation method used for drugs analysis. Active hydrogens on an analyte contained in -OH, -SH, -NH_2, or -NHR functional groups are replaced with an acyl group (–CO–R) and converted to esters, thioesters or amides using the action of a carboxylic acid or carboxylic acid derivative. There are three main types of reagents used for acylation derivatisation: acid anhydrides, acyl halides and reactive acyl derivatives. The acylation reagents used most often are acid anhydrides such as acetic acid anhydride (acetic anhydride, AA), heptafluorobutyric acid anhydride (HFBA), pentafluoropropionic acid anhydride (PFPA) and trifluoroacetic acid anhydride (TFA) because the excess reagent, which can affect the stability of the derivatives, is easier to remove[24-26]. Moreover, pyridine is a common solvent used as a basic catalyst in the acylation reaction.

3.1.1 Acetic anhydride

Acetic anhydride is an acylation reagent with the formula $(CH_3CO)_2O$, see Figure 3-1. Acetic anhydride is usually used with pyridine which acts as an accepter for the reaction by-products. Acylation with acetic anhydride has been used in systematic forensic toxicology analysis to derivatise a wide range of drugs due to the significant improvement to their chromatographic properties[25]. A common procedure for acetic anhydride derivatisation is incubation of the dry analyte with a mixture of acetic anhydride and pyridine at the optimum temperature and time for each analyte.

3.1.2 Pentafluoropropionic anhydride (PFPA)

Pentafluoropropionic anhydride (PFPA) is a widely used acylation reagent with the formula $(CF_3CF_2CO)_2O$, see Figure 3-1. Derivatisation with PFPA can not only improve the volatility and thermal stability of an analyte, but also usually gives mass spectra with abundant ions at high *m/z* values. Moreover, derivatisation with PFPA produces a volatile acid by-product that is easily removed before analysis by GC. PFP derivatives can be prepared using PFPA with or without a

basic catalyst or using perfluoro-acylimidazoles[25]. As is the case for acetic anhydride, the derivatisation procedure needs to be optimised for each analyte.

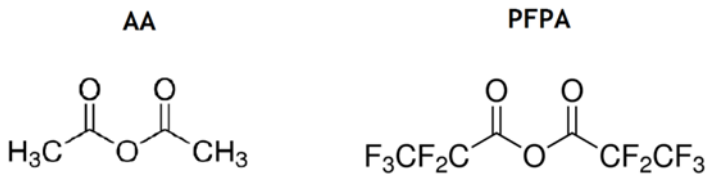


Figure 3-1: Chemical structures of acetic anhydride and PFPA.

3.2 Silylation

Silylation is another common derivatisation technique used for drugs analysis. Active protons on analytes are replaced with an alkylsilyl group, most commonly the trimethylsilyl (TMS, $-Si(CH_3)_3$) group but can also be others such as the dimethyl-(*tert*-butyl)-silyl group (TBDMS,$-Si(CH_3)_2-C(CH_3)_3$) to produce silyl ethers, esters or amines[27]. This reaction can be applied to alcohols, carboxylic acids, amines, amides, thiols, and the enol form of carbonyl compounds. The ability of active functional groups to form silyl derivatives is in the following order: alcohols > phenols > carboxylic acids > amines > amides. Because both the silylation reagents and the derivatives are sensitive to water, silylation must be performed under anhydrous conditions to prevent the degradation of reagent or derivatives[24,25].

There are many reagents developed for silylation reactions with different functional groups and products such as TMS-amines, TMS-amides and TMS-esters. The common silylation reagents include BSTFA, MSTFA, TMSCl and TMSI.

3.2.1 *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA)

N-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide or MSTFA is one of the most commonly used silylation reagents. It is the most volatile TMS-amide which is offered as a liquid reagent. MSTFA is a highly reactive TMS donor and can produce a volatile by-product, *N*-methyltrifluoroacetamide, which often elutes with the GC solvent peak, see Figure 3-2. A common procedure for MSTFA

derivatisation is incubation of the dry analyte with MSTFA at 60°C for 1 h. However, the derivatisation temperature and time should be optimised for each analyte[24-26].

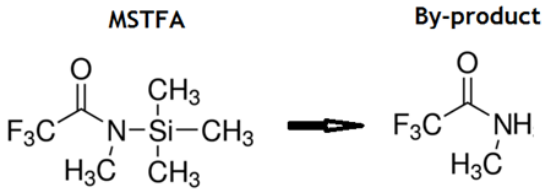


Figure 3-2: Chemical structure of MSTFA and its by-product.

Chapter 4 Method validation

4.1 Introduction

Reliable analytical data are very important in forensic and clinical toxicology. Toxicological investigations depend on reliable information for correct interpretation. Unreliable results can lead to misinterpretation or wrong clinical treatment[32]. Routine analytical methods in forensic toxicology include methods that are able to differentiate drugs in analytical specimens, especially drugs in the same class which have similar chemical properties and similar chemical structures, such as amphetamine, methamphetamine, MDMA, MDA, or new drug groups, such as synthetic cathinones or synthetic cannabinoids. To ensure reliable results, a developed method must be validated before application in routine analysis. Method validation parameters such as sensitivity, selectivity, precision and accuracy need to be examined to ensure that the method is suitable for forensic toxicology investigation.

4.2 Selectivity and specificity

Selectivity is defined as the ability of the bioanalytical method to determine the analyte(s) in the analysed matrices without interference. Specificity is defined as the ability of the bioanalytical method to measure unequivocally and differentiate the analyte(s) in the presence of other components, which may be expected to be present. Typically, these might include metabolites, impurities, degradants and matrix components[32]. Both quantitative and qualitative methods need to be evaluated with respect to their selectivity and specificity to confirm that they are able to differentiate analytes of interest from the matrix components, endogenous compounds and other drugs.

Selectivity of the method can be determined using a blank matrix whereas specificity of the method can be determined using a blank matrix spiked with drugs of interest. A lack of response in a blank matrix or a lack of response interfering with the signal of analytes of interest and internal standards prove that the method is selective and specific. However, a small degree of

interference is accepted as long as accuracy and precision (see below) at the lower limit of quantitation (LLOQ) are within the acceptance criteria (±20%). Ten to twenty different sources of blank samples are recommended for checking the absence of interference from endogenous compounds and matrix components. Spiked samples (blank samples spiked with common drugs or compounds of interest) are recommended for checking for interference from xenobiotic compounds that are expected to be present in authentic specimens, particularly in forensic cases that often contain many different drugs and poisons[33,34]. Moreover, the number of compounds spiked in blank samples depends on the purpose of the method.

4.3 Linearity

To obtain a reliable quantification, the relationship between the measuring signals and the concentration of analytes in the sample needs to be determined. Linearity is defined as the ability of a method to obtain a corresponding response proportional to the concentration of the analyte[35]. The working range of a method is defined as "the interval over which the method provides results with an acceptable uncertainty"[36]. For any quantitative method it is necessary to confirm that the method can be used over the working range.

The range of the calibration curve is chosen depending on the purpose of the analytical method, such as the concentration of drugs found in the body (therapeutic or toxic range). Five to eight different concentrations of calibrator across the calibration range is recommended to assess the calibration curve. Spiked calibration standards in blank samples are analysed using the optimised method and the corresponding response of each target analyte is plotted versus the corresponding concentration. To calculate concentrations of unknown samples, regression analysis is used to obtain the regression equation, which expresses the relation of corresponding response to analyte concentration[37]. The equation of a linear relationship can be written as below (Equation 4-1). Note that non-linear calibration curves can also meet the validation criteria.

Equation 4-1 $y = mx+b$

where *y* is the corresponding response, *x* is the analyte concentration, m is the slope and b is the *y*-intercept.

Linear regression is used to provide the relationship between the *x* and *y* variables in a data set (concentration of an analyte and its corresponding response from an analytical technique)[38,39]. The linear relationship between *x* and *y* can be determined by the linear correlation coefficient (R^2), the value of which should be close to 1. For quantitative analysis, a correlation coefficient (R^2) of 0.99 or better is required. However, a correlation coefficient of 0.98 is minimally acceptable[40].

4.4 Lower limit of quantitation and limit of detection

The lower limit of quantification (LLOQ) is defined as the lowest concentration of an analyte in a specimen that can be quantified with acceptable precision and accuracy[32]. The recommended acceptance criteria for precision and accuracy at the LLOQ is ±20%. The limit of detection (LOD) is the lowest concentration of analyte at which the analyte can reliably be detected in the sample. LLOQ can be determined with different procedures. The traditional procedure is to determine the LLOQ based on the signal-to-noise ratio (S/N). S/N ratios ≥10 and ≥3 are required for the LLOQ and LOD, respectively[36]. However, this method is quite difficult if matrix peaks are eluted close to the peak of interest.

An alternative procedure is to determine the LLOQ based on the specific calibration curve. For chromatographic assays, LOD and LLOQ can also be defined in terms of the lowest concentrations of calibrator which are within the acceptable precision and accuracy if results are reported above the value of the lowest calibrator. Moreover, calibration curves used in the method must be in the determination range with acceptable precision and accuracy, and therefore LOD and LLOQ do not need to be determined experimentally[40].

4.5 Accuracy and precision

Accuracy is defined as the closeness of a test result to a reference value. To determine the accuracy of method, both systematic and random effects need to

be assessed. Therefore, the measurement of accuracy normally includes both "precision" for random error and "trueness" for systematic error[36].

Precision is defined as the closeness of agreement between independent test results obtained under stipulated conditions[37,41]. Precision is the measurement of random error and is not the measurement of true value. Measurements of precision are carried out using two main conditions; "repeatability" also called "intra-assay precision" and "reproducibility" also called "inter-day precision". Repeatability expresses the precision under the same analysis conditions such as replicate single analyses on one instrument over a short timescale whereas reproducibility expresses the precision on different instruments, with different analysts, over an extended period of time or between laboratories. The precision of a method is measured in terms of the percentage relative standard deviation (%RSD), with an acceptable limit of less than ±20%[40].

Trueness is defined as closeness of agreement between the average value obtained from a series of test results and an accepted reference value[41]. Trueness is usually determined in terms of bias. Bias is usually measured in terms of percentage deviation from the accepted reference value (%bias), with an acceptable limit of less than ±20%.

Equation 4-2 $$\text{Bias}(\%) = \frac{\overline{X}-\mu}{\mu} \text{x}100$$

Where $\overline{X}$ is the observed mean value and μ is the reference value[32].

Precision and bias can be determined by analysing quality control (QC) samples on the same day and between days using the same extraction and analytical method. QC samples should include low, medium and high concentrations of analyte related to the method working range.

4.6 Matrix effects

One parameter that needs to be assessed for LC/MS analysis is the matrix effect. This phenomenon was first described by Kebarle and Tang in 1993. Matrix effects

(also known as ion suppression or enhancement) occur in the ionisation step of LC/MS, especially in the electrospray ionisation (ESI) interface. Co-eluting compounds can alter the ionisation efficiency by suppression or enhancement of the signal depending on the sample preparation procedure, sample matrix, mobile phase additives or ionisation interface[42-45]. Because matrix effects may affect validation parameters such as LOD, LLOQ, precision and accuracy, studies of ion suppression or ion enhancement need to be an integral part of the validation of an LC/MS method. Matrix effects can be examined using two methods. The first approach is post-column infusion of a solution of the analyte via a post-column tee connection with a syringe pump. However, this approach does not provide a quantitative level of matrix effect. A second approach was published in 2003 by Matuszewski *et al.*[46] who determined the peak areas of analyte in three different sets of samples. Set *A* is neat standards concentration, set *B* is blank matrix spiked after extraction and set *C* is blank matrix spiked before extraction. This method can calculate matrix effect (ME), recovery (RE) and process efficiency (PE) according to the equations below (Equations 4-3 to 4-5).

Equation 4-3 $$ME\ (\%) = \frac{B}{A} \times 100$$

Equation 4-4 $$RE\ (\%) = \frac{C}{B} \times 100$$

Equation 4-5 $$PE\ (\%) = \frac{C}{A} \times 100$$

Matrix effect assessment in this study was determined using the second approach. Replicate neat standards and blank samples obtained from five different sources spiked after and before extraction at low and high concentrations were evaluated.

Chapter 5 Gas chromatography/mass spectrometry (GC/MS)

5.1 Introduction

Gas chromatography (GC) is one of the most widely used separation and quantitation techniques for volatile and thermally stable compounds in analytical chemistry. Not only separating and analysing compounds, GC can also be used as a sample preparation technique to purify compounds from a mixture. The mass spectrometer (MS) is one of the most powerful GC detectors, that provides high selectivity and sensitivity, and the mass spectrum can be used for spectral library matching.

Both GC and MS were developed in the beginning of the 1900s and the first application of the gas-liquid chromatography (GLC) technique was published in 1952 by James and Martin[47]. Nowadays, gas chromatography in combination with a mass spectrometric detector (GC/MS) is the most frequently used chromatographic technique in modern forensic toxicology laboratories, especially in amphetamine-type stimulants determination[47-49].

5.2 Gas chromatography

Chromatography ("colour writing") is a generic term for separation processes involving differential partitioning of the components to be separated between a mobile and stationary phase. The chromatography technique was developed by Russian scientist Mikhail Tswett for the separation of plant pigments in the early 1900s.

Gas chromatography is a separation technique in which the mobile phase is a carrier gas such as helium or nitrogen and the stationary phase is a solid support, usually coated with liquid polymer. GC is usually used for separating small, volatile and thermally stable compounds. The volatility of a compound depends on the size and polarity of the molecule. Therefore, GC techniques are suitable for nonpolar or weakly polar compounds with a molecular mass and boiling point

below 600 Da and 500^{o}C, respectively. A GC system includes the carrier gas supply, injector, column, column oven and connection to the detector.

To separate the compound, a sample is introduced to the GC column via the injector and heated at a temperature in the inlet sufficient to volatilise the sample into the gas phase. The sample is transferred to the analytical column (containing the stationary phase) and carried to the detector by a carrier gas (usually helium). Each compound is eluted from the GC column at a different time resulting in a different "retention time" for each of the individual analytes. The GC separation is dependent on the vaporisation of compounds, the polarity of the stationary phase (column) and the temperature that is applied to the GC oven (usually up to 350^{o}C). Today, the most widely used GC columns are fused silica capillary columns; a long narrow capillary tube coated on the inner wall of the tube with a thin film of a highly viscous polymeric liquid (usually chemically bonded to the surface of the silica) or a thin layer of an adsorbent[50].

There are many detectors that can be used with GC of which some are destructive such as the flame ionisation detector (FID), the nitrogen-phosphorus detector (NPD), the flame photometric detector (FPD) and the mass spectrometer (MS) and some are non-destructive such as the thermal conductivity detector (TCD) and the electron capture detector (ECD)[51]. The FID has been the most common GC detector since 1958. The FID is virtually a universal detector for organic compounds whereas the NPD is only suitable for nitrogen and phosphorus-containing compounds. The TCD is also a universal detector that is usually used for compounds that do not give a signal in the FID. However, only the mass spectrometer was used in this work.

5.3 Mass spectrometry

5.3.1 Introduction

Mass spectrometry is an analytical method that is often used to identify and confirm substances. Mass spectrometry was first developed in 1910 by the physicist J.J. Thompson. There are three major components of a mass spectrometer: the ion source, mass analyser, and detector. In GC/MS, a fourth

component is the interface. After analytes are eluted from the GC column, the interface transfers as much of the analytes as possible into the mass spectrometer. Analytes are ionised in the ion source (usually by electron impact ionisation, EI) to form a charged molecular ion and subsequent fragment ions. Ions are transferred to the mass analyser to separate them according to their mass-to-charge ratio (*m/z*) values before they arrive at the detector. The fragmentation pattern of each compound is characteristic depending on the structure of the molecule and the height of each spectral peak relative to other peaks provides the details used to distinguish compounds – the so-called "mass spectrum pattern".

5.3.2 Electron impact ionisation

Electron impact ionisation was first introduced by Dempster in 1921 for the measurement of lithium and magnesium isotopes. It is the most common technique to form ions in a mass spectrometer that is connected with a gas chromatograph (GC/MS instrument). The gaseous molecules enter the ion source from the GC/MS interface and ions are produced by directing an electron beam into the gaseous molecules. The electron beam is emitted from a heated filament made from rhenium, tungsten or platinum. An ionisation energy of 70 eV is currently used as standard in all commercial GC/MS instruments. This energy is enough to lead to the formation of the molecular ion ($M^{+\cdot}$) and to initiate fragmentation processes. Fragmentation mass spectra obtained from EI at 70 eV are now generally reproducible between instruments and can be used for mass spectral library searching and compound identification[52,53].

5.3.3 Quadrupole mass analyser

A mass analyser is an instrument that can separate ions according to their mass-to-charge ratios. There are many types of mass analyser used in forensic toxicology such as the quadrupole, ion trap or time-of-flight analysers. However, the mass analyser that is usually connected to a gas chromatograph is the quadrupole. The principles of the quadrupole mass filter were published in 1953. A basic component of the quadrupole mass analyser is a set of four rods

(quadrupoles) working with a combination of direct-current (DC) and radio-frequency (RF) potentials[49,53].

The ions created during ionisation are transferred to the mass analyser and are detected according to their mass-to-charge (*m/z*) ratios. Ions are allowed to pass through the quadrupole mass analyser depending on the selected mass range or *m/z* ratio by adjusting the DC potential and RF frequency. However, the mass range available with a quadrupole mass analyser is limited (typically 25-1,000 amu). The ions are filtered by the mass analyser and directed toward the detector.

Although GC/MS is usually applied in forensic toxicology as a standard technique for routine analysis, there are some limitations which apply to this technique for non-volatile or thermally labile compounds.

Chapter 6 Liquid chromatography/tandem mass spectrometry

6.1 Introduction

In the last decade, liquid chromatography/mass spectrometry (LC/MS) has become more important as an identification and confirmation technique than GC/MS in many routine analyses. From the early 2000s, the accuracy, resolution and sensitivity of LC/MS have been improved due to the development of atmospheric pressure ionisation (API) and electrospray ionisation (ESI) techniques[54]. These techniques offered solutions to the determination of compounds having a low concentration, thermal sensitivity, or high polarity.

The increase in routine applications of LC/MS in forensic toxicology is due to the ability of LC/MS to separate components of a complex matrix, which assists in the identification of a wide range of drugs. Moreover, LC/MS is usually applied as a routine method for the analysis of drugs that are not detectable by GC/MS due to the properties of the compound, such as thermal instability and/or high polarity or due to the limitation of other techniques, such as poor selectivity. LC separation coupled with a triple quadrupole mass analyser is a common technique and gold standard method for trace analysis or quantitative analysis of low concentrations of substances in complex mixtures such as drugs in hair. The LC/MS system consists of two main components: the LC system and the mass analyser.

6.2 Liquid chromatography

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid and the stationary phase is a solid or liquid held on solid support particles. Liquid chromatography can be applied to the analysis of any compound with solubility in a liquid that can be used as a mobile phase. LC separation is based on differential partitioning between the mobile and stationary phases. Differences in the solubility of compounds in the mobile phase give different partition (K) or distribution (D) coefficients resulting in differential retention on

the LC column (containing the stationary phase). Nowadays, LC is referred to as column chromatography in which the stationary phase is packed within a tube, the “column”.

Nowadays, reversed-phase chromatography (RPC) is the most commonly-used technique in forensic and clinical toxicology. RPC is a liquid chromatography technique in which the mobile phase is significantly more polar than the stationary phase. The separation principle of this technique depends on the polarity of analytes in which more polar molecules are more soluble in the mobile phase and tend to be eluted earlier. Non-polar molecules in the mobile phase tend to absorb on the relatively non-polar packing material in the stationary phase which typically contains C8 or C18 carbon chains bonded to silica of a chosen particle size and require a less polar mobile phase (usually containing an aqueous organic solvent and buffer) for elution.

The basic components of an LC system are the pump, injector, column and detector. The mobile phase is delivered through the system via an LC pump, typically at a flow rate of 0.1–1 ml/min depending on the type of LC system. The majority of pumps currently used in LC are dual piston-type pumps. Extracted samples are placed in an autosampler and subsequently injected by syringe into the injection port containing a fixed-volume loop held in a six-port valve[55,56]. The injection volume depends on the size of the sample loop which is typically in the range 10–100 µl. The sample is carried into the mobile phase and flows directly to the LC column which is connected to the MS analyser via the electrospray ionisation interface.

The important parts in an LC method are the mobile phase and column. Both a single solvent (isocratic elution) and a variable mixture of organic solvents and water (gradient elution) can be used as the mobile phase depending on the solubility of the compounds and their range of polarities. Separation of analytes occurs in the analytical column. LC columns are made from stainless steel tubes packed with stationary phase particles inside. There are many types of LC column depend on the packing material and chromatography mode such as RP columns or ion-exchange columns. For reverse-phase LC, a common analytical

column is a C18 substituted silica-based (octadecasilyl, ODS) column which is offered in a wide range of particle and pore sizes[56].

6.3 Triple quadrupole mass spectrometer

6.3.1 Introduction

The principle of LC/MS is to generate ions from either organic or inorganic compounds in the MS ion source interface such as the ESI or APCI interfaces, to separate these ions by their mass-to-charge (*m/z*) ratios and to detect them by their corresponding *m/z* and abundances. Ion separation by *m/z* can be effected by static or dynamic electric or magnetic fields depending on the type of MS analyser[57]. However, soft ionisation techniques such as ESI usually produce only a single ion - the protonated molecular ion - and no fragment ions. This causes problems with respect to identification and quantitation of analytes and so LC/MS developed into LC/MS/MS by the introduction of additional components in the mass analyser which induce fragmentation of the molecular ion.

An Agilent 6420 Triple Quad LC/MS/MS instrument was used for identification and quantitation of analytes in this work. The instrument has three main components: the LC system, ion source (ESI) and mass analyser (triple quadrupole mass analyser, QqQ).

6.3.2 Electrospray ionisation

Electrospray ionisation (ESI) is one amongst the group of atmospheric pressure ionisation (API) techniques. ESI was first introduced in 1968 by Dole and his colleagues and was first coupled to MS by Yamashita and Fenn in 1984[53]. ESI is a soft ionisation technique that is able to produce gas-phase ions with no, or little, spontaneous decomposition, and is therefore suitable for large, non-volatile and chargeable molecules[58,59]. ESI is a common ion source for LC/MS instruments in forensic toxicology because of their applicability to a wide range of compounds.

Modern ESI sources (so-called “ESI interfaces”) are based on the ESI designed by Fenn and colleagues in the mid-1980s. LC eluent is introduced into the ESI

interface through a capillary tube from the LC column by connecting the tube with the ESI inlet. Eluent is nebulised through the fine capillary needle which has applied to it a very high negative or positive potential of 3–4 kV and forms highly charged liquid droplets ("Taylor cone") before passing through a countercurrent stream of hot nitrogen gas to vaporise the solvent. The droplets decrease in size and split into micro-droplets ("Coulombic explosion") and subsequently form gaseous ions. Finally, only charged molecules can pass through the ion transfer capillary and set of skimmers into the mass analyser. Nowadays, solvents are evaporated by either a heated transfer capillary or a countercurrent stream of hot nitrogen, the "curtain gas"[58]. Moreover, a trace of formic acid is often added to the mobile phase to aid protonation of the sample molecules.

The Agilent® ESI source consists of a needle, nebulising gas and heated nitrogen drying gas. To achieve the maximum sensitivity, several parameters such as capillary voltage, nebuliser, gas flow and gas temperature need to be optimised.

6.3.3 Triple quadrupole mass analyser

The word "quadrupole" describes the array of four cylindrical rods that is used to generate two electric potentials: radiofrequency (RF) and direct current (DC). The potential difference from two of the opposite parallel rods can create an oscillating electrical field and only a specified value of *m/z* will pass through this field by adjusting the DC potential and RF on the rods[60].

The term "tandem mass spectrometry" is defined as the techniques in which mass-selected ions are subjected sequentially (or in tandem) to a second mass spectrometric analysis (MS/MS). A triple quadrupole mass analyser (TQMS or QqQ) is a tandem-in-space instrument consisting of two quadrupole mass analysers (Q1 and Q3) and a collision-induced dissociation (CID) chamber in the second quadrupole located between Q1 and Q3. A triple quadrupole mass spectrometer works on the same principle as a single quadrupole mass analyser. It is a widely used instrument in bioanalysis. The first quadrupole (Q1) is used to select target ions from the charged species leaving the ionisation source. The selected ions are fragmented in the hexapole or octapole (second quadrupole,

"q") which works as a collision cell with an inert gas such as helium or nitrogen. Fragmented ions are selected according to their *m/z* ratios in the second quadrupole mass filter (Q3) and then travel on to the detector. The most common detector is the electron multiplier[61-64]. The detector in the Agilent 6420 triple quadrupole MS model is the high energy dynode detector. Ions are converted to electrons by dynodes before impacting the multiplier to produce more electrons. The signal current is converted by an A/D converter and transferred to the computer of the MS data acquisition system.

In tandem mass spectrometry, ion fragmentation can be obtained by four main mechanisms: (1) collisions of ions with a gas such as collision induced dissociation (CID), (2) interactions of ions with electrons such as electron capture dissociation (ECD), (3) interactions of ions with surfaces and (4) interactions of ions with photons such as infrared multiphoton dissociation (IRMPD). However, the most prominent fragmentation technique is CID. Collision induced dissociation is a technique to induce fragmentation of gaseous ions. The selected ions are usually accelerated and collide with neutral molecules, generally helium, nitrogen or argon. Ion fragmentation generated by CID depends on a collision energy[64].

Tandem mass spectrometry can be performed in different modes such as product ion scan mode, in which a parent ion is selected with a given *m/z* value and its product ions are detected selectively, precursor ion scan mode, in which all precursor ions of a given fragment ion are detected, and neutral loss scan mode[64].

Selected reaction monitoring (SRM) is one of the methods used in tandem mass spectrometry in which a particular ion is selected in Q1 of a tandem mass spectrometer and a product ion from the precursor ion is selected for detection in Q3. Multiple reaction monitoring (MRM) is the application of SRM to select multiple fragment ions from one or more precursor ions within a single run. MRM provides high sensitivity and selectivity for identification and quantitation, especially with triple quadrupole instruments[62].

Chapter 7 New psychoactive substances

7.1 Introduction

In 2013, almost a quarter of a billion people between the ages of 15 and 64 years were estimated by the United Nations Office on Drugs and Crime (UNODC) to use an illicit drug and approximately 27 million people suffered from problem drug use, including drug dependence or drug-use disorders[65]. In recent years, the use and production trend of new psychoactive substances (NPS) has been increasing globally, especially in Europe, Asia and the Americas[66]. In 2015, 101 NPS were reported to the EU Early Warning System, which was an increase of 25 % compared to 2013[67].

"Designer drugs" or "new psychoactive substances" refers to synthetic analogues of controlled substances, usually prepared by modifying the chemical structures of existing drugs to varying degrees, which are created and marketed to circumvent legislative controls[68-70]. The term designer drugs has been known since 1984 and originally referred to heroin-like synthetic substances[71]. Designer drugs were defined as analogues or chemical cousins of controlled substances that are designed to produce effects similar to the controlled substances they mimic[72]. Examples of classical designer drugs are amphetamine derivatives such as methylenedioxyamphetamine, methylenedioxymethamphetamine, *para*-methoxyamphetamine and fentanyl derivatives such as α-methylfentanyl.

New psychoactive substances are defined as narcotic or psychotropic drugs that are not scheduled under the United Nations 1961 or 1971 Conventions, but which may pose a threat to public health comparable to scheduled substances[72]. The word "new" means newly misused or available rather than newly invented which distinguishes them from classical illicit drugs such as amphetamine, heroin, cocaine and cannabis. There are various different structural types of NPS, such as substituted cathinones, synthetic cannabinoids and synthetic hallucinogens. In the early 2000s, NPS were subject to a huge explosion in internet marketing and were sold not as drugs but as "research chemicals". Nowadays, NPS are available as powders or tablets and have been advertised and sold as "legal highs" with a variety of brand names and usually labelled with the phrase "not for human

consumption"[69]. However, almost all of those early substances have already been controlled in some countries such as UK, USA, Australia and Japan[73,74].

In the global NPS market, UNODC reported in 2014 that synthetic cannabinoids continued to be ranked as the first and accounted for 39% of NPS, followed by phenethylamine derivatives, accounting for 18%, and synthetic cathinones, accounting for 15% of the total number of NPS reported. 450 NPS substances have been reported to the UNODC early warning system from 95 member states and territories, an increase of 20 new substances from the 430 substances reported in 2013[65]. This indicates that the NPS market is very dynamic. NPS can be divided into six main groups according to the active drug substances they contain, i.e. phenethylamines, synthetic cathinones, synthetic cannabinoids, ketamine, piperazines and plant-based substances[75]. Ketamine is included because, although it has long been known as an anaesthetic, it has more recently been subject to abuse in a way similar to other NPS. However, data on NPS substances are still limited due to the large number of different NPS available on the market, and because almost all NPS have no history of medical use.

7.2 Phenethylamine derivatives

Phenethylamine derivatives or substituted phenethylamines refer to a class of compounds derived from phenethylamine with documented psychoactive and stimulant effects. Phenethylamine itself is not used as a prescribed drug because it is rapidly metabolise with a half-life of 5-10 min. The classical examples of compounds in this group are amphetamine-type stimulants such as amphetamine, methamphetamine, MDMA and MDA (see Figure 7-1), which have been widely abused for more than 20 years.

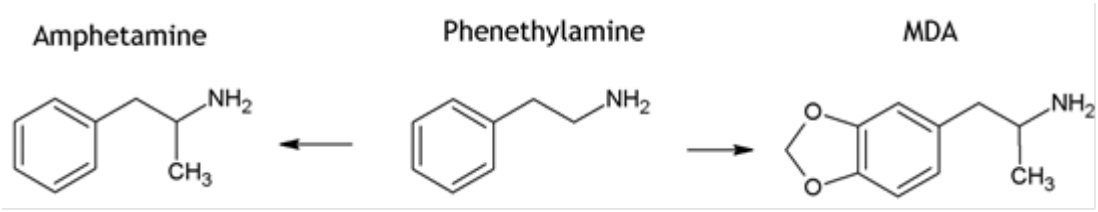


Figure 7-1: Structures of amphetamine, phenethylamine and MDA.

The new drugs in this class include ring-substituted substances such as 2,5-dimethoxyphenethylamines (the so-called 2C-X series), the D series drugs e.g. 2,5-dimethoxy-4-iodoamphetamine (DOI), 2,5-dimethoxy-4-chloroamphetamine (DOC) and *p*-methoxy-methamphetamine (PMMA)[76]. In the 2C-X series, X means an atom or chemical group substituted at the 4-position of 2,5-dimethoxyphenethylamine, such as 2C-B (4-bromo-), 2C-I (4-iodo-) and 2C-T-2 (4-ethyl-thio-)[68]. The psychoactive effects of 2C series drugs have been reported to be dose dependent, ranging from mere stimulant effects at lower doses to stimulant plus hallucinogenic and entactogenic effects at higher doses[77]. Moreover, the number of drugs in this class increased after the publication of the book "PIHKAL" (Phenethylamines I Have Known And Loved, 1991) which presented synthesis instructions and pharmacological explorations of hundreds of substances by Dr Alexander and Ann Shulgin[78]. Although some classical phenethylamine derivatives such as amphetamine, MDMA, PMA, 2C-B and 4-methylthioamphetamine (4-MTA) are listed in Schedules I and II of the UN Convention on Psychotropic Drugs 1971, most of the new drugs such as the 2C-series and the D-Series are not under international control. Among the phenethylamines, PMA, PMMA and 4-methylthioamfetamine (4-MTA) have more often been reported in association with drug-related deaths than other drugs[75].

The following sections deal with phenethylamines which were selected for study in the present work.

7.2.1 3,4-Methylenedioxymethamphetamine (MDMA)

3,4-Methylenedioxy-methamphetamine (MDMA, also known as "Adam", "ecstasy", "beans", "E", "X" "XTC" or "the love drug") is a psychoactive compound. It was first synthesised by the German pharmaceutical company Merck under the name of "methylsafrylamin" in 1912 and was approved as a precursor for therapeutically active compounds[79,80]. MDMA was used as a therapeutic drug for couples therapy and anxiety disorders by psychotherapists in the mid-1970s and was first identified in street use by Gaston and Rasmussen in 1972[77]. Abuse of MDMA become widespread from the late 1970s and early 1980s primarily in California, Florida, New York and Texas as a so-called "wonder drug"[81]. Because of the growing abuse of MDMA, it was categorised in

1977 under the UK Misuse of Drugs Act 1971 as a class A drug and placed in Schedule I of the Controlled Substances Act on May 31, 1985 by the US Drugs Enforcement Administration.

7.2.1.1 Chemical properties

MDMA is a ring-substituted amphetamine derivative structurally similar to methamphetamine and mescaline (a phenethylamine-type hallucinogen, see Figure 7-2). It is a semi-synthetic substance that can be synthesised from plant oils including saffron, sassafras, mace, nutmeg, dill, vanilla bean and parsley[82]. MDMA has a chiral centre with two enantiomeric forms (*S*-(+) MDMA and *R*-(-) MDMA) which have different stereoselective metabolism, different pharmacologic activity and different body disposition. Compared with the *R*-isomer, *S*-MDMA is responsible for the empathic and psychostimulant effects[83]. However, MDMA is commonly sold in the illicit drug market as tablets that contain 50-150 mg of a racemic mixture of MDMA·HCl in a variety of shapes, colours, commercial logos or stamped figures[70,84,85].

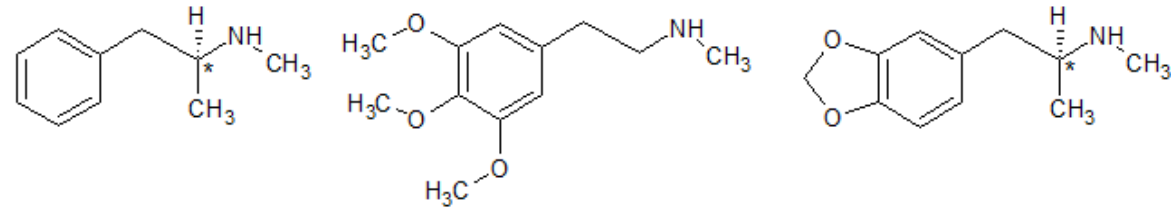


Figure 7-2: Structures of methamphetamine, mescaline and MDMA (* chiral centre).

7.2.1.2 Metabolism and excretion

MDMA is typically taken by oral administration and is well absorbed from the gastrointestinal tract. The peak plasma concentration is obtained approximately 2-3 h after administration and the plasma half-life ranges from 6 to 9 h[86]. MDMA is metabolised by cytochromes P450 2D6, 1A2, 2B6 and 3A4 through two main metabolic pathways. The major metabolic pathway in humans is preliminary demethylenation of the methylenedioxy ring to the intermediate metabolite 3,4-dihydroxymethamphetamine (HHMA) followed by *O*-methylation to 4-hydroxy-3-

methoxymethamphetamine (HMMA) catalysed by catechol-*O*-methyltransferase (COMT), see Figure 7-3. A minor pathway is *N*-dealkylation at the side chain to form a primary amine (3,4-methylenedioxyamphetamine, MDA) followed by demethylenation of the methylenedioxy ring to the intermediate metabolite 3,4 dihydroxyamphetamine (HHA) and further *O*-methylation to 4-hydroxy-3-methoxyamphetamine (HMA) catalysed by COMT[87]. CYP2D6 is the most important isoenzyme of cytochrome P450 regulating the demethylenation reaction[88]. The dihydroxy-metabolites, HHMA and HHA can undergo sulphate conjugation, while HMMA and HMA can be conjugated with both glucuronide and sulphate. These metabolites are mostly present in plasma and urine as their conjugated forms[83]. However, MDMA is excreted primarily in the unchanged form in human urine and HMMA and HHMA are reported to be the major urinary metabolites.

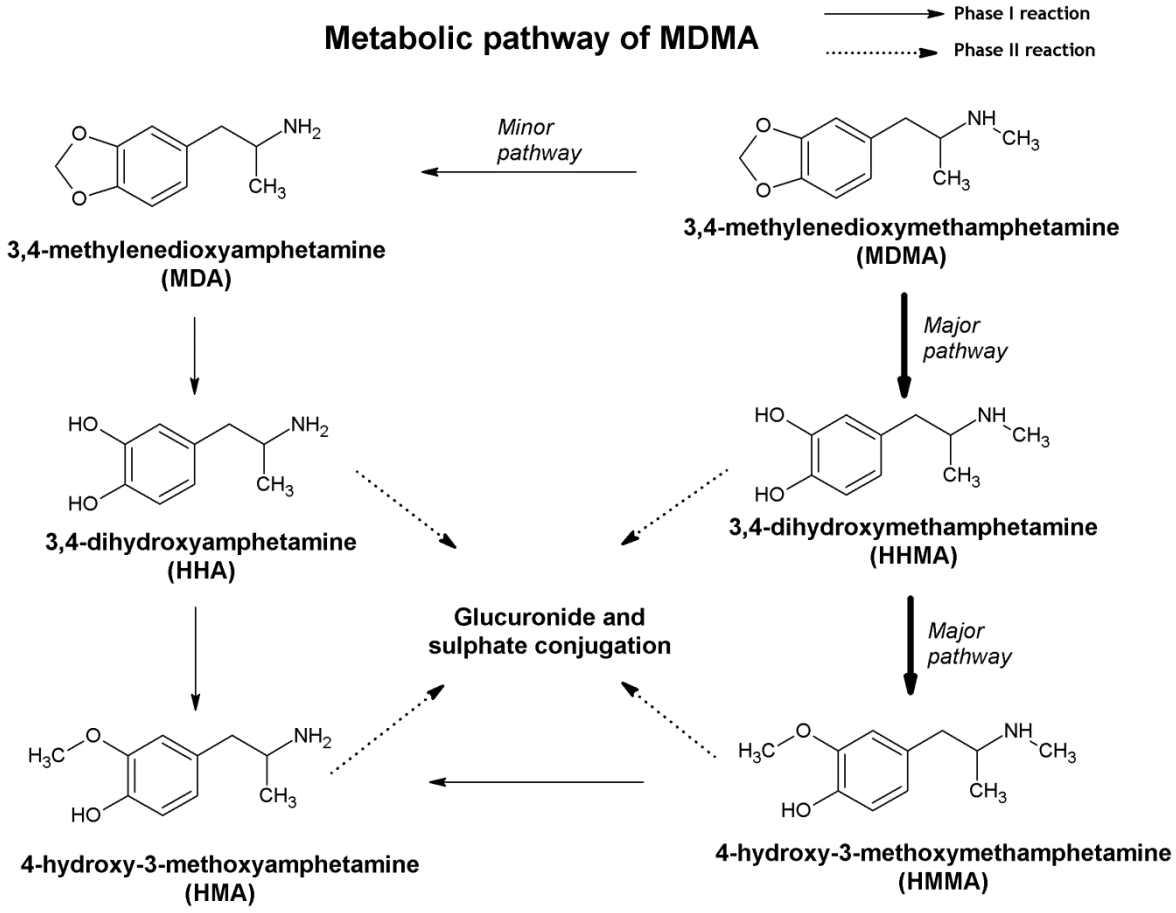


Figure 7-3: Chemical structures of MDMA and its metabolites.

Studies of MDMA and its metabolites have been reported and published since the early 1980s. MDA was identified as an MDMA metabolite in humans in 1988 by

Verebey *et al.*[89] In 1996, Helmlin *et al.*[90] reported an analytical method for MDMA and its metabolites in human plasma. Plasma and urine samples were obtained from male and female patients over the period 0-530 min after oral administration of 1.5 mg MDMA per kg body-weight. MDMA was detected in plasma 15-30 min after administration and reached peak plasma levels 120 min after administration. The results showed that the major urinary metabolite of MDMA in human is 4-hydroxy-3-methoxymethamphetamine (HMMA). 4-hydroxy-3-methoxyamphetamine (HMA) and 3,4-dihydroxymethamphetamine (HHMA) are formed as intermediate metabolites.

Segura *et al.*[91] described an *in vivo* metabolism study of MDMA in humans. Plasma and urine samples were obtained from four healthy male volunteers before and at intervals 0-24 h after the oral administration of a single 100 mg dose of MDMA. HHMA was reported as one of the major metabolites of MDMA and contributed approximately 18% of total MDMA dose recovery. The C_{max} of HHMA was 0.155 µg/ml and AUC_{0-24h} was 1.99 µg·h/ml which were similar to those obtained for MDMA (0.18 µg/ml and 1.47 µg·h/ml).

In 2004, Jenkins *et al.*[92] reported the determination of MDMA and its metabolites (MDA and HMMA) in three case studies. MDMA in human urine was detected at concentrations ranging from 6.8-12.6 µg/ml, MDA from 0.1-0.7 µg/ml and HMMA from 1.1-6.1 µg/ml. In 2006, Pirnay *et al.*[93] developed a GC/MS method for measurement of MDMA and its metabolites (MDA, HMA, and HMMA) in human urine. A participant received 1.6 mg/kg oral MDMA and a urine specimen was collected at 1.2 h after administration. MDMA was detected in the urine at a concentration of 1.46 µg/ml, MDA at 0.047 µg/ml, HMA at 0.029 µg/ml and HMMA at 4.26 µg/ml.

Shima *et al.*[94] reported the determination of MDMA main metabolites, including the sulphate and glucuronide conjugates of HHMA, in humans and rats. Human urine samples were obtained from 25 MDMA users. Rat urine samples were obtained from two male Wistar rats that were administered a single oral dose of 3 mg/kg MDMA and urine was collected for 24 h. The human urinary concentrations ranged from 2.1-134 µg/ml for MDMA, 0.29-8.1 µg/ml for MDA, 0.04-2.3 µg/ml for HMMA, <0.006-0.14 µg/ml for 3-OH-4-MeO-MA, 0.5-32.3

µg/ml for HMMA-Gluc and 1.8-55.6 µg/ml for HMMA-Sul. The concentrations of free-form 3-OH-4-MeO-MA were ten times lower than those of HMMA. The concentration of HMMA-Sul was higher than HMMA-Gluc in all samples. The ratio of HMMA-Sul to HMMA-Gluc ranged from 1.6 to 9.9 and the conjugated percentage of HMMA ranged from 72.7-99.3%.

7.2.1.3 Toxicity of MDMA

MDMA is a central nervous system (CNS) and sympathetic nervous system (SNS) stimulant which acts by releasing serotonin, dopamine and norepinephrine and by inhibiting their reuptake[86]. Consumption of MDMA is well known to be associated with acute or long-term neurotoxic effects and it is the most concerning issue of MDMA misuse[95]. Neurotoxic effects of MDMA include confusion, anxiety, depression and the loss of some cognitive functions such as memory and performance of complex tasks[7,83,96]. In animal experiments, direct injection of MDMA into rat brain does not generate the neurotoxic effects that were observed after peripheral administration[8,97,98]. These results suggest that neurotoxicity of MDMA results from its metabolites rather than from MDMA itself.

7.2.2 *para*-Methoxyamphetamine (PMA)

para-Methoxyamphetamine (PMA), also known as 4-methoxyamphetamine or "Death", appeared in the illicit drugs market and was reported in 1973. It was produced by a clandestine laboratory in Canada. Even though PMA appeared more than 40 years ago, it was not included in the controlled substance legislation in some countries such as Israel[99]. PMA is one of a group of methoxylated phenethylamine derivatives that has strong hallucinogenic properties[100] and is more toxic than MDMA[101]. It has been used as an illicit drug and has approximately 5 times the potency of mescaline[102].

7.2.2.1 Chemical properties

PMA is an amphetamine derivative with a methoxy substituent at the 4-position of the benzene ring, see Figure 7-4. PMA alone or in combination with other drugs has frequently been found on the black market as "ecstasy". PMA is

typically administered both orally and intravenously. It has different forms such as powder, pill or capsule. The usual dose in one tablet or capsule ranges from 25-100 milligrams[103]. PMA is also one of the metabolites of amphetamine[104] and PMMA[104,105]. However, few metabolism studies of PMA have been published.

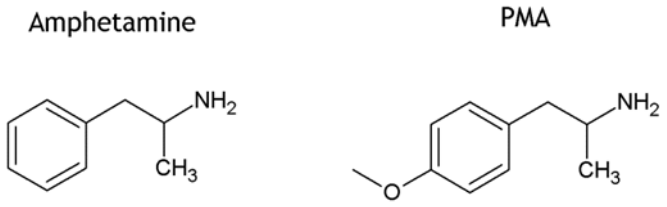


Figure 7-4: Chemical structures of amphetamine and PMA.

7.2.2.2 Metabolism and excretion

Previous studies on PMA and *para*-methoxymethamphetamine (PMMA) metabolism have shown that PMA has a variety of metabolic pathways similar to amphetamine. PMA is metabolised by cytochromes P450 2D6, 1A2 and 3A4[106]. The major metabolic pathway of PMA is *O*-demethylation to *para*-hydroxyamphetamine (PHA), followed by hydroxylation at the 3´-position and then methylation by COMT of the 3´- or 4´-hydroxy group (4-HMA or 3-HMA)[107,108]. A minor metabolic pathway is beta-hydroxylation of the side chain to form *B*-hydroxy-PMA, followed by 4-hydroxylation to 4-hydroxynorephedrine (see Figure 7-5)[105]. PHA (free and conjugated) has been reported as a major metabolite of PMA excreted in human urine[109].

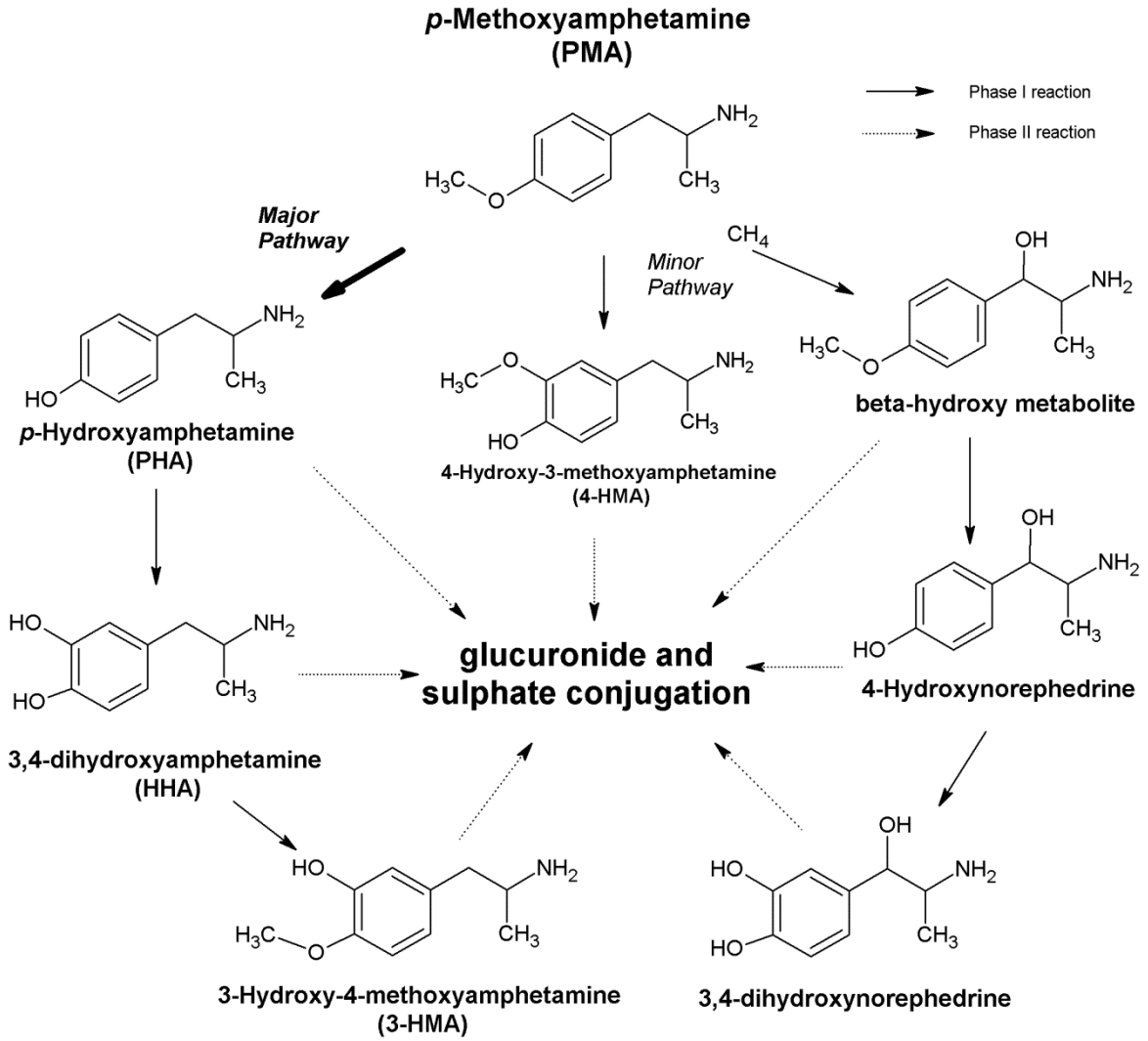


Figure 7-5: Chemical structures of PMA and its metabolites.

Studies of PMA metabolites were first reported and published in the early 1970s. PHA was identified as a major metabolite of PMA in humans in 1979 by Kitchen *et al.*[109] However, no *in vivo* metabolism studies of PMA were published after 1979 until 2015. Lai *et al.*[110] reported *in vitro* drug metabolism studies on seven phenethylamine-based designer drugs in humans using HLM. PHA was reported as the main phase I metabolite of PMA in HLM samples. Although no metabolism studies of PMA were published from 1979 until 2015, deaths related to PMA have been reported as recently as 2012[99].

7.2.2.3 Toxicity of PMA

Like MDMA, PMA is a central nervous system (CNS) stimulant that can produce hallucinogenic effects. The toxic effects of PMA have sympathomimetic and

serotonergic symptoms such as nausea, anxiety, agitation, hyperthermia and arrhythmia. Ling *et al.*[111] reported a retrospective study of PMA poisoning cases. Symptoms of patients with PMA poisoning were tachycardia, hyperthermia, seizures, arrhythmias and coma. Moreover, two patients presented with severe hypoglycaemia with hyperkalaemia. PHA has been reported to be more potent than the parent compound in evoking neurotransmitter release and inhibiting dopamine reuptake[112].

Deaths from PMA were first reported in 1974 by Cimbura[113]. Nine deaths due to a new street drug, PMA, were found in Ontario. PMA was reported to have a powerful hallucinogenic effect with high toxicity. In 1998, Felgate *et al.*[114] reported six deaths involving PMA in Australia. Femoral blood PMA levels were reported in the range from 0.24 to 4.9 µg/ml (mean, 2.3 µg/ml) and liver PMA levels ranged from 1.4 to 21 mg/kg (mean, 8.9 mg/kg). Moreover, blood PMA levels greater than 0.5 µg/ml were reported to be associated with toxic effects. Kraner *et al.*[106] also reported three fatalities related to PMA in the USA in 2001. Post mortem blood PMA concentrations were 1.07, 0.60 and 1.90 µg/ml and symptoms that were observed included hyperthermia, agitation, bruxism, convulsion and haemorrhage. In 2001, Martin reported three fatality cases from PMA overdose. Peripheral PMA blood levels were 0.6, 0.6 and 1.3 µg/ml[115].

In 2003, Refstad[116] reported a lethal case from PMA poisoning in Norway. PMA was found to be more toxic than MDMA and to have a delayed effect after intake compared with MDMA that led to hypoglycaemia and hyperkalaemia. Moreover, an outbreak of PMA and PMMA poisoning cases in Israel was reported in 2012 by Luria *et al.*[99] Twenty-four deaths involved with PMA and PMMA were reported and mean post mortem PMA and PMMA blood concentrations were 2.72±1.67 and 0.35±0.24 µg/ml, respectively.

7.3 Synthetic cathinones

Cathinone is a principal psychostimulant ingredient in the leaves of the *Catha edulis* plant, commonly known as khat. It is the prototype of a wide range of psychoactive substances that have been developed, the so-called “synthetic cathinones”. The original habitat of the khat shrub was in the Horn of Africa and

the Arabian Peninsula and it is still particularly associated with Yemen. It has been known for several hundred years that chewing khat leaves produces psychostimulant effects such as euphoria[117,118]. The active substance "cathine" was first isolated from khat leaves in 1930 and it has the same chemical structure as d-norpseudoephedrine (see Figure 7-6), an alkaloid present in *Ephedra* species. Methcathinone, the first of the cathinone derivatives, was originally synthesised for therapeutic purposes in 1928 and was marketed as an antidepressant during the 1930s and 1940s. In 1975, cathinone was isolated from the fresh leaves of khat and is 7 to 10 fold more potent than cathine but is less potent than amphetamine or methamphetamine. However, cathinone is unstable and degrades rapidly. It can undergo transformations to complex substances by rearrangement to cathine or isocathinones and form cyclic pyrazines. Abuse of methcathinone (so-called "Cat" or "Jeff") has been widespread in the Soviet Union from the 1970s and subsequently in the USA, particularly in Michigan, from the early 1990s. Finally, cathinone, cathine and methcathinone were controlled under Schedule I of the UN Convention on Psychotropic Substances in the early 1990s[119].

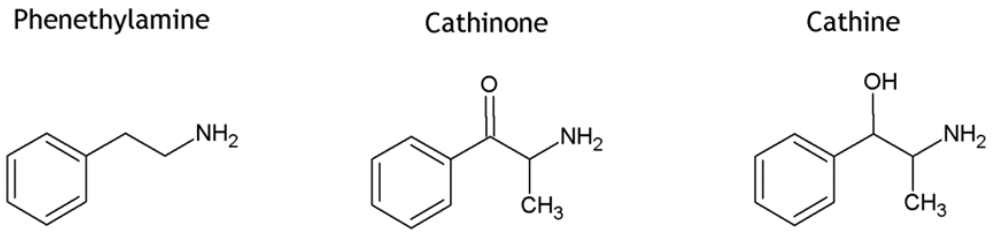


Figure 7-6: Structures of phenethylamine, cathinone and cathine.

The characteristic feature of substances in this group is the presence of a ketone group attached at the beta position on the side chain of the phenethylamine moiety (*β*-keto (bk) substituted phenethylamines or *β*-keto amphetamines derived from cathinone). There is no published clinical trial of current synthetic cathinones in humans, only some *in vitro* metabolism data are available. Pharmacologically synthetic cathinones are similar to amphetamine, MDMA and cathinone due to the shared features of their chemical structures[120]. The effects of synthetic cathinones include euphoria, increased energy, increased blood

pressure, vasoconstriction, increased heart rate, hyperthermia, agitation and aggression[121].

In the mid-2000s, synthetic cathinones appeared on the illicit drug market and increased in popularity due to their psychostimulant effects, similar to amphetamine-type stimulants, and their low prices compared with cocaine or MDMA. Methylone, the first synthetic cathinone, was reported to the European Monitoring Centre on Drugs and Drug Addiction (EMCDDA) in 2005.[75] Nowadays, synthetic cathinones represent approximately 15% of the NPS available in illicit drug markets and most of them have been produced in China and South East Asia countries.

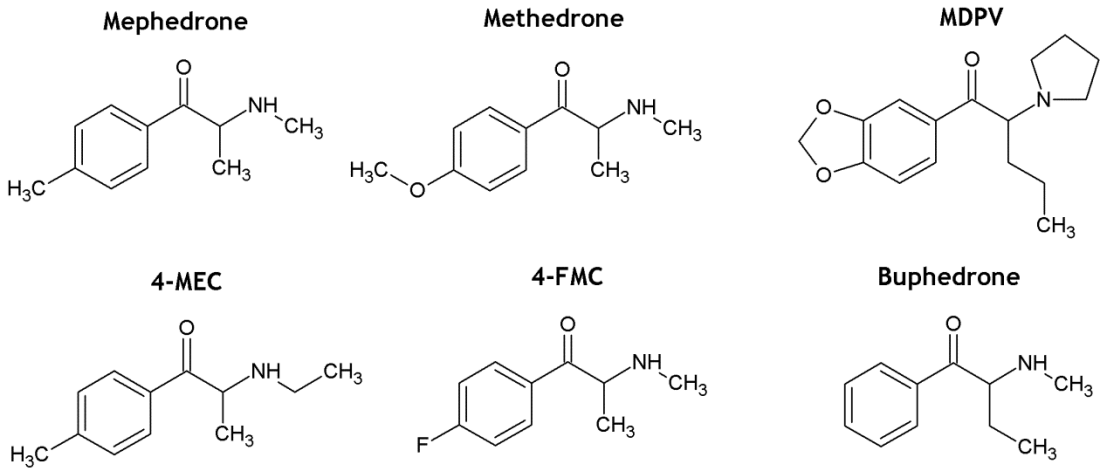


Figure 7-7: Chemical structures of well-known synthetic cathinones.

Some well-known synthetic cathinones are mephedrone (4-methylmeth-cathinone), methylone (3,4-methylenedioxy-methcathinone), butylone (*β*-keto-*N*-methyl-3,4-benzodioxyoly-butanamine), methedrone (4-methoxymeth-cathinone), MDPV (methylenedioxy-pyrovalerone), 4-MEC (methylethcathinone), 3-FMC (3-fluoromethcathinone), 4-FMC (4-fluoromethcathinone), buphedrone (*α*-methylaminobutyrophenone) and naphyrone (naphthylpyrovalerone), see Figure 7-7. Synthetic cathinones have been sold in internet shops as “legal highs”, “bath salts” or “plant food” with “not for human consumption” on the label to circumvent the Medicines Act 1968[122,123].

7.3.1 Methylone

Methylone was first synthesised by Jacob and Shulgin as an antidepressant and anti-Parkinsonian agent in 1996 and first appeared in the illicit drug market mixed with mCCP (meta-chlorophenylpiperazine) under the trade name "Explosion".

7.3.1.1 Chemical properties

Methylone (3,4-methylenedioxy-*N*-methylcathinone or bk-MDMA) is a ring-substituted *N*-methylcathinone derivative and it is a *β*-keto analogue of MDMA. Methylone hydrochloride salt is a white powder which is soluble in water. Because of the chiral centre at the C-2 carbon, methylone has two enantiomers, *R*-methylone and *S*-methylone, of which the *S*-form is more potent than the *R*-form, similar to MDMA[124].

7.3.1.2 Metabolism and excretion

A few previous studies on the metabolism of methylone found that methylone is metabolised through two main metabolic pathways, similar to MDMA. The major metabolic pathway in humans is preliminary demethylenation of the methylenedioxy ring to 3,4-dihydroxymethcathinone, followed by catechol-*O*-methyltransferase (COMT)-catalysed methylation to 3-hydroxy-4-methoxymethcathinone or 4-hydroxy-3-methoxymethcathinone plus subsequent conjugation with (mostly) glucuronide or sulphate. A minor pathway is *N*-dealkylation at the side chain to form 3,4-methylenedioxycathinone, a primary amine, which is then partially conjugated (see Figure 7-8). *In vitro* studies found that methylone is metabolised mainly via CYP2DC and to a lesser extent via CYP1A2, CYP2B6 and CYP2C19[120].

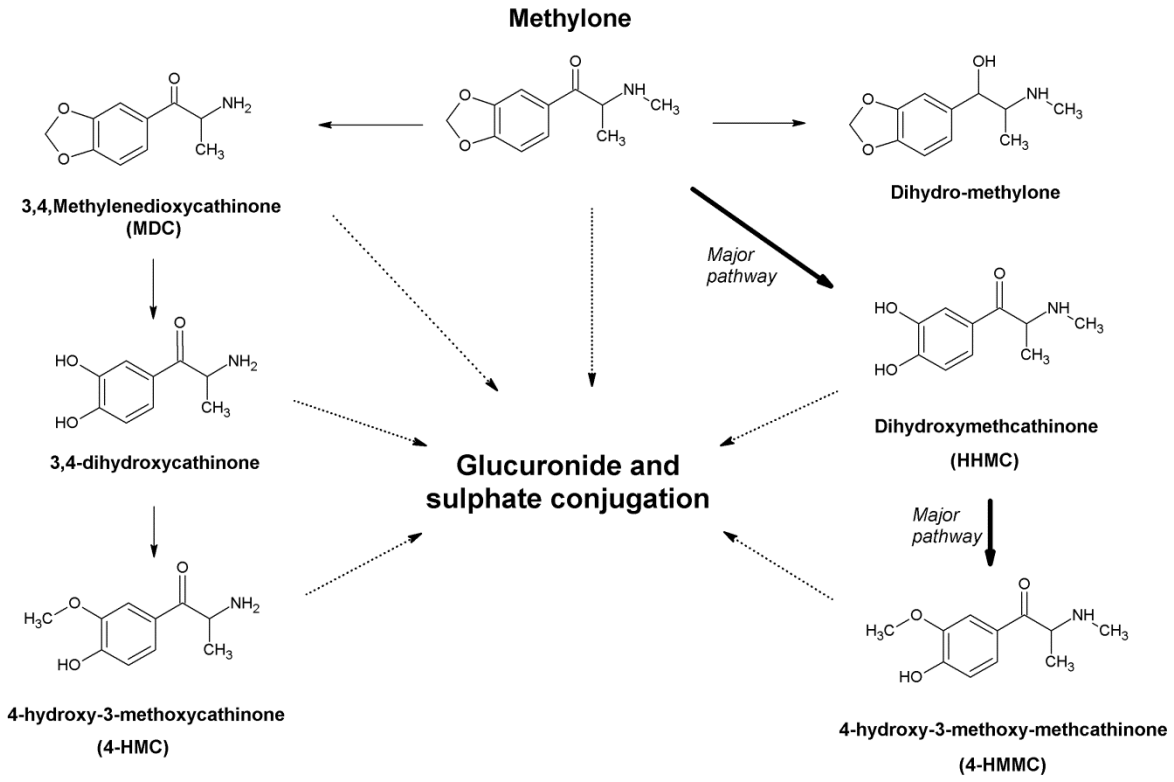


Figure 7-8: Chemical structures of methylone and its metabolites.

Phase I metabolism of methylone in humans and rats was first studied by Kamata *et al.*[125] in 2006. In human urine, 4-hydroxy-3-methoxymethcathinone (HMMC) and 3-hydroxy-4-methoxymethcathinone (3-OH-4-MeO-MC) were reported as major urinary metabolites and 3,4-methylenedioxycathinone (MDC) as a minor metabolite. In addition, no trace of *β*-keto reduction metabolite 3,4-methylenedioxyephedrine (MDEP) was detected. Moreover, this study suggested that not only HMMC and 3-OH-4-MeO-MC can conjugate with both glucuronide and sulphate but also methylone and MDC. In 2010, Meyer *et al.*[126] found an additional methylone metabolite, 4-hydroxy-3-methoxycathinone (4-HMC) in human urine samples.

In 2012, MDC, dihydro-methylone and *N*-hydroxy-methylone were reported as *in vitro* methylone metabolites in HLM by Mueller and Rentsch[127]. Pederson *et al.*[128] also reported the *in vitro* metabolism of methylone using HLM and human liver S9 fraction in 2013. 3,4-Dihydroxymethcathinone (HHMC), the intermediate metabolite leading to HMMC, was first determined as a main metabolite of methylone. Moreover, *N*-hydroxy-methylone, dihydro-methylone and nor-methylone were also detected in the *in vitro* experiments.

In 2015 Chen[129] reported the metabolism of four designer drugs using rat liver microsome S9 fraction with analysis using LC-ion trap MS. An aliphatic hydroxy metabolite (3-OH-4-MeO-methcathinone) was identified as the main metabolite of methylone in rats. Moreover, Ellefsen *et al.*[130] also identified HHMC, HMMC and MDC as methylone metabolites in rat urine.

7.3.1.3 Toxicity of methylone

Methylone is a central nervous system (CNS) stimulant that can produce hallucinogenic effects by increasing the concentrations of monoamine neurotransmitters in the synaptic cleft. The presence of the *β*–keto moiety in the structure of synthetic cathinones increases their polarity and decreases their ability to cross through the blood-brain barrier[131]. The effects of methylone are comparable to MDMA but are slightly milder. Cozzi *et al.*[132] reported that methylone is less potent than its phenylalkylamine analogue MDMA in an *in vitro* neurotransmitter uptake inhibition test. Methylone was more effective against serotonin uptake but less effective at inhibiting dopamine and norepinephrine uptake than was methamphetamine[124].

Elliott and Evans[133] reviewed case data for a three-year period and reported methylone concentrations of 0.1, 4.31 and 11.0 µg/ml in post mortem blood. The second of these involved a 25-year old female who died after a party. A high concentration (5.83 µg/ml) of 4-MEC, see Figure 7-7, was also found in the blood. No additional information is available on the other two cases.

McIntyre *et al.*[134] reported a case involving A 19-year-old woman who was known to use illicit drugs who was found floating in the ocean 100 yards from the beach. The autopsy findings were consistent with drowning. Post mortem blood screened positive for methamphetamine by ELISA and methylone was subsequently detected at concentrations of 3.4 µg/ml, 3.4 µg/ml, 4.3 µg/ml, 11 mg/kg and 1.7 mg in the peripheral blood, central blood, vitreous, liver and gastric contents respectively. No other ATS were detected.

Cawrse *et al.*[135] reported 4 post mortem cases involving methylone. The first involved a 19-year old who died during exercise. The cause of death was cardiac

arrest associated with methylone which was found in cardiac blood at a concentration of 0.74 µg/ml, in peripheral blood at 0.67 µg/ml and in urine at 38 µg/ml. The second incident involved the suicide by gunshot of a 38-year old male and the homicide of his 35-year old girlfriend. In the male, methylone was found in heart blood at 0.11 µg/ml and in urine at 0.25 µg/ml and in the female it was found in heart blood at 1.1 µg/ml. MDPV was also found in blood from the female at a concentration of 0.03 µg/ml. The fourth death involved the suicidal drowning of a female following a police car chase. Methylone was found in heart blood at a concentration of 0.06 µg/ml. MDPV was also found at a concentration of 0.47 µg/ml.

A case of sudden cardiac death was reported by Carbone *et al.*[136] involving a previously healthy 19-year-old man who collapsed while jogging outdoors in warm weather. Methylone was detected in post mortem blood at a concentration of 0.7 µg/ml.

Pearson *et al.*[137] reported three fatal intoxications due to methylone, all of which displayed symptoms of seizure-like activity and elevated body temperatures before death. Peripheral blood methylone concentrations were 0.84, 3.3 and 0.56 µg/ml. The authors concluded that peripheral blood methylone concentrations in excess of 0.5 µg/ml may result in death due to its toxic properties, which can include elevated body temperature and other sympathomimetic-like symptoms.

7.3.2 Butylone

7.3.2.1 Chemical properties

Butylone (2-methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one or bk-MBDB) is a ring-substituted *N*-methylcathinone derivative. Butylone is a *β*-keto analog of 1,3-benzodioxolyl-*N*-methylbutanamine (MBDB). Butylone has appeared in illicit drug markets as a methylone alternative drug and has been controlled in some countries such as Japan as a "designated substance"[74].

7.3.2.2 Metabolism and excretion

A few previous studies[126,127,138] on the metabolism of butylone found that it is metabolised through three main metabolic pathways. The major metabolic pathway in humans is preliminary demethylenation of the methylenedioxy ring (dihydroxy metabolite), followed by catechol-*O*-methyltransferase (COMT)-catalysed methylation (4-OH-3-MeO metabolite or 3-OH-4-MeO metabolite) and subsequent conjugation with (mostly) glucuronide or sulphate. A minor pathway is *N*-dealkylation at the side chain to form a primary amine (nor-butylone or bk-BDB) which is then partially conjugated, see Figure 7-9. Both metabolic pathways are similar to the metabolic pathway of MDMA. Moreover, the other major metabolic pathway that was reported by Zaitsu *et al.* is *β*-ketone reduction to amino-alcohol, *β*-OH-MBDB[138].

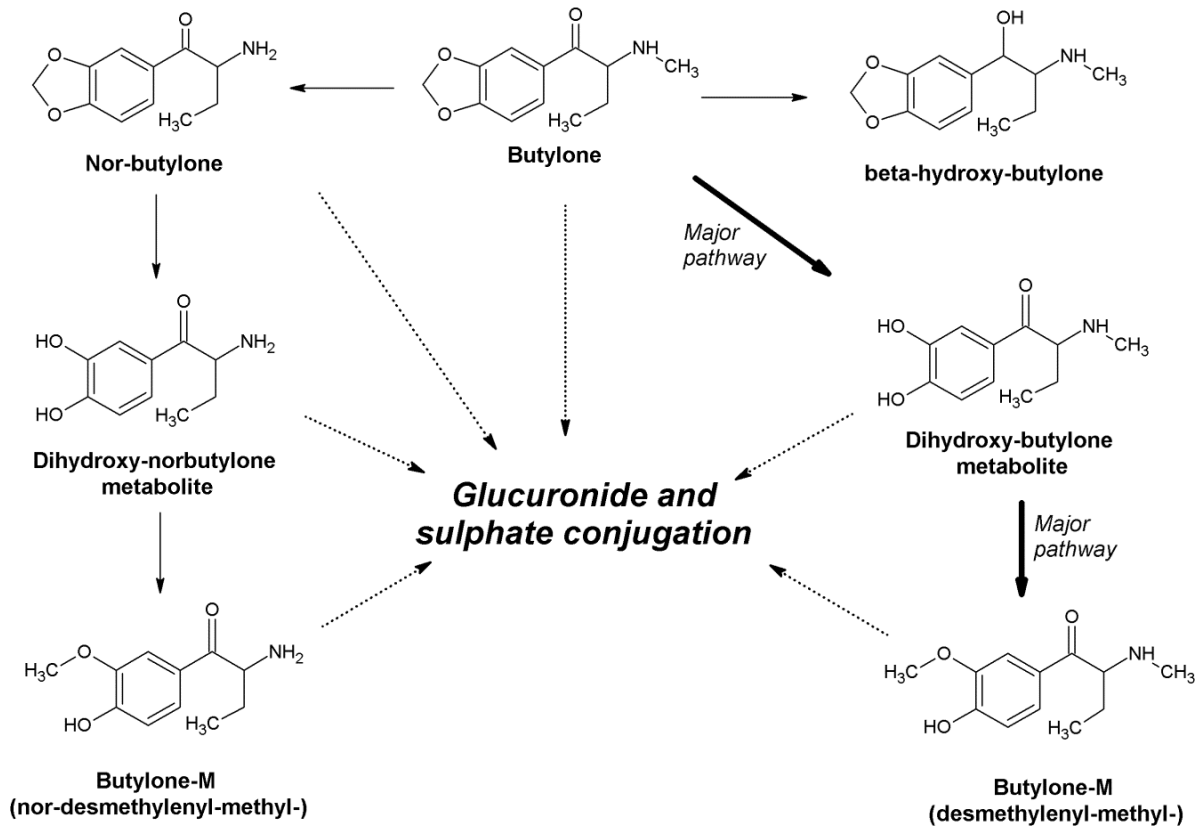


Figure 7-9: Chemical structures of butylone and its metabolites.

Only a few studies have been reported for butylone and its metabolites. Phase I metabolites of butylone in human urine were first investigated by Zaitsu *et al.*[138] in 2009. A 4-OH-3-MeO metabolite and its isomer 3-OH-4-MeO metabolite were

reported as major metabolites and nor-butylone (bk-BDB) as a minor metabolite. However, the urinary ratios of 3-OH-4-MeO metabolite to 4-OH-3-MeO metabolite seemed higher than the urinary ratio of 3,4-HMMA to 4,3-HMMA the corresponding metabolites of MDMA. In addition, the *β*-ketone-reduced metabolite (*β*-OH-MBDB or dihydro-butylone) was identified in hydrolysed human urine samples.

Mayer *et al.*[126] reported a detection method for mephedrone, butylone and methylone and their metabolites in human and rat urine using GC/MS with derivatisation. Nor-butylone, butylone-metabolite (demethylenyl-methyl-) and dihydro-butylone were detected in both rat and human urine following analysis of their acetyl derivatives. All metabolites that were reported in that paper are consistent with the previously published work by Zaitsu *et al.*[138]

In 2012 Mueller and Rentsch[127] also studied the metabolism of 11 cathinones using automated online human liver microsomes with subsequent identification by LC/MS(n). A nor-metabolite and a hydroxy-metabolite were found as butylone and ethylone metabolites. However, the keto-reduction metabolites were not observed.

7.3.2.3 Toxicity of butylone

Little pharmacological literature for butylone is available. However, based on its structural similarity to methylone, butylone is likely to have stimulant effects on CNS along with hallucinogenic properties. Some drug forums report personal effects from butylone which are similar to methylone such as increase in energy, stimulation and euphoria[139]. An on-line drug forum reports doses in the range 20-210 mg (threshold to strong doses, respectively)[140].

Some reports have appeared in the literature describing bioanalysis of butylone, often in combination with other drugs, but most do not contain quantitative analytical results[118,141-143].

A non-fatal case was reported by Wood *et al*.[142], involving a 31 year old male who ingested 1 g of a powder containing butylone and 3,4-methylenedioxy-

pyrovalerone (MDPV). Prior to arriving at a hospital emergency department, he had experienced an "intense high" and "racing heart" which persisted for several hours, along with insomnia, sweating, abdominal discomfort and "fluctuating body temperature. On hospital admission, 30 h after the ingestion, he had difficulty in sleeping, anxiety, agitation and dilated pupils. He was discharge 4 h after admission.

A case of acute liver failure was reported in Ireland involving a 28 year old male who presented at the emergency department with symptoms which included acute psychosis, tachycardia, hypertension, hyperthermia and profuse sweating[144]. He had ingested twelve tablets containing butylone and MDPV although a detail of the composition of these is not known. The patient developed rhabdomyolysis and then acute liver failure, two days after admission. However, he was able to be discharged four days after admission. Preparations containing butylone and MDPV have also been reported in the UK[145].

A fatal case was reported by Warrick *et al.*[146] dealing with simultaneous ingestion by a healthy 24 year old female of two capsules, each containing an estimated dose of 422 mg methylone and 53 mg butylone. The patient was admitted in an comatose state with hyperthermia, tachycardia, tachypnoea and hypertension. Despite treatment she subsequently died 48 h after admission due to multiple organ failure and death was attributed to serotonin syndrome. Qualitative urinalysis found only methylone and butylone.

Another fatal case was reported in Poland, following the suicidal ingestion of twelve tablets containing butylone by a 21 year old male[147]. On hospital admission, he was conscious but confused, with tachycardia, hypertension, dilated pupils, hyperthermia and cariac arrhythmia. He subsequently developed seizures and died four hours and forty minutes after admission. Toxicological analysis gave butylone concentrations of 20 mg/l blood and 33 mg/kg liver.

Elliott and Evans[133] reviewed case data for a three-year period and reported butylone concentrations of <0.125 and 1.72 µg/ml in post mortem blood, but no additional information is available on these two cases.

No fatal cases involving butylone have been reported by Forensic Medicine and Science, University of Glasgow.

Chapter 8 *In vitro* and *in vivo* metabolism studies on MDMA using human liver microsomes and LC/MS/MS with chemical derivatisation

8.1 Introduction

In the past decade, the United Nations Office on Drugs and Crime (UNODC) has reported that amphetamine-type stimulants such as amphetamine, methamphetamine and MDMA are amongst the most widely used illicit drugs globally[66]. MDMA is listed under the Misuse of Drugs Act 1971 as a Class A, Schedule 1 drug (for example, it has a high potential for abuse, with no accepted medical application)[148]. MDMA has been reported to be safe by psychotherapists and users, however. Five deaths associated with the use of MDMA were reported in 1987 by Dowling *et al.*[149] Moreover, in 1992 Henry *et al.*[150] reported seven deaths from MDMA misuse in which the MDMA toxicity pattern included convulsions, fulminant hyperthermia, disseminated intravascular coagulation, rhabdomyolysis, and acute renal failure. The numbers of MDMA-related fatalities and emergency room admissions have led to pharmacology and toxicity studies on this substance. Previous studies[7] in animals suggested that metabolites of MDMA have developed selective neurotoxic effects on the central serotonergic system. Moreover, MDA itself has been popular as a drug of abuse during the 1960s although several deaths related to MDA overdose have been reported[151].

For MDMA analysis, the usual screening method involves immunoassay, which is generally not sufficiently specific to distinguish between MDMA and its metabolites. For example, the immunoassay method for MDMA in the forensic toxicology laboratory, University of Glasgow uses Immunalysis® ELISA kits and the antibodies of these kits cross-react with both MDA and HMA. Routine analysis of MDMA in biological specimens such as serum, blood or urine detected only MDMA itself and MDA as an MDMA metabolite. However, MDA is a minor metabolite of

MDMA in humans whereas major metabolites in humans are HHMA and HMMA. Abraham *et al.*[152] reported that HMMA was detected in urine samples longer than its parent MDMA in all participants after single dose oral administration of MDMA at 1.0 mg/kg (low dose, *n* = 8) and 1.6 mg/kg (high dose, *n* = 9). HMMA was detectable until 6 days after administration whereas the last detection of MDMA was 4 days after high dose administration. MDA and HMA were detectable for as long as 2.5 and 3 days, respectively. This result has shown that analysis of HMMA in urine specimen can extend the detection window of positive urine up to 6 days after administration, which is consistent with the MDMA metabolites analysis method developed in this chapter. Determination of the other major MDMA metabolites, HMMA and HHMA, in urine samples is more useful than detecting only MDMA and MDA.

8.2 Aims

Although methods for quantitative MDMA and its active metabolites have been described in the literature, few methods have been developed for the determination of all its metabolites (MDA, HHMA, HMMA and HMA). The primary aim of the present study was to identify the metabolites of MDMA in humans both *in vitro* and *in vivo*. The secondary aim was to develop a reliable, sensitive, and selective method for identification and quantitation of MDMA and its metabolites in human urine by reversed-phase LC/MS/MS with derivatisation.

8.3 Review of previous analytical methods for the determination of MDMA and its metabolites

Analytical methods which have been described and used in forensic toxicology for the quantitation of MDMA and its metabolites (MDA, HHMA, HMMA and HMA) are reviewed below. These are summarised in Table 8-1.

Analytical methods for MDMA and MDA were reported and published in the late 1980s after widespread abuse of the drugs in 1985[81,153-155]. In the first instance, all MDMA and MDA analytical methods were based on gas chromatography-mass spectrometry (GC/MS). Other active metabolites of MDMA in the rat such as 4-hydroxy-3-methoxymethamphetamine or 3,4-dihydroxymethamphetamine were

reported and published in the early 1990s[156,157]. In 1996, Helmlin *et al.*[90] reported an analytical method for MDMA and its metabolites in human plasma and urine using high-performance liquid chromatography with diode array detection (HPLC/DAD) and GC/MS. (±)-3,4-Dihydroxymethamphetamine (HHMA), (±)-3,4-dihydroxyamphetamine (HHA), (±)-4-hydroxy-3-methoxymethamphetamine (HMMA) and (±)-4-hydroxy-3-methoxyamphetamine (HMA) were synthesised and used as analytical standards. Urine was hydrolysed with 37% hydrochloric acid or enzymatic hydrolysis with 6,000 units of *β*-glucuronidase/sulphatase (from *Helix pomatia*, type H-1) before extracting with a cation exchange SPE column (Adsorbex SCX, 100 mg). Analytes were separated by HPLC using a Spherisorb ODS-1 column (4.6 mm x 150 mm, 3 µm) and detected with DAD at 220 nm. For GC/MS, extracted samples were derivatised with heptafluorobutyric anhydride (HFBA) before analysis by GC/MS with a DB-5 bonded-phase capillary column (20 m x 0.18 mm i.d., 0.40 µm) and detected by MS in full-scan mode (range from *m/z* 33-780) or SIM mode at *m/z* 389, 254, 210, 162, 135 for MDMA-HFBA; *m/z* 375, 240, 162, 135 for MDA-HFBA; *m/z* 587, 360, 254, 210 for HMMA-di-HFBA; *m/z* 573, 360, 240, 163 for HMA-di-HFBA; *m/z* 769, 542, 515, 254, 210 for HHMA-tri-HFBA; and *m/z* 755, 542, 515, 240, 210 for HHA-tri-HFBA. HMMA and HMA were detected and quantitated by HPLC/DAD in human urine and plasma after hydrolysis. However, HHMA was only identified in urine by HPLC and HHA was not detected due to the unstable nature of the dihydroxylated metabolite.

The detection of dihydroxylated metabolites (HHMA and HHA) in biological samples has been limited by their catecholamine-like chemical properties that result in high reactivity and low stability. In 2001, Segura *et al.*[91] described the first validated method for determination of HHMA in plasma and urine using strong cation-exchange solid-phase extraction followed by high-performance liquid chromatography/electrochemical detection (HPLC/ED). 3,4-Dihydroxy-methamphetamine (HHMA) was synthesised and used as an analytical standard. Plasma and urine samples were obtained from four healthy male volunteers. Sodium bisulphite was added to all samples to prevent the oxidation of HHMA. Plasma and urine were hydrolysed with hydrochloric acid before extraction with Bond Elut® SCX columns (containing benzenesulphonic acid substituents). Analytes of interest were eluted with methanol/HCl (99:1 v:v) containing EDTA

and sodium bisulphite. Analytes were separated using a Kromasil® 100 C4 column (4.6 mm x 250 mm) and detected with a dual porous graphite electrode cell set at +0.05 V and +0.35 V. Recovery of HHMA was around 50% and the LLOQ and LOD were 31.8 µg/l and 10.5 µg/l, respectively.

Jenkins *et al.*[92] developed a mixed-mode solid phase extraction method for determination of MDMA and its metabolites in human urine using LC/MS, LC/UV or GC/NPD. Urine samples were hydrolysed with *β*-glucuronidase before being extracted with Oasis® MCX SPE columns followed by elution with 10% ammonium hydroxide in methanol. For LC/MS, MDMA, MDA and HMMA were separated using an XTerra® MS C18 column (2.1 mm x 150 mm x 0.3 µm) and detected with MS using in-source CID in the SRM mode at *m/z* 194.11>163.08 and 194.11>135.00 for MDMA, 180.10>163.08 and 180.10>135.00 for MDA, 196.16>165.08 and 196.08>137.00 for HMMA and 199.20>165.10 and 199.20>137.00 for MDMA-d_5 (IS). The recovery for all analytes ranged from 83.9% to 108.0% and the LLOQ was 0.1 µg/ml for MDMA and MDA and 0.04 µg/ml for HMMA. Quadratic calibration curves were reported over the range 0.2-20 µg/ml. For LC/UV, analytes were separated using an XTerra® MS C18 column (4.6 mm x 100 mm x 0.3 µm) and detected with a dual-wavelength UV detector at 210 and 280 nm. Linear calibration curves were reported over the range 0.2-20 µg/ml and the LLOQ was 1 µg/ml for MDMA, 0.5 µg/ml for MDA and 2 µg/ml for HMMA. However, an interference was observed for HMMA detection. For GC-NPD, samples were derivatised with MBTFA before being separated with an RTX-5 capillary column (30 m x 0.32 mm i.d. x 0.25 µm). The LLOQ was 1 µg/ml for MDMA and MDA and 0.4 µg/ml for HMMA.

Pirnay *et al.*[93] developed a sensitive GC/MS method for measurement of MDMA and its metabolites MDA, HMA, and HMMA in human urine. HHMA and HHA were not included in this study. Analyte conjugates of interest were hydrolysed with hydrochloric acid and the pH adjusted to 5-6.5 with sodium hydroxide and phosphate buffer (pH 6.0) before being extracted with Clean Screen® ZSDAU020 SPE cartridges. The SPE cartridges were conditioned with methanol, deionised water and phosphate buffer (pH 6.0) and washed with deionised water, acetic acid and methanol. Analytes were eluted with methylene chloride/iso-propanol/ammonium hydroxide; (78/20/2 v/v/v). MDMA and its metabolites

were derivatised with HFBA before being separated with a DB-35MS capillary column (15 mm x 0.32 mm i.d. x 0.25 mm) and detected by MS using EI ionisation in the SIM mode at *m/z* 254, 210, 162 for MDMA, *m/z* 162, 135, 375 for MDA, *m/z* 240, 360, 333 for HMA, *m/z* 360, 254, 210 for HMMA, *m/z* 258, 213 for MDMA-d_5 (IS), and *m/z* 167, 244 for MDA-d_5 (IS). Recoveries for all analytes at concentrations of 0.030, 0.3 and 3 µg/ml were higher than 85.5%. Linear calibration curves were reported over the range 0.025-5 µg/ml and the LLOQ was 0.025 µg/ml for all analytes. Moreover, Pirnay *et al.*[158] also developed and optimised hydrolysis conditions for quantification of urinary metabolites of MDMA. Urine samples were hydrolysed with 10,000 units/ml of *β*-glucuronidase from *H. pomatia* (type HP-2) and from *Escherichia coli* (*E. coli*, type XI-A) and these were compared with acid hydrolysis using concentrated hydrochloric acid at incubation temperatures of 100, 110, 120, 140 or 160^{o}C for 20, 30, 40 or 60 min. The optimal hydrolysis conditions for MDMA metabolites was acid hydrolysis with 100 µl of HCl to 1 ml of urine and incubation at 120^{o}C for 40 min.

Mueller *et al.*[159] reported a validated LC/ESI-MS method for simultaneous determination of MDMA and its metabolites (MDA, HHMA and HMMA) in squirrel monkey plasma. Plasma samples were hydrolysed by glucuronidase from *H. pomatia* and extracted by protein precipitation using perchloric acid. Analytes of interest were separated using a Zorbax® 300-SCX column (2.1 mm x 150 mm, 5 µm) with a mixture of aqueous ammonium formate (pH 3) and acetonitrile as mobile phase and detected with MS in positive ion SRM mode with the following transitions: *m/z* 194>163 for MDMA, 180>163 for MDA, 182>151 for HHMA, 196>165 for HMMA, 199>165 for MDMA-d_5(IS) and 185>168 for MDA-d_5(IS). Linear calibration curves were reported over the range 0.02-1 µg/ml for MDMA, HHMA and HMMA and from 0.01-0.5 µg/ml for MDA. The LLOQ was 0.02 µg/ml for MDMA, HHMA and HMMA and 0.01 µg/ml for MDA.

Shima *et al.*[94] reported the urinary excretion in humans and rats of HMMA, the main metabolite of MDMA, including the sulphate and glucuronide conjugates. HMMA and 3-OH-4-MeO-MA were synthesised according to the procedures of Lim and Foltz[154]. HMMA-glucuronide (HMMA-Gluc) and HMMA-sulphate (HMMA-Sul) were synthesised following the method of Shima *et al.*[160] and used as analytical standards. Urine samples were extracted with methanol and redissolved in

water. Analytes of interest were separated using an L-column® ODS (1.5 mm x 150 mm, 5 µm) with a linear gradient of methanol and 10 mM ammonium formate buffer, pH 3.5 (15-35% methanol) as mobile phase. Mass detection was performed in the positive ion SIM mode with the following ions for quantitative analysis: *m/z* 194 for MDMA, *m/z* 180 for MDA, *m/z* 196 for HMMA and 3-OH-4-MeO-MA, *m/z* 276 for HMMA-Sul and *m/z* 372 for HMMA-Gluc. The following ions were also monitored in the SIM mode as qualifier ions: *m/z* 163 and 135 for MDMA and MDA, *m/z* 165 and 137 for HMMA and 3-OH-4-MeO-MA and *m/z* 196 and 165 for HMMA-Sul. Calibration curves were prepared over the range 0.017-8.96 µg/ml for MDA, 0.037-18.5 µg/ml for HMMA-Gluc and 0.027-13.75 µg/ml for HMMA-Sul, 0.058-3.90 µg/ml for HMMA and 3-OH-4-MeO-MA and 1.93-193.25 µg/ml for MDMA.

Kolbrich *et al.*[161] developed a two-dimensional GC/MS method with cryofocusing for simultaneous quantification of MDMA, MDA, HMMA, HMA, and MDEA in human plasma. Samples were hydrolysed with HCl and extracted using Polymeric Styre Screen DBX SPE columns before being derivatised with HFBA and back-extracted with pH 7.4 tris buffer. Analytes were separated using 2D-GC with 2 capillary GC columns in series with cryogenic capture of analytes at the inlet of the second column. The primary column was DB-1MS (15 m x 0.25 mm i.d. x 0.25 µm) and the secondary column was ZB-50 (30 m x 0. 32 mm i.d. x 0.25 µm). Analytes were monitored using one quantitative ion (bold) and 2 qualifier ions as follows: *m/z* **254**, 210, 162 for MDMA, *m/z* **162**, 135, 375 for MDA, *m/z* **210**, 254, 360 for HMMA, *m/z* **240**, 163, 360 for HMA, *m/z* **258**, 213 MDMA-d_5 and *m/z* **167**, 380 for MDA-d_5. Linear calibration curves were reported over the range 1-100 µg/l for MDA, 2.5-100 µg/l for HMA and 2.5-400 µg/l for MDMA and HMMA. The LLOQ of each analyte was the lowest concentration on the calibration curve. Recoveries were 85.6% to 107.2% for all analytes.

Mueller *et al.*[162] described a hydrolysis method for the determination of MDMA metabolite conjugates in human, squirrel monkey and rat plasma. They used a similar sample preparation method as previously published[159] except acid hydrolysis with 0.5 M hydrochloric acid for 80 min at 100°C was performed before extraction by protein precipitation. MDMA and its metabolites MDA, HHMA, HMMA, HHMA-gluc and HMMA-gluc were separated by LC using a Zorbax®

300-SCX column (narrow-bore 2.1 mm x 150 mm, 5 µm) with a mobile phase consisting of 5 mM aqueous ammonium formate adjusted to pH 3 with formic acid and acetonitrile (70:30, v/v). HHMA-sul and HMMA-sul were separated by LC using a Spherisorb® C18 column (4.6 mm x 150 mm, 3 µm) with a mobile phase of 5 mM aqueous ammonium formate adjusted to pH 9 with sodium hydroxide and acetonitrile (70:30, v/v). Mass detection was performed in the positive ion SIM mode with the following ions: *m/z* 182 and 151 for HHMA, *m/z* 196 and 165 for HMMA, *m/z* 358 and 182 for HHMA-gluc, *m/z* 372 and 196 for HMMA-gluc and negative ion SIM mode with *m/z* 260 and 180 for HHMA-sul and *m/z* 274 and 194 for HMMA-sul. Only HMMA-gluc, HHMA-sul and HMMA-sul were detected in authentic human plasma.

Fonsart *et al.*[163] described a pharmacokinetic study of MDMA and its metabolite MDA in rats. (±)-HMA, (±)-HMMA, (±)-HHA, (±)-MDA and (±)-MDMA were synthesised and used as analytical standards. Plasma and urine samples were hydrolysed with hydrochloric acid and the pH was adjusted to 6-6.5 with ammonium carbonate before extraction with Oasis® WCX SPE cartridges. SPE cartridges were conditioned with methanol and deionised water, washed with deionised water and methanol and eluted with acetonitrile/methanol/10% TFA in water (63/27/10, v/v/v). Analytes were separated using a Nucleodur Pyramid® C18 column (2.1 mm x 250 mm, 5 µm) and each compound was detected by ESI-MS in positive ion SIM mode at *m/z* 168 ± 0.5 for HHA, 182 ± 0.5 for HHMA and HMA, 196 ± 0.5 for HMMA, 180 ± 0.5 for MDA, 185 ± 0.5 for MDA-d_5, 194 ± 0.5 for MDMA and 199 ± 0.5 for MDMA-d_5. Linear calibration curves were reported over the range 2.5-375 µg/l for MDA, MDMA, HMMA and HMA, 12.5-750 µg/l for HHMA and 150-2250 µg/l for HHA and LLOQs were similar to the lowest calibration concentration.

Schwaninger *et al.*[164] developed validated LC-high resolution mass spectrometry (LC-HRMS) and GC/MS with negative ion chemical ionisation (GC-NICI-MS) methods for determination of MDMA and its phase I and II metabolites in human urine. *R/S*-HHMA sulphate, *R/S*-HMMA sulphate and single diastereoisomers of HMMA-gluc and the derivatisation reagent *S*-heptafluoro-butyrylprolyl chloride (*S*-HFBPCl) were synthesised and used. An LC-HRMS method was used to determine glucuronide and sulphate metabolites. Urine samples were extracted

with Isolute C18 SPE cartridges and separated using a Phenomenex Chirex 3012 column (4.6 mm x 25 mm, 5 µm). Analytes were detected by HRMS in positive ion SIM mode with the following accurate masses: *m/z* 262.0744 for HHMA-sul, *m/z* 276.0900 for HMMA-sul and *m/z* 372.1653 for HMMA-gluc. No interference peaks were detected in blank samples and recoveries were over 75% for all analytes. Linear calibration curves were reported over the range 0.074-9.2 µg/ml for *S* and *R*-HMMA-gluc, 0.054-18.3 µg/ml for *S*/*R*-HMMA-sul and 0.13-19.4 µg/ml for *S*/*R*-HHMA 3-sul and *S*/*R*-HHMA 4-sul. The LLOQ of each analyte was the lowest concentration on the calibration curve. A GC-NICI-MS method was used to determine sulphate metabolites after cleavage. Urine samples were hydrolysed with sulphatase from *Aerobacter aerogenes* before being extracted and derivatised with *S*-HFBPCl and separated using an HP-5 MS column (30 m x 0.25 mm i.d., 0.25 µm). Analytes were detected by NICI-MS in SIM mode with the following ions (quantifier ions are in bold): *m/z* **446**, 426, 466 for MDMA, *m/z* **432**, 392, 412 for MDA, *m/z* **486**, 271, 414 for HMA, *m/z* **420**, 251, 492 for HHA, *m/z* **434**, 356, 506 for HHMA, *m/z* **520**, 448, 500 for HMMA, *m/z* **437**, 397, 417 for MDA-d_5 and *m/z* **451**, 431, 471 for MDMA-d_5. Quadratic calibration curves were reported over the range 0.02-4.47 µg/ml for *S* and *R*-MDA, 0.02-4.52 µg/ml for *S* and *R*-HMA, 0.04-4.17 µg/ml for *S* and *R*-HHA, 0.02-24.12 µg/ml for *S* and *R*-MDMA, 0.02-24.37 µg/ml for *S* and *R*-HMMA and 0.04-6.78 µg/ml for *S* and *R*-HHMA. The LLOQ of each analyte was the lowest concentration on the calibration curve.

Ramaley *et al.*[165] described an *in vitro* study of MDMA in human hepatocytes. Female liver hepatocytes were incubated with 0.0625, 0.25 and 1 mM MDMA at 37°C for 4 h. The supernatant was extracted with solvent B (95% acetonitrile, 5% H_2O, 10 mM ammonium acetate, 10 mM acetic acid) before being analysed by LC/MS. MDMA and its metabolites were separated using an Atlantis HILIC® column (2.0 mm x 50 mm, 3 µm) and detected by QTrap® MS/MS in enhanced product ion scan mode at *m/z* 194 for MDMA, *m/z* 499 for MDMA-glutathione and *m/z* 487 for HHMA-glutathione.

Table 8-1: Previous GC/MS and LC/MS methods for MDMA and its metabolites.

Analytes	Matrix	Chromatography	Extraction Method	Year
MDMA MDA HHMA, HHA HMMA, HMA	Human plasma & urine	**HPLC-DAD** Column: Spherisorb ODS-1 (4.6 mm x 150 mm) Mobile phase: acetonitrile-water (96:904, v/v) + orthophosphoric acid and hexylamine. Flow rate: 1 ml/min. **GC/MS** Column: DB-5 capillary (20 m x 0.18 mm i.d. x 0.40 µm)	Enzymatic hydrolysis 6,000 units SPE: Adsorbex SCX (100 mg) GC/MS: Derivatised with HFBA	**1996**[90]
MDMA HHMA HMMA	Human plasma & urine	**HPLC/ED** Column: Kromasil C4 (4.6 mm x 250 mm) Mobile phase: 18% acetonitrile and 82% 0.1 M sodium acetate pH 3.1 **Flow Rate:** 1 ml/min	Acid hydrolysis SPE: Bond Elut SCX	**2001**[91]
MDMA MDA HMMA	Urine	**LC/MS** Column: XTerra® MS C18 (2.1 mm x 150 mm, 3.5 µm) Mobile phase: 30% methanol and 70% 20 mM ammonium bicarbonate buffer pH 9; Flow Rate: 0.2 ml/min **GC/NPD** Column: RTX-5 capillary (30 m x 0.32 mm i.d. x 0.25 µm)	Enzymatic hydrolysis SPE: Oasis MCX GC-NPD: Derivatised with MBTFA	**2004**[92]
MDMA MDA HMMA HMA	Human urine	**GC/MS (SIM)** Column: DB-35MS capillary column (15 mm x 0.32 mm i.d. x 0.25 mm)	Acid hydrolysis SPE: Clean Screen® ZSDAU020 Derivatised with HFBA	**2006**[93]
MDMA MDA HHMA HMMA	Monkey plasma	**LC/MS** Column: Zorbax 300-SCX (2.1 mm x 150 mm, 5 µm) Mobile phase: 5 mM aqueous ammonium formate adj. pH 3 with formic acid and acetonitrile in gradient mode	Enzymatic hydrolysis Protein precipitation	**2007**[159]
MDMA MDA HMMA HMMA-gluc HMMA-sul	Human & rat urine	**LC/MS (SIM)** Column: L-column ODS (1.5 mm x 150 mm, 5 µm) Mobile phase: 10 mM ammonium formate buffer pH 3.5 and methanol in gradient mode	Liquid/liquid extraction with MeOH	**2008**[94]

Table 8-1 (cont.): Previous GC/MS and LC/MS methods for MDMA and its metabolites.

Analytes	Sample	Chromatography	Extraction Method	Ref.
MDMA, MDA HMMA, HMA	Plasma	**2D-GC/MS** Column1: DB-1MS (15 m x 0.25 mm i.d. x 0.25 µm) Column2: ZB-50 (30 m x 0.32 mm i.d. x 0.25 µm)	Acid hydrolysis SPE: Polymeric Styre Screen DBX Derivatised with HFAA	**2008**[161]
MDMA, MDA HHMA, HMMA HHMA-gluc HHMA-sul HMMA-gluc HMMA-sul	Human, monkey & rat plasma	**LC/MS (glucuronide)** Column: Zorbax 300-SCX (narrow bore 2.1 mm x 150 mm, 5 µm) Mobile phase: 70% (v/v) 5 mM aqueous ammonium formate adj. pH 3 with formic acid and 30% acetonitrile. **LC/MS (sulphate)** Column: Spherisorb C18 (4.6 mm x 150 mm, 3 µm) Mobile phase: 70% (v/v) 5 mM aqueous ammonium formate adj. pH 9 with NaOH and 30% acetonitrile.	Acid hydrolysis with HCl & enzymatic hydrolysis Protein precipitation	**2009**[162]
MDMA, MDA HHMA, HHA HMMA, HMA	Rat blood & urine	**LC/MS (Ion trap)** Column: Nucleodur Pyramid C18 (2.1 mm x 250 mm, 5 µm) Mobile phase: 0.05% (V/V) TFA in water and acetonitrile in gradient mode Flow Rate: 0.2 ml/min	Hydrolysis (Urine) SPE: Oasis® WCX	**2009**[163]
MDMA, MDA HHMA, HMMA HHMA-sul HMMA-gluc HMMA-sul	Urine	**LC-HRMS** Column: Phenomenex Chirex 3012 (4.6 mm x 250 mm, 5 µm) Mobile phase: ammonium formate buffer with 0.1% formic acid and acetonitrile with 0.1% formic acid; Flow Rate: 1 ml/min **GC-NICI-MS** Column: HP-5 MS column (30 m x 0.25 mm i.d., 0.25 µm)	Hydrolysed with *Aerobacter aerogenes* SPE: Isolute C18	**2011**[164]
MDMA MDMA-glutathione HHMA-glutathione	Human hepatocytes	**LC/MS (QTrap®)** Column: Atlantis HILIC (2.0 mm x 50 mm, 3 µm) Mobile phase: gradient from 70 - 50% B, flow rate 0.3 ml/min A: 95% isopropyl alcohol, 5% H_2O, 10 mM ammonium acetate, 10 mM acetic acid B: 95% acetonitrile, 5% H_2O, 10 mM ammonium acetate, 10 mM acetic acid	Incubated at 37°C for 4 h Liquid extraction with solvent B	**2014**[165]

8.4 GC/MS method development

To evaluate MDMA and its metabolites (MDA, HHMA, HMMA and HMA), an extraction method was developed and identification methods based on GC/MS and LC/MS were investigated.

8.4.1 Derivatisation methods

To identify MDMA and its hydrophilic metabolites by GC/MS, a derivatisation method was developed. The derivatising agents involved in this study were chosen from those used in previous studies and from the routine method for MDMA analysis in the forensic toxicology laboratory, University of Glasgow. Three types of derivatising agents were compared for the derivatisation of MDMA and its metabolites including HHMA, HMMA and HMA: acetic anhydride, pentafluoropropionic anhydride and *N*-methyl-*N*-(trimethylsilyl)-trifluoro-acetamide.

Subsequently, acetylation derivatisation was also used for LC/MS/MS analysis to improve the capacity factors of hydrophilic metabolites and the chromatographic separation of MDMA and its metabolites (see Section 8.5.1.1).

8.4.1.1 Acetic anhydride (AA)

Acetylation of MDMA and its metabolites was carried out using acetic anhydride (AA). 50 µl of AA: pyridine (3:2, v:v) was added to each dried extract in a screw-capped vial, and heated at 60^{o}C for 30 min. All samples were then evaporated to dryness at room temperature under a stream of nitrogen and residues were reconstituted in 200 µl of 10% acetonitrile in deionised water containing 0.1% formic acid for LC/MS or 100 µl of ethyl acetate or dry toluene for GC/MS and transferred to labelled 1.5 ml screw-capped autosampler vials with inserts before analysis by LC/MS/MS or GC/MS.

8.4.1.2 Pentafluoropropionic anhydride (PFPA)

PFP derivatives of MDMA and its metabolites were prepared using pentafluoropropionic anhydride (PFPA). 50 µl of PFPA:ethyl acetate (2:1, v:v) was added to each dried extract in a screw-capped vial, and heated at 60°C for 30 min. All samples were then evaporated to dryness at room temperature under a stream of nitrogen and residues were reconstituted in 100 µl of ethyl acetate or dry toluene and transferred to labelled 1.5 ml screw-capped autosampler vials with inserts before analysis by GC/MS.

8.4.1.3 *N*-Methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA)

Trimethylsilyl derivatives of MDMA and its metabolites were prepared using *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide. 50 µl of MSTFA was added to each dried extract in a screw-capped vial, and heated at 60°C for 30 min. All samples were transferred to labelled 1.5 ml screw-capped autosampler vials with inserts before analysis by GC/MS.

8.4.2 Identification method

8.4.2.1 Gas chromatography/mass spectrometry conditions

GC/MS analysis was carried out using an Agilent 7890A gas chromatograph equipped with an Agilent 7693 autosampler and connected to an Agilent 5975C InertXL MSD with Triple-Axis mass selective detector, operated in the EI positive ion mode. Chromatographic separation was performed on an HP-5MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness, 0.25 µm, Agilent Technologies Inc.). Helium gas at a constant flow of 1 ml/min was used as the carrier gas. The injection port temperature was 225°C, and the transfer line temperature was 280°C. The injection volume was set at 1 µl in the splitless mode.

The mass-selective detector was operated in the full scan mode with a mass range between *m/z* 40 and 500 and was tuned and calibrated with perfluorotributylamine. The ion source and quadrupole temperatures were 250°C and 150°C, respectively. A solvent delay was set at 6 min to protect the

filament. All compounds were identified by their retention times (t_R) and mass spectral fragmentation patterns.

8.4.2.2 GC/MS method for TMS-derivatives

The initial column temperature of 90°C was held for 1 min, followed by a temperature ramp of 10°C/min to 280°C, which was held for 2 min. The total separation run time was 22 min. The ion source was maintained at 250°C and quadrupole at 150°C. The MS detector was operated in the full scan mode with a mass range between *m/z* 40 to 700 and in the selected ion monitoring (SIM) mode with the ions summarised in Table 8-2.

Table 8-2: SIM conditions for TMS-derivatives of MDMA and its metabolites.

Compound	Retention Time	Selected Ions
MDA-TMS	11.66	116, 135, 236
MDMA-TMS	12.65	130, 250, 264
HMA-2TMS	13.16	116, 179, 209, 310
HMM-2TMS	14.07	130, 179, 324
HHMA-3TMS	14.59	130, 179, 382

8.4.2.3 GC/MS method for PFPA-derivatives

The initial column temperature of 90°C was held for 1 min, followed by a temperature ramp of 10°C/min to 280°C. After that, the temperature ramp was hold at 280°C for 2 min. The total separation run time was 22 min. The ion source was maintained at 250°C and quadrupole at 150°C. The MS detector was operated in the full scan mode with a mass range between *m/z* 40 to 700 and in the selected ion monitoring mode (SIM) with the ions summarised in Table 8-3.

Table 8-3: SIM conditions for PFPA-derivatives of MDMA and its metabolites.

Compound	Retention Time	Selected Ions
HMA-2PFP	8.5	190, 283, 310
MDA-PFP	9.6	135, 162, 190, **325**
HHMA-3PFP	9.6	160, 204, 442
HMM-2PFP	10.5	160, 204, 310
MDMA-PFP	11.2	135, 162, 204, **339**

Bold = M^+

8.4.2.4 GC/MS method for Ac-Derivatives

The initial column temperature of 90°C was held for 1 min, followed by a temperature ramp of 10°C/min to 250°C. After that, the temperature ramp was increased to 20°C/min to 300°C. The total run time was 19.5 min. The ion source was maintained at 250°C and quadrupole at 150°C. The selected ion monitoring mode was used and the ions were as summarised in Table 8-4.

Table 8-4: SIM conditions for Ac-derivatives of MDMA and its metabolites.

Compound	Retention Time	Selected Ions
MDA-Ac	14.16	44, 162, 135, **221**
MDMA-Ac	15.10	58, 100, 135, 162, **235**
HMA-2Ac	15.83	86, 137, 164, 206
HMM-2Ac	16.60	58, 100, 164, 206
HHMA-3Ac	17.57	58, 100, 150, 234

Bold = M^+

8.4.3 Results of derivatisation with GC/MS analysis

8.4.3.1 Optimised GC/MS conditions for trimethylsilyl derivatives

To optimise the GC/MS conditions and to select ions for use in the SIM mode, standards of MDA, MDMA, HMA, HMMA and HHMA were derivatised with MSTFA and analysed by GC/MS following the method previously described (Section

8.4.2.2) in full scan MS mode over the mass range from *m/z* 40-700. The EI mass spectra of the TMS-derivatives are shown in Figure 8-1.

The TMS-derivatives of MDA, MDMA, HMA, HMMA and HHMA were eluted at 11.66, 12.65, 13.16, 14.07 and 14.59 min, respectively. However, the ion at *m/z* 73, corresponding to the TMS group, is prominent in all TMS spectra and is not selective for a SIM method. Two large peaks in the mass spectra of MDMA-TMS, HMMA-2TMS and HHMA-3TMS were at *m/z* 73 and 130 and for MDA-TMS and HMA-2TMS were at *m/z* 73 and 116. The mass spectra of MDA-TMS, MDMA-TMS and HHMA-3TMS had only a few characteristic peaks - two large peaks and small fragment ion peaks which were insufficient for quantification.

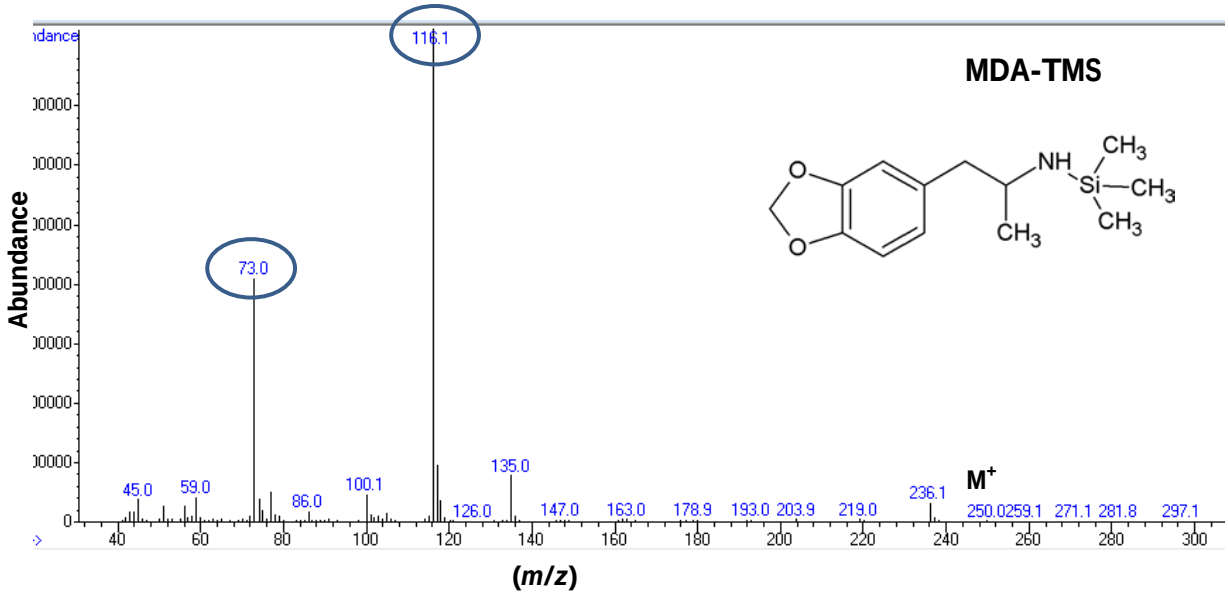


Figure 8-1: Mass spectral patterns of TMS-derivatives of MDMA and its metabolites.

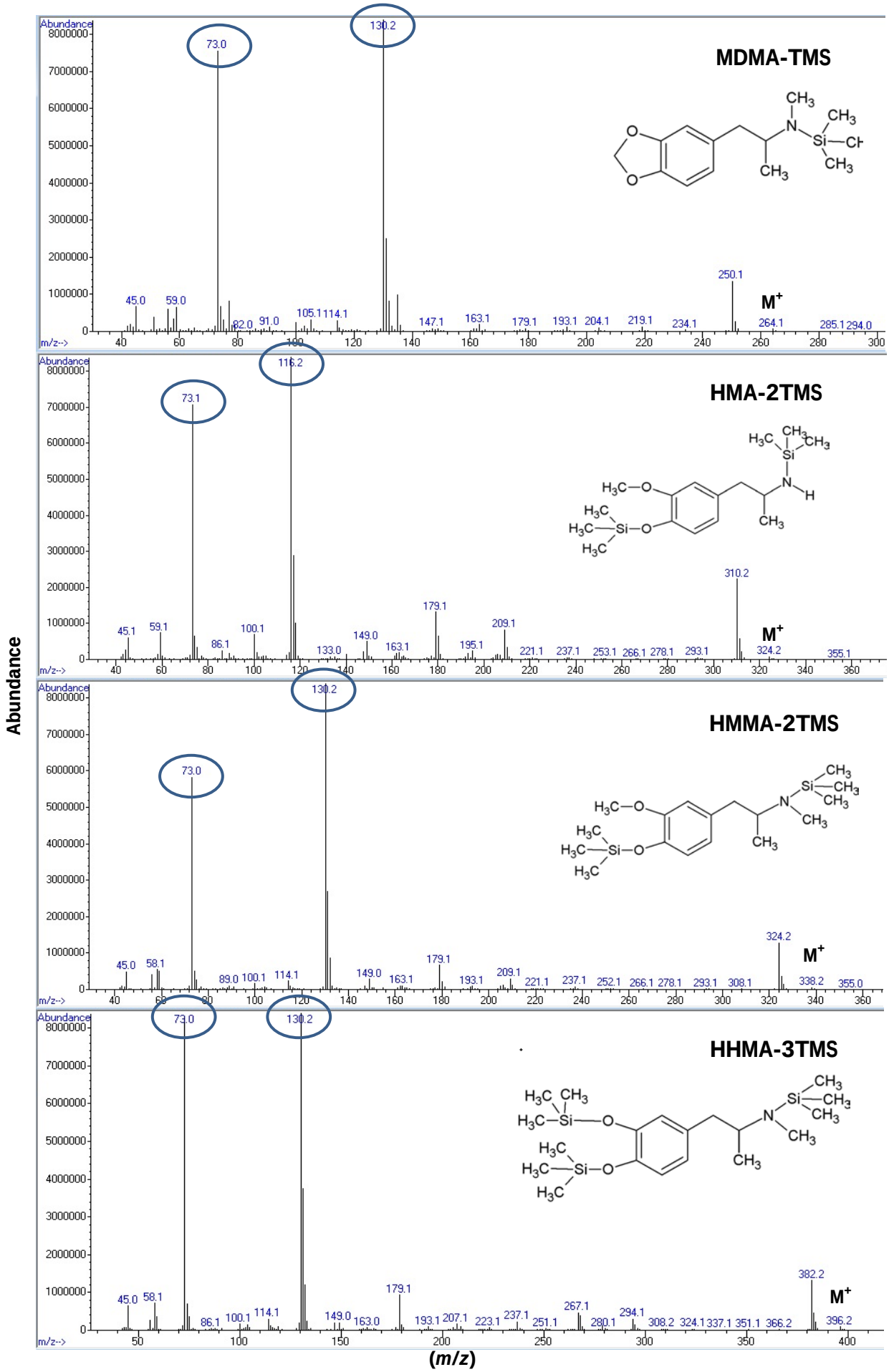


Figure 8-1 (cont.): Mass spectral patterns of TMS-derivatives of MDMA and its metabolites.

8.4.3.2 Optimised GC/MS conditions for acetate derivatives

To optimise the GC/MS conditions and select ions for use in the SIM mode, standards of MDA, MDMA, HMA, HMMA and HHMA were derivatised with acetic anhydride: pyridine (3:2, v:v) and analysed by GC/MS following the method previously described (Section 8.4.2.4) in full scan MS mode over the mass range from *m/z* 40-700. Acetate derivatives of MDA, MDMA, HMA, HMMA and HHMA were well separated at 14.16, 15.10, 15.83, 16.60 and 17.57 min, respectively. However, the ions at *m/z* 44 and 58 that were prominent in nearly all acetate mass spectra of MDMA and its metabolites are not sufficiently selective for use in a SIM method. The EI mass spectra of Ac derivatives are shown in Figure 8-2.

Two large peaks in the mass spectra of MDMA-Ac, HMMA-2Ac and HHMA-3Ac were at *m/z* 58 and 100 and the largest peak for MDA-Ac and HMA-2Ac was at *m/z* 44. The mass spectrum of HHMA-3Ac had only a few characteristic peaks, large peaks at *m/z* 58 and 100 and small fragment ion peaks at *m/z* 123, 150, 192 and 234.

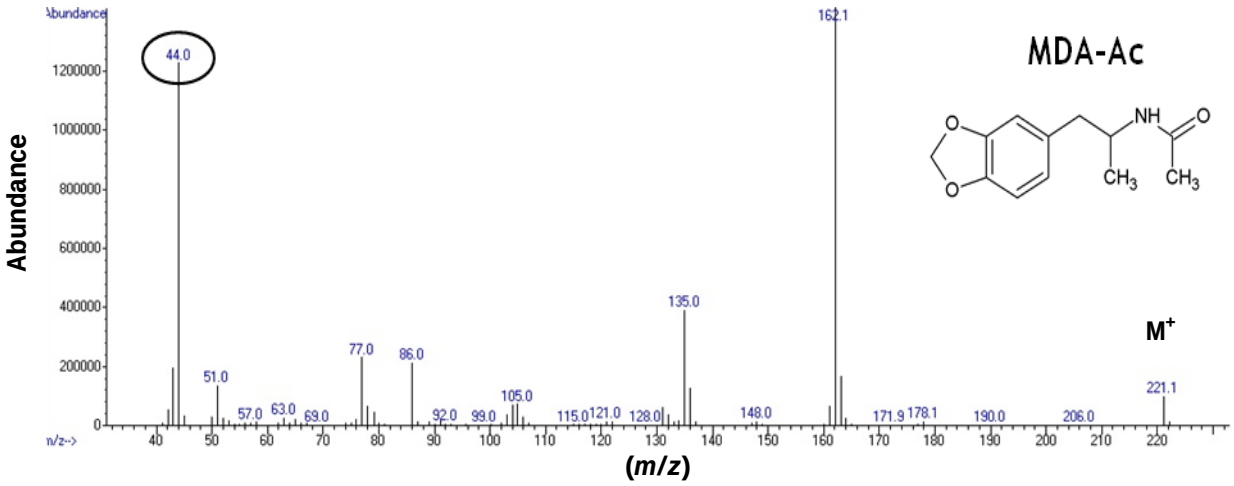


Figure 8-2: Mass spectral patterns of Ac-derivatives of MDMA and its metabolites.

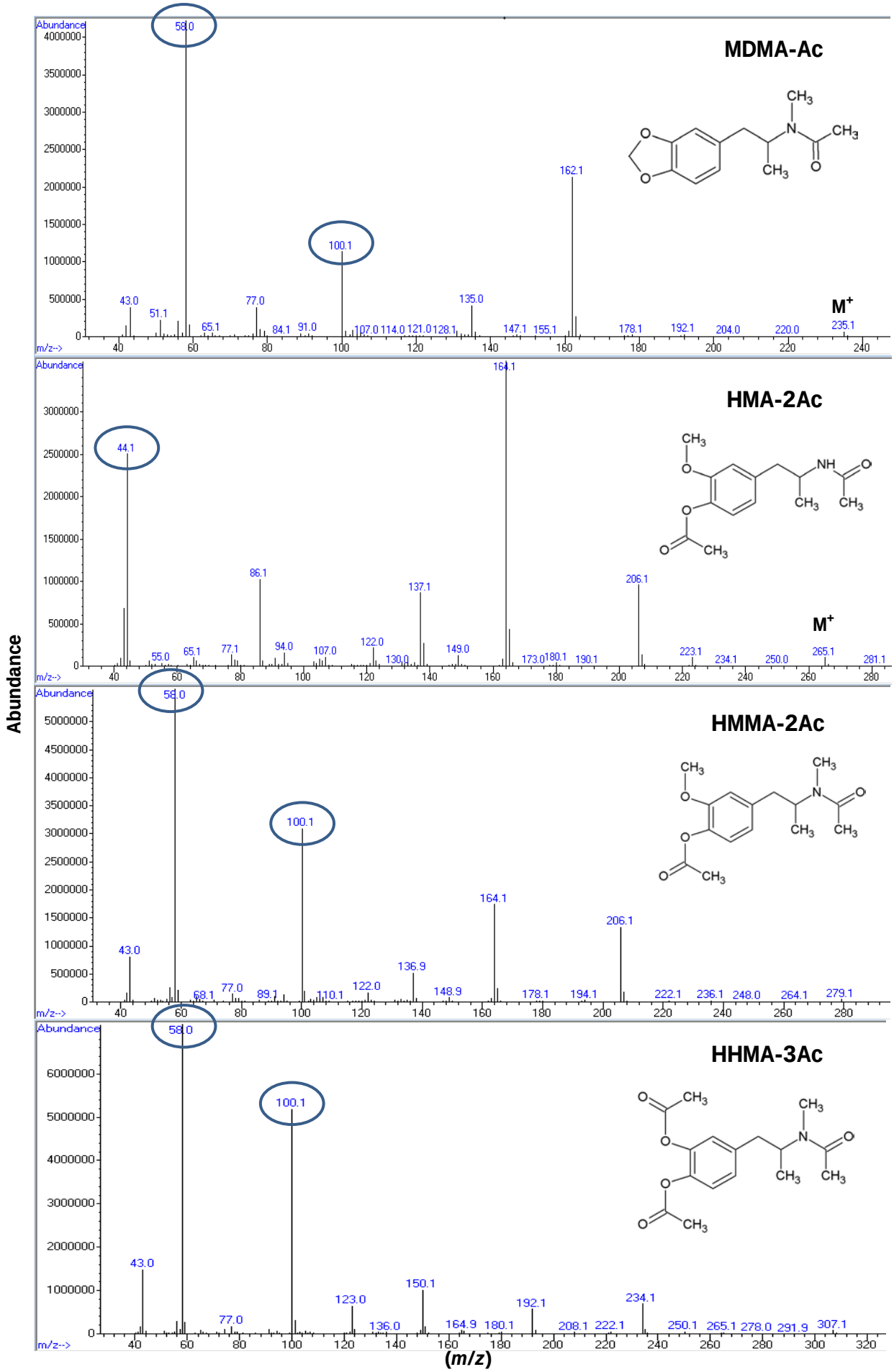


Figure 8-2 (cont.): Mass spectral patterns of Ac-derivatives of MDMA and its metabolites.

8.4.3.3 Optimised GC/MS conditions for PFPA derivatives

To optimise the GC/MS conditions and select ions for use in the SIM mode, standards of MDA, MDMA, HMA, HMMA and HHMA were derivatised with PFPA: ethyl acetate (2:1, v:v) and analysed by GC/MS following the method previously described (Section 8.4.2.3) in full scan MS mode over the mass range from *m/z* 40-1000. PFP-derivatives of HMA, HHMA, MDA, HMMA and MDMA were eluted at 8.5, 9.6, 9.6, 10.5 and 11.2 min, respectively. The perfluoroacyl derivatives had mass spectra containing ions at high *m/z* values which were more selective than those for the TMS and Ac derivatives. However, the GC method needs to be further developed to separate HMA, HHMA and MDA. The EI mass spectra of the PFP-derivatives are shown in Figure 8-3.

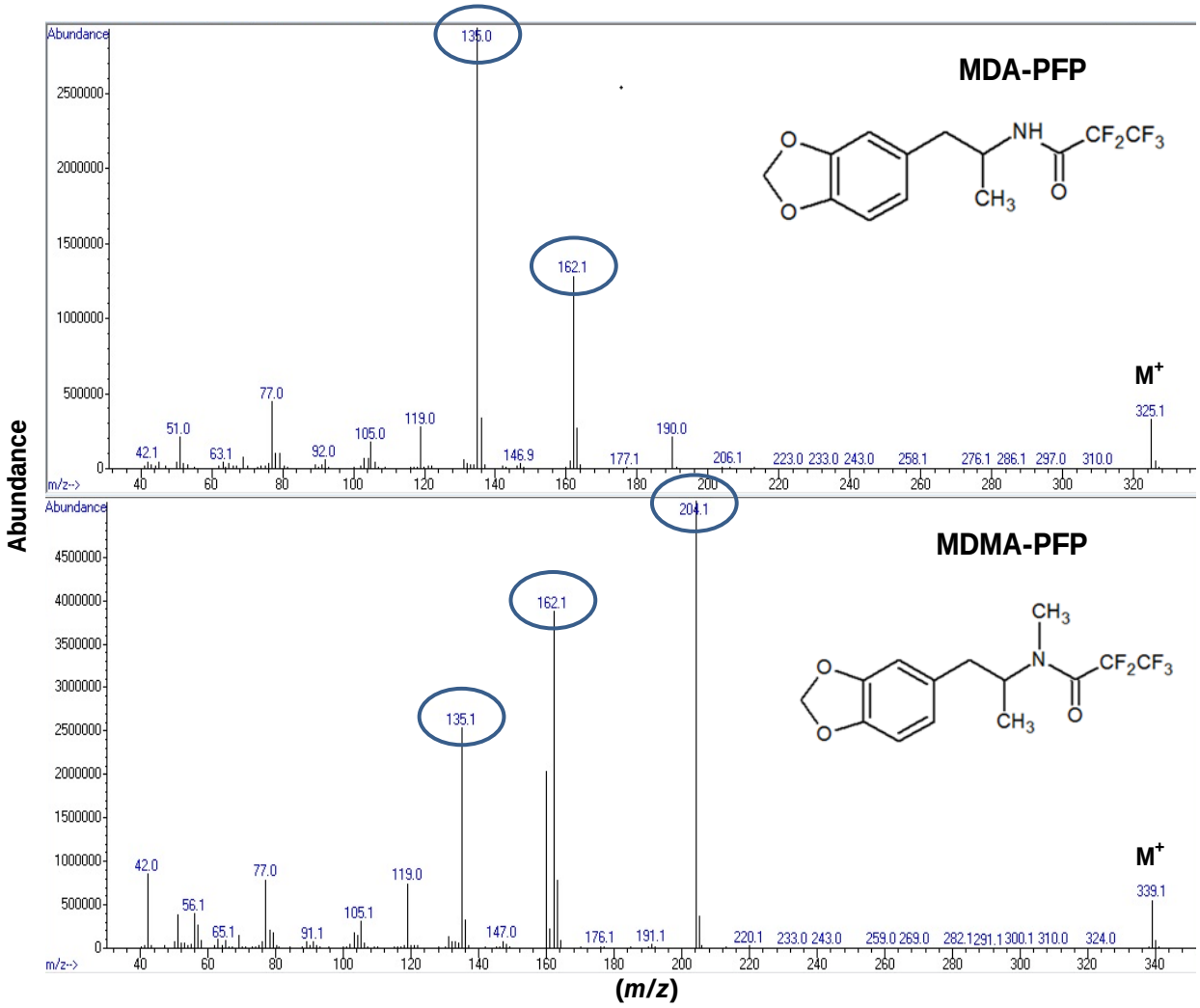


Figure 8-3: Mass spectral patterns of PFP-derivatives of MDMA and its metabolites**.**

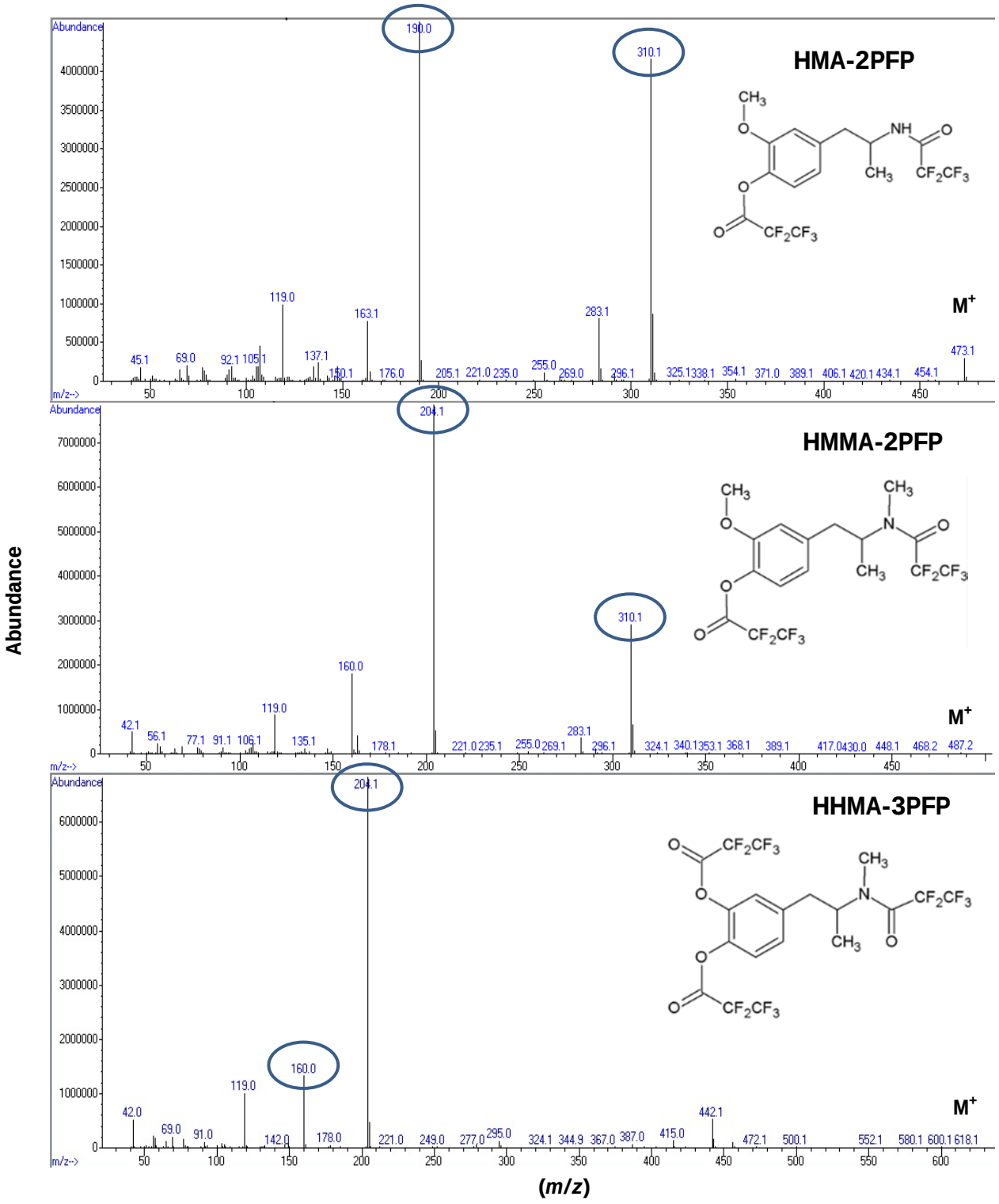


Figure 8-3 (cont.): Mass spectral patterns of PFP-derivatives of MDMA and its metabolites.

The mass spectrum of HMA-2PFP had two large peaks at *m/z* 190 and 310, small fragment ion peaks at *m/z* 119, 163 and 283 and a low intensity molecular ion peak at *m/z* 473. The mass spectrum of HHMA-3PFP had a base peak at *m/z* 204 and small fragment ion peaks at *m/z* 119, 160 and 442. The mass spectrum of

MDA-PFP had two large peaks at *m/z* 135 and 162, small fragment ion peaks at *m/z* 119 and 190 and a small molecular ion peak at *m/z* 325. The mass spectrum of HMMA-2PFP had three large peaks at *m/z* 160, 204 and 310 and a small fragment ion peak at *m/z* 119. The mass spectrum of MDMA-PFP had three large peaks at *m/z* 135, 162 and 204, small fragment ion peaks at *m/z* 77 and 119 and a small molecular ion peak at *m/z* 339. Therefore, the PFP-derivatives of MDMA and its metabolites were suitable for quantification.

8.4.3.4 Comparison of derivatisation methods

To evaluate the response intensity of the three derivatisation methods, 5 µg standards of HHMA, HMMA, HMA and MDMA were derivatised with MSTFA, PFPA and acetic anhydride and analysed by GC/MS. Response factors (RF) for each analyte were calculated following the equation below.

Response Factor (RF) = Peak Area / weight injected (µg)

To compare the response intensity between TMS and PFP-derivatives and Ac-derivatives, relative response factors (RRFs) were calculated following the equation below.

Relative Response Factor (RRF) = Response Factor A / TMS Response Factor

Table 8-5: Comparison of response intensity of the three derivatisation methods.

Compound	Ac-derivative		PFP-derivative		TMS-derivative
	RF	RRF_{Ac}	RF	RRF_{PFP}	RF
HHMA	5.88×10^6	**0.85**	3.56×10^6	**0.51**	6.93×10^6
HMA	3.77×10^6	**0.56**	7.24×10^6	**1.08**	6.69×10^6
HMM	6.16×10^6	**0.89**	9.12×10^6	**1.32**	6.89×10^6
MDMA	5.70×10^6	**0.79**	6.68×10^6	**0.93**	7.20×10^6

*Response Factor (RF)
**Relative Response Factor (RRF_x)

The results showed that TMS-derivatives gave a good response for all analytes. This is why they were chosen for the denominator. However, the mass spectra obtained from TMS-derivatives were unsuitable for the development of a quantitative method. PFP-derivatives were selected after comparing their responses with Ac-derivatives due to their good response factors and the availability of peaks which had high *m/z* values in their mass spectra. However, this GC/MS method could not separate HHMA and MDA which are major and minor MDMA metabolites.

In an attempt to improve the separation of PFP-derivatives of HMA, HHMA and MDA, the initial GC column temperature was decreased to 80^{o}C and held for 1 min, followed by a temperature ramp of 20^{o}C/min to 170^{o}C. Because the PFP-derivatives of MDMA and its metabolites were eluted from the GC column at temperatures in the range 170^{o}C to 200^{o}C, temperature ramps of 3^{o}C, 4^{o}C, and 4.5^{o}C/min were applied over this range followed by a ramp of 20^{o}C/min to 300^{o}C. The MS detector was operated in the full scan mode with a mass range between *m/z* 40 to 700.

With temperature ramps of 4^{o}C, and 4.5^{o}C/min, HHMA-3PFP and MDA-PFP co-eluted at 9.88 and 9.76 min, respectively. With a temperature ramp of 3^{o}C/min, HHMA-3PFP and MDA-PFP were partially separated, eluting at 9.27 and 9.31 min. The chromatograms of PFP-derivatives under these conditions are shown in Figure 8-4.

In conclusion, because HHMA-3PFP derivative still overlapped with MDA-PFP derivative even with a decreased oven temperature ramp of 3oC/min, acetate derivatives were judged to be potentially the most useful for a GC/MS method. However, in parallel, an LC/MS method was also developed to identify and quantitate MDMA and its metabolites in urine samples which ultimately was found to be superior to the GC/MS method. Finally, the LC/MS method was chosen and validated. The GC-MS method was not further developed, for example by using lower temperature programme rates or by evaluating other GC columns, and was not subsequently validated for the purposes of this study.

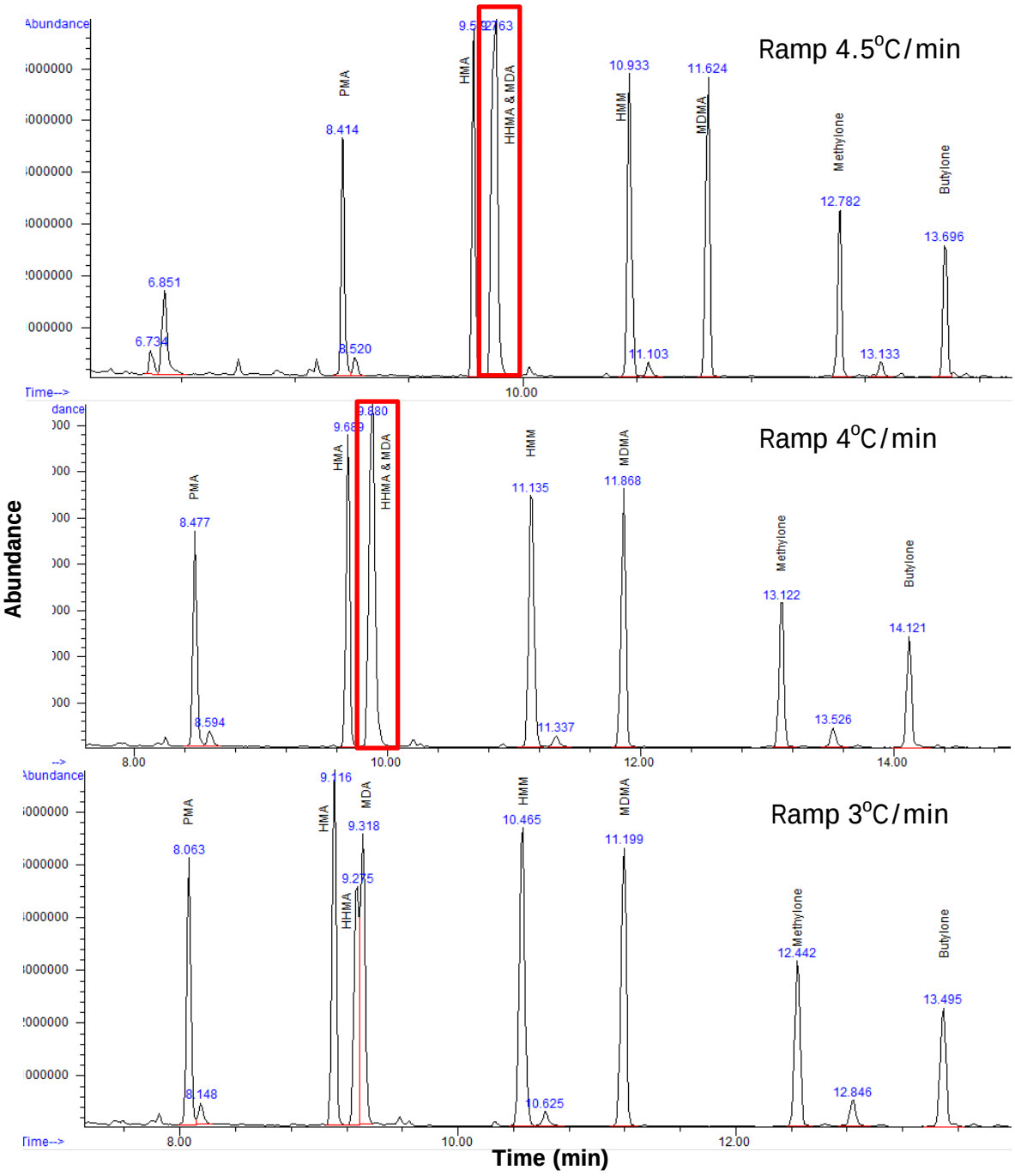


Figure 8-4: TIC chromatograms of PFP-derivatives of MDMA and its metabolites with temperature ramps of 3, 4 and 4.5°C/min.

8.5 LC/MS method development

8.5.1 Optimisation of LC/MS conditions

For the analysis of target compounds in biological samples that have high complexity matrices, mass spectrometers are often coupled to liquid

chromatography to gain a physical separation of the compounds. Separation by LC of bases on the stationary phase (column) depends on the mobile phase and the physical properties of the molecules, such as hydrophobicity. The investigation of MDMA and its metabolites depends on the ability of the method to separate all analytes including hydrophobic (MDMA and MDA), less hydrophobic (HMA and HMMA) and hydrophilic compounds (HHMA). Physico-chemical properties of MDMA and its metabolites are listed in Table 8-6.

Table 8-6: Physico-chemical properties of MDMA and its metabolites.

Compound	Formula	MW	Log *P* octanol/water	pK_a
MDMA	$C_{11}H_{15}NO_2$	193.24	1.806	10.32
MDA	$C_{10}H_{13}NO_2$	179.22	1.667	9.94
HMMA	$C_{11}H_{17}NO_3$	195.26	0.913	9.84 & 10.63
HMA	$C_{10}H_{15}NO_2$	181.23	0.774	9.70 & 10.05
HHMA	$C_{10}H_{15}NO_2$	181.23	0.606	9.70 & 10.34

8.5.1.1 LC separation

To separate MDMA and its metabolites, standards of MDMA, MDA, HHMA, HMMA and MDA were analysed by LC/MS using a Gemini C18 column. LC separation started with isocratic elution using a low concentration of organic solvent (9% acetonitrile containing 0.1% formic acid in water) at flow rate of 0.35 ml/min. HHMA, HMMA and HMA split into two peaks with retention (capacity) factors (*k'*) in the range 0.7 to 1.26. Figure 8-5 shows the poor separation of HHMA, HMMA and HMA that was obtained on the Gemini C18 column.

A HILIC column or normal phase liquid chromatography was considered for the analysis of hydrophilic compounds such as HHMA. However, the aim of this study was to develop an LC/MS method using a conventional C18 HPLC column that is a routinely-available column in all laboratories that perform HPLC and/or LC/MS analysis.

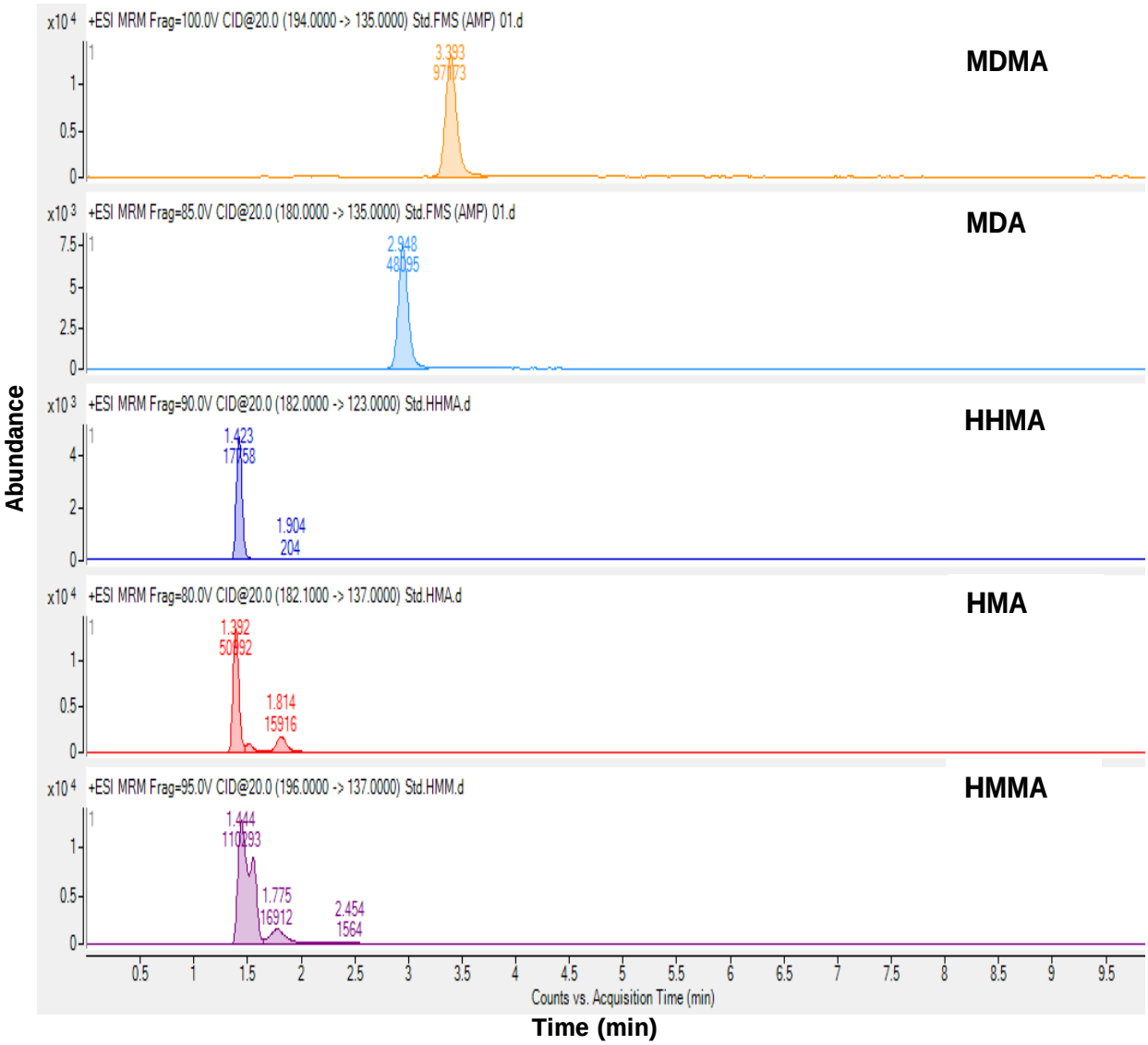


Figure 8-5: LC/MS chromatograms of underivatised MDMA and its metabolites using a Gemini C18 column.

To improve the LC separation, acylation with acetic anhydride was used to derivatise MDMA and its metabolites that contain active functional groups (OH and NH) to form larger, more hydrophobic, compounds. Derivatives have the potential to improve LC separations by reverse-phase HPLC and increase sensitivity in mass spectrometry. Moreover, HHMA and HMA have different chemical structures but the same molecular weights, whereas their Ac-derivatives have different molecular weights because HHMA accepts three acyl groups whereas HMA only accepts two. The structures of HHMA, HMA and their Ac-derivatives are shown in Figure 8-6.

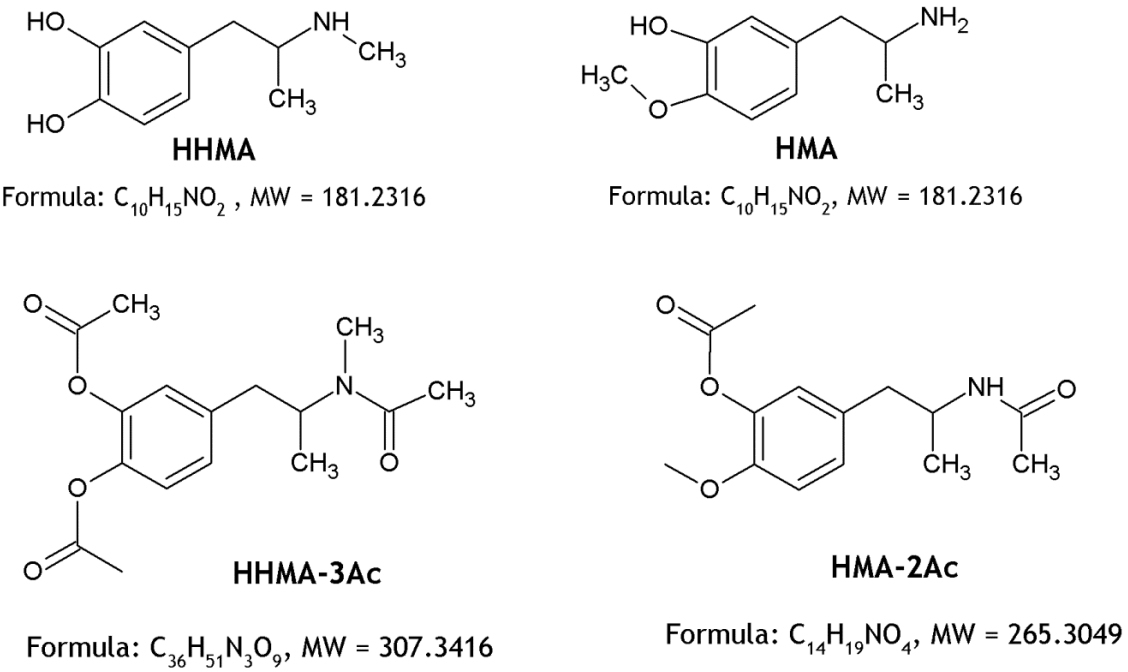


Figure 8-6: Structures of HHMA, HMA and their Ac-derivatives.

A mobile phase containing 33% of 0.1% formic acid in methanol and 67% of 0.1% formic acid in water was tried for separation of the Ac-derivatives of MDMA and its metabolites but was found to have a significant adverse effect on the column pressure. However, good chromatography was obtained using an isocratic mobile phase containing 33% of 0.1% formic acid in acetonitrile and 67% of water containing 0.1% formic acid with a flow rate of 0.35 ml/min. All analytes were separated using this mobile phase on a conventional C18 column with satisfactory retention factors (*k'*) in the range 3.3 to 5.6. The retention factor was 3.3 for HMA-2Ac (the most hydrophilic), 3.5 for MDA-Ac, 4.7 for HHMA-3Ac, 4.9 for HMMA-2Ac, 5.4 for MDMA-d_5-Ac and 5.6 for MDMA-Ac (the least hydrophilic), see Figure 8-7. The hold-up time (t_0 = 0.8 min) was determined by injecting pure methanol. It was concluded that acetylation improved the peak shapes and retention factors of MDMA and its metabolites in reverse-phase LC/MS.

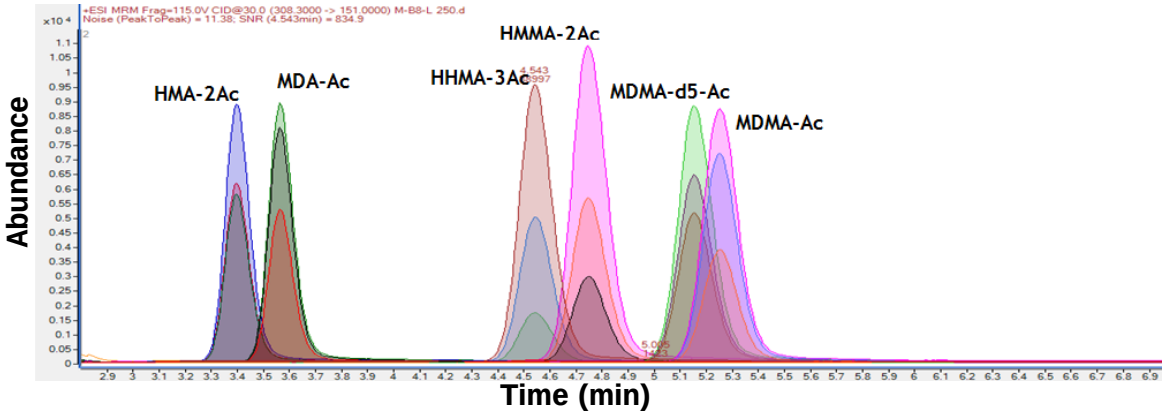


Figure 8-7: LC/MS/MS MRM chromatograms for MDMA-Ac and its metabolites following extraction from urine (concentration 0.25 µg/ml).

8.5.1.2 MRM optimisation

Liquid chromatography coupled with tandem mass spectrometry in multiple reaction monitoring (MRM) mode is a highly sensitive and selective detection method for targeted analysis. The sensitivity of MRM-MS depends critically on the appropriate tuning of instrument parameters such as fragmentor voltage (FV), collision energy (CE) and/or cone voltage in order to generate optimal precursor and product ions. Precursor ions, product ions, FVs and CEs for MDMA-Ac, MDA-Ac, HHMA-3Ac, HMMA-2Ac, HMA-2Ac and MDMA-d_5-Ac were obtained from direct injection of Ac-derivatives of standards in methanol into the mass spectrometer interface using a syringe pump with positive ESI and the optimised parameters are summarised below in Table 8-8. To obtain a high degree of certainty in the identification method, 4 identification points (IPs) are required[166]. One quantifier and two qualifier ions were applied for MDMA and its metabolites.

The fragmentation patterns of the acetate derivatives of MDMA and its metabolites in LC/MS are rather different from the type of ion reactions found in GC/MS. Mass spectral data for all analytes were obtained in the positive ESI mode. Representative fragmentation reactions of derivatives of MDMA and its metabolites at a CE of 20 eV are illustrated in Figure 8-8. Under optimised conditions, most ion reactions had different optimum CEs and a CE of 20 eV was chosen for Figure 8-8 as a mid-range value which demonstrates the patterns, although ion ratios under optimised conditions are different.

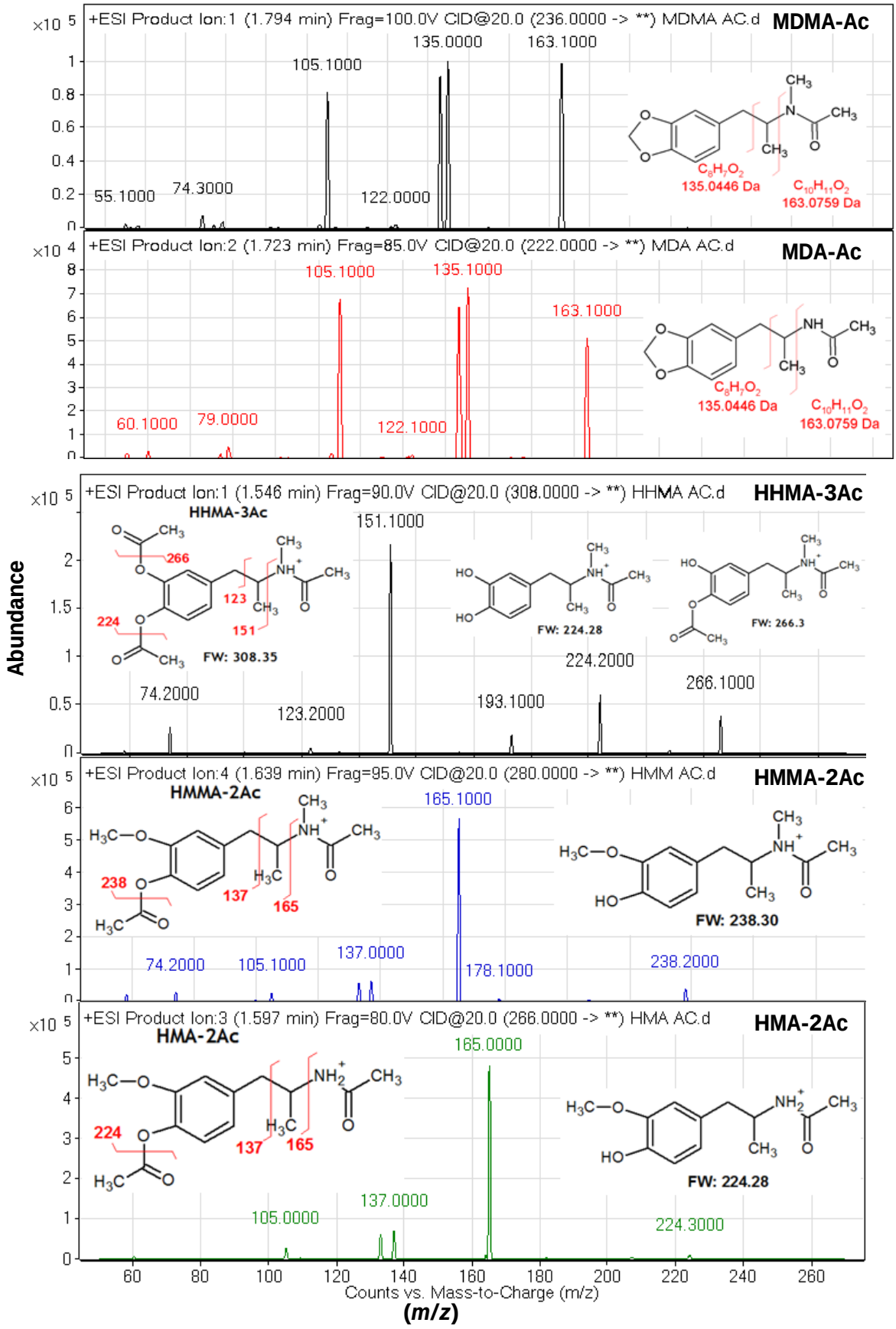


Figure 8-8: Mass spectra of Ac-derivatives of MDMA and its metabolites at a CE of 20 eV in product ion scan mode. Under optimised conditions, most ion reactions have different optimum CEs. The spectra illustrated here demonstrate typical patterns, although the ion ratios under optimised conditions may be different.

The protonated molecular ion $(M-H)^+$ of MDMA-Ac at *m/z* 236 was used as precursor ion and its fragment ions at *m/z* 105, 135 and 163 were selected as product ions. The fragment ion at *m/z* 163, the most intense peak in the mass spectrum of MDMA-Ac, was used as the quantifier ion and the ions at *m/z* 105 and 135 were used as qualifiers ions.

The protonated molecular ion of MDA-Ac at *m/z* 222 was used as precursor ion and its fragment ions at *m/z* 105, 135 and 163 were selected as product ions. The ion at *m/z* 163, the most intense peak of MDA-Ac, was used as the quantifier ion and the ions at *m/z* 105 and 135 were used as qualifiers ions.

The protonated molecular ion of HHMA-3Ac at *m/z* 308 was used as precursor ion and its fragment ions at *m/z* 151, 224 and 266 were selected as product ions. The product ions of the Ac-derivative of HHMA at *m/z* 266 and *m/z* 244 resulted from loss of ketene groups ($COCH_2$, 42 Da) from the protonated molecular ion. The ion at *m/z* 151, the most intense peak in the mass spectrum of HHMA-3Ac, was used as the quantifier ion and the ions at *m/z* 224 and 266 were used as qualifiers ions.

The protonated molecular ion of HMMA-2Ac at *m/z* 280 was used as precursor ion and its fragment ions at *m/z* 137, 165 and 238 were selected as product ions. The product ion of the Ac-derivative of HMMA at *m/z* 238 resulted from loss of a ketene group ($COCH_2$, 42 Da) from the protonated molecular ion. The ion at *m/z* 165, the most intense peak of HMMA-2Ac, was used as the quantifier ion and the ions at *m/z* 137 and 238 were used as qualifier ions.

The protonated molecular ion of HMA-2Ac at *m/z* 266 was used as precursor ion and its fragment ions at *m/z* 105, 137 and 165 were selected as product ions. The ion at *m/z* 165, the most intense peak of HMA-2Ac, was used as a quantitative ion and ion at *m/z* 105 and 137 were used as qualifier ions.

The protonated molecular ion of MDMA-d_5-Ac at *m/z* 241 was used as precursor ion and its fragment ions at *m/z* 107, 135 and 165 were selected as product ions. The ion at *m/z* 165, the most intense peak in the mass spectrum of MDMA-d_5-Ac, was used as the quantifier ion.

In conclusion, because the mass spectra of acetate derivatives of MDMA and its metabolites were not sufficiently selective for use in a GC/MS SIM method, acetylation with LC/MS analysis was chosen for further *in vitro* and *in vivo* metabolite studies.

8.5.2 Solid phase extraction

Liquid-liquid extraction is not suitable for the extraction of hydrophilic compounds such as HHMA and, to decrease the matrix background from biological samples, solid phase extraction methods were developed. The SPE cartridges involved in this study were chosen for evaluation from amongst those used in previous studies and also included the one used in the routine method for MDMA analysis in the forensic toxicology laboratory, University of Glasgow.

Three types of SPE cartridge (Oasis® MCX, Oasis® WCX and Clean Screen® DAU) were compared for the extraction of MDMA and its hydrophilic metabolites, focussing on HHMA, HMMA and HMA. MDA was not included in this study, which was primarily concerned with obtaining good recoveries of polar metabolites, as its polarity is similar to that of MDMA: Log *P* values for MDMA and its metabolites are listed in Table 8-6.

8.5.2.1 Extraction method

8.5.2.1.1 Oasis® MCX and Clean Screen® DAU

The SPE cartridges were sequentially conditioned with 3 ml methanol, 3 ml deionised water and 2 ml 0.1 M phosphate buffer pH 6. After transferring samples (n = 2) to the column and passing them through the cartridge completely, the cartridges were washed with 3 ml deionised water, 2 ml 0.1 M acetate buffer pH 4.5 and 3 ml methanol and dried under full vacuum for 10 min. New labelled 4 ml glass vials were placed in a rack within the vacuum work station. Analytes were eluted with 3 ml of dichloromethane/ isopropanol/NH_3 (78:20:2 v:v:v) and the eluent were transferred to labelled 3.5 ml vials and evaporated to dryness by a gentle flow of nitrogen gas at ambient temperature before performing derivatisation.

8.5.2.1.2 Oasis® WCX

The SPE cartridges were sequentially conditioned with 3 ml methanol and 3 ml deionised water. After transferring samples (*n* = 2) to the column and passing them through the cartridge completely, the cartridges were washed with 3 ml of deionised water and 3 ml of methanol. New labelled 4 ml glass vials were placed in a rack within the vacuum manifold. Analytes were eluted with 3 ml of acetonitrile/methanol/10% NH_3 (63:27:10 v:v:v) and the eluent were transferred to labelled 3.5 ml vials and evaporated to dryness by a gentle flow of nitrogen gas at ambient temperature before performing derivatisation.

8.5.2.2 Results of solid phase extraction

8.5.2.2.1 Clean Screen® DAU

Clean Screen DAU was tested according to the method described in Section 8.5.2.1.1 which was developed for routine analysis of amphetamines in blood and urine in the forensic toxicology laboratory, University of Glasgow. Clean Screen DAU is a mixed-mode column exhibiting both hydrophobic and cation exchange characteristics which was designed for forensic work. The cartridges were tested using a mixture of standards of MDMA, HHMA, HMMA, HMA and MDMA-d_5, each at a concentration of 1 µg/ml in blank HLM incubation matrix. The extracted samples were analysed using LC/MS/MS. The results showed that HHMA-3Ac was not retained on the column although the other analytes were retained strongly (recoveries higher than 85% for HMMA-2Ac and higher than 90% for MDMA-Ac, HMA-2Ac and MDMA-d_5-Ac). Figure 8-9 displays LC/MS/MS MRM chromatograms for the HHMA ions, with a complete absence of a peak at 3.6 min, the retention time of HHMA-3Ac. Figure 8-10 displays the corresponding chromatograms for HMA, HMMA and MDMA, with satisfactory peak shapes and peak separations.

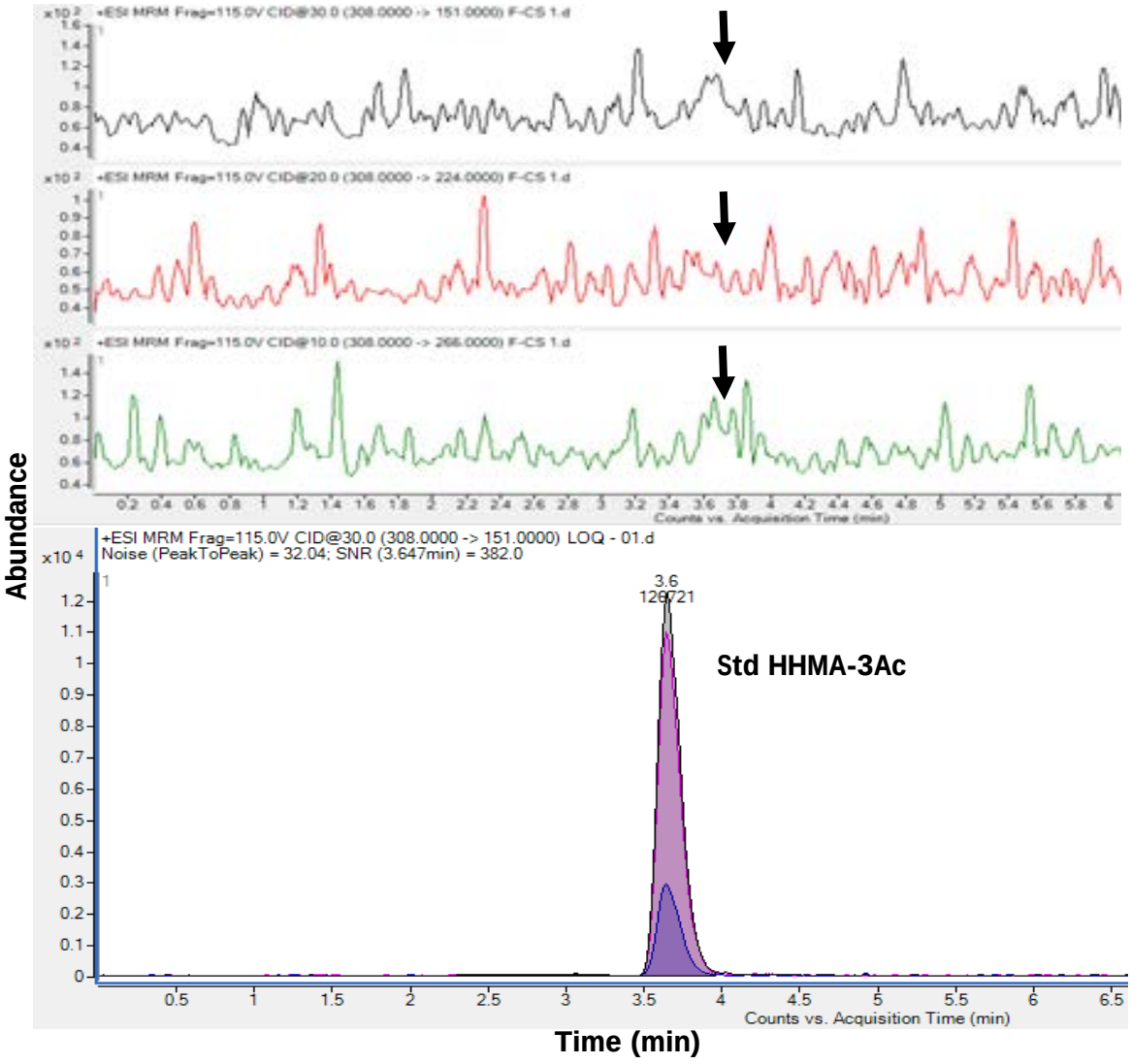


Figure 8-9: LC/MS/MS MRM chromatograms of HHMA-3Ac ions after extraction with Clean Screen® DAU. The retention time of HHMA-3Ac is 3.6 min on Gemini column (arrowed).

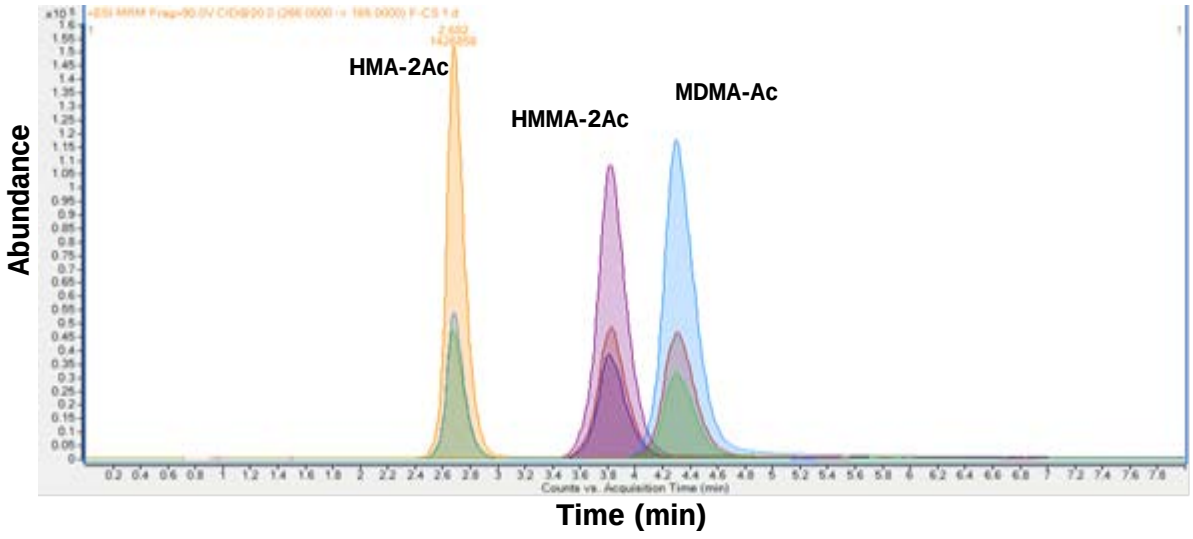


Figure 8-10: LC/MS/MS MRM chromatograms for HMA-2Ac, HMMA-2Ac and MDMA-Ac after extraction with Clean Screen® DAU.

8.5.2.2.2 Oasis® MCX

Oasis® MCX was also tested using the method described in Section 8.5.2.1.1. Oasis® MCX is a mixed-mode reversed-phase/strong cation-exchange polymer column designed for basic compounds. The cartridges were tested using a mixture of standards of MDMA, HHMA, HMMA and HMA, each at a concentration of 1 µg/ml in blank HLM incubation matrix. The extracted samples were analysed using LC/MS/MS. The results showed that HHMA-3Ac was not retained on this column either (see Figure 8-11) although the recovery of MDMA-Ac was 63% and HMMA-2Ac and HMA-2Ac were retained strongly (recoveries higher than 100%, Figure 8-12). Previous work by Menet *et al.*[167] has shown that HHMA and HMA were poorly retained on mixed-mode and strong cation exchange cartridges (Bond Elut Certify® and Chromabond SCX®). The efficiency of mixed-mode SPE columns in retaining hydrophilic compounds by ionic interaction is dependent on the relative number of SCX moieties compared to C18 moieties.

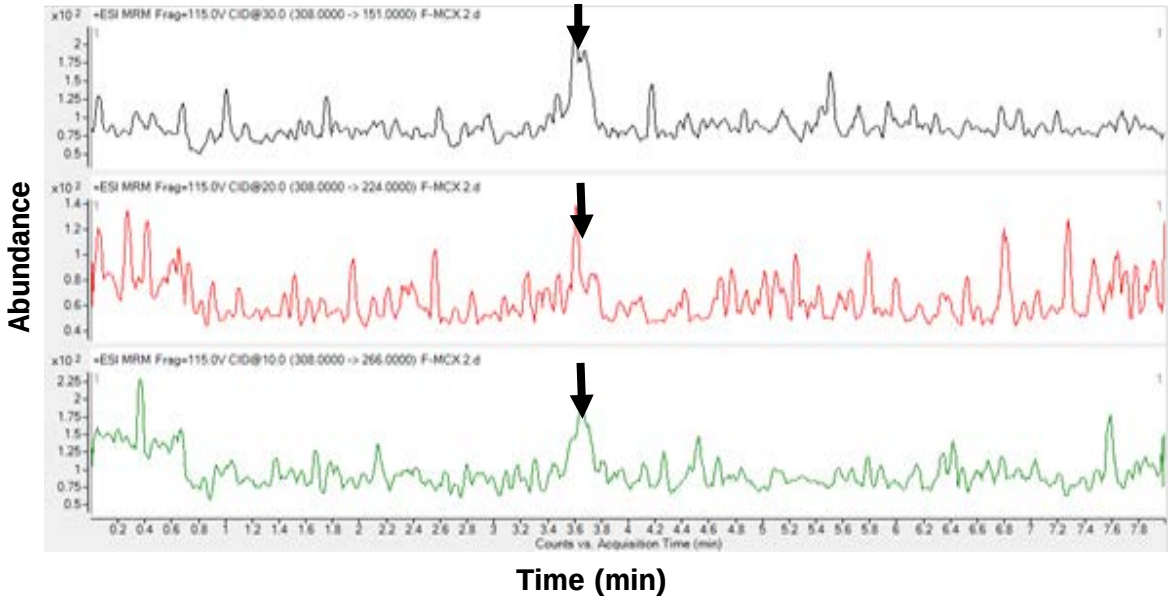


Figure 8-11: LC/MS/MS MRM chromatograms of HHMA-3Ac ions after extraction with Oasis® MCX. The retention time of HHMA-3Ac is 3.6 min on Gemini column (arrowed).

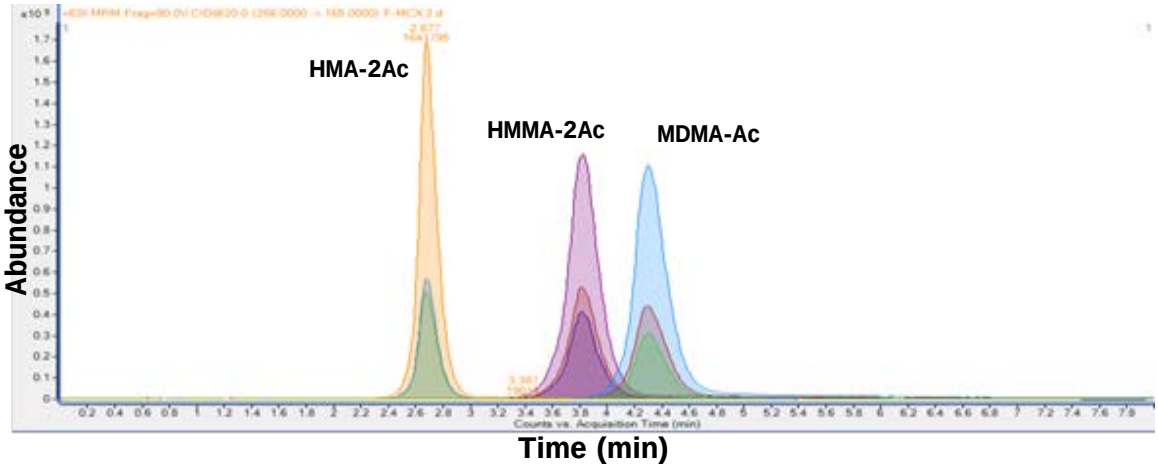


Figure 8-12: LC/MS/MS MRM chromatograms for HMA-2Ac, HMMA-2Ac and MDMA-Ac after extraction with Oasis® MCX.

8.5.2.2.3 Oasis® WCX

Oasis® WCX was tested using the method described in Section 8.5.2.1.2 according to Fonsart *et al.*[163] with some modifications. Oasis® WCX is a mixed-mode reversed-phase/weak cation-exchange polymer column designed to retain and release quaternary amines and strongly basic compounds. The cartridges were tested using standards of MDMA, HHMA, HMMA and HMA at a concentration of 1 µg/ml in blank HLM incubation matrix. The extracted samples were analysed using LC/MS/MS. The results for this cartridge also showed that HHMA was poorly retained on the column (recoveries lower than 15%, Figure 8-13) whereas the recovery of MDMA was 57% (Figure 8-14). HMMA and HMA were retained strongly (recoveries higher than 70%) on the column. It shows that hydrophilic analytes were not completely retained by the WCX column. However, this result is in contrast with a previous study by Menet *et al.*[167] which found that Oasis® WCX gave good recoveries for MDMA and its five metabolites in a comparison of three SPE types (Bond Elut® Certify, Chromabond® SCX and Oasis® WCX). The most likely explanation of these differences is that the eluent used in this study was different from that used in the previous study by Menet *et al.*, which would have affected the recovery of HHMA. In that study, the recovery of HHMA was 63.3% at a low concentration (250 ng/ml) and 69.0% at a high concentration (1 µg/ml).

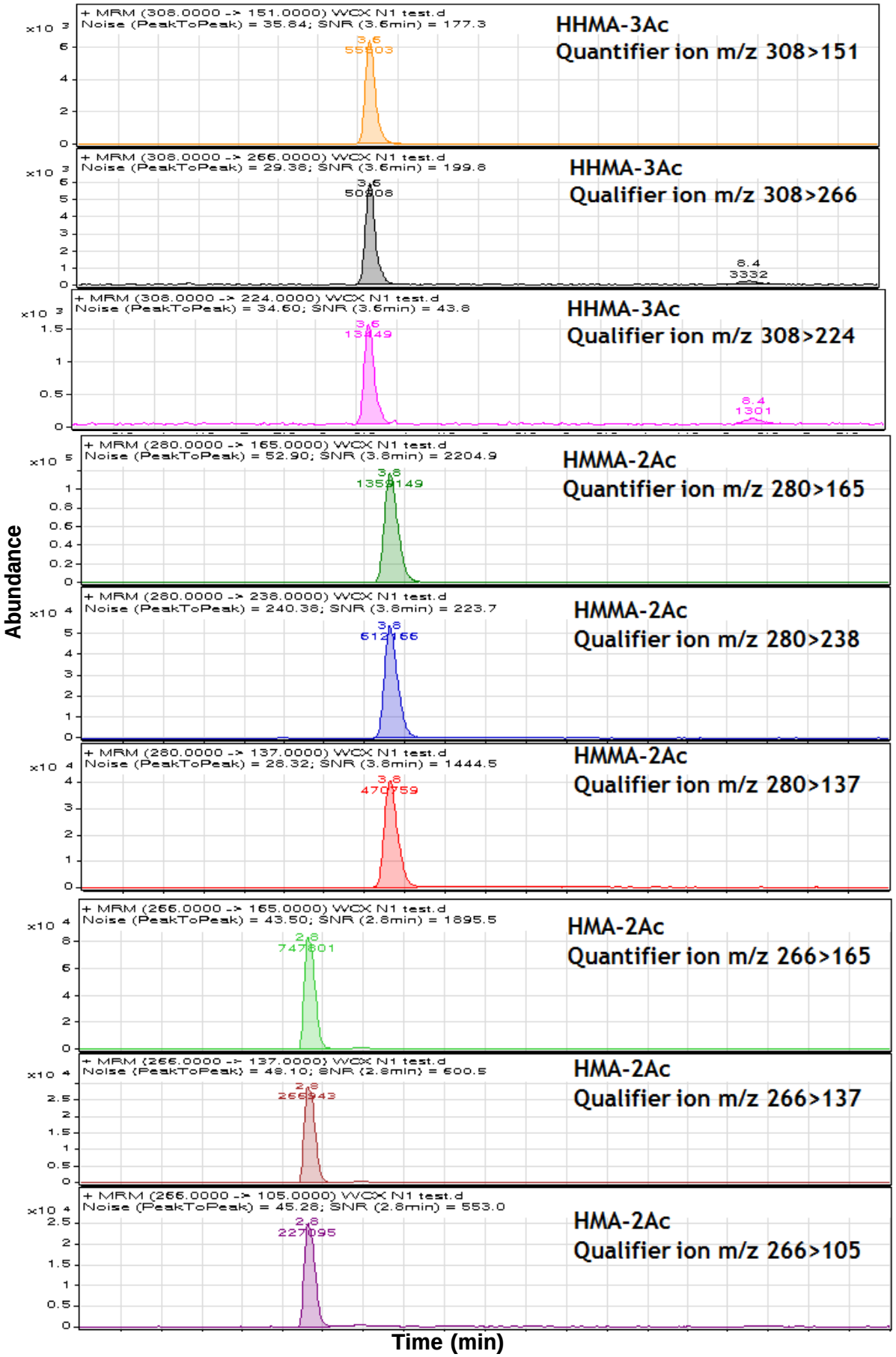


Figure 8-13: LC/MS/MS MRM chromatograms of HHMA-3Ac, HMMA-2Ac and HMA-2Ac after extraction with SPE Oasis® WCX.

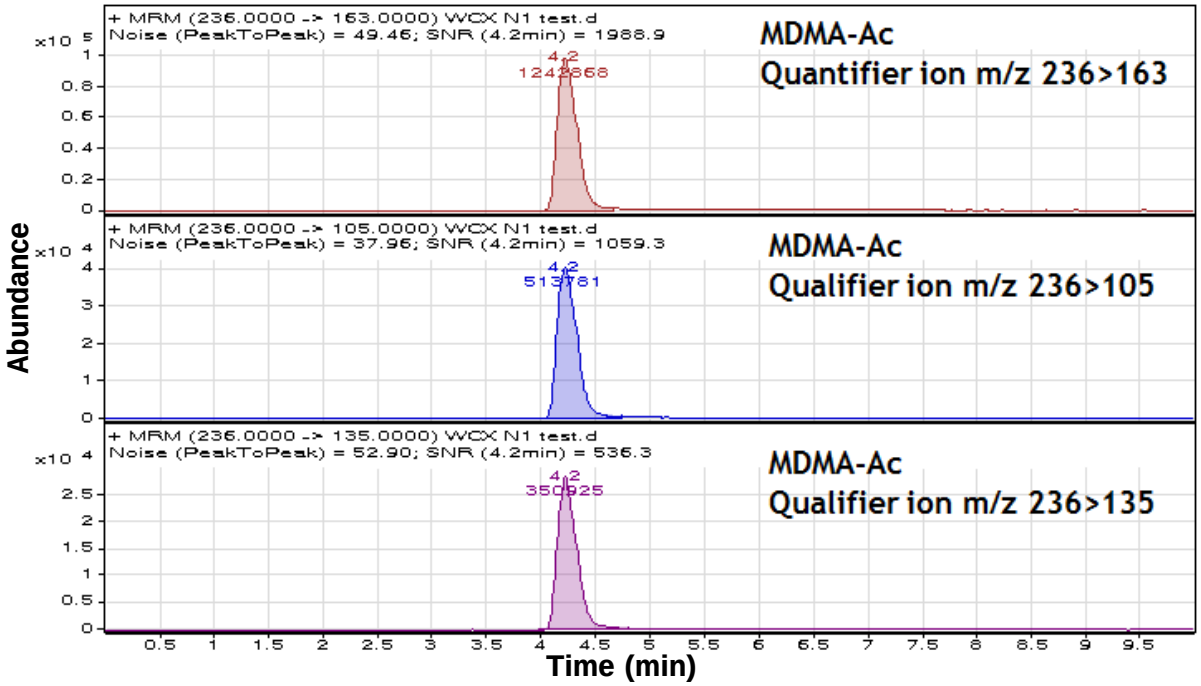


Figure 8-14: LC/MS/MS MRM chromatograms of MDMA-Ac after extraction with SPE Oasis® WCX.

8.5.2.3 Comparison of SPE methods

Oasis® WCX columns were considered to be the best compromise after comparing them with Oasis® MCX (Waters) and Clean Screen DAU (UCT Inc.) columns. Oasis® MCX and Clean Screen® DAU were excluded due to significant losses of HHMA during extraction although MDMA and other metabolites were retained with good recoveries. The Oasis® WCX columns gave the best recovery of HHMA amongst the three types of SPE cartridges evaluated, see Table 8-7.

Table 8-7: SPE recoveries of MDMA and its metabolites.

Drug	Average recovery % (*n* = 2)		
	Oasis® MCX	Clean Screen® DAU	Oasis ®WCX
HHMA	ND	ND	13%
HMMA	115%	100%	74%
HMA	100%	87%	97%
MDMA	63%	113%	57%

ND = Not detected

However, although SPE methods can reduce the matrix background from biological samples, HHMA was poorly retained on the Oasis® WCX column (recovery was 13%). The analyst's time to perform SPE of 24 samples and dry the extracts was approximately 2-3 h while the sample preparation time for protein precipitation was only 1 h for 24 samples. In addition, the major financial difference between SPE and liquid-liquid extraction or protein precipitation methods was the purchase of SPE columns for each extraction (£2.45 per cartridge for Oasis® WCX).

In the end, protein precipitation with acetonitrile was developed and used subsequently as the extraction method for MDMA and its metabolites in incubation medium and in urine samples (see Sections 8.6.2 and 8.6.3).

8.6 Methods and materials

8.6.1 Reagents and standards

Acetonitrile, ethyl acetate and methanol (HPLC grade) were obtained from VWR International Ltd (UK). Acetic anhydride, formic acid, anhydrous pyridine, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), pentafluoropropionic acid anhydride (PFPA), sodium chloride and anhydrous toluene were obtained from Sigma-Aldrich Co, LLC (UK). Ammonia solution 28% w/v, dichloromethane, 2-propanol, potassium chloride, sodium acetate trihydrate, sodium phosphate dibasic, potassium dihydrogen phosphate, disodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and sodium dihydrogen orthophosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) were obtained from BDH (UK).

3,4-Methylenedioxymethamphetamine-HCl (MDMA), 3,4-methylenedioxy-methamphetamine-d_5 (MDMA-d_5), 3,4-methylenedioxyamphetamine (MDA), human liver microsomes (HLM), *B*-Nicotinamide adenine dinucleotide phosphate hydrate (*B*-NADP), D-glucose 6-phosphate dipotassium salt hydrate (G6P), *B*-glucuronidase from *Helix pomatia* and glucose-6-phosphate dehydrogenase (G6PD) from baker's yeast (*S. cerevisiae*) were purchased from Sigma-Aldrich Co, LLC (UK). 4-Hydroxy-3-methoxymethamphetamine-HCl (HMMA), 3,4-dihydroxy-methamphetamine-HCl (HHMA), and 4-hydroxy-3-methoxyamphetamine-HCl

(HMA) were purchased from Lipomed (supplied by Kinesis, Ltd UK). HLM was subdivided on receipt into 50 µl aliquots and were stored at -80^{o}C until used.

Stock standard solutions were prepared by dissolving each compound in methanol to achieve a concentration of 1 mg/ml of drug substance. Working standard solutions were prepared by serial dilution of stock standard solutions with methanol. All stock and working standard solutions were stored at 4^{o}C.

8.6.1.1 Preparation of 0.1 M phosphate buffer pH 6

1.7 g of disodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and 12.14 g of sodium dihydrogen orthophosphate monohydrate (NaH_2PO_4) were weighed out and added to a 1 L volumetric flask and 800 ml of deionised water was added. The pH was adjusted to pH 6 using 0.1 M monobasic sodium phosphate or 0.1 M dibasic sodium phosphate. The volume was then made up to 1 L with deionised water.

8.6.1.2 Preparation of 0.1 M acetate buffer pH 4.5

5.86 g of sodium acetate trihydrate was weighed out and added to a 1 L volumetric flask and 800 ml of deionised water and 3.24 ml of glacial acetic acid were added. The pH was adjusted to pH 4.5 using 0.1 M acetic acid or 0.1 M sodium acetate. The volume was then made up to 1 L with deionised water.

8.6.1.3 Preparation of phosphate buffer saline (PBS) pH 7.4

4.00 g of sodium chloride (NaCl), 0.10 g of potassium chloride (KCl), 0.72 g of sodium phosphate dibasic (Na_2HPO_4) and 0.12 g of potassium dihydrogen phosphate (KH_2PO_4) were weighed out and added to a 500 ml volumetric flask. The volume was then made up to 500 ml with deionised water.

8.6.1.4 Preparation of 0.1 M sodium acetate buffer pH 5

13.6 g of sodium acetate was weighed out and added to a 1 L volumetric flask and 800 ml of deionised water and 6 ml of glacial acetic acid were added. The

pH was adjusted to pH 5 using acetic acid. The volume was then made up to 1 L with deionised water.

8.6.1.5 Preparation of NADPH generating system

β-NADP was prepared as a 10 mg/ml solution in water and G6P as a 0.1 M solution in water; both were stored at -20^{o}C until used. The G6PD was dissolved in buffer to achieve an activity of 1,000 IU/ml and was stored at -20^{o}C until used.

8.6.1.6 Preparation of MDMA in phosphate buffer pH 7.4

Stock solutions of MDMA for use in metabolism studies were freshly prepared at 10 µg/ml in pH 7.4 phosphate buffer and stored at -20 ^{o}C until used.

8.6.1.7 Preparation of 0.1 M acetic acid

800 ml of deionised water was measured into a 1L volumetric flask and 5.7 ml of glacial acetic acid was added. The volume was then made up to 1 L with deionised water.

8.6.1.8 Preparation of 0.1 M sodium acetate

1.36 g of sodium acetate trihydrate was weighed out and added to a beaker and dissolved in 80 ml of deionised water. This was then transferred to a 100 ml volumetric flask and the volume was made up to 100 ml with deionised water.

8.6.1.9 Preparation of 0.1 M monobasic sodium phosphate

2.76 g of sodium dihydrogen orthophosphate was weighed out and added to a beaker and dissolved in 100 ml of deionised water. This was then transferred to a 200 ml volumetric flask and the volume was made up to 200 ml with deionised water.

8.6.1.10 Preparation of 0.1 M dibasic sodium phosphate

2.84 g of disodium hydrogen orthophosphate (anhydrous) was weighed out and added to a beaker and dissolved in 100 ml of deionised water. This was then transferred to a 200 ml volumetric flask and the volume was made up to 200 ml with deionised water.

8.6.1.11 Preparation of quality control samples

The quality control samples were prepared by spiking mixed standard HHMA, HMA, HMMA, MDA, MDMA and MDMA-d_5 in blank urine samples to obtain the concentrations of 0.1 µg/ml (low), 0.5 µg/ml (medium) and 1 µg/ml (high).

8.6.2 *In vitro* assay (human liver microsomes)

The method used was adapted from previous work by Mueller and Rentsch[127]. All reagents were held at room temperature to thaw. 50 µl HLM were used in the incubation mixture with 400 µl of drug substance at 10 µg/ml (i.e. 4 µg) in pH 7.4 phosphate buffer per incubation. The NADPH generating system (NGS) was used to generate the NADPH for the reaction. It consisted of 50 µl of 0.1M G6P, 25 µl of 10 mg/ml *B*-NADP and 2 µl of 40 IU/ml G6PD. The NGS mixture was pre-incubated for 3 min at 37^{o}C before being added to the incubation mixture to start the reaction. The mixture was incubated in a shaking incubator at 37^{o}C, 30 rpm for 90 min. To maintain the reaction, 77 µl more NGS system was added to the incubation mixture after 30 min. The reaction was stopped at 90 min by the addition of 600 µl of ice-cold acetonitrile to the incubation mixture. After vortexing and centrifuging at 13,000 x g for 10 min, the supernatant was transferred to a 3.5 ml glass vial before performing the extraction of metabolites.

8.6.3 *In vivo* assays (MDMA positive urine samples)

Human urine samples which were known to be positive for MDMA were obtained from Glasgow Royal Infirmary for routine toxicology service analysis and the results of the analyses described below were reported as part of the case

investigation. The West of Scotland Research Ethics Committee confirmed that separate ethical permission was not required as the work was related to hospital service development. All samples were stored at 4°C prior to processing. Each urine sample was analysed for MDMA and its metabolites (MDA, HHMA, HMMA and HMA) using the two methods described below.

8.6.3.1 Non-hydrolysed extraction method

A 0.4 ml portion of each urine sample was mixed with 0.8 ml of acetonitrile and centrifuged at 13,000 rpm for 10 min. 400 µl of the top organic layer was transferred to a 3.5 ml glass vial and evaporated to dryness by a gentle flow of nitrogen gas at ambient temperature before performing derivatisation. Residues were reconstituted in 1 ml of 10% acetonitrile in deionised water containing 0.1% formic acid.

8.6.3.2 Enzymatically hydrolysed extraction method

A 0.4 ml portion of each urine sample was mixed with 70 µl of 0.1M sodium acetate buffer pH 5 and 40 µl of *β*-glucuronidase. The mixture was incubated in a shaking incubator at 56°C, 10 rpm for 60 min. 0.8 ml of acetonitrile was added before vortexing and centrifuging at 13,000 x g for 10 min. 400 µl of the top organic layer was transferred a 3.5 ml glass vial and evaporated to dryness by a gentle flow of nitrogen gas at ambient temperature before performed derivatisation. Residues were reconstituted in 1 ml of 10% acetonitrile in deionised water containing 0.1% formic acid.

8.6.4 Derivatisation

8.6.4.1 Acetic anhydride derivatisation (AA)

This was as described in Section 8.4.1.1.

8.6.4.2 Stability of derivatives of standards

The stability of derivatives in LC mobile phase was determined using acetyl derivatives of MDMA, MDA, HHMA, HMMA and HMA standards in the concentration

range from 0.01-2 µg/ml (0.01, 0.25, 0.50, 0.1, 0.25, 0.5, 1 and 2 µg/ml). The derivatives were investigated for short-term stability using the LC/MS method described below. Derivatised samples were stored in the LC/MS autosampler at room temperature after derivatisation and were analysed at 6, 12, 18, 24, 30 and 96 h.

8.6.5 Liquid chromatography/mass spectrometry conditions

Chromatographic separation was performed using an Agilent Binary SL 1200 LC from Agilent Technologies Inc. Isocratic elution was performed on a Gemini C18 column (150 x 2.0 mm, 5 µm) protected by a Gemini C18 Security Guard Pre-Column (4 x 2.0 mm, 5 µm) from Phenomenex Inc., which was maintained at 40°C with a flow rate of 0.35 ml/min. The injection volume was 20 µl and the mobile phase was composed of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (67:33 v:v) and the run time was 10 min per injection.

Analysis of MDMA and its metabolites was performed using an Agilent 6410 Triple Quad LC/MS/MS instrument (Agilent Technologies Inc.) equipped with an Agilent Binary SL 1200 LC system interface. Nitrogen was from a nitrogen generator (In House Gas (Manufacturing) Ltd, UK).

Ionisation of analyses of interest was carried out using ESI. The mass spectrometer was operated in the positive ion multiple reaction monitoring (MRM) mode. Nitrogen was used as nebuliser gas at 30 psi. The gas temperature was set to 350^{o}C and the gas flow to 11 ml/min. The MRM transitions, FVs and CEs for derivatised MDMA and MDMA metabolites were optimised separately by direct infusion into the MS of each derivatised standard solution at a concentration of 5 µg/ml in mobile phase (50:50 v:v). All analytes were identified and quantified based on their retention time and quantifier and qualifier ion ratios that were obtained from MRM transitions. The MRM transitions, FVs and CEs used for the measurement of MDMA and its metabolites are summarised in Table 8-8.

Table 8-8: MRM conditions for derivatives of MDMA and metabolites.

Name	Precursor Ion (*m/z*)	Fragmentor Voltage (eV)	Product Ion* (*m/z*)	Collision Energy (eV)	Qualifier/ quantifier ion ratio
MDA-Ac	222.2	90	**163**	10	-
			135	20	60%
			105	25	91%
MDMA-Ac	236.2	90	**163**	10	-
			135	30	44%
			105	30	83%
MDMA-d_5-Ac	241.3	90	165	10	-
			135	20	-
			107	30	-
HMA-2Ac	266.3	90	**165**	20	-
			137	30	66%
			105	40	70%
HMMA-2Ac	280.3	115	238	10	27%
			165	20	-
			137	30	51%
HHMA-3Ac	308.3	115	266	10	53%
			224	20	18.5%
			151	30	-

**m/z* values in bold were used as quantifier ions

8.6.6 Method validation

8.6.6.1 Blank urine samples

Existing blank urine samples from a previous research project, obtained with informed consent and appropriately stored at -20°C in Forensic Medicine and Science, were used. The chair of the University Medical Research Ethics Committee confirmed that approval would not be needed for these samples.

8.6.6.2 Selectivity/specificity

The selectivity of the described method was evaluated using 10 different sources of blank urine. The specificity of the described method was evaluated using blank urine spiked with a mixture of drugs that are routinely detected in forensic toxicology cases (amphetamine, methamphetamine, mephentermine, paramethoxyamphetamine, alprazolam, diazepam, flunitrazepam, midazolam, codeine, morphine, hydrocodone, hydromorphone, oxycodone, butylone and methylone) at a concentration of 1 µg/ml. Blank and spiked samples were

extracted and analysed by LC/MS to determine if there were any signals interfering with the signals of the analytes or the internal standard from blank matrix or other common drugs.

8.6.6.3 Linearity

Calibration curves for MDMA, MDA, HHMA, HMMA and HMA were prepared in blank urine which was extracted by the described method, covering low and high concentrations in the range from 0.025 to 1.5 µg/ml (0.025, 0.050, 0.1, 0.25, 0.50, 0.75, 1 and 1.5 µg/ml). MDMA-d_5 at a concentration of 0.25 µg/ml was spiked in all samples as internal standard. Blank urine with internal standard was included with each run as zero concentration standard samples. Calibration curves were plotted of the resulting response (the area ratio of analytes versus internal standard) versus the corresponding concentrations. The linear correlation coefficient (R^2) was obtained for each regression curve.

8.6.6.4 Lower limit of quantitation

The sensitivity of the method was assessed by determining the Lower Limit of Quantitation (LLOQ) of each analyte. Spiked urine samples at a concentration of 0.025 µg/ml (the lowest concentration standard in the calibration curves) and blank spiked with internal standard (n = 6) was analysed for this purpose. The signal-to-noise (S/N) ratio of LLOQ, calculated by comparing the peak area of analyte with the baseline (noise), is required to be equal or greater than 10. The lowest concentration standard in the calibration curve was used as a lower limit of quantitation for the described method.

8.6.6.5 Accuracy and precision

Three different concentrations of the quality control samples (QCs) were used to evaluate the intra-assay and inter-assay precision. The concentration of 0.1 µg/ml was used as low QC, 0.5 µg/ml as medium QC and 1 µg/ml as high QC. Intra-assay precision of the method for MDMA and its metabolites was determined using QCs (n = 3) in one day. The calibration curve of each analyte of interest was prepared using the concentration ranges mentioned previously.

Inter-assay precision was assessed using a similar procedure to the intra-assay precision but over six different days. Accuracy was estimated in terms of bias and was calculated using the equation described in Chapter 4.

8.6.6.6 Matrix effects

Matrix effects were measured using the method described by Matuszewski *et al.*[46] Neat standards at concentrations of 0.1 and 1 µg/ml were obtained by diluting 10 µg/ml and 100 µg/ml stock standards in 10% of acetonitrile plus 0.1% formic acid in deionised water. Blank urine samples were obtained from five different sources and spiked after un-hydrolysed extraction with analytes of interest at 0.1 and 1 µg/ml. Matrix effects of endogenous compounds were calculated by comparing the peak areas of spiked samples with the peak areas of neat standards.

8.6.7 Post mortem case materials

Three post mortem urine samples known to be positive for MDMA were analysed and reported as part of the medico-legal investigation of the three cases. Ethical approval and consent are not required if the analytical results are reported to the Procurator Fiscal. In addition, the Head of the Scottish Fatalities Investigation Unit was informed and his permission was obtained in an email to publish the analytical results in this thesis. All samples were stored at 4^{o}C prior to processing. Urine samples were analysed for MDMA and its metabolites (MDA, HHMA, HMMA and HMA) using the method described earlier

8.7 Results and discussion

8.7.1 Stability of Ac-derivatives

To develop a quantitative LC/MS method using chemical derivatisation, one parameter that needed to be assessed was the stability of the derivatives in the mobile phase. Standards of MDMA-Ac, HHMA-3Ac, HMMA-2Ac and HMA-2Ac at 0.01, 0.025, 0.050, 0.1, 0.25, 0.5, 1 and 2 µg/ml were analysed by LC/MS and then maintained at room temperature on the autosampler for re-analysis. For routine analysis, samples should be stable before analysis in the autosampler for

24 h. Analytes were evaluated at 0, 6, 12, 18, 24, 30 and then at 96 h. Area ratios of analytes and internal standard were calculated and compared with that obtained with freshly prepared standards.

The stability of underivatised and derivatised MDMA and its metabolites, including MDA, HMMA and HMA has been studied and data is available in the literature[93,159,164,168,169]. Pirnay *et al.*[93] reported that HFBA-derivatives of MDMA, MDA, HMMA and HMA were stable for 3 freeze/thaw cycles, 14 h at room temperature before analysis, 72 h in the refrigerator before analysis and 72 h at ambient temperature on the GC/MS autosampler. Moreover, only one study on the stability of HHMA has been reported. Mueller *et al.*[159] found less than a 10% loss of underivatised HHMA in mobile phase after 14 h in the autosampler at room temperature. However, this is the first time that the stability of acetate derivatives of MDMA, MDA, HHMA, HMMA and HMA in LC mobile phase has been reported.

Plots of analyte concentration versus storage time in the autosampler are shown in Figure 8-15. Acetates of MDMA, HHMA, HMMA and HMA were stable in the HPLC mobile phase for 30 h at room temperature at all concentrations, which was acceptable for a regular analytical run. Additional measurements were made at 96 h to determine if any decomposition occurred over an extended period. These analyses indicated that analyte concentrations decreased by up to almost 50% over 96 h. Further work on stability would be necessary if samples were likely to be left routinely in the autosampler for more than 30 h. These results have shown that derivatisation with acetic anhydride is suitable for the development of a routine method for quantitation of MDMA and its metabolites in urine samples.

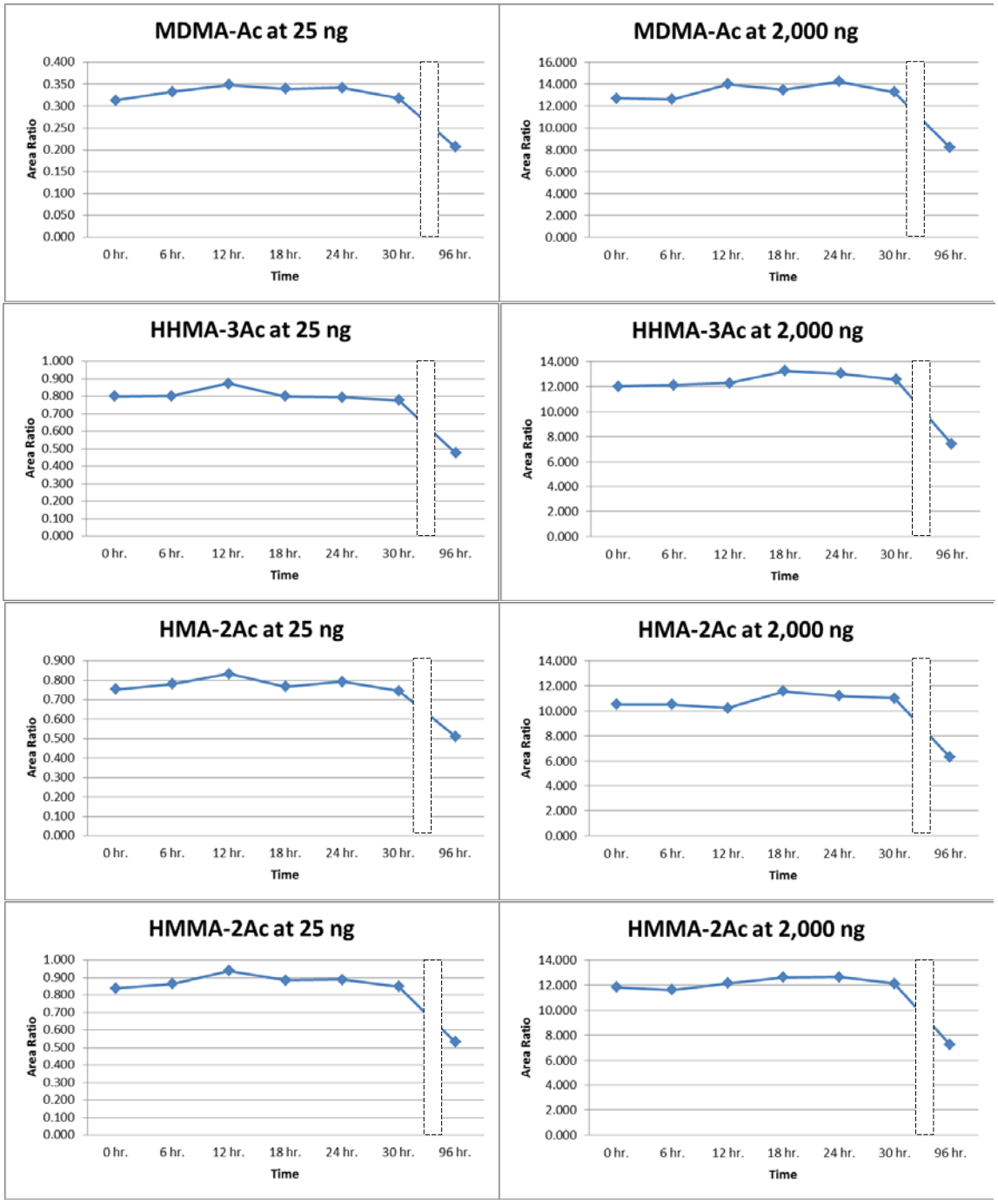


Figure 8-15: Stability of Ac-derivatives of standards of MDMA and its metabolites in the HPLC mobile phase at low and high concentrations over a 96 h period. A non-linear scale has been applied to the x-axis to improve visibility of the points up to 30 h.

8.7.2 Method validation

8.7.2.1 Selectivity/specificity

Specificity of the method was evaluated using blank urine spiked with a mixture of the most common drugs detected in forensic cases such as amphetamines, benzodiazepines and opiates. To investigate the selectivity of the method from endogenous compounds, 10 blank urine specimens from different individuals were analysed to evaluate chromatographic interference. Blank urine spiked with internal standard was also included with each batch. The absence of analyte ions in blank urines spiked with internal standard proved that internal standard did not contain relevant amounts of unlabelled analytes (Figure 8-16). Also, no interference peaks were detected for any of the analytes in extracts of spiked urine samples (Figure 8-17).

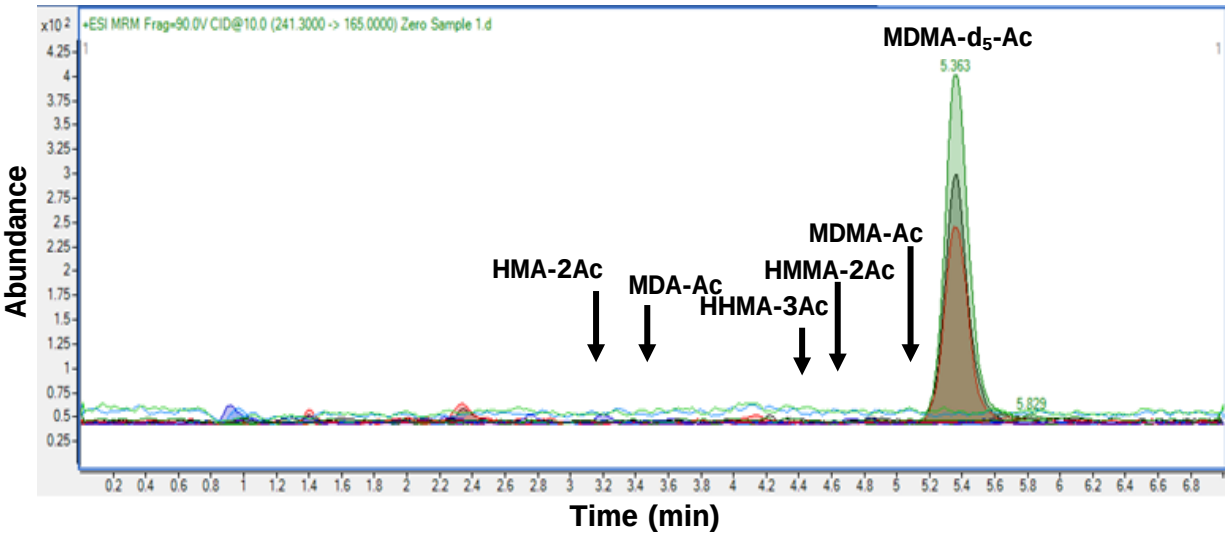


Figure 8-16: MRM chromatograms obtained by LC/MS/MS analysis of an acetylated extract of blank urine spiked with internal standard MDMA-d_5, showing absence of interferences at the retention times of MDMA and its metabolites (selectivity).

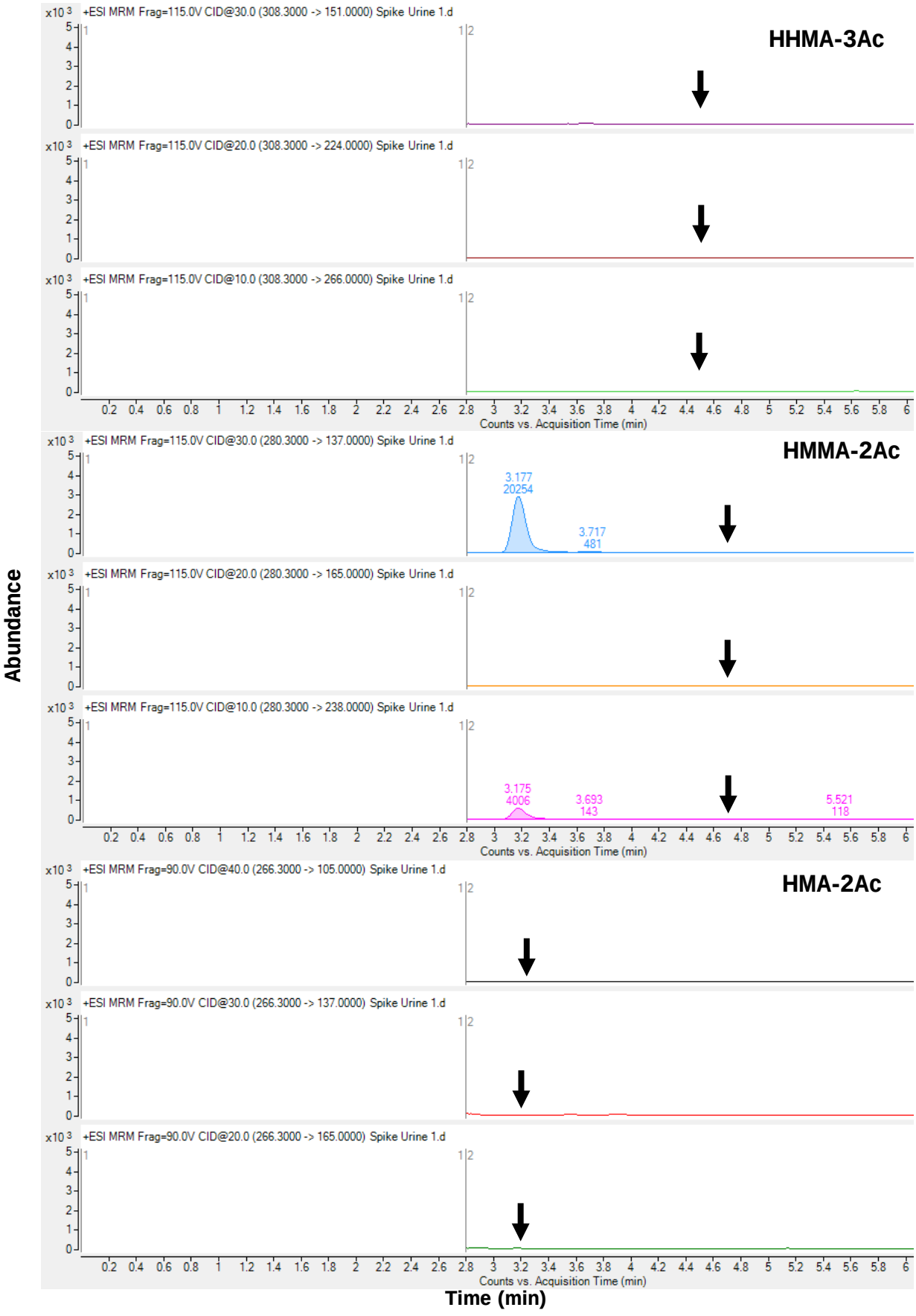


Figure 8-17: MRM chromatograms obtained by LC/MS/MS analysis of a spiked urine sample. Retention times of analytes are indicated (arrowed).

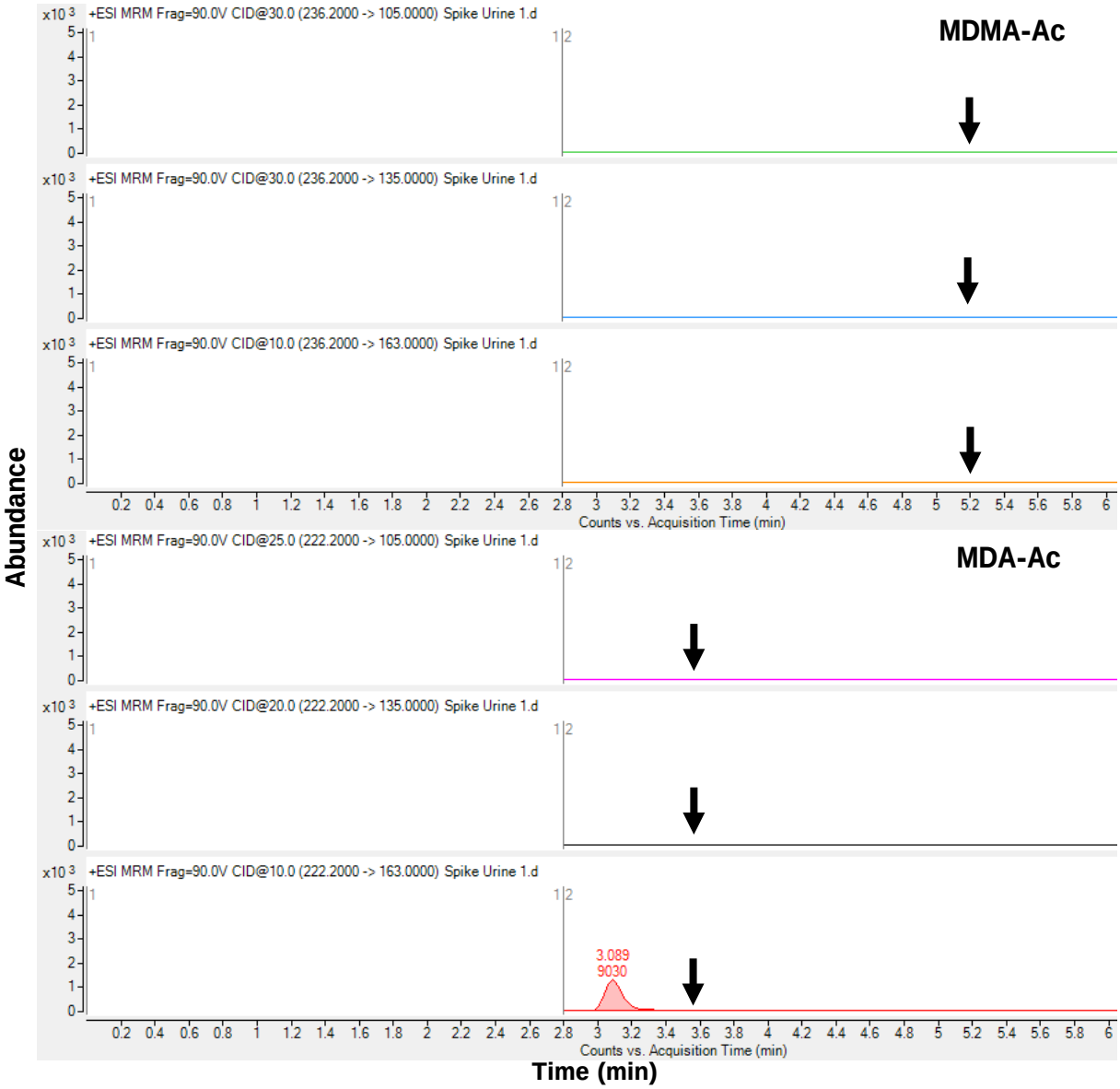


Figure 8-17 (cont.): MRM chromatograms obtained by LC/MS/MS analysis of a spiked urine sample. Retention times of analytes are indicated (arrowed).

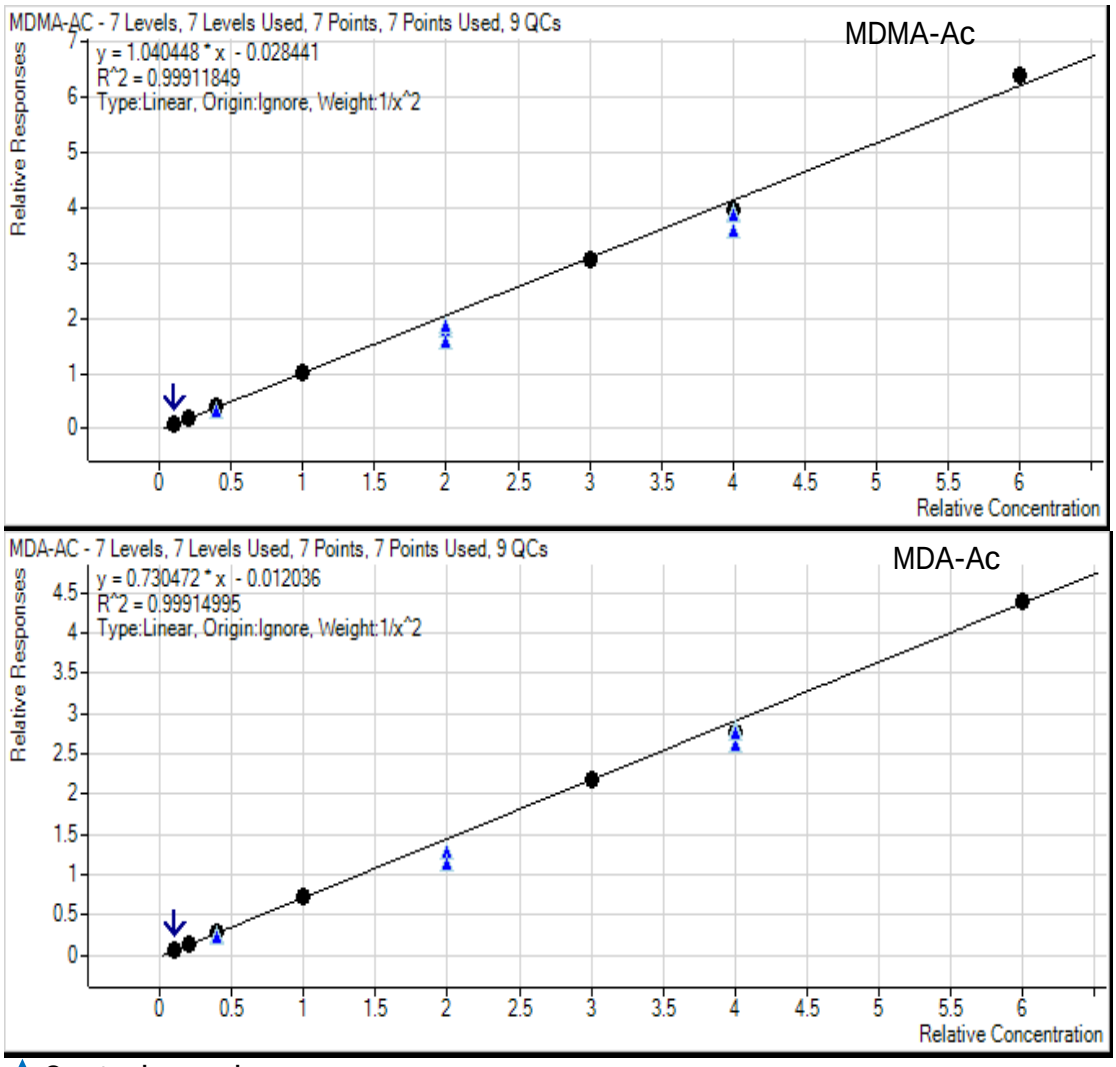
8.7.2.2 Linearity

The concentration range for calibration curves was chosen based on previous studies of urine concentrations of MDMA and its metabolites in humans[90,148,152]. Calibration curves using eight concentration levels with six replicates each were constructed to assess the calibration model. A weighted ($1/x^2$) calibration model was used to allow for unequal variances across the calibration range (0.025 to 1.5 µg/ml). Regression equations and linear correlation coefficients (R^2) are given in Table 8-9. The linear regression equations for MDMA and MDA had linear correlation coefficient (R^2) values greater than 0.999, which is very satisfactory, and for HHMA, HMMA and HMA had values greater than 0.98, which is sufficient.

Table 8-9: Regression models, correlation coefficients (R^2) and LLOQs for LC/MS/MS analysis of acetate derivatives of MDMA and its metabolites in urine.

Analytes	Regression	R^2	LLOQ* (µg/ml)
MDMA-Ac	$y = 1.040x - 0.0284$	0.999	0.025
MDA-Ac	$y = 0.730x - 0.0120$	0.999	0.025
HHMA-3Ac	$y = 0.957x - 0.0126$	0.980	0.025
HMMA-2Ac	$y = 1.149x - 0.0070$	0.982	0.025
HMA-2Ac	$y = 0.680x - 0.0070$	0.985	0.025

*LLOQ was set at the concentration of the lowest standard in the calibration curve (0.025 µg/ml).



▲ Control samples

Figure 8-18: Calibration curves (0.025-1.5 µg/ml) for LC/MS/MS analysis of acetate derivatives of MDMA and its metabolites in urine. LLOQs are indicated (arrowed). Concentrations of standards are 0.025, 0.050, 0.1, 0.25, 0.50, 0.75, 1 and 1.5 µg/ml.

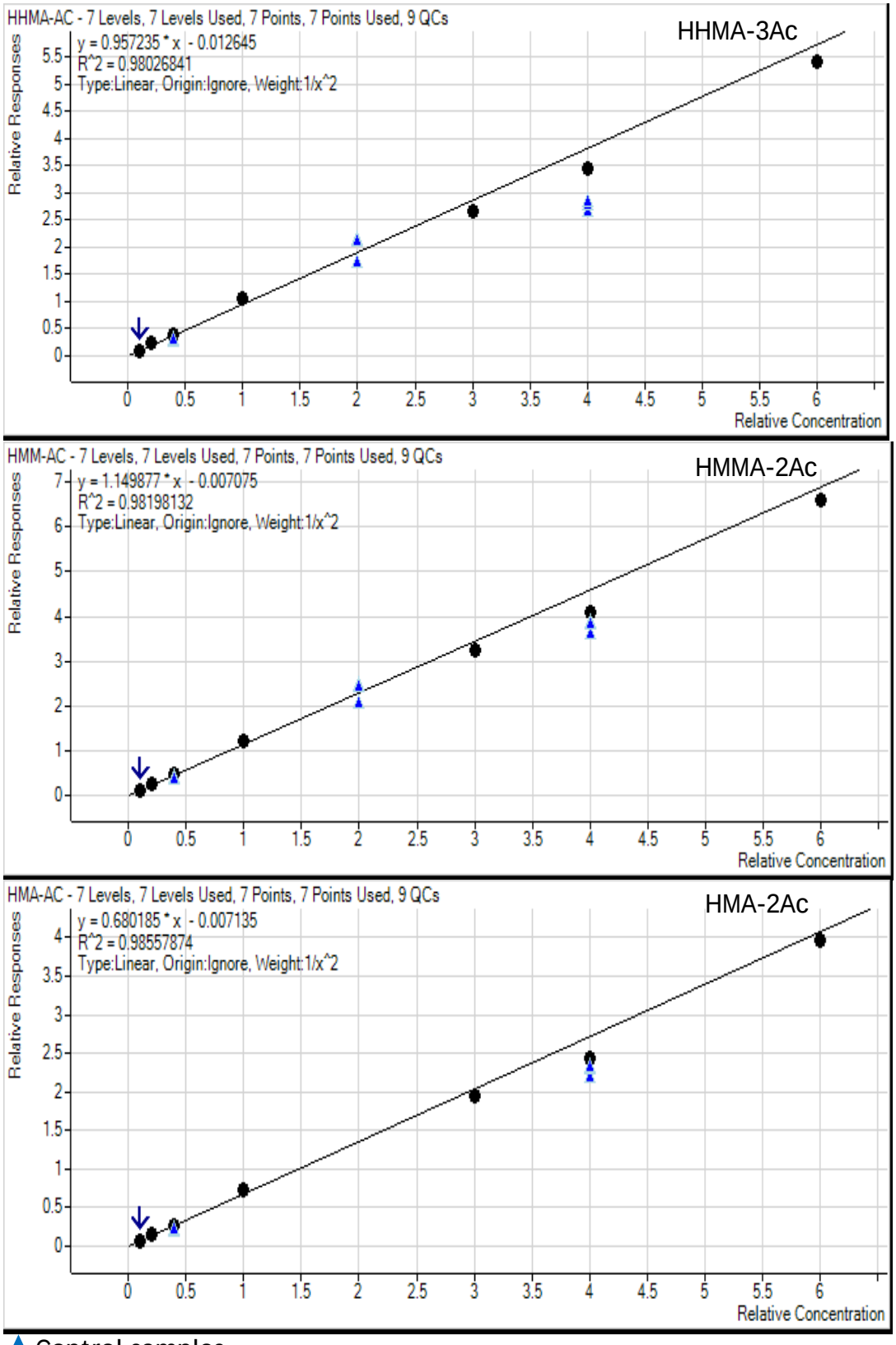


Figure 18-18 (cont.): Calibration curves (0.025-1.5 µg/ml) for LC/MS/MS analysis of acetate derivatives of MDMA and its metabolites in urine. LLOQs are indicated (arrowed). Concentrations of standards are 0.025, 0.050, 0.1, 0.25, 0.50, 0.75, 1 and 1.5 µg/ml.

8.7.2.3 Lower limit of quantitation

Calculation of the lower limit of quantification (LLOQ) has been carried out by different methods but in the present study the lower point of the calibration curve at 0.025 µg/ml was used as the LLOQ. It can be seen from Figure 8-19 that the signal-to-noise ratios at that concentration are higher than 10, the minimum value. The signal-to-noise (S/N) ratio of MDMA-Ac was 39, MDA-Ac was 34, HHMA-3Ac was 44, HMMA-2Ac was 64 and HMA-2Ac was 49, which suggest that the LLOQs are actually lower than 0.025 µg/ml. The LLOQs of the method were in the same range as in previously published methods[91,93]. However, the urine sample volume used in this method was significantly lower (400 µl) than in those studies. Based on the Substance Abuse and Mental Health Services Administration (SAMHSA) confirmatory test cut-offs at 250 ng/ml for MDMA and MDA in urine, the detection limits and the linearity ranges of this method are suitable for routine analysis of MDMA and its metabolites in urine samples.

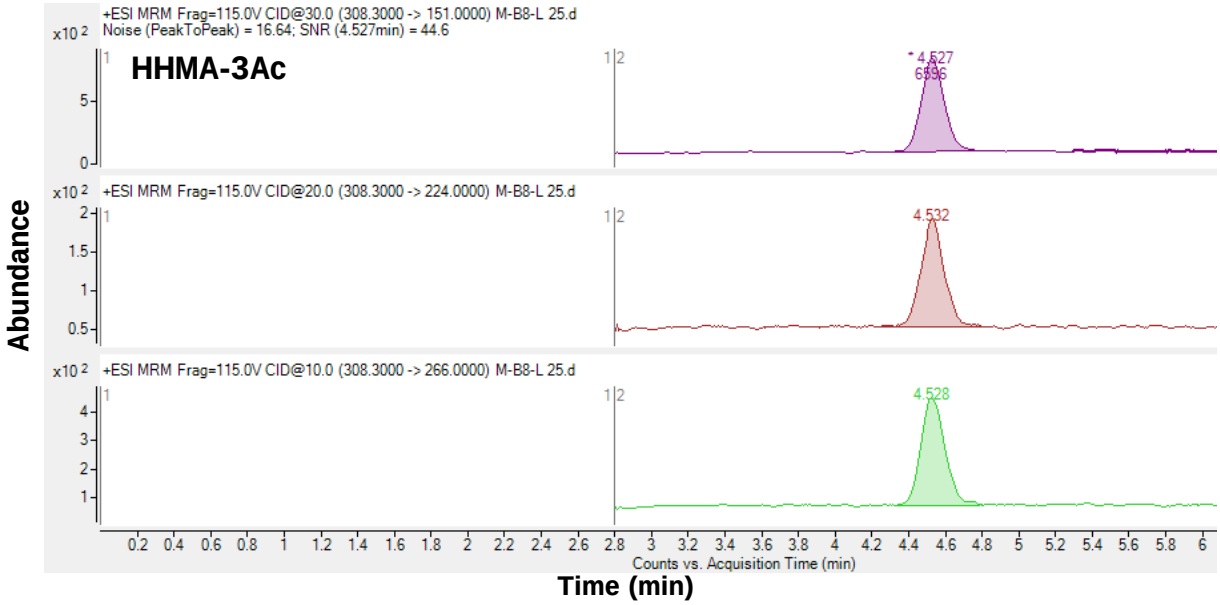


Figure 8-19: LC/MS/MS MRM chromatograms for the analysis of acetate derivatives of MDMA metabolites from urine at a concentration of 0.025 µg/ml (LLOQ). The S/N ratios are greater than 10 in each chromatogram.

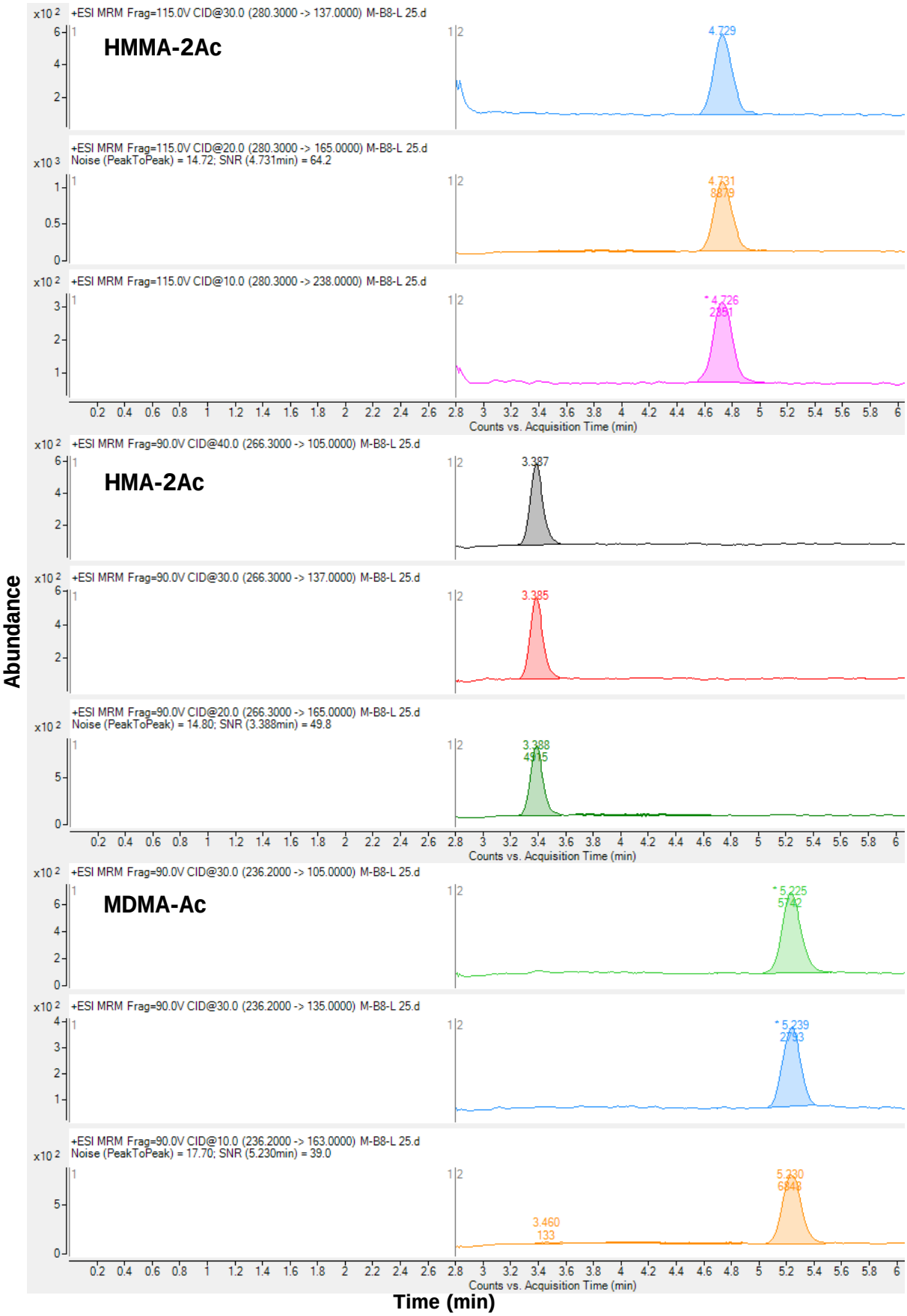


Figure 8-19 (cont.): LC/MS/MS MRM chromatograms for the analysis of acetate derivatives of MDMA metabolites from urine at a concentration of 0.025 µg/ml (LLOQ). The S/N ratios are greater than 10 in each chromatogram.

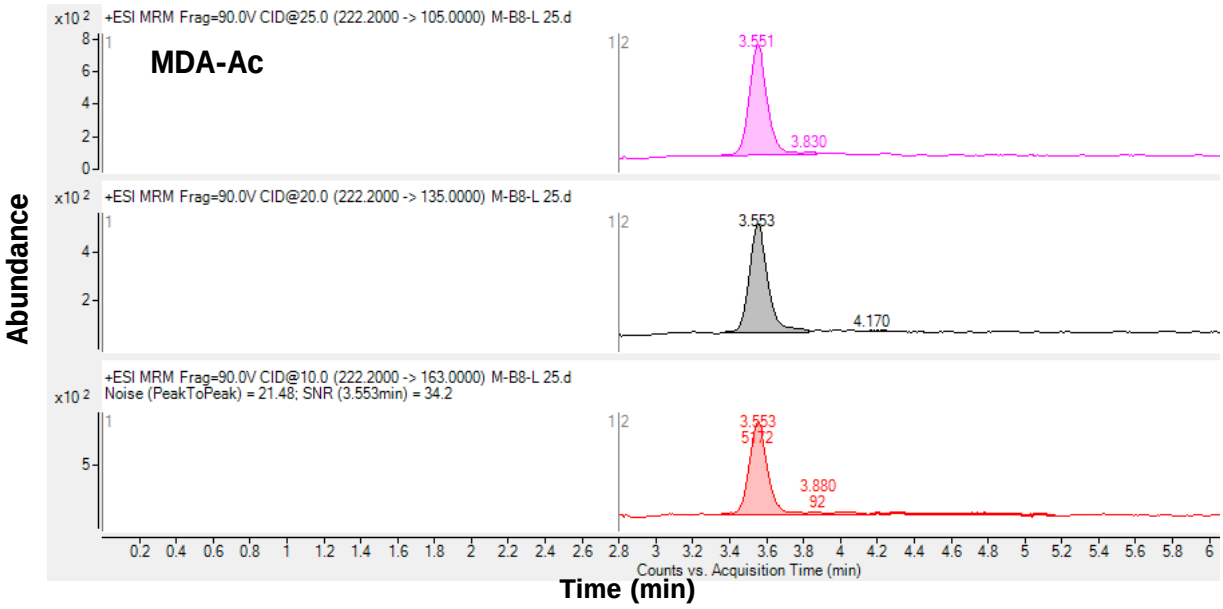


Figure 8-19 (cont.): LC/MS/MS MRM chromatograms for the analysis of acetate derivatives of MDMA metabolites from urine at a concentration of 0.025 µg/ml (LLOQ). The S/N ratios are greater than 10 in each chromatogram.

8.7.2.4 Accuracy and precision

Precision and accuracy for intra-day and inter-day assay were examined by analysing urine quality control samples (QCs) containing the analytes at three concentrations (LOW, MED and HIGH, *n* = 6 in each case). The measured concentrations of QCs were determined from daily calibration curves. The precision of the method was assessed as the percentage relative standard deviation (%RSD) and accuracy was assessed as the percentage deviation from the accepted reference value (%bias). Good values for intra-day precision (%RSD) were obtained for all analytes in the range of 0.8 to 5.7%, which were inside the acceptance criteria of ±15%. Values for inter-day precision (%RSD) were 6.1 to 18.5% and for accuracy (bias) were -14.5 to 17.2%. The higher values of %RSD and bias were due to the measured concentrations of HHMA-3AC and HMA-2Ac in MED QCs, that were above the acceptance criteria, while other QCs were inside the acceptance limits. However, the method was sufficiently accurate and precise for forensic toxicology analysis of MDMA and its metabolites in authentic urine samples. Inter-day precision and accuracy for all analytes are listed in Table 8-10.

Good precision and accuracy were obtained at the LLOQ for all analytes, in the range of 1.6 to 3.2 %RSD and -3.2 to -14.7 %bias respectively, which were inside the acceptance criteria of ± 20%. Accuracy and precision data for each compound at the LLOQs are listed in Table 8-11.

Table 8-10: Inter-day assay precision and accuracy for determination of MDMA and its metabolites in urine (*n* = 6).

Analyte	QC Sample	Expected Concentration, ng/ml	Observed Concentration, ng/ml	Accuracy (%Bias)	Precision (%RSD)
MDMA-Ac	LOW	100	97.4	-2.6	7.0
	MED	500	506	1.2	9.6
	HIGH	1000	1066	6.6	12.0
MDA-Ac	LOW	100	97.7	-2.3	7.3
	MED	500	514	2.8	9.5
	HIGH	1000	1089	8.9	13.5
HHMA-3Ac	LOW	100	92.7	-7.3	6.1
	MED	500	586	17.2	18.5
	HIGH	1000	885	-14.5	11.8
HMMA-2Ac	LOW	100	97.1	-2.9	6.9
	MED	500	567	13.4	14.9
	HIGH	1000	932	-6.8	10.0
HMA-2Ac	LOW	100	95.0	-5.0	9.8
	MED	500	581	16.3	14.6
	HIGH	1000	970	-3.0	11.6

Table 8-11: Inter-day assay accuracy and precision at the LLOQs (*n* = 6).

Analyte	Expected Concentration, ng/ml	Observed Concentration, ng/ml	Accuracy (%Bias)	Precision (%RSD)
MDMA-Ac	25	24.2	-3.2	1.9
MDA-Ac	25	24.1	-3.5	1.6
HHMA-3Ac	25	21.3	-14.7	3.2
HMMA-2Ac	25	21.7	-13.0	1.9
HMA-2Ac	25	22.2	-11.4	2.1

8.7.2.5 Matrix effects

The assessment of matrix effects showed the occurrence of ion suppression or ion enhancement of each analyte in extracts of urine from 5 different sources. A matrix effect value of <100% demonstrates ion suppression whereas a value of >100% indicates ion enhancement. The matrix slightly inhibited the ionisation of all derivatised analytes at low concentrations. However, no remarkable matrix effects were observed for any of the analytes at high concentrations. Matrix effects data are listed in Tables 8-12 and 8-13.

Table 8-12: Urine matrix effects at low analyte concentrations (0.1 µg/ml) as percent of signal obtained with pure reference standards.

Drug	Source 1	Source 2	Source 3	Source 4	Source 5	Mean	%CV
HHMA	98	91	98	87	66	88	13
HMMA	95	91	91	88	70	87	10
HMA	96	87	79	80	62	81	14
MDMA	106	87	91	85	68	88	14
MDA	105	90	82	89	61	86	17

Table 8-13: Urine matrix effects at high analyte concentrations (1 µg/ml) as percent of signal obtained with pure reference standards.

Drug	Source 1	Source 2	Source 3	Source 4	Source 5	Mean	%CV
HHMA	98	97	98	103	103	100	2
HMMA	99	98	97	102	102	100	2
HMA	99	98	90	100	103	98	4
MDMA	101	97	95	99	103	99	3
MDA	100	98	88	107	103	99	7

8.7.3 *In vitro* metabolism studies

Pooled human liver microsomes (HLM) were incubated with MDMA standard and extracted and analysed using the LC/MS/MS method described previously. 3,4-Dihydroxymethamphetamine (HHMA) and 3,4-methylenedioxyamphetamine

(MDA), the two key metabolites of MDMA, were expected to be produced by human liver microsomes in the *in vitro* metabolism procedure described earlier. Derivatives of standards were identified at the following retention times: MDMA-Ac at 4.7 min, HMMA-2Ac at 4.2 min, HHMA-3Ac at 4.0 min, MDA-Ac at 3.2 min and HMA-2Ac at 3.0 min. Slight variations in retention times occur in HPLC depending on a number of factors, for example, history of column use by other researchers and aging of mobile phase. Each analyte was confirmed by assessing the qualifier/quantifier ion ratios, with ± 20% tolerance compared to the un-extracted standards.

Three phase I metabolites (both major and minor metabolites) of MDMA were detected in HLM samples. The retention times and the mass ratios of MDMA and its metabolites obtained from HLM samples were consistent with those that were observed with the standards. MDMA derivative (MDMA-Ac) was eluted at 4.7 min and was identified and confirmed by quantifier and qualifier ions at *m/z* 236>163, 236>135 and 236>105. The qualifier/quantifier ion ratio was 39% for *m/z* 135 and was 73% for *m/z* 105 which were in the acceptable range, see Figure 8-20.

3,4-Dihydroxymethamphetamine derivative (HHMA-3Ac) was eluted at 4.0 min and was identified and confirmed by MRM transitions at *m/z* 308>266, 308>224 and 308>151. The ratio of qualifier/quantifier ions was 55% for *m/z* 266 and 21% for *m/z* 224 which were in the acceptable range.

3,4-Methylenedioxyamphetamine derivative (MDA-Ac) was eluted at 3.2 min and was identified and confirmed by MRM transitions at *m/z* 222>163, 222>135 and 222>105. The ratio of qualifier/quantifier ions was 54% for *m/z* 135 and was 83% for *m/z* 105 which were in the acceptable range.

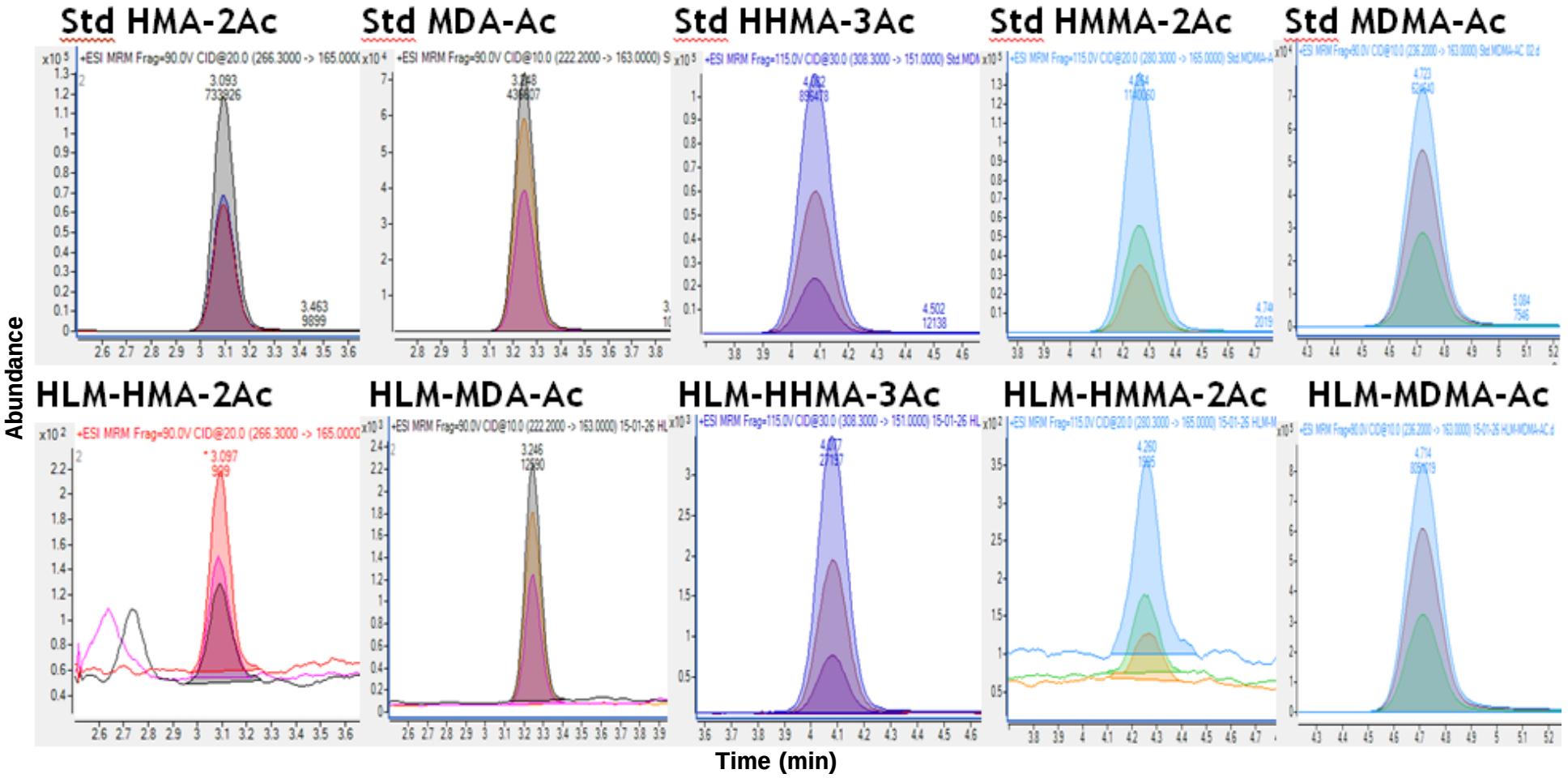


Figure 8-20: LC/MS/MS MRM chromatograms of MDMA metabolites in HLM samples compared with Ac-derivatives of reference standards.

A small peak of 4-hydroxy-3-methoxymethamphetamine derivative (HMMA-2Ac) was detected in HLM samples at 4.2 min and was identified and confirmed by MRM transitions at *m/z* 280>238, 280>165 and 280>137. The ratio of qualifier/ quantifier ion was 23% for *m/z* 238 which was in the acceptable range. However, the ratio of qualifier/quantifier ion for *m/z* 137 was 37%, which was out of the acceptable range (±20%) probably because the chromatographic peaks corresponding to HMMA-2Ac were small in size, which can affect the ion ratios.

A small peak of 4-hydroxy-3-methoxyamphetamine derivative (HMA-2Ac) was detected in HLM samples at 3.0 min and was identified and confirmed by MRM transitions at *m/z* 266>105, 266>137 and 266>165. The ratio of qualifier/ quantifier ions was 57% for *m/z* 137 and was 63% for *m/z* 105 which were in the acceptable range. LC/MS MRM chromatograms of MDMA metabolites in an HLM sample are shown in Figure 8-21.

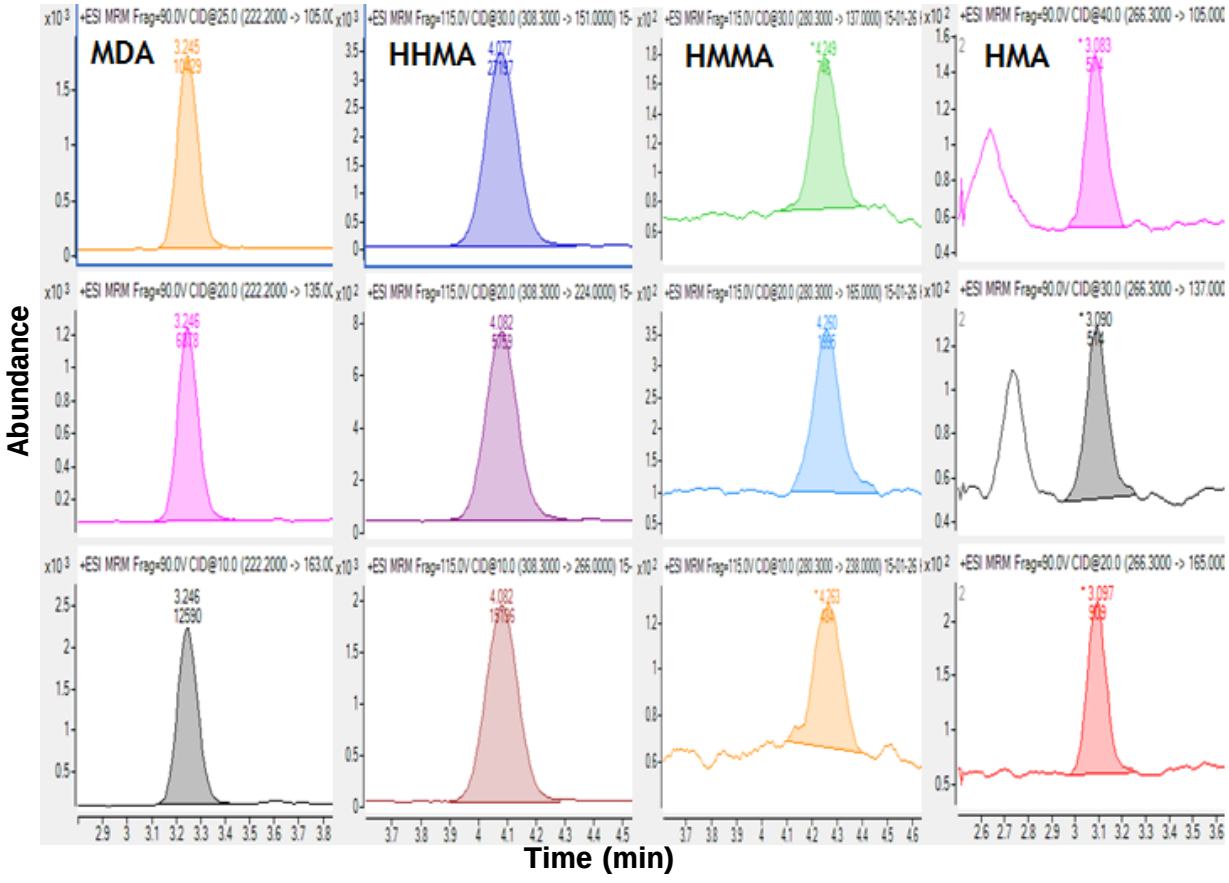


Figure 8-21: LC/MS MRM chromatograms of MDMA metabolites in an HLM sample.

In vitro results have shown that HHMA appears to be a major MDMA metabolite in HLM samples whereas MDA was found as a minor metabolite. This result is in agreement with previously published data about MDMA metabolites in humans and confirmed that HHMA is an intermediate metabolite of MDMA in humans[90,91,162,164,170].

Because HMMA and HMA are produced from HHMA and MDA, small peaks of HMMA and HMA were also found in HLM samples. Finally, this experiment confirmed that human liver microsomes can be used to simulate drug metabolism in humans.

8.7.4 *In vivo* metabolism studies

8.7.4.1 Non-hydrolysed urine analysis

Ten positive human urine samples obtained from Glasgow Royal Infirmary for routine toxicology service analysis were analysed for MDMA and its metabolites (MDA, HHMA, HMMA and HMA) using the method described previously without a hydrolysis procedure in the sample preparation step. MDMA, MDA, HHMA and HMMA were detected in all samples. The concentrations were reported as µg/ml urine. Concentrations outside the calibration range were estimated by extrapolation and are reported here for research comparison purposes only. Concentrations of MDMA and its metabolites in non-hydrolysed urine samples are summarised in Table 8-14.

MDMA, MDA, HHMA and HMMA concentrations were found in the ranges 9.2-89.2 µg/ml, 0.23-5.4 µg/ml, 0.12-2.90 µg/ml and 0.15-4.25 µg/ml, respectively. HMA was found at concentrations greater than the method LLOQ in only one of ten non-hydrolysed urine specimens (U-01) and at concentrations lower than the calibration range in six of ten non-hydrolysed urine samples.

The concentrations of free HHMA were found to be higher than MDA in three samples (U-02, U-03 and U-08) and the concentrations of free-HMMA were also found to be higher than MDA in three samples (U-02, U-07 and U-08). Moreover,

the concentration of MDA was found to be lower than the combined concentrations of free HHMA and free HMMA in seven samples.

Table 8-14: Concentrations of analytes in non-hydrolysed urine samples (µg/ml).

Name	HMA-2Ac	MDA-Ac	HHMA-3Ac	HMMA-2Ac	MDMA-Ac**
U-01	0.052	5.41**	2.32**	1.39	76.1
U-02	<0.025	0.67	2.90**	4.25**	45.4
U-03	ND*	0.60	0.65	0.47	89.2
U-04	<0.025	1.27	0.49	0.53	21.5
U-05	ND	0.23	0.21	0.23	9.20
U-06	<0.025	1.68**	1.24	1.11	53.2
U-07	<0.025	0.32	0.16	1.84**	14.2
U-08	<0.025	0.35	1.46	1.38	12.6
U-09	ND	0.51	0.12	0.15	10.8
U-10	<0.025	1.52	1.22	0.87	69.5

*ND = not detected
**Concentrations outside the calibration range were estimated by extrapolation for research comparison purposes only.

The concentrations of MDA, free HHMA and free HMMA in ten urine samples were found to be within the expected ranges with the exception of U-01 and U-02, which had concentrations of MDA and HMMA which were higher than the expected ranges. Moreover, the concentration of free HMA was found to be lower than expected and in all samples MDMA concentrations were higher than the calibration range.

8.7.4.2 Hydrolysed urine analysis

Some of the MDMA metabolites, such as HHMA, HMMA and HMA, were expected to be excreted in conjugated forms (mostly glucuronide or sulphate conjugates). To evaluate the conjugated forms of MDMA metabolites, a hydrolysis procedure was required in order to release HHMA, HMMA and HMA from their conjugates. Ten positive human urine samples, the same samples as used in Section 8.7.4.1, were analysed for MDMA and its metabolites (MDA, HHMA, HMMA and HMA) using the method described previously with the addition of an enzymatic hydrolysis

method in the sample preparation step. Total MDMA, MDA, HHMA, HMMA and HMA concentrations (µg/ml) in hydrolysed urine samples are summarised in Table 8-15. Concentrations outside the calibration range were estimated by extrapolation and are reported here for research comparison purposes only.

Total MDMA, MDA, HHMA, HMMA and HMA concentrations were found in the ranges 8.8-87.3 µg/ml, 0.20-5.47 µg/ml, 0.16-2.91 µg/ml, 0.56-5.27 µg/ml and 0.025-0.27 µg/ml, respectively. HMA was found at concentrations greater than the method LLOQ in six of ten hydrolysed samples (U-01, U-02, U-04, U-06, U-08 and U-10) and at concentrations lower than the calibration range in the other urine samples.

The concentrations of total HMMA were found to be higher than MDA in eight samples (U-02, U-03, U-05, U-06, U-07, U-08, U-09 and U-10). Moreover, the concentration of MDA was found to be lower than the combined concentrations of total HHMA and HMMA in all samples, suggesting that HHMA and HMMA are major metabolites of MDMA in human.

Table 8-15: Concentration of analytes in hydrolysed urine samples (µg/ml).

Name	HMA-2Ac	MDA-Ac	HHMA-3Ac	HMMA-2Ac	MDMA-Ac**
U-01	0.269	5.47**	2.91**	3.19**	67.5
U-02	0.044	0.77	2.22**	5.27**	43.6
U-03	<0.025	0.52	0.72	0.93	87.3
U-04	0.063	1.21	0.46	0.87	20.8
U-05	<0.025	0.20	0.57	0.56	8.84
U-06	0.078	1.48	1.38	4.12**	59.7
U-07	<0.025	0.33	0.16	1.81**	14.7
U-08	0.053	0.48	0.95	1.73	12.3
U-09	<0.025	0.34	0.24	0.96	11.4
U-10	0.025	1.13	0.92	1.60**	74.0

**Concentrations outside the calibration range were estimated by extrapolation for research comparison purposes only.

Concentrations of MDA, total HHMA and total HMMA in ten urine samples were found to be within the expected range with the exception of U-01, which had high concentrations of MDA, HHMA and HMMA, U-02 which had high concentrations of HMMA and HHMA and U-06 which had a high concentration of HMMA above the calibration range. Moreover, the concentration of MDMA in all urine samples was found to be higher than the expected range while the concentration of total HMA was found to be lower than expected.

No information was available concerning the dose(s) or time(s) of MDMA administration in these cases, although given the circumstances that the patients were admitted to the hospital emergency department because of acute intoxication, it is likely that the samples were collected proximal to the last administration of MDMA. In each case, MDMA was the main analyte detected, with the maximum metabolite/MDMA ratio being 0.14 for HMMA in case U-08 and the ratio of free (MDA+HHMA+HMMA+HMA)/MDMA ranging from 0.02-0.26. These findings contrast with results published by Segura *et al.* who found that, after a single administration of 100 mg MDMA to four volunteers, both HMMA and HHMA exceeded MDMA in urine at all intervals up to 24 h after administration[91]. The percentages of administered dose excreted over the first 24 h in the form of MDMA, MDA, HMMA and HHMA were 15.0%, 1.5%, 22.7% and 17.7% respectively. However, no data is given for the ratios of metabolites to MDMA after the first 24 h.

One possible explanation for the differences between this study and that of Segura *et al.*[91] is that repeated or multiple doses were taken by the users in the present study. Metabolite/MDMA ratios may decrease following administration of more than one dosing. Farré *et al.*[171] found that metabolic ratios in plasma of MDA/MDMA, (MDA+HMA+HMMA)/MDMA, (HMMA+HMA)/(MDA+MDMA) and (HMA+HMMA)/MDMA after a single dose were 0.05, 1.67, 1.54 and 1.52 but after repeated dose were 0.05, 0.86, 0.77 and 0.81, respectively. The lower ratios after the second dose were due to lower conversion of MDMA to HMMA and HMA, which was attributed to autoinhibition of CYP2D6 by MDMA. However, Farré's ratios were for plasma and may not be reliable indicators of ratios in urine.

An alternative explanation of the differences is that the method used in this study is different from that of Segura *et al.*[91] and resulted in different relative amounts of MDMA and its metabolites, for example as a result of differences in the efficiency of the hydrolysis procedure. In the absence of commercially-available standards of metabolite conjugates, this would need to be resolved by comparative analysis of samples by the two methods.

8.7.4.3 Comparison of hydrolysed and non-hydrolysed urine samples

The same urine samples were analysed before and after hydrolysis, allowing direct comparison of analyte concentrations under the two conditions. As shown in Table 8-16, the urinary concentrations of HMMA and HMA dramatically increased after enzymatic hydrolysis using the *Β*-glucuronidase and sulphatase derived from *Helix pomatia*, see Figure 8-22. Compared to non-hydrolysed urine samples, HMMA and HMA concentrations were significantly higher (p<0.05) after enzymatic hydrolysis (139% ± 84.5% and 783% ± 112%, respectively). There were no significant differences in MDMA, MDA and HHMA concentrations between non-hydrolysed and hydrolysed samples. The differences in concentrations of MDMA and its metabolites found in this study between hydrolysed and non-hydrolysed urine samples are in agreement with previously reported data[158].

Pirnay *et al.*[158] found that HMMA and HMA recoveries were significantly increased by 4768% ± 1128% and 1310% ± 366% following acid hydrolysis (n = 5). MDA recoveries were significantly increased by *E coli Β*-glucuronidase by 33.0% ± 8.4% whereas no significant differences were detected for either MDMA or MDA between non-hydrolysed urine samples and acid and *H. pomatia* enzymatically hydrolysed specimens. Acid hydrolysis was recommended in that study as the hydrolysis method to evaluate HMMA, HMA and MDA in urine samples based on recoveries, time efficiency, and cost.

Table 8-16: Percentage differences in analyte concentrations between hydrolysed and non-hydrolysed urine samples (µg/ml).

Sample	% differences in analyte concentrations between hydrolysed and non-hydrolysed urine samples				
	HMA-2Ac	**MDA-Ac**	**HHMA-3Ac**	**HMMA-2Ac**	**MDMA-Ac**
U-01	416	1.01	25.7	130	-11.3
U-02	264	14.1	-23.3	23.9	-4.05
U-03	0	-13.7	10.5	98.7	-2.15
U-04	245	-4.26	-6.3	64.89	-3.55
U-05	0	-9.94	166	144	-3.98
U-06	1790	-11.7	12.0	271	12.1
U-07	350	1.04	1.24	-1.55	3.01
U-08	520	38.3	-35.1	24.9	-2.57
U-09	0	-34.1	92.8	551	5.27
U-10	1580	-25.6	-24.3	85.1	6.47
Mean	**738%**	**-4.49%**	**22.0%**	**139.2%**	**-0.08%**

Mueller *et al.*[162] found that recoveries of HHMA and HMMA after acid hydrolysis were significantly higher compared to enzymatic hydrolysis (glucuronidase and sulphatase). They also suggested that acid hydrolysis proved less efficient in cleaving glucuronides than sulphates whereas enzymatic hydrolysis proved less efficient in cleaving sulphates than glucuronides.

Schwaninger *et al.*[21] reported that HHMA, HHMA-sulphates, HMMA, HMMA-sulphates and HMMA-glucuronides were excreted in human urine. Moreover, Shima *et al.*[160,172] found that in phase II metabolism of MDMA, sulphation is quantitatively more significant than glucuronidation for HMMA in humans.

Enzymatic hydrolysis in this study used *β*-glucuronidase from *Helix pomatia* which contained ≥100,000 units/ml of *β*-glucuronidase and ≤7,400 units/ml of sulphfatase. The results showed no significant differences in HHMA concentrations between non-hydrolysed and *H. pomatia* enzymatically hydrolysed samples. One possible explanation for this is that sulphation of HHMA may predominate in humans in phase II metabolism.

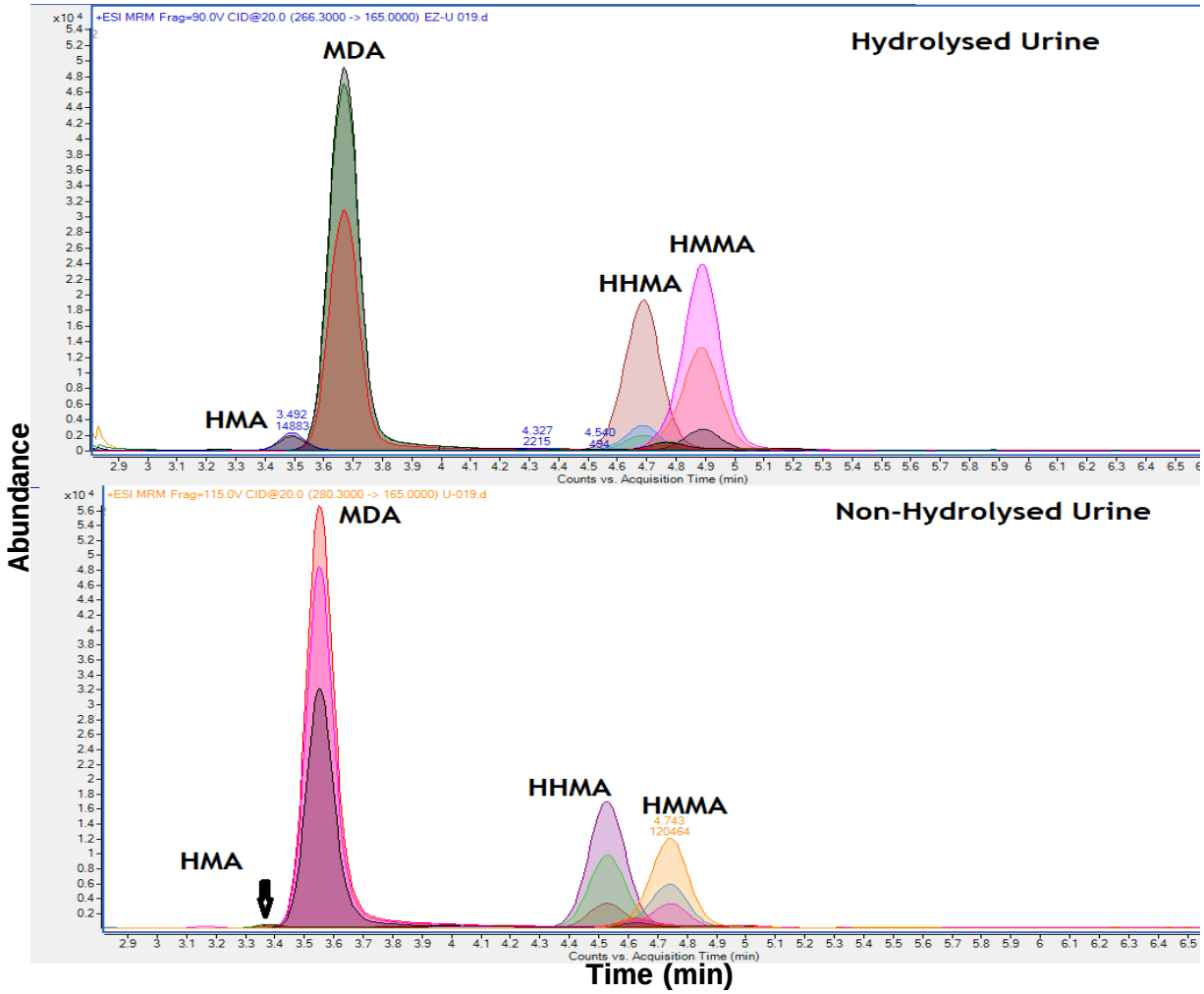


Figure 8-22: LC/MS/MS MRM chromatograms of hydrolysed and non-hydrolysed urine, showing increased concentrations of free HMA and HMMA after hydrolysis.

8.7.5 Post mortem case samples

Three urine samples which were known to be positive for MDMA by ELISA screening were obtained from Forensic Medicine and Science, University of Glasgow as part of the investigation of post mortem medico-legal cases. All samples were analysed for MDMA and their metabolites in un-hydrolysed urine using the LC/MS method described above. Concentrations outside the calibration range were estimated by extrapolation and are reported here for research comparison purposes only.

MDMA and its metabolites were detected in all case samples (see Table 8-17). MDMA, MDA, HHMA and HMMA concentrations were found in the ranges 9.13-32.6 µg/ml, 0.20-1.25 µg/ml, 0.55-1.50 µg/ml and 0.29-0.50 µg/ml, respectively. The concentrations of MDMA in the urine samples were found to be higher than the

calibration range. However, the concentrations of HHMA, HMMA and MDA in urine were found to be within the expected ranges (see Figure 8-23).

Table 8-17: Concentrations of MDMA and its metabolites in non-hydrolysed urine from MDMA-positive post mortem cases.

Sample	MDMA** (µg/ml)	MDA (µg/ml)	HHMA (µg/ml)	HMMA (µg/ml)	HMA (µg/ml)
Case 1	9.13	0.20	0.90	0.50	<0.025
Case 2	32.6	1.25	1.50	0.38	<0.025
Case 3	23.6	0.72	0.55	0.29	ND

*ND = not detected
**Concentrations outside the calibration range were estimated by extrapolation for research comparison purposes only.

As noted earlier for hospital samples (Section 8.7.4.2), MDMA was present in these post mortem urine samples at much higher concentrations than any of its metabolites, in contrast to the findings of Segura *et al.* [91] However, the ratio of MDA/MDMA in the present study, in both clinical and post mortem cases is in agreement with those reported by Segura *et al.*[91] and others, for example, Kunsman *et al*[148] reported MDMA and MDA concentrations in randomly collected urine specimens from active-duty military members ranging from 0.38 to 96.2 µg/ml and from 0.15 to 8.6 µg/ml respectively. The ratio of MDA/MDMA in urine is therefore not reliable as an indicator of an acute sudden death due to administration of MDMA.

The method developed and validated in this chapter was successfully applied to routine post mortem case samples. However, dilution of samples or a higher calibration range should be considered for MDMA.

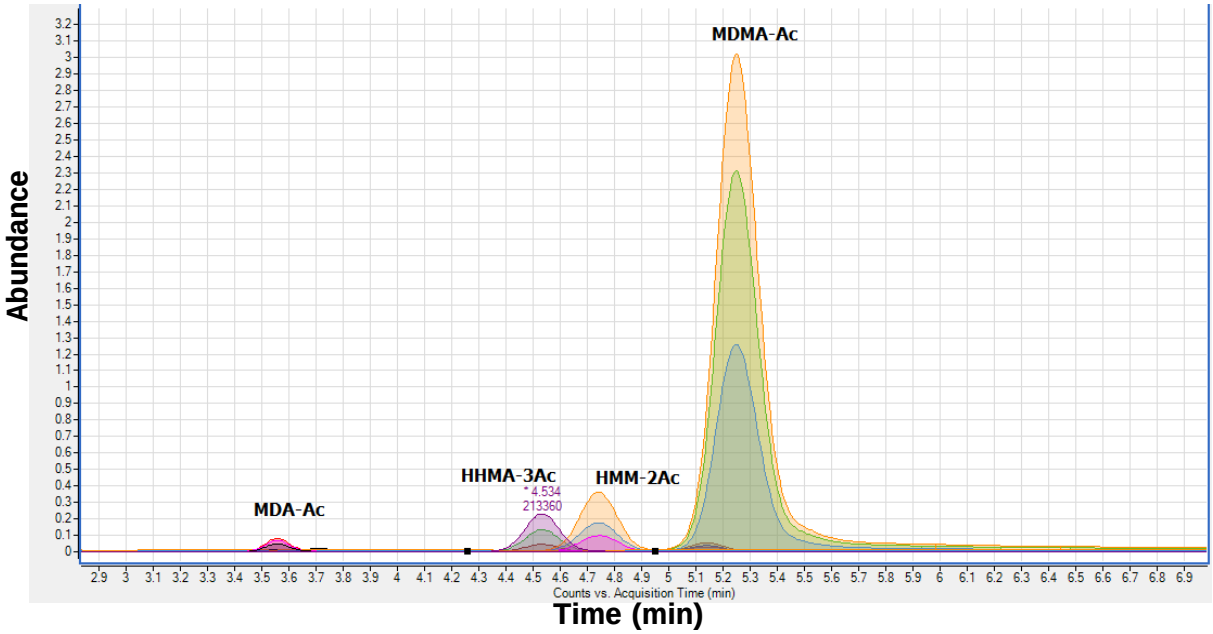


Figure 8-23: LC/MS/MS chromatograms of acetate derivatives of MDMA and its metabolites extracted from a post mortem human urine case sample.

8.8 Conclusions

This study presents a description of an *in vitro* metabolism study of MDMA using human liver microsomes (HLM) and a comparison of the metabolite pattern with that found in human urine. The study has confirmed that HLM can be used to simulate drug metabolism in humans and to provide chromatographic and mass spectrometric data on metabolites. Protein precipitation plus derivatisation coupled with LC/MS/MS provides a highly sensitive and selective assay procedure for detecting the dihydroxy metabolite of MDMA (HHMA). An analytical method was developed for MDMA and its metabolites in urine and has shown sufficient specificity by removing interfering compounds, including endogenous compounds. The method was validated and achieved an adequate sensitivity for analysis of MDMA and its metabolites in urine samples. Moreover, the method has shown adequate accuracy and precision for use in routine analysis. A standard enzymatic hydrolysis procedure using *β*-glucuronidase/sulfatase from *Helix pomatia* was successful for the recovery of total HMMA and total HMA form urine samples.

In summary, the results show that HHMA and HMMA are major phase I metabolites of MDMA in humans. HHMA has a high reactivity due to its chemical

structure. However, chemical derivatisation can decrease the polarities of hydrophilic metabolites and improve their analysis on conventional reversed phase C18 columns. Acetate derivatives were stable in LC mobile phase up to 30 h. Finally, this method was found to be suitable for application as a routine quantitative method for MDMA and its metabolites in both clinical and post mortem urine samples.

Chapter 9 *In vitro* metabolism studies on methylenedioxy-substituted amphetamines using human liver microsomes and LC/MS/MS with chemical derivatisation

9.1 Introduction

The chemistry and pharmacology of selected phenethylamine derivatives were covered in Chapter 7. Analysis of cathinones such as methylone and butylone in biological specimens is not a routine procedure in forensic toxicology laboratories. Only a few laboratories have identification methods for the detection of methylone and butylone but these do not cover their metabolites. Kamata *et al.*[125] reported that methylone metabolite (HMMC) was detected in rat urine samples longer than methylone after a single dose of 5.0 mg/kg. HMMC was detectable up to 48 h post-dosing whereas methylone was detectable only up to 36 h post-dosing. This result has shown that analysis of HMMC in urine specimens can extend the detectable time of positive urine.

9.2 Aims

Based on the knowledge of the toxicity of MDMA and its metabolites, previous studies[120,121,173] reported that *β*-keto amphetamines (cathinones) have similar neurotoxicity to MDMA. Limited data are available on the metabolism of methylone and butylone. A significant problem for the forensic toxicologist is a lack of information on the pharmacology and toxicology of NPS and a lack of reference standards of the metabolites and even of the parent drugs themselves. Ethical considerations preclude administration of NPS to human subjects, so any advance in the elucidation of NPS metabolism in humans will depend on the availability of alternative methods that can simulate human metabolic pathways. *In vitro* studies with human liver microsomes provide one potential alternative approach to metabolism studies. Furthermore, some phase I metabolites such as dihydroxy-metabolites (HHMA or HHMC) have a high polarity,

basicity and high water solubility. As noted in Chapter 8, an additional challenge to the forensic toxicologist is the analysis of these polar metabolites using conventional C18 HPLC columns, which result in low retention volumes and poor resolution of metabolites. Conversion of metabolites to more hydrophobic compounds by derivatisation has the potential to improve separations by reversed-phase HPLC and increase sensitivity in mass spectrometry, because mobile phases with a higher organic solvent content can be used. The need for a sensitive technique for detection and identification of methylone, butylone and their metabolites is essential.

The initial aim of this work was to apply the *in vitro* method described in Chapter 8 to simulate the human metabolic pathways of methylendioxy-substituted cathinones, using methylone and butylone as two representative examples of this type of drug, see Figure 9-1. The second part was aimed to develop a reliable, sensitive, and selective method for identification of methylone, butylone and their metabolites using reversed-phase LC/MS/MS.

9.3 Review of previous analytical methods for the determination of methylone, butylone and their metabolites in biological samples

Only a few analytical methods have been described and used in forensic toxicology for the quantitation of methylone and butylone and their metabolites in biological samples and these are reviewed below and summarised in Table 9-1.

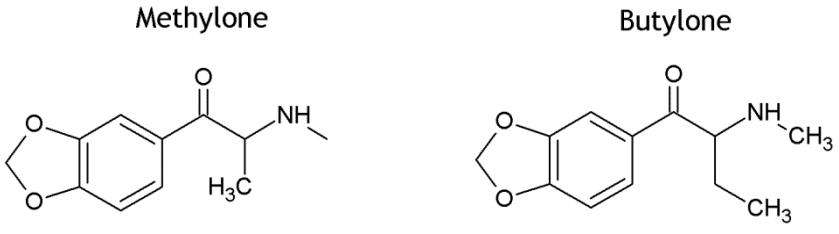


Figure 9-1: Chemical structures of methylone and butylone.

Table 9-1: Previous GC/MS and LC/MS methods for the analysis of methylone, butylone and their metabolites in biological samples.

Analytes	Samples	Chromatography	Extraction Method	Year
MDMC, MDC, HMMC, 3-OH-4-MeO-MC	Human & rat urine	**GC/MS** Column: DB-5MS capillary (30 m x 0.25 mm i.d. x 0.25 µm) **LC/MS** Column: L-column ODS (1.5 mm x 150 mm) Mobile phase: 10 mM ammonium formate buffer pH 3.5 and methanol in gradient mode	Acid hydrolysis LLE: chloroform: 2-propanol (3:1, v/v) Derivatised with TFA	2006[125]
bk-MBDB bk-BDB ß-OH-MBDB 4-OH-3-MeO-Met.	Urine	**GC/MS** Column: DB-5MS capillary (30 m x 0.25 mm i.d. x 0.25 µm) **LC/MS** Column: L-column ODS2 (1.5 mm x 150 mm, 5 µm) Mobile phase: 10 mM ammonium formate buffer pH 3.5 and methanol in gradient mode; flow Rate: 0.1 ml/min	**GC/MS** Acid hydrolysis LLE: CH_2Cl_2 /IPA (4:1) Derivatised with TFA **LC/MS** Acid hydrolysis Protein precipitation with DBA MeOH	2009[138]
MDMC, bk-MBDB MDC, bk-BDB HMMC, HMC 4-OH-3-MeO-Met. Di-OH butylone Met	Human & rat urine	**GC/MS** Column: HP-1 capillary (12 m x 0.2 mm i.d. x 0.33 µm)	Enzymatic hydrolysis SPE: Isolute HCX Derivatised with Ac and/or Diazomethane	2010[126]
MDMC, MDC Dihydro-methylone bk-MBDB bk-BDB, OH-MBDB	HLM	**LC/MS** Column: Betasil phenyl/ hexyl (3 mm x 100 mm, 3 µm) Mobile phase: 5 mM ammonium acetate + 0.1% formic acid in water and in 5 mM ammonium acetate + 0.5% formic acid methanol	HLM Incubation	2012[127]
MDMC, MDC, HHMC, *N*-OH-Met., Dihydro-Met.	HLM, human liver S9	**UHPLC-MS/MS** Column: UHPLC BEH C18 (2.1 mm x 100 mm, 1.7 µm) Mobile phase: acidic water and acetonitrile + 0.05% formic acid Flow Rate: 0.6 ml/min	HLM Incubation	2013[128]

Table 9-1 (cont.): Previous GC/MS and LC/MS methods for the analysis of methylone, butylone and their metabolites in biological samples.

Analytes	Samples	Chromatography	Extraction Method	Year
MDMC, MDC, HMMC, 3-OH-4-MeO-MC, 3-OH-MDMC	Rat blood	**LC-MS** Column: Luna HST C18 (2 mm x 100 mm, 2.5 µm) Mobile phase: 0.1% formic acid in water and MeOH in gradient mode Flow Rate: 0.15 ml/min	Protein precipitation with methanol	2013[174]
MDMC, MDC, HHMC, HMMC	Rat & human plasma	**LC/MS** Column: Synergi Polar-RP (2 mm x 100 mm, 2.5 µm) Mobile phase: 0.1% formic acid in water and acetonitrile in gradient mode Flow Rate: 0.3 ml/min	Enzymatic hydrolysis Protein precipitation: Perchloric acid SPE: SOLA SCX	2015[130]
MDMC, 3-OH-4-MeO-MC	RLM S9	**LC/MS** Column: Gold ODS (2.1 mm x 150 mm, 5 µm) Mobile phase: 10 mM ammonium acetate + 0.1% acetic acid in water and methanol in gradient mode Flow Rate: 0.2 ml/min	RLM Incubation	2015[129]

Met = metabolite

Metabolites of methylone in humans and rats were reported and published in 2006 by Kamata *et al.*[125] using GC/MS with TFA derivatisation and LC-ESI/MS. Methylone, 3-hydroxy-4-methoxymethcathinone (3-OH-4-MeO-MC), 4-hydroxy-3-methoxymethcathinone (HMMC) and 3,4-methylenedioxycathinone (MDC) were synthesised and used as analytical standards. Acid hydrolysis was used as a sample preparation method for urine samples for LC-ESI/MS and the extracts were then derivatised with TFA for GC/MS. The GC/MS method was used to differentiate and identify methylone and its metabolites. The ion at *m/z* 149 and the molecular ion (M^+) were selected to identify methylone and MDC and ions at *m/z* 154 and 247 were selected for HMMC and 3-OH-4-MeO-MC. LC-ESI/MS was additionally performed for the confirmation of methylone and its metabolites detected by GC/MS. Protonated molecules with $M\text{-}H^+$ at *m/z* 194 for MDC, 208 for methylone, 210 for HMMC and 3-OH-4-MeO-MC were selected for LC/MS.

Metabolites of butylone (bk-MBDB) in human urine were reported and published in 2009 by Zaitsu *et al.*[138] Butylone, nor-butylone (bk-BDB), bk-MBDB-M1 (4-OH-3-MeO metabolite), bk-MBDB-M2 (3-OH-4-MeO metabolite), and *β*-OH-MBDB were synthesised and used as analytical standards. Acid hydrolysis and derivatisation was used as a sample preparation method for GC/MS. Analytes were separated using a fused-silica capillary column DB-5MS and detected with MS in full scan mode over the range from *m/z* 40-500. For LC/MS, deproteinisation with dibenzylamine methanol was used as a sample preparation for un-hydrolysed and hydrolysed urine. Analytes were separated using an L-column ODS2 semi micro column and detected in full scan mode. All butylone metabolites (bk-BDB, bk-MBDB-M1, bk-MBDB-M2 and *β*-OH-MBDB) in human urine samples were identified by GC/MS and confirmed with LC/MS.

Mayer *et al.*[126] described a detection method for mephedrone, butylone and methylone and their metabolites in human and rat urine using GC/MS. Urine samples were hydrolysed with a mixture of glucuronidase and arylsulfatase from *Helix pomatia* before being extracted by solid phase extraction using Isolute Confirm HCX cartridges. Analytes were eluted in two separate fractions: a non-basic compounds fraction was eluted with methanol and a basic compounds fraction was eluted with a freshly prepared mixture of methanol: aqueous ammonia (98:2, v/v). Both fractions were derivatised by methylation with

diazomethane in diethyl ether, acetylation with acetic anhydride: pyridine (3:2, v/v), or combined methylation and acetylation. Analytes were separated using a cross-linked dimethyl silicone capillary column HP-1 (12 m x 0.2 mm i.d., 0.33 µm phase thickness) and detected with MS in full scan mode over the mass range *m/z* 50-500 and in the SIM mode. Ions at *m/z* 149, 114, 72, and 58 were used to identify butylone and its metabolites and ions at *m/z* 149, 100, 86, and 58 were used to identify methylone and its metabolites.

In 2012, Mueller and Rentsch[127] described an offline and automated online metabolism methods using HLM applied to 11 cathinones with analysis by LC/MS(n). The offline method consisted of 500 pmol of total CYP substances diluted in phosphate buffer pH 7.4 and NADPH generating system. The mixture was incubated at 37°C for 3 h and the reaction was stopped by adding ice-cold acetonitrile. The online method was performed using an HTC PAL autosampler. The incubation mixture was introduced into a 200 µl injection loop and heated to 37°C using the column oven for 50 min. After incubation, the mixture was transferred to the online extraction columns using Cyclone® and C18 XL columns under alkaline conditions. Analytes were separated using a Betasil phenyl/ hexyl column (3 mm x 100 mm, 3 µm) and identified using their MS^2 and MS^3 mass spectra and neutral-loss scans.

Pederson *et al.*[128] described a method for the identification of methylone and its metabolites using *in vitro* studies. Various recombination enzymes (P450 CYP: 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5 and supermix) and different liver fractions (UltraPool HLM and UltraPool human liver S9) were applied to the incubation mixture which was incubated at 37°C for 140 min. The reaction was stopped with ice-cold acidic acetonitrile containing 0.5% formic acid and the supernatant was analysed directly by UPLC-TOF/MS. HHMC was synthesised and characterised using ^{1}H-NMR before being applied as a reference standard. Analytes were separated using a UHPLC BEH C18 column (2.1 mm x 100 mm, 1.7 µm), identified using UHPLC-TOF/MS in positive ion scan mode and quantified using UPLC-MS/MS with two multiple-reaction-monitoring (MRM) transitions. Methylone was determined using the 208>160 (quantifier) and 208>132 transitions and HHMC using the *m/z* 196>160 (quantifier) and 196>132 transitions.

In 2015, Ellefsen *et al.*[130] described the quantitative analysis of methylone and its metabolites in rat and human plasma using LC/MS. Samples were hydrolysed with *β*-glucuronidase before extraction by protein precipitation. Analytes were separated using a Phenomenex Synergi Polar-RP column (2.0 mm x 100 mm, 2.5 µm) and quantitated with a 3200 QTRAP mass spectrometer. Methylone was determined with the *m/z* 208>160 (quantifier) and 208>132 transitions, MDC with the *m/z* 164>146 (quantifier) and 194>118 transitions, HHMC with the *m/z* 196>160 (quantifier) and 196>132 transitions and HMMC with the *m/z* 210>160 (quantifier) and 210>132 transitions.

Chen[129] described an *in vitro* method for the determination of four designer drugs (MDPV, 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP), methylone and methcathinone) and their major metabolites by LC/MS. Rat liver microsomes S9 fraction was incubated with each target drug and NADPH at 37^{o}C for 120 min. The reaction was stopped with acetonitrile and the supernatants were analysed by LC/MS. Analytes were separated using a Thermo Gold ODS column (2.1 mm x 150 mm, 5 µm) and quantitated by an LXQ ion trap mass spectrometer. Methylone was identified from its protonated molecular ion, M-H^{+}, at *m/z* 208 and from the base peak fragment ion at *m/z* 190.1, which was chosen as the quantitation ion. 3-OH-4-MeO-MC was identified with an M-H^{+} at *m/z* 210.1 in MS^{1}, at *m/z* 192.1 in MS/MS and at *m/z* 160.1 in MS^{3}.

9.4 Methods and materials

9.4.1 Reagents and standards

Methylone-HCl was purchased from Sigma-Aldrich Co, LLC (UK). Butylone-HCl was purchased from Lipomed (Kinesis, Ltd UK). All other reagents were the same as those described in Section 8.6.1.

Individual stock standard solutions were prepared by dissolving each compound in methanol to achieve a concentration of 1 mg/ml of drug substance. Working standard solutions were prepared by serial dilution of stock standard solutions with methanol. All stock and working standard solutions were stored at 4^{o}C.

9.4.1.1 Preparation of phosphate buffer saline (PBS) pH 7.4

This was the same as described in Section 8.6.1.3.

9.4.1.2 Preparation of NADPH generating system

This was the same as described in Section 8.6.1.5.

9.4.1.3 Preparation of methylone and butylone in phosphate buffer pH 7.4

Stock solution of test standards (methylone and butylone) for use in metabolism studies were freshly prepared at 10 µg/ml in pH 7.4 phosphate buffer.

9.4.2 *In vitro* assay (human liver microsomes)

This was the same as described in Section 8.6.2.

9.4.3 Derivatisation

Acetylation of methylone, butylone and their metabolites was carried out using acetic anhydride. 50 µl of acetic anhydride: pyridine (3:2, v:v) was added to each of the dried HLM sample extracts, and heated at 60^{o}C for 30 min. All samples were then evaporated to dryness at room temperature under a stream of nitrogen and residues were reconstituted in 200 µl of 10% acetonitrile in deionised water containing 0.1% formic acid and transferred to labelled 1.5 ml autosampler vials with inserts before analysis by LC/MS/MS.

9.4.4 Liquid chromatography/mass spectrometry conditions

9.4.4.1 Liquid chromatography conditions

Chromatographic separation was performed on the same instrument and conditions as described in Section 8.6.5. The injection volume was 20 µl and the runtime was extended to 15 min per injection to allow butylone to be eluted between 9-10 min.

9.4.4.2 Mass spectrometry conditions

Mass spectrometric analysis was performed on the same instrument as described in Section 8.6.5. The MRM transitions, FVs and CEs for derivatised methylone and butylone were optimised separately by direct infusion of each acetate derivative standard solution at a concentration of 5 µg/ml in mobile phase (50:50 v:v) into the MS. All analytes were identified and quantified based on their retention times and quantifier and qualifier ion ratios that were obtained from the MRM transitions.

The MRM transitions, fragmentation voltages and collision energies used for the measurement of methylone and its metabolites were developed and are summarised in Table 9-2 and for butylone and its metabolites in Table 9-3.

Table 9-2: MRM conditions for acetate derivatives of methylone and its metabolites.

Name	Precursor Ion (*m/z*)	Fragmentor Voltage (eV)	Product Ions (*m/z*)	Collision Energy (eV)
MDC-Ac	236	80	176	15
			146	30
			118	35
Methylone-Ac	250	90	208	10
			190	20
			160	25
HMMC-2Ac & *β*-OH-M	294	95	234	15
			160	30
			132	35
HHMC-3Ac	322	100	280	10
			178	30
			160	30

β-OH-M = *β*-hydroxy-methylone

Table 9-3: MRM conditions for acetate derivatives of butylone and its metabolites.

Name	Precursor Ion (*m/z*)	Fragmentor Voltage (eV)	Product Ions (*m/z*)	Collision Energy (eV)
bk-BDB-Ac	250	80	190	15
			160	25
			132	30
Butylone-Ac	264	90	222	10
			204	20
			174	25
4-OH-3MeO-M-2Ac	308	100	248	15
			174	30
			162	30
β-OH-M-2Ac	308	100	248	15
			174	30
			162	30
Di-OH-M-3Ac	336	100	294	10
			192	20
			174	30

9.5 Results and discussion

9.5.1 Optimisation of LC/MS conditions

9.5.1.1 LC separation

In Chapter 8, MDMA, HHMA, HMMA, MDA and HMA were analysed in both HLM cultures and urine samples. The same LC method was used for the analysis of methylone, butylone and their metabolites in HLM samples but the MS-MRM method needed to be further developed. Target analytes in this study included hydrophilic compounds, the dihydroxy-metabolites of methylone and butylone and derivatisation with acetic anhydride was applied to HLM extracts for the purpose of improving LC separations by reversed-phase HPLC.

The retention factors were 5.3 for methylone-Ac and 10.2 for butylone-Ac and the hold-up time (t_0 = 0.8 min) was determined by injecting pure methanol. Figure 9-2 shows the separation of standards of methylone-Ac and butylone-Ac on the Gemini C18 column.

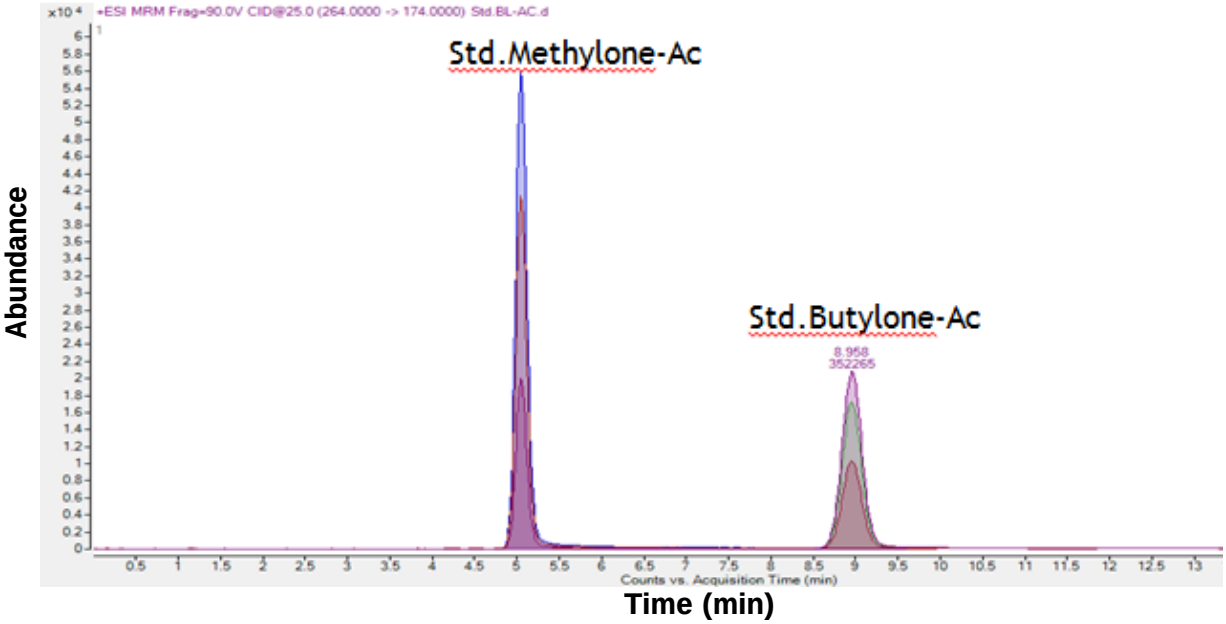


Figure 9-2: Chromatograms of methylone-Ac and butylone-Ac on a Gemini C18 column.

9.5.1.2 MRM optimisation

To optimise MRM conditions, standards of methylone and butylone were derivatised with acetic anhydride and re-dissolved in methanol. The Ac-derivatives of the standards were directly injected into the mass spectrometer interface using a syringe pump with positive ESI to obtain the precursor ion, product ions, FVs and CEs. Because of unavailability of reference standards of methylone and butylone metabolites, a minimum of four identification points (IPs)[166] was required. One quantifier and two or three qualifier ions were used to identify target analytes. FVs and CEs of derivative butylone and methylone metabolites were developed based on derivatives of standards of methylone and butylone and are summarised in Tables 9-2 and 9-3 respectively. Under optimised conditions, most ion reactions had different optimum CEs. For Figures 9-4 and 9-6, CEs of 10 and 20 eV were chosen to demonstrate the patterns, although ion ratios at other CEs are different.

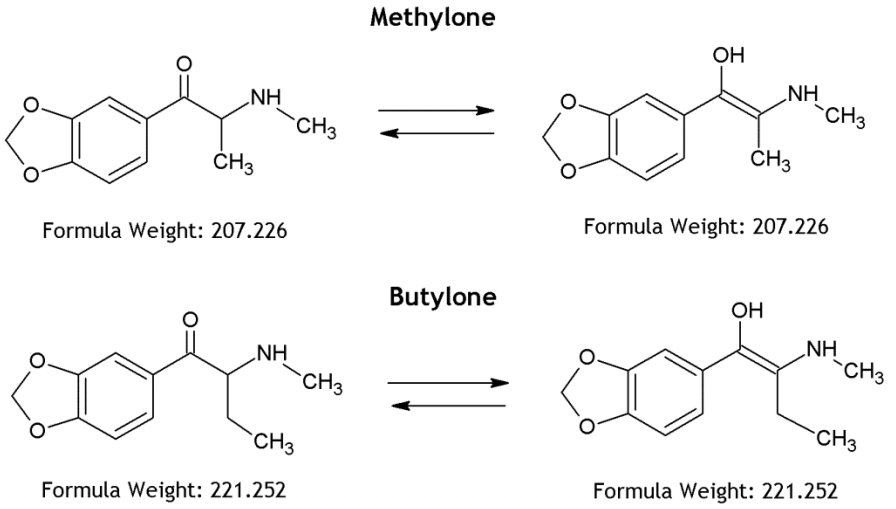


Figure 9-3: Keto–enol tautomerism of methylone and butylone.

The fragmentation patterns of derivatives of methylone and butylone in LC-MS are rather different from the types of ion reaction found in GC-MS. Previous studies have shown that ketones can isomerise to alcohols by keto-enol tautomerism, see Figure 9-3. Zuba[68] found that in ESI, a proton was attached to the oxygen atom of cathinone (in its enol-form) to form a protonated molecule ($M\text{-}H^+$) and the first fragmentation was the loss of water (H_2O).

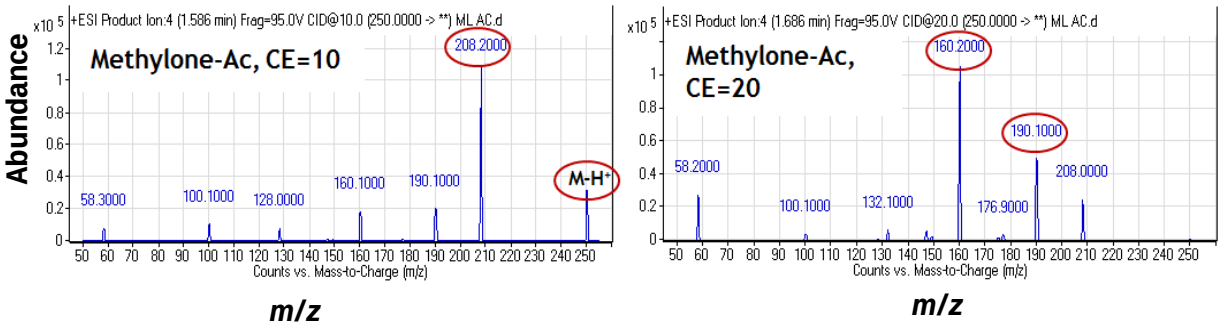


Figure 9-4: Product ion scan spectra of the Ac-derivative of a methylone standard at CEs of 10 and 20 eV. The optimum CE values for each ion are listed in Table 9-2.

Figure 9-4 shows the product ion scan spectra of the Ac-derivative of methylone standard using LC/MS/MS. When the CE was 10 eV, the mass spectrum of methylone-Ac had a base peak at *m/z* 208 and a small peak for the protonated molecule ($M\text{-}H^+$, *m/z* 250). At a higher CE of 20 eV, the protonated molecular ion was absent, the fragment ion peak of methylone-Ac at *m/z* 208 decreased in intensity and the peaks at *m/z* 160 and 190 increased. The first fragmentation of

methylone-Ac was the loss of ketene ($COCH_2$, 42 Da) from the protonated molecular ion to give the ion at *m/z* 208 followed by the loss of water to give the ion at *m/z* 190.

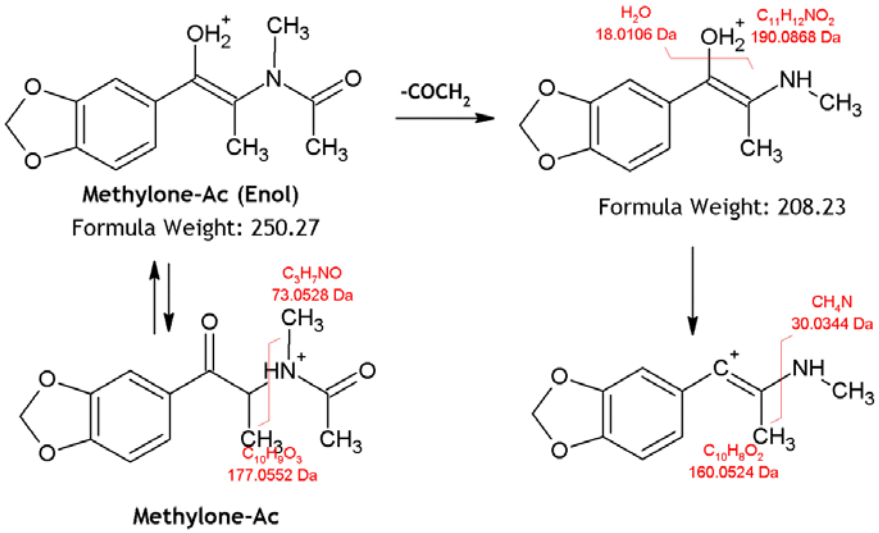


Figure 9-5: Mass spectral fragmentation of methylone-Ac.

From MRM optimisation, the protonated molecular ion of methylone-Ac at *m/z* 250 was used as the precursor ion and its fragment ions at *m/z* 208, 190 and 160 were selected as product ions. The most intense product ion peak at *m/z* 160 was chosen as a quantifier ion and the ratio of qualifier/quantifier at the optimised CEs were approximately 71% for *m/z* 208 and 34% for *m/z* 190. The mass spectral fragmentation of methylone-Ac is shown in Figure 9-5.

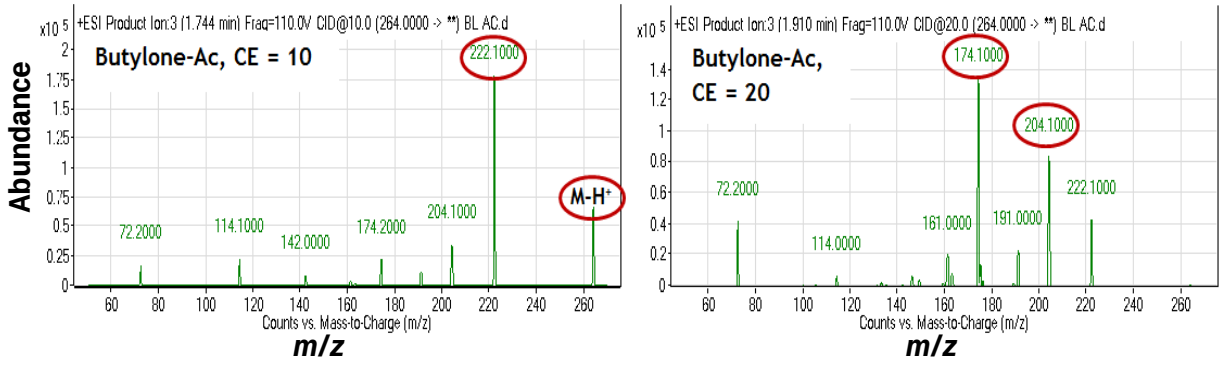


Figure 9-6: Product ion scan spectra of the Ac-derivative of a butylone standard at CE of 10 and 20 eV. The optimum CE values for each ion are listed in Table 9-3.

Butylone-Ac was found to fragment in a similar way to methylone-Ac. Figure 9-6 shows the product ion scan spectra of the Ac-derivative of butylone standard using LC/MS/MS. When the CE was 10 eV, the mass spectrum of butylone-Ac had a base peak at *m/z* 222 and a small peak for the protonated molecular ion (*m/z* 264, $M\text{-}H^+$). At a higher CE of 20 eV, the fragment ion peak of butylone-Ac at *m/z* 222 decreased in intensity and the peaks at *m/z* 174 and 204 increased. The first fragmentation of butylone-Ac was the loss of the ketene group ($COCH_2$, 42 Da) from the protonated molecular ion to give the fragment at *m/z* 222 followed by the loss of water to give *m/z* 204, the same pattern as that obtained for methylone.

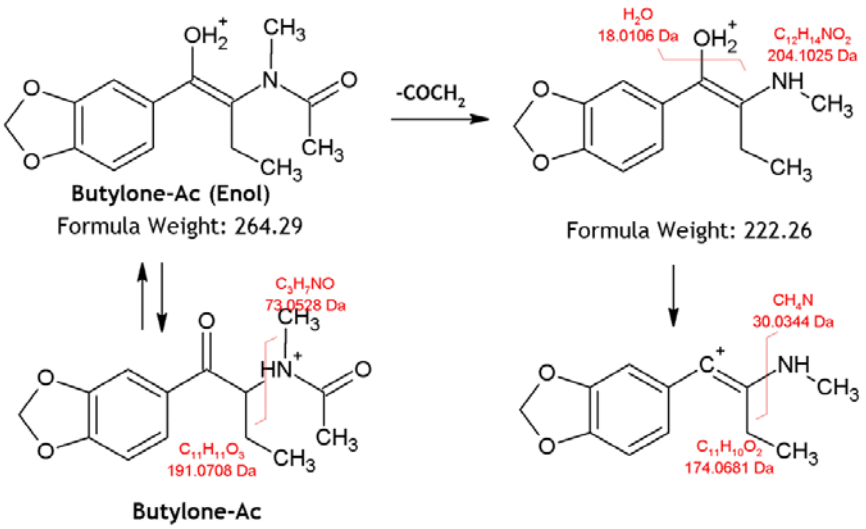


Figure 9-7: Mass spectral fragmentation of butylone-Ac.

From MRM optimisation, the protonated molecular ion of butylone-Ac at *m/z* 264 was used as the precursor ion and its fragment ions at *m/z* 174, 204 and 222 were selected as product ions. The most intense product ion peak at *m/z* 174 was chosen as the quantifier ion and the ratio of qualifier/quantifier at the optimised CEs were approximately 81% for *m/z* 204 and 49% for *m/z* 222. Mass spectral fragmentation of butylone-Ac is shown in Figure 9-7.

9.5.2 General approach for metabolite identification

Because methylone and butylone have both methylenedioxy and *β*-ketone groups within their structure, their metabolic pathways are likely to resemble those of MDMA and cathinone. Demethylenation of the methylenedioxy ring followed by

O-methylation of either the 3- or 4-hydroxy group on the benzene ring has been reported as the primary metabolic route whereas *N*-dealkylation has been reported as a minor metabolic pathway of methylone and butylone in humans[74,126,138]. In addition, reduction of the *β*-ketone has been reported as one of the major metabolic pathways of butylone in humans[74,138].

Part of the purpose of this study derived from the fact that metabolites of methylone including HHMC, HMMC and MDC and metabolites of butylone including dihydroxy-metabolite, *β*-hydroxy-butylone and nor-butylone were not commercially available and *in vitro* metabolism provides one route to obtaining them. However, the concentrations of metabolites obtained from HLM are too low for full scan LC/MS/MS analysis and the MRM mode has to be used, which means the product ions must be specified in advance. As a result, the mass spectral characteristics of acetate-derivatives of methylone and butylone metabolites were predicted based on the mass spectra of the acetate derivatives of methylone and butylone and of MDMA metabolites (HHMA-3Ac, HMMA-2Ac and HMA-2Ac). Moreover, multiple reaction monitoring (MRM) ions were reviewed and compared with mass spectra of synthesised metabolite reference standards such as HHMC, MDC, HMMC and nor-butylone described in the previous studies referred to above.

9.5.3 Methylone metabolite identification

Metabolism studies of methylone in humans were first published by Kamata *et al.* in 2006. However, most were *in vivo* studies and only a few *in vitro* studies have been reported[127-129]. Both *in vivo* and *in vitro* studies on the metabolism of methylone in humans found that methylone has several metabolic pathways, similar to MDMA, including *N*-demethylation and demethylenation followed by *O*-methylation of either a 3- or 4-hydroxy group on the benzene ring. In addition, *β*-keto reduction to the corresponding amino-alcohols that was partly involved in the metabolism of cathinone was not observed in the previous studies of methylone metabolism[125]. 3,4-dihydroxymethcathinone (HHMC), the intermediate metabolite leading to 4-hydroxy-3-methoxymethcathinone (HMMC) was expected to be the major metabolite of methylone produced by human liver

microsomes whereas 3,4-methylenedioxycathinone (MDC) was expected to be a minor metabolite of methylone in this study.

9.5.3.1 Predicted mass spectral fragmentation of methylone metabolites

Fragment ions in LC/MS arise from simple cleavages of the chemical bonds and some from rearrangements, which are different from the mass spectra obtained in GC/MS. Kamata *et al.*[125] reported the identification method of methylone metabolites. MDC, HMMC and 3-OH-4-MeO-MC were synthesised and analysed with both GC/MS and LC/MS/MS. For methylone, the LC/MS mass spectrum shows the base peak to be the protonated molecular ion (*m/z* 208, $M\text{-}H^+$) and small fragment peaks are present at *m/z* 132, 160 and 190. For MDC, the base peak is the protonated molecule (*m/z* 194, $M\text{-}H^+$) and small fragment peaks are present at *m/z* 118, 146 and 176. For HMMC and 3-OH-4-MeO-MC, the base peaks in their mass spectra are also the protonated molecular ions (both at *m/z* 210, $M\text{-}H^+$) with smaller fragment peaks at *m/z* 132, 160 and 192. However, the mass spectra of isomers, HMMC and 3-OH-4-MeO-MC, were very similar. Small fragment ions that correspond to the loss of water ($M\text{-}H^+\text{-}H_2O$) appeared in all mass spectra (*m/z* 190 for methylone, 176 for MDC and 192 for HMMC and 3-OH-4-MeO-MC). These spectra are in agreement with the ESI-LC/MS and LC/MS/MS spectra described by Zaitsu *et al.*[74] and Chen[129].

Moreover, Ellefsen *et al.*[130] reported the LC/MS/MS positive ESI MRM ions for methylone and its metabolites in rat and human plasma. Standards of HHMC, HMMC and MDC were synthesised and analysed. Protonated molecular ions were chosen as precursor ions, at *m/z* 196.1 for HHMC, 210.1 for HMMC and 194.1 for MDC. For product ions, those at *m/z* 160.1 and 132.2 were selected for HHMC, 160.2 and 132.3 for HMMC and 146.0 and 118.1 for MDC.

In the present *in vitro* study, HHMC was expected to be a major metabolite and MDC was expected to be a minor metabolite in HLM samples. Predicted mass spectral fragmentation of MDC, HHMC and HMMC acetates was postulated based on the data mentioned above. As described in the previous section, the first fragmentation of acetate derivatives is the loss of ketene ($-COCH_2$, 42 Da). The

molecular ions of MDC-Ac, HMMC-2Ac and HHMC-3Ac were at *m/z* 236, 294 and 322, respectively. The product ions from the loss of the ketene would be at *m/z* 194 for MDC (Figure 9-8), 252 for HMMC and 280 for HHMC. Other product ions at *m/z* 146 and 118 for MDC, *m/z* 160 and 132 for HMMC and *m/z* 178,160 and 132 for HHMC were expected, based on the spectra from Kamata and Ellefsen. Each predicted fragment ion was subsequently included in the initial LC/MS/MS analysis of HLM samples to identify which metabolites were present and the fragment ions they actually produced.

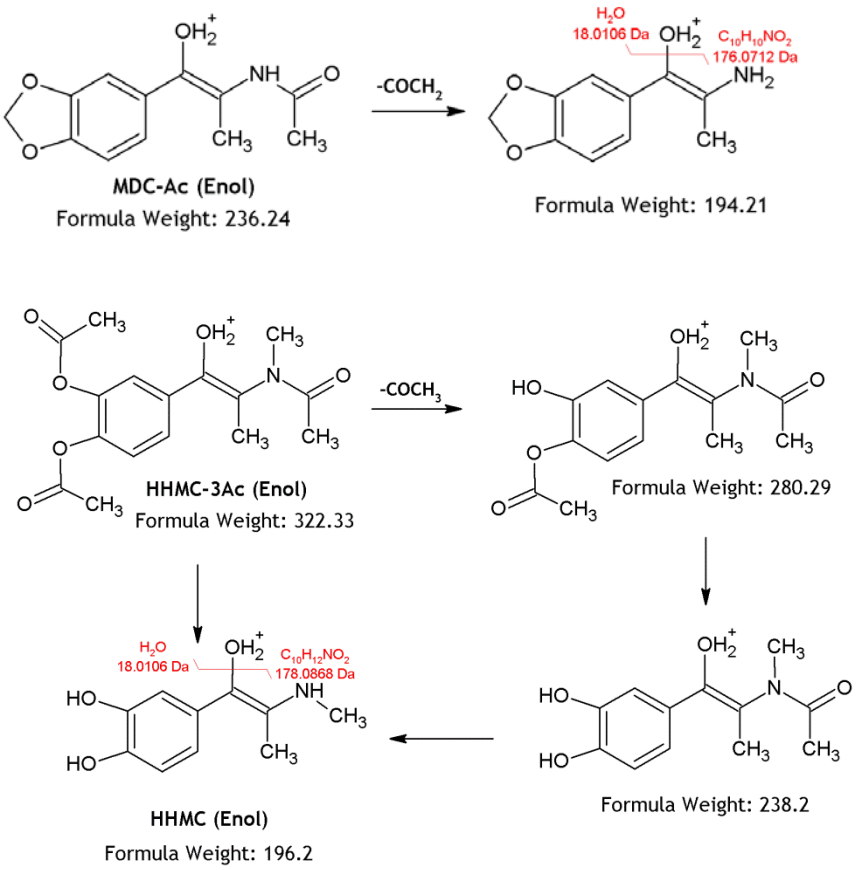


Figure 9-8: Mass spectral fragmentation of MDC-Ac and HHMC-3Ac.

The MRM method for analysis of acetate derivatives of methylone and its metabolites finally adopted was as summarised in Table 9-2, in Section 9.4.4.2 above.

9.5.3.2 Residual methylone identification in HLM samples

Pooled human liver microsomes were incubated with methylone and extracted and analysed using the LC/MS/MS method described previously. The acetate

derivative of a methylone standard was identified at retention time of 5.0 min and confirmed by assessing the qualifier/quantifier ion ratios with ± 20% tolerance.

The un-metabolised methylone acetate (MDMC-Ac) was identified in HLM samples. The retention time and the mass ratio of methylone obtained from HLM samples were consistent with those observed for the methylone standard. Methylone derivative (MDMC-Ac) was eluted at 5.0 min and was identified and confirmed by quantifier and qualifier ions at *m/z* 250>208, 250>190 and 250>160. The qualifier/quantifier ion ratio was 74% for *m/z* 208 and was 35% for *m/z* 190 which were in the acceptable range. LC-MS/MS MRM chromatograms of methylone-Ac in HLM sample compared with the Ac-derivative of a standard are shown in Figure 9-9.

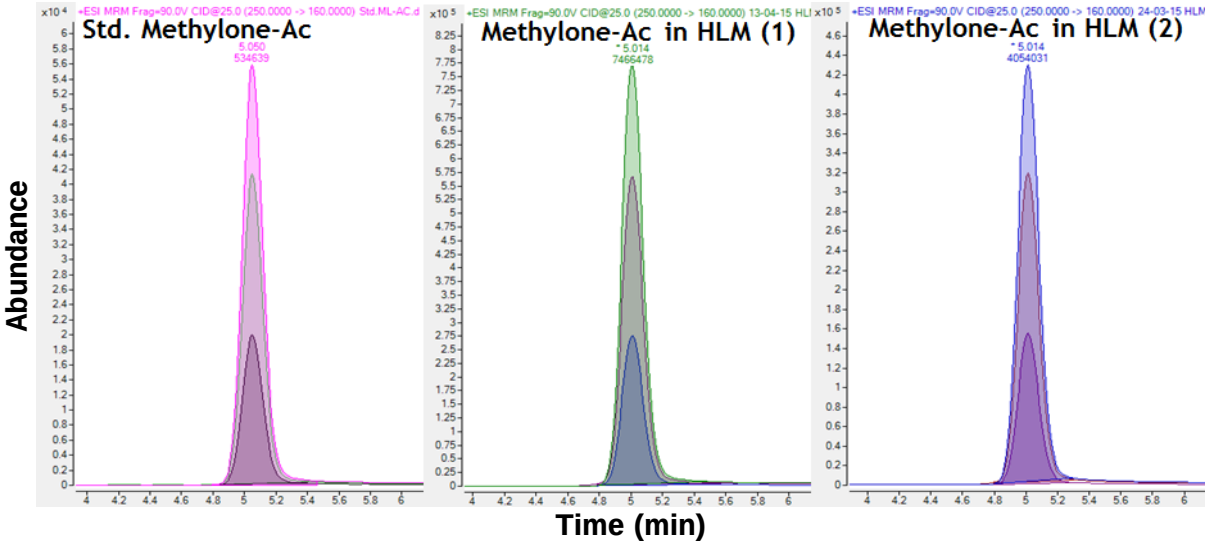


Figure 9-9: Specimen LC-MS/MS MRM chromatograms of methylone-Ac in HLM samples compared with the Ac-derivative of a methylone standard.

9.5.3.3 *In vitro* nor-methylone (MDC) identification

The acetate of nor-methylone (3,4-methylenedioxycathinone, MDC-Ac, a minor metabolite) was identified in HLM samples by MRM transitions at *m/z* 236>176, 236>146 and 236>118 and was eluted at 3.2 min. The MRM transition at *m/z* 236>118 gave the highest corresponding peak area for MDC-Ac in all HLM samples, followed by the transitions *m/z* 236>146 and 236>176. LC/MS/MS MRM chromatograms of nor-methylone-Ac in HLM samples are shown in Figure 9-10.

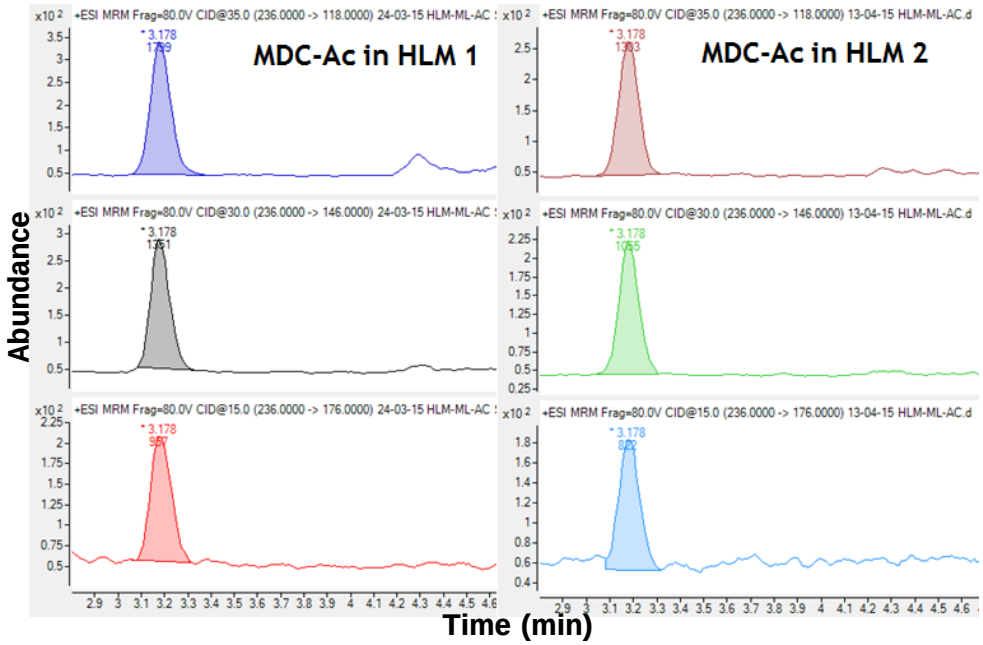


Figure 9-10: Specimen LC/MS/MS MRM chromatograms for nor-methylone-Ac in HLM samples.

9.5.3.4 *In vitro* HHMC identification

The derivative of 3,4-dihydroxymethcathinone (HHMC-3Ac), a major metabolite, was identified in HLM samples by MRM transitions at *m/z* 322>280, 322>178 and 322>160 and was eluted at 5.7 min. The MRM transition at *m/z* 322>280 gave the highest corresponding peak area for HHMC-3Ac in all HLM samples, followed by the transitions *m/z* 322>178 and 322>160. LC/MS/MS MRM chromatograms of HHMC-3Ac in HLM samples are shown in Figure 9-11.

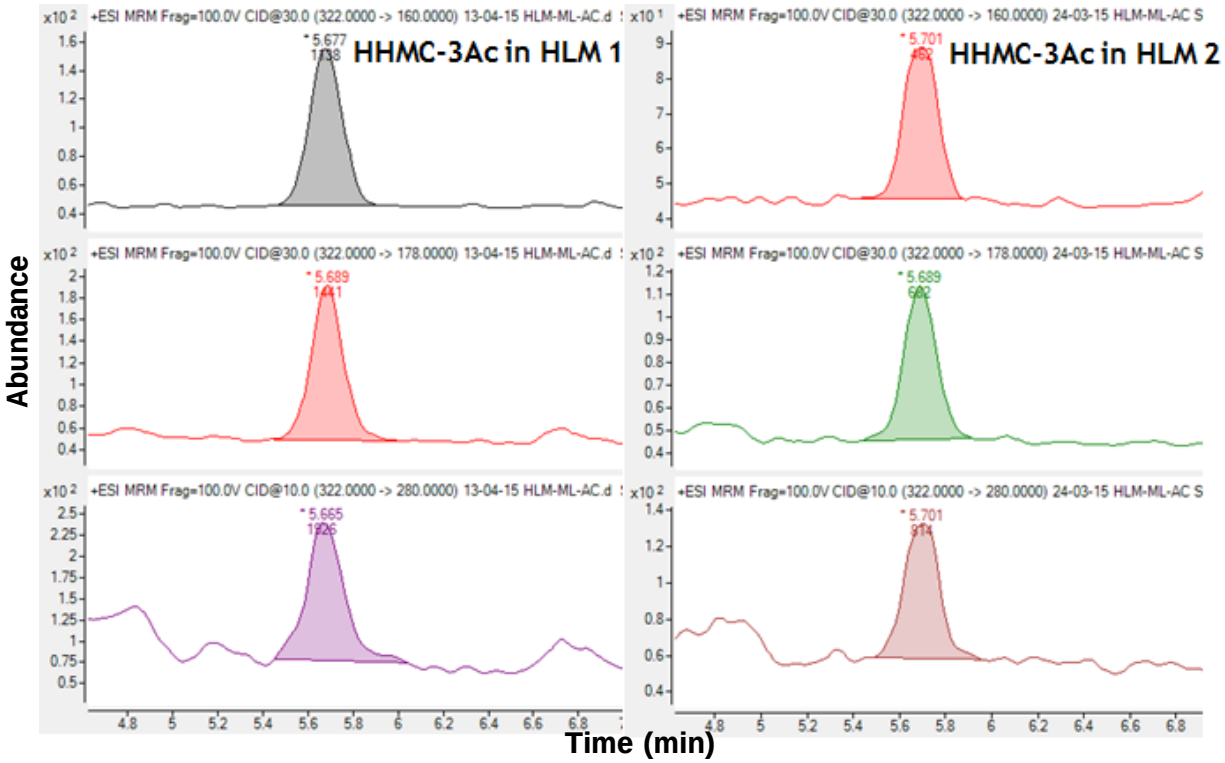


Figure 9-11: Specimen LC-MS/MS MRM chromatograms for dihydroxy-methcathinone-3Ac in HLM samples.

9.5.3.5 *In vitro* HMMC identification

The acetate of 4-hydroxy-3-methoxymethcathinone (HMMC-2Ac) was identified in HLM samples by MRM transitions at *m/z* 294>234, 294>160 and 294>132 and was eluted at 5.5 min. The MRM transition at *m/z* 294>234 gave the highest corresponding peak area for HMMC-2Ac in all HLM samples, followed by the transitions *m/z* 294>160 and 294>132. LC/MS/MS MRM chromatograms of 4-hydroxy-3-methoxymethcathinone-2Ac in HLM samples are shown in Figure 9-12.

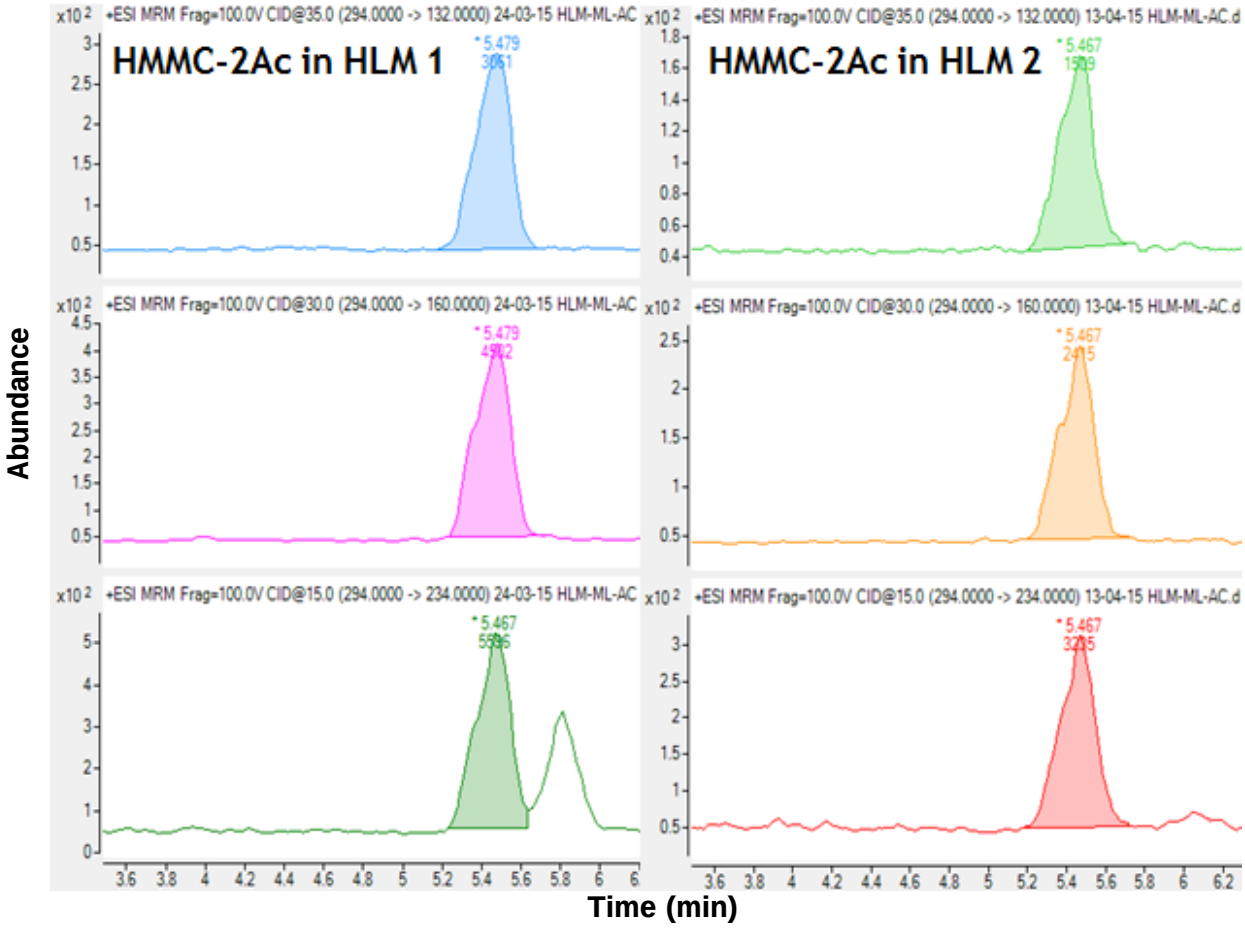


Figure 9-12: Specimen LC/MS/MS MRM chromatograms for 4-hydroxy-3-methoxymethcathinone-2Ac in HLM samples.

Moreover, 4-hydroxy-3-methoxymethcathinone and *β*-hydroxy-methylone are structural isomers which have similar fragmentation ions (Figure 9-13). Previous studies of methylone metabolites in human urine reported that the ketone-reduction metabolite was not observed[74,125,138]. However, Mueller and Rentsch[127] reported that the ketone-reduction metabolite and hydroxy metabolite of methylone were both detected in *in vitro* study on the metabolism of 11 cathinones using HLM.

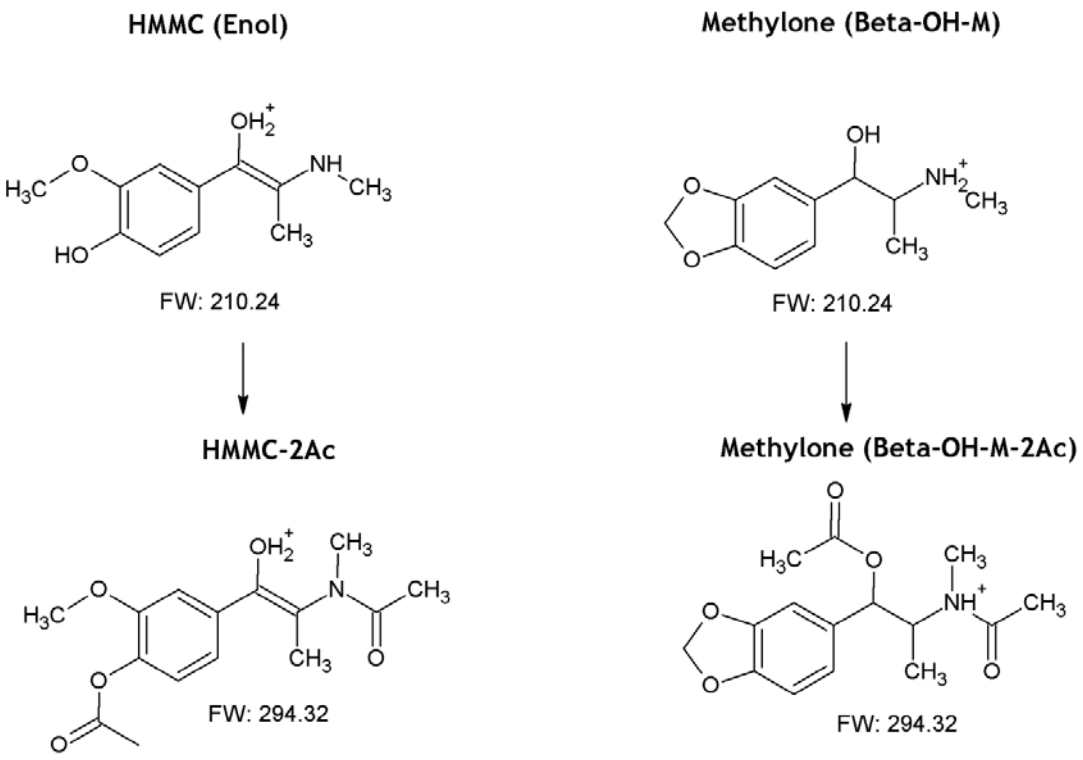


Figure 9-13: Structures of HMMC and methylone ketone-reduction metabolites.

Because HMMC and *β*-hydroxy-methylone are structural isomers, transitions for HMMC and *β*-hydroxy metabolite would be the same. In the present study, MRM transitions at *m/z* 294>234, 294>160 and 294>132 for HMMC would also be valid for *β*-hydroxy metabolite identification. The problem was then to decide on which isomer was actually present in the absence of standards.

There is evidence that the isomer present is the *β*-hydroxy metabolite, as these transitions had the highest corresponding peak areas among the three metabolites (Figure 9-14), i.e. this was the main metabolite. The previous study on MDMA, reported in Chapter 8, showed that the major metabolite of MDMA generated by HLM is HHMA and only a trace of HMMA was detected in MDMA-HLM samples. Because HHMA is an intermediate metabolite leading to HMMA, concentrations of HHMC detected in HLM samples should, by analogy, be higher than HMMC. It is unlikely that the metabolic pattern would be reversed for methylone such that HMMC was the major metabolite. It was therefore tentatively concluded that this was the *β*-hydroxy metabolite.

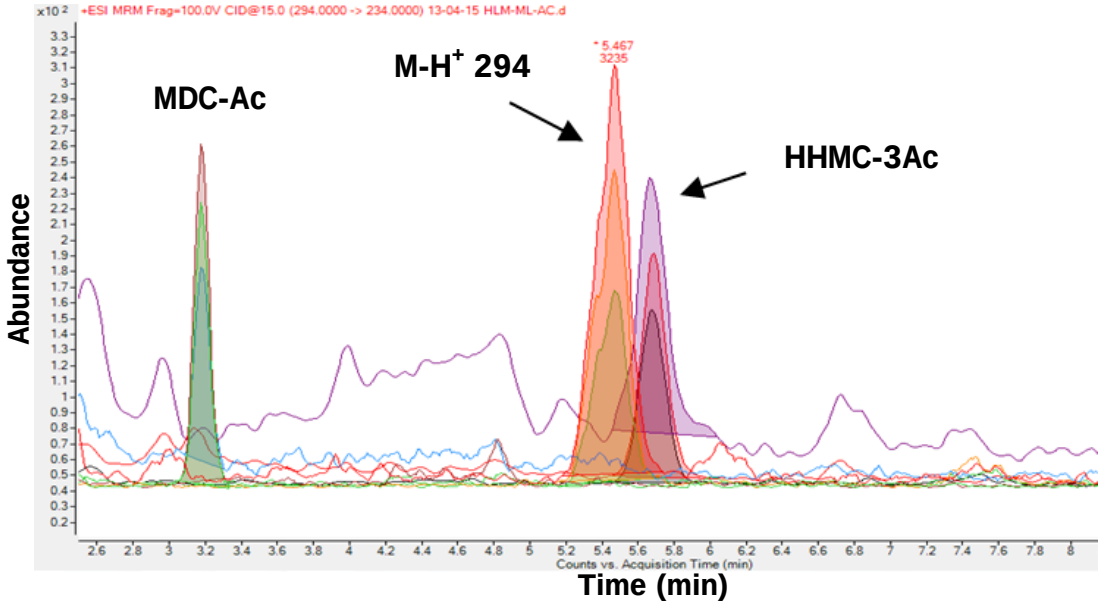


Figure 9-14: Specimen LC-MS/MS MRM chromatograms for methylone metabolites in an HLM sample.

These results suggest that the ketone-reduction product is one of the major metabolites of methylone in humans. However, authentic standards of HMMC and ketone-reduction metabolite are required to confirm the identification of both metabolites.

9.5.4 Butylone metabolite identification

The 3,4-dihydroxy-metabolite (di-OH-M) and *β*-OH-metabolite (*β*-OH-M) were expected to be the major metabolites of butylone formed by human liver microsomes whereas nor-butylone (bk-BDB) was expected to be a minor metabolite of butylone in this study. Multiple reaction monitoring (MRM) ions were reviewed and compared with mass spectral characteristics of synthesised reference standards of bk-BDB and 4-OH-3-MeO-M described in previous studies by other workers which are summarised below.

The first metabolism study of butylone was reported by Zaitsu *et al.*[138] in 2009. All metabolites in urine samples were determined based on GC/MS with TFA derivatisation. In 2011, Zaitsu *et al.*[74] reported a review of methylone, butylone and ethylone metabolism in humans. Butylone metabolites (bk-BDB, 4-OH-3-MeO-M and 3-OH-4-MeO-M) were synthesised and analysed by both GC/MS and LC/MS/MS.

For butylone, the mass spectrum shows large fragment ion peaks at *m/z* 174, 146, 131 and 204 and a small peak for the protonated molecule (*m/z* 222, $M\text{-}H^+$). For bk-BDB, the mass spectrum shows large fragment ion peaks at *m/z* 117 and 160 and small fragment ion peaks at *m/z* 132 and 190. For 4-OH-3-MeO-M and 3-OH-4-MeO-M, the mass spectrum shows large fragment peaks at *m/z* 134, 174 and 162 and small fragment peaks at *m/z* 146, 206 and 224 (the protonated molecule, $M\text{-}H^+$). However, the mass spectra of the isomers, 4-OH-3-MeO-M and 3-OH-4-MeO-M, were very similar. As in the case of methylone, the small fragment ions that correspond to the loss of water ($M\text{-}H^+\text{-}H_2O$) were present in all mass spectra (*m/z* 204 for butylone, 190 for bk-BDB and 206 for 4-OH-3-MeO-M and 3-OH-4-MeO-M).

Since 2009, only four metabolism studies of butylone have been reported. Three of them were *in vivo* studies in human urine whereas only one was an *in vitro* study with human liver microsomes (HLM). Mueller and Rentsch[127] reported the metabolism of 11 cathinones using human liver microsomes with LC-MS(n) analysis. Nor-butylone and demethylenyl-diol-metabolites were reported as phase I metabolites produced by HLM and the *β*-ketone reduction metabolite that was reported by Zaitsu *et al.*[74] and Meyer *et al.*[126] was not observed. However, information on the MS^2 and MS^3 spectra of butylone metabolites was not provided in this paper.

9.5.4.1 Identification of residual butylone in HLM samples

Pooled human liver microsomes (HLM) were incubated with butylone and extracted and analysed using the LC/MS/MS method described previously. The acetate derivative of a standard of butylone was identified at a retention time of 8.95 min and confirmed by assessing the qualifier/quantifier ion ratios with ± 20% tolerance.

The derivative of un-metabolised butylone (bk-MBDB-Ac) was detected in extracts. The retention time and the mass ratio of butylone obtained from HLM samples were consistent with those observed in the standard. Butylone acetate derivative (bk-MBDB-Ac) was eluted at 9.0 min and was identified and confirmed by quantifier and qualifier ion reactions at *m/z* 264>222, 264>204 and 264>174. The qualifier/quantifier ion ratio was 82% for *m/z* 222 and 49% for *m/z* 204

which were in the acceptable range. LC/MS/MS MRM chromatograms of butylone-Ac in HLM extracts compared with the Ac-derivative of a standard are shown in Figure 9-15.

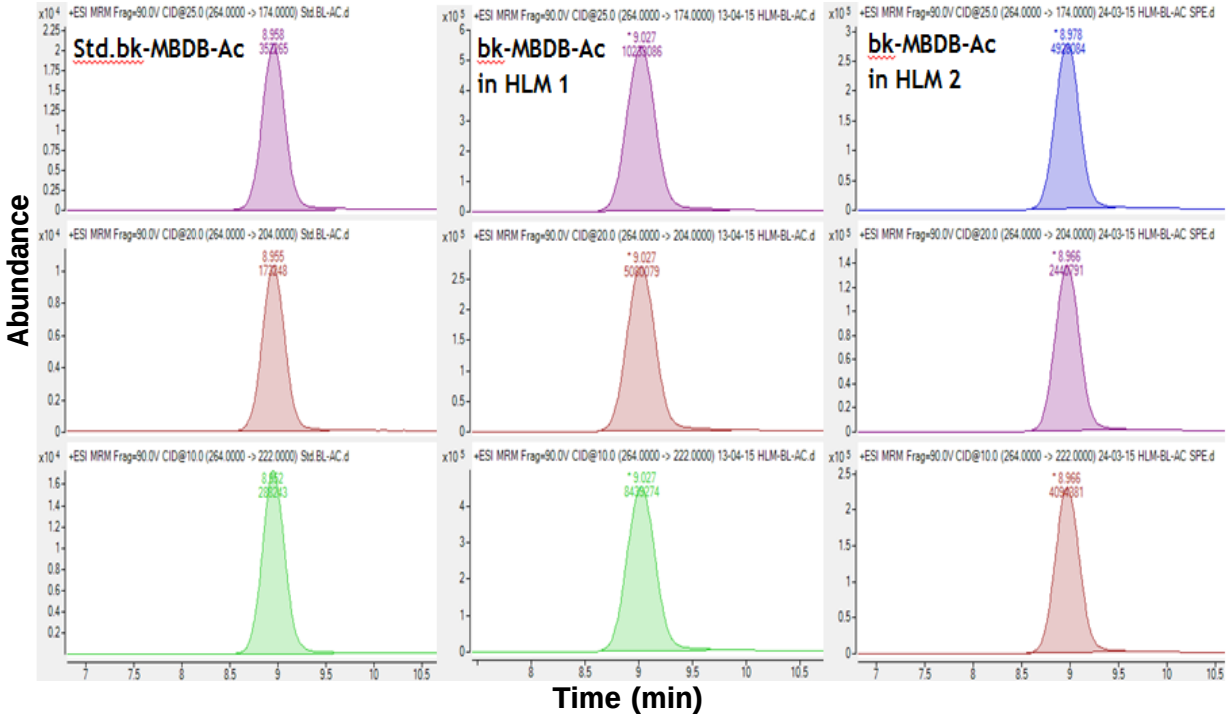


Figure 9-15: Specimen LC/MS/MS MRM chromatograms of butylone-Ac in HLM sample compared with the Ac-derivative of a butylone standard.

9.5.4.2 *In vitro* nor-butylone identification

9.5.4.2.1 Predicted mass spectral fragmentation of nor-butylone (bk-BDB)

As described for methylone metabolites, the first fragmentation of acetate derivatives is the loss of ketene ($COCH_2$, 42 Da). The protonated molecular ion of bk-BDB-Ac would be at *m/z* 250 and the product ion from the loss of ketene would be at *m/z* 208. Other product ions at *m/z* 190, 160 and 132 for bk-BDB are predicted, based on Zaitsu *et al.*[74] A comparison of butylone and nor-butylone LC/MS/MS mass spectra in Zaitsu's paper shows that both of them have the same fragmentation pattern. The first fragment of both is the loss of H_2O (*m/z* 204 for bk-MBDB and *m/z* 190 for bk-BDB). The second fragment is the loss of *m/z* 30 (CH_2O, *m/z* 174 for bk-MBDB and *m/z* 160 for bk-BDB). The next fragment is the

loss of *m/z* 28 (*m/z* 146 for bk-MBDB and *m/z* 132 for bk-BDB). These results indicate that the LC/MS/MS fragmentation occurs at the methylenedioxy ring rather than on the side chain.

9.5.4.2.2 Identification of nor-butylone in HLM samples

The acetate derivative of nor-butylone (bk-BDB-Ac, a minor metabolite) was identified in HLM extracts by MRM transitions at *m/z* 250>190, 250>160 and 250>132 and was eluted at 4.7 min. The MRM for *m/z* 250>160 gave the highest corresponding peak area for bk-BDB-Ac in all HLM samples, followed by transitions at *m/z* 250>132 and 250>190. LC/MS/MS MRM chromatograms of nor-butylone-Ac in HLM samples are shown in Figure 9-16. The small peak that was observed at 5.0 min in HLM sample was expected to be a peak of a nor-butylone isomer, as yet unidentified.

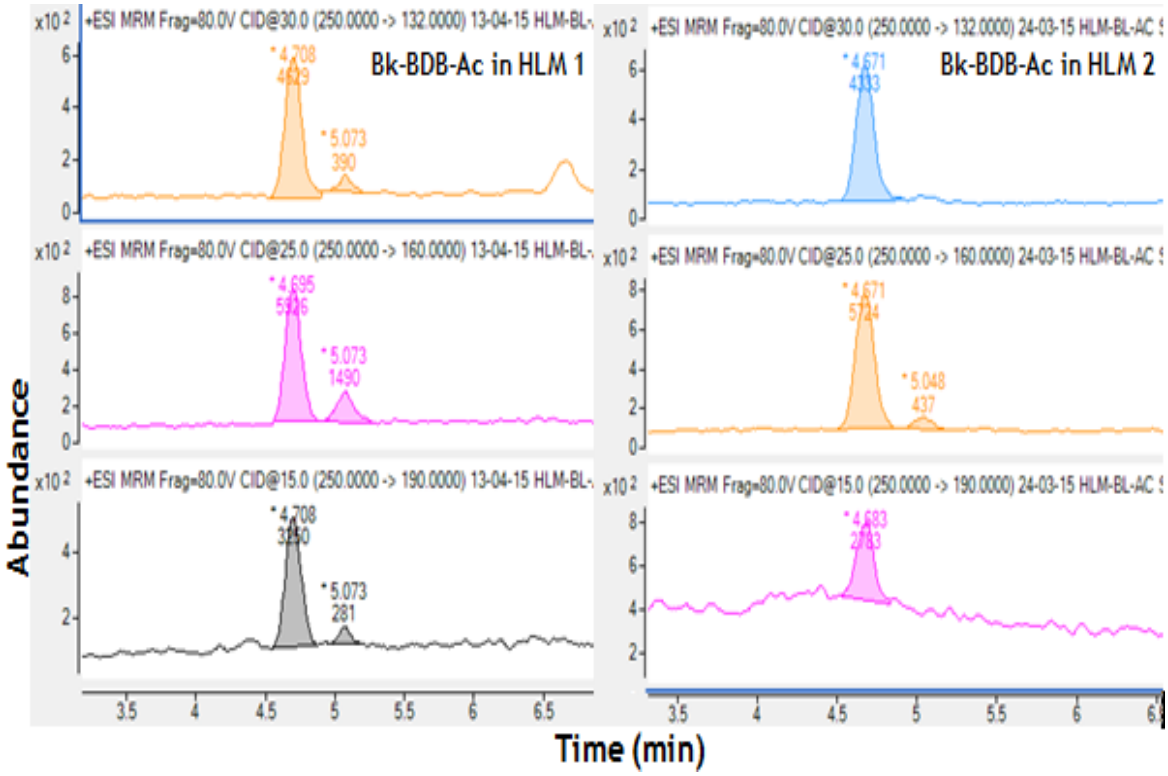


Figure 9-16: Specimen LC-MS/MS MRM chromatograms of nor-butylone-Ac in HLM samples.

9.5.4.3 *In vitro* dihydroxy metabolite of butylone identification

9.5.4.3.1 Predicted mass spectral fragmentation of the dihydroxy metabolite of butylone

For the dihydroxy metabolite of butylone, no information on the LC/MS/MS mass spectrum and MRM transitions have been published before. The protonated molecular ion of di-OH-M-3Ac would be at *m/z* 336 and the product ions from the loss of ketene groups would be at *m/z* 294, 252 and 210. A predicted fragment ion at *m/z* 192 would come from the loss of H_2O from the dihydroxy metabolite itself ($M\text{-}H^+$, *m/z* 210). Moreover, previous studies on methylone metabolites have shown that synthesised standards of HHMC and HMMC gave the same fragment ions at *m/z* 160 and 132. A standard of 4-OH-3-MeO-butylone metabolite has been synthesised by Zaitsu *et al.*[74] and fragment ions at *m/z* 174 and 146 were observed in its mass spectrum. By analogy with the two methylone-derived compounds, these two ions are also expected to be present in the mass spectrum of the dihydroxy metabolite of butylone.

The predicted ESI-LC/MS/MS fragmentation patterns of the acetate derivatives of the dihydroxy metabolite are summarised in Table 9-4 and shown in Figure 9-17. The table also indicates which ions were subsequently detected in HLM samples and three MRM transitions from amongst these that provided high response peak areas were chosen for the identification method described in Section 9.4.4.2 above.

Table 9-4: The predicted ESI-LC/MS/MS fragmentation patterns of a dihydroxy metabolite of butylone.

Compound	$M\text{-}H^+$ (*m/z*)	Product ion (*m/z*)	Predicted losses		Detection in HLM sample
			Da	Possible composition	
di-OH-M-3Ac	336	**294**	-42	$COCH_2$	Y
		252	-42	294-$COCH_2$ (2Ac)	Y
		234	-18	252–H_2O	N
		210	-42	252-$COCH_2$ (3Ac, di-OH-M)	N
		192	-18	H_2O	Y
		174	-18	H_2O, Ref*[74]	Y
		162	-12	C	N
		146	-16	NH_2, Ref*[74]	Y

Ref* = predicted from literature data
Bold are selected ions

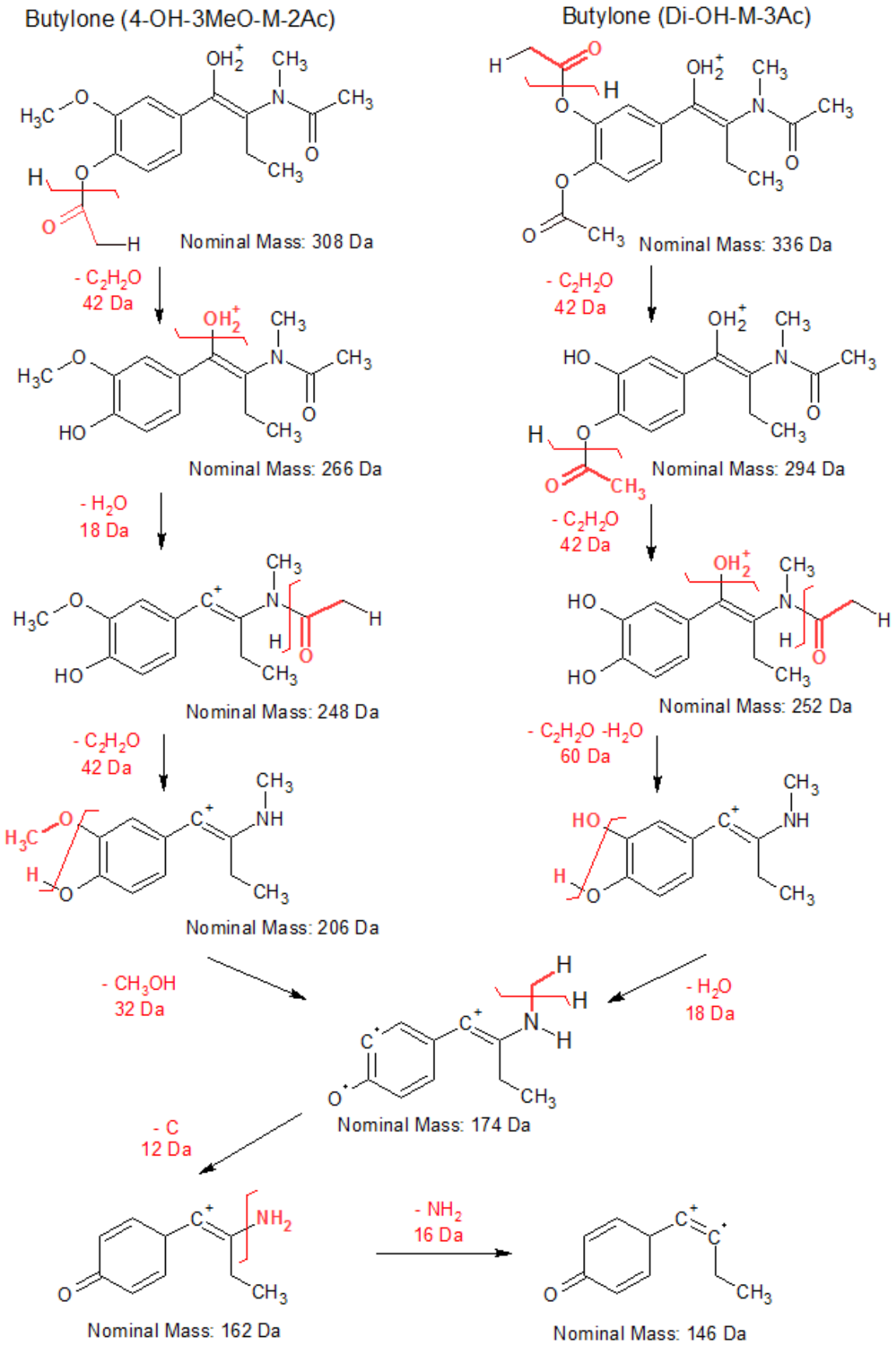


Figure 9-17: Predicted ESI LC/MS/MS fragmentation patterns of acetate derivatives of butylone metabolites (4-OH-3-MeO-M-2Ac and di-OH-M-3Ac). Fragmentations are marked in red. Hydrogen transfers are indicated by an "H" adjacent to the bond which is broken.

9.5.4.3.2 Identification of the dihydroxy metabolite of butylone in HLM samples

The acetate derivative of the 3,4-dihydroxy metabolite (di-OH-M-3Ac), a major butylone intermediate metabolite, was identified in extracts by MRM transitions at *m/z* 336>294, 336>252, 336>192, 336>174 and 336>146. This compound was expected to elute after butylone-Ac itself, which eluted at 9.0 min (Section 9.5.4.1). It was in fact detected in HLM samples and was eluted at approximately 10.1 min. Amongst the predicted MRM transitions, the transition at 336>294 gave the highest corresponding peak area for di-OH-M-3Ac in all HLM samples, followed by 336>174, 336>192, 336>252 and 336>146, respectively. LC/MS/MS MRM chromatograms of di-OH-M-3Ac in HLM samples are shown in Figure 9-18.

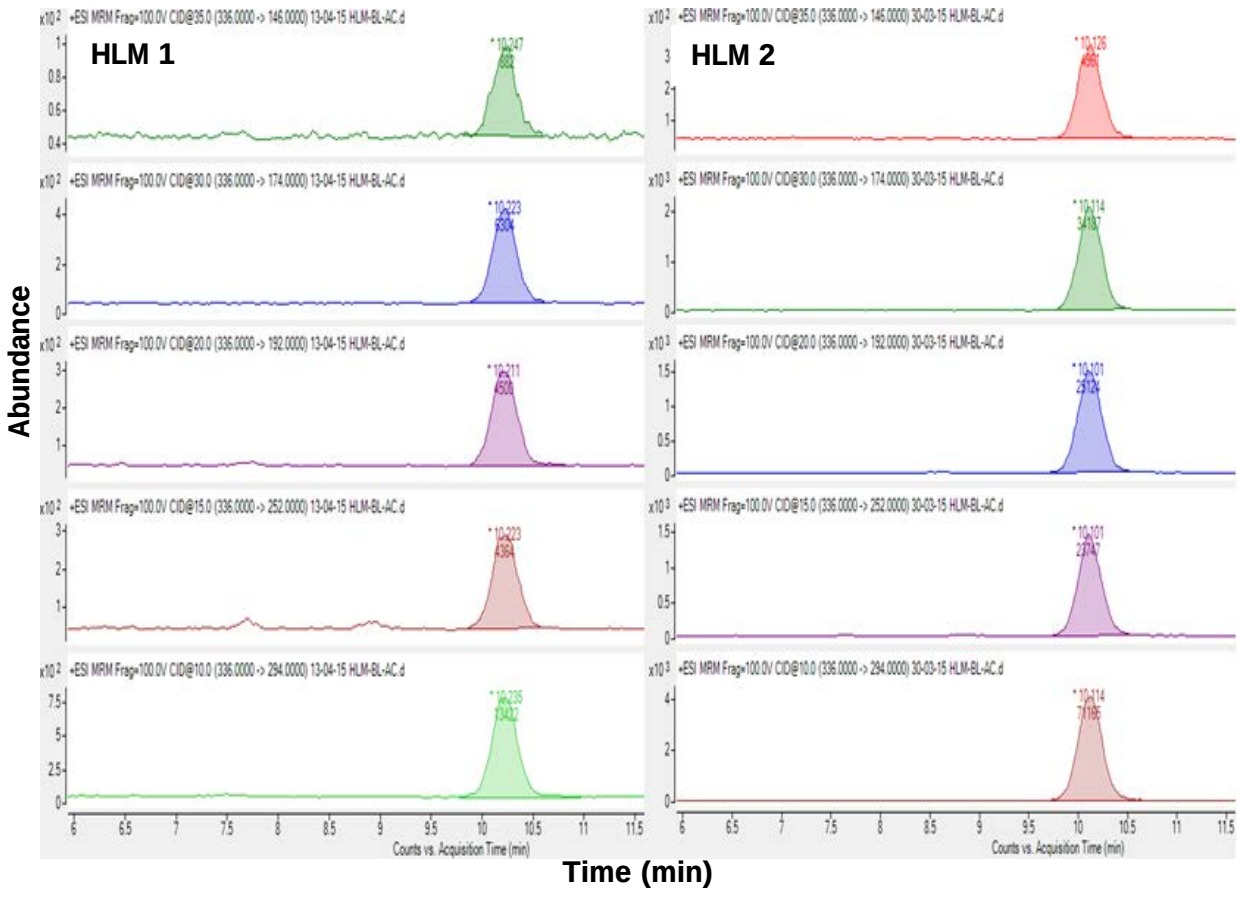


Figure 9-18: Specimen LC/MS/MS MRM chromatograms for di-OH-M-3Ac in HLM samples.

9.5.4.4 *In vitro* Β-OH and 4-OH-3-MeO metabolites of butylone identification

9.5.4.4.1 Predicted mass spectral fragmentation of Β-OH and 4-OH-3-MeO metabolites of butylone

Because the Β-hydroxy-butylone and 4-OH-3-MeO butylone metabolites are structural isomers, predicted LC/MS/MS fragmentation patterns of both acetate metabolites contain quite similar fragment ions.

The molecular ion of Β-OH-M-2Ac would be at *m/z* 308 and the product ions from the loss of ketene groups would be at *m/z* 266 and 224. A product ion at *m/z* 248 was predicted from the loss of one ketene and H_2O. A predicted fragment ion at *m/z* 206 would come from the loss of H_2O from the Β-OH-metabolite itself ($M\text{-}H^+$, *m/z* 224). Other product ions at *m/z* 174, 162 and 134 were chosen from predicted fragmentation patterns.

The molecular ion of 4-OH-3-MeO-M-2Ac would be at *m/z* 308. The product ions in the mass spectrum of the 4-OH-3-MeO-M-2Ac from the loss of two molecules of ketene would be at *m/z* 248 and 224. Other product ions at *m/z* 206, 174, 162 and 134 were predicted following Zaitsu *et al.*[74] The product ion at *m/z* 206 would come from the loss of H_2O from the 4-OH-3-MeO-M itself ($M\text{-}H^+$, *m/z* 224).

The predicted ESI-LC/MS/MS fragmentation patterns of the acetate derivatives of Β-hydroxy metabolite 4-OH-3-MeO-M are summarised in Table 9-5 and shown in Figure 9-19. The table also indicates which ions were subsequently detected in HLM samples and three of these MRM transitions that provide high response peak areas were chosen for the identification method, described in Section 9.4.4.2 above.

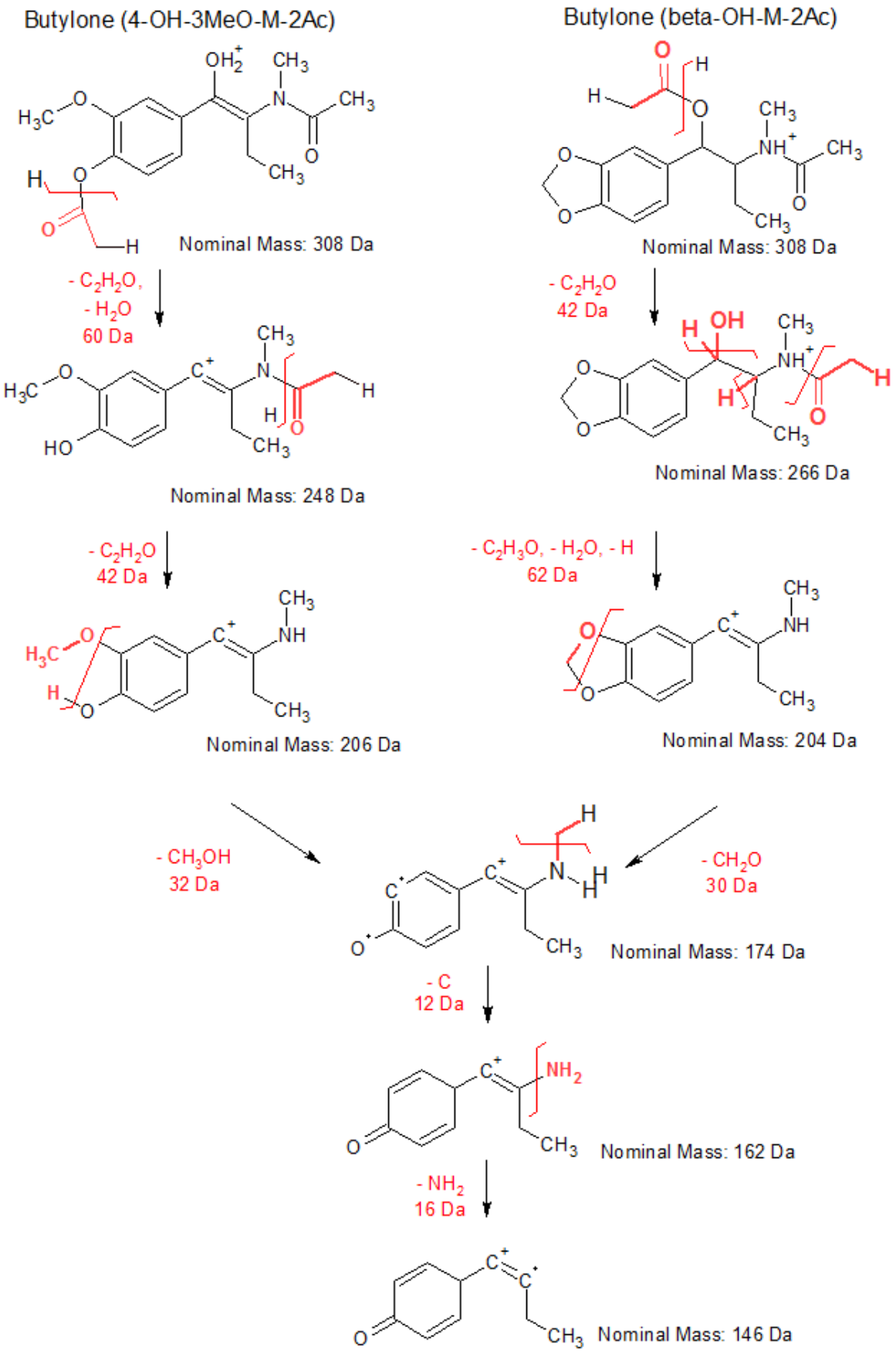


Figure 9-19: Predicted ESI LC/MS/MS fragmentation patterns of acetate derivatives of butylone metabolites (4-OH-3-MeO-M-2Ac and *β*-OH-M-2Ac). Fragmentations are marked in red. Hydrogen transfers are indicated by an "H" adjacent to the bond which is broken.

Table 9-5: The predicted ESI-LC/MS/MS fragmentation patterns of *β*-hydroxy and 4-OH-3-MeO metabolites of butylone.

Compound	M-H$^+$ (*m/z*)	Product ion (*m/z*)	Predicted losses		Detection in HLM sample
			Da	Possible composition	
β-OH-M-2Ac & 4-OH-3-MeO-M	308	266	-42	$COCH_2$	N
		248	-18	266-H_2O	Y
		224	-42	266–$COCH_2$ (2Ac)	N
		206	-18	224-H_2O, Ref*	N
		174	-32	CH_3OH, Ref*[74]	Y
		162	-12	C, Ref*[74]	Y
		146	-16	NH_2, Ref*[74]	Y

Ref* = predicted from literature data
Bold are selected ions

9.5.4.4.2 Identification of *β*-OH and 4-OH-3-MeO metabolites of butylone in HLM samples

The ketone-reduction metabolite of butylone has been identified and reported by Zaitsu *et al.*[74,138] and Meyer *et al.*[126] The acetate derivatives of the *β*-hydroxy metabolite (*β*-OH-M-2Ac) and 4-hydroxy-3-methoxy-metabolite (4-OH-3MeO-M-2Ac) were identified in extracts by MRM transitions at *m/z* 308>248, 308>174, 308>162 and 308>134. The corresponding peaks for the molecular ion at *m/z* 308 were detected in HLM samples at approximately 8.8 and 10.3 min. The MRM transition at *m/z* 308>248 gave the highest corresponding peak area in all HLM samples, followed by the transitions at *m/z* 308>174, 308>162 and 308>134, respectively. LC/MS/MS MRM chromatograms of *β*-OH-M-2Ac and 4-OH-3MeO-M-2Ac in HLM samples are shown in Figure 9-20.

As mentioned above in Section 9.5.4.4.1, the 4-hydroxy-3-methoxy-metabolite and the *β*-hydroxy metabolite of butylone are structural isomers. MRM transitions set up for determination of *β*-OH-M-2Ac and 4-OH-3MeO-M-2Ac were detected in all HLM samples, with the highest corresponding peak area amongst the three metabolites. Also, as mentioned in Section 9.5.3.5 for the identification of *β*-hydroxy-methylone metabolite in HLM samples and concerning MDMA-HLM results in which only a trace of HMMA was detected, all information obtained in these studies supported the conclusion that the peak detected at 10.2 min was the ketone-reduction metabolite rather than the 4-

hydroxy-3-methoxy-metabolite. The earlier peak eluted at 8.8 min was expected to be 4-OH-3MeO-M-2Ac. However, confirmation of *β*-OH-Met and 4-OH-3-MeO-Met will depend on the availability of authentic reference standards.

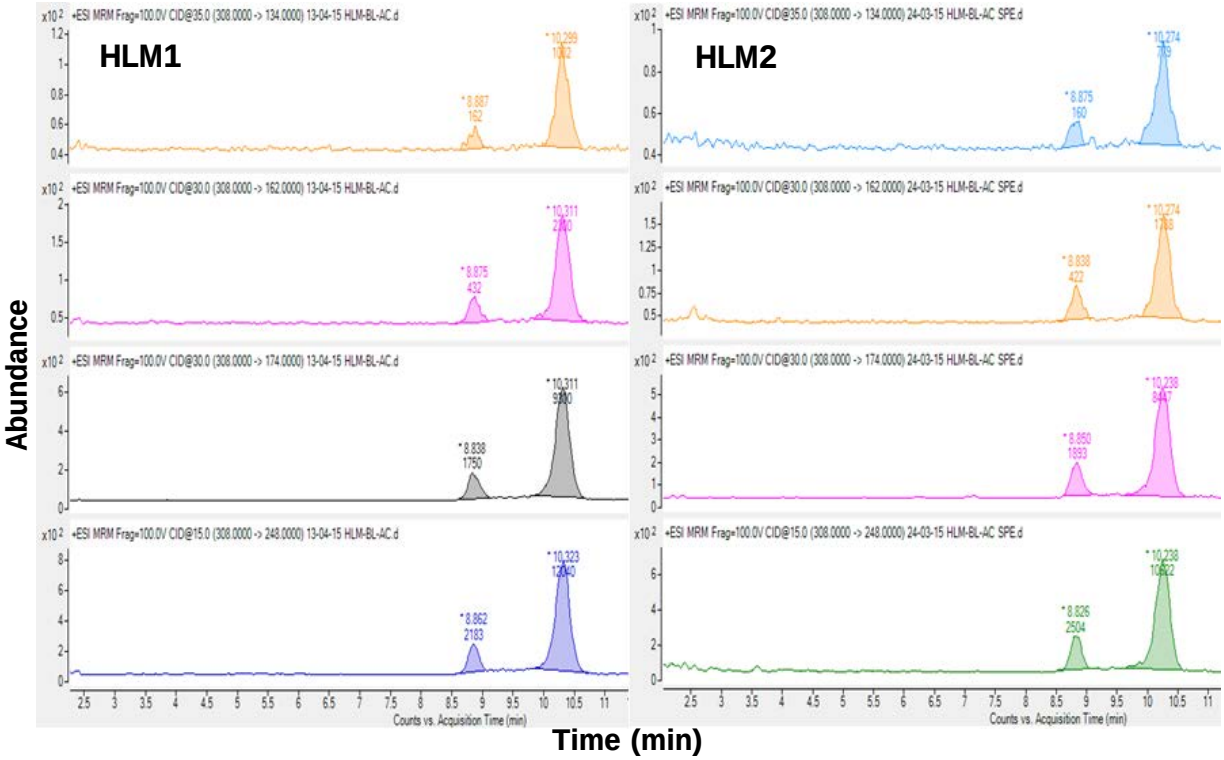


Figure 9-20: Specimen LC/MS/MS MRM chromatograms for *β*-OH-M-2Ac (at 10.3 min) and 4-OH-3MeO-M-2Ac (at 8.8 min) in two HLM samples.

9.6 Conclusions

Knowledge of the metabolic pathways of methylone and butylone is important for method development in forensic toxicology analysis, especially for the analysis of blood and urine specimens. This study has confirmed that human liver microsomes can be used to simulate drug metabolism in humans and to provide chromatographic and mass spectrometric data on drug metabolites. The analytical method for metabolites was developed based on reviewed data and MRM transitions predicted from drug chemical structures which achieved an adequate sensitivity for the analysis and identification of methylone, butylone and their metabolites in HLM samples.

In summary, the results show that ketone-reduction and demethylenation are major phase I metabolic pathways of methylone and butylone in humans whereas

N-demethylation is a minor pathway. To our knowledge, this is the first method designed for the identification of the dihydroxy metabolite of butylone. However, both methylone and butylone major metabolites (ketone-reduction and 4-OH-3-MeO metabolites) are structural isomers. To apply this method for routine analysis, reference standards of both metabolites are required. Finally, this method could be developed and applied as a routine quantitative method to detect and confirm abuse of butylone and methylone in authentic samples. Future studies *in vitro* and/or *in vivo* on phase II metabolites of *β*-keto-amphetamines are required.

Chapter 10 *In vitro* metabolism study on PMA using human liver microsomes and LC/MS/MS with chemical derivatisation

10.1 Introduction

para-Methoxyamphetamine (PMA), a classic amphetamine-type designer drug, has been implicated in deaths since the 1970s[101,113]. PMA is a toxic mono-methoxy ring-substituted amphetamine derivative with strong stimulant and hallucinogenic properties. PMA has been controlled in the UK since 1977 and was added to the 1971 UN Convention on Psychotropic Substances in 1986[175]. Although studies of PMA have been carried out since the late 1970s, few metabolism studies of PMA have been reported. Moreover, deaths related to PMA alone or PMA in conjunction with other drugs such as MDMA have been reported until 2013. In 1998, six deaths related to PMA were reported in Australia[114]. Three years later, three fatalities were reported in the Midwest of the United Stated of America[106] and three cases of PMA overdose were reported in Canada[115]. In 2012, the National Programme on Substance Abuse Deaths reported that the number of UK deaths from "Legal highs" had risen to at least 68 cases and that 17 of these were linked to PMA[175]. Moreover, seven deaths linked to fake ecstasy containing PMA were reported in Scotland in 2013[176].

A few previous metabolism studies of PMA have shown that the major metabolic pathway in humans is *O*-demethylation to 4-hydroxyamphetamine (PHA) which is then partially conjugated. A minor metabolic pathway is beta-hydroxylation to *β*-hydroxy-PMA, followed by *O*-demethylation to 4-hydroxynorephedrine[109]. Furthermore, PHA is also found as an important metabolite of both amphetamine and methamphetamine[177-179]. Identification and confirmation of PMA is included in routine analysis of the amphetamines group in forensic toxicology laboratories. However, routine analysis of PMA has regularly detected only PMA itself in both blood and urine samples[180,181]. To confirm the misuse of PMA from urine samples in some cases such as in workplace drugs testing, PMA metabolites

need to be identified and confirmed. Moreover, Hubbard *et al.*[182] reported that PHA was detected in monkey urine samples at concentrations higher than those of PMA itself 24 h after administration of a single dose of 0.75 mg/kg. This result has shown that the simultaneous analysis of PMA and PHA in biological samples is more useful than analysis of PMA alone.

10.2 Aims

Although methods for quantitative analysis of PMA have been published, most methods for the identification of drugs in the amphetamines group are based on GC/MS. Few LC/MS methods have been developed for quantitative analysis of PMA and its metabolites (PHA, *β*-hydroxy-PMA and 4-hydroxy-norephedrine). The primary aim of the present study was to identify the metabolites of PMA in humans with the assistance of an *in vitro* metabolism model using human liver microsomes. Moreover, there have been no studies performed for identification of *in vitro* metabolites of these drugs using triple quadrupole LC-MS. Then, the second aim of this study was to develop a reliable, sensitive, and selective method for identification of PMA and its metabolites in biological samples by reversed-phase LC/MS/MS with chemical derivatisation.

10.3 Review of previous analytical methods for the determination of PMA and its metabolites

Early analytical methods for PMA were reported and published in the late 1970s, all based on GC/MS. Previous analytical methods for identification and quantification of PMA and its metabolites in biological samples are reviewed below and summarised in Table 10-1.

A metabolism study of PMA in humans was first reported and published in 1971 by Schweitzer *et al.*[183] In humans, little PMA was reported to be excreted in unchanged form following oral administration. In 1974, Beckett *et al.*[184] described the identification of four metabolite products of PMA in rabbit, pig and rat liver fractions: 1-(*p*-methoxyphenyl)-propan-2-one, 1-(*p*-methoxyphenyl)-propan-2-ol, 1-(*p*-methoxyphenyl)-propan-2-one oxime and *N*-

hydroxy-*p*-methoxy-amphetamine were determined using TLC, GC/FID and GC/MS. All metabolites were separated using three different GC columns and detected with a flame ionisation detector and mass spectrometry in full scan mode at an electron energy of 70 eV. 1-(*p*-Methoxyphenyl)-propan-2-one oxime was found as a decomposition product of *N*-hydroxy-*p*-methoxyamphetamine.

Hubbard *et al.*[182] described the *in vitro* metabolism of PMA in the dog and monkey using GC/MS with trifluroacetyl derivatisation. PHA and *p*-hydroxybenzoic acid (PHBA) were separated using a silanised spiral glass column (1.8 m x 2.0 mm i.d.) packed with 5% OV-225 on 80-10 mesh Chromosorb W-AW-DMSC and detected with FID and MS. *O*-Demethylation was found to be an important process in the *in vitro* biotransformation of PMA in the dog and monkey. Only 3% of unchanged PMA was excreted in the monkey and the excretion of PHA was 44% of the dose in 24 h.

In 1979, Kitchen *et al.*[109] described a detection method for PMA and its metabolites in humans using a radiochemical method. PHA and hydroxy-norephedrine were synthesised and used as analytical standards. PHA (free and conjugated) was found as a major metabolite and 4-hydroxy-norephedrine as a minor metabolite in urine.

In 2015, Lai *et al.*[110] described a liquid chromatography-quadrupole/time-of-flight mass spectrometry (QTOF-MS) method for screening *in vitro* drug metabolites of seven phenethylamine-based designer drugs in humans, including PMA. The HLM method consisted of 100 mM phosphate buffer pH 7.4, HLM 0.5 mg/ml and substrate. The mixture was pre-incubated at 37°C for 5 min and the reaction was initiated by addition of 10 µl of NADPH solution and incubated for 2 h. The reaction was stopped by adding ice-cold acetonitrile. Analytes were separated using a C8 Zorbax Eclipse Plus column (2.1 mm x 150 mm, 3.5 µm) and identified using QTOF-MS in scan mode over the range *m/z* 50 - 1000. Only the *para*-hydroxy-metabolite (PHA) was found in phase I metabolism of PMA in HLM samples. The molecular ion of PHA was detected at *m/z* 152.1063 and major fragment ions were at *m/z* 135.0799, 107.0495, 91.0552, 77.0390 and 65.0364.

Table 10-1: Previous GC/MS and LC/MS methods for PMA and its metabolites.

Analytes	Matrices	Chromatography	Extraction Method	Year
1-(*p*-Methoxyphenyl)-propan-2-one, 1-(*p*-Methoxyphenyl)-propan-2-ol, 1-(*p*-Methoxyphenyl)-propan-2-one oxime, *N*-hydroxy-*p*-methoxyamphetamine	Rabbit, pig, rat liver fraction	**TLC** **GC-FID and GC/MS** Column I: Gas Chrom Q (1 m x 6.4 mm o.d.) Column II: Gas Chromsorb W (2 m x 6.4 mm o.d.) Column III: Gas Chrom W (2 m x 6.4 mm o.d.)	Incubated PMA with liver LLE: diethyl ether (3x3 ml) Reduction with $TiCl_3$	1974[184]
PHA, *p*-hydroxybenzoic acid (PHBA)	Dog and monkey urine	**GC/MS** Column: silanised spiral glass column (1.8 m x 2.0 mm i.d.) packed with 5%OV-225 on 80-10 mesh Chromosorb W-A W-DMSC	Enzymatic and acid hydrolysis LLE: Methylene Chloride Derivatised with TFA	1977[182]
PMA, PHA	HLM	**LC-TOF-MS** Column: C8 Zorbax Eclipse Plus column (2.1 mm x 150 mm, 3.5 µm) Mobile phase: gradient of 5 mM ammonium acetate in ultrapure water and acetonitrile Flow Rate: 0.2 ml/min	HLM Incubation	2015[110]

10.4 Methods and materials

10.4.1 Reagents and standards

A standard of PMA hydrochloride was obtained from Sigma-Aldrich Co, LLC (UK). A standard of 4-hydroxy-3-methoxyamphetamine-HCl (HMA) was purchased from Lipomed (supplied by Kinesis, Ltd UK). All other reagents were the same as those described in Section 8.6.1.

10.4.2 *In vitro* assay (human liver microsomes)

This was the same as described in Section 8.6.2.

10.4.3 Derivatisation

This was the same as described in Section 9.4.3.

10.4.4 Liquid chromatography/mass spectrometry conditions

10.4.4.1 Liquid chromatography conditions

Chromatographic separation was performed using an Agilent Binary SL 1200 LC from Agilent Technologies Inc. Isocratic elution was performed on a Gemini C18 column (150 x 2.0 mm, 5 µm) protected by a Gemini C18 Security Guard Pre-Column (4 x 2.0 mm, 5 µm, Phenomenex Inc.), which was maintained at 40ºC with a flow rate of 0.35 ml/min. The injection volume was 20 µl and the mobile phase was composed of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (67: 33 v:v) giving a run time of 10 min per injection.

10.4.4.2 Mass spectrometry conditions

Analysis of PMA and its metabolites was performed using an Agilent 6410 Triple Quad LC/MS/MS instrument (Agilent Technologies Inc.) equipped with an Agilent Binary SL 1200 LC system interface. Nitrogen was from a nitrogen generator (In House Gas (Manufacturing) Ltd, UK).

Ionisation of analytes of interest was carried out using ESI. The mass spectrometer was operated in the positive ion multiple reaction monitoring (MRM) mode. Nitrogen was used as nebuliser gas at 30 psi. The gas temperature was set to 350°C and the gas flow to 11 ml/min. The MRM transitions, FVs and CEs for the acetate derivative of PMA were optimised by direct infusion of acetate solutions of derivatised standards at a concentration of 5 µg/ml in mobile phase (50:50 v:v) into the MS. All analytes were identified and quantified based on their retention times and quantifier and qualifier ion ratios obtained from the MRM transitions.

The MRM transitions, FVs and CEs used for the measurement of PMA and its metabolites are summarised in Table 10-2.

Table 10-2: MRM conditions for acetate derivatives of PMA and its metabolites.

Name	Precursor Ion (*m/z*)	Fragmentor Voltage (eV)	Product Ions (*m/z*)	Collision Energy (eV)
PMA-Ac	208	90	149	5
			121	20
			91	30
PHA-Ac	236	90	194	5
			135	20
			107	40
4-HMA-2Ac	266	90	165	20
			137	30
			105	40
β-OH-PMA-2Ac	266	95	224	10
			165	20
			91	40

10.5 Results and discussion

10.5.1 Optimisation of LC/MS conditions

10.5.1.1 LC separation

The LC method used for detection of MDMA and its metabolites was used for the analysis of PMA and its metabolites in HLM samples. To separate Ac-derivatives

of PMA and its metabolites, LC separation was applied on a Gemini C18 column with isocratic elution with an organic solvent containing 0.1% formic acid in aqueous acetonitrile at flow rate of 0.35 ml/min. The retention factor was 4.2 for PMA-Ac and the hold-up time (t_0 = 0.8 min) was determined by injecting pure methanol. Figure 10-1 shows the chromatogram for a standard of PMA-Ac on the Gemini C18 column. However, the MS-MRM method needed to be developed.

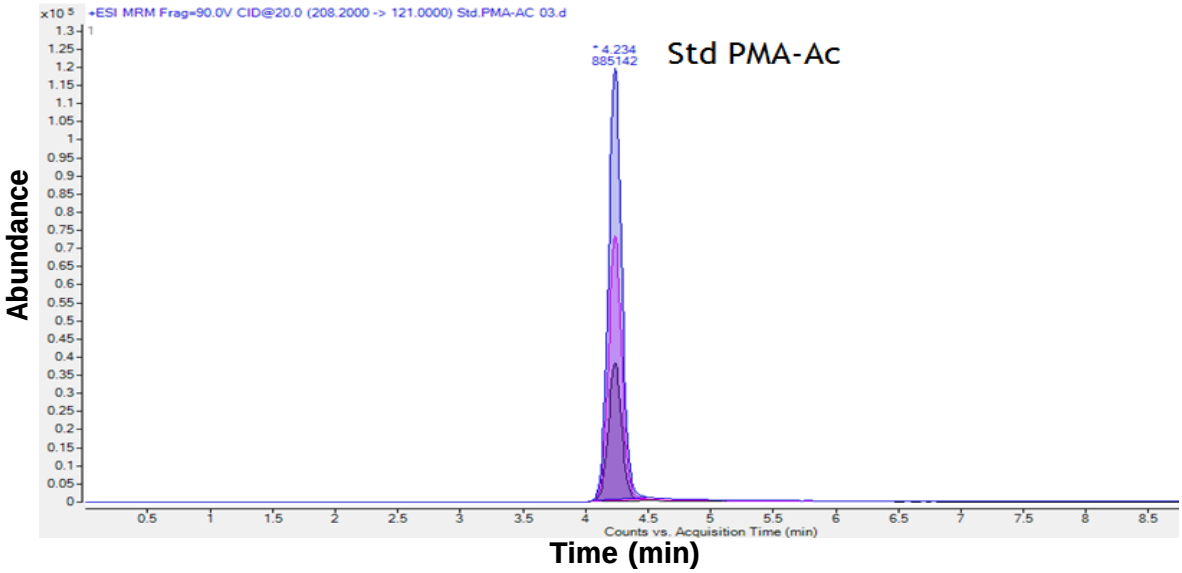


Figure 10-1: LC/MS/MS MRM chromatograms of a PMA-Ac standard on a Gemini C18 column.

10.5.1.2 MRM optimisation

To optimise the MRM conditions, PMA standard was derivatised with acetic anhydride and re-dissolved in methanol. The Ac-derivative of PMA was injected directly into the mass spectrometer interface using a syringe pump with positive ESI to obtain the precursor ions, product ions, FVs and CEs. Because of unavailability of reference standards of PMA metabolites, a minimum of four identification points (IPs) was required for evaluating PMA metabolites. One quantifier and two or three qualifier ions were used to identify target analytes. FVs and CEs of derivative PMA metabolites were developed based on derivatised standard PMA.

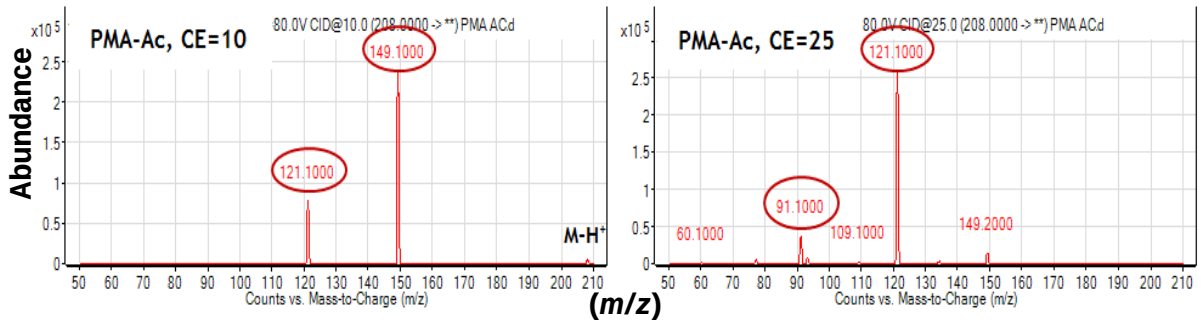


Figure 10-2: LC/MS/MS product ion scan spectra of the Ac-derivative of PMA standard at CE of 10 and 25 eV. The optimum CE values for each ion are listed in Table 10-2.

Figure 10-2 shows the LC/MS/MS product ion scan spectra of the Ac-derivative of PMA standard. When the CE was 10 eV, the mass spectrum of PMA-Ac had two large peaks at *m/z* 149 and 121 and a small peak for the protonated molecule (*m/z* 208, M-H$^+$). At a higher CE of 25 eV, the fragment ion peak at *m/z* 149 was decreased and the peaks at *m/z* 121 and 91 were increased. The optimised CEs values for PMA-Ac which were finally selected are listed in Table 10-2. The LC/MS mass spectral fragmentation of PMA-AC is shown in Figure 10-3.

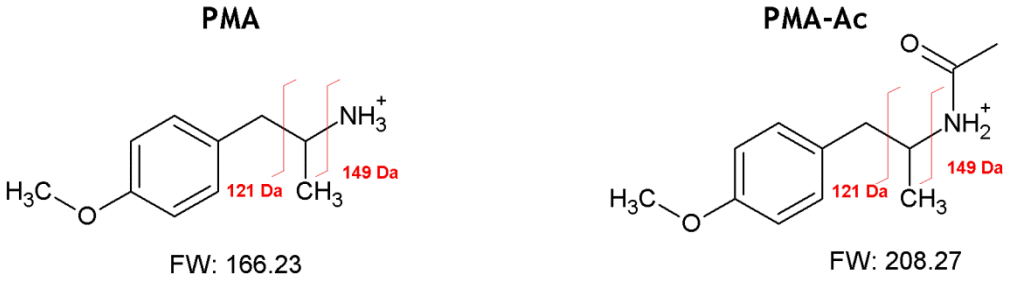


Figure 10-3: Mass spectral fragmentation of PMA and PMA-Ac.

After MRM optimisation, the protonated molecular ion of PMA-Ac at *m/z* 208 was used as a precursor ion and its fragment ions at *m/z* 149, 121 and 91 were selected as product ions. When the optimised CE values were used, the most intense product ion peak was at *m/z* 121 and was chosen as a quantifier ion and the ratio of qualifier/quantifier was approximately 61% for *m/z* 149 and 32% for *m/z* 91.

10.5.2 Predicted mass spectral fragmentation of PMA metabolites

O-Demethylation of PMA to PHA has been reported as a primary metabolic pathway whereas *β*-hydroxylation of the side chain to *β*-hydroxy-PMA, followed by loss of the methyl group to give 4-hydroxy-norephedrine has been reported as a minor metabolic pathway of PMA in humans[109]. PHA was expected to be the major metabolite and *β*-hydroxy-PMA (*β*-OH-PMA) a minor metabolite of PMA resulting from metabolism by human liver microsomes. However, PHA and *β*-OH-PMA were commercially unavailable. In order to identify both metabolites in HLM samples by LC/MS/MS, the mass spectral characteristics of acetate-derivatives of PHA and *β*-OH-PMA were predicted based on the mass spectral characteristics obtained from the acetate derivative of PMA. Moreover, possible multiple reaction monitoring (MRM) ions were reviewed and compared with mass spectral characteristics of synthesised reference standards of PHA described in previous studies[110,185].

Although metabolism studies of PMA have been published from the late 1900s, all of them were restricted to GC/MS analysis. PMA has some metabolites similar to amphetamine/methamphetamine such as PHA and 4-hydroxynorephedrine and some similar to MDMA such as 3- or 4-HMA[104,186,187]. However, not all amphetamine metabolites are included in routine analysis and basic analytical methods for amphetamine-type stimulants (ATS) by GC/MS. Only a few LC/MS methods for the determination of PMA and other ATS have been published. Hendrickson *et al.*[185] reported a quantitative method for the determination of methamphetamine and its metabolites by LC/MS. A standard of PHA, one of the active metabolites of methamphetamine, was obtained from NIDA and was analysed by LC/MS with MRM at *m/z* 152>107. Lai *et al.*[110] also investigated PMA metabolites produced by HLM with liquid chromatography-quadrupole time-of-flight mass spectrometry. PHA was found in HLM samples and was identified with a precursor ion at *m/z* 152.1070 ($M\text{-}H^+$) and product ions at *m/z* 135.0810, 107.0497, 91.0548 and 77.0386.

As described in Sections 9.5.3 and 9.5.4, dealing with predicted butylone and methylone metabolites, the first fragmentation of acetate derivatives is the loss

of ketene ($COCH_2$, 42 Da). The molecular ion of PHA-2Ac would be at *m/z* 236 and the product ion from the loss of the ketene group would be at *m/z* 194. Other product ions at *m/z* 135 and 107 are expected based on the fragmentation of PMA-Ac and on reviewed data from Hendrickson *et al.* and Lai *et al.* The fragment ion at *m/z* 135 results from the loss of $[NH_2]^+$ from the side chain of PHA itself and next fragment ion at *m/z* 107 comes from the loss of 28 Da (C_2H_4) from the side chain. The predicted ESI-LC/MS/MS fragmentation pattern for the acetate derivative of PHA is shown in Figure 10-4.

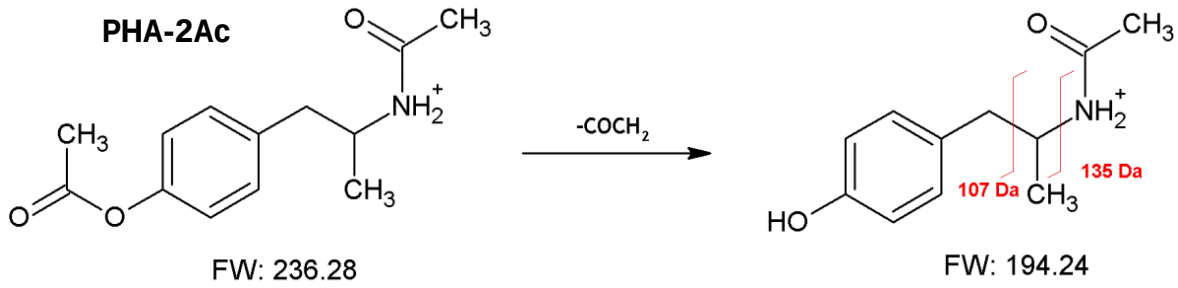


Figure 10-4: Postulated ESI LC/MS fragmentation pattern of PHA-2Ac.

For *β*-hydroxy-PMA, no information on the LC/MS mass spectrum and MRM transitions has been published before. The molecular ion of *β*-OH-PMA-2Ac would be at *m/z* 266 and the product ion from the loss of ketene would be at *m/z* 224. Predicted fragment ions are at *m/z* 165 and 147 from the loss of side chain of *β*-OH-PMA itself and *m/z* 91 that is expected based on the fragment ion of PMA-Ac. The predicted ESI-LC/MS/MS fragmentation pattern of *β*-OH-PMA-2Ac is shown in Figure 10-5.

Other possible PMA metabolites are 3´-hydroxy-4-methoxyamphetamine (3-HMA) and 4´-hydroxy-3-methoxyamphetamine (4-HMA). The MRM transitions for HMA-2Ac identification were predicted based on reference standard spectra as explained in Section 8.5.1.2. Each predicted fragment ion was subsequently included in the initial LC/MS/MS analysis of HLM samples to identify which metabolites were present and the fragment ions they actually produced.

Finally, an MRM method for the expected acetate derivatives of PMA and its metabolites was as summarised in Table 10-2, in Section 10.4.4.2 above.

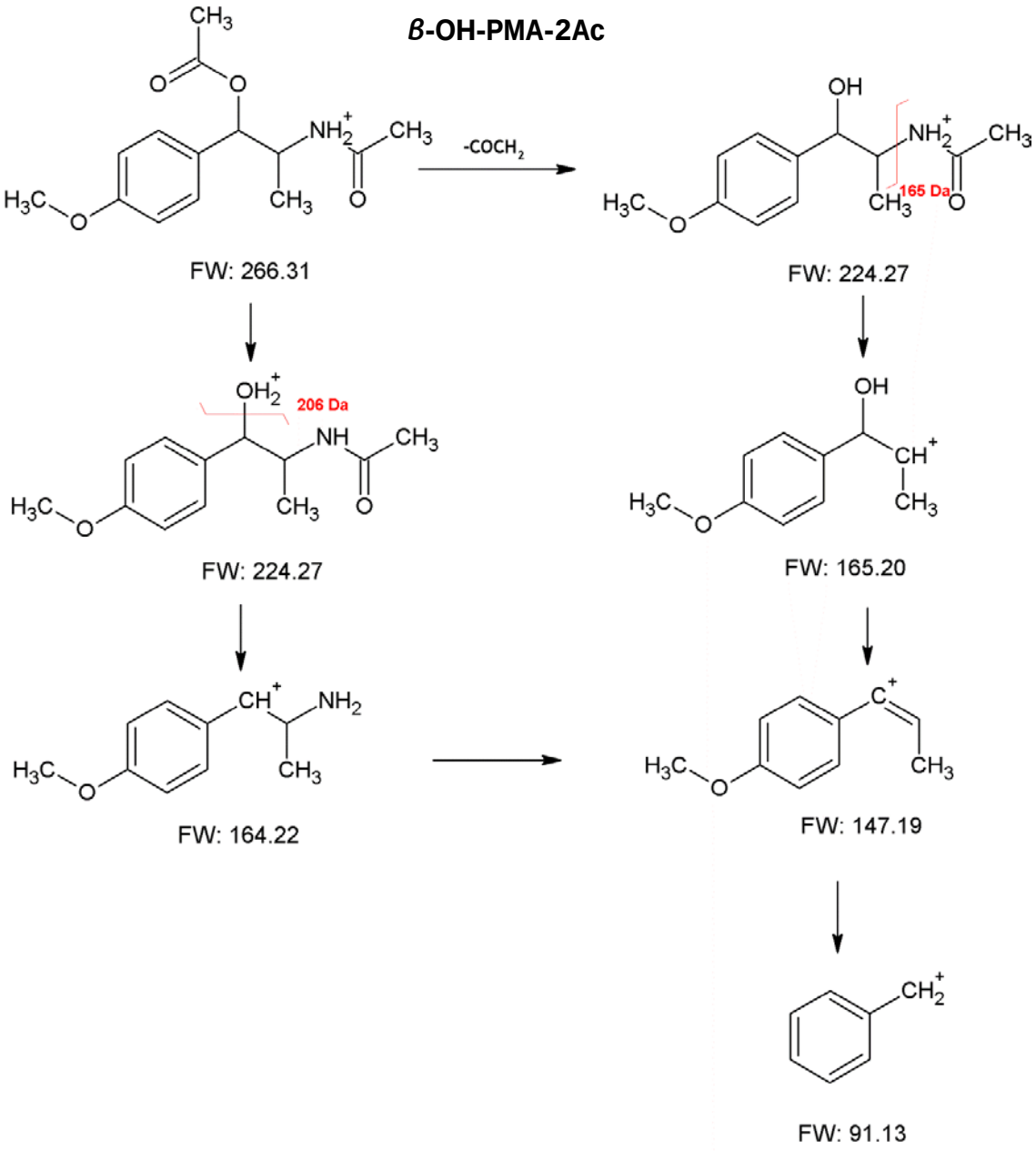


Figure 10-5: Postulated ESI LC/MS/MS fragmentation pattern of *β*-OH-PMA-2Ac.

10.5.3 *In-vitro* metabolism studies of PMA

Pooled human liver microsomes (HLM) were incubated with a PMA standard and extracted and analysed using the LC/MS/MS method described previously. PHA, the major metabolite of PMA, and *β*-hydroxy-PMA (a minor metabolite) were expected from the experimental procedure involving *in-vitro* metabolism with human liver microsomes. The acetate derivative of a PMA standard was identified at 4.2 min and confirmed by assessing the qualifier/quantifier ion ratios with ± 20% tolerance.

The derivative of un-metabolised PMA (PMA-Ac) was identified in extracts by MRM transitions at *m/z* 208>149, 208>121 and 208>91. The acetate derivative of PHA (PHA-2Ac, a major metabolite), was identified by MRM transitions at *m/z* 236>194, 236>135 and 236>107 and the acetate derivative of the *β*-hydroxy metabolite (*β*-OH-PMA-2Ac, a minor metabolite) was identified by MRM transitions at *m/z* 266>224, 266>165 and 266>91. Another metabolite, the acetate derivative of 4-hydroxy-3-methoxyamphetamine (4-HMA-2Ac) was identified by MRM transitions at *m/z* 266>165, 266>137 and 266>105.

Three phase I metabolites (both major and minor metabolites) of PMA were detected in HLM samples. The retention time and the mass ratios for PMA obtained from HLM samples were consistent with those observed for the standard. Residual PMA as its acetate derivative (PMA-Ac) was eluted at 4.2 min and was identified and confirmed. The qualifier/quantifier ion ratio was 61% for *m/z* 149 and 32% for *m/z* 91 which were in the acceptable range. LC-MS/MS MRM chromatograms of PMA-Ac in HLM samples compared with the Ac-derivative of a standard are shown in Figure 10-6.

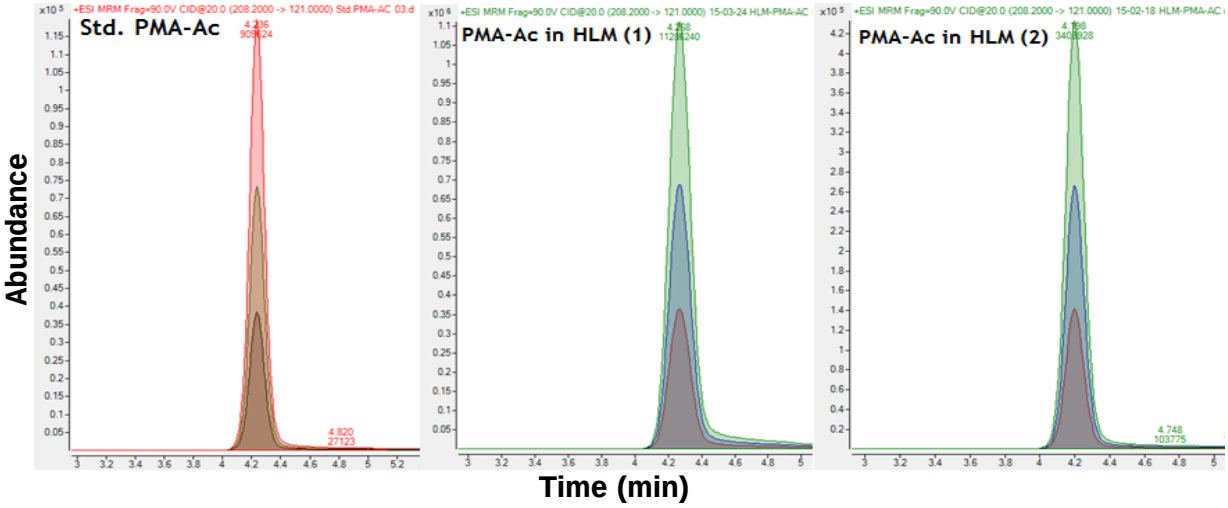


Figure 10-6: Examples of LC/MS/MS MRM chromatograms for PMA-Ac in two HLM samples compared with the Ac-derivative of a PMA standard.

Derivatives of both major and minor PMA metabolites were identified in HLM samples by the MRM transitions explained in Section 10.5.2. The derivative of PHA-2Ac was detected in HLM samples and was eluted at 3.7 min. The MRM at *m/z* 236>107 gave the highest corresponding peak area for PHA-2Ac in all HLM samples, followed by those at *m/z* 236>135 and 236>194.

Because the corresponding peak area of PHA in HLM samples gave quite a high response, it was possible to analyse HLM samples by LC/MS using product ion scan mode to fragment the target ion at *m/z* 236 with FV at 90 eV and CE at 20 eV. LC/MS ESI spectra obtained at 3.64 min showed the fragmentation pattern of PHA-2Ac that correlated with the predicted PHA-2Ac fragmentation in Section 10.5.2. The LC-MS spectrum of PHA-2Ac in HLM samples is shown in Figure 10-8 and LC-MS/MS MRM chromatograms of PHA-2Ac in HLM samples are shown in Figure 10-7.

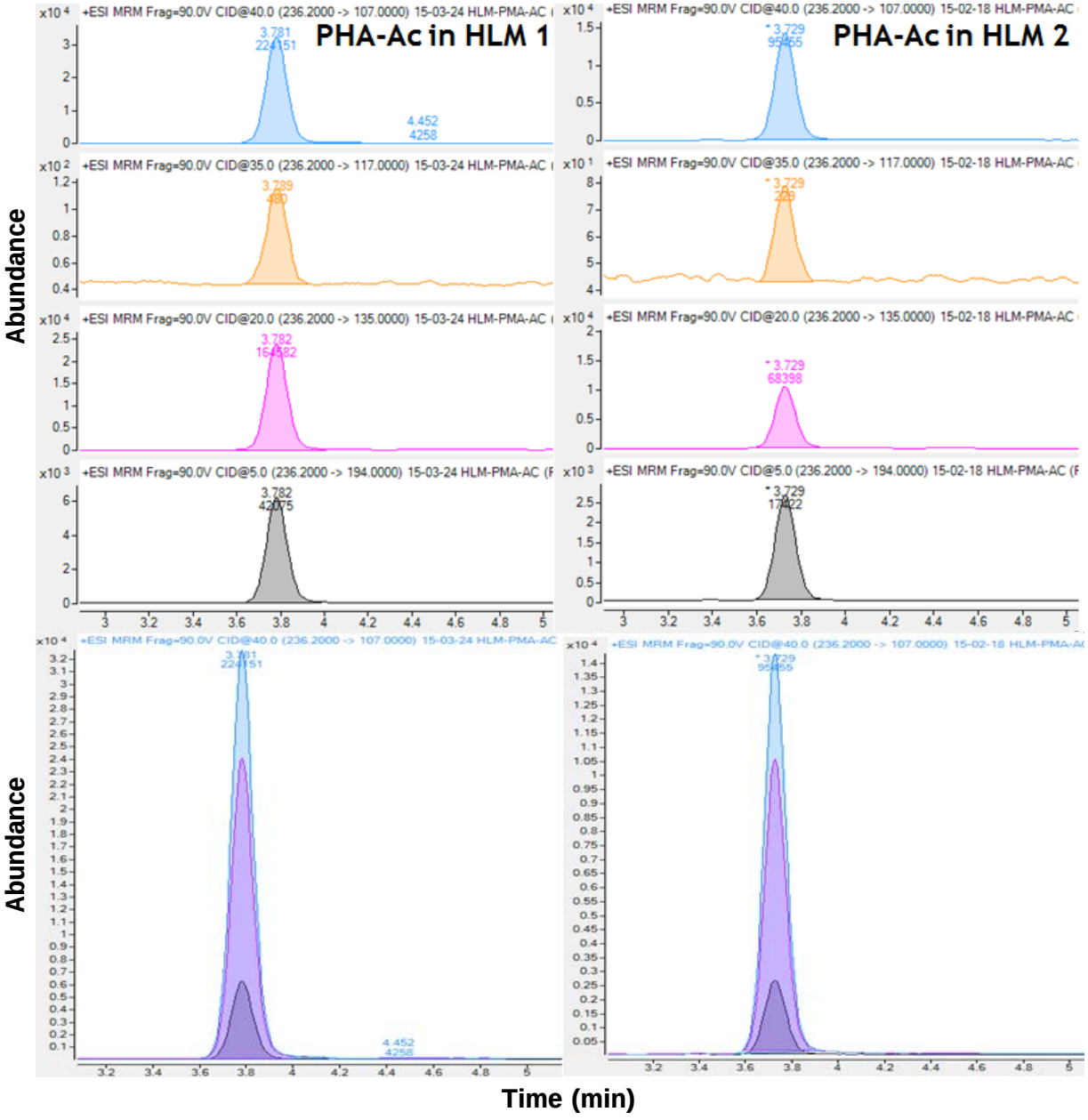


Figure 10-7: Examples of LC/MS/MS MRM chromatograms for PHA-2Ac in two HLM samples.

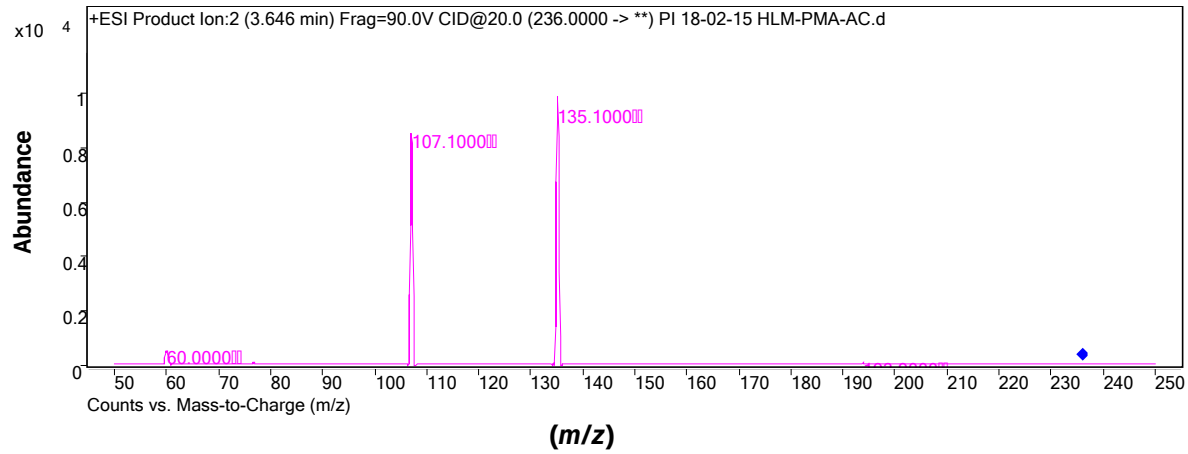


Figure 10-8: Mass spectrum of the Ac-derivative of PHA-2Ac in HLM samples at CE of 20 eV in product ion scan mode.

The acetate derivative of the *β*-hydroxy metabolite of PMA (*β*-OH-PMA-2Ac) was detected in HLM samples and was eluted at approximately 2.6 min. The transition at *m/z* 266>91 gave the highest corresponding peak area for *β*-OH-PMA-2Ac in all HLM samples, followed by those at *m/z* 266>224 and 266>165. LC/MS/MS MRM chromatograms for *β*-OH-PMA-2Ac in HLM samples are shown in Figure 10-9.

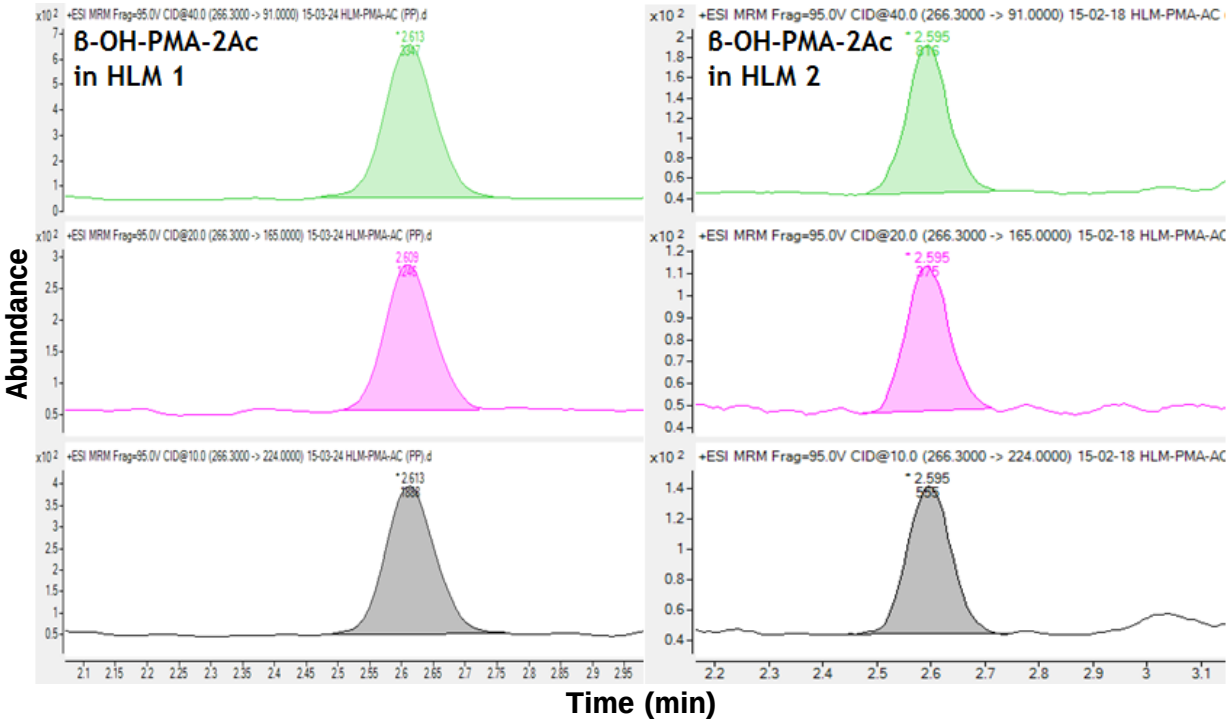


Figure 10-9: Examples of LC/MS/MS MRM chromatograms for *β*-OH-PMA-2Ac in HLM samples.

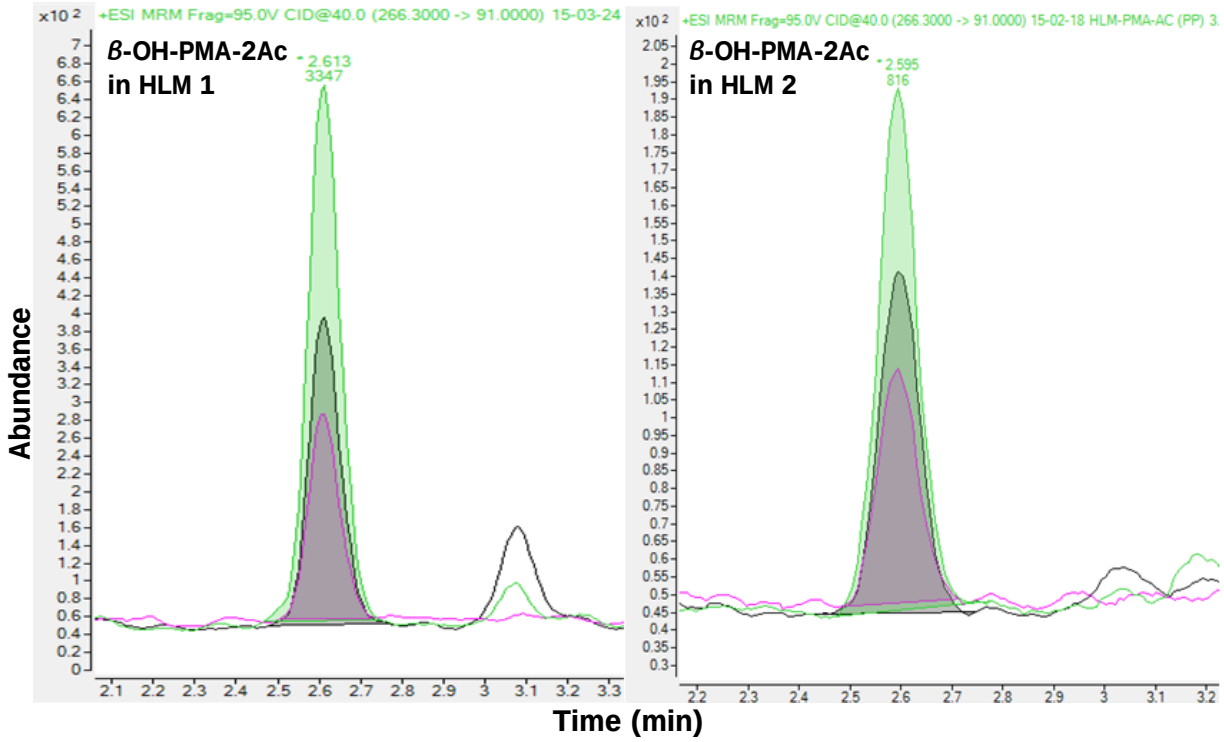


Figure 10-9 (cont.): Examples of LC/MS/MS MRM chromatograms for *β*-OH-PMA-2Ac in HLM samples.

The acetate derivative of a 4-HMA-2Ac standard was identified at a retention time of 3.8 min. The ion ratios obtained for the 4-HMA-2Ac standard were 52% for *m/z* 137 and 57% for *m/z* 105. 4-HMA-2Ac was also detected in HLM samples at 3.7 min. To confirm the detection of 4-HMA-2Ac, quantifier/qualifier ion ratios needed to be assessed. The ion ratio obtained from HLM samples was 74% for *m/z* 137 and 80% for *m/z* 105 which were out of the acceptable range (±20%). However, peaks corresponding to 4-HMA-2Ac were small and this can affect the ion ratios. Moreover, 4-HMA has an isomer, 3-HMA, the standard of which is not commercially available. The peak corresponding to MRM transitions at *m/z* 266>165, 266>137 and 266>105 may be 3-HMA rather than 4-HMA. The peak might also be a mixture of the two. So, finally, the peak eluting at 3.7 min cannot be confirmed as 4-HMA alone. LC/MS/MS MRM chromatograms for the possible metabolite 4-HMA-2Ac in an HLM sample compared with a 4-HMA-2Ac standard are shown in Figure 10-10.

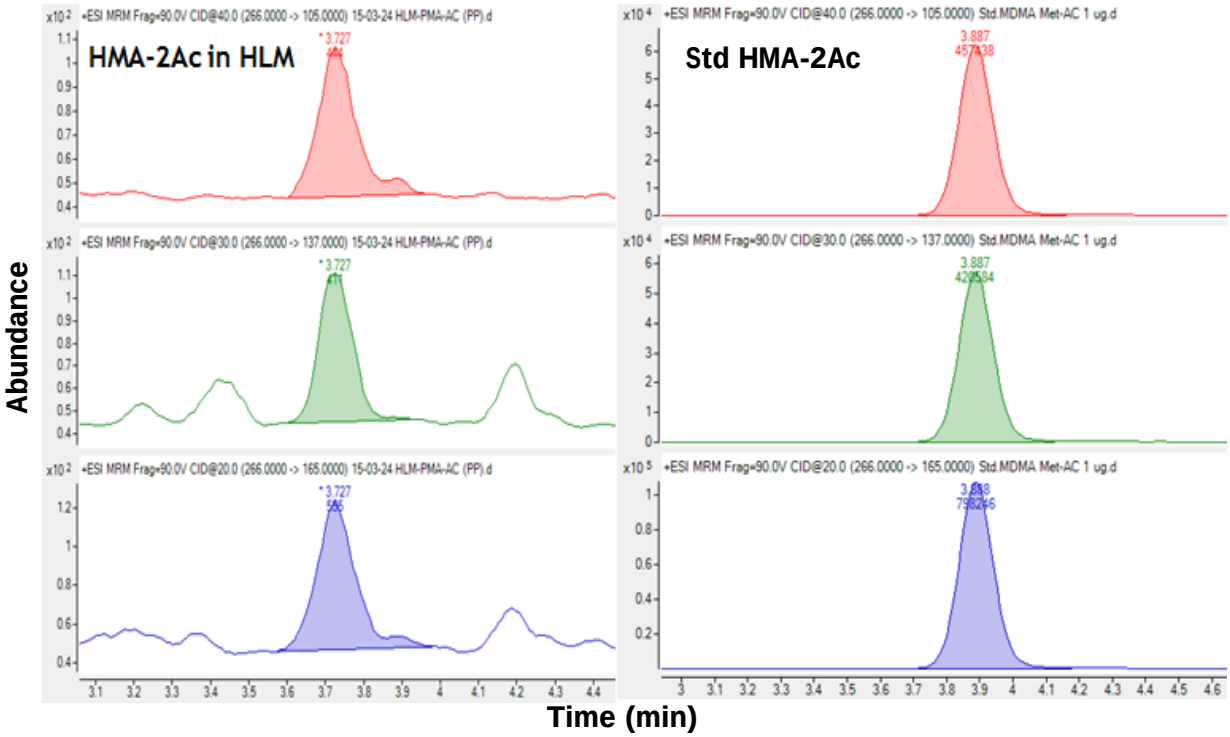


Figure 10-10: Examples of LC/MS/MS MRM chromatograms for 4-HMA-2Ac in an HLM sample compared with the 4-HMA-2Ac standard.

10.6 Conclusions

This chapter describes an *in vitro* metabolism study of PMA using human liver microsomes with LC/MS/MS analysis. The results show that loss of the methyl group from the 4-position is a major phase I metabolic pathway and *β*-hydroxylation and COMT-methylation to 4-HMA/3-HMA are minor metabolic pathways of PMA in humans. To our knowledge, this is the first LC/MS method designed for identification and confirmation of both major and minor metabolites of PMA (PHA, *β*–hydroxy PMA and 4-HMA/3-HMA). The LC/MS method described in Section 10.4.4 could be applied and used in routine analysis for the determination of PMA and its metabolites in biological samples such as blood and urine. However, reference standards of all metabolites are required. Future *in vivo* studies on phase II metabolites of PMA are required.

Chapter 11 Formaldehyde/formalin

11.1 Chemical properties

Formaldehyde is an important precursor in many chemical industries and has many uses, including as an embalming fluid for tissue-fixation in pathology and anatomy labs. Formaldehyde was first synthesised in 1859 by Aleksandr Butlerov and its structure was identified as an aliphatic aldehyde in 1867 by August Wilhelm von Hofmann[188]. The chemical formula of formaldehyde is CH_2O with a relative molecular mass of 30.03, see Figure 11-1. It is a colourless flammable gas at room temperature and has a characteristic, irritating aroma. Formaldehyde dissolves rapidly in water, alcohols, and other polar fluids. It is a highly reactive substance and breaks down at 150^{o}C into methanol and carbon monoxide. The solid form of formaldehyde is $(CH_2O)_3$, commercially known as trioxane or paraformaldehyde, which is a mixture of polymers of formaldehyde that contains 91 percent formaldehyde or more. Solid paraformaldehyde is commonly used for room disinfection by slow release of formaldehyde vapour[189-191].

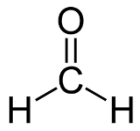


Figure 11-1: Chemical structure of formaldehyde.

Formalin is the name of commercial solutions of formaldehyde gas in water that have been used as a chemical disinfectant and embalming fluid for biological specimens. 100% formalin contains a 37-40% saturated aqueous solution of formaldehyde and 12-15% methanol which is added as a stabiliser to delay polymerisation of the formaldehyde[188]. Formaldehyde can be both reduced and oxidised[192].

Formaldehyde ⟶ oxidation ⟶ Formic acid

Formaldehyde ⟶ reduction ⟶ Methanol

11.2 Pharmacokinetics of formaldehyde

Formaldehyde is a highly toxic substance to animals and is well known as a human carcinogen. Formaldehyde can be absorbed into the body by ingestion, inhalation, skin absorption and eye contact. Ingestion and eye contact are not significant routes of exposure to formaldehyde whereas inhalation and dermal absorption of formaldehyde are major routes of occupational exposure for workers in the industry. Formaldehyde inhalation can cause several symptoms which vary from headaches (small exposure) to severe difficulty in breathing, dependent on the formaldehyde concentration, length of exposure and individual sensitivity. For example, inhaling formaldehyde at 10 to 20 parts per million (ppm) may cause cough, difficulty in breathing, heavy lachrymation and burning of the nose and throat. As well as dermal absorption, skin contact with formaldehyde can cause a range of effects from acne or dermal irritation to severe allergic dermatitis[191].

When formaldehyde is absorbed into the blood circulation, it binds reversibly with glutathione to form *S*-(hydroxymethyl) glutathione, a non-enzymatic hemiacetal adduct. After that, formaldehyde dehydrogenase oxidises this adduct compound to *S*-formylglutathione, which is further metabolised to formate, releasing free glutathione, by *S*-formylglutathione hydrolase. Formate, the metabolite of formaldehyde, can be incorporated into proteins and nucleic acids via one-carbon pathways or can be metabolised to CO_2 and water and eliminated in exhaled air (as CO_2) and urine[188].

11.3 Tissue fixation

Historically, the majority of fixation procedures and fixative solutions were developed for histological studies. Tissue fixation is a chemical process that is used for preserving biological tissues from post mortem decomposition. From the early 1900s, formalin has been the most commonly used fixative solution for permanent preservation of tissue specimens and autopsy materials. Previous studies indicated that formaldehyde preserves tissue by reversibly cross-linking with primary amino groups and thiols in biological macromolecules such as

proteins, nucleic acids and polysaccharides through a CH_2 linkage. Moreover, formaldehyde can also form reactive hydroxymethyl groups, a product between the aldehyde and reactive amino groups. Reversible cross-linking by formaldehyde usually occurs at neutral pH. Therefore, neutral buffered formalin solution can induce more cross-link reactions in contrast with acid formalin solution[193]. The concentration and pH of formalin solution used in tissue fixation may vary between laboratories. A 10% buffered formalin solution (4% formaldehyde) is most frequently used as an embalming fluid in pathology and histology laboratories. A 10% buffered formalin solution may be prepared by adding 100 ml of 37-40% formaldehyde to 900 ml of water containing 6.5 g of dibasic sodium phosphate (anhydrous) and 4 g of monobasic sodium phosphate[194].

11.4 Previous studies on the reactions between drugs and formalin

In forensic toxicology it is sometimes necessary to analyse biological samples that have been treated with formalin, usually when no other samples are available, and the problem is to assess the original identities and concentrations of drugs or poisons in the samples.

The study of the stability and the reactions between drugs and formalin solution was first reported in 1990 by Dettling *et al.*[195] Nortriptyline was unstable in formalin solution dependent on the pH and concentration of formaldehyde. A high percentage of formaldehyde and basic pH (9.5) caused more decomposition of nortriptyline. Amitriptyline was detected in this experiment and reported as an *N*-methylated product of nortriptyline from formaldehyde. However, the loss of nortriptyline did not correlate with the formation of corresponding amounts of amitriptyline.

The second study about the stability of several compounds in formalin-fixed tissues and formalin-blood solution was published in the same year by Winek *et al.*[196] This study demonstrated that diazepam and phenytoin decreased by at least 41% and 31%, respectively in buffered formalin blood after 30 days. Phenobarbital and desipramine showed losses of more than 60% in liver tissue

fixed in formalin solution, whereas carbon monoxide and cyanide were not detected after adding buffered formalin solution to blood.

Winek *et al.*[197] also reported the stability of four major tricyclic antidepressants (amitriptyline, nortriptyline, desipramine, and imipramine) in formalin-fixed human liver tissue and formalin solutions. All drugs were unstable in 10% buffered formalin fixed tissue and in formalin solution and some methylation of secondary amines (nortriptyline and desipramine) to tertiary amines (amitriptyline and imipramine) was observed. Moreover, this study suggested that acidic compounds are more stable than basic drugs in formalin samples and that drugs analysed in formalin-fixed liver and formalin medium may be detected for up to 22 months. Xiang *et al.*[198] also reported the stability of tetramine, morphine and meperidine in formalin solution. The results showed that concentrations of these three drugs decreased in formalin-fixed tissues (liver, kidney, lung and heart) compared with frozen tissues.

Cingolani *et al.*[199] reported a quantitative method to detect morphine in fixed tissue and in formalin solutions. Morphine was detected at approximately 30% to 36% of the original concentrations in kidney and liver tissues stored in 10% pH 7 buffered formalin solutions and at 42% to 75% of the original concentrations in formalin solution after 12 weeks. This study suggested that morphine has good stability in fixed tissue, which can be used in forensic toxicology diagnosis in cases of morphine poisoning.

Studies of the reaction of drugs with formaldehyde were more often published from 2001. Gannett *et al.*[200] reported the *in vitro* reaction of formaldehyde with fenfluramine. Fenfluramine is unstable in formalin solution and is converted to its *N*-methyl derivative, *N*-methyl fenfluramine, based on the Eschweiler-Clarke reaction, which involves the methylation of primary and secondary amines to tertiary amines by formaldehyde. Fenfluramine was totally converted to *N*-methyl fenfluramine in 24h at pH 9.5 in 20% formaldehyde solution. Moreover, Gannett *et al.*[201] also reported the *in vitro* reaction of three barbiturates (phenobarbital, pentobarbital, and secobarbital) in formalin solution. The results showed that decomposition of barbiturates occurred rapidly at higher pH and phenobarbital rapidly decomposed under basic conditions (pH 9.5). 2-

Phenylbutyric acid was reported as the main decomposition product in formalin. Moreover, this study suggested that analysis for the parent drug and its predicted decomposition product may reduce the likelihood of false negative reports.

Tracy *et al.*[202] reported the stability of 10 benzodiazepines in formaldehyde solutions over a 30 day period. Alprazolam, chlordiazepoxide, diazepam, flunitrazepam, flurazepam, lorazepam, midazolam, oxazepam, prazepam and triazolam were applied in this study. The study found that the decomposition rate of benzodiazepines depended on both the pH and formaldehyde concentration, the same as previous studies. Diazepam and prazepam were the most stable benzodiazepines in formalin solution (approximately 40% decrease in concentration after 30 days) whereas alprazolam and midazolam were not stable in acidic pH formalin solution. This study suggested that determination of benzodiazepines in post mortem embalming samples must be done with caution because benzodiazepines can react with formaldehyde to form conversion products.

In 2005, Tirumalai *et al.*[203] reported that *N,N*-dimethylamphetamine (DMA), the product of methamphetamine in formalin solution, was observed at both neutral (pH 7) and basic pH (pH 9.5) after a few days. At pH 9.5 in 20% formalin solution, 90% of the methamphetamine was rapidly converted to DMA in 24 h. This study suggested that determination of methamphetamine in post mortem embalming tissue can give misleading results due to conversion of methamphetamine to DMA. Shakleya *et al.*[204] studied the methylation of methamphetamine in formalin-fixed liver tissue. The results showed that DMA was detected only in liver tissues treated with formalin.

The conversion of secondary amines to tertiary amines in formalin solution was confirmed when Shakleya *et al.*[205] reported that MDMA is methylated, producing 3,4-methylenedioxydimethylamphetamine (MDDMA) in formalin solution and Suma and Prakash[206] reported that sertraline can be converted to *N*-methyl-sertraline in embalming fluid. Moreover, Ramagiri *et al.*[207] also reported that fluoxetine was totally converted to *N*-methyl-fluoxetine in formalin solution after 30 days in 20% formalin at pH 9.5.

Viel *et al.*[208] reported the stability of cocaine in formalin solution and fixed tissues (brain and liver) over a period of 30 days. Cocaine was stable in unbuffered formalin (pH 3.5) over 30 days and unstable in buffered formalin (pH 7.4) in which cocaine was hydrolysed to benzoylecgonine. This study also found that both cocaine and benzoylecgonine were rapidly extracted into the formalin solution from the embalmed tissues. However, the study suggested that the hydrolysis of cocaine did not occur by reaction with formalin.

In 2013, Takayasu[192] and Nikolaou *et al.*[209] reviewed the toxicological analysis of formalin-fixed tissue and solution. Finally in 2015, Uekusa *et al.*[210] reported the analysis of drugs in tissues in formalin solutions and in fixatives. Drug-positive autopsy liver and kidney were preserved in formalin solution for 1, 3, 6 and 13 months and applied in this study. Concentrations of chlorpromazine, levomepromazine, and promethazine decreased to 23–39% of their starting values in embalmed liver after 1 month and bromazepam was not detected after 13 months. Moreover, chlorpromazine sulfoxide and levomepromazine sulfoxide, which were not observed before preservation, were detected in fixed-liver tissues and formalin solution at all analysed time periods.

Chapter 12 *In vitro* reactions of formaldehyde with drugs and poisons: introduction and experimental section

12.1 Introduction

In South-East Asia and in Buddhist countries such as Thailand, cremation is a common practice for disposal of the dead. Cremation is accepted in Buddhism, Hinduism, Jainism, Sikhism, and Shintoism[211]. In Thailand, the corpse is kept for three to seven days in the temple to allow distant relatives to attend a memorial and funeral rite before cremation. In some cases, especially for an important or wealthy person, the corpse is kept for a year or more in a special building at a temple[212]. To maintain a body longer in tropical wet and dry climates, formalin is injected into the body as an embalming solution to reduce body decomposition. Therefore, all drugs present in the body will be exposed to formaldehyde, a highly reactive substance that can react chemically with drugs of interest resulting in decreasing concentrations of the original compounds and production of new substances.

In some cases, forensic toxicologists are required to perform analyses on samples from an embalmed body or of embalmed tissue stored in formaldehyde. An analysis of an embalmed sample or of embalming fluid may create a false negative or false positive because of the reaction between drugs and formalin. Not only methamphetamine and MDMA but the degradation products of other drugs in formalin solution also need to be identified and reported. Information about the reaction and degradation of drugs in formalin is currently insufficient and needs to be identified and reported and also presents a continuing challenge in the forensic toxicology field.

12.2 Aims

Post mortem forensic toxicology involves the detection and quantification of drugs and poisons, including pesticides, in autopsy blood and other tissues in

order to determine their possible role in the cause of death. In some jurisdictions, as mentioned above, fatalities are subject to embalming before blood can be obtained at autopsy for toxicological analysis. This presents problems of identification of poisons in blood because the embalming process used in hot climates involves the chemically-reactive material formalin. This modifies drugs or other substances such that they are chemically different from the starting materials and are no longer easily detectable by conventional means. There is a need to identify markers of the original drugs or poisons which can be used to indicate the original species in the blood.

The primary aim of the present study was to identify the degradation products of drugs and pesticides in formalin solution. In order to evaluate the effects of formaldehyde on drugs and poisons, a range of model compounds was used, including amphetamine-type stimulants (amphetamine, methamphetamine, and MDMA), benzodiazepines (alprazolam and diazepam), opiates (morphine, hydromorphone, codeine, and hydrocodone) and a carbamate insecticide (carbosulfan), which are often associated with cases of suicide, homicide, accidental poisoning and road traffic accident because of their toxicity and potential for abuse. Typical approaches to this involve *in vitro* simulation of the reactions between analytes and formalin followed by LC/MS analysis. The second aim was to examine the conversion products in formalin-blood to which the target analytes have been added. These steps evaluated which reaction products might serve as markers of the original substances.

12.3 Amphetamine-type Stimulants (ATS)

In the last 10 years, abuse of Amphetamine-type Stimulants or ATS such as amphetamine, methamphetamine, MDMA and MDEA has become a global problem especially in East and South-East Asia where consumption of methamphetamine is dominant. The United Nations Office on Drugs and Crime (UNODC) reported in the World Drug Report 2015 that treatment for ATS use is increasing globally, especially in Asia, and ATS, excluding ecstasy, comprise one of the two most widely used types of illicit drugs globally. In parts of North America and Europe, the use of crystalline methamphetamine is also increasing[65]. ATS are a group of substances, mostly synthetic in origin, that are

structurally derived from *β*-phenethylamine[213]. ATS can be divided into three major sub-groups by structural characteristics following substitution patterns on the aromatic ring. The first group has no substituents on the aromatic ring, such as amphetamine, methamphetamine, and fenethylline. The second group features methylenedioxy-substitution on the aromatic ring and includes MDMA and its analogues. The third group contains other substitution patterns which usually include one or more alkyloxy groups, such as 2,5-dimethoxy-4-bromophenethylamine (2C-B) or 4-methyl-2,5-dimethoxyamphetamine (DOM)[213].

12.3.1 Mechanism of action

ATS are a group of CNS stimulant drugs that act on the cerebral cortex producing effects similar to adrenaline. Amphetamines, which have a chemical structure similar to dopamine (functions as a neurotransmitter in brain), can cross through the blood-brain barrier and act on the monoamine neurotransmitter system, which involves neuromodulators and neuro-transmitters that contain one amino group, such as dopamine, noradrenaline and serotonin. Amphetamines can bind to and also act as substrates for a low-affinity monoamine transporter protein for dopamine, noradrenaline and serotonin, resulting in increasing levels of neurotransmitter. Whereas MDMA has more potent effects on the serotonin system than on the dopamine and noradrenaline systems, for amphetamine and methamphetamine the opposite is true[213]. ATS may produce one or more dose-related symptoms, including increased alertness and euphoria, increased heart rate, blood pressure, respiration and body temperature. Agitation, tremors, hypertension, memory loss, hallucinations, psychotic episodes, paranoid delusions and violent behavior can result from chronic abuse[214].

12.3.2 Amphetamine

Amphetamine was first synthesised in 1887 and has been used since 1935 for treatment of obesity, narcolepsy, hypertension and attention deficit hyperactivity disorder (ADHD), with different trade names; Adderall®, Benzedrine® and Dexedrine®[215]. Because of its addiction potential, it is controlled under Schedule II of the United Nations Convention on Psychotropic Substances. Amphetamine is a phenethylamine derivative which has two

enantiomers, levorotatory (l-) and dextrorotatory (d-) that have different plasma half-lives[216]. Amphetamine is available as the l-, d- or dl-racemic forms, of which the d-isomer is 3-4 times more potent than the l-form. The chemical formula of amphetamine is $C_9H_{13}N$ with a relative molecular mass of 135.20, see Figure 12-1. It is commonly produced as tablets and can be administered orally, injected, snorted, or smoked[102].

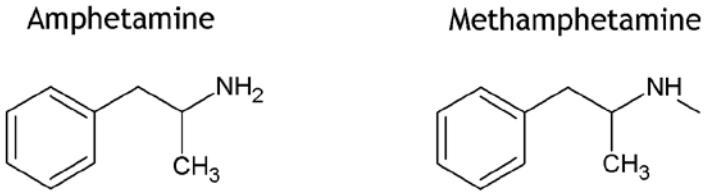


Figure 12-1: Chemical structures of amphetamine and methamphetamine.

Amphetamine is rapidly absorbed from the gastrointestinal tract. It is a lipophilic compound that can cross through the blood-brain barrier and which is also concentrated in the lung, kidney, cerebrospinal fluid, and brain. The major metabolic pathway of amphetamine involves hydroxylation to *para*-hydroxyamphetamine and deamination to phenylacetone by the CYP2D6 enzyme, a member of the cytochrome P450 mixed-function oxidase system. Peak plasma levels occur within 1 to 3 h and complete absorption occurs in 4 to 6 h after oral administration. It is eliminated in urine via the renal system and approximately 30% is excreted unchanged in 24 h. However, the elimination rate of amphetamine is dependent on the urinary pH: acidic urine (pH 5.5 to 6.0) can eliminate amphetamine faster than basic urine (pH 7.5 to 8.0)[102,217].

12.3.3 Methamphetamine

Methamphetamine is the *N*-methyl derivative of amphetamine. It is a strong CNS stimulant and is controlled under Schedule II of the United Nations Convention on Psychotropic Substances. Methamphetamine hydrochloride was first synthesised in 1919 and has been used for treatment of obesity and ADHD with trade names such as Desoxyn® and Methedrine®[102]. Methamphetamine is produced in a variety of forms (powder, tablets, capsules or crystals) that may be ingested orally, injected, snorted, or smoked. Similar to amphetamine,

methamphetamine has both d- and l-forms although illicit methamphetamine is usually sold as a racemic dl-mixture. The chemical formula of methamphetamine is $C_{10}H_{15}N$, with a relative molecular mass of 149.24.

Methamphetamine is rapidly absorbed from the gastrointestinal tract and readily moves through the blood-brain barrier due to its high lipophilicity. The average peak plasma level of methamphetamine is reached approximately 3 h after oral administration and the plasma half-life is approximately 10 h in humans[218]. The major metabolic pathway of methamphetamine involves *N*-demethylation to amphetamine, a major active metabolite, and *p*-hydroxylation to 4-hydroxymethamphetamine. Minor metabolites of methamphetamine include 4-hydroxyamphetamine, 4-hydroxynorephedrine, 4-hydroxyphenylacetone, benzoic acid, norephedrine, phenylacetone and hippuric acid. Approximately 43% of methamphetamine is eliminated as unchanged drug and about 4-7% as amphetamine in the 24 h period after oral administration[102].

12.3.4 MDMA

See Section 7.2.1.

12.4 Benzodiazepines

Benzodiazepines are psychoactive drugs that have been widely used for the treatment of psychoneurological disease since the 1960s. Chlordiazepoxide, the first medicinal benzodiazepine, was discovered accidentally in 1955 by Leo Sternbach[219]. The core chemical structure of this group is the fusion of a benzene ring and a diazepine ring (Figure 12-2). There are more than 50 different medicinal drugs in this group. All of the benzodiazepines that are precribed in the UK are controlled under the Misuse of Drugs Act 1971 as Class C drugs.

Benzodiazepines are commonly misused and are often implicated in crimes such as suicide, murder, sexual assault and traffic accidents and are usually taken in combination with other drugs of abuse. Most forensic laboratories have routine screening and confirmation assays for benzodiazepines in urine and post mortem

blood. However, analysis of benzodiazepines in biological samples is quite difficult because of the similar chemical structures of drugs in this group, which provide similar mass spectra and retention times in chromatographic methods. Some previous studies[196,202] of benzodiazepines in formalin-fixed tissue and formalin solution suggested that benzodiazepines are not stable in formalin solution. Nevertheless, further study of the reactions of benzodiazepines and formalin solution is needed.

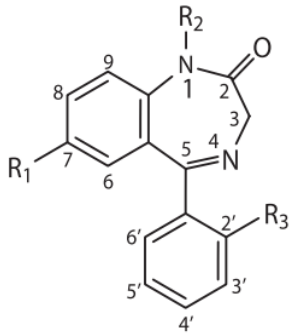


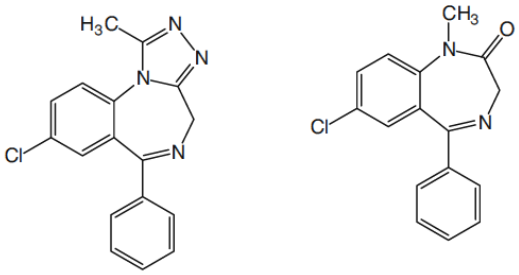
Figure 12-2: Core chemical structure of benzodiazepines.

12.4.1 Mechanism of action

Benzodiazepines are positive modulators of the neurotransmitter gamma-aminobutyric acid (GABA). Benzodiazepines act by binding to a specific site at the $GABA_A$ receptor, a ligand-gated chloride-selective ion channel, in the CNS. Benzodiazepines have special receptors, BZ1 and BZ2 receptors, which differ from $GABA_A$ subunit receptors[220]. However, drugs in this class can interact with different affinities to each receptor, resulting in corresponding differences in their pharmacology. Benzodiazepines are highly lipophilic compounds that have high absorption rates and fast onset of effects, the main ones being sedative, hypnotic, muscle relaxant and decreased anxiety. However, high doses of benzodiazepines may cause impairment of memory and cognition[221]. Benzodiazepines are used for treating insomnia and anxiety, as anticonvulsants, and for other purposes such as anesthetic adjuncts and alcohol withdrawal[222]. Benzodiazepines can be classified by their elimination half-life as short-, intermediate- and long-acting.

12.4.2 Alprazolam

Alprazolam (also known as Xanax®), a triazolobenzodiazepine derivative, was first clinically used in 1976 as an anxiolytic agent. The chemical formula of alprazolam is $C_{17}H_{13}ClN_4$ with a relative molecular mass of 308.77, see Figure 12-3. Today, it is commonly used for the short-term treatment of agoraphobia, anxiety disorders and panic disorder[223]. Alprazolam is available as a free base tablet of 0.25-1 mg and as a capsule[102]. It is one of the most misused benzodiazepines and is controlled under Schedule IV of the United Nations Convention on Psychotropic Substances.



Alprazolam **Diazepam**

Figure 12-3: Chemical structures of alprazolam and diazepam.

Similar to other benzodiazepines, alprazolam binds to specific sites on the $GABA_A$ receptor and produces anxiolytic, sedative, hypnotic and skeletal muscle relaxant effects. Adverse effects of alprazolam include drowsiness, hypotension, confusion, nausea and tachycardia. Alprazolam is a short-acting benzodiazepine that has a fast onset of action as well as a short elimination half-life. Peak plasma concentrations of alprazolam occur 1-2 h following oral administration. The average plasma half-life of alprazolam is 12-15 h. Alprazolam is metabolised by oxidation to two active metabolites (4-hydroxyalprazolam and α-hydroxy-alprazolam) via by CYP3A4 followed by conjugation with glucuronic acid and excretion in urine[102,222].

12.4.3 Diazepam

Diazepam (also known as Valium®) was first used clinically in 1963 and was the second medicinal benzodiazepine derivative. The chemical formula of diazepam is $C_{16}H_{13}ClN_2O$ with a relative molecular mass of 284.7, see Figure 12-3. Diazepam is one of the most frequently prescribed benzodiazepines and is controlled under Schedule IV of the United Nations Convention on Psychotropic Substances. It is commonly used for anxiety disorders, insomnia and the short-term treatment of acute alcohol withdrawal, acute agitation and tremor[223]. Common adverse effects of diazepam are fatigue, drowsiness, and muscle weakness. Diazepam is available as both a free base tablet for oral administration and as a solution for injection.

Diazepam also acts on the $GABA_A$ receptor. It is a long-acting benzodiazepine with an average plasma half-life of 24-48 h. Peak plasma concentrations of diazepam occur at 30-90 min after oral administration. Diazepam is metabolised by *N*-demethylation to nordiazepam via CYP3A4 and CYP2C19 and then both parent and metabolite are further metabolised to the active metabolites oxazepam and temazepam, which are subsequently conjugated with glucuronide[223]. Diazepam is excreted in urine as oxazepam glucuronide approximately 33% and 20% conjugated nordiazepam and temazepam.

12.5 Opioids

The term “opioids” refers to compounds that act on the nervous system in a similar way to opiates whereas “opiates” are natural alkaloids that are found in the opium poppy plant. Opium is the dried milky juice (latex) obtained from the unripe seed pods of *Papaver somniferum L.*, more commonly referred to as the opium or oil poppy[224]. The major important opiates are morphine, codeine and thebaine whereas hydromorphone, hydrocodone and buprenorphine are semi-synthetic opioids derived from morphine, codeine and thebaine, respectively. Morphine and codeine are used to treat mild to moderate and chronic severe pain and codeine is also used to relieve cough and diarrhoea. However, both opiates and opioids have a high addiction potential[225]. Previous studies on the

detection and quantitation of morphine in fixed tissues and formalin solutions suggested that morphine is unstable in these matrices.

12.5.1 Mechanism of action

Opioids act by binding to specific opioid receptors (mu, delta and kappa) located within various nervous system sites and also found in the spinal cord and peripheral sensory and autonomic nerves. The mu (μ) receptor, which has three subtypes μ1, μ2 and μ3, is the site at which the prototype opiate morphine binds whereas the delta (δ) and kappa (κ) receptors are the site for the endogenous opioid ligand enkephalin[226]. Some opioids act as agonists or partial agonists on one type of receptor and antagonists at another, resulting in a very complicated pharmacological action. Mu receptors produce respiratory depression, euphoria, reduced gastrointestinal tract motility and vasodilation whereas delta and kappa receptors have analgesic properties[227]. Adverse effects of opioids include sedation, respiratory depression and euphoria[225]. High enough doses of opioids can cause death from respiratory failure.

12.5.2 Morphine and hydromorphone

Morphine is a natural alkaloid found in latex of the opium poppy (10-17% by weight) and was first isolated from opium in 1803 by Friedrich Sertürner. It acts directly on the central nervous system to decrease the feeling of pain. It is the prototype of narcotic analgesic drugs and is still made from opium. Morphine is available as the sulphate salt for oral, subcutaneous, intramuscular and intravenous administration. Oral morphine is recommended by the World Health Organization (WHO) for the treatment of chronic cancer pain. The chemical formula of both morphine and hydromorphone is $C_{17}H_{19}NO_3$ with a relative molecular mass of 285.3, see Figure 12-4. Because of their high addictive potential, morphine and hydromorphone are controlled under Schedule I of the United Nations Convention on Narcotic Drugs.

Morphine acts predominantly on mu opioid receptors (strongly binding) and is also a kappa and delta opioid receptor agonist. Adverse effects of morphine are nausea, delirium and constipation. Morphine is primarily metabolised by

conjugation to form morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), of which M3G is the main metabolite (45-55%). A minor metabolic route is *N*-demethylation to normorphine via CYP3A4 and CYP2C8. Other minor metabolites include normorphine-6-glucuronide, normorphine-3-glucuronide and codeine[228].

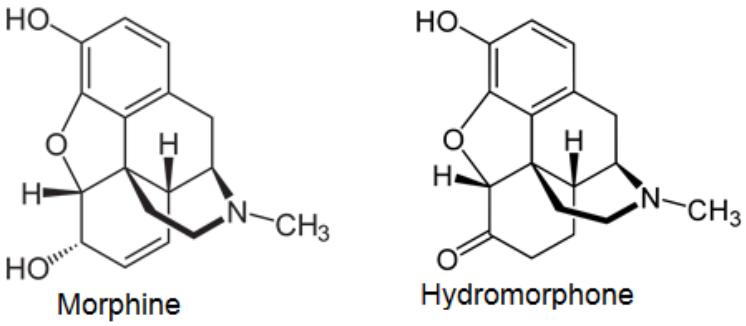


Figure 12-4: Chemical structures of morphine and hydromorphone.

Hydromorphone (a hydrogenated ketone of morphine) is a semi-synthetic opioid agonist derived from morphine. It was first synthesised in 1921 and was first clinically used in 1926[229]. The structure of hydromorphone is similar to morphine only differing in the hydrogenation of the double bond at the C7-C8 position and the presence of a 6-keto group[230]. Hydromorphone is used as a second-line drug to morphine in the treatment of moderate to severe pain and is commonly used in hospital, mostly intravenously[230]. It is approximately 4-8 times more potent than morphine[229].

Hydromorphone acts primarily on mu opioid receptors and to a lesser degree on delta receptors but does not act on kappa receptors. It has a fast onset of action, approximately 30 min, and the average plasma half-life is 2.5h. Unlike other opioids, hydromorphone is not metabolised via CYP450 enzyme[230]. Metabolites of hydromorphone are dihydromorphine, dihydro-isomorphine and conjugated metabolites, hydromorphone-3-glucuronide and hydromorphone-6-glucuronide[229].

12.5.3 Codeine and hydrocodone

Codeine, a methylated morphine derivative, is a natural alkaloid found in latex of the opium poppy (0.7-4% by weight) that was first isolated from opium in 1832 by Pierre Robiquet in France. It is a weak opiate. Codeine is used to treat pain and diarrhoea and is also included in cough medicine[227]. It is a weak base and is available as the phosphate or sulphate salt for oral, subcutaneous and intravenous administration[102]. Hydrocodone (a potent analogue of codeine) is a semi-synthetic opioid derived from codeine used to treat moderate to severe pain and used as an antitussive to treat cough. It is available as the bitartrate salt and in combination with ibuprofen and acetaminophen. Hydrocodone is 6 times more potent than codeine with respect to its analgesic property. Pure codeine and hydrocodone are prescribed drugs, although some weaker formulations of codeine are pharmacy medicines, and are controlled under the United Nations Convention on Narcotic Drugs, in which hydrocodone is under Schedule I and codeine is under Schedule III. The chemical formula of both codeine and hydrocodone is $C_{18}H_{21}NO_3$ with a relative molecular mass of 299.36, see Figure 12-5.

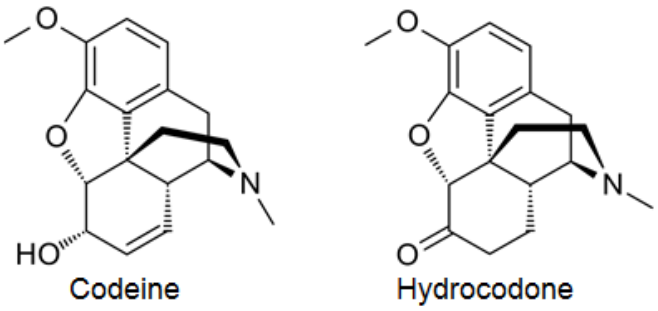


Figure 12-5: Chemical structures of codeine and hydrocodone.

Codeine is rapidly absorbed following either oral or intramuscular administration. Average peak plasma concentrations of codeine occur 2 h after oral administration and the average plasma half-life is 2-4 h. Metabolism of codeine is by *O*-demethylation to morphine via CYP2D6 and by *N*-demethylation to nor-codeine. All three compounds are further conjugated with glucuronide and excreted in urine[102,231].

A major metabolic route of hydrocodone is by *N*-demethylation to nor-hydrocodone which is predominantly formed by CYP3A4. Hydrocodone is also metabolised by *O*-demethylation to hydromorphone via CYP2D6 and keto-reduction to hydrocodol which is further metabolised to hydromorphol before conjugation and excretion in urine. Peak plasma concentrations of hydrocodone occur at 1.5 h following oral administration and the average plasma half-life is 3-8 h[102].

12.6 Carbamate Insecticides

Carbamates, including aldicarb, carbosulfan, and methomyl are a class of synthetic pesticides that contain esters of carbamic acid in their structures (Figure 12-6). They are the most commonly used pesticides in agriculture around the world. The first carbamate compound was isolated in the mid-1860s from calabar beans of the plant *Physostigma venenosum*. Most carbamate pesticides were synthesised in the 1960s and 1970s and carbaryl was the first carbamate compound that was used as an insecticide. Today, carbamates have replaced organophosphates for pesticide use because organophosphates were found to be extremely toxic to mammals[232].

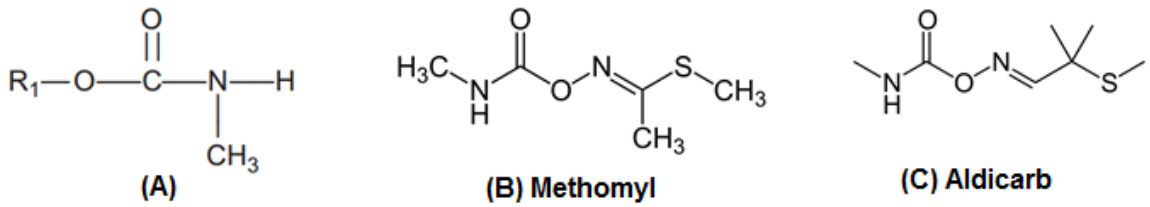


Figure 12-6: General structure of carbamates (A), methomyl and aldicarb.

Some carbamate insecticides such as carbosulfan and methomyl were banned by the European Union because of their high toxicity to humans. In agricultural countries, including Thailand, carbamates are commonly used in the fruit and vegetables industry. Carbosulfan, carbofuran and methomyl were reported as pesticide residues found in fruit and vegetables originating from Southeast Asia[233]. In 2008, more than ten thousand tonnes of carbofuran were imported into Thailand[234]. Moreover, these substances are also commonly encountered in the author's laboratory in Thailand in cases of suicide[235]. However, information

about the stability and reactivity of carbamate insecticides in formalin solution has not previously been published.

12.6.1 Mechanism of action

The primary target organ of carbamates is the nervous system. Acetylcholine (ACh) is the neurotransmitter found at the neuromuscular junction to activate muscles and which is also released at synapses in the ganglia of the visceral motor system and in the CNS[236,237]. ACh is released from storage vesicles in nerve endings and quickly binds to ACh receptors in the post-synaptic membrane. The neurotransmitter is usually deactivated through hydrolytic cleavage into choline and acetic acid by the enzyme acetylcholinesterase (AChE), as shown in Figure 12-7. During this cleavage reaction, the acetate group binds to the active site of the enzyme but is easily released again. Cleavage of ACh is required to allow a cholinergic neurone to return to its resting state after activation. Similarly, for a muscle to relax after a contraction is triggered by ACh at a neuromuscular junction, rather than persisting in a contracted state, ACh must be broken down by AChE.

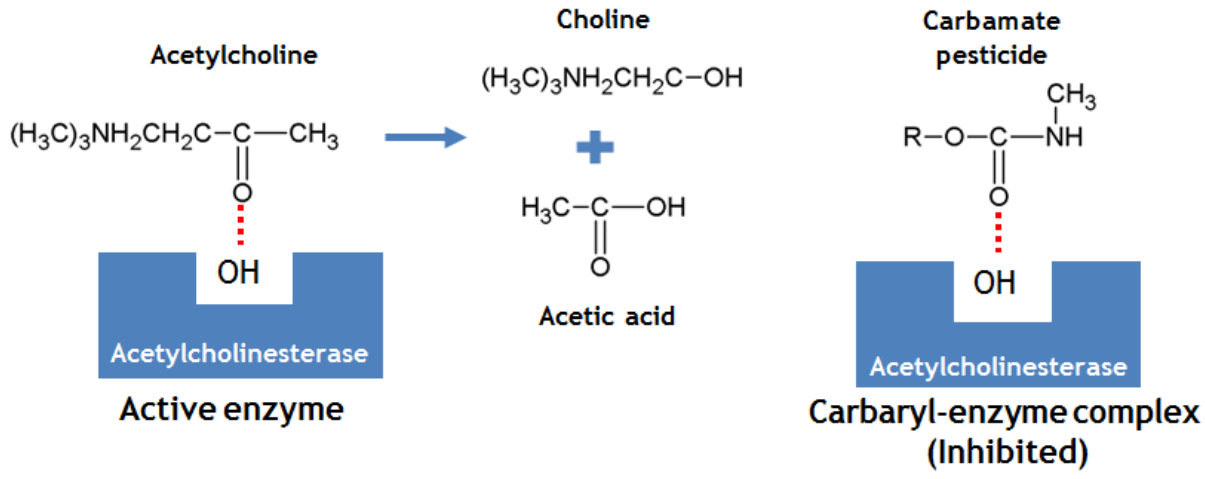


Figure 12-7: The mechanism of inhibition of AChE with carbamate pesticides. The diagram on the left shows the normal mode of action of the enzyme, through which ACh is cleaved into choline plus acetic acid. When a carbamate is cleaved by AChE, the carbamoyl group binds (reversibly) to the active site and blocks its action, as shown in the diagram on the right.

Carbamates are anti-AChE substrates which can reversibly inhibit the enzyme at the synapses in the brain and at neuromuscular junctions, resulting in accumulation of acetylcholine and leading in turn to the overstimulation of

muscarinic and nicotinic acetylcholine receptors[232]. Cleavage of a carbamate by AChE results in binding of the carbamate moiety to the active site of the enzyme and inhibiting its activity. However, the carbamylation process resulting in anti-AChE action is quickly reversible, unlike the phosphorylation of AChE by an organophosphate, which is only very slowly reversible. For this reason, carbamates are less toxic than organophosphates and have largely replaced the latter as pesticides.

Carbamates are rapidly absorbed, metabolised in the liver and eliminated in the urine. Effects associated with muscarinic and nicotinic receptors are salivation, urination, diarrhoea, tremors and convulsions[238].

12.6.2 Carbosulfan

Carbosulfan is a widely used insecticide. The chemical formula of carbosulfan is $C_{20}H_{32}N_2O_3S$ with a relative molecular mass of 380.55, as shown in Figure 12-8. Carbosulfan is available as dusts, granular formulations and emulsifiable concentrates[239].

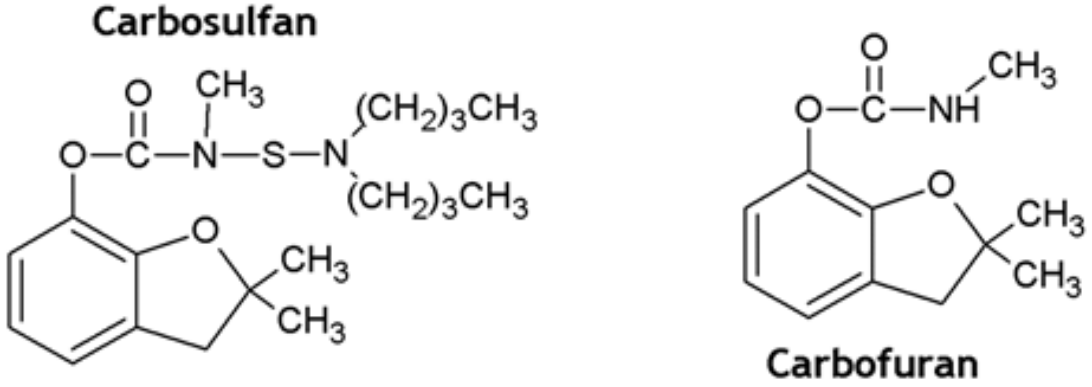


Figure 12-8: Chemical structures of carbosulfan and carbofuran.

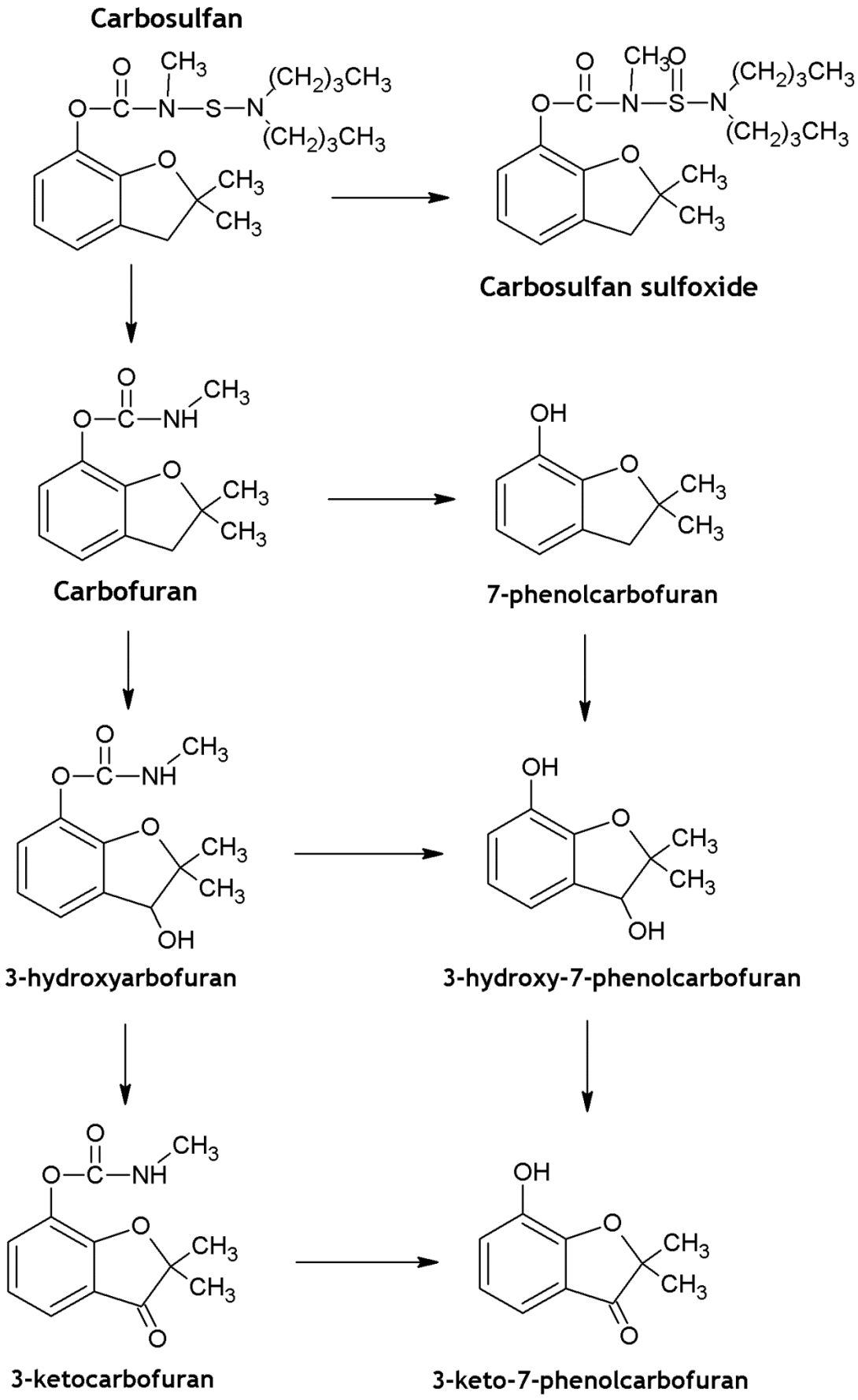


Figure 12-9: The overall scheme of carbosulfan metabolism.

Hepatic metabolism of carbosulfan involves CYP450 isozymes through which sulfoxidation of carbosulfan is mediated mainly by CYP3A4 enzymes. The major metabolic pathway of carbosulfan in humans is the cleavage of the nitrogen sulfur bond (N–S) to carbofuran (Figure 12-9), which is itself used as a pesticide, and the minor pathway is formation of carbosulfan sulfoxide[240]. Carbofuran is further hydroxylated to 3-hydroxycarbofuran and 3-ketocarbofuran. Other metabolites are 3-hydroxy-7-phenol-carbofuran, 3-keto-7-phenolcarbofuran and 7-phenolcarbofuran. Whereas all carbosulfan metabolites are toxic compounds, carbofuran is found to be more toxic than its parent, carbosulfan. 3-Hydroxycarbofuran was reported as the main metabolite in all species[241,242].

12.7 Methods and materials

12.7.1 Reagents and standards

Acetonitrile, ethyl acetate and methanol (HPLC grade) were obtained from VWR International Ltd (UK). Standards of *N,N*-dimethylamphetamine (DMA) and 3,4-methylenedioxy-*N,N*-dimethylamphetamine (MDDMA) were obtained from Lipomed (supplied by Kinesis, Ltd UK). Standard 3,4-methylenedioxymethamphetamine (MDMA), hydrocodone, carbosulfan, carbofuran, alprazolam-d_5, hydromorphone-d_3, hydrocodone-d_3, 3-hydroxycarbofuran, 3-ketocarbofuran and formaldehyde solution in water were obtained from Sigma-Aldrich, Co., LLC (UK).

12.7.1.1 Preparation of stock standard solutions

Individual stock standard solutions were prepared by dissolving each compound in methanol to achieve a concentration of 1 mg/ml of drug or pesticide substance. Working standard solutions were prepared by serial dilution of stock standard solutions with methanol. All stock and working standard solutions were stored at 4^{o}C.

12.7.1.2 Preparation of formalin solutions

10% and 20% formalin solutions were prepared by adding 10 ml or 20 ml, respectively, of formaldehyde solution into a 100 ml volumetric flask and making up to 100 ml with deionised water.

12.7.2 Reaction of formalin solution with drugs and pesticide

12.7.2.1 Spiked samples in formalin solution

Spiked samples in 10% formalin solution were prepared separately for each of the following substances: amphetamine, methamphetamine, MDMA, alprazolam, diazepam, morphine, codeine, hydromorphone, hydrocodone and carbosulfan. Drug concentrations were set at 20 µg/ml for LC/MS analysis and carbosulfan was at 50 µg/ml for subsequent GC/MS analysis. All samples were stored at room temperature prior to processing.

12.7.2.2 Spiked samples in control solution

Spiked samples in water were prepared separately for each of the following substances: amphetamine, methamphetamine, MDMA, alprazolam, diazepam, morphine, codeine, hydromorphone, hydrocodone and carbosulfan. Drug concentrations were set at 20 µg/ml for LC/MS analysis and carbosulfan was at 50 µg/ml for subsequent GC/MS analysis. All samples were stored at room temperature prior to processing.

12.7.3 Reaction of formalin blood with drugs

12.7.3.1 Spiked samples in formalin blood

Spiked samples in 10% formalin blood were prepared separately for each of the following substances: amphetamine, methamphetamine, MDMA, alprazolam, diazepam, morphine, codeine, hydromorphone, hydrocodone and carbosulfan. Drug concentrations were set at 20 µg/ml for LC/MS analysis and carbosulfan was at 50 µg/ml for subsequent GC/MS analysis. The appropriate amount of

standard was added to blood which was then carefully mixed with an equal volume of 20% formalin solution. All samples were stored at room temperature prior to processing.

12.7.3.2 Spiked samples in whole blood

Spiked blood samples were prepared were prepared separately for each of the following substances: amphetamine, methamphetamine, MDMA, alprazolam, diazepam, morphine, codeine, hydromorphone, hydrocodone and carbosulfan. Drug concentrations were set at 20 µg/ml for LC/MS analysis and carbosulfan was at 50 µg/ml for subsequent GC/MS analysis. All samples were stored at room temperature prior to processing.

12.7.4 Liquid-liquid extraction

12.7.4.1 Liquid-liquid extraction for carbosulfan

0.5 ml of a spiked sample containing 50 µg/ml of carbosulfan was extracted with 2 ml of ethyl acetate. The organic layer was transferred to a labelled 4 ml screw-capped vial and evaporated by a gentle flow of nitrogen gas at ambient temperature, and residues were reconstituted in 200 µl of ethyl acetate and transferred to labelled 1.5 ml screw-capped vials with inserts before analysis by GC/MS.

12.7.4.2 Liquid-liquid extraction for LC/MS

200 µl of spiked samples containing 20 µg/ml of amphetamine, methamphetamine, MDMA, alprazolam, diazepam, morphine, codeine, hydromorphone or hydrocodone were treated with 400 µl of acetonitrile and centrifuged at 12,000 rpm for 10 min. 200 µl of the supernatant layer was transferred to a labelled 1.5 ml capped vial with insert before analysis by LC/MS.

12.7.5 Instrumentation

12.7.5.1 Gas chromatography/mass spectrometry conditions

GC/MS analysis was carried out using an Agilent 7890A gas chromatograph equipped with an Agilent 7693 autosampler and connected to an Agilent 5975C inertXL MSD with Triple-Axis mass selective detector, operated in the EI positive ion mode. Chromatographic separation was performed on an HP-5MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness, 0.25 µm, Agilent Technologies Inc.). Helium gas at a constant flow of 1 ml/min was used as the carrier gas. The injection port temperature was 225^{o}C, and the transfer line temperature was 250^{o}C. The injection volume was set at 1 µl in the splitless mode. The oven temperature was maintained at 80^{o}C for 2 min, and then it was increased at a rate of 8^{o}C/min and finally held at 250^{o}C for 1 min.

The mass-selective detector was operated in the full scan mode with a mass range between *m/z* 40 and 500 and was tuned and calibrated with perfluorotributylamine. The ion source and quadrupole temperatures were 250^{o}C and 150^{o}C, respectively. A solvent delay was set at 5 min to protect the filament. All compounds were identified by their retention times (t_R) and mass spectral fragmentation patterns. The molecular ion at *m/z* 323, base peak ion at *m/z* 160 and fragment ions at *m/z* 118 and 135 were monitored for carbosulfan. The molecular ion at *m/z* 221, base peak ion at *m/z* 164 and fragment ions at *m/z* 149 and 131 were monitored for carbofuran.

12.7.5.2 Liquid chromatography/mass spectrometry conditions

Chromatographic separation was performed using an Agilent Binary SL 1200 LC from Agilent Technologies Inc. Gradient elution was performed with a flow rate of 0.3 ml/min on a Gemini C18 column (150 x 2.0 mm, 5 µm) protected by a Gemini C18 Security Guard Pre-Column (4 x 2.0 mm, 5 µm) from Phenomenex Inc., which were maintained at 40ºC. The injection volume was 10 µl and the mobile phase was composed of 2 mM ammonium acetate in water (A) and 2 mM ammonium acetate in methanol (B). The initial condition was maintained at 15% B for 2 min, increased to 50% B within 3 min and then increased to 90% B within

5 min and held for 5 min. Finally, the initial condition was restored within 0.5 min and held for 6.5 min to re-equilibrate the system: the total run time was 22 min.

Analysis of all analytes was performed using an Agilent 6420 Triple Quad LC/MS/MS instrument (Agilent Technologies Inc.) equipped with an Agilent Binary SL 1200 LC system interface. Nitrogen was from a nitrogen generator (In House Gas (Manufacturing) Ltd, UK).

Ionisation of analytes of interest was carried out using ESI. The mass spectrometer was operated in both positive ion full scan and multiple reaction monitoring (MRM) modes. Nitrogen was used as nebuliser gas at 30 psi. The gas temperature was set to 300^{o}C and the gas flow to 11 ml/min. For full scan mode, the mass range was set at *m/z* 50 to 500 and the FV at 20 eV. The MRM transitions, FVs and CEs for each analyte were optimised separately by direct infusion into the MS of each standard solution at a concentration of 5 µg/ml in mobile phase (50:50 v:v). All analytes were identified and quantified based on their retention times and quantifier and qualifier ion ratios that were obtained from MRM transitions. The MRM transitions, FVs and CEs used for the measurement of amphetamines, benzodiazepines, opiates and carbosulfan are summarised in Table 12-1.

Table 12-1: MRM conditions for amphetamines, benzodiazepines, opiates and carbosulfan.

Name	Precursor Ion (*m/z*)	Product Ions (*m/z*)	Fragmentor Voltage (eV)	Collision Energy (eV)
AP	136.1	91.2	80	20
	136.1	119.1		5
MA	150.0	91.1	90	20
	150.0	119.1		10
MDMA	194.2	163.1	100	10
	194.2	135.2		20
MDDMA	208.2	163.1	110	10
	208.2	72.2		10
DMA	164.1	91.2	100	20
	164.1	119.2		10
Morphine	286.3	201.1	170	25
	286.3	153.2		45
Hydromorphone	286.3	185.1	170	30
	286.3	157.1		45
Codeine	300.3	165.2	160	45
	300.3	215.1		21
Hydrocodone	300.3	199.2	170	30
	300.3	128.2		60
Alprazolam	309.2	281.2	180	30
	309.2	205.1		45
Diazepam	285.2	193.2	180	30
	285.2	154.2		25
Carbosulfan	381.2	160.2	120	15
	381.2	118.2		20
Carbofuran	222.1	165.1	120	10
	222.1	123.0		15
3-Hydroxy	238.1	220.0	80	5
Carbofuran	238.1	163.1		10
3-keto	236.0	165.0	100	5
Carbofuran	236.0	151.0		10

Chapter 13 *In vitro* reaction of formaldehyde with drugs and poisons: identification of the conversion products

13.1 Introduction

This chapter will provide the results of the reactions of formaldehyde with drugs and poisons obtained using the methods described in Chapter 12.

13.2 Reactions of formaldehyde with ATS

13.2.1 Reactions of ATS in formalin solution

Based on the Eschweiler-Clarke reaction, amphetamine, which is a primary amine, is expected to react with formalin solution to form methamphetamine and DMA. Methamphetamine, which is a secondary amine, is expected to form DMA. Also, MDMA, which is a secondary amine, is expected to form MDDMA. To confirm these reactions, amphetamine, methamphetamine and MDMA were spiked in 10% formalin solution and also in water as a control.

The aim of these tests was to identify the products of reactions with formalin. In these initial tests, spiked samples were analysed qualitatively after spiking (day 0) and at day 1 and day 7. Because the samples were analysed qualitatively rather than quantitatively and no internal standards were added, comparisons of peak areas between chromatograms acquired on different days is subject to larger errors than if the analyses had been quantitative, as the sensitivity of the instrument varied from day to day. However, comparison of peak area ratios, that is, relative amounts of each analyte in a sample, can be made. Further stability studies based on quantitative analysis will be required to prepare true stability curves over a period of several days.

The formation of MA and DMA was confirmed by comparing the retention times, M-H^+ ESI mass spectra and MRM transitions of the products with those of standards. Standards of amphetamine, methamphetamine and DMA were detected in full scan mode at 5.8, 6.0 and 6.1 min, respectively using the molecular ions at *m/z* 136, 150 and 164, as shown in Figure 13-1.

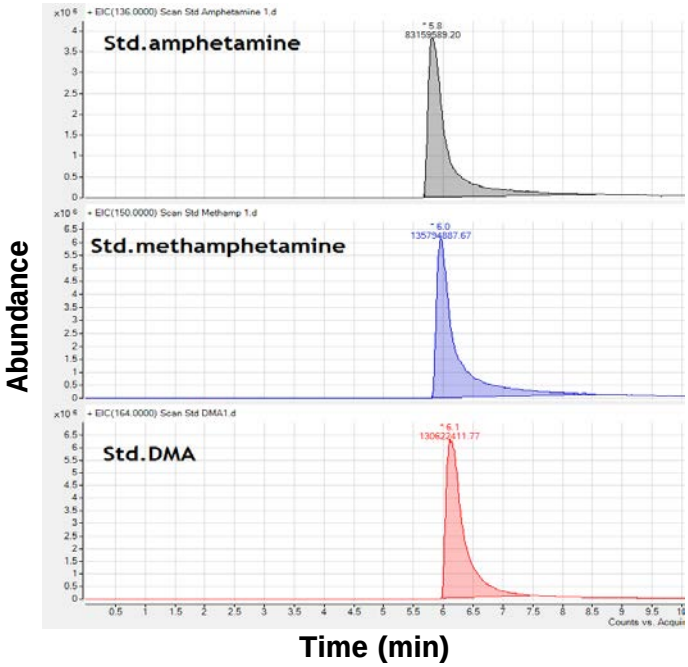


Figure 13-1: EICs of unextracted standards of amphetamine, methamphetamine and DMA analysed by LC/MS.

In amphetamine-spiked samples in 10% formalin solution (with no pH adjustment), amphetamine was rapidly converted to methamphetamine after spiking, which was clearly visible in chromatograms acquired on days 1 and 7 (Figure 13-2) but which was also detected even on day 0. The peak area ratios of methamphetamine/amphetamine in formalin solution on days 0, 1 and 7 were approximately 0.66%, 3.06% and 12.58%, respectively. No DMA was detected in any of the samples.

By contrast, amphetamine in water was not converted to methamphetamine at all, indicating that the reaction product obtained in formalin was due only to the formalin. It should be noted that the peak area of amphetamine in water on day 7 is approximately 50% of the area on day 1 and that this is due to a change in the absolute sensitivity of the instrument rather than to a 50% loss of

amphetamine. Future quantitative analyses would be required to confirm the stability of amphetamine in water.

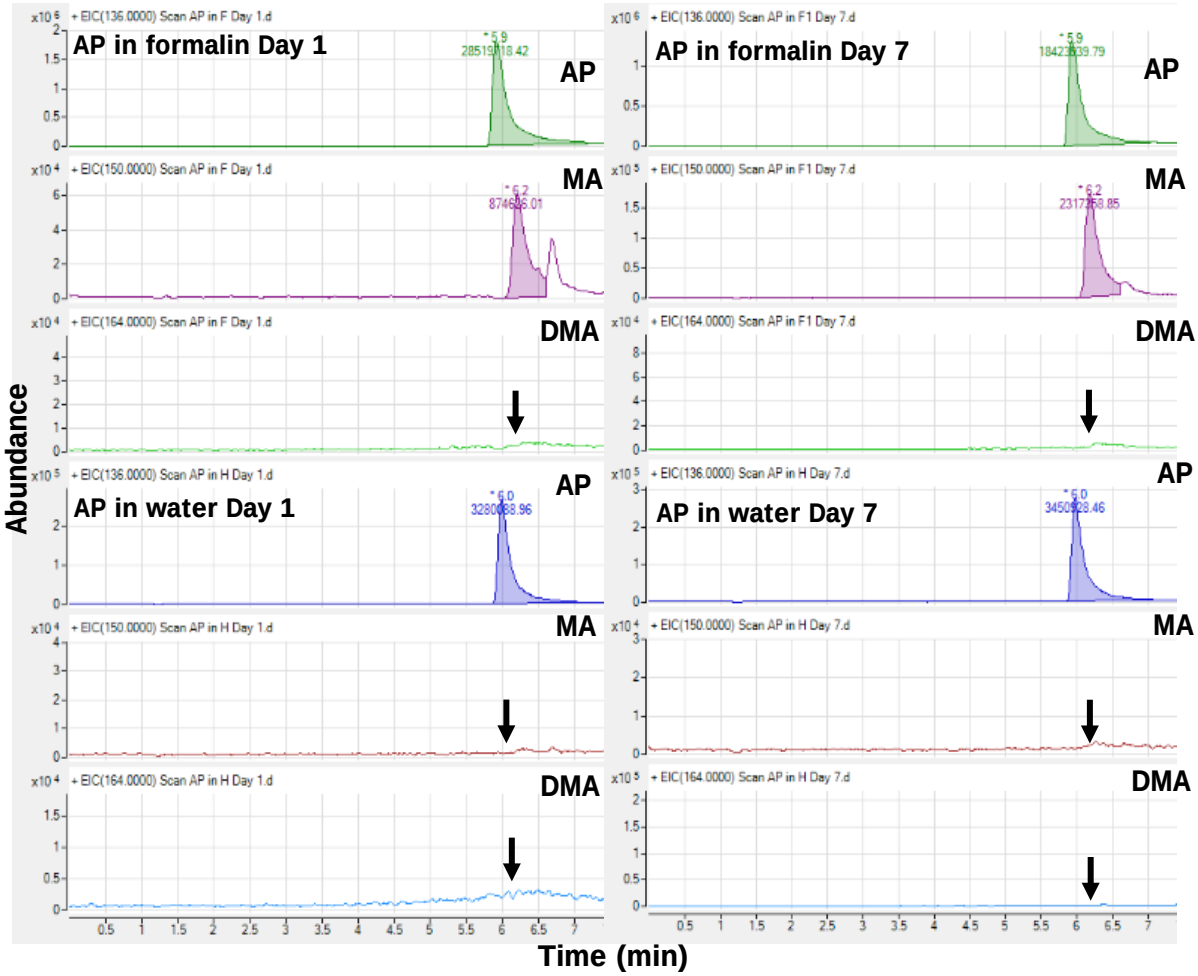


Figure 13-2: MRM chromatograms from LC/MS/MS analysis of amphetamine (AP) spiked samples in 10% formalin and in water at days 1 and 7 showing the formation of methamphetamine (MA) on days 1 and 7.

In methamphetamine-spiked samples in 10% formalin solution (with no pH adjustment), methamphetamine was rapidly converted after spiking to DMA, which was clearly visible on both day 1 and day 7 (Figure 13-3) but which was also detected even on day 0. The peak area ratios of DMA/methamphetamine in formalin solution on days 0, 1 and 7 were approximately 0.76%, 0.78% and 0.83%, respectively.

By contrast, methamphetamine in water was not converted to DMA at all, indicating that the reaction product obtained in formalin was due only to the formalin.

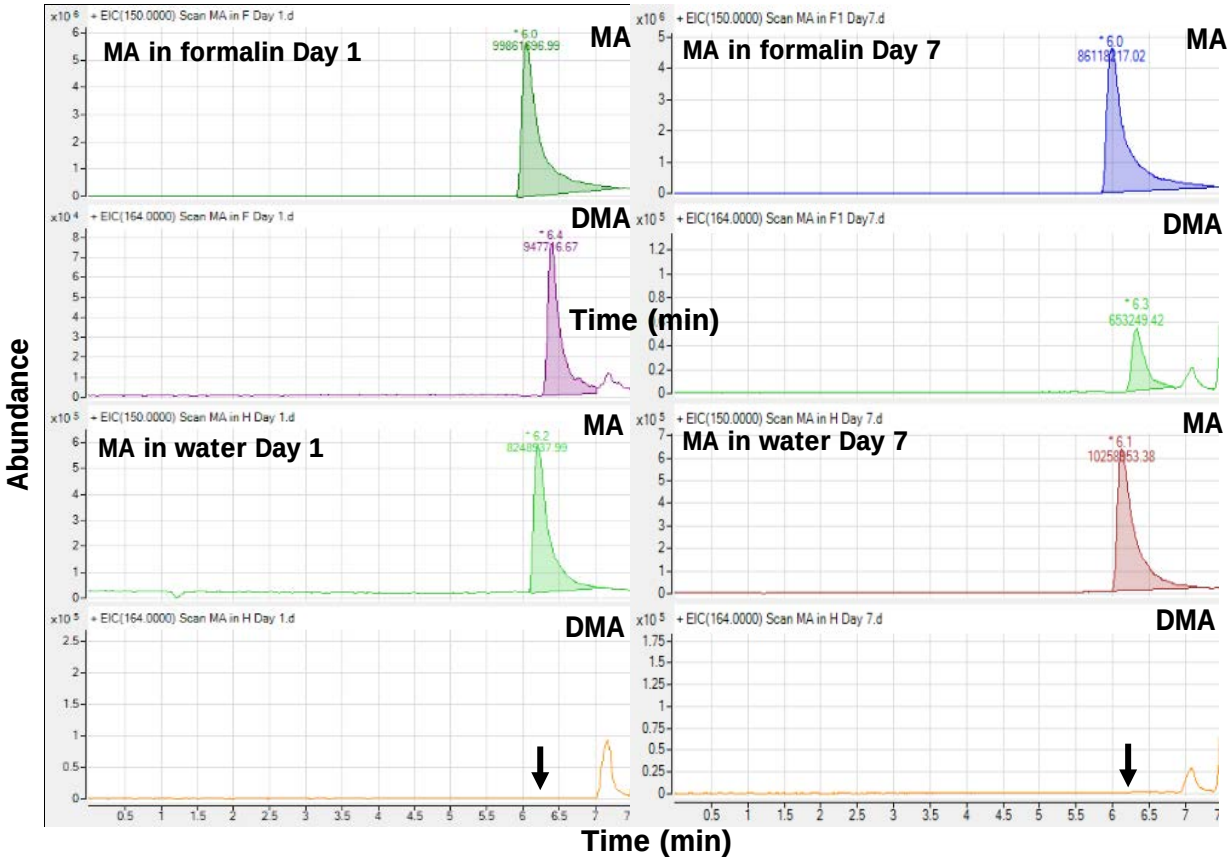


Figure 13-3: LC/MS/MS MRM chromatograms of methamphetamine-spiked samples in 10% formalin and in water at days 0 and 7.

Similar to amphetamine and methamphetamine, MDMA was rapidly converted to MDDMA after spiking. Detection of MDMA and MDDMA was confirmed by comparing the retention times and the M-H$^+$ ESI mass spectra with those of standards. Standards of MDMA and MDDMA were detected in full scan mode at 6.0 and 6.3 min, respectively, using the molecular ions at *m/z* 194 and 208 (Figure 13-4). MDDMA was clearly visible on both day 1 and day 7 (Figure 13-5) but was also detected on day 0. The peak area ratios of MDDMA/MDMA in 10% formalin solution on days 0, 1 and 7 were approximately 2.4%, 2.2% and 5.7%, respectively. By contrast, MDMA in water was not converted to MDDMA, indicating that the reaction product obtained in formalin was due only to the formalin.

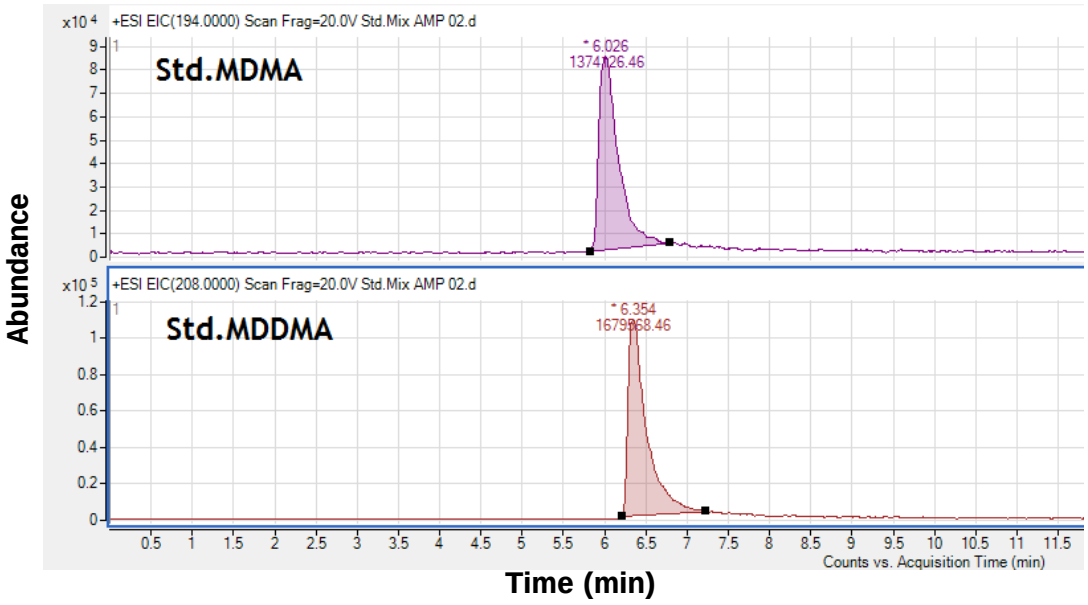


Figure 13-4: LC/MS EIC chromatograms of unextracted standards of MDMA and MDDMA.

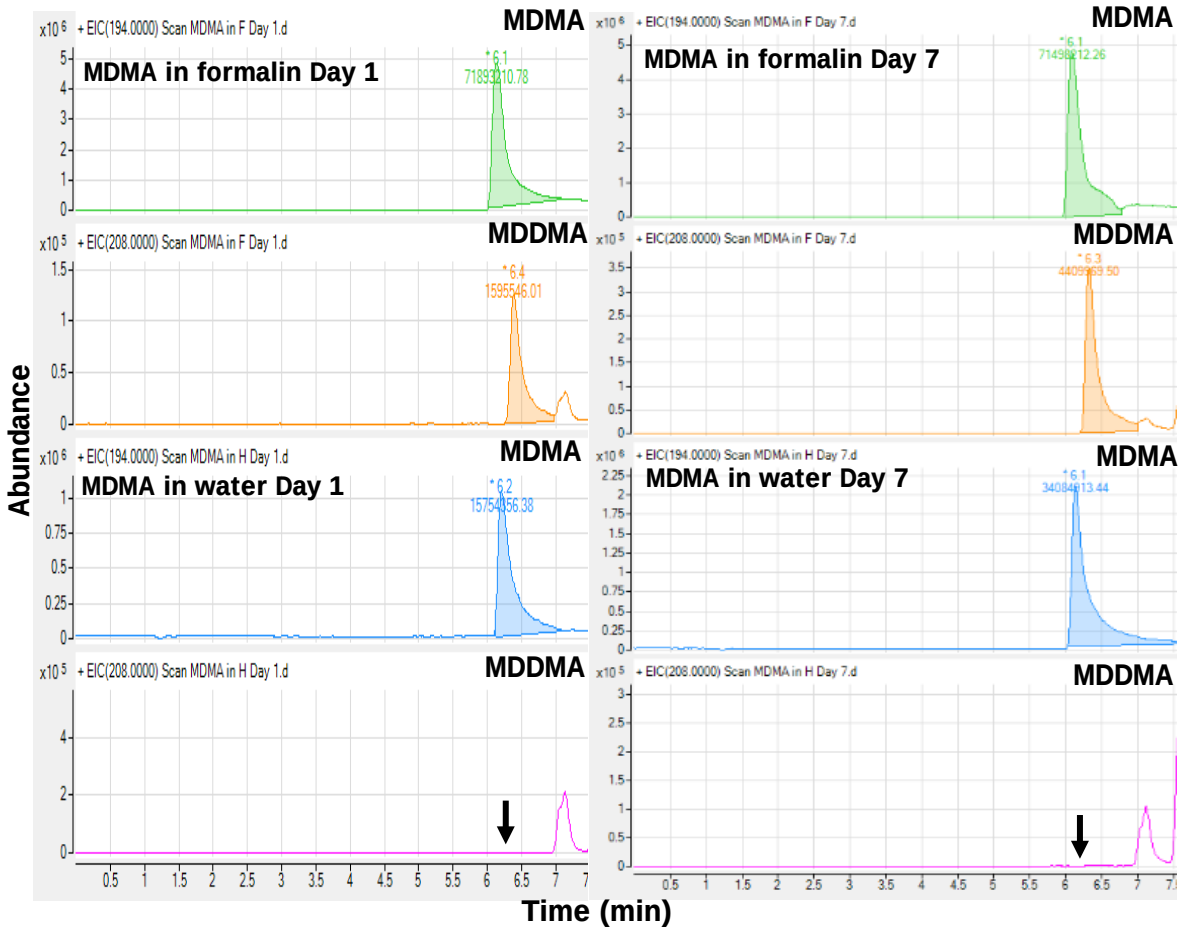


Figure 13-5: LC/MS/MS MRM chromatograms of MDMA-spiked samples in 10% formalin and in water at days 0 and 7.

However, amphetamine, methamphetamine and MDMA do not appear to be completely converted by the Eschweiler-Clarke reaction. Results from 10% formalin spiked samples indicated that the sum of the peak areas of amphetamine and the conversion product detected at days 1 and 7 comprised approximately 42% and 30% of the peak area of the original starting material, respectively. Similarly, methamphetamine and its conversion product DMA comprised approximately 54% and 59% of the starting material at days 1 and 7, respectively and MDMA and its conversion product, MDDMA, comprised approximately 68% and 70% of the starting material at days 1 and 7, respectively.

These discrepancies in peak areas suggest that other chemical reactions have taken place in addition to the Eschweiler-Clarke reactions which have not yet been identified but which should be the subject of future quantitative studies.

13.2.2 Reactions of ATS in formalin blood

To evaluate the Eschweiler-Clarke reaction in formalin blood, which has a different matrix from formalin solution, amphetamine, methamphetamine and MDMA were spiked in 10% formalin blood and control blood (whole blood with no formalin). Spiked samples were analysed after spiking (day 0) and at days 1, 7 and 20.

The results showed that amphetamine, methamphetamine and MDMA are slowly methylated in 10% formalin blood. Conversion products were not detected after spiking or after 24 h. At day 7, traces of conversion products were detected but only with the more sensitive MRM method (Figures 13-6 and 13-7). Amphetamine was identified with MRM transitions at *m/z* 136>119 and 136>91. Methamphetamine was identified with MRM transitions at *m/z* 150>119 and 150>91 and DMA was identified with MRM transitions at *m/z* 164>119 and 164>91. MDMA was identified with MRM transition at *m/z* 194>163 and 194>135 and MDDMA was identified with MRM transitions at *m/z* 208>163 and 208>72. However, in formalin blood samples at day 20, the results showed that amphetamine, methamphetamine and MDMA had been converted to methamphetamine, DMA and MDDMA in yields of approximately 4.4%, 0.24% and 0.68%, respectively, bearing in mind the limitations of these comparisons in the

absence of internal standards. In control blood, no conversion products were detected in any of the samples.

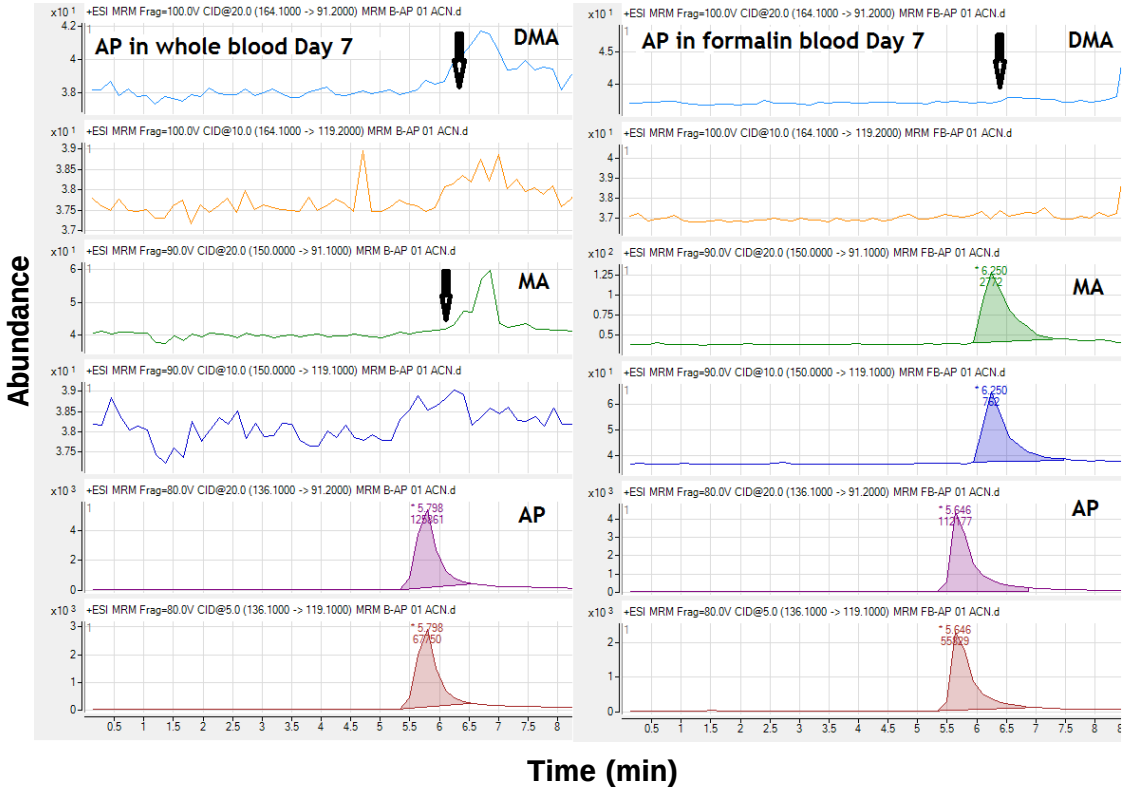


Figure 13-6: LC/MS/MS MRM chromatograms of amphetamine (AP)-spiked samples in 10% formalin blood and in control (whole) blood at day 7. Arrows indicate retention times of conversion products methamphetamine (MA) and DMA.

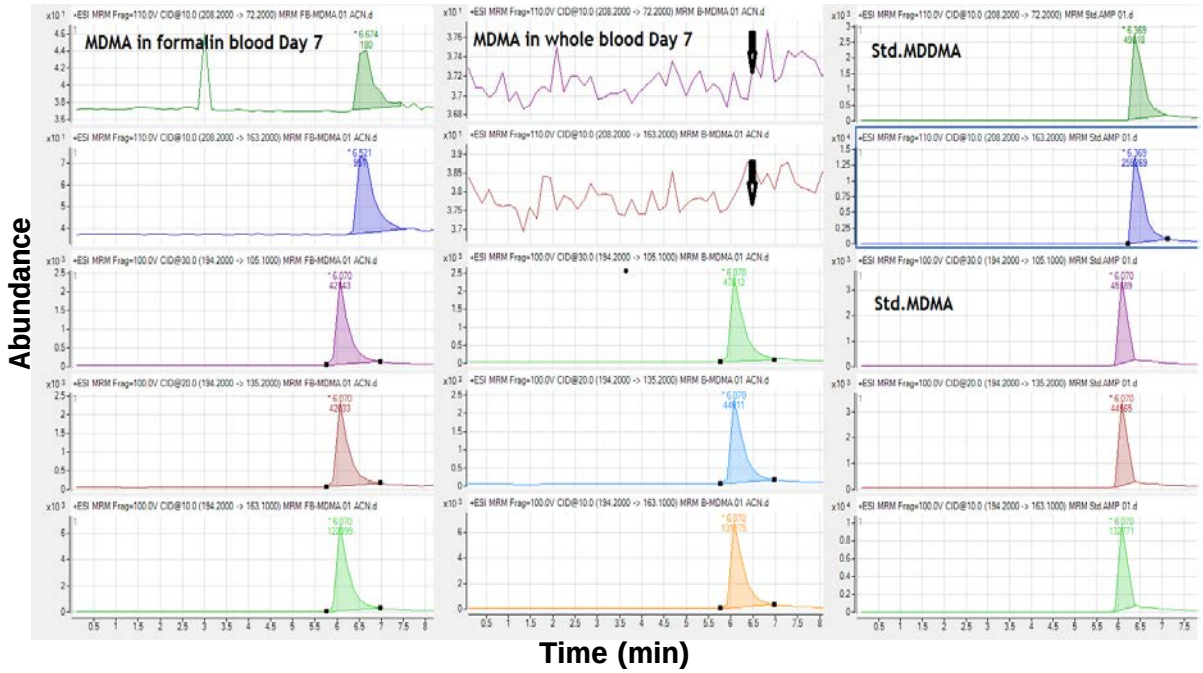


Figure 13-7: LC/MS/MS MRM chromatograms of MDMA-spiked samples in 10% formalin blood and in control (whole) blood at day 7. Arrows indicate retention time of the conversion product MDDMA.

Comparing the results with those obtained using spiked formalin solution samples, amphetamine, methamphetamine and MDMA are more rapidly methylated in formalin solution than in formalin blood. Results obtained from spiked samples in formalin solution are in agreement with previous studies[203-205] that amine-containing compounds react with formaldehyde by the Eschweiler-Clarke reaction. Tirumalai *et al.*[203] reported that conversion products of methamphetamine were not observed in 5%, 10% or 20% formalin solution with no pH adjustment over 30 days. In contrast, results obtained in this study found that amphetamine, methamphetamine and MDMA are rapidly methylated in 10% formalin solution after spiking. However, Shakleya *et al.*[204,205] reported that methamphetamine and MDMA were extensively methylated within 24 h to DMA and MDDMA, respectively, in formalin fixed-liver tissue.

An additional observation in the present study was that the formalin blood did not produce any additional interferences in the LC/MS/MS analyses.

13.3 Reactions of formaldehyde with benzodiazepines

13.3.1 Reactions of benzodiazepines in formalin solution

To evaluate the reactions between benzodiazepines and formaldehyde, alprazolam and diazepam were spiked in both 10% formalin solution and, as controls, in water. Because a previous study[202] found that alprazolam rapidly decreased in six days and was not detected in 10% and 20% formaldehyde after 30 days, spiked samples were analysed after spiking (day 0) at days 1, 5 and 30. The formation of conversion products was identified using LC/MS in full scan mode.

In the present study, alprazolam appeared to be unstable in both formalin solution and water and rapidly decomposed after spiking. The chromatographic peak area was approximately 50% of that of the starting material immediately after spiking and was 38% on day 30 in formalin solution. These changes should be taken as indicative as no internal standards were used. In control samples in water, alprazolam appeared to decompose in 24 h to approximately 55% of the starting material and slowly decomposed to 49% at days 30 (Figure 13-8). A

standard of alprazolam was monitored by LC/MS using the molecular ion M-H+ at *m/z* 309 and was identified at a retention time of 12.9 min.

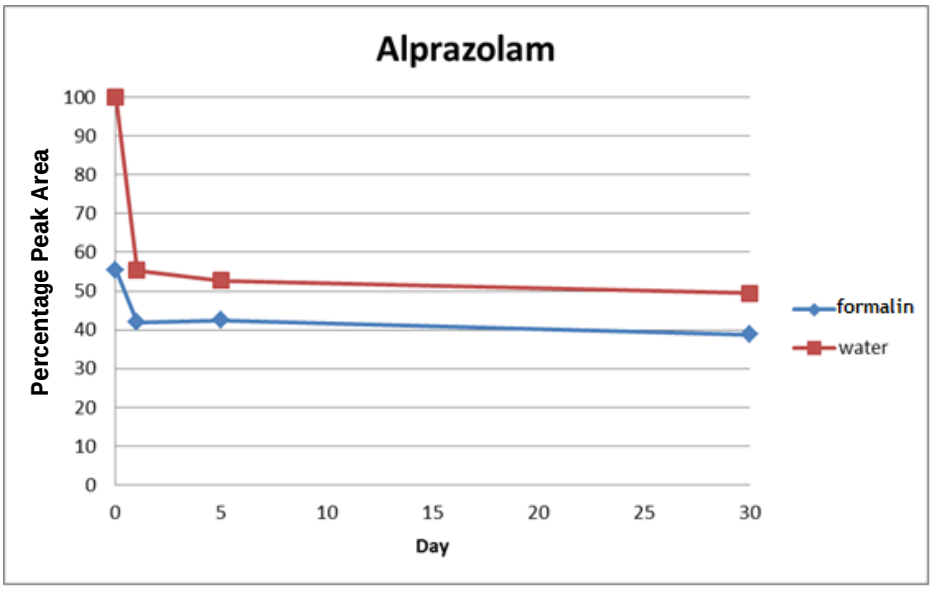


Figure 13-8: Relative peak area of alprazolam in 10% formalin and water over 30 days as a percent of the peak area of the starting material.

Comparing the total ion chromatograms obtained from spiked samples in formalin solution with spiked samples in controls, extra peaks were observed at 11.3, 13.1 and 13.5 min with M-H$^+$ at *m/z* 327, 429 and 399, respectively (see Figure 13-9). A previous study reported by Maślanka *et al.*[243] found that triazolbenzophenone, (open-ring alprazolam) is one of the major hydrolysis products of alprazolam and can be identified at M-H$^+$ *m/z* 327.32. The hydrolysis of benzodiazepines has previously been studied and reported[244-246]. The diazepinone ring in benzodiazepines is highly sensitivity to hydrolysis[245]. Hydrolysis pathways can follow either 1,2 or 4,5 position cleavage, see Figure 12-2. The 4,5 position hydrolysis was reported to occur under acidic conditions whereas 1,2 cleavage is more important under basic conditions[247]. The diazepinone ring opening can occur by fragmentation of the C=N bond. The formation of open-ring alprazolam in spiked formalin samples was confirmed by the observation of a molecular ion (M-H$^+$) at *m/z* 327. Open-ring alprazolam increased after spiking and was clearly observed by day 30, see Figure 13-10.

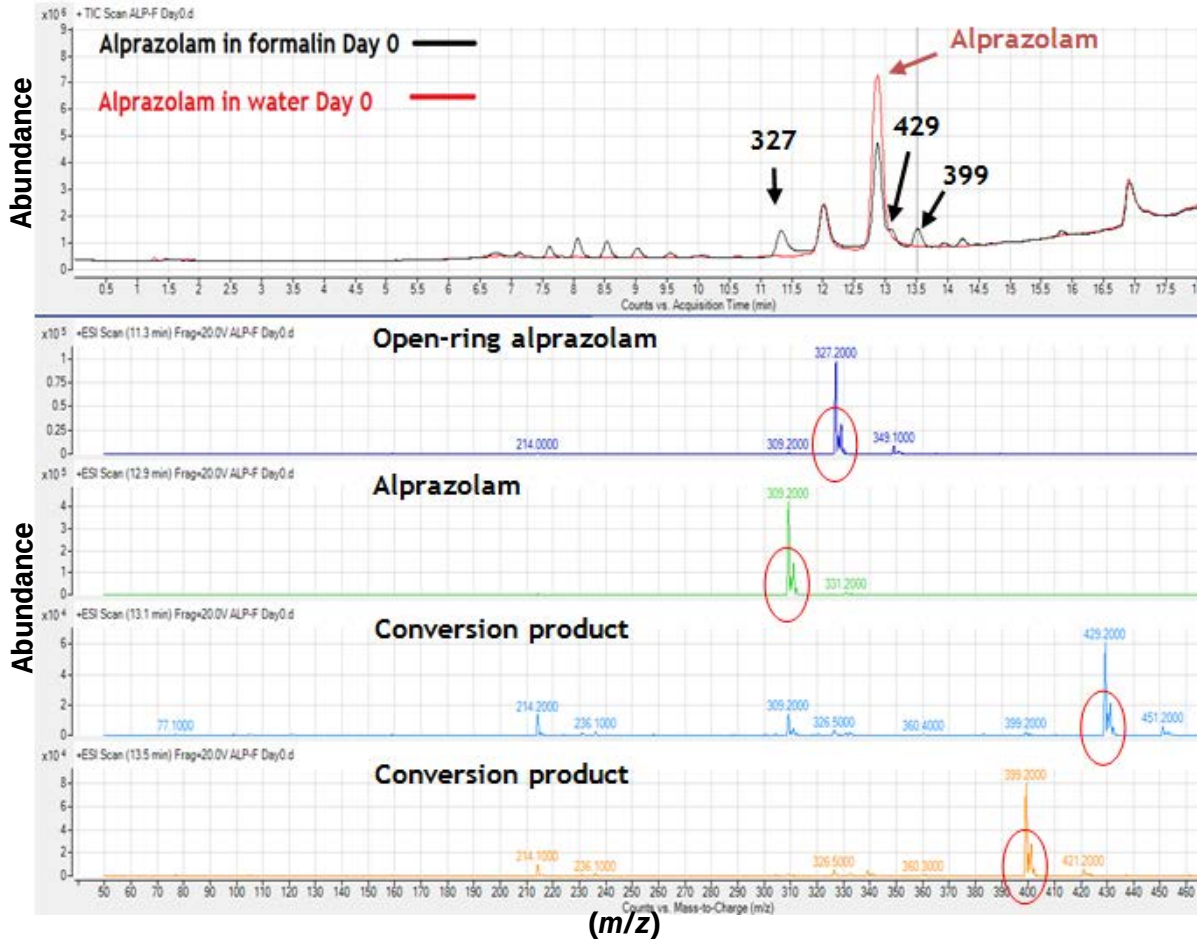


Figure 13-9: LC/MS chromatograms of alprazolam samples in 10% formalin after spiking (day 0).

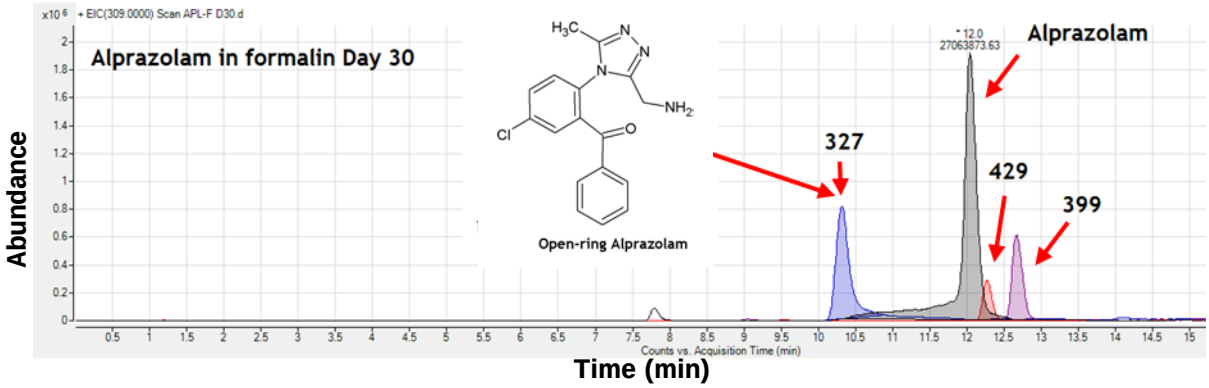


Figure 13-10: LC/MS chromatograms of alprazolam samples in 10% formalin at day 30 and chemical structure of open-ring alprazolam.

Furthermore, the results for spiked samples also suggested that alprazolam was converted to compounds which had ESI molecular ions ($M\text{-}H^+$) at *m/z* 399 and 429, which were higher than alprazolam by 90 Da (for *m/z* 399) and by 120 Da (for *m/z* 429). Because alprazolam is a chlorine-containing compound, the isotopic pattern of the chlorine isotopes (^{35}Cl:^{37}Cl = 3:1) was used to identify the

degradation products. The isotopic pattern was observed in both degradation products. Neither of these conversion products were detected in control samples over 30 days.

To confirm the formation of these conversion products, deuterated alprazolam-d_5 standard was spiked in formalin solution and water and was analysed after spiking (day 0) and at days 1 and 7. Alprazolam-d_5 was detected at 11.6 min with an M-H^+ ion at *m/z* 314, and open-ring alprazolam-d_5 was detected at 10.7 min with an M-H^+ ion at *m/z* 332. A peak of the other conversion product was observed at 11.9 min with an M-H^+ ion at *m/z* 404 which was higher than that of alprazolam-d_5 by 90 Da. However, the conversion product at *m/z* 434 was not observed (see Figure 13-11).

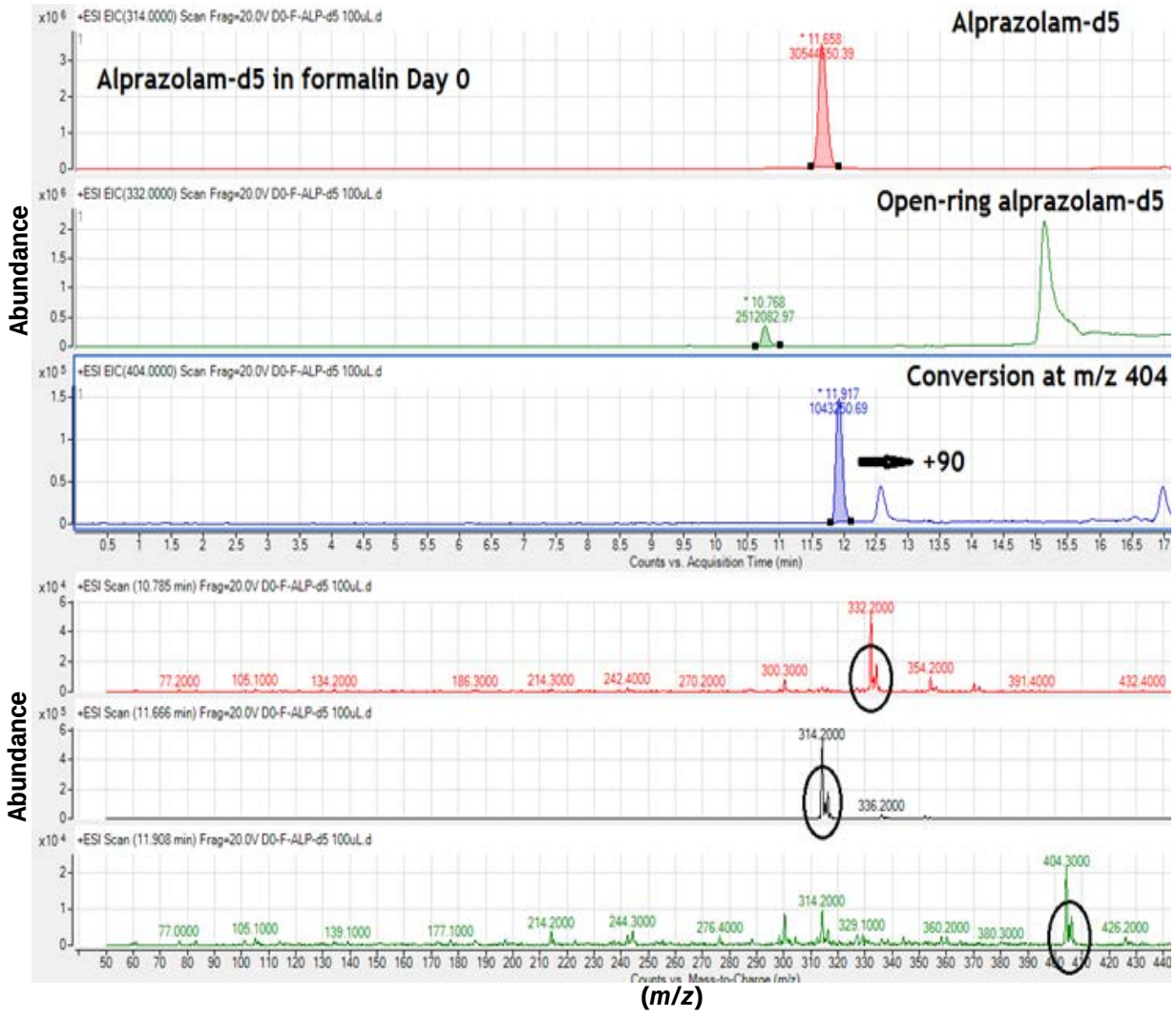


Figure 13-11: LC/MS chromatograms of alprazolam-d_5 samples in 10% formalin after spiking (day 0) and mass spectra of open-ring alprazolam-d_5, alprazolam-d_5 and the conversion product with M-H^+ at *m/z* 404.

By contrast with alprazolam, diazepam was found to be stable in both formalin solution and water over 30 days. It was detected in both samples at approximately 90% of the starting material at day 30, see Figure 13-12. This result is in general agreement with a previous study by Tracy *et al.*[202] who reported that diazepam is stable throughout a 30 day period in control solution and that it was detected at approximately 65% of the starting amount in 10% formalin solution with no pH adjustment after 30 days.

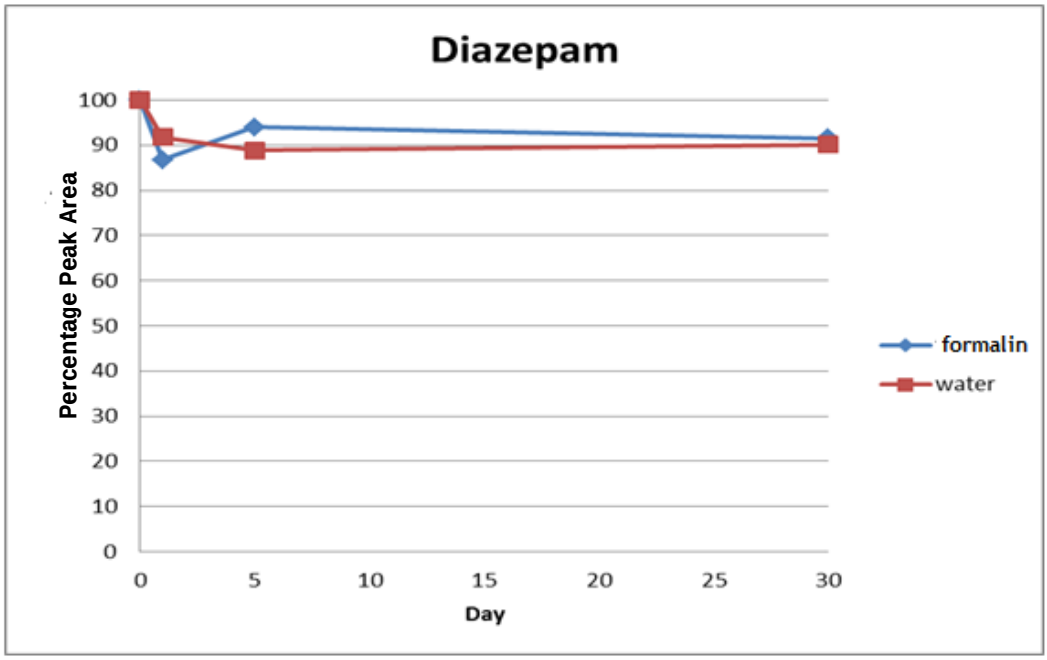


Figure 13-12: Relative peak area of diazepam in 10% formalin and water over 30 days as a percent of the peak area of the starting material.

Traces of conversion products of diazepam in formalin solution were observed in all samples at a retention time of 10.9 min, with an M-H$^+$ ion at *m/z* 303, and at 14.3 min, with two M-H$^+$ ions at *m/z* 315 and 375, respectively. A previous study by Maslanka *et al.*[243] also reported that 2-glycyloamino-5-chloro-benzophenone (open-ring diazepam) is one of the major hydrolysis products of diazepam and can be identified at M-H$^+$ *m/z* 303.3. The isotopic pattern of ^{35}Cl and ^{37}Cl (3:1) was also observed in the other two degradation products with M-H$^+$ ions at *m/z* 315 and 375, which are higher than that of diazepam by 30 Da and 90 Da respectively (see Figure 13-13).

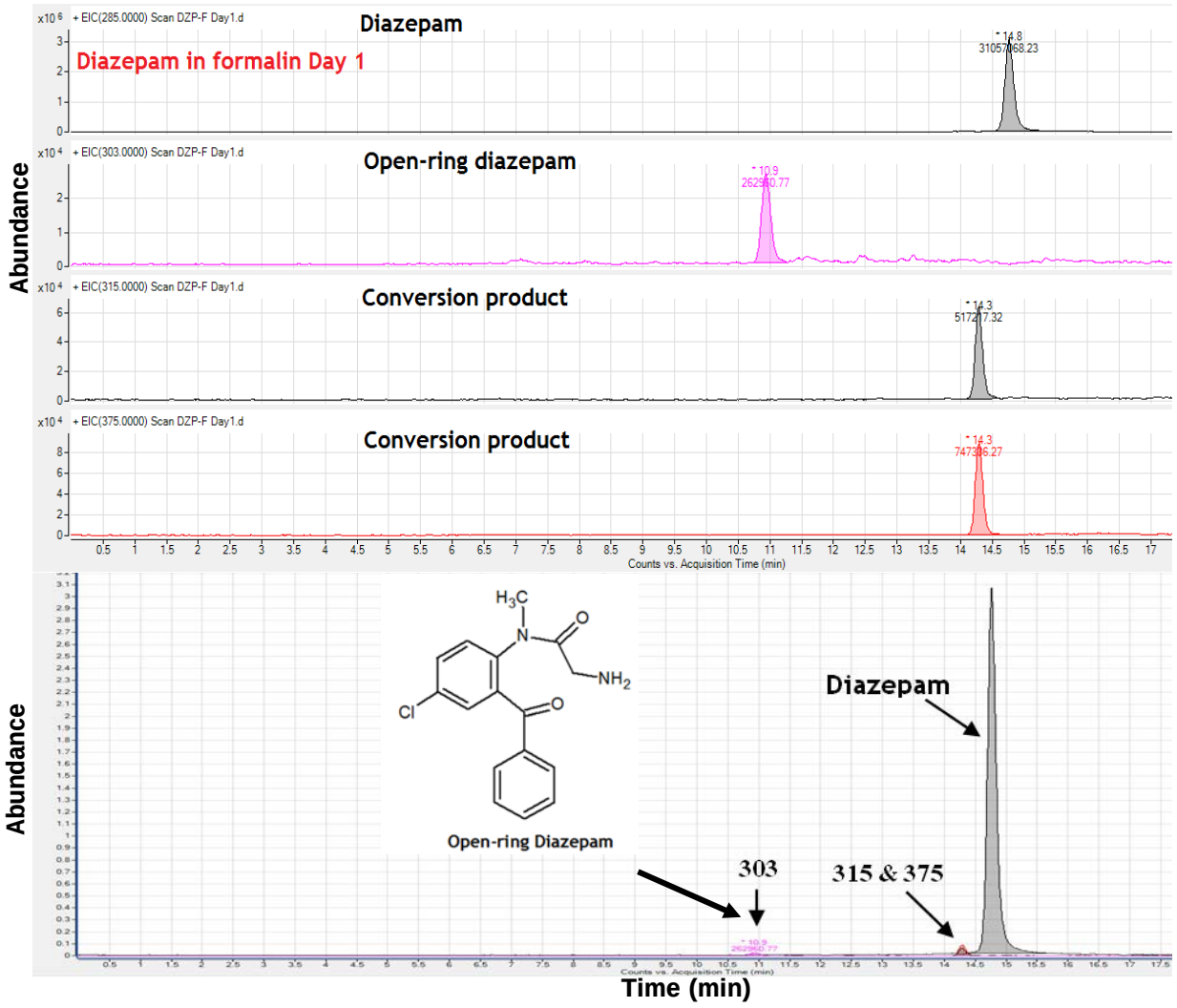


Figure 13-13: LC/MS chromatograms of diazepam samples in 10% formalin at day 1 and chemical structure of open-ring diazepam.

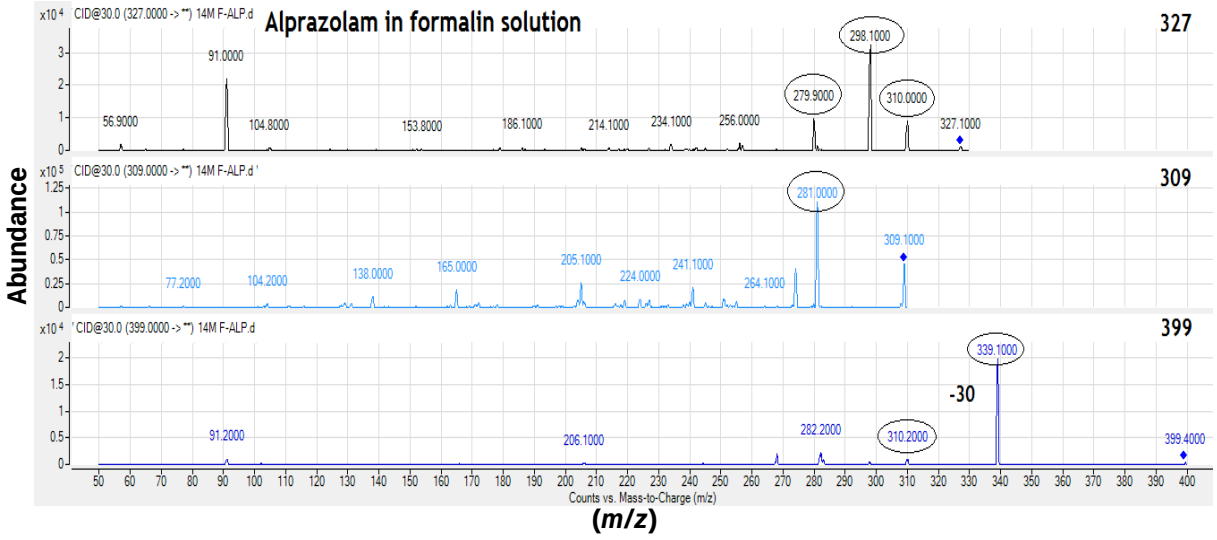


Figure 13-14: LC/MS/MS product ion scans of alprazolam conversion products in 10% formalin solution.

Furthermore, more information on the degradation products was obtained using LC/MS/MS product ion scan mode. In alprazolam samples in formalin solution, the results showed that the product with M-H^+ *m/z* 399 fragmented to ions at *m/z* 339 and 310, whereas open-ring alprazolam (M-H^+ at *m/z* 327) fragmented to ions at *m/z* 310, 298 and 280 (see Figure 13-14). A previous study by Gonsalves *et al.*[246] showed that open-ring alprazolam was fragmented to ions at *m/z* 310 and 298 which is in agreement with the fragment ions obtained here for alprazolam samples in formalin solution. It can be concluded that the conversion product with M-H^+ at *m/z* 327 is open-ring alprazolam. However, this method could not be applied to diazepam because of the low concentrations of its conversion products.

The data available indicate that the conversion product of alprazolam, with M-H+ at *m/z* 399, is alprazolam to which is attached formaldehyde as paraformaldehyde (plus 30, 60, 90 Da by the addition of 1, 2 or 3 formaldehyde molecules). However, the structure of the conversion compounds will be the subject of future work which needs to apply high resolution mass spectrometry and NMR to confirm the chemical structures.

13.3.2 Reactions of benzodiazepines in formalin blood

Because diazepam was found to be stable in formalin solution and only traces of conversion products were observed, alprazolam alone was spiked in 10% formalin blood and control blood to observe the conversion products at days 1, 3, 7, 10, 15 and 30.

Alprazolam was found to be stable in control blood but unstable in formalin blood. Conversion products of alprazolam were observed in formalin blood from day 1 to day 30. Open-ring alprazolam, with M-H^+ at *m/z* 327, as detected at day 1 in approximately 13% yield compared to alprazolam and then increased to 36% yield at day 30. Similarly, the product with M-H^+ at *m/z* 399 was detected in approximately 39% yield at day 1 which increased to 78% at day 30 (see Figure 13-15). A trace of another conversion product with a small peak area was observed in formalin solution with M-H^+ at *m/z* 429 but only at days 7, 10 and 15 and was not included in Figure 13-15.

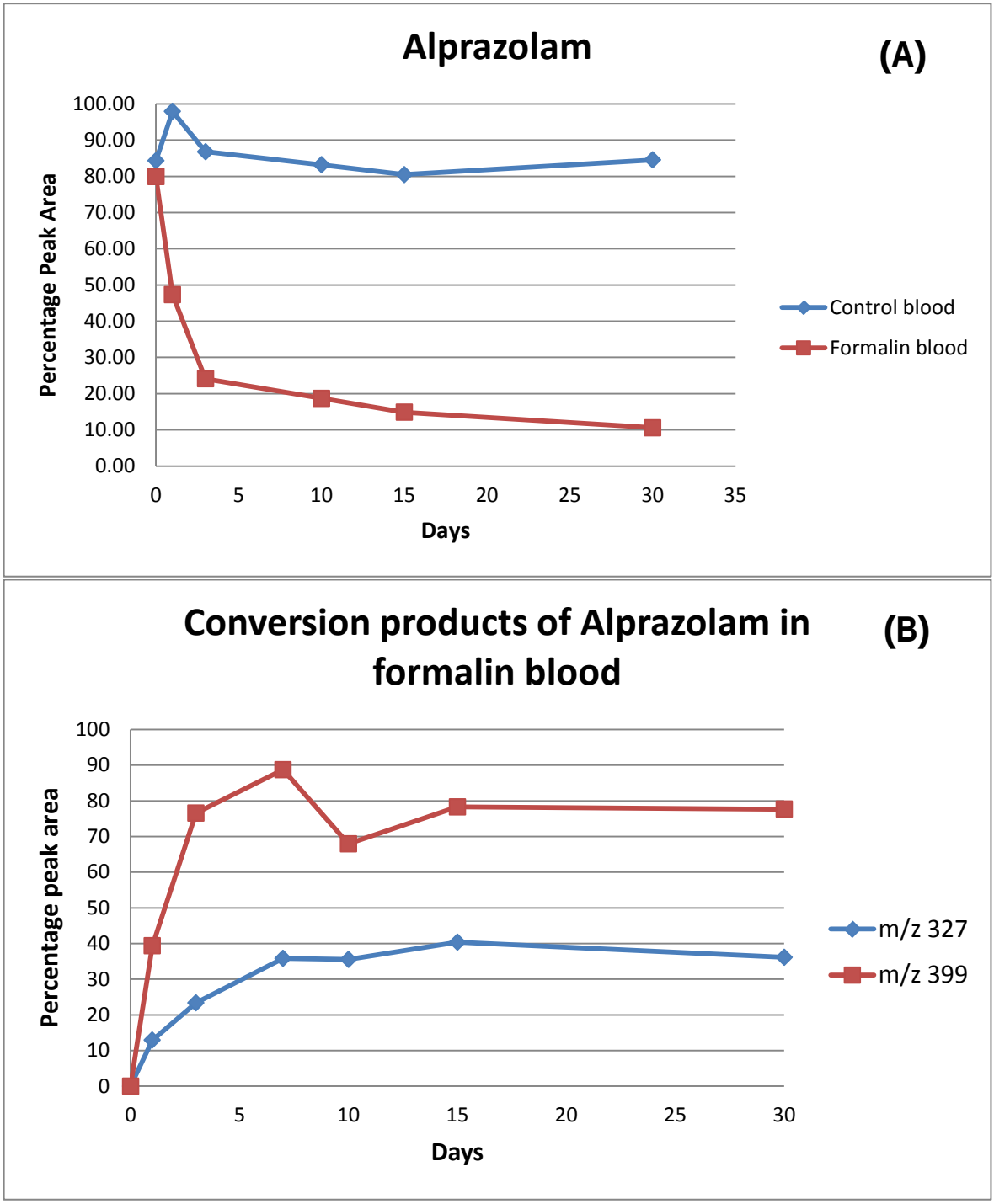


Figure 13-15: (A) Percentage peak area of alprazolam in 10% formalin blood and control blood over 30 days. (B) Percentage peak area of alprazolam conversion products formed in 10% formalin blood compared with alprazolam. The EIC of *m/z* 327 is for open ring alprazolam and of *m/z* 399 is for the adduct of alprazolam with formaldehyde.

13.4 Reactions of formaldehyde with opiates

13.4.1 Reactions of opiates in formalin solution

Four drugs (codeine, morphine, hydrocodone and hydromorphone) were included in this study to identify the reactions between opiates and formaldehyde. Standards of opiates were spiked in 10% formalin solution and water as controls and were analysed after spiking, at days 0, 1, 5 and 30. The formation of conversion products was identified using LC/MS in full scan mode.

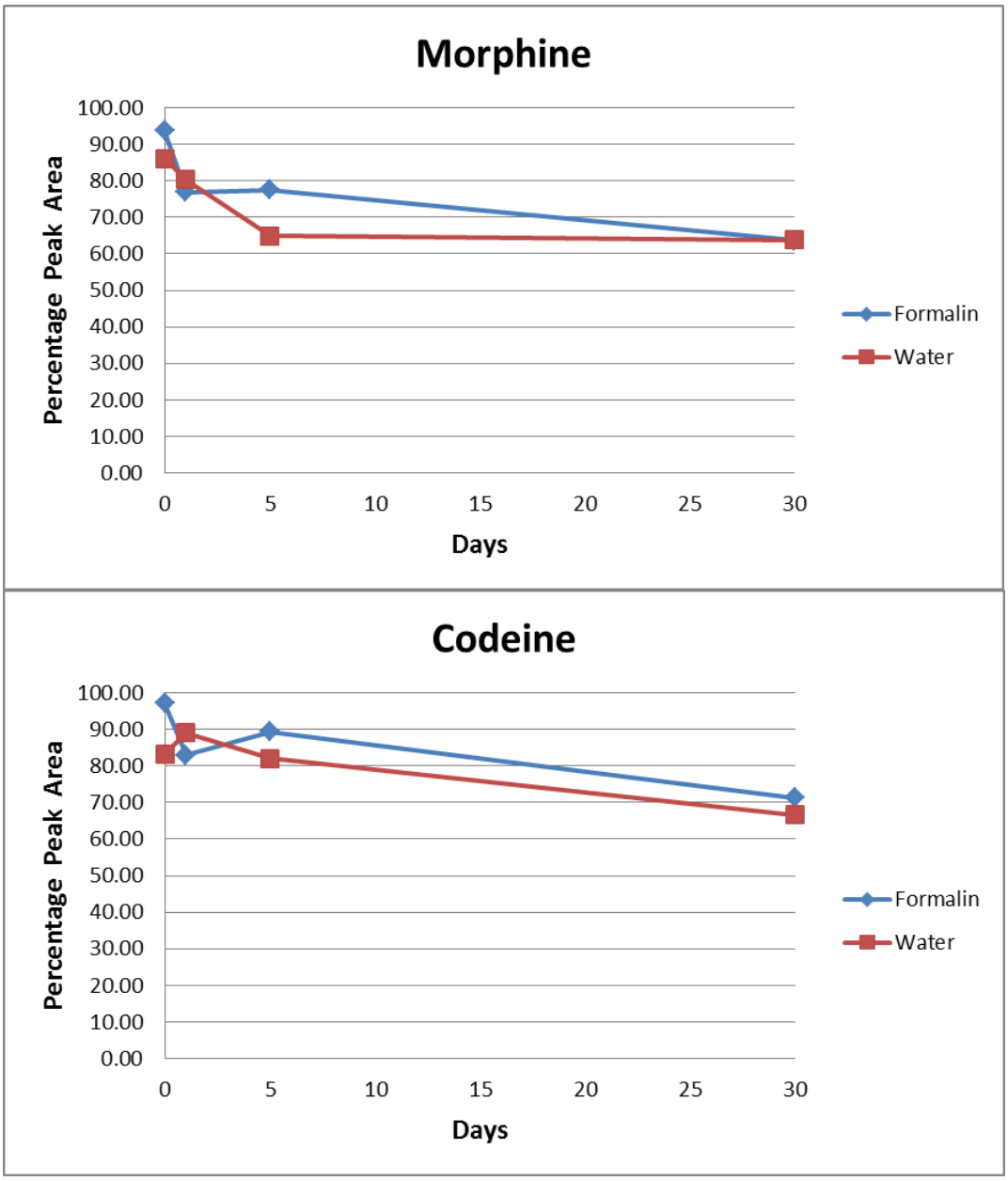


Figure 13-16: Percentage peak areas of codeine and morphine in 10% formalin and water over 30 days.

These initial results indicate that morphine and codeine appear to be more stable in formalin solution than hydromorphone and hydrocodone, see Figures 13-16 and 13-17. After 30 days, codeine was still observed in both formalin solution and water and the peak areas relative to the peak area of the starting materials were 66% in water and 71% in formalin solution. Likewise, morphine was observed at 63% of the starting peak area in both formalin solution and water. No conversion products were observed in spiked-codeine and spiked-morphine in formalin solution and water samples. Standard morphine and codeine were identified by LC/MS using their molecular ions ($M\text{-}H^+$) at *m/z* 286 and 300 and were detected at 6.6 and 7.8 min, respectively.

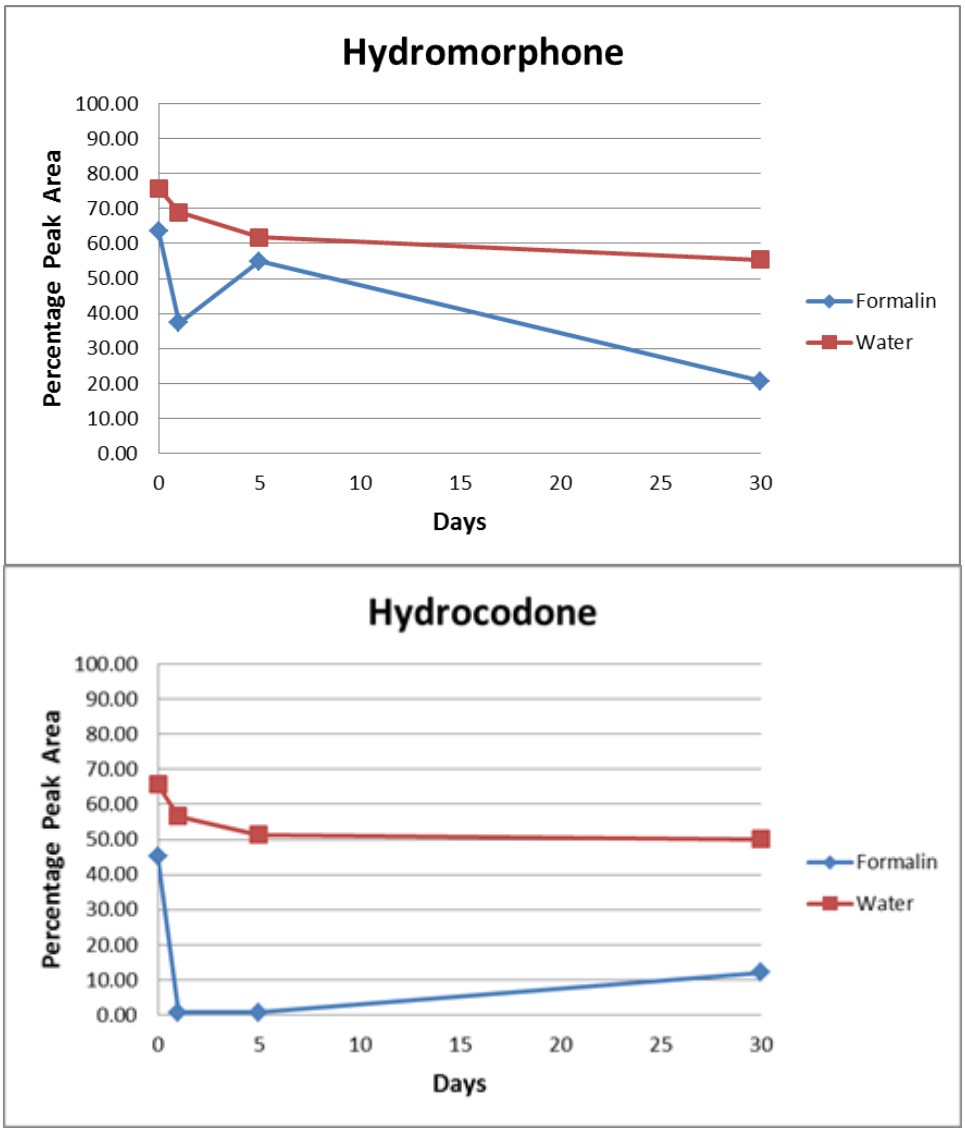


Figure 13-17: Percentage peak area of hydrocodone and hydromorphone in 10% formalin and water over 30 days.

Hydromorphone and hydrocodone were found to be more stable in water than formalin solution. After 30 days, hydromorphone was still observed in both formalin solution and water at a peak area of 55% of the starting peak area in water and 20% in formalin solution. Likewise, hydrocodone was observed at 12% of the starting peak area in formalin solution and 50% in water, see Figure 13-17. Standard hydromorphone and hydrocodone were identified using their molecular ions ($M\text{-}H^+$) at *m/z* 286 and 300 and were detected at 6.6 and 7.6 min, respectively.

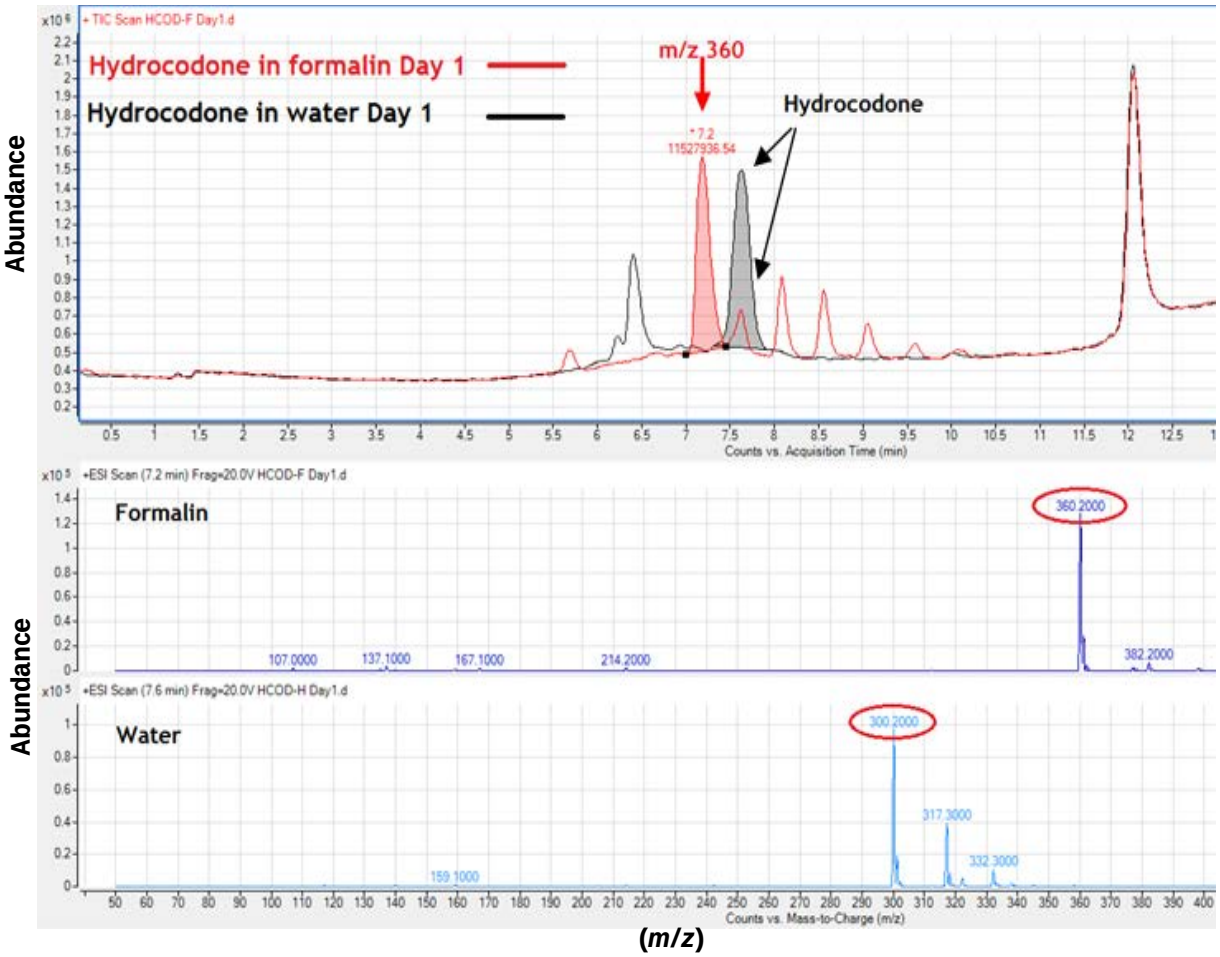


Figure 13-18: LC/MS overlay chromatograms and mass spectra of hydrocodone samples in 10% formalin at day 1.

Hydrocodone had decomposed by day 1 and only a trace amount was detected. The dip in the graph on days 1 and 5 is most likely due to a decrease in instrument sensitivity rather than a true decrease. Degradation products of hydrocodone were observed after spiking and were clearly identifiable at day 1, see Figure 13-18. The molecular ion peak ($M\text{-}H^+$) of the product detected at 7.2 min was at *m/z* 360, which is higher than that of hydrocodone by 60 Da. A second degradation product was detected at 7.5 min with $M\text{-}H^+$ at *m/z* 330,

which is higher than that of hydrocodone by 30 Da. All conversion compounds were detected in formalin solution over 30 days and the peak areas of both compounds were higher than that of hydrocodone after day 1. In contrast, no degradation products were observed in spiked-control samples over 30 days.

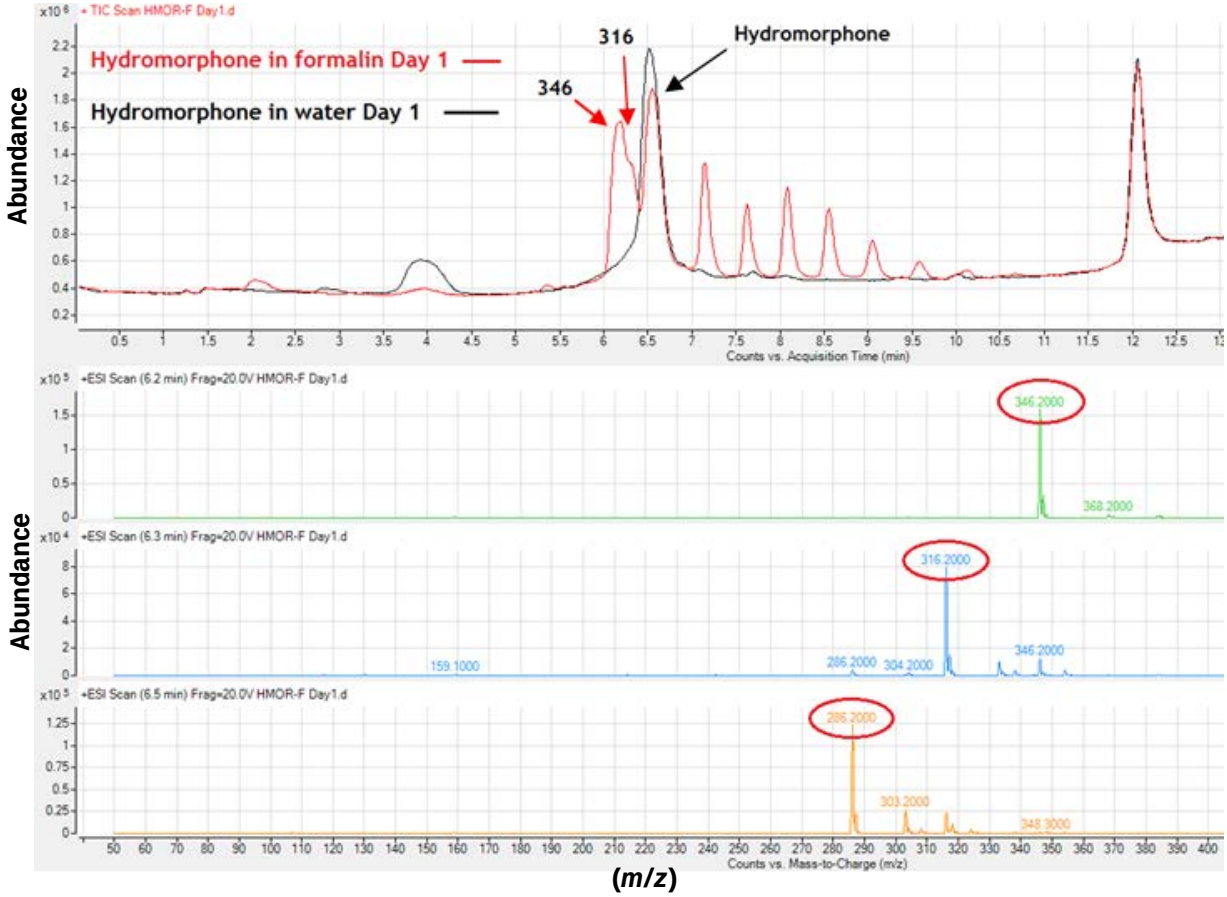


Figure 13-19: LC/MS overlay chromatograms and mass spectra of hydromorphone samples in 10% formalin at day 1.

Similar to hydrocodone, degradation products of hydromorphone were observed after spiking and were clearly identifiable by day 1, see Figure 13-19. Products with molecular ion peaks ($M\text{-}H^+$) at *m/z* 316 and 346, which are higher than that of hydromorphone by 30 Da and 60 Da, were detected at 6.3 and 6.2 min, respectively. Both conversion compounds were detected in formalin solution over 30 days and the peak area of the degradation compound with $M\text{-}H^+$ at *m/z* 316 was higher than that of hydromorphone by day 30. No degradation products were observed in spiked-control samples in water over 30 days.

Furthermore, more information on the degradation compounds was obtained using LC/MS in the product ion scan mode. In hydrocodone samples in formalin solution, the results showed that the compound with $M\text{-}H^+$ at *m/z* 360 gave

fragment ions at *m/z* 330, 312 and 300 whereas the compound with M-H$^+$ at *m/z* 330 gave fragment ions at *m/z* 312 and 300. In hydromorphone samples in formalin solution, results showed that the compound with M-H$^+$ at *m/z* 316 produced fragment ions at *m/z* 298 and 286 whereas the one with M-H$^+$ at *m/z* 346 was fragmented to ions at *m/z* 316, 298 and 286. These indicate that conversion products found in formalin solution are hydrocodone or hydromorphone to which was attached to formaldehyde in the form of paraformaldehyde (plus 30, 60, 90 Da), see Figure 13-20. However, the detailed structure of hydrocodone and hydromorphone conversion compounds will also be the subject of future work which needs to apply high resolution mass spectrometry and NMR to confirm their chemical structures.

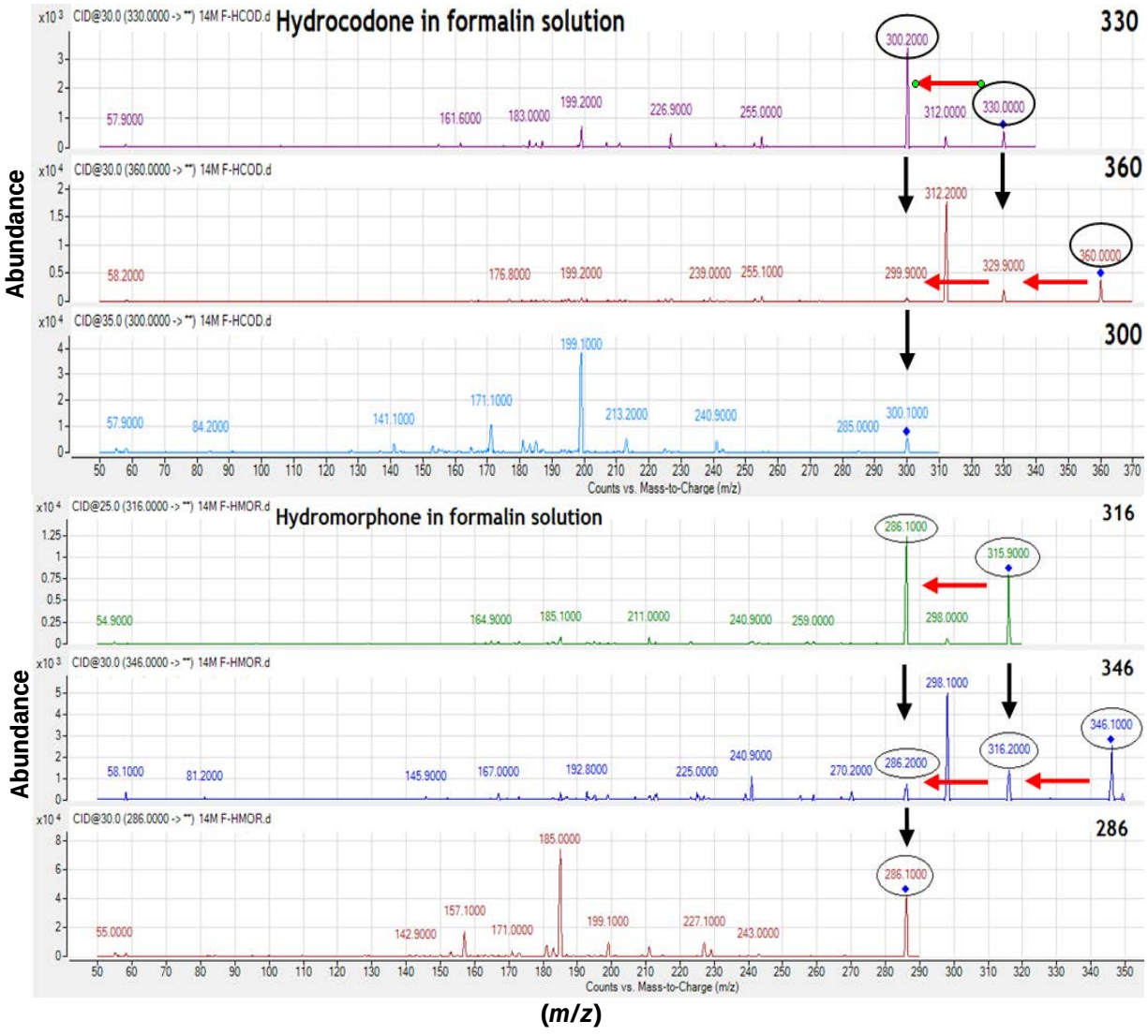


Figure 13-20: LC/MS product ion scans of conversion products of hydrocodone and hydromorphone in 10% formalin.

13.4.2 Reactions of opiates in formalin blood

To evaluate the reactions of opiates with formalin blood, which is a different matrix from formalin solution, codeine, hydrocodone, hydromorphone and morphine were spiked in 10% formalin blood and control blood. Spiked samples were analysed after spiking (day 0) and at days 1, 3, 7, 10, 15 and 30. In these initial studies no internal standards were added and relative peak areas obtained over a period of 30 days would have had larger variations due to changes in instrumental performance than might have been obtained when using internal standards, which might explain the fluctuations observed in drug concentrations.

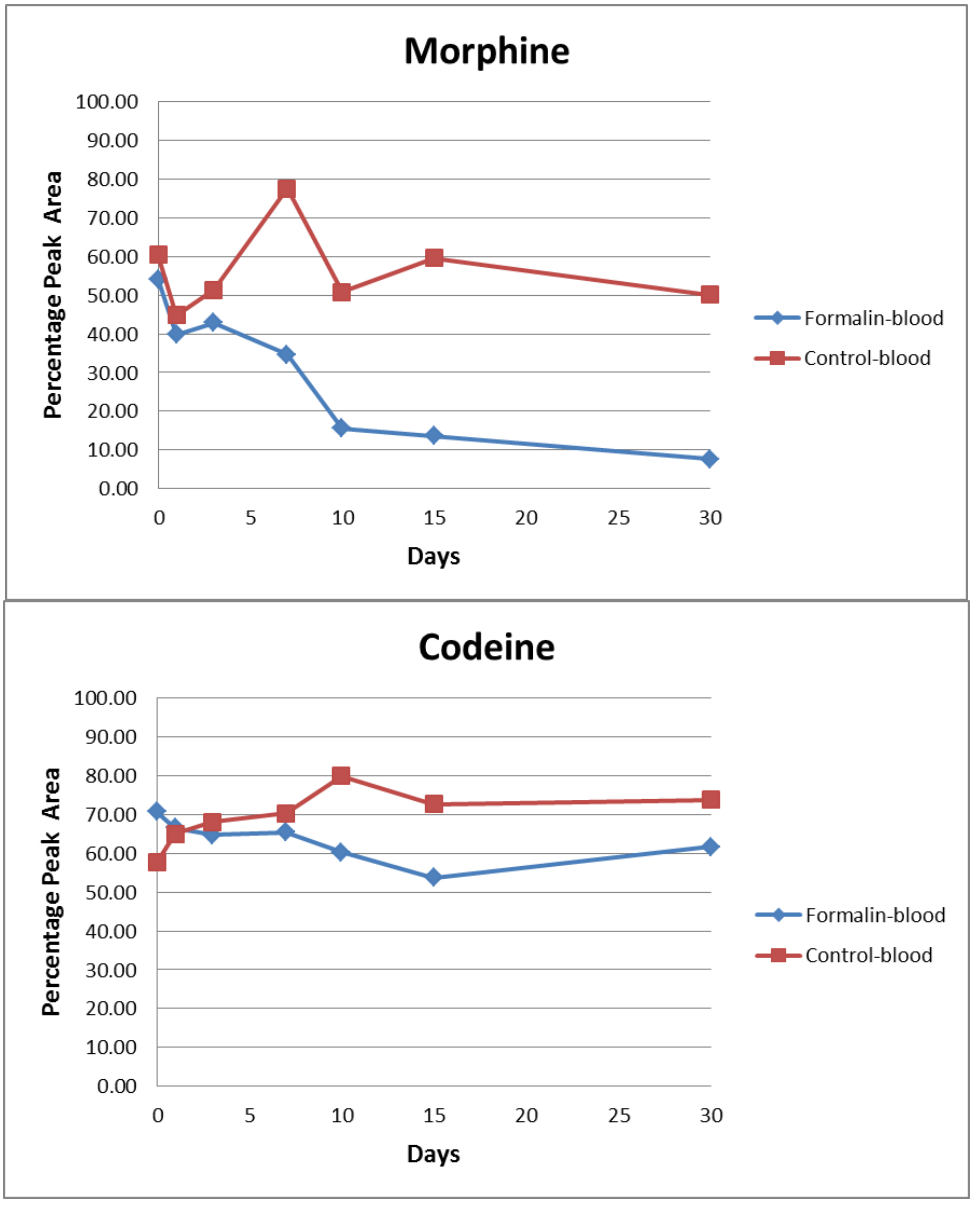


Figure 13-21: Percentage of codeine and morphine in 10% formalin blood and control blood over 30 days.

The results showed that codeine is more stable in formalin blood than hydrocodone. At day 30, codeine was still observed in both formalin blood and control blood and the peak area was 74% in control blood and 62% in formalin blood compared with the initial spiked peak area. Morphine was observed at 50% of the starting peak area in control blood but it was observed at only 7% in formalin blood at day 30, see Figure 13-21. No conversion products were observed in spiked-codeine and spiked-morphine in formalin blood and control blood samples.

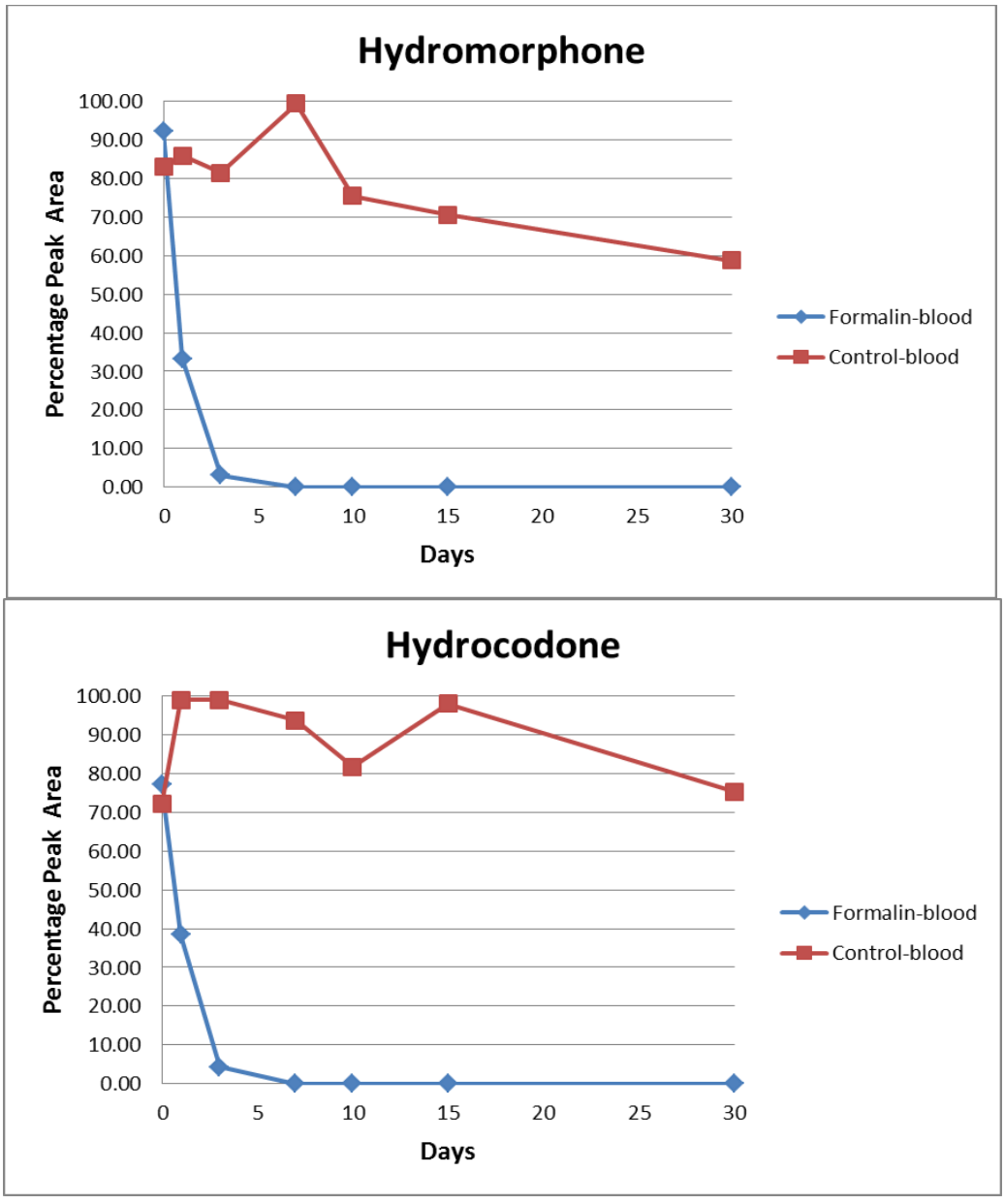


Figure 13-22: Percentage of hydrocodone and hydromorphone in 10% formalin blood and control blood over 30 days.

Hydrocodone and hydromorphone were more stable in control blood than formalin blood, see Figure 13-22. At day 30, hydrocodone and hydromorphone were detected in control blood at 75% and 59% compared with the initial spiked peak area, respectively. In contrast, hydrocodone and hydromorphone in formalin blood rapidly decreased within 24 h and could not be detected in MS full scan mode after 2 days and in MRM mode after 7 days. Moreover, traces of the conversion products of hydrocodone with M-H^+ ions at *m/z* 330 and 360 were detected in formalin blood samples within 3 days but were not detected after day 7 whereas a trace of the conversion product of hydromorphone that gave the M-H^+ ion at *m/z* 346 was detected within 7 days but was not detected after day 10.

The results indicate that reactions continued to progress between the drugs and formalin and possibly also with blood components over the period of study. For both hydrocodone and hydromorphone reaction products were observed initially which were similar to the formalin adducts observed in formalin solution, but subsequently these could not be detected, suggesting that further changes had occurred. These studies need to be repeated to identify the reaction sequences which occur and the initial and subsequent products they produce.

13.5 Reactions of formaldehyde with a carbamate insecticide

13.5.1 Reactions of carbosulfan in formalin solution

To evaluate the reactions between carbosulfan and formaldehyde, a standard of carbosulfan was spiked in 10% formalin solution and in water as a control. A pilot study by GC/MS found that carbosulfan rapidly decomposed in formalin solution within 7 days. Subsequently, spiked samples were analysed after spiking and at 24 and 48 h. The formation of the conversion product was identified using GC/MS in full scan mode. A standard of carbosulfan eluted at 14.5 min when analysed by GC/MS. The GC/MS chromatogram (A) and EI^+ mass spectrum (B) of carbosulfan are shown in Figure 13-23.

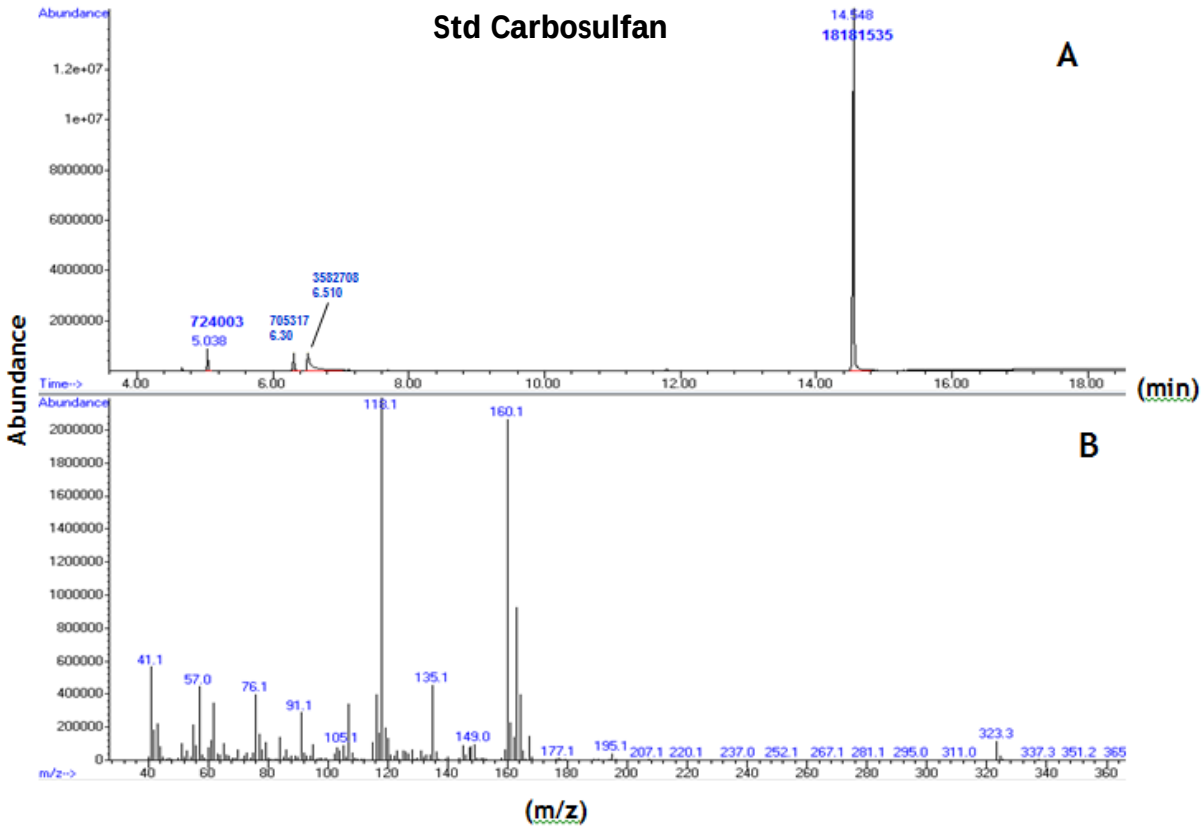


Figure 13-23: GC/MS chromatogram (A) and EI^+ mass spectrum (B) of carbosulfan.

The results showed that carbosulfan reacted with formalin solution and was completely hydrolysed to 7-phenolcarbofuran within 24 h whereas carbosulfan in water was stable up to 48 h, see Figure 13-24.

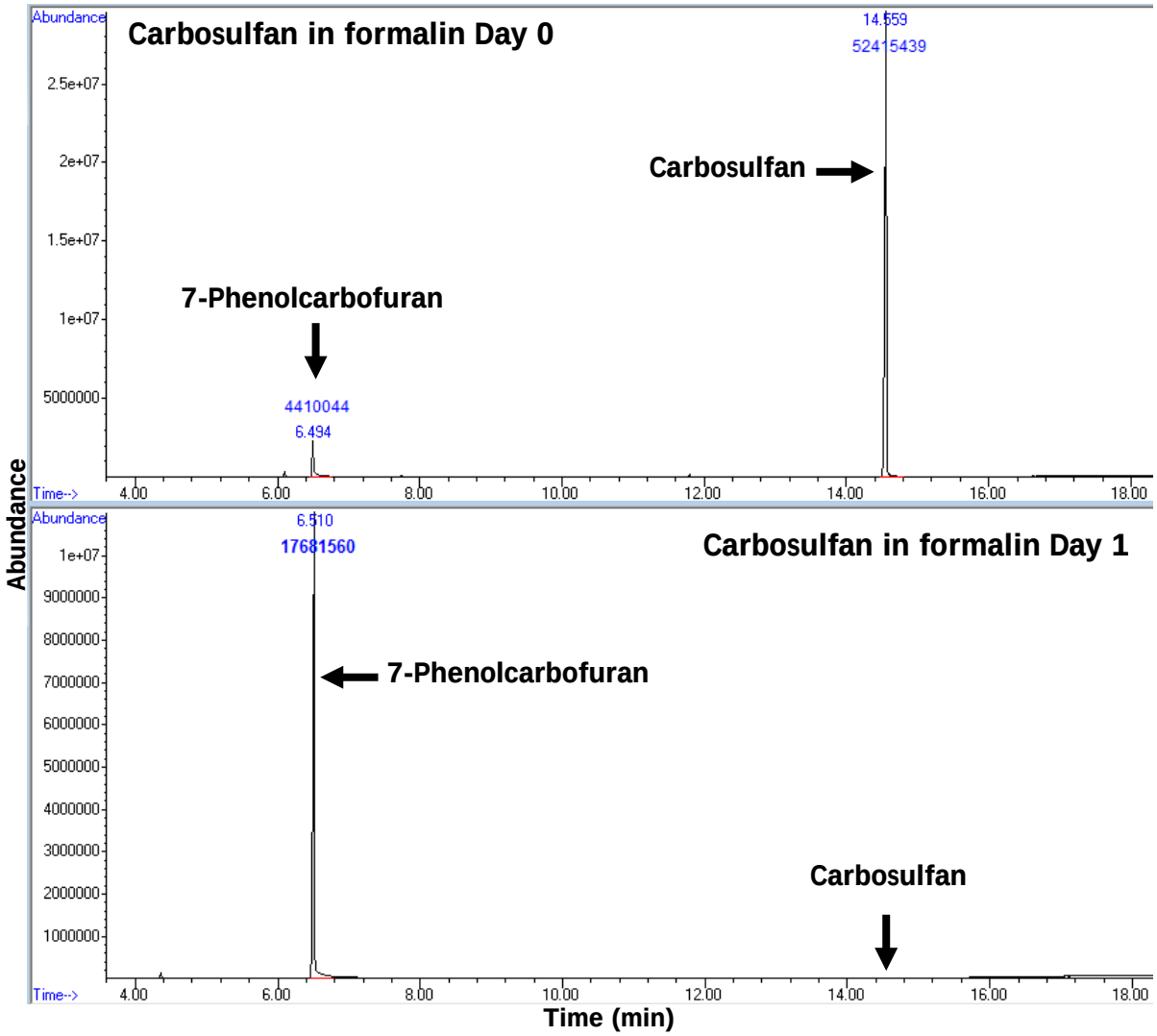


Figure 13-24: GC/MS chromatograms of carbosulfan in 10% formalin solution at days 0 and 1. The degradation product at 6.5 min is 7-phenolcarbofuran.

The mass spectrum showed that the peak at 6.5 min corresponds to 7-phenolcarbofuran, see Figure 13-25, and ions at *m/z* 164, 149 and 131 were subsequently monitored. 7-phenolcarbofuran which was observed at 6.5 min was confirmed by comparing the EI^+ mass spectral fragmentation pattern with a library spectrum. However, 7-phenolcarbofuran was also observed in the carbosulfan standard. It may be an artifact produced in the GC inlet.

Previous studies found that carbosulfan is metabolised to carbofuran, 3-hydroxycarbofuran, 3-ketocarbofuran, 3-hydroxy-7-phenolcarbofuran, 3-keto-7-phenolcarbofuran and 7-phenolcarbofuran[248]. The EI^+ mass spectrum of 7-phenolcarbofuran gives a molecular ion at *m/z* 164 and a base peak ion at *m/z* 149 by the loss of a CH_3 radical via α-cleavage from the molecular ion. The subsequent fragment ion at *m/z* 131 is produced by loss of an H_2O molecule[249].

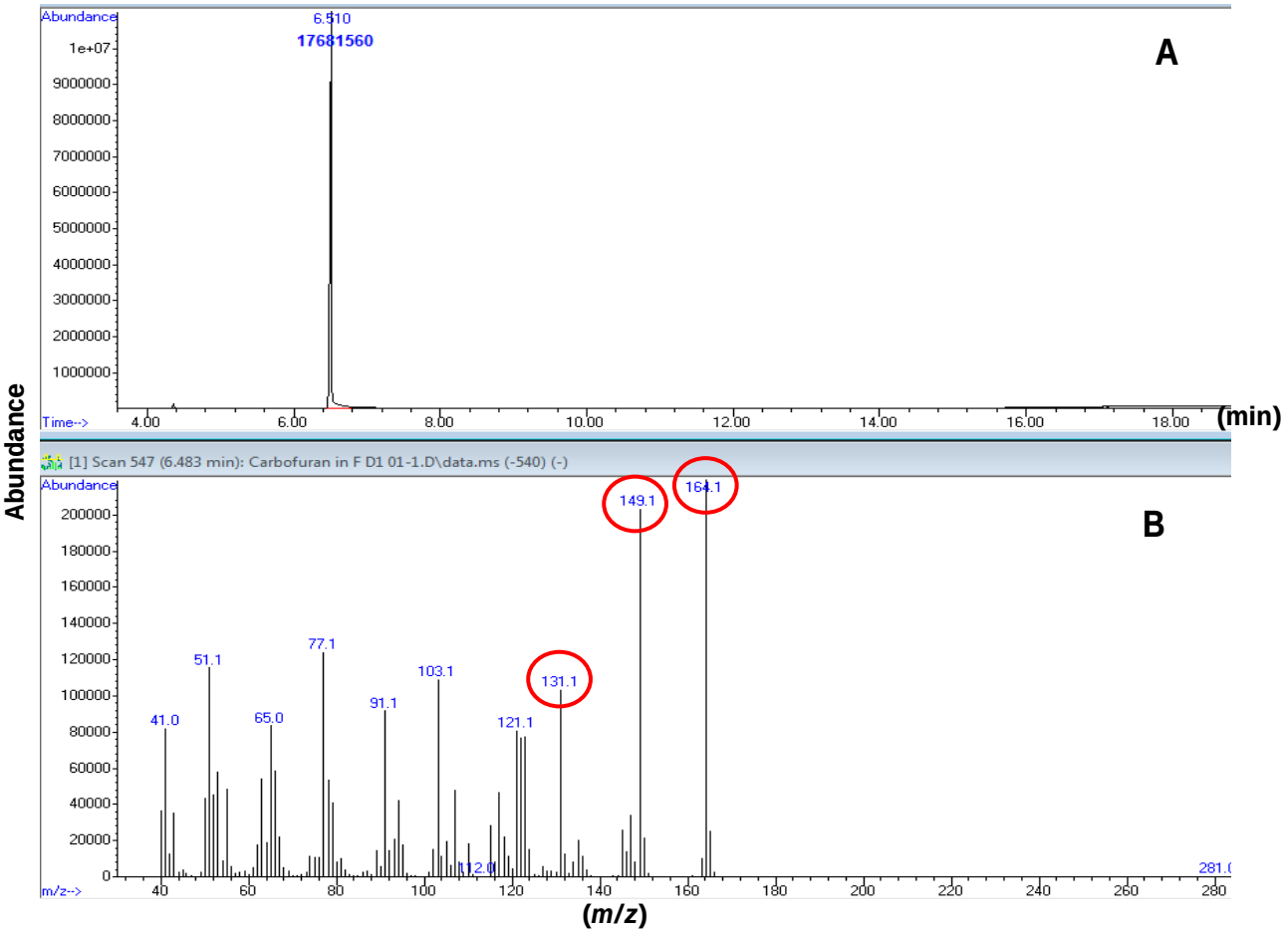


Figure 13-25: GC/MS chromatogram (A) and EI^+ mass spectrum (B) of 7-phenolcarbofuran formed in GC inlet in a spiked formalin sample at day 1.

To confirm the carbosulfan degradation products, standard carbosulfan was spiked in 10% formalin solution and water as a control and was analysed using LC/MS after spiking and at 24 and 48 h and then left for 15 and 30 days. The degradation products formed were identified using LC/MS in full scan mode and by MRM transitions compared with reference standards. Standard 3-hydroxy-carbofuran, 3-ketocarbofuran, carbofuran and carbosulfan were identified using their molecular ions ($M\text{-}H^+$) at *m/z* 238, 236, 222 and 381 and were eluted at 8.1, 9.5, 10.3 and 15.6 min, respectively.

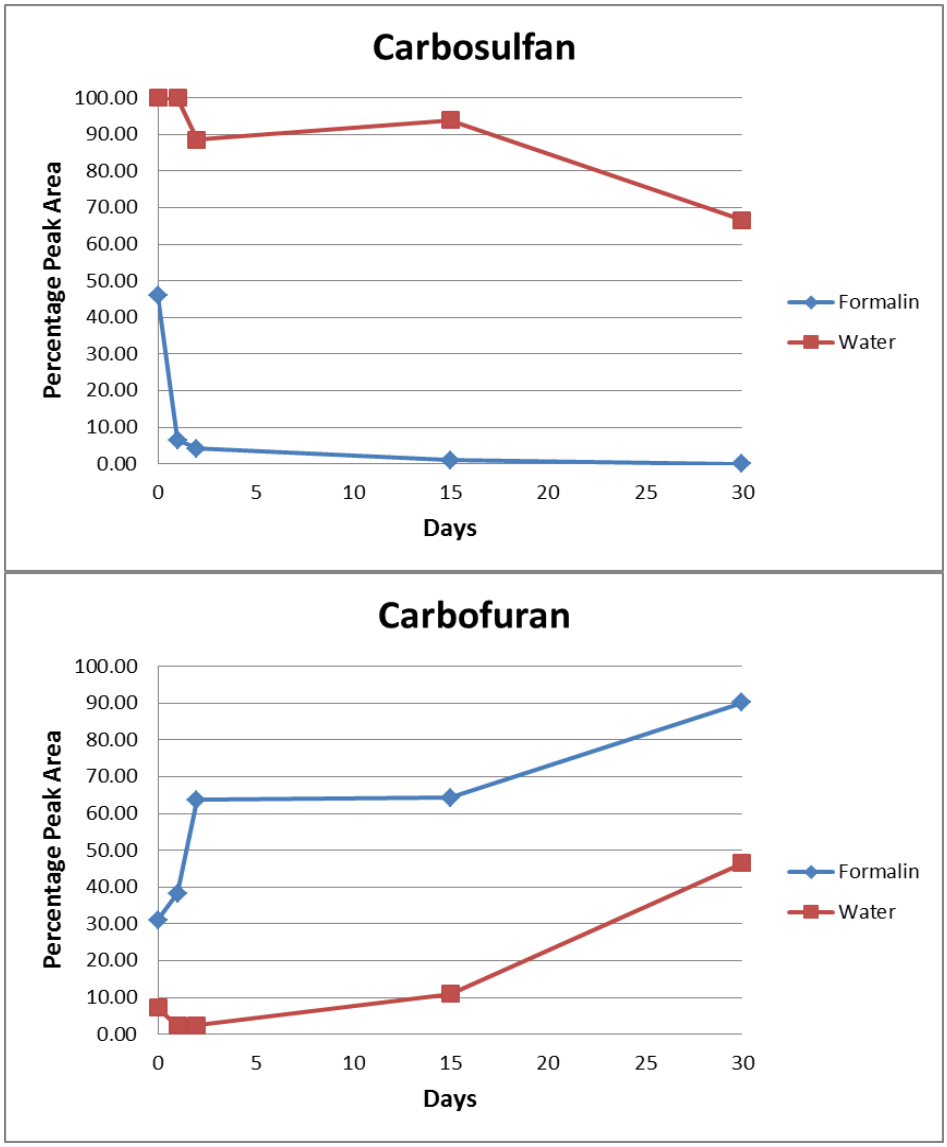


Figure 13-26: Percentage peak area of carbosulfan and its degradation compound (carbofuran) in 10% formalin and water over 30 days.

The results from LC/MS analysis showed that carbosulfan is more stable in water than formalin solution. Carbosulfan was observed in water over 30 days and the peak area was 66% of the starting peak area at day 30. Similar to the results obtained from GC/MS, carbosulfan rapidly decomposed to carbofuran in 24 h in formalin solution. It was detected at 8% after 24 h, 2.5% at day 15 and was not detected at day 30, see Figures 13-26 and 13-27.

However, as noted earlier in section 13.4.2, these initial studies were aimed at identifying the conversion products: only single replicates were used and no internal standards were added. Relative peak areas obtained over a period of 30 days would have had larger variations due to changes in instrumental

performance than might have been obtained when using internal standards, which might explain the fluctuations observed in drug concentrations.

Degradation compounds, carbofuran and 3-ketocarbofuran, were detected in both formalin solution and water after spiking. In 10% formalin solution, carbofuran was detected after spiking at a peak area of 31% relative to carbosulfan and increased to 90% at day 30. It was also detected after spiking at a peak area of 7% relative to starting material and increased to 46% at day 30 in spiked control samples. Traces of 3-ketocarbofuran were detected in all samples over 30 days. Moreover, the peak area of carbofuran detected at day 30 was higher than the peak area detected at day 15. One explanation of this is that carbosulfan is not converted directly to carbofuran but *via* intermediates which have yet to be identified.

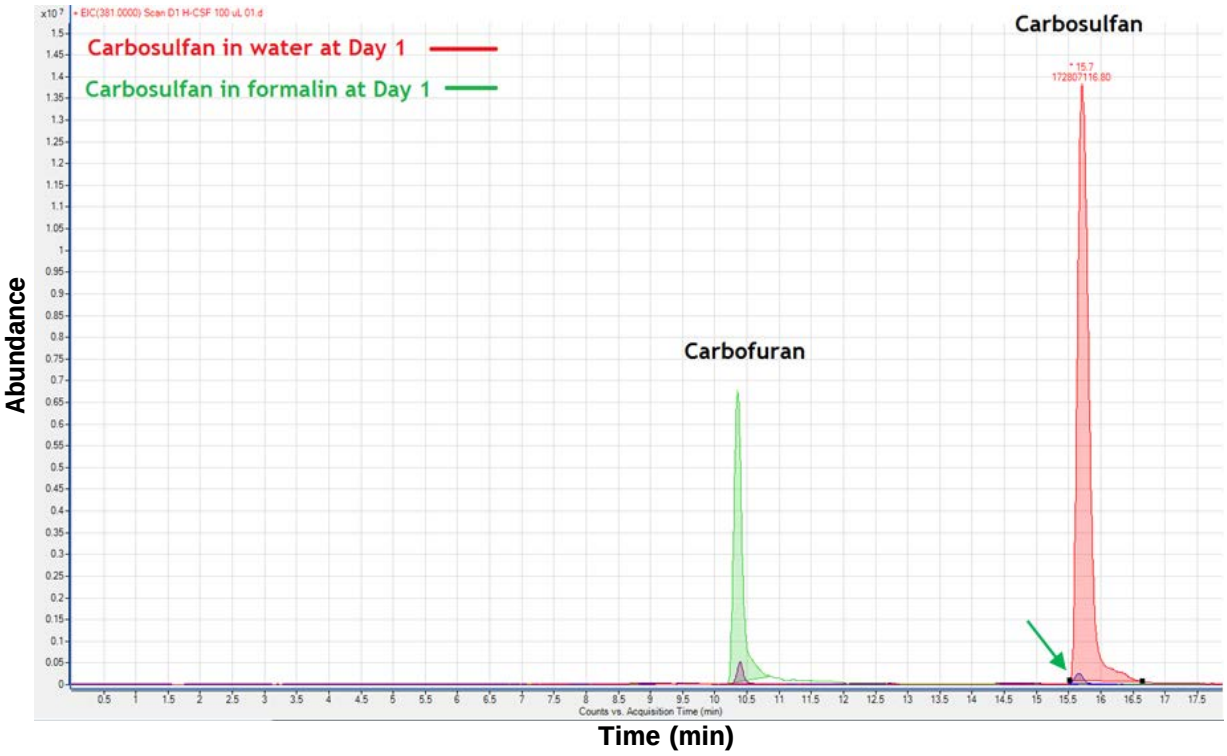


Figure 13-27: LC/MS overlay chromatograms of carbosulfan in spiked formalin and control samples at day 1.

13.5.2 Reactions of carbosulfan in formalin blood

To evaluate the reaction between carbosulfan and formalin blood, standard carbosulfan was spiked in 10% formalin blood and control blood. Spiked samples

were analysed using LC/MS after spiking and at days 1, 2, 3 and then again after 15 days.

The results obtained from carbosulfan spiked in 10% formalin blood were different from those obtained with 10% formalin solution in water. Carbosulfan slowly hydrolysed to carbofuran in spiked-blood samples and was still obviously present at day 15 whereas in formalin solution carbosulfan was not detected after 15 days, see Figure 13-28. Also, when compared with the initial spiked peak area, carbosulfan is more stable in control blood than formalin blood until 72 h. Another degradation compound, 3-ketocarbofuran, was detected in both control blood and formalin blood after 24 h.

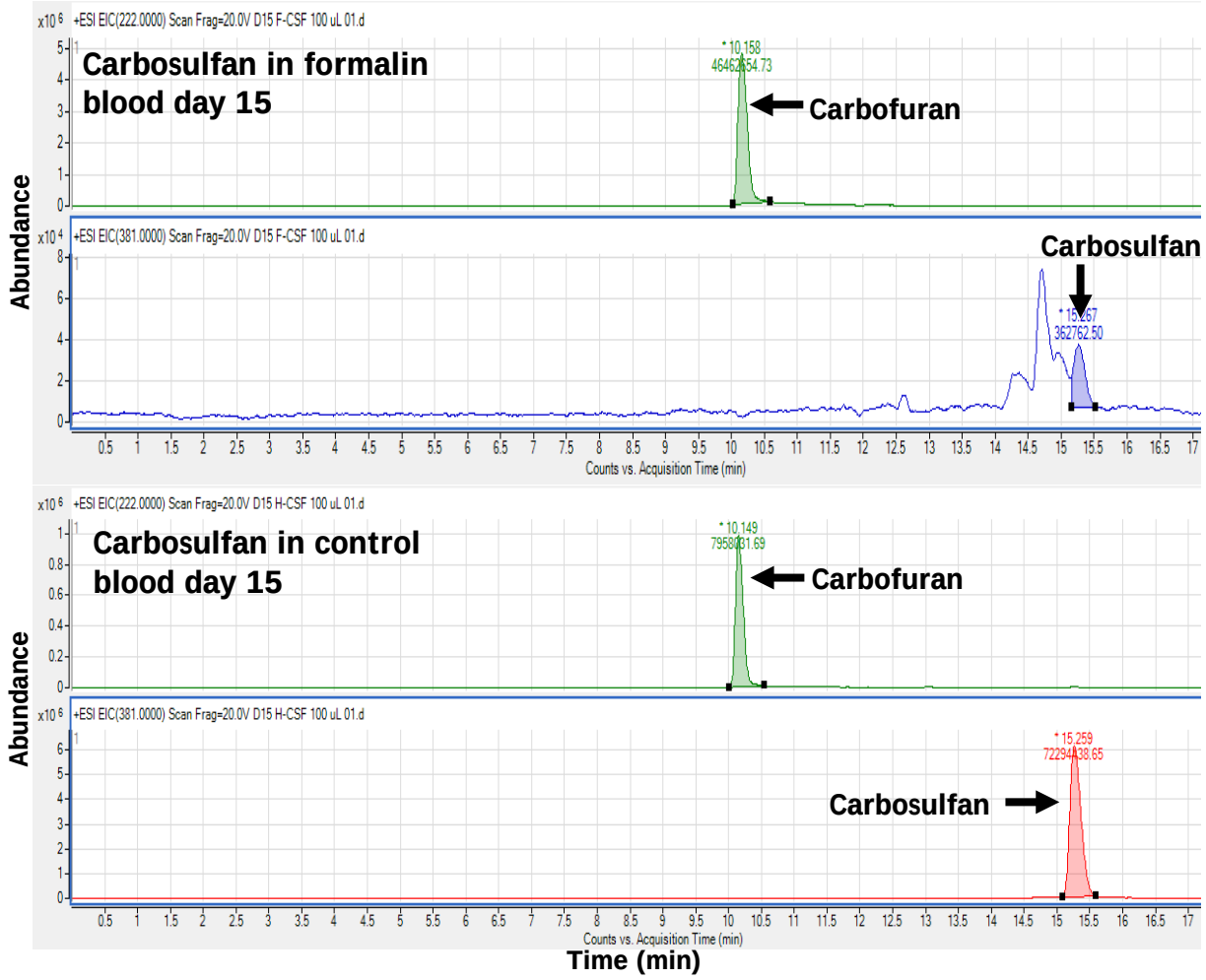


Figure 13-28: LC/MS chromatograms of carbosulfan and carbofuran in formalin-blood and control-blood samples at day 15.

13.6 Conclusions

This Chapter presents the results of an *in vitro* study of the reactions of drugs and poisons with formaldehyde. It has confirmed that formaldehyde reacts with primary and secondary amines to produce tertiary amines by the Eschweiler-Clarke reaction. However, amphetamine, methamphetamine and MDMA do not appear to be 100% converted in formalin solution and formalin blood by the reaction. Alprazolam, diazepam and carbosulfan are hydrolysed in formalin samples. Moreover, other reaction products of formaldehyde with drugs were observed. Alprazolam, diazepam, hydromorphone and hydrocodone react with formaldehyde and converted to conversion products which have molecular ions ($M\text{-}H^+$) higher than those of the parent drugs by increments of 30, 60, 90 or 120 Da. However, the rates of reaction are difficult to determine and the structures of all conversion products will require to be confirmed by additional work in future using high resolution mass spectrometry and NMR to confirm their chemical structures.

The identification of drugs and poisons in formalin-fixed samples, formalin blood or formalin solution will be of great value when fresh biological samples or untainted autopsy samples are not available. Forensic toxicologists should be aware, when performing a quantitative analysis of drugs in formalin blood, formalin-fixed tissue or the formalin solution in which tissue is stored, that the concentrations of drugs are likely to decrease as a result of the reactions between the drugs and the formalin solution. In the case that original compounds cannot be observed in samples, identification of degradation compounds may provide evidence of the original presence of the parent drugs.

Chapter 14 General conclusions

The focus of the current work was to study *in vitro* transformation reactions related to forensic toxicology. Part I focused on an *in vitro* method to identify drug metabolites using human liver microsomes whereas Part II focused on the identification of products of the reaction between formaldehyde and drugs and poisons using *in vitro* experimental procedures.

Human liver microsomes were found to be useful for producing drug metabolites when commercial reference standards of metabolites are not available. Moreover, derivatisation was found to be selective for converting hydrophilic compounds to more lipophilic compounds which are better suited to analysis by LC/MS with conventional reverse-phase columns.

In Chapter 8, a method for analysing MDMA and its metabolites in urine was developed and validated in order to study the metabolic pathway of MDMA by HLM compared with *in vivo* samples. Metabolites produced by HLM were confirmed by comparing with reference standards and all metabolites were detected in real urine samples. HHMA and HMMA were found as major metabolites of MDMA whereas MDA and HMA were found as minor metabolites. The LC/MS method in combination with derivatisation was found to be sensitive and selective for the quantitation of MDMA and its metabolites in urine samples with limits of quantitation of 0.025 µg/ml.

In the work described in Chapter 9, the aim was to develop a method to study metabolic pathways of cathinones using HLM and to identify the cathinone metabolites by LC/MS. Metabolites of butylone and methylone were obtained using HLM and were identified by LC/MS using MRM transitions which, in the absence of standards, were predicted based on data from previous studies. Ketone-reduction and demethylenation were found to be major phase I metabolic pathways of cathinones whereas *N*-demethylation is a minor pathway in humans. The LC/MS method developed was found to be selective for the identification of butylone, methylone and their metabolites. Moreover, this method was designed for the identification of the dihydroxy metabolite of butylone which has not previously been studied.

In Chapter 10, the metabolic pathway of PMA was determined and its metabolites were identified using LC/MS. Metabolites of PMA were prepared using HLM and were identified by LC/MS using MRM transitions which were predicted based on reviewed data. Loss of the methyl group from the methoxy substituent was found to be a major phase I metabolic pathway of PMA in humans whereas *β*-hydroxylation is a minor pathway. The LC/MS method which was developed to identify PMA and its metabolites was found to be selective and has not previously been published.

In addition, Chapter 12 described methods used to evaluate the *in vitro* reaction of formaldehyde with amphetamines, benzodiazepines, opiates and a carbamate insecticide. The results obtained from these experiments were described in Chapter 13. For the amphetamines group, which are representative of amine-containing compounds, the Eschweiler-Clarke reaction was found to be a major reaction of formaldehyde with these compounds. For benzodiazepines, alprazolam and diazepam were hydrolysed when reacted with formaldehyde. Open-ring alprazolam and open-ring diazepam were detected as decomposition compounds. Other conversion products formed by the addition of 1 or more formaldehyde molecules to the drugs were detected for both compounds. For the opiates, hydromorphone and hydrocodone were unstable in formalin solution whereas morphine and codeine were more stable. Formaldehyde adduct conversion products of hydromorphone and hydrocodone were identified in formalin solution. Finally, carbosulfan was less stable in formalin solution than water. The major decomposition product of carbosulfan is carbofuran. Other decomposition products, 3-ketocarbofuran and 3-hydrocarbofuran, which are carbofuran metabolites, were also detected.

The combination of LC/MS with derivatisation is suitable for the analysis of hydrophilic compounds such as drug metabolites which are difficult to analyse by LC/MS with a conventional C18 column. The detection of parent drugs and their metabolites as well as parent drugs and their conversion products in formalin samples is very useful for the investigation and interpretation of cases in forensic toxicology.

Chapter 15 Future work

In the Part I studies described in Chapters 8-10, drug metabolism could be simulated using HLM and all metabolites including highly polar compounds such as dihydroxy-metabolites could be identified by the combination of a derivatisation technique with LC/MS. However, this study only focused on phase I metabolic pathways of some new psychoactive substances which are increasingly abused worldwide.

Further work should be carried out to investigate phase I metabolism using other liver preparations such as S9 fraction or hepatocytes to allow comparison with the results obtained from HLM. Phase II metabolic pathways including conjugation reactions which convert parent compounds to highly hydrophilic compounds also need to be a subject for future work. Moreover, other new psychoactive substances for which increasing abuse has been reported also need to be studied with respect to their metabolic pathways. It is necessary in forensic toxicology to develop sensitive and selective methods to identify new drugs and their metabolites for the increasing scope of analyses and increasing numbers of drug of abuse cases submitted to the laboratory.

In the Part II studies described in Chapters 12-13, all compounds were found to be unstable in formalin solution and formalin blood. However, few previous studies have tried to identify what conversion products are formed or what reactions are occurring. In the present work, only the conversion products of amphetamine, methamphetamine, MDMA and carbosulfan were confirmed by comparing with reference standards whereas other conversion or decomposition products of benzodiazepines and opiates were tentatively identified due to the lack of standards.

Future work should therefore also be carried out on the identification and confirmation of decomposition or conversion compounds of benzodiazepines and opiates in formalin solution using high resolution mass spectrometry to provide elemental compositions of the products and nuclear magnetic resonance (NMR) techniques, which will provide the chemical structure of these compounds.

List of references

(1) Traub, S.: Toxicology. In *Handbook of Bioterrorism and Disaster Medicine*; Antosia, R., Cahill, J., Eds.; Springer New York, 2006; pp 239-242.

(2) Dettmeyer, R.; Verhoff, M.; Schütz, H.: Forensic Toxicology. In *Forensic Medicine*; Springer Berlin Heidelberg, 2014; pp 495-542.

(3) Drummer, O.: Forensic toxicology. In *Molecular, Clinical and Environmental Toxicology*; Luch, A., Ed.; Birkhäuser Basel, 2010; Vol. 100; pp 579-603.

(4) Kostakis, C.; Harpas, P.; Stockham, P.: Chapter 10 - Forensic Toxicology. In *Liquid Chromatography*; Salvatore, F., Paul, R. H., Colin, F. P., Peter, S., David, L., Eds.; Elsevier Amsterdam, 2013; pp 249-293.

(5) Pathways of Biotransformation — Phase I Reactions. In *Drug Metabolism*; Ionescu, C., Caira, M., Eds.; Springer Netherlands, 2005; pp 41-128.

(6) Huidobro, A. L.; Rupérez, F. J.; Barbas, C.: Isolation, identification and determination of the major degradation product in alprazolam tablets during their stability assay. *Journal of Pharmaceutical and Biomedical Analysis* 2007, *44*, 404-413.

(7) de la Torre, R.; Farré, M.: Neurotoxicity of MDMA (ecstasy): the limitations of scaling from animals to humans. *Trends in Pharmacological Sciences* 2004, *25*, 505-508.

(8) Green, A. R.; Cross, A. J.; Goodwin, G. M.: Review of the pharmacology and clinical pharmacology of 3,4-methylenedioxymethamphetamine (MDMA or “Ecstasy”). *Psychopharmacology* 1995, *119*, 247-260.

(9) Coleman, M. D.: Introduction. In *Human Drug Metabolism*; John Wiley & Sons West Sussex, 2010; pp 1-11.

(10) Drug Metabolism in Context. In *Drug Metabolism*; Ionescu, C., Caira, M., Eds.; Springer Netherlands, 2005; pp 1-40.

(11) Tallarida, R.; Raffa, R.; McGonigle, P.: Drug Metabolism (Biotransformation). In *Principles in General Pharmacology*; Springer New York, 1988; pp 61-95.

(12) Kadlubar, S.; Kadlubar, F.: Enzymatic Basis of Phase I and Phase II Drug Metabolism. In *Enzyme- and Transporter-Based Drug-Drug Interactions*;

Pang, K. S., Rodrigues, A. D., Peter, R. M., Eds.; Springer New York, 2010; pp 3-25.

(13) Asha, S.; Vidyavathi, M.: Role of human liver microsomes in *in vitro* metabolism of drugs—A review. *Applied Biochemistry and Biotechnology* 2010, *160*, 1699-1722.

(14) Dudda, A.; Kuerzel, G.: Metabolism studies *in vitro* and *in vivo*. In *Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays*; Vogel, H. G., Maas, J., Hock, F., Mayer, D., Eds.; Springer Berlin Heidelberg, 2013; pp 1053-1094.

(15) Jancova, P.; Anzenbacher, P.; Anzenbacherova, E.: Phase II drug metabolizing enzymes. *Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia* 2010, *154*, 103-116.

(16) Portenoy, R. K.; Thaler, H. T.; Inturrisi, C. E.; Friedlander-Klar, H.; Foley, K. M.: The metabolite morphine-6-glucuronide contributes to the analgesia produced by morphine infusion in patients with pain and normal renal function. *Clinical Pharmacology and Therapeutics* 1992, *51*, 422-431.

(17) Parrott, A. C.: MDMA, serotonergic neurotoxicity, and the diverse functional deficits of recreational "Ecstasy" users. *Neuroscience & Biobehavioral Reviews* 2013, *37*, 1466-1484.

(18) Daumann, J.; Gouzoulis-Mayfrank, E.: Chapter 60 - Neuropharmacology of Ecstasy (MDMA) and Other Designer Drugs. In *Biological Research on Addiction*; Miller, P. M., Ed.; Academic Press San Diego, 2013; pp 615-623.

(19) Sinz, M.: Drug metabolism in preclinical development. In *Applications of Pharmacokinetic Principles in Drug Development*; Krishna, R., Ed.; Springer New York, 2004; pp 75-132.

(20) Peters, F. T.; Meyer, M. R.: *In vitro* approaches to studying the metabolism of new psychoactive compounds. *Drug Testing and Analysis* 2011, *3*, 483-495.

(21) Schwaninger, A. E.; Meyer, M. R.; Barnes, A. J.; Kolbrich-Spargo, E. A.; Gorelick, D. A.; Goodwin, R. S.; Huestis, M. A.; Maurer, H. H.: Stereoselective urinary MDMA (ecstasy) and metabolites excretion kinetics following controlled MDMA administration to humans. *Biochemical Pharmacology* 2012, *83*, 131-138.

(22) Costa, A.; Sarmento, B.; Seabra, V.: An evaluation of the latest *in vitro* tools for drug metabolism studies. *Expert Opinion on Drug Metabolism & Toxicology* 2014, *10*, 103-119.

(23) Vermeire, T. G.; Baars, A. J.; Bessems, J. G. M.; Blaauboer, B. J.; Slob, W.; Muller, J. J. A.: Toxicity testing for human health risk assessment. In *Risk Assessment of Chemicals*; van Leeuwen, C. J., Vermeire, T. G., Eds.; Springer Netherlands, 2007; pp 227-280.

(24) Danielson, N. D.; Gallagher, P. A.; Bao, J. J.: Chemical reagents and derivatization procedures in drug analysis. In *Encyclopedia of Analytical Chemistry*; John Wiley & Sons New Jersey, 2006.

(25) Segura, J.; Ventura, R.; Jurado, C.: Derivatization procedures for gas chromatographic-mass spectrometric determination of xenobiotics in biological samples, with special attention to drugs of abuse and doping agents. *Journal of chromatography. B* 1998, *713*, 61-90.

(26) Dettmer-Wilde, K.: Derivatization. In *Practical Gas Chromatography*; Dettmer-Wilde, K., Engewald, W., Eds.; Springer Berlin Heidelberg, 2014; pp 603-632.

(27) Kopka, J.: Gas Chromatography Mass Spectrometry. In *Plant Metabolomics*; Saito, K., Dixon, R., Willmitzer, L., Eds.; Springer Berlin Heidelberg, 2006; Vol. 57; pp 3-20.

(28) Xu, F.; Zou, L.; Liu, Y.; Zhang, Z.; Ong, C. N.: Enhancement of the capabilities of liquid chromatography–mass spectrometry with derivatization: General principles and applications. *Mass Spectrometry Reviews* 2011, *30*, 1143-1172.

(29) Santa, T.: Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry. *Biomedical Chromatography* 2011, *25*, 1-10.

(30) Santa, T.: Derivatization in LC-MS Bioanalysis. In *Handbook of LC-MS Bioanalysis*; John Wiley & Sons New Jersey, 2013; pp 239-248.

(31) Moeder, M.: Gas Chromatography-Mass Spectrometry. In *Practical Gas Chromatography*; Dettmer-Wilde, K., Engewald, W., Eds.; Springer Berlin Heidelberg, 2014; pp 303-350.

(32) Peters, F. T.: Method Validation using LC/MS. In *Application of LC-MS in Toxicology*; Polettini, A., Ed.; Pharmaceutical Press London, 2006; pp 71-96.

(33) Dadgar, D.; Burnett, P. E.: Issues in evaluation of bioanalytical method selectivity and drug stability. *Journal of Pharmaceutical and Biomedical Analysis* 1995, *14*, 23-31.

(34) Peters, F.; Maurer, H.: Bioanalytical method validation and its implications for forensic and clinical toxicology — A review. In *Validation in Chemical Measurement*; De Bièvre, P., Günzler, H., Eds.; Springer Berlin Heidelberg, 2005; pp 1-9.

(35) Wenclawiak, B.; Hadjicostas, E.: Validation of Analytical Methods – to be Fit for the Purpose. In *Quality Assurance in Analytical Chemistry*; Wenclawiak, B. W., Koch, M., Hadjicostas, E., Eds.; Springer Berlin Heidelberg, 2010; pp 215-245.

(36) Magnusson, B.; Örnemark, U.: *Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics*; 2nd edn, 2014; 19-36.

(37) Reichenbächer, M.; Einax, J.: Validation of Method Performance. In *Challenges in Analytical Quality Assurance*; Springer Berlin Heidelberg, 2011; pp 117-239.

(38) Catalano, T.: Statistical Concepts for the Analytical Chemist. In *Essential Elements for a GMP Analytical Chemistry Department*; Springer New York, 2013; pp 111-136.

(39) Nahler, G.: Linear regression. In *Dictionary of Pharmaceutical Medicine*; Springer Vienna, 2009; pp 106-106.

(40) SOFT/AAFS: Forensic Toxicology Laboratory Guidelines. Seattle, 2006.

(41) Danzer, K.: Analytical Performance Characteristics. In *Analytical Chemistry*; Springer Berlin Heidelberg, 2007; pp 177-216.

(42) Peters, F. T.; Drummer, O. H.; Musshoff, F.: Validation of new methods. *Forensic Science International* 2007, *165*, 216-224.

(43) Rogatsky, E.; Stein, D.: Evaluation of matrix effect and chromatography efficiency: New parameters for validation of method development. *Journal of the American Society for Mass Spectrometry* 2005, *16*, 1757-1759.

(44) Taylor, P. J.: Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clinical Biochemistry* 2005, *38*, 328-334.

(45) Van Eeckhaut, A.; Lanckmans, K.; Sarre, S.; Smolders, I.; Michotte, Y.: Validation of bioanalytical LC–MS/MS assays: Evaluation of matrix effects. *Journal of Chromatography B* 2009, *877*, 2198-2207.

(46) Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M.: Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. *Analytical Chemistry* 2003, *75*, 3019-3030.

(47) Hübschmann, H.-J.: Introduction. In *Handbook of GC/MS*; Wiley-VCH Weinheim, 2008; pp 1-6.

(48) Sparkman, O. D.; Penton, Z. E.; Kitson, F. G.: Chapter 1 - Introduction and History. In *Gas Chromatography and Mass Spectrometry;* 2nd edn; Sparkman, O. D., Penton, Z. E., Kitson, F. G., Eds.; Academic Press Amsterdam, 2011; pp 2-13.

(49) Khan, J.; Kennedy, T.; Christian, D., Jr.: Chromatography and Mass Spectrometry. In *Basic Principles of Forensic Chemistry*; Humana Press London, 2012; pp 107-125.

(50) Engewald, W.; Dettmer-Wilde, K.; Rotzsche, H.: Columns and Stationary Phases. In *Practical Gas Chromatography*; Dettmer-Wilde, K., Engewald, W., Eds.; Springer Berlin Heidelberg, 2014; pp 59-116.

(51) Sparkman, O. D.; Penton, Z. E.; Kitson, F. G.: Chapter 2 - Gas Chromatography. In *Gas Chromatography and Mass Spectrometry;* 2nd edn; Sparkman, O. D., Penton, Z. E., Kitson, F. G., Eds.; Academic Press Amsterdam, 2011; pp 15-83.

(52) Hübschmann, H.-J.: Fundamentals. In *Handbook of GC/MS*; Wiley-VCH Weinheim, 2008; pp 7-292.

(53) Westman-Brinkmalm, A.; Brinkmalm, G.: A Mass Spectrometer's Building Blocks. In *Mass Spectrometry*; John Wiley & Sons New Jersey, 2008; pp 15-87.

(54) Kruve, A.; Rebane, R.; Kipper, K.; Oldekop, M.-L.; Evard, H.; Herodes, K.; Ravio, P.; Leito, I.: Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part I. *Analytica Chimica Acta* 2015, *870*, 29-44.

(55) Ismail, B.; Nielsen, S. S.: Basic Principles of Chromatography. In *Food Analysis*; Springer New York, 2010; pp 473-498.

(56) Reuhs, B.; Rounds, M.: High-Performance Liquid Chromatography. In *Food Analysis*; Springer New York, 2010; pp 499-512.

(57) Gross, J.: Introduction. In *Mass Spectrometry*; Springer Berlin Heidelberg, 2011; pp 1-20.

(58) Gross, J.: Electrospray Ionization. In *Mass Spectrometry*; Springer Berlin Heidelberg, 2011; pp 561-620.

(59) Hiraoka, K.: Fundamentals of Electrospray. In *Fundamentals of Mass Spectrometry*; Hiraoka, K., Ed.; Springer New York, 2013; pp 145-171.

(60) Smith, J. S.; Thakur, R.: Mass Spectrometry. In *Food Analysis*; Springer New York, 2010; pp 457-470.

(61) Yost, R. A.; Fetterolf, D. D.: Tandem mass spectrometry (MS/MS) instrumentation. *Mass Spectrometry Reviews* 1983, *2*, 1-45.

(62) Westman-Brinkmalm, A.; Brinkmalm, G.: Tandem Mass Spectrometry. In *Mass Spectrometry*; John Wiley & Sons New Jersey, 2008; pp 89-103.

(63) Gross, J.: Tandem Mass Spectrometry. In *Mass Spectrometry*; Springer Berlin Heidelberg, 2011; pp 415-478.

(64) Giorgi, G.: Mass Spectrometry and Tandem Mass Spectrometry: An Overview. In *Detection of Chemical, Biological, Radiological and Nuclear Agents for the Prevention of Terrorism*; Banoub, J., Ed.; Springer Netherlands, 2014; pp 17-31.

(65) UNODC: *World Drug Report 2015*; United Nations: New York, 2015.

(66) UNODC: *World Drug Report 2014*; United Nations: New York, 2014.

(67) EMCDDA: *European Drug Report trends and developments 2015*; European Monitoring Centre for Drugs and Drug Addiction: Spain, 2015.

(68) Zuba, D.: Identification of cathinones and other active components of "legal highs" by mass spectrometric methods. *Trends in Analytical Chemistry* 2012, *32*, 15-30.

(69) Weaver, M.; Hopper, J.; Gunderson, E.: Designer drugs 2015: assessment and management. *Addiction Science & Clinical Practice* 2015, *10,* 1-9.

(70) Fu, S.; Stojanovska, N.: Designer Drugs. In *Encyclopedia of Forensic Sciences*; Siegel, J. A., Saukko, P. J., Eds.; Academic Press Waltham, 2013; pp 36-44.

(71) Freye, E.: History of Designer Drugs. In *Pharmacology and Abuse of Cocaine, Amphetamines, Ecstasy and Related Designer Drugs*; Springer Netherlands, 2010; pp 183-189.

(72) King, L.; Kicman, A.: A brief history of "new psychoactive substances". *Drug Testing and Analysis* 2011, *3*, 401-403.

(73) Home Office and The Rt Hon Norman Baker. New psychoactive substances review: report of the expert panel, https://www.gov.uk/government/publications/new-psychoactive-substances-review-report-of-the-expert-panel (accessed Jan 28, 2016).

(74) Zaitsu, K.; Katagi, M.; Tatsuno, M.; Sato, T.; Tsuchihashi, H.; Suzuki, K.: Recently abused *β*-keto derivatives of 3,4-methylenedioxyphenylalkylamines: a review of their metabolisms and toxicological analysis. *Forensic Toxicology* 2011, *29*, 73-84.

(75) UNODC: *The challenge of new psychoactive substances*; United Nations Publication, 2013.

(76) Meyer, M. R.; Maurer, H. H.: Metabolism of designer drugs of abuse: An updated review. *Current Drug Metabolism* 2010, *11*, 468-482.

(77) Buchanan, J. F.; Brown, C. R.: "Designer drugs". A problem in clinical toxicology. *Medical Toxicology and Adverse Drug Experience* 1988, *3*, 1-17.

(78) Shulgin, A.; Shulgin, A.: *PiHKAL: A Chemical Love Story*; Transform Press Berkeley, 1991.

(79) Capela, J.; Carmo, H.; Remião, F.; Bastos, M.; Meisel, A.; Carvalho, F.: Molecular and cellular mechanisms of ecstasy-induced neurotoxicity: An overview. *Molecular Neurobiology* 2009, *39*, 210-271.

(80) Freudenmann, R. W.; Öxler, F.; Bernschneider-Reif, S.: The origin of MDMA (ecstasy) revisited: the true story reconstructed from the original documents. *Addiction (Abingdon, England)* 2006, *101*, 1241-1245.

(81) Fitzgerald, R. L.; Blanke, R. V.; Rosecrans, J. A.; Glennon, R. A.: Stereochemistry of the metabolism of MDMA to MDA. *Life Sciences* 1989, *45*, 295-301.

(82) Lyles, J.; Cadet, J. L.: Methylenedioxymethamphetamine (MDMA, Ecstasy) neurotoxicity: cellular and molecular mechanisms. *Brain Research. Brain Research Reviews* 2003, *42*, 155-168.

(83) de la Torre, R.; Farre, M.; Roset, P. N.; Pizarro, N.; Abanades, S.; Segura, M.; Segura, J.; Cami, J.: Human pharmacology of MDMA: pharmacokinetics, metabolism, and disposition. *Therapeutic Drug Monitoring* 2004, *26*, 137-144.

(84) Katagi, M.; Tsuchihashi, H.: 3,4-Methylenedioxyamphetamines. In *Drugs and Poisons in Humans*; Springer Berlin Heidelberg, 2005; pp 229-239.

(85) Porto, S. K.; Nogueira, T.; Blanes, L.; Doble, P.; Sabino, B. D.; do Lago, C. L.; Angnes, L.: Analysis of ecstasy tablets using capillary electrophoresis with capacitively coupled contactless conductivity detection. *Journal of Forensic Sciences* 2014, *59*, 1622-1626.

(86) McCann, U. D.; Ricaurte, G. A.: Chapter Fifteen - Effects of MDMA on the Human Nervous System. In *The Effects of Drug Abuse on the Human Nervous System*; Madras, B., Kuhar, M., Eds.; Academic Press Boston, 2014; pp 475-497.

(87) Maurer, H. H.; Bickeboeller-Friedrich, J.; Kraemer, T.; Peters, F. T.: Toxicokinetics and analytical toxicology of amphetamine-derived designer drugs ("Ecstasy"). *Toxicology Letters* 2000, *112–113*, 133-142.

(88) Lin, L. Y.; Di Stefano, E. W.; Schmitz, D. A.; Hsu, L.; Ellis, S. W.; Lennard, M. S.; Tucker, G. T.; Cho, A. K.: Oxidation of methamphetamine and methylenedioxymethamphetamine by CYP2D6. *Drug Metabolism and Disposition: The Biological Fate of Chemicals* 1997, *25*, 1059-1064.

(89) Verebey, K.; Alrazi, J.; Jaffe, J. H.: The complications of "ecstasy" (MDMA). *Journal of the American Medical Association* 1988, *259*, 1649-1650.

(90) Helmlin, H.-J.; Bracher, K.; Bourquin, D.; Vonlanthen, D.; Brenneisen, R.; Styk, J.: Analysis of 3,4-Methylenedioxymethamphetamine (MDMA) and its metabolites in plasma and urine by HPLC-DAD and GC-MS. *Journal of Analytical Toxicology* 1996, *20*, 432-440.

(91) Segura, M.; Ortuño, J.; Farré, M.; McLure, J. A.; Pujadas, M.; Pizarro, N.; Llebaria, A.; Joglar, J.; Roset, P. N.; Segura, J.; de la Torre, R.: 3,4-Dihydroxymethamphetamine (HHMA). A major *in vivo* 3,4-methylenedioxymethamphetamine (MDMA) metabolite in humans. *Chemical Research in Toxicology* 2001, *14*, 1203-1208.

(92) Jenkins, K. M.; Young, M. S.; Mallet, C. R.; Elian, A. A.: Mixed-mode solid-phase extraction procedures for the determination of MDMA and metabolites in urine using LC-MS, LC-UV, or GC-NPD. *Journal of Analytical Toxicology* 2004, *28*, 50-58.

(93) Pirnay, S. O.; Abraham, T. T.; Huestis, M. A.: Sensitive gas chromatography-mass spectrometry method for simultaneous measurement of

MDEA, MDMA, and metabolites HMA, MDA, and HMMA in human urine. *Clinical Chemistry* 2006, *52*, 1728-1734.

(94) Shima, N.; Katagi, M.; Kamata, H.; Zaitsu, K.; Kamata, T.; Nishikawa, M.; Miki, A.; Tsuchihashi, H.; Sakuma, T.; Nemoto, N.: Urinary excretion of the main metabolites of 3,4-methylenedioxymethamphetamine (MDMA), including the sulfate and glucuronide of 4-hydroxy-3-methoxymethamphetamine (HMMA), in humans and rats. *Xenobiotica* 2008, *38*, 314-324.

(95) O'Leary, G.; Nargiso, J.; Weiss, R.: 3,4-methylenedioxymethamphetamine (mdma): A review. *Current Psychiatry Reports* 2001, *3*, 477-483.

(96) Baylen, C. A.; Rosenberg, H.: A review of the acute subjective effects of MDMA/ecstasy. *Addiction (Abingdon, England)* 2006, *101*, 933-947.

(97) McCann, U. D.; Ricaurte, G. A.: Major metabolites of(±)3,4-methylenedioxyamphetamine (MDA) do not mediate its toxic effects on brain serotonin neurons. *Brain Research* 1991, *545*, 279-282.

(98) Burgess, C.; O'Donohoe, A.; Gill, M.: Agony and ecstasy: a review of MDMA effects and toxicity. *European Psychiatry* 2000, *15*, 287-294.

(99) Lurie, Y.; Gopher, A.; Lavon, O.; Almog, S.; Sulimani, L.; Bentur, Y.: Severe paramethoxymethamphetamine (PMMA) and paramethoxyamphetamine (PMA) outbreak in Israel. *Clinical Toxicology* 2012, *50*, 39-43.

(100) Lapoint, J.; Dargan, P. I.; Hoffman, R. S.: Chapter 7 - Synthetic Amphetamine Derivatives. In *Novel Psychoactive Substances*; Wood, D. M., Dragan, P.I. Ed.; Academic Press Boston, 2013; pp 161-178.

(101) Barratt, M. J.; Allen, M.; Lenton, S.: "PMA Sounds Fun": negotiating drug discourses online. *Substance Use & Misuse* 2014, *49*, 987-998.

(102) Baselt, R. C.: *Disposition of toxic drugs and chemicals in man*; 10th edn; Biomedical Publications Foster, 2014.

(103) Caldicott, D. G. E.; Edwards, N. A.; Kruys, A.; Kirkbride, K. P.; Sims, D. N.; Byard, R. W.; Prior, M.; J. Irvine, R.: Dancing with "Death": *p*-Methoxyamphetamine overdose and its acute management. *Journal of Toxicology: Clinical Toxicology* 2003, *41*, 143-154.

(104) Gorrod, J. W.: The metabolism and excretion of "amphetamines" in man. *Biochemical Pharmacology* 1974, *23*, 780-785.

(105) Staack, R. F.; Fehn, J.; Maurer, H. H.: New designer drug *p*-methoxymethamphetamine: studies on its metabolism and toxicological detection in urine using gas chromatography–mass spectrometry. *Journal of Chromatography B* 2003, *789*, 27-41.

(106) Kraner, J. C.; McCoy, D. J.; Evans, M. A.; Evans, L. E.; Sweeney, B. J.: Fatalities caused by the MDMA-related drug paramethoxyamphetamine (PMA). *Journal of Analytical Toxicology* 2001, *25*, 645-648.

(107) Ruprah, M.: Amphetamine and its analogs: Psychopharmacology, toxicology and abuse. *Journal of Applied Toxicology* 1994, *14*, 458-458.

(108) Rohanova, M.; Balikova, M.: Studies on distribution and metabolism of *para*-methoxymethamphetamine (PMMA) in rats after subcutaneous administration. *Toxicology* 2009, *259*, 61-68.

(109) Kitchen, I.; Tremblay, J.; André, J.; Dring, L. G.; Idle, J. R.; Smith, R. L.; Williams, R. T.: Interindividual and interspecies variation in the metabolism of the hallucinogen 4-methoxyamphetamine. *Xenobiotica* 1979, *9*, 397-404.

(110) Lai, F. Y.; Erratico, C.; Kinyua, J.; Mueller, J. F.; Covaci, A.; van Nuijs, A. L. N.: Liquid chromatography-quadrupole time-of-flight mass spectrometry for screening in vitro drug metabolites in humans: investigation on seven phenethylamine-based designer drugs. *Journal of Pharmaceutical and Biomedical Analysis* 2015, *114*, 355-375.

(111) Cimbura, G.: PMA deaths in Ontario. *Canadian Medical Association Journal* 1974, *110*, 1263-1267.

(112) Felgate, H. E.; Felgate, P. D.; James, R. A.; Sims, D. N.; Vozzo, D. C.: Recent paramethoxyamphetamine deaths. *Journal of Analytical Toxicology* 1998, *22*, 169-172.

(113) Martin, T. L.: Three cases of fatal paramethoxyamphetamine overdose. *Journal of Analytical Toxicology* 2001, *25*, 649-651.

(114) Refstad, S.: Paramethoxyamphetamine (PMA) poisoning; a "party drug" with lethal effects. *Acta Anaesthesiologica Scandinavica* 2003, *47*, 1298-1299.

(115) Ling, L. H.; Marchant, C.; Buckley, N. A.; Prior, M.; Irvine, R. J.: Poisoning with the recreational drug paramethoxyamphetamine ("death"). *The Medical Journal of Australia* 2001, *174*, 453-455.

(116) Kaminskas, L.; Irvine, R.; Callaghan, P.; White, J.; Kirkbride, P.: The contribution of the metabolite *p*-hydroxyamphetamine to the central actions of *p*-methoxyamphetamine. *Psychopharmacology* 2002, *160*, 155-160.

(117) Nichols, T.; Khondkar, P.; Gibbons, S.: The psychostimulant drug khat (*Catha edulis*): A mini-review. *Phytochemistry Letters* 2015, *13*, 127-133.

(118) Valente, M.; Guedes de Pinho, P.; de Lourdes Bastos, M.; Carvalho, F.; Carvalho, M.: Khat and synthetic cathinones: a review. *Archives of Toxicology* 2014, *88*, 15-45.

(119) Kelly, J. P.: Cathinone derivatives: A review of their chemistry, pharmacology and toxicology. *Drug Testing and Analysis* 2011, *3*, 439-453.

(120) Karch, S. B.: Cathinone neurotoxicity ("The 3Ms"). *Current Neuropharmacology* 2015, *13*, 21-25.

(121) Prosser, J.; Nelson, L.: The Toxicology of bath salts: A review of synthetic cathinones. *Journal of Medical Toxicology* 2012, *8*, 33-42.

(122) Karila, L.; Megarbane, B.; Cottencin, O.; Lejoyeux, M.: Synthetic cathinones: A new public health problem. *Current Neuropharmacology* 2015, *13*, 12-20.

(123) Davidson, C.; Schifano, F.: The potential utility of some legal highs in CNS disorders. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 2016, *64*, 267-274.

(124) WHO "Methylone (bk-MDMA) Critical Review Report Agenda item 4.14," World Health Organization, Geneva, 2014.

(125) Kamata, H. T.; Shima, N.; Zaitsu, K.; Kamata, T.; Miki, A.; Nishikawa, M.; Katagi, M.; Tsuchihashi, H.: Metabolism of the recently encountered designer drug, methylone, in humans and rats. *Xenobiotica* 2006, *36*, 709-723.

(126) Meyer, M.; Wilhelm, J.; Peters, F.; Maurer, H.: Beta-keto amphetamines: studies on the metabolism of the designer drug mephedrone and toxicological detection of mephedrone, butylone, and methylone in urine using gas chromatography–mass spectrometry. *Analytical and Bioanalytical Chemistry* 2010, *397*, 1225-1233.

(127) Mueller, D.; Rentsch, K.: Generation of metabolites by an automated online metabolism method using human liver microsomes with subsequent identification by LC-MS(n), and metabolism of 11 cathinones. *Analytical and Bioanalytical Chemistry* 2012, *402*, 2141-2151.

(128) Pedersen, A. J.; Petersen, T. H.; Linnet, K.: *In vitro* metabolism and pharmacokinetic studies on methylone. *Drug Metabolism and Disposition* 2013, *41*, 1247-1255.

(129) Chen, X.: Simultaneous determination of four designer drugs and their major metabolites by liquid chromatography–mass spectrometry. *Journal of Chromatography B* 2015, *992*, 1-7.

(130) Ellefsen, K.; Concheiro, M.; Suzuki, M.; Rice, K.; Elmore, J.; Baumann, M.; Huestis, M.: Quantification of methylone and metabolites in rat and human plasma by liquid chromatography-tandem mass spectrometry. *Forensic Toxicology* 2015, 1-11.

(131) Hill, S. L.; Thomas, S. H. L.: Clinical toxicology of newer recreational drugs. *Clinical Toxicology* 2011, *49*, 705-719.

(132) Cozzi, N. V.; Sievert, M. K.; Shulgin, A. T.; Jacob Iii, P.; Ruoho, A. E.: Inhibition of plasma membrane monoamine transporters by *β*-ketoamphetamines. *European Journal of Pharmacology* 1999, *381*, 63-69.

(133) Elliott, S.; Evans, J.: A 3-year review of new psychoactive substances in casework. *Forensic science international* 2014, *243*, 55-60.

(134) McIntyre, I. M.; Hamm, C. E.; Aldridge, L.; Nelson, C. L.: Acute methylone intoxication in an accidental drowning-A case report. *Forensic science international* 2013, *231*, e1-e3.

(135) Cawrse, B. M.; Levine, B.; Jufer, R. A.; Fowler, D. R.; Vorce, S. P.; Dickson, A. J.; Holler, J. M.: Distribution of methylone in four postmortem cases. *Journal of analytical toxicology* 2012, *36*, 434-439.

(136) Carbone, P. N.; Carbone, D. L.; Carstairs, S. D.; Luzi, S. A.: Sudden cardiac death associated with methylone use. *The American journal of forensic medicine and pathology* 2013, *34*, 26-28.

(137) Pearson, J. M.; Hargraves, T. L.; Hair, L. S.; Massucci, C. J.; Frazee, C. C., 3rd; Garg, U.; Pietak, B. R.: Three fatal intoxications due to methylone. *Journal of analytical toxicology* 2012, *36*, 444-451.

(138) Zaitsu, K.; Katagi, M.; Kamata, H. T.; Kamata, T.; Shima, N.; Miki, A.; Tsuchihashi, H.; Mori, Y.: Determination of the metabolites of the new designer drugs bk-MBDB and bk-MDEA in human urine. *Forensic Science International* 2009, *188*, 131-139.

(139) Butylone experiences. (2013).from https://www.reddit.com/r/Drugs/comments/1710z1/butylone_experiences/ (accessed Apr 28, 2016).

(140) Butylone. (2016). from http://wiki.bluelight.org/index.php/Butylone (accessed Jun 24, 2016).

(141) Helander, A.; Beck, O.; Hagerkvist, R.; Hulten, P.: Identification of novel psychoactive drug use in Sweden based on laboratory analysis-initial experiences from the STRIDA project. *Scandinavian journal of clinical and laboratory investigation* 2013, *73*, 400-406.

(142) Wood, D. M.; Davies, S.; Cummins, A.; Button, J.; Holt, D. W.; Ramsey, J.; Dargan, P. I.: Energy-1 ("NRG-1"): don't believe what the newspapers say about it being legal. *Emergency medicine journal* 2011, *28*, 1068-1070.

(143) Uralets, V.; Rana, S.; Morgan, S.; Ross, W.: Testing for designer stimulants: metabolic profiles of 16 synthetic cathinones excreted free in human urine. *Journal of analytical toxicology* 2014, *38*, 233-241.

(144) Frohlich, S.; Lambe, E.; O'Dea, J.: Acute liver failure following recreational use of psychotropic "head shop" compounds. *Irish journal of medical science* 2011, *180*, 263-264.

(145) Brandt, S. D.; Sumnall, H. R.; Measham, F.; Cole, J.: Analyses of second-generation "legal highs" in the UK: initial findings. *Drug Testing and Analysis* 2010, *2*, 377-382.

(146) Warrick, B. J.; Wilson, J.; Hedge, M.; Freeman, S.; Leonard, K.; Aaron, C.: Lethal serotonin syndrome after methylone and butylone ingestion. *Journal of Medical Toxicology.* 2012, *8*, 65-68.

(147) Rojek, S.; Klys, M.; Strona, M.; Maciow, M.; Kula, K.: "Legal highs"-toxicity in the clinical and medico-legal aspect as exemplified by suicide with bk-MBDB administration. *Forensic Science International* 2012, *222*, e1-e6.

(148) Kunsman, G. W.; Levine, B.; Kuhlman, J. J.; Jones, R. L.; Hughes, R. O.; Fujiyama, C. I.; Smith, M. L.: MDA-MDMA concentrations in urine specimens. *Journal of Analytical Toxicology* 1996, *20*, 517-521.

(149) Dowling, G. P.; McDonough, E. T.; Bost, R. O.: "Eve" and "Ecstasy": A report of five deaths associated with the use of MDEA and MDMA. *journal of the American Medical Associtation* 1987, *257*, 1615-1617.

(150) Henry, J. A.; Jeffreys, K. J.; Dawling, S.: Toxicity and deaths from 3,4-methylenedioxymethamphetamine ("ecstasy"). *The Lancet* 1992, *340*, 384-387.

(151) Simpson, D. L.; Rumack, B. H.: Methylenedioxyamphetamine. clinical description of overdose, death, and review of pharmacology. *Archives of Internal Medicine* 1981, *141*, 1507-1509.

(152) Abraham, T. T.; Barnes, A. J.; Lowe, R. H.; Kolbrich Spargo, E. A.; Milman, G.; Pirnay, S. O.; Gorelick, D. A.; Goodwin, R. S.; Huestis, M. A.: Urinary MDMA, MDA, HMMA, and HMA excretion following controlled MDMA administration to humans. *Journal of Analytical Toxicology* 2009, *33*, 439-446.

(153) Fitzgerald, R. L.; Blanke, R. V.; Glennon, R. A.; Yousif, M. Y.; Rosecrans, J. A.; Poklis, A.: Determination of 3,4-methylenedioxyamphetamine and 3,4-methylenedioxymethamphetamine enantiomers in whole blood. *Journal of Chromatography B* 1989, *490*, 59-69.

(154) Lim, H. K.; Foltz, R. L.: *In vivo* and *in vitro* metabolism of 3,4-(methylenedioxy)methamphetamine in the rat: identification of metabolites using an ion trap detector. *Chemical Research in Toxicology* 1988, *1*, 370-378.

(155) Lim, H. K.; Foltz, R. L.: Identification of metabolites of 3,4-(methylenedioxy)methamphetamine in human urine. *Chemical Research in Toxicology* 1989, *2*, 142-143.

(156) Yousif, M. Y.; Fitzgerald, R. L.; Narasimhachari, N.; Rosecrans, J. A.; Blanke, R. V.; Glennon, R. A.: Identification of metabolites of 3,4-methylenedioxymethamphetamine in rats. *Drug and Alcohol Dependence* 1990, *26*, 127-135.

(157) Lim, H. K.; Foltz, R. L.: *In vivo* formation of aromatic hydroxylated metabolites of 3,4-(methylenedioxy)methamphetamine in the rat: Identification by ion trap tandem mass spectrometric (MS/MS and MS/MS/MS) techniques. *Biological Mass Spectrometry* 1991, *20*, 677-686.

(158) Pirnay, S. O.; Abraham, T. T.; Lowe, R. H.; Huestis, M. A.: Selection and optimization of hydrolysis conditions for the quantification of urinary metabolites of MDMA. *Journal of Analytical Toxicology* 2006, *30*, 563-569.

(159) Mueller, M.; Peters, F. T.; Ricaurte, G. A.; Maurer, H. H.: Validated liquid chromatographic–electrospray ionization mass spectrometric assay for simultaneous determination of 3,4-methylenedioxymethamphetamine and its

metabolites 3,4-methylenedioxyamphetamine, 3,4-dihydroxymethamphetamine, and 4-hydroxy-3-methoxymethamphetamine in squirrel monkey plasma. *Journal of Chromatography B* 2007, *855*, 262-270.

(160) Shima, N.; Kamata, H.; Katagi, M.; Tsuchihashi, H.; Sakuma, T.; Nemoto, N.: Direct determination of glucuronide and sulfate of 4-hydroxy-3-methoxymethamphetamine, the main metabolite of MDMA, in human urine. *Journal of Chromatography B* 2007, *857*, 123-129.

(161) Kolbrich, E. A.; Lowe, R. H.; Huestis, M. A.: Two-dimensional gas chromatography/electron-impact mass spectrometry with cryofocusing for simultaneous quantification of MDMA, MDA, HMMA, HMA, and MDEA in human plasma. *Clinical Chemistry* 2008, *54*, 379-387.

(162) Mueller, M.; Kolbrich-Spargo, E. A.; Peters, F. T.; Huestis, M. A.; Ricaurte, G. A.; Maurer, H. H.: Hydrolysis of 3,4-methylenedioxymethamphetamine (MDMA) metabolite conjugates in human, squirrel monkey, and rat plasma. *Analytical and Bioanalytical Chemistry* 2009, *393*, 1607-1617.

(163) Fonsart, J.; Menet, M.-C.; Debray, M.; Hirt, D.; Noble, F.; Scherrmann, J.-M.; Declèves, X.: Sprague–Dawley rats display sex-linked differences in the pharmacokinetics of 3,4-methylenedioxymethamphetamine (MDMA) and its metabolite 3,4-methylenedioxyamphetamine (MDA). *Toxicology and Applied Pharmacology* 2009, *241*, 339-347.

(164) Schwaninger, A. E.; Meyer, M. R.; Huestis, M. A.; Maurer, H. H.: Development and validation of LC-HRMS and GC-NICI-MS methods for stereoselective determination of MDMA and its phase I and II metabolites in human urine. *Journal of Mass Spectrometry* 2011, *46*, 603-614.

(165) Ramaley, C.; Leonard, S. C.; Miller, J. D.; Wilson, D. T.; Chang, S. Y.; Chen, Q.; Li, F.; Du, C.: *In vitro* metabolism of 3,4-methylenedioxymethamphetamine in human hepatocytes. *Journal of Analytical Toxicology* 2014, *38*, 249-255.

(166) Laurent, R.: Identification and comfirmation criteria for LC-MS. In *Applications of LC-MS in Toxicology*; Aldo, P., Ed.; Pharmaceutical Press London, 2006; pp 97-109.

(167) Menet, M.-C.; Fonsart, J.; Hervé, F.; Fompeydie, D.; Galliot-Guilley, M.; Noble, F.; Scherrmann, J.-M.: Determination of 3,4-methylenedioxymethamphetamine and its five main metabolites in rat urine by

solid-phase extraction and high performance liquid chromatography with on line mass spectrometry. *Journal of Chromatography B* 2010, *878*, 2905-2910.

(168) Jamali, B.; Ardakani, Y. H.; Foroumadi, A.; Kobarfard, F.; Rouini, M. R.: Determination of MDMA and its three metabolites in the rat perfused liver. *Journal of Analytical Toxicology* 2013, *37*, 357-361.

(169) Steuer, A. E.; Schmidhauser, C.; Liechti, M. E.; Kraemer, T.: Development and validation of an LC-MS/MS method after chiral derivatization for the simultaneous stereoselective determination of methylenedioxy-methamphetamine (MDMA) and its phase I and II metabolites in human blood plasma. *Drug Testing and Analysis* 2015, *7*, 592-602.

(170) Schwaninger, A. E.; Meyer, M. R.; Zapp, J.; Maurer, H. H.: Sulfation of the 3,4-methylenedioxymethamphetamine (MDMA) metabolites 3,4-dihydroxymethamphetamine (DHMA) and 4-hydroxy-3-methoxymethamphetamine (HMMA) and their capability to inhibit human sulfotransferases. *Toxicology Letters* 2011, *202*, 120-128.

(171) Farre, M.; Tomillero, A.; Perez-Mana, C.; Yubero, S.; Papaseit, E.; Roset, P. N.; Pujadas, M.; Torrens, M.; Cami, J.; de la Torre, R.: Human pharmacology of 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) after repeated doses taken 4 h apart Human pharmacology of MDMA after repeated doses taken 4 h apart. *European neuropsychopharmacology* 2015, *25*, 1637-1649.

(172) Shima, N.; Tsutsumi, H.; Kamata, T.; Nishikawa, M.; Katagi, M.; Miki, A.; Tsuchihashi, H.: Direct determination of glucuronide and sulfate of *p*-hydroxymethamphetamine in methamphetamine users' urine. *Journal of Chromatography B* 2006, *830*, 64-70.

(173) López-Arnau, R.; Martínez-Clemente, J.; Pubill, D.; Escubedo, E.; Camarasa, J.: Comparative neuropharmacology of three psychostimulant cathinone derivatives: butylone, mephedrone and methylone. *British Journal of Pharmacology* 2012, *167*, 407-420.

(174) López-Arnau, R.; Martínez-Clemente, J.; Carbó, M. l.; Pubill, D.; Escubedo, E.; Camarasa, J.: An integrated pharmacokinetic and pharmacodynamic study of a new drug of abuse, methylone, a synthetic cathinone sold as "bath salts". *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 2013, *45*, 64-72.

(175) King, L. A.; Nutt, D. J.: Deaths from "legal highs": a problem of definitions. *The Lancet* 2014, *383*, 952.

(176) Caroline, D.: Warning over fake ecstasy tablets after seven people die in Scotland. The Guardian, 2013; 10 July 2013.

(177) Honecker, H.; Coper, H.: Kinetics and metabolism of amphetamine in the brain of rats of different ages. *Naunyn-Schmiedeberg's Archives of Pharmacology* 1975, *291*, 111-121.

(178) Shimosato, K.; Tomita, M.; Ijiri, I.: Urinary excretion of *p*-hydroxylated methamphetamine metabolites in man. *Archives of Toxicology* 1986, *59*, 135-140.

(179) Cheung, S.; Nolte, H.; Otton, S. V.; Tyndale, R. F.; Wu, P. H.; Sellers, E. M.: Simultaneous gas chromatographic determination of methamphetamine, amphetamine and their *p*-hydroxylated metabolites in plasma and urine. *Journal of Chromatography B* 1997, *690*, 77-87.

(180) Concheiro, M.; Simões, S. M. d. S. S.; Quintela, Ó.; de Castro, A.; Dias, M. J. R.; Cruz, A.; López-Rivadulla, M.: Fast LC–MS/MS method for the determination of amphetamine, methamphetamine, MDA, MDMA, MDEA, MBDB and PMA in urine. *Forensic Science International* 2007, *171*, 44-51.

(181) del Mar Ramírez Fernández, M.; Wille, S. M. R.; di Fazio, V.; Gosselin, M.; Samyn, N.: Analysis of amphetamines and metabolites in urine with ultra performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography B* 2010, *878*, 1616-1622.

(182) Hubbard, J. W.; Midha, K. K.; Cooper, J. K.: The metabolism of *p*-methoxyamphetamine in dog and monkey. O-demethylation as a major route. *Drug Metabolism and Disposition* 1977, *5*, 329-334.

(183) Schweitzer, J. W.; Friedhoff, A. J.; Angrist, B. M.; Gershon, S.: Excretion of *p*-methoxyamphetamine administered to humans. *Nature* 1971, *229*, 133-134.

(184) Beckett, A. H.; Midha, K. K.: The Identification of four metabolic products after incubation of *p*-Methoxyamphetamine with liver preparations of various species. *Xenobiotica* 1974, *4*, 297-311.

(185) Hendrickson, H.; Laurenzana, E.; Owens, S. M.: Quantitative determination of total methamphetamine and active metabolites in rat tissue by liquid chromatography with tandem mass spectrometric detection. *Journal of the American Association of Pharmaceutical Scientists* 2006, *8*, E709-E717.

(186) Goldstein, M.; Anagnoste, B.: The conversion *in vivo* of d-amphetamine to (+)-*p*-hydroxynorephedrine. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1965, *107*, 166-168.

(187) Sever, P. S.; Caldwell, J.; Dring, L. G.; Williams, R. T.: The metabolism of amphetamine in dependent subjects. *European Journal of Clinical Pharmacology* 1973, *6*, 177-180.

(188) Vulimiri, S. V.; Pratt, M. M.; Kulkarni, S.; Beedanagari, S.; Mahadevan, B.: Chapter 23 - Reproductive and developmental toxicology: toxic solvents and gases. In *Reproductive and Developmental Toxicology*; Gupta, R. C., Ed.; Academic Press San Diego, 2011; pp 303-315.

(189) WHO.: *Environmental Health Criteria 89: Formaldehyde*; World Health Organization: Finland, 1989.

(190) U.S.EPA: Formaldehyde TEACH Chemical Summary. Last revised 20/09/2007; U.S.EPA, 2007; pp 1-11.

(191) Berry, C.: Industry Guide 31: A guide to Formaldehyde. N.C. Department of Labor Occupational Safety and Health Program, 2013.

(192) Takayasu, T.: Toxicological analyses of medications and chemicals in formalin-fixed tissues and formalin solutions: A review. *Journal of Analytical Toxicology* 2013, *37*, 615-621.

(193) Kuhlmann, W. D.: Fixation of biological specimens. 2009. from http://www.immunologie-labor.com/cellmarker_files/IET_tissue_02.pdf. (accessed Jan 28, 2016).

(194) Waters, B.: Fixation and Transport of Autopsy Material. In *Handbook of Autopsy Practice*; Waters, B., Ed.; Humana Press New Jersey, 2009; pp 145-149.

(195) Dettling, R. J.; Briglia, E. J.; Dal Cortivo, L. A.; Bidanset, J. H.: The production of amitriptyline from nortriptyline in formaldehyde-containing solutions. *Journal of Analytical Toxicology* 1990, *14*, 325-326.

(196) Winek, C. L.; Esposito, F. M.; Cinicola, D. P.: The stability of several compounds in formalin fixed tissues and formalin-blood solutions. *Forensic Science International* 1990, *44*, 159-168.

(197) Winek, C. L.; Zaveri, N. R.; Wahba, W. W.: The study of tricyclic antidepressants in formalin-fixed human liver and formalin solutions. *Forensic Science International* 1993, *61*, 175-183.

(198) Xiang, P.; Shen, M.; Bu, J.; Huang, Z.: The stability of tetramine, morphine and meperidine in formalin solution. *Forensic Science International* 2001, *122*, 159-162.

(199) Cingolani, M.; Froldi, R.; Mencarelli, R.; Mirtella, D.; Rodriguez, D.: Detection and quantitation of morphine in fixed tissues and formalin solutions. *Journal of Analytical Toxicology* 2001, *25*, 31-34.

(200) Gannett, P. M.; Hailu, S.; Daft, J.; James, D.; Rybeck, B.; Tracy, T. S.: *In vitro* reaction of formaldehyde with fenfluramine: Conversion to N-methyl fenfluramine. *Journal of Analytical Toxicology* 2001, *25*, 88-92.

(201) Gannett, P. M.; Daft, J. R.; James, D.; Rybeck, B.; Knopp, J. B.; Tracy, T. S.: *In vitro* reaction of barbiturates with formaldehyde. *Journal of Analytical Toxicology* 2001, *25*, 443-449.

(202) Tracy, T. S.; Rybeck, B. F.; James, D. G.; Knopp, J. B.; Gannett, P. M.: Stability of benzodiazepines in formaldehyde solutions. *Journal of Analytical Toxicology* 2001, *25*, 166-173.

(203) Tirumalai, P. S.; Shakleya, D. M.; Gannett, P. M.; Callery, P. S.; Bland, T. M.; Tracy, T. S.: Conversion of methamphetamine to N-methyl-methamphetamine in formalin solutions. *Journal of Analytical Toxicology* 2005, *29*, 48-53.

(204) Shakleya, D. M.; Kraner, J. C.; Kaplan, J. A.; Gannett, P. M.; Callery, P. S.: Identification of N,N-dimethylamphetamine formed by methylation of methamphetamine in formalin-fixed liver tissue by multistage mass spectrometry. *Forensic Science International* 2006, *157*, 87-92.

(205) Shakleya, D. M.; Kraner, J. C.; Kaplan, J. A.; Callery, P. S.: Methylation of 3,4-methylenedioxymethamphetamine in formalin-fixed human liver tissue. *Journal of Analytical Toxicology* 2005, *29*, 715-717.

(206) Suma, R.; Prakash, P. K. S.: Conversion of sertraline to N-methyl sertraline in embalming fluid: A forensic implication. *Journal of Analytical Toxicology* 2006, *30*, 395-399.

(207) Ramagiri, S.; Shukla, S. K.; Sai Prakash, P. K.: Stability study of fluoxetine in formalin-fixed liver tissue. *Journal of Analytical Toxicology* 2006, *30*, 692-696.

(208) Viel, G.; Nalesso, A.; Cecchetto, G.; Montisci, M.; Ferrara, S. D.: Stability of cocaine in formalin solution and fixed tissues. *Forensic Science International* 2009, *193*, 79-83.

(209) Nikolaou, P.; Papoutsis, I.; Dona, A.; Spiliopoulou, C.; Athanaselis, S.: Toxicological analysis of formalin-fixed or embalmed tissues: A review. *Forensic Science International* 2013, *233*, 312-319.

(210) Uekusa, K.; Hayashida, M.; Ohno, Y.: Forensic toxicological analyses of drugs in tissues in formalin solutions and in fixatives. *Forensic Science International* 2015, *249*, 165-72.

(211) Smith, T. O.; Gitsham, P.; Donell, S. T.; Rose, D.; Hing, C. B.: The potential dangers of medical devices with current cremation practices. *European Geriatric Medicine* 2012, *3*, 97-102.

(212) Buddhist Funeral Rites in Thailand & other South East Asian Countries. http://www.buddhanet.net/bfuneral.htm (accessed Jan 28, 2016).

(213) Meyer, J. S.; Piper, B. J.: Chapter 26 - Developmental neurotoxicity of abused drugs. In *Reproductive and Developmental Toxicology*; Gupta, R. C., Ed.; Academic Press San Diego, 2011; pp 341-353.

(214) UNODC: *Recommended methods for the identification and analysis of amphetamine, methamphetamine and their ring-substituted analogues in seized Materials (revised and updated)*; United Nations Publications: Austria, 2006.

(215) Schuckit, M. A.; Stimulants: Amphetamines and Cocaine. In *Drug and Alcohol Abuse*; Springer New York, 2006; pp 137-163.

(216) EMCDDA: Amphetamine drug profile. European Monitoring Centre for Drugs and Drug Addiction, http://www.emcdda.europa.eu/publications/drug-profiles/amphetamine (accessed Jan 28, 2016).

(217) INCHEM: Amphetamine. Chemical Safety Information from Intergovernmental Organizations, http://www.inchem.org/documents/pims/pharm/pim934.htm (accessed Jan 28, 2016).

(218) Dwoskin, L.; Glaser, P. A.; Bardo, M.: Methamphetamine. In *Addiction Medicine*; Johnson, B. A., Ed.; Springer New York, 2011; pp 1049-1061.

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

(220) Lo, J.; Kaye, A.: Benzodiazepines and Muscle Relaxants. In *Essentials of Pharmacology for Anesthesia, Pain Medicine, and Critical Care*;

Kaye, A. D., Kaye, A. M., Urman, R. D., Eds.; Springer New York, 2015; pp 167-178.

(221) Olkkola, K. T.; Ahonen, J.: Midazolam and Other Benzodiazepines. In *Modern Anesthetics*; Schüttler, J., Schwilden, H., Eds.; Springer Berlin Heidelberg, 2008; Vol. 182; pp 335-360.

(222) Dasgupta, A.: Pharmacology of Commonly Abused Drugs. In *Beating Drug Tests and Defending Positive Results*; Humana Press New York, 2010; pp 11-28.

(223) Gerlach, M.; Warnke, A.: Anxiolytics and Sedative-Hypnotics. In *Psychiatric Drugs in Children and Adolescents*; Gerlach, M., Warnke, A., Greenhill, L., Eds.; Springer Vienna, 2014; pp 219-255.

(224) UNODC: *Recommended methods for testing opium, morphine and heroin: Manual for use by national drug testing Laboratories*; United Nations Publications: Austria, 1998.

(225) Cox, B.: Pharmacology of Opioid Drugs. In *The Opiate Receptors*; Pasternak, G. W., Ed.; Humana Press New York, 2011; pp 23-57.

(226) Freye, E.; Levy, J.: Mechanism of Action of Opioids and Clinical Effects. In *Opioids in Medicine*; Springer Netherlands, 2008; pp 85-187.

(227) Satterly, M.; Anitescu, M.: Opioids and Substance Abuse. In *Substance Abuse*; Kaye, A. D., Vadivelu, N., Urman, R. D., Eds.; Springer New York, 2015; pp 179-192.

(228) Sverrisdóttir, E.; Lund, T. M.; Olesen, A. E.; Drewes, A. M.; Christrup, L. L.; Kreilgaard, M.: A review of morphine and morphine-6-glucuronide's pharmacokinetic–pharmacodynamic relationships in experimental and clinical pain. *European Journal of Pharmaceutical Sciences* 2015, *74*, 45-62.

(229) Gregory, T. B.: Hydromorphone: Evolving to meet the challenges of today's health care environment. *Clinical Therapeutics* 2013, *35*, 2007-2027.

(230) Murray, A.; Hagen, N. A.: Hydromorphone. *Journal of Pain and Symptom Management* 2005, *29*, 57-66.

(231) Wu, X.; Zhang, W.; Bai, Y.; Guo, T.; Gu, J.: Simultaneous analysis of codeine and its active metabolites in human plasma using liquid chromatography–tandem mass spectrometry: Application to a pharmacokinetic study after oral administration of codeine. *Journal of Pharmaceutical and Biomedical Analysis* 2013, *78–79*, 261-268.

(232) Gupta, R. C.; Milatovic, D.: Chapter 45 - Organophosphates and carbamates. In *Veterinary Toxicology;* 2nd edn; Gupta, R. C., Ed.; Academic Press Boston, 2012; pp 573-585.

(233) Skretteberg, L. G.; Lyrån, B.; Holen, B.; Jansson, A.; Fohgelberg, P.; Siivinen, K.; Andersen, J. H.; Jensen, B. H.: Pesticide residues in food of plant origin from Southeast Asia – A Nordic project. *Food Control* 2015, *51*, 225-235.

(234) Carbofuran. Alternative Agriculture Network – Esan (2016). from https://aanesan.wordpress.com/2009/11/23/ความจริงของ-คาร์โบฟูราน/ (accessed Jun 27, 2016)

(235) Lotrakul, M.: Suicide in Thailand during the period 1998-2003. *Psychiatry and Clinical Neurosciences* 2006, *60*, 90-95.

(236) Zhai, R. G.: The Architecture of the presynaptic release site. In *Molecular Mechanisms of Neurotransmitter Release*; Wang, Z.-W., Ed.; Humana Press Totowa, 2008; pp 1-16.

(237) Lüllmann, H.; Preuner, J.; Schaube, H.: A kinetic approach for an interpretation of the acetylcholine — d-tubocurarine interaction on chronically denervated skeletal muscle. *Naunyn-Schmiedeberg's Archives of Pharmacology* 1974, *281*, 415-426.

(238) Gupta, R. C.; Malik, J. K.; Milatovic, D.: Chapter 37 - Organophosphate and carbamate pesticides. In *Reproductive and Developmental Toxicology*; Gupta, R. C., Ed.; Academic Press San Diego, 2011; pp 471-486.

(239) INCHEM: Pesticide Residues in Food - 1984. Chemical Safety Information from Intergovernmental Organizations, http://www.inchem.org/documents/ehc/ehc/ehc104.htm (accessed Jan 28, 2016).

(240) Kim, J. H.; Chang, C.H.; Moon, J.K.; Lee, H.G.; Lee, J.H.; Park, H.W.; Park, B.S.; Hwang, E.C.; Lee, H.S.; Liu, K.H.: Comparative metabolism of insecticide carbosulfan by human, mouse and rat liver microsomes. *Toxicology Letters* 2007, *172,* S211.

(241) Abass, K.; Reponen, P.; Mattila, S.; Pelkonen, O.: Metabolism of carbosulfan. I. Species differences in the *in vitro* biotransformation by mammalian hepatic microsomes including human. *Chemico-Biological Interactions* 2009, *181*, 210-219.

(242) Abass, K.; Reponen, P.; Mattila, S.; Pelkonen, O.: Metabolism of carbosulfan II. Human interindividual variability in its *in vitro* hepatic biotransformation and the identification of the cytochrome P450 isoforms involved. *Chemico-Biological Interactions* 2010, *185*, 163-173.

(243) Maślanka, A.; Krzek, J.; Szlósarczyk, M.; Żmudzki, P.; Wach, K.: Dependence of the kinetic and thermodynamic parameters on hydrophilic–lipophilic character of alprazolam, clonazepam, diazepam, doxepin and haloperidol in alkaline environment. *International Journal of Pharmaceutics* 2013, *455*, 104-112.

(244) Nudelman, N. S.; Cabrera, C. G.: Isolation and structural elucidation of degradation products of alprazolam: Photostability studies of alprazolam tablets. *Journal of Pharmaceutical Sciences* 2002, *91*, 1274-1286.

(245) Cabrera, C. G.; de Waisbaum, R. G.; Nudelman, N. S.: Kinetic and mechanistic studies on the hydrolysis and photodegradation of diazepam and alprazolam. *Journal of Physical Organic Chemistry* 2005, *18*, 156-161.

(246) Gonsalves, A. R.; Pineiro;, M.; José Manuel Martins; Barata, P. A.; Menezes, J. C.: Identification of alprazolam and its degradation products using LC-MS-MS. *ARKIVOC: Online Journal of Organic Chemistry*, *2010*, 128-141.

(247) Tracy, T. S.; Rybeck, B. F.; James, D. G.; Knopp, J. B.; Gannett, P. M.: Stability of benzodiazepines in formaldehyde solutions. *Journal of Analytical Toxicology* 2001, *25*, 166-173.

(248) Soler, C.; Hamilton, B.; Furey, A.; James, K. J.; Manes, J.; Pico, Y.: Liquid chromatography quadrupole time-of-flight mass spectrometry analysis of carbosulfan, carbofuran, 3-hydroxycarbofuran, and other metabolites in food. *Analytical Chemistry* 2007, *79*, 1492-1501.

(249) Hong, J.; Shin, C.-M.; Kim, Y. H.; Yoo, J. S.: Electron impact fragmentation of carbofuran and its major photolytic transformation metabolites. *Rapid Communications in Mass Spectrometry* 1999, *13*, 354-361.

Appendices: Publications in support of this Thesis

Conferences

1. *In vitro* reaction of formaldehyde with drugs and poisons: identification of the conversion products. Proceedings of World Forensic Festival 2014 meeting, Seoul, Korea, 2014 (poster).

2. *In vitro* reaction of formaldehyde with drugs and poisons: identification of the conversion products. Proceedings of The International Association of Forensic Toxicologist meeting, Buenos Aires, Argentina, 2014 (poster).

3. *In vitro* metabolism studies on methylenedioxy-substituted amphetamines using human liver microsomes and LC/MS/MS with chemical derivatization. Proceedings of the Society of Forensic Toxicologists meeting, Atlanta, United States of America, 2015 (poster).

In vitro reaction of formaldehyde with drugs and poisons: identification of the conversion products.

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Abstract

In tropical countries, formalin, the active ingredient of which is formaldehyde, is injected into cadavers as an embalming solution to reduce body decomposition and preserve the body for a longer time. All drugs present in the body will be exposed to formaldehyde, a highly reactive substance that can chemically react with analytes, resulting in decreasing concentrations of the original compounds and producing one or more new substances. Occasionally, in some cases, formalin-blood, formalin-fixed tissue or embalming fluid is required for drugs analysis, and an analysis of these samples may create a false negative or false positive because of the reaction between drugs and formaldehyde. Previous studies on the chemical reactivity between formalin solution and drugs demonstrated that many drugs are unstable in formalin-fixed tissue and or the formalin solution in which the tissue is stored. In particular, primary and secondary amines can react with formaldehyde to form secondary and tertiary amines by the Eschweiler–Clarke reaction.

The present study was aimed at quantification of the original drugs and identification of their degradation products in formalin solution. In order to evaluate the effect of formaldehyde on drugs and poisons, a range of model compounds was used, including amphetamine-type stimulants (amphetamine, methamphetamine, and 3,4-methylenedioxy-N-methamphetamine (MDMA)), benzodiazepines (alprazolam and diazepam), opiates (morphine, hydromorphone, codeine, and hydrocodone) and a carbamate insecticide (carbosulfan), which are often associated with cases of suicide, homicide, accidental poisoning and road traffic accident because of their toxicity and potential for abuse.

Spiked samples in 10% formalin solution (with no pH adjustment) were stored at room temperature and analysed at selected times over 30 days. The original drugs were quantified and their degradation products in formalin solution were identified and confirmed by gas chromatography-mass spectrometry (GC/MS) and liquid chromatography - triple quadrupole mass spectrometer (LC/MS/MS).The results show that amphetamine (a primary amine) is methylated producing methamphetamine and methamphetamine (secondary amine) produces dimethylamphetamine (DMA). MDMA is methylated producing 3,4-methylenedioxy-*N,N*-dimethylamphetamine (MDDMA). For opiates, hydrocodone rapidly changes to a more polar compound that gives an MH^+ at *m/z* 360. Hydromorphone also changes to more polar compounds with MH^+ at *m/z* 316 and 346. Codeine and morphine are stable in formalin solution. Furthermore, open-ring alprazolam (5-chloro-[2-(3-aminomethyl-5-methyl-1,2,4-triazol-4-yl]benzophenone) was observed as the hydrolysis product of alprazolam. Carbosulfan is completely hydrolysed to carbofuran within 24 h.

The forensic toxicologist should consider the degradation products as potential target analytes when performing drugs analysis in embalming samples.

Keywords: formalin-blood, degradation product, stability studies, LC-MS-MS, amphetamines, benzodiazepines, opiates, carbosulfan.

In vitro reaction of formaldehyde with drugs and poisons: identification of the conversion products.

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Abstract

In tropical countries, formalin is injected into cadavers as an embalming solution to reduce body decomposition and preserve the body for a longer time. All drugs present in the body will be exposed to formaldehyde, a highly reactive substance that can chemically react with analytes, resulting in decreasing concentrations of the original compounds and in the production of one or more new substances. ***Aims:*** The present study was aimed at quantification of the original drugs and identification of their degradation products in formalin solution and formalin blood. ***Methods:*** In order to evaluate the effect of formaldehyde on drugs and poisons, a range of model compounds was used, including amphetamine-type stimulants, benzodiazepines, opiates and carbamate insecticides. Spiked samples in 10% formalin solution (with no pH adjustment) and 10% formalin-blood were stored at room temperature and analysed at selected times over 30 days. The original drugs were quantified and their degradation products in formalin solution/blood were identified and confirmed by gas chromatography-mass spectrometry (GC/MS) and liquid chromatography - triple quadrupole mass spectrometer (LC/MS/MS). ***Result:*** In both, formalin solution and formalin blood, the results show that amphetamine is methylated producing methamphetamine and methamphetamine produces *N,N*-dimethylamphetamine (DMA). MDMA is methylated producing 3,4-methylenedioxy-*N,N*-dimethylamphetamine (MDDMA). For opiates, hydrocodone rapidly changes to a more polar compound that gives an MH+ at *m/z* 360. Hydromorphone also changes to more polar compounds with MH+ at *m/z* 316 and 346. Codeine and morphine are stable in formalin solution/blood. Furthermore, open-ring alprazolam (5-chloro-[2-(3-aminomethyl-5-methyl-1,2,4-triazol-4-yl]benzophenone) was observed as the hydrolysis product of alprazolam and a compounds that gives an MH+ at *m/z* 399 was observed as a conversion product in both formalin solution and blood. Finally, carbosulfan is rapidly hydrolysed to

carbofuran and its metabolites, 3-ketocarbofuran and 3-hydroxycarbofuran within 24 h. ***Conclusions:*** The forensic toxicologist should consider the degradation products as potential target analytes when performing drugs analysis in embalming samples.

Keywords: formalin-blood, degradation product, stability studies, LC-MS-MS, amphetamines, benzodiazepines, opiates, carbosulfan.

In vitro metabolism studies on methylenedioxy-substituted amphetamines using human liver microsomes and LC/MS/MS with chemical derivatization

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Abstract

The abuse of ß-keto derivatives of MDMA such as butylone and methylone has been reported since the mid-2000s. The forensic toxicologist faces problems with these drugs, because of lack of information on their metabolism and unavailability of reference standards of the metabolites and the parent drugs themselves. Ethical considerations usually preclude human metabolism studies under controlled conditions and *in vitro* methods provide potential alternatives. A few previous studies showed that ß-keto derivatives of MDMA have similar metabolic pathways to MDMA, resulting in metabolites formed by demethylenation, demethylenation plus *O*-methylation (4-OH-3-MeO metabolites) and N-demethylation. Another challenge is the analysis of these polar metabolites using conventional C18 HPLC columns, resulting in low retention volumes. One rapid and simple solution to this is chemical derivatization. Conversion of metabolites to more hydrophobic compounds by derivatization can improve separations by reversed-phase HPLC and increase sensitivity in mass spectrometry.

Aims: To identify metabolites of butylone and methylone in humans using human liver microsomes (HLM) *in vitro* and reversed-phase LC/MS/MS with derivatization. MDMA was used in this study as a model compound as its metabolites are commercially available.

Method: Pooled human liver microsomes were incubated with NADPH regenerating system and MDMA, butylone or methylone in 0.1 M phosphate buffer pH 7.4 in a shaking incubator at 37^{o}C for 90 min. Each reaction was stopped by the addition of ice-cold acetonitrile and extracts were derivatised with acetic anhydride/pyridine (3:2 v:v) for 30 min at 60^{o}C. Derivatised

metabolites were identified by LC/MS/MS using multiple reaction monitoring (MRM). Ten MDMA positive urines were also analysed to compare the metabolite patterns with those obtained *in vitro* with HLM.

Results: Three phase-I metabolites (both major and minor metabolites) of MDMA, butylone and methylone were detected. For MDMA, 3,4-dihydroxymethamphetamine derivative (HHMA-3Ac) was identified by MRM transitions at *m/z* 308>266, 308>224 and 308>151, 4-hydroxy-3-methoxymethamphetamine derivative (HMMA-2Ac) at *m/z* 280>238, 280>165 and 280>137 and MDA-Ac at *m/z* 222>163, 222>135 and 222>105. For methylone, dihydroxymethcathinone (DHMC), a major metabolite, was identified by MRM transitions at *m/z* 322>280 and 322>178 (DHMC-3Ac). Nor-methylone (a minor metabolite) was identified by MRM transitions at *m/z* 236>146 and 236>118 (bk-MDC-Ac). Another metabolite, 4-hydroxy-3-methoxymethcathinone (4-HMMC) was identified by MRM transitions at *m/z* 294>234 and 294>160 (4-HMMC-2Ac). Furthermore, a major metabolite of butylone (demethylenation metabolite) was identified by MRM transitions at *m/z* 336>294 and 336>174 (butylone-M dihydroxy-3Ac). Nor-butylone was identified by MRM transitions at *m/z* 250>160 and 250>132 (bk-BDB-Ac). Finally, the 4-OH-3-MeO metabolite was identified by MRM transitions at *m/z* 308>248 and 308>174 (butylone-M (demethylenyl-methyl-) 2Ac). Derivatives of MDMA and its metabolites were stable in the HPLC mobile phase for 30 h at room temperature and were quantified in all 10 positive human urine samples. HMMA was the major metabolite in human urine samples whereas HHMA was the major metabolite by HLM. However, HHMA is an intermediate metabolite leading to HMMA. This result confirms that demethylenation of the methylenedioxy ring, followed by catechol-*O*-methyltransferase (COMT)-catalysed methylation is a major metabolic pathway of MDMA in humans.

Conclusions: Human liver microsomes can be used to simulate drug metabolism in humans and to provide chromatographic and mass spectrometric data on metabolites. Acetate derivatives result in higher molecular weights, providing more specific ions for identification, and in decreased polarities of metabolites, improving their analysis on reversed phase C18 columns. This method would be suitable for routine analysis of urine to detect and confirm abuse of methylenedioxy-substituted amphetamines.

Keywords: Methylenedioxy-substituted amphetamines, *in vitro* metabolism, human liver microsomes.