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The Analysis of Recreational Drugs in Biological Specimens using Liquid Chromatography Mass Spectrometry

A thesis
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of the requirements for the degree
of
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Abstract

In the last few years, the prevalence of legal party pills in New Zealand has risen dramatically. These pills contain new piperazine designer drugs, two of the more common being 1-benzylpiperazine (BZP) and *m*-trifluoromethylphenylpiperazine (TFMPP). This thesis describes an optimised LC-MS/MS method for the detection of BZP and TFMPP in whole blood, using an automated solid phase extraction (SPE) for sample clean-up. The method was validated on three different days using five replicate samples each day. The standard curve was linear from 7 – 7000 ng/mL for BZP and 10 – 10,000 ng/mL for TFMPP, with coefficients of variation (CV) below 10%, and accuracy greater than 90% for both drugs. The method was used to quantitate samples provided by the Medical Research Institute of New Zealand. Blood levels were used to show concentrations in the blood over time, and relate these to performance of subjects on a driving simulator. The study was stopped after 41% of the participants who received BZP and TFMPP had adverse reactions to the pills, including vomiting and migraines.

The LC-MS/MS method was also used to detect and quantitate methamphetamine, amphetamine, methylenedioxymethamphetamine, methylenedioxyamphetamine, morphine, codeine and 6-monoacetylmorphine in hair. The drugs were extracted from 20 mg of hair using hydrochloric acid in a water bath overnight, then purified using SPE. Validation on three days with five replicate samples gave coefficients of variation (CV) below 12% and acceptable accuracy for all drugs. The method was tested on three samples, previously reported by Environmental Science and Research (ESR) using gas chromatography mass spectrometry (GC-MS) giving results in good agreement.

This thesis describes a sensitive, accurate, reproducible LC-MS/MS method easily adapted to analyse drugs of abuse in different biological matrices. It demonstrates the versatility of LC-MS/MS and its applications in forensic work.

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List of Abbreviations

5-HT	Serotonin
6-MAM	6-Monoacetylmorphine
ACTH	Adrenocorticotropin
APCI	Atmospheric Pressure Chemical Ionisation
BZP	1-Benzylpiperazine or *N*-Benzylpiperazine
ACN	Acetonitrile
CRH	Corticotrophin releasing hormone
CSA	Controlled Substances Act (America)
CV	Co-efficient of Variation
CYP	Cytochrome P-450 Enzymes
DA	Dopamine
DAT	Dopamine Transporter
DC	Direct Current
DEA	Drug Enforcement Agency (America)
EGYT-2760	*N*-Benzylpiperazine
EGYT-475	*N*-Benzylpiperazine-picolinyl-fumarate
ESI	Electrospray Ionisation
ESR	Environmental Science Research
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
LC	Liquid Chromatography
LLE	Liquid-liquid Extraction
LOD	Limit of Detection
m/z	Mass to Charge Ratio
MA	Methamphetamine

mCPP	*m*-Chlorophenyl piperazine
MDA	3,4-Methylenedioxyamphetamine
MDBP	1-(3,4-methylenedioxybenzyl)piperazine
MDMA	Methylenedioxymethamphetamine
MeOPP	1-(4-methoxyphenyl)piperazine
MHRA	Medicines and Health Care Products Regulatory Agency
MoH	Ministry of Health
MRINZ	Medical Research Institute New Zealand
MRM	Mass Reaction Monitoring
MS	Mass Spectrometry
NDPSC	National Drugs and Poisons Schedule Committee (Australia)
NPD	Nitrogen-Phosphorus Detector
RF	Radio Frequency
SCX	Strong Cation Exchange
SERT	Sertonin Transporter
SNS	Sympathetic Nervous System
SPE	Solid Phase Extraction
STANZ	Social Tonics Association of New Zealand
SUSDP	Standard for Uniform Scheduling of Drugs and Poisons (Australia)
TFMPP	*m*-trifluoromethylphenylpiperazine or 1-(3-trifluoromethylphenyl)piperazine
UV	Ultra-Violet (Detection)

Chapter 1: Introduction/Literature Review

1.1. Introduction

Recently, piperazine analogues, a new class of designer drugs, have caught the attention of the public, authorities and media, with many questions being raised in regard to both their safety and legal status. 1-Benzyl-piperazine (BZP) and *m*-trifluoromethylphenyl-piperazine (TFMPP) are two of the more common drugs in this class. With comparisons being made to amphetamine and ecstasy (methylenedioxymethamphetamine, MDMA), they have been classed as stimulants, reported and advertised to give feelings of euphoria, alertness, and a desire to socialise.[1] Side effects include a 'hangover' (similar to that of alcohol), dry mouth and urine retention. The legal status of these drugs varies throughout the world and little scientific research on them exists. Of what research is available, studies have been conducted on their detection, metabolism and neuropharmacology. Due to the legal status of these designer drugs in New Zealand, there is plenty of opportunity for study, and the current interest they have generated makes the prospect for further study in this field exciting and unique.

The detection of these, and other recreational drugs, is important in workplace and roadside drug testing, forensic casework and court disputes, for example, child custody cases. It is essential to have a method that is simple, robust, accurate and reproducible. Traditionally, gas chromatography mass spectrometry (GC-MS) is the method of choice for the analyses mentioned above and it has a long history of use in the forensic field. GC-MS however, requires time-consuming sample preparation or is otherwise limited to volatile compounds.[2] Liquid chromatography tandem mass spectrometry (LC-MS/MS) allows the detection and quantitation of many different drugs in biological matrices and often requires less sample preparation. According to the review by Maurer[3] LC-MS/MS has the potential to become the 'golden standard' for forensic and clinical analysis.

This thesis describes an optimised LC-MS/MS method, with solid phase extraction, developed for the detection of BZP and TFMPP in whole blood. The method has been used to quantitate levels of the drugs in human blood samples, taken during research to determine their effects on human driving performance. The results from this project will give an idea of the typical levels that can be found in users, and provide some insight into the effects that these drugs have on humans.

It also examines the use of the above LC-MS/MS method for the analysis of these and other commonly abused, recreational drugs in hair. This includes drugs such as the amphetamines (methamphetamine (MA), methylenedioxymethamphetamine (MDMA)) and the opiates (morphine and codeine). Hair testing is becoming a common type of analysis, especially in workplace drug testing, drug facilitated sexual assaults and child custody disputes. Although hair cannot determine the exact time of exposure, or level of impairment, it does offer long-term exposure information not offered by other biological specimens such as blood and urine.

1.1.1. Biological Matrix

There are many different forms of samples that can be collected for use in drug analysis. These include blood (and its components e.g. plasma, serum), urine, oral fluid (saliva), sebum, hair, nails, and skin.

The use of alternative specimens such as hair and nails can be of assistance in determining drug use patterns. Examples of uses of alternative specimens are: workplace drug testing (hair, sweat, oral fluid), criminal investigations (hair, sweat, oral fluid) child custody disputes and divorce cases (hair), and roadside testing for drug-impaired drivers (oral fluid).[4] The use of hair, nails and oral fluid for drug testing is becoming more popular due to its non-invasive manner.

Figure 1-1 shows the various biological specimens and the time period in which they are useful for the detection of drugs. Blood and oral fluid are

useful for immediate detection, and can give an indication of levels of impairment in a user. Drugs can be detected in urine (depending on the drug) for days. Hair and nails are proving valuable as they can give a history of a person's drug use, but cannot give information of the level of impairment, or the precise time of ingestion.

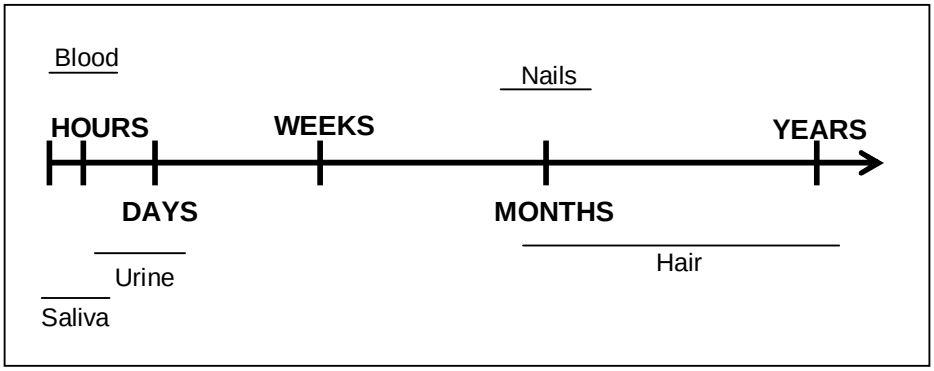


Figure 1-1: Timeline of Detection of Drugs in Biological Specimens

1.1.1.1. Blood and Urine

Urine is the most widely used biological sample for toxicological analysis. Urine demonstrates use of a drug but gives little or no idea of when the drug was taken and the magnitude of any pharmacological effect, and therefore cannot be used to determine the level of impairment. Urine analysis can also be lengthy as there is often a need to identify metabolites due to transformation of the drug during metabolism. It is possible to detect drugs/metabolites in urine from hours until days (Figure 1-1).

Blood has many advantages as a biological matrix as it can provide information about distribution, metabolism, and pharmacokinetics and can be used to measure levels almost immediately after administration.[5] Plasma is often the most common choice for blood analysis, although in autopsy cases plasma often cannot be obtained, so whole blood is used. With major advances in sample preparation, chromatography, and detectors, the use of whole blood as a matrix for quantification and identification has become widespread.[5]

1.1.1.2. Hair

The earliest testing of hair for toxins began with heavy metals in the 1950's and escalated in the late 1970s. It is proving valuable in toxicological analysis, as it can give an idea of a person's drug history (months to years, see Figure 1-1) and is also non-invasive. Unfortunately it cannot determine the level of impairment and can only give an estimate to the time of exposure. It is particularly useful in cases where a urine or blood sample was not taken soon enough.[6]

The methods of incorporation of drugs in hair are still a bit unclear. The most simple explanation is by way of passive transfer from the blood into the hair follicle during formation. However, the multi-compartmental model (demonstrated by Henderson[7]) seems to be the most widely accepted. Here the hair is transferred by multiple mechanisms.[7,8]

Passive Transfer: Occurs via passive diffusion from bloodstream into growing hair cells at the base of the follicle. The drug is retained in the interior of the hair (medulla), during keratogenesis (formation of keratin, hardening of hair). Hair grows approximately 1 cm every 28 days,[8] so roughly, drugs deposited into the hair will be found approximately 1 cm from the scalp, one month after the drug was taken.

Sweat and Sebum: Drugs are transferred into the hair after formation through sweat and sebum. Drugs have been found in sweat in higher concentrations than found in the blood, so this offers an explanation to the higher concentrations sometimes found in the hair.[7]

External Environment: Drugs are passed into the hair from the environment. This could be through the air, water or hair treatments (dying, perming etc.). Drugs such as amphetamine, cannabis, heroin and cocaine are often smoked and hence transferred into the hair.

Intradermal Transfer: Very lipid-soluble drugs such as tetrahydrocannibinol (THC, cannabis) are deposited into skin layers and transferred to hair.

Melanin: Drugs could possibly bind to melanin-related sites in the skin, which could result in drug uptake in the hair.

For analysis using hair, decontamination of the surface of the hair is a vital step. It is important to clear the hair of drugs deposited via air or hair treatments as mentioned in the multi-compartmental model above. The reviews by Pragst *et al.*, Musshoff *et al.*, and Boumba *et al.* give comprehensive overviews of the methods of drug administration of hair mentioned above, as well as the structure of hair and methods of drug detection.[8,9,10]

1.1.2. Solid Phase Extraction

Biological samples such as blood, urine and serum are extremely complex. Injecting them directly onto expensive and sensitive equipment such as LC and GC columns is not a very advisable move. By using an effective method such as solid phase extraction (SPE) to clean-up samples, the life of the instrument is prolonged, and downtime (for source cleaning) of equipment is decreased.[11]

Liquid-liquid extraction (LLE) is a direct method used to extract compounds of interest from complex biological matrices by transferring it from one liquid (solvent) to another. It requires two immiscible solvents, often one is aqueous and the other organic and depends on the difference in solubility of the compound in the different solvents. LLE is frequently used as an efficient separation technique, however solid phase extraction is becoming increasingly popular. Solid phase extraction (SPE) is an indirect chromatography method used to separate an analyte from a liquid, using a solid phase. The analyte/s are adsorbed onto the column, and then eluted using a solvent, often one which can easily be evaporated or can be used directly for analysis by liquid chromatography.

SPE tends to have several advantages over LLE. SPE can easily be automated which generally gives better results, has greater reproducibility, a higher recovery, saves time by increased throughput and reduces errors. SPE uses less solvent and the time analysts spend exposed to organic solvents is

decreased. The general process for the SPE, used for sample clean-up is pictured in Figure 1-2. First the column is conditioned to prepare it for the sample, and then the sample is loaded. The column is washed (to remove unwanted analytes not retained by the sorbent), dried and the analytes of interest eluted.

The extraction of drugs from hair samples is more complex than from whole blood. As drugs and other contaminants can get on to the surface of the hair, a decontamination step is required. This usually involves soaking the hair in a solvent overnight to remove dirt, externally deposited drugs and other contaminants. Once removed, the hair sample can be transferred into solvent for the extraction of the drugs from within the sample. The most common methods are via chemical hydrolysis, enzymatic digestion or solvent extraction. In this study, drugs are extracted from the hair using acid. After extraction of the drugs from the hair, the samples still remain fairly dirty, therefore SPE is used to clean up the samples.

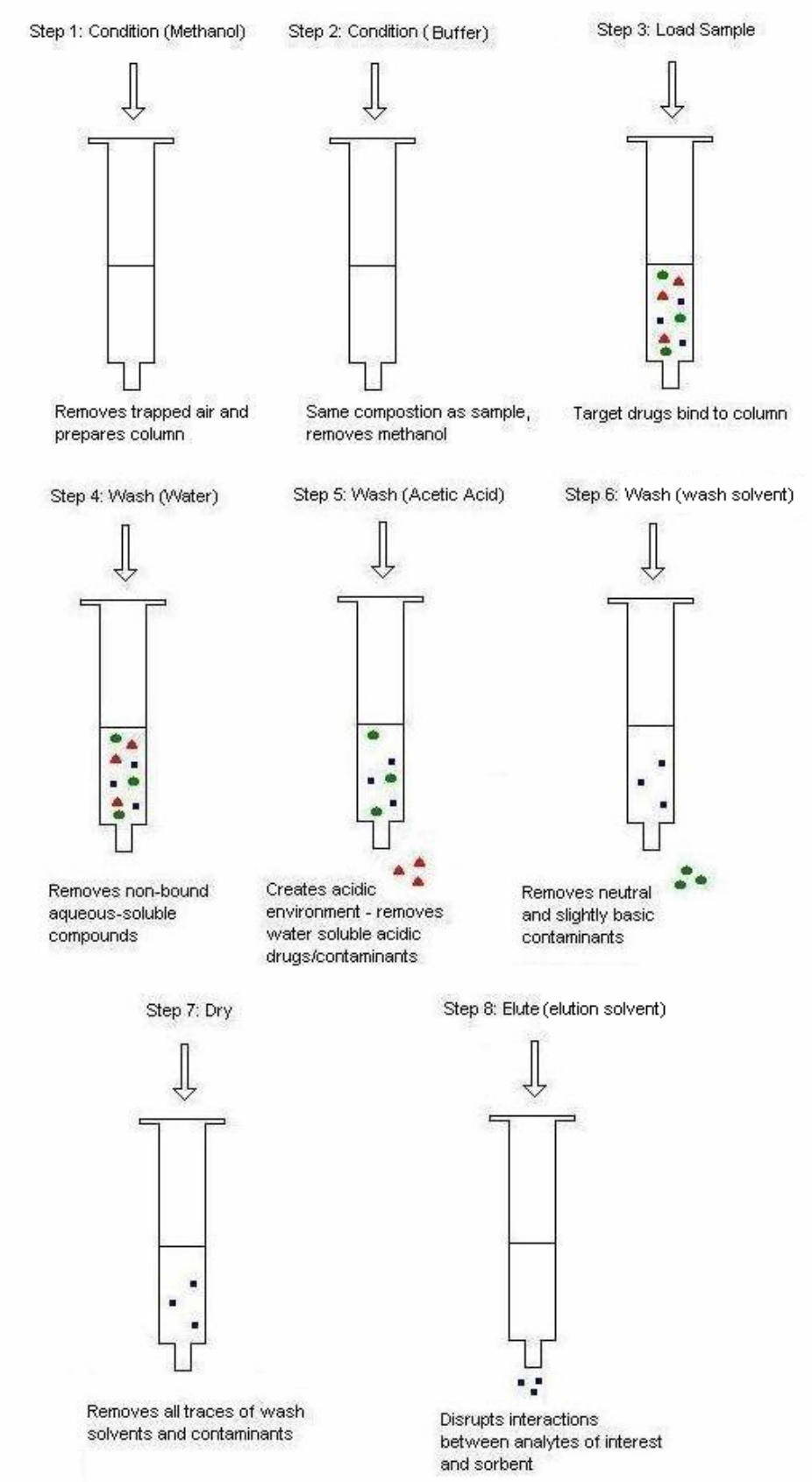


Figure 1-2: Diagram Of Solid Phase Extraction Process

1.1.3. Liquid Chromatography – Mass Spectrometry

Liquid chromatography (LC) and Gas Chromatography (GC) are chromatographic analytical techniques used to separate particular analytes in solution. They both consist of a mobile phase and a stationary phase, which adsorbs and desorbs the analyte of interest. However, in LC the mobile phase is a liquid (normally a mixture of aqueous buffer and organic solvent) and the stationary phase usually consists of surface modified silica or polymer particles. With GC, the mobile phase is an inert carrier gas and the stationary phase can be either an inert porous solid or a solid support coated with a non-volatile liquid.

Traditionally GC-MS is the method of choice for toxicological analysis, however with LC-MS being so diverse it is proving to be a very powerful tool in many aspects of drug analysis.[12] Recent reviews have shown LC-MS to be very significant in pharmaceutical research and clinical and forensic toxicology. It has proved especially valuable for the detection and quantification of more polar, thermally labile, or low-dosed drugs in blood plasma,[3] mainly as they do not require derivatisation. It is highly sensitive and selective.

The LC column used in this thesis is a strong cation-exchange (SCX) column. Ion chromatography separates analytes on the basis of charged particles in solution. The stationary phase consists of a polymer that carries a charge (in the case of cation-exchange this will be negative). Freely moving counter ions pair with the polymer in order to neutralise the charged particles. When the solution is introduced, the analytes of interest replace the counter ions and are retained by the column. The mobile phase elutes the analytes by providing competition for binding sites on the polymer. Cation-exchange can be represented by the following equation:[13]

$$M^-C^+ + A^+ \leftrightarrows M^-A^+ + C^+$$

(Where M^- is the negatively charged stationary phase, C^+ is the counterion and A^+ is the analyte of interest)

Mass spectrometry measures and identifies molecules by measuring the mass to charge ratio (*m/z*) of ions. It can be used to quantify and determine chemical and structural information about atoms and molecules. An important part of the technique is the distinctive fragmentation patterns produced by collisions (with gas), which provide significant structural information that helps to identify components of the molecule.

In this study, samples will be analysed by LC-MS/MS, using a triple quadrupole mass spectrometer. This consists of three quadrupoles arranged sequentially, two of which are mass analysing and the third of which is used as a collision cell. A quadrupole is made up of four parallel electrodes/poles which have DC (direct current) and RF (radio frequency) potentials applied. The poles oscillate and attract and repel ions. Only ions with a stable trajectory pass through, and unwanted ions hit the poles and lose charge. During application, the RF is held constant, and the RF and DC voltages are scanned (ratio of RF: DC remains the same). Whether or not an ion has a stable motion depends upon the DC potential, the amplitude and frequency of the RF field and the *m/z* of the ion.[14]

Tandem MS gives better specificity because daughter ions originate only from selected parent ions.[15] The advantage of tandem mass spectrometry is that the first quadrupole selects a specific molecular ion; the second quadrupole often referred to as the collision chamber, introduces a gas, which collides with the molecular ion producing increased fragmentation; the third chamber scans the predominant ion fragment produced, and hence is highly specific.[16] Finally, the fragments are passed on to a detector. This is often an electron multiplier in which electrons impact on a plate, generating more electrons, which impact

on another plate, producing more electrons and so on. The amount of electrons produced is proportional to the signal intensity.

A problem with coupling LC and MS, is the relatively high volume of vapour that is generated by the evaporation of the mobile phase, and hence, the need for high-capacity pumps in the source of the MS. The use of low-dispersion tubing, unique interfaces and a range of different ionisation techniques have overcome this problem.[17] The application of an interface is highly important in this area and development has taken a long time.

The most common ionisation method employed in this field of research is atmospheric ionisation, which involves removing large amounts of solvent, to leave just the ion. This could be by thermospray, electrospray (ESI), or atmospheric pressure chemical ionisation (APCI). In recent years, the techniques of ESI and APCI have superseded that of thermospray. In electrospray, the solution is passed along a stainless steel capillary, which has a high voltage applied to the outlet tip (around 3-5 kV). When the solution reaches the end of the tube, it is nebulised by the high current and a fine spray of ionised droplets emerges. The droplets then pass into a heated evaporation chamber where solvent is evaporated from them – aided by flowing nitrogen gas at the end of the capillary. The droplets get smaller, which causes them to fissure into smaller droplets, until eventually, the droplet is so small that it becomes de-solvated. It is then able to pass into the mass analyser.[18] In APCI, the solvent molecules are ionised by a corona discharge to form chemically ionised reagent plasma. In the plasma, protons are transferred to form positive and negative ions, due to collisions and charge transfer reactions occurring between the solvent and analyte. ESI is regarded as a soft method of ionisation, and is a lot more versatile than APCI, as it is able to ionise extremely polar, non-volatile molecules, which can be difficult for APCI.[16] It is vital to have a good ionisation source for clean MS spectra, sensitive detection, robustness and reliable operation.[19]

There are two interfaces used in this report, as during the running of these projects, the LC-MS/MS system was upgraded. The original source

TurboIonspray, a pneumatically-assisted ESI method, which allows higher flow rates with improved sensitivity, is pictured in Figure 1-3 below. Both the turbo probe and the ionspray probe are directed at angles of 45° and 135° accordingly, with respect to the curtain plate. The turbo probe produces a jet of heated dry nitrogen gas, which collides with the spray, produced by the ionspray probe (LC-effluent) at an angle of approximately 90°, near the orifice. This interaction helps focus the turbo ionspray stream and increases the rate of droplet evaporation, resulting in an increased ion signal.[20]

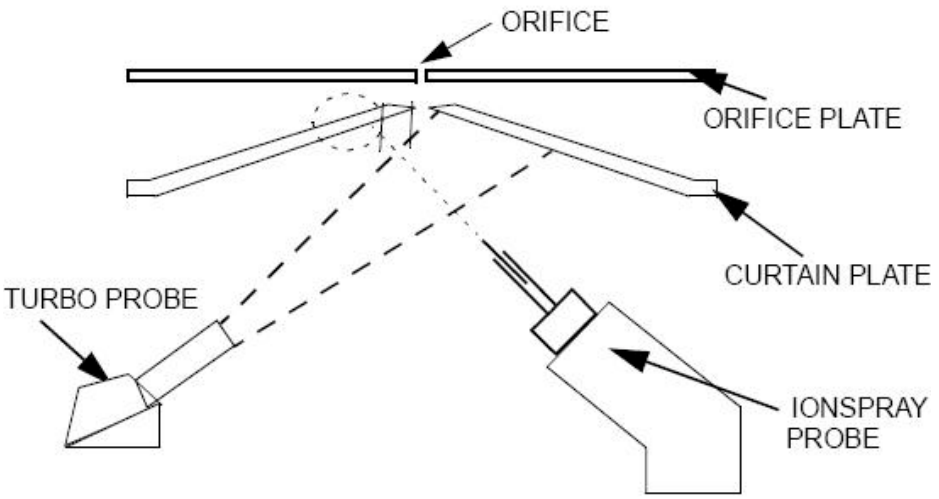


Figure 1-3: Turbo Ionspray Setup[20]

However, the upgraded model employed two interfaces. It used the original TurboIonspray interface coupled to a Hot Source-Induced Desolvated (HS-ID) (Ionics, pictured Figure 1-4). The HSID interface was a relatively inexpensive way of upgrading the MS, which enabled an increase in sensitivity (at least double) and a decrease in background noise. HSID is an atmospheric pressure interface which uses orthogonal nebulisation to reduce any non-desolvated residues fouling up the ion optics, therefore increasing signal intensity and performance of the MS. In the HSID interface, desolvation occurs via direct heat transfer from the hot gas as the ions cross a flow path that consists of three laminar flow regions and four 90˚ bends, prior to being extracted into the low pressure region of the mass spectrometer.[21]

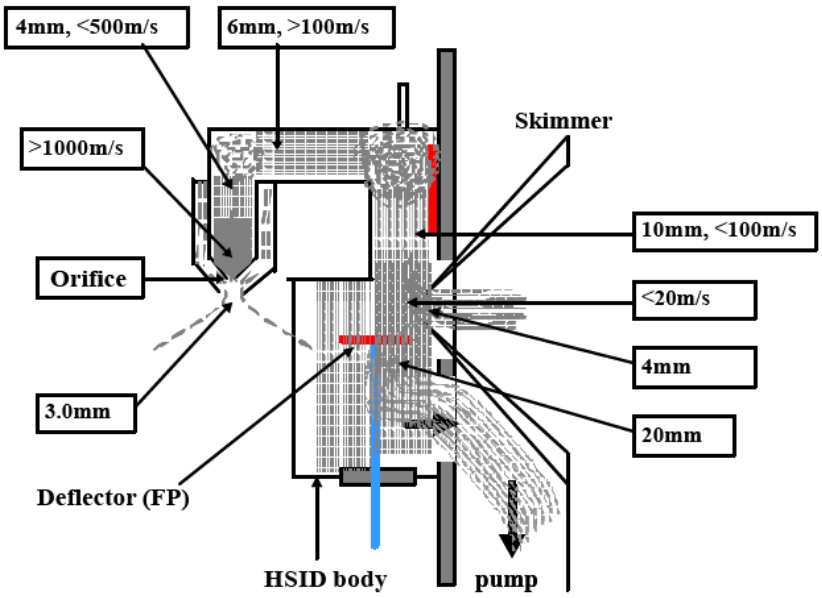


Figure 1-4: Hot Source-Induced Desolvated (HSID) Interface[21]

In clinical and toxicological studies, LC-MS and LC-MS/MS (tandem mass spectrometers) are increasingly used because of their versatility for high-throughput determinations of drug concentrations in biological samples.[3] Operating in mass reaction monitoring (MRM) mode, low levels of compounds in complex biological matrices can be identified and the best selectivity is ensured. Recent studies have proved LC-MS/MS (with APCI) to be useful in the screening of drugs of abuse, including BZP, in urine. It has shown LC-MS/MS to generate reliable results with high sensitivity.[22]

1.2. The Detection and Quantitation of BZP and TFMPP in Blood

1.2.1. Application of Study

The aim of the study was for the Medical Research Institute of New Zealand (MRINZ) to provide information to the Ministry of Health (MoH) on the acute health effects of BZP and TFMPP measuring cardiovascular and psychological functioning, driving performance, and sleep. MoH provided funding for this study. Ethics approval was given by the Central Regional Ethics Committee and by the University of Waikato Ethics Committee.

Volunteers were recruited through MRINZ by way of posters, advertising at shops, university etc. Volunteers were required to be 20 years old or over and to have taken party pills containing BZP and TFMPP on at least three occasions. People excluded were those with a history of diagnosed psychiatric conditions, epilepsy, moderate to severe asthma, hypertension, glaucoma, thyroid disorders, diabetes, urinary difficulties, cardiovascular disease or lactose intolerance. Those using any medication with an effect on serotonin or dopamine were also excluded. This criteria was assessed on an initial visit to the hospital.

Subjects were required to avoid the use of any recreational drugs from one week before the test day until one week after; to avoid the use of alcohol from 8 p.m. on the night before the test day, and to fast for a minimum of six hours before the start of their test day.

The study required 64 volunteers in total. Each subject was required to participate for one full day where they received either (16 subjects each group):

- Two doses of BZP and TFMPP and alcohol
- Two doses of BZP and TFMPP and orange juice
- Two doses of a placebo and alcohol
- Two doses of a placebo and orange juice

Volunteers receiving the first and second combinations (above) consumed one party pill at time zero and a second two hours later. The total dose of BZP and TFMPP received reflected the average recommended consumption. The capsules the subjects received were formulated by the hospital pharmacy. Three different types of capsules bought over-the-counter at a Wellington party pill shop were analysed to quantify levels of BZP and TFMPP. The final formulation was a mixture of two of the bought party pills. Methods and results for this analysis can be found in Appendix A. The capsules contained 75 mg of BZP.2HCl and 18 mg of TFMPP.2HCl. Subjects receiving alcohol were given six units in total, spread out over three hours (30 mL of vodka = one unit).

The volunteers were subjected to numerous tests, including a driving simulator and physical tests, to examine the effects of the party pills alone and in combination with alcohol. Blood samples were taken before administration (baseline) then at 3.5, 6.5 and 10 hrs. These were analysed for alcohol (ethanol), BZP and TFMPP levels.

The primary aim of this thesis was to develop, validate, and utilise an LC-MS/MS method for the detection of BZP and TFMPP in whole blood. A total of 256 blood samples were expected to be analysed (for BZP and TFMPP) as part of this project using the method developed in this study.

1.2.2. Benzylpiperazine and *m*-Trifluoromethylphenylpiperazine Literature Review

BZP and TFMPP are new designer drugs which have caused controversy, especially in New Zealand, due to their legality. According to Encyclopaedia Britannica “Designer drugs usually are synthesised for the first time in an attempt to create a chemical whose molecular structure differs only slightly from that of some well-known controlled substance but whose effects are essentially the same. Because of the difference in molecular structure, the designer drug, unlike the controlled substance, ordinarily will not be

specifically listed as illicit by law-enforcement organisations".[23] A review of the limited literature available on BZP and TFMPP is provided below.

1.2.2.1. Legal Status

In New Zealand, BZP and TFMPP are found in a large and diverse range of party pills ('herbals', 'energy' pills) marketed under 150 (or more) brand names including Charge, Frenzy, Rapture, Jet and Bliss. Quantities of BZP range from approximately 60 mg to 500 mg per tablet and a recommended dosage of about 200-300 mg has been given by some manufacturers. The pills are readily sold at a variety of different retail outlets such as liquor stores, dairies, petrol stations and the internet. It is estimated that 2-3 million servings (serving size is based on package recommendations) were sold in New Zealand in the year ending 2004.[24,25] It was this magnitude of popularity that prompted the endorsement of The Misuse Of Drugs Amendment No.3 Bill in June 2005, creating a new category of controlled, but not illegal substances, known as Schedule D. BZP is on this Schedule – making it illegal to sell to people under 18 years old, and imposing restrictions on advertising.[26]

The government has decided to provide funding for research into BZP and TFMPP in order to make a more considered decision on the legality of the drugs. However there is a lot of controversy from opposing members of parliament and the public, which has resulted in petitions and other movements attempting to make the drugs illegal. The drugs are marketed for use as harm-minimisation treatment for users addicted to methamphetamine ('P') and ecstasy. However, the conflicting dispute, argues that they could provide a gateway to harder/illegal drug use.

In the United Kingdom, piperazines are classed as a prescription only medicine. The status of BZP is yet to be determined, however sales of the drugs have been suspended by the MHRA (Medicines and Health Care Products Regulatory Agency), until further notice. The drugs could be purchased from 'head shops' and the internet, marketed as 'pep' pills. In the

United States, the Drug Enforcement Administration (DEA) made a final decision to place BZP including its salts, isomers and salts of isomers into Schedule 1 (drugs which have no medical use and a high level of abuse potential) of the Controlled Substances Act (CSA).[27] TFMPP was temporarily scheduled, but later removed, as it does not demonstrate the same abuse potential as BZP. After an increased number of encounters of BZP and TFMPP in Japan, it was banned under the Narcotics and Psychotropics Control Law, 2003,[28] and it is regulated (under the Act on the Prohibition of Certain Goods Dangerous to the Health) in Sweden.[29]

On 1st September 2006, Australia added BZP and TFMPP to Schedule 9 (prohibited substances) of the Standard for Uniform Scheduling of Drugs and Poisons (SUSDP) as decided by the National Drugs and Poisons Schedule Committee (NDPSC).[30] Victoria was the last State in Australia to illegalise BZP following this ruling.

1.2.2.2. History

BZP was first synthesised by Wellcome Research Laboratories for use as an anthelminthic (anti-parasitic) mainly used in cattle. Subsequent studies found it reversed the sedative effects of tetrabenazine (a dopamine depleting drug) in rats and mice, leading to the suggestion that BZP use may exhibit antidepressant activity as it affects monoamine accumulation.[31]

Trials (performed by EGIS Pharmaceutical Works, Hungary) were carried out on its effectiveness as an antidepressant. The trial drug was known as Trelibit, its main constituent being EGYT-475 or *N*-benzyl-piperazine-picolinylfumarate. However, studies on its biochemical mode of action found that its metabolite EGYT-2760 or BZP had a high-affinity uptake of noradrenaline, dopamine and serotonin. Hence Trelibet itself was regarded as inactive and BZP was concluded to be more like amphetamine, exhibiting a serotonin uptake inhibition and receptor agonistic effect.[32,33] After studies concluded its effects were in fact very similar to that of dexamphetamine, clinical research on BZP was stopped.[34]

It was not until the 1990s that the use of BZP as a recreational drug became more apparent and with its legal status undetermined, use spread worldwide. In New Zealand, the drug was formally marketed by Matt Bowden with the creation of pharmaceutical company Stargate International (estabilished circa 1999), involved in the manufacture and distribution of BZP and similar products, as harm-minimisation tools. This actuated the establishment of the Social Tonics Association of New Zealand (STANZ), which provided recommendations to the Health Select Committee advising regulations regarding the use of new legal recreational drugs.[35] The BZP party pills industry has grown incredibly from this point forward and it is this escalation in prevalence which has led to the need for intensive research to be carried out on the pharmacological effects of these drugs.

1.2.2.3. Neurological and Health Effects

BZP is a sympathomimetic drug,[25] meaning, it produces physiological effects which resemble those caused by the stimulation of the sympathetic nervous system (SNS). The SNS is responsible for secreting adrenaline and norepinephrine (or noradrenaline) in a sympathico-adrenal response (fight or flight response).

Research has shown that BZP has three main mechanisms of action. It affects the reuptake of noradrenaline (NA), the reuptake inhibition and stimulation-independent release of dopamine (DA), and exhibits an uptake-inhibition effect and a receptor agonistic effect on serotonin (5-hydroxytryptamine, 5-HT)[33]. NA, DA and 5-HT are all monoamine neurotransmitters, which are responsible for nerve responses in the body and brain. The effect on DA appears to be the main action of BZP, with 5-HT and NA playing a lesser role, with the latter mainly being responsible for the peripheral effects being the main mediator of the SNS.[36]

Tests on EGYT-475 and its metabolite EGYT-2760 using rat stomach fundus for the study of serotoninergic mechanisms, indicated BZP had 5-HT

agonistic and antagonistic actions. BZP was found to cause a dose-dependent inhibition of the 5-HT induced contractions and at higher concentrations displayed agonistic activity on the rat fundus preparation.[37] BZP also demonstrated a strong inhibitory action on the high-affinity uptake of 5-HT in rat hippocampus,[33] and increased both the resting and the nerve-evoked release of NA in peripheral sympathetic nerve fibres (of rabbits).[38]

Tests comparing MDMA and piperazines BZP and TFMPP in rats showed that *in vivo* BZP released [^{3}H]MPP$^+$ (DA transporter (DAT) mediated release assay) and also caused elevations in 5-HT (however to a much lesser extent). TFMPP was the opposite and released [^{3}H]5-HT (serotonin transporter (SERT) mediated release assay) with no effect on DA. When administered together they mimicked the monoamine release of MDMA, however to a much lesser extent (3-fold less potent).[39,40] It has been noted that manufacturers of the drugs combine the two stimulants to recreate the effects of ecstasy. Tablets are usually found to contain varying ratios of BZP and TFMPP with a 3:1 ratio usually found in the U.S.A.[41]

TFMPP was found to display full agonist activity at multiple 5-HT receptors, partial agonist or antagonist activity at 5-HT2A receptors, and SERT mediated 5-HT releasing activity.[40] There have been a few studies on the serotoninergic properties of TFMPP, for example its ability to moderately reduce the frequency of spontaneous motor seizures in rats with pilocarpine-induced epilepsy.[42] In rats, TFMPP increased concentrations of the hormones corticosterone (in plasma), adrenocorticotropin (ACTH) and prolactin and decreased CRH (corticotrophin releasing hormone) in the hypothalamus (in rats) by way of 5-HT antagonist-susceptible mechanism.[43] These hormones are normally released in response to physical, emotional or chemical stress. Also, in rats, TFMPP has been found to produce dose-dependent decreases in food intake, body temperature, and spontaneous ambulatory motor activity. Detailed information on the neurochemical, physiological, cardiovascular and locomotor effects of TFMPP can be found in the review by Murphy *et al*.[43]

Some tests on behaviour (in humans, rats and primates) have been conducted, which showed BZP produces considerable increases in heart rate, systolic blood pressure, diastolic blood pressure (to a lesser extent) and pupil size. Other side effects (fewer cases) were flushing and sweating. In a double-blind placebo controlled study, former amphetamine addicts found BZP and dexamphetamine indistinguishable, administered in a 10:1 ratio.[44,34] At a high intake level, monkeys experienced involuntary head movements, jaw chattering, bizarre body postures and hyperactivity. The behavioural effects were suggested to be long-lasting in the monkeys.[31] Supporting this, BZP was also found to have significant effect on ambulation (continuous circling of the cage, and sniffing) in rats.[40] In tests where TFMPP and BZP were administered together at high dosages (10mg/kg in rats) several rats were found to develop short-lived seizures. This was not observed after administration of the individual drugs, suggesting that BZP and TFMPP may have a synergistic effect when administered together *in vivo*, possibly due to pharmacokinetic factors.[39]

BZP was self-administered by primates, although when administered with TFMPP, self-administration was significantly decreased. When TFMPP was administered twenty minutes prior to cocaine self administration, the pre-treatment suppressed the cocaine-maintained response.[31] In studies using rats, BZP induced place preference, which leads to the conclusion that the drug possesses rewarding properties.[45] This suggests BZP may exhibit abuse liability of the amphetamine type – although this is not the case for TFMPP.

A study carried out in New Zealand suggested that early BZP use caused heightened anxiety later on in rats.[46] BZP was administered to rats at the periadolescent stage (equivalent to adolescence in humans) at a dose of 10 mg/kg for ten days. Seventeen days after the last dose of BZP, the rats were subjected to behavioural tests. The preliminary results indicated that the BZP administered during adolescence caused higher anxiety levels in the rats during adulthood.

In a recent study (2005) carried out in Christchurch, it was revealed that during a five month period, 61 patients on 80 occasions were admitted to the emergency ward (Christchurch Hospital) after ingestion of party pills, containing various amounts of BZP. None of these cases proved fatal but a few resulted in the patients being admitted to an intensive care unit. The study described the toxic effects as palpitations, agitation, nausea, vomiting, confusion and collapse, with a few severe cases even resulting in seizures.[25] However the concentrations of BZP and/or TFMPP in patients are not known. It is also worth noting that during this period retailers were supplying party pills containing up to 1000 mg of BZP in Christchurch, possibly contributing to the severity of the adverse reactions in these cases.

This study and a survey carried out in the emergency department at Waikato Hospital[47] suggest that the main age range for consumption of these drugs is from about fifteen years old through to early twenties. The use of alcohol was also a common factor, with the reports showing that 49% and 66.4% respectively of users had mixed the two substances. The effect of the consumption of alcohol (which is not recommended, according to package/manufacturer specifications) with the party pills is currently unknown. A national (New Zealand) survey carried out by the Centre for Social and Health Outcomes Research and Evaluation (SHORE)[48] at Massey University supports these findings. The level of party pill use was the highest for people aged 18-24 years old, with 33.9% of them having used party pills in the preceding year. Nearly nine out of ten users self-reported that they used other substances with the party pills, with 91% reporting having used alcohol.

Two severe adverse effects have been reported in one-off cases. One user (17 years old) of the party pills (who also consumed a small amount of alcohol) was admitted to the emergency ward at Waikato hospital presenting with symptoms of acute renal failure. It was postulated that the kidney failure was a direct result of the toxicity of the party pills, however use of party pills was not confirmed by analysis.[49] A 20 year old male developed an acute psychotic episode (including delusional beliefs and auditory and visual hallucinations) that led to him setting fire to his room. The man was admitted to an acute

inpatient psychiatric unit, and symptoms had completely subsided within 48 hours. It is thought that the ingestion of four tablets of rapture (self-reported) was the main cause of this episode, however, the man had also reported using nitrous oxide and a small quantity of cannabis.[50]

There have been two reported deaths where the use of BZP has been noted. A 23 year old woman (in Switzerland) was hospitalised 11 hours after ingestion of BZP and 7 hours after ingestion of MDMA, plus large quantities of water. She died shortly after hospitalisation due to swelling of the brain caused by hyponatriemia, (a drop in sodium levels, in plasma) due to water intoxication.[51] However, the contribution that BZP made to the death cannot be confirmed, as these are common adverse effects relating to the intake of MDMA. The second case was in Sweden, where a 22 year old male died. GC-MS analysis showed a level of 1.7 μg/g blood of BZP; ecstasy, cannabis and 3,4-methylenedioxyamphetamine (MDA, a psychotropic drug said to be similar to ecstasy) were also detected. MDA is also a metabolite of ecstasy.[29] BZP was said to have contributed to the case but the exact role was not clear.

1.2.2.4. Structure, Detection and Metabolism

BZP is an entirely synthetic, N-monosubstituted piperazine with a structure similar to that of amphetamine as shown in Figure 1-5. The piperazine (1,4-diazacyclohexane) functional group is a six-membered heterocycle that contains two secondary amine groups. Piperazines have many medicinal uses such as anti-allergy, antibiotics, analgesics and cancer treatments, for example imatinib mesylate (Figure 1-5) is used for the treatment of leukaemia. However more recently, piperazines have been found in the form of recreational drugs, such as *m*-chlorophenylpiperazine (mCPP), 1-(3,4-methylenedioxybenzyl)piperazine (MDBP), 1-(4-methoxyphenyl)piperazine (MeOPP) and the more common BZP and TFMPP. BZP is normally found as a base (yellow-green liquid) or a hydrochloride salt (white solid). BZP in the free base form is corrosive and causes burns; it is also an irritant to the eyes and respiratory system.[52] TFMPP is found as a hydrochloride salt (yellowish solid) or a colourless liquid. Both substances are primarily used as chemical

intermediates in the production of certain detergents and pharmaceuticals,[28] but have no known human medicinal uses.

In the early nineties, due to its stimulating properties, BZP became increasingly widespread as a 'rave' drug. With increasing popularity, came escalating concern regarding the safety of these drugs. The danger with new recreational drugs is that they have not been subjected to clinical tests and therefore the safety level of the drugs cannot be certain. Research has been documented over the last 10-15 years on different aspects of these new drugs, however this is insufficient to draw conclusions on their appropriate legal status.

Various analytical procedures for the detection of recreationally used piperazines were explored in 2001. This included immunoassays, HPLC-UV, GC-NPD and GC-MS, in which the latter two methods proved useful for determining the contents of a BZP capsule.[53] More recent methods for the detection of BZP and TFMPP in biological samples have been documented. These include GC-MS for the detection and quantitation of the drugs in plasma,[54] GC-MS,[55] LC-ESI-MS[55] and LC-MS-MS[22,56] for the detection of BZP and other abuse-liable drugs in urine and chiral capillary electrophoresis for the separation of amphetamines and piperazines in spiked urine and synthetic samples.[57]

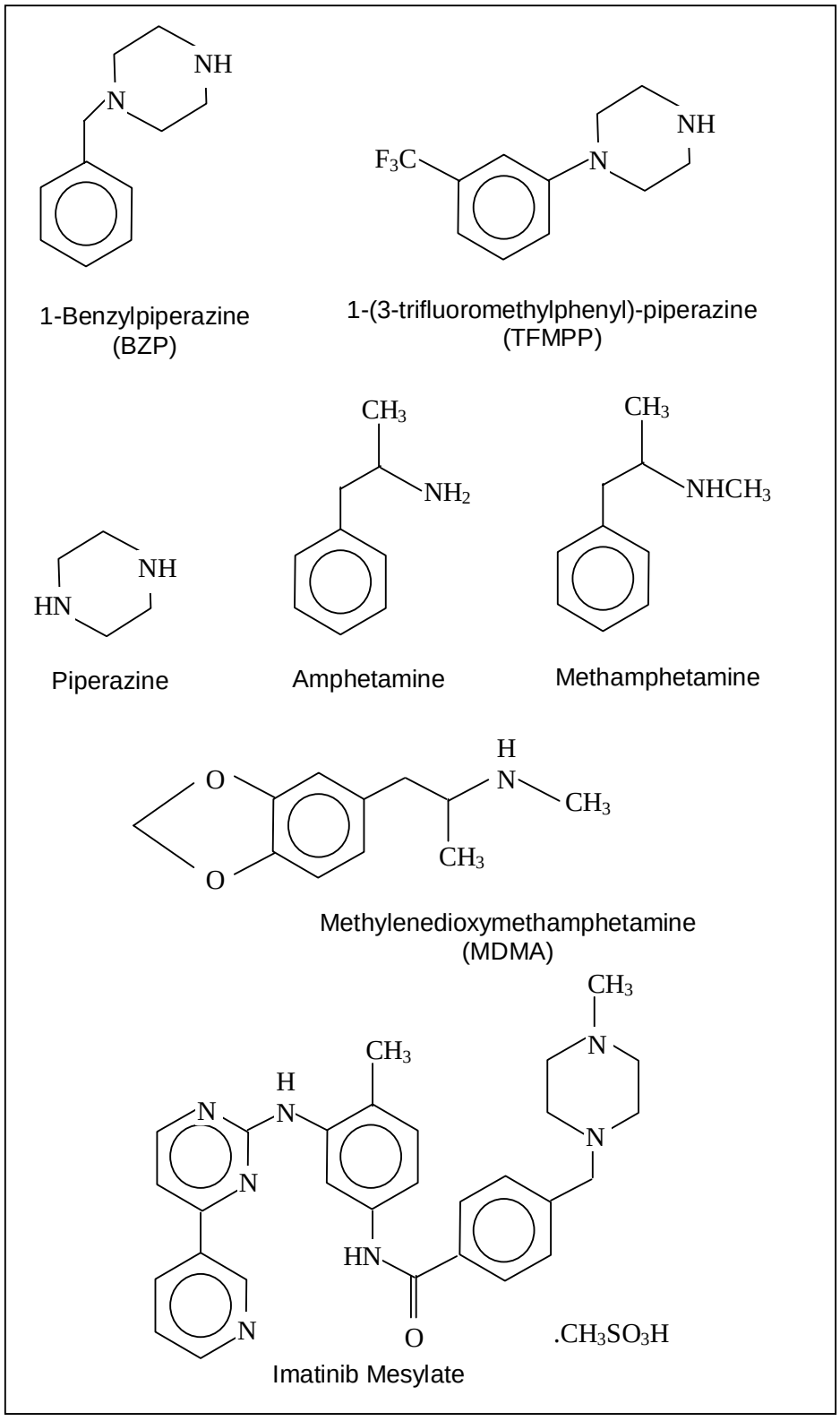


Figure 1-5: Structures of BZP, TFMPP, Piperazine, Amphetamine, Methamphetamine, MDMA, and Imatinib Mesylate

Studies on the metabolism (in rats) of BZP were carried out by Staack *et al.* in about 2002,[58] and further studies using GC-MS and LC-MS have confirmed the major metabolites.[55] The identified metabolites indicated that BZP was hydroxylated in the aromatic ring and that the piperazine moiety is metabolically degraded. Apart from the principal metabolite 4-hydroxy-BZP (**2**), 3-hydroxy-BZP (**3**), 4-hydroxy-3-methoxy-BZP (**4**), piperazine (**5**), benzylamine (**6**), and N-benzylethylene diamine (**7**)[58] were also identified (Figure 1-6). The primary metabolite 4-hydroxy-BZP is conjugated into the glucuronide and a smaller portion is metabolised into the sulphate.[59]

By comparison with BZP, the metabolism of TFMPP has been more extensively researched. The metabolic pathway of TFMPP (in rats) is as shown in Figure 1-7. TFMPP is extensively metabolised, and almost exclusively excreted as metabolites. The main metabolite is hydroxy-TFMPP (**2**) formed by aromatic hydroxylation, catalysed by cytochrome P-450 enzymes (CYP, or more specifically CYP2D6)[60] followed by glucuronidation (about 70%) or sulphation.[28,61] However in humans the conjugation of phenol into the sulphate is generally more extensive, so it is possible that this may be the phase II metabolic pathway for TFMPP and also BZP.[28] For reference, Phase I of drug metabolism involves the oxidation, reduction or hydrolysis of a molecule to form a polar functional group. Phase II reactions are known as conjugation reactions and usually involve the polar functional groups formed at Phase I.

Other minor metabolic pathways of TFMPP are the degradation of the piperazine heterocycle by double *N*-dealkylation of TFMPP to *N*-(3-trifluoromethylphenyl)-ethylenediamine (**3**) or to 3-trifluoromethylaniline (**6**); degradation of hydroxy-TFMPP to *N*-(hydroxy-3-trifluoromethylphenyl)-ethylenediamine (**4**) or to hydroxy-3-trifluoromethylaniline (5) followed by partial *N*-acetylation of the anilines (**7 - 8**).[62]

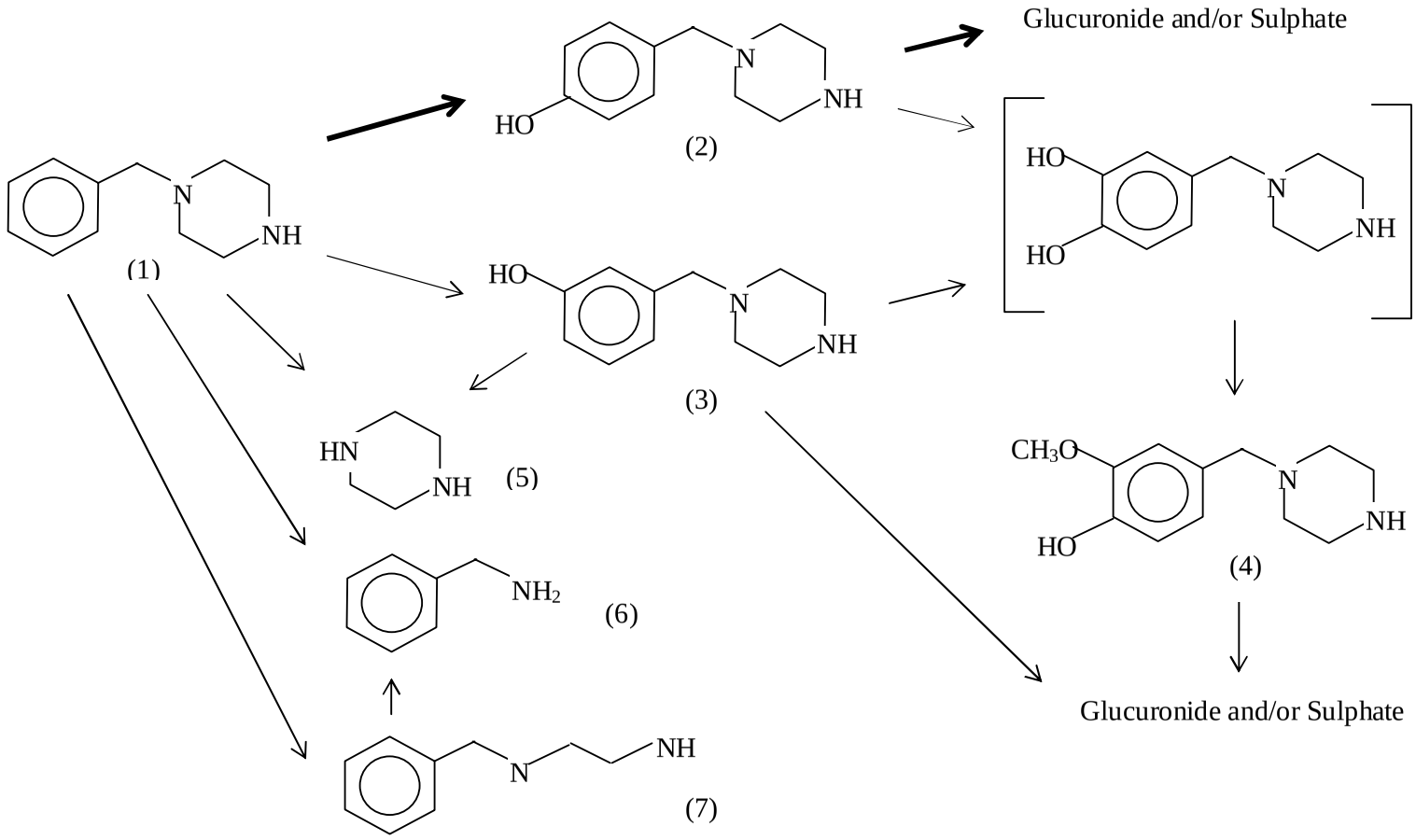


Figure 1-6: Proposed Metabolic Pathway of BZP (adapted from references 28 & 61)

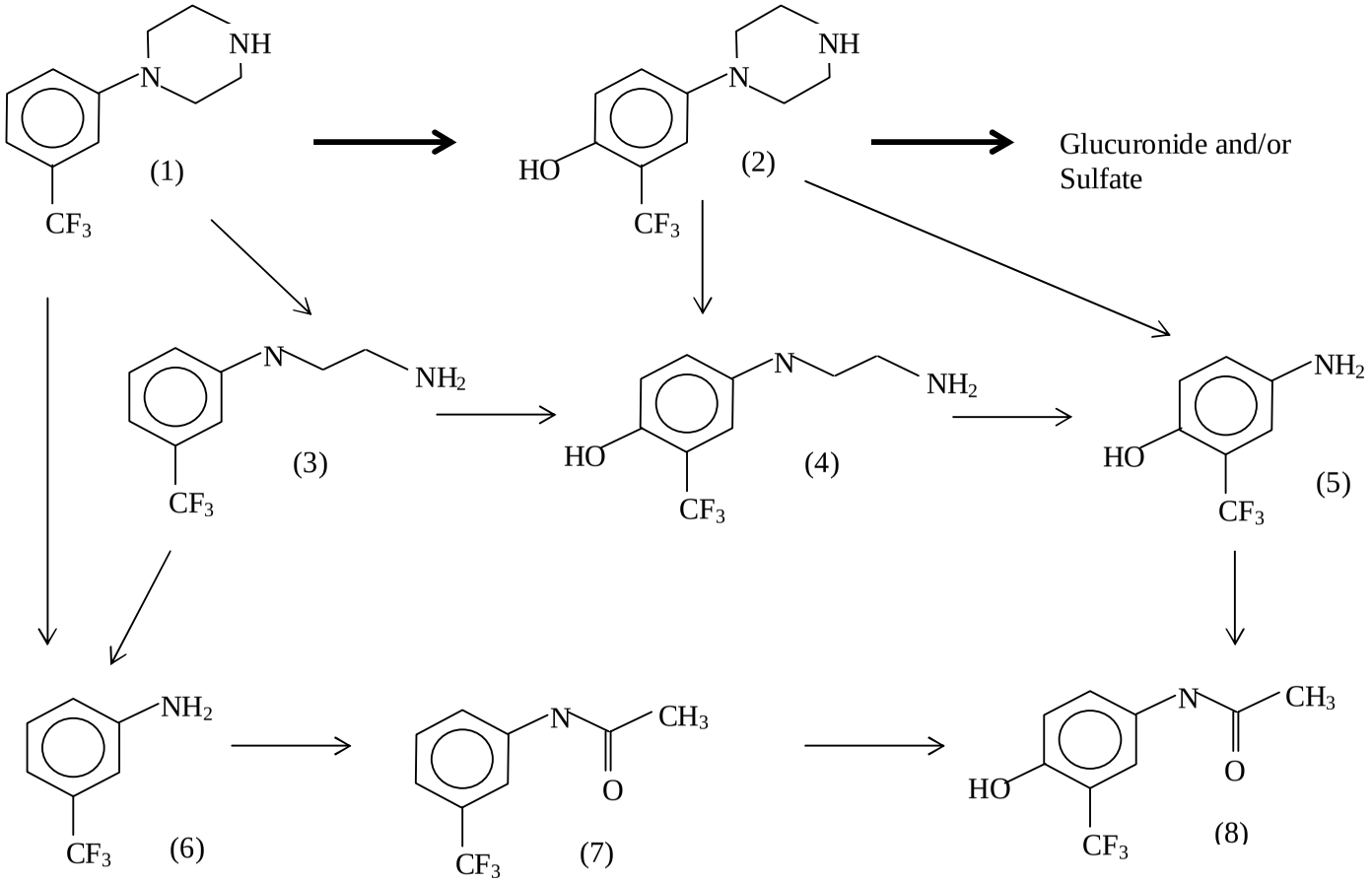


Figure 1-7: Proposed Metabolic Pathway of TFMPP (adapted from references 28 & 61)

1.3. Detection of Multiple Recreational Drugs in Hair

1.3.1. Application of Study

Detection of drugs in hair is proving very valuable as it is non-invasive and can provide a history of drug use. Determining a person's drug history is important in drug-related deaths, as it can help establish whether or not they were a chronic or naive user, which can often shed some light as to why they might have died. Also, detection in hair can help determine whether someone may have been using particular drugs at the time of an earlier (criminal) incident. This can help support or refute other information. Other applications of hair analysis involve child custody disputes and drink spiking incidents.[6]

GC-MS is a popular choice for multiple drug analysis, as well as other forensic applications. Many successful GC-MS methods for the detection of multiple drugs in biological specimens have been documented,[63,64,65,66] due to its sensitivity and also due to the availability of large libraries of standardised mass spectra. LC-MS is becoming more popular due to its ability to analyse a wide range of compounds with great sensitivity and a variety of literature (including a detailed review by Maurer[3]) exists showing its versatility to detect multiple classes of drugs in plasma and oral fluid[3,15,67,68,69] as well as in hair.[6,70] It appears that GC-MS is the most common type of analysis for the detection of drugs in hair, with very few methods published using LC-MS.

The secondary aim of this thesis is to develop a method ideally to simultaneously detect and quantitate multiple recreational drugs in hair using LC-MS/MS. Drugs to be analysed are methamphetamine, amphetamine, MDMA, MDA, BZP, morphine, codeine and 6-MAM.

1.3.2. Illegal Recreational Drugs

There are many types of illegal drugs used/abused for recreational purposes, however the most common are the amphetamine type compounds (methamphetamine, MDMA) and the opiates. The United Nations Office on

Drugs and Crime, World Drugs Report 2006,[71] details the worldwide use of these illegal drugs and is summarised in Figure 1-8.

Although New Zealand's main illegal drug use is associated with cannabis (marijuana), the use of stimulants such as ecstasy and amphetamines and the use of opiates, is growing. Figure 1-9 shows different drug types and the proportion of New Zealanders that have ever tried or used them in the last 12 months,[72] including the legal drugs alcohol and tobacco. The need for reliable detection of these illicit drugs is important, and is often used in court for charges relating to possession and manufacture, workplace drug testing and toxicology.

Vast amounts of research have been carried out on the above-mentioned drugs (especially in comparison to BZP and TFMPP, which are relatively new to the drug scene). A brief introduction to the drugs that can be detected in hair by LC-MS/MS is included below along with their structures and mechanisms of action.

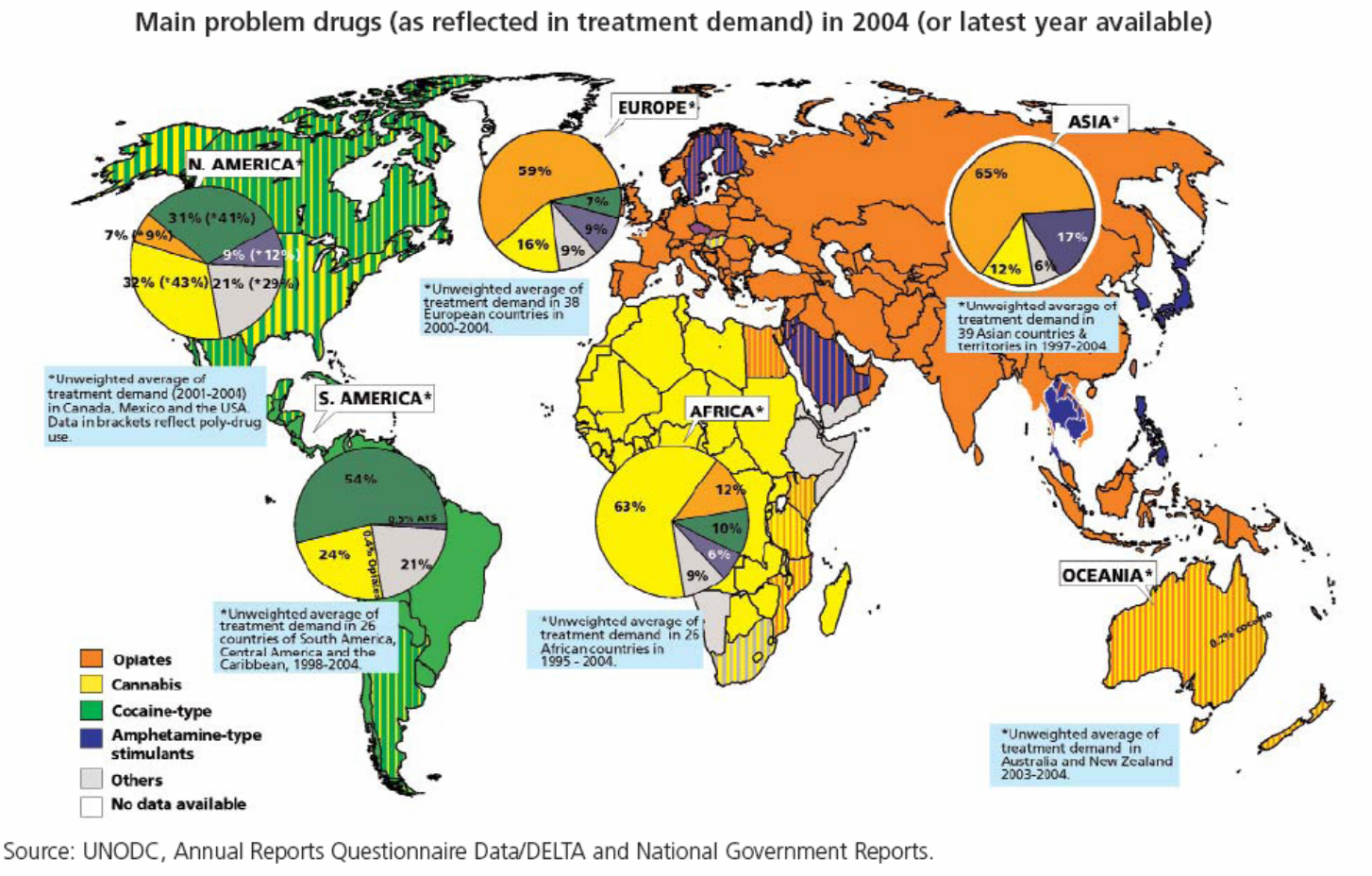


Figure 1-8: Global Drug Use: As shown in the United Nations Office on Drugs and Crime World Drugs Report 2006.[71]

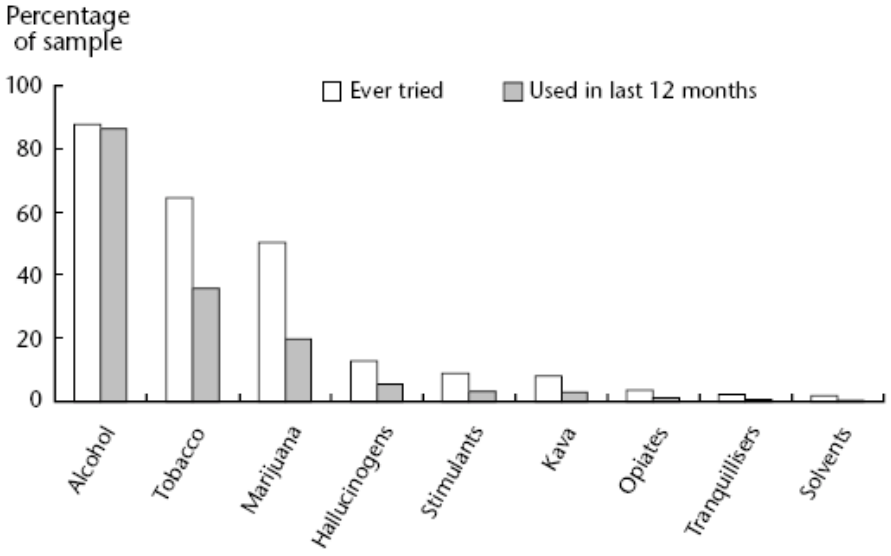


Figure 1-9: New Zealand Drug Statistics 1998.[72] Proportion of New Zealanders who have ever tried or recreationally used different types of drugs, in the last 12 months.

1.3.2.1. Amphetamine Derived Compounds

Methamphetamine (MA) (the structures of amphetamine, methamphetamine and MDMA are shown in Figure 1-5, page 23) is a synthetic, white, bitter-tasting powder or crystalline salt. L-methamphetamine (levo-methamphetamine) is widely used in nasal decongestants, such as the Vicks Inhaler, however it has no stimulant or addictive properties. The D- (dextro-methamphetamine) isomer is known to be a very addictive stimulant drug and use appears to be widespread. Short-term effects include increased heart rate and blood pressure, increased activity, a euphoric or 'rush' feeling and a loss of appetite.[73]

Methamphetamine has been called a problem drug in some countries, due to its highly addictive properties and its ability to cause aggressive tendencies or psychosis in humans.[74] In 2002, New Zealand's MA use dramatically increased (according to household drug surveys), and in 2003 MA was reclassified (from Class B) to a Class A drug in New Zealand (under the Misuse of Drugs Act 1975).[75] In the United States however, it is a Schedule II drug on the CSA, as it possesses some medicinal properties. MA is prescribed

in the United States for the treatment of narcolepsy, attention deficit disorder and for short-term treatment of obesity.[73]

Methylenedioxymethamphetamine (MDMA, ecstasy) is a synthetic, psychoactive drug with hallucinogenic properties. It is normally formulated into tablets which often contain harmful adulterants.[76] The physical effects of MDMA are similar to those of MA, although users also feel an increased level of sociability, sense of wellness and insightfulness. MDMA was legal up until 1985 and was commonly found in bars and gay clubs and increasingly found at 'raves'.[77] It is currently illegal in most countries; it is a Class A drug in the United Kingdom and New Zealand, and a Schedule I drug in the United States.

3,4-Methylenedioxyamphetamine (MDA, 'love drug', Figure 1-10) is also a stimulant which is commonly abused. It is a metabolite of MDMA, and hence has the same legal status as MDMA. Initially it was used as a cough suppressant, ataractic (sedative) and anorexigenic (appetite suppressant) drug. In the 1970s, abuse became widespread with reports of deaths in California and Canada.[78] It is commonly found in combination with MDMA. Often so-called "ecstasy" tablets contain a mixture of MDMA, MDA, methamphetamine and other adulterants. This cocktail can be lethal and often users of these drugs are unaware of what they are taking.

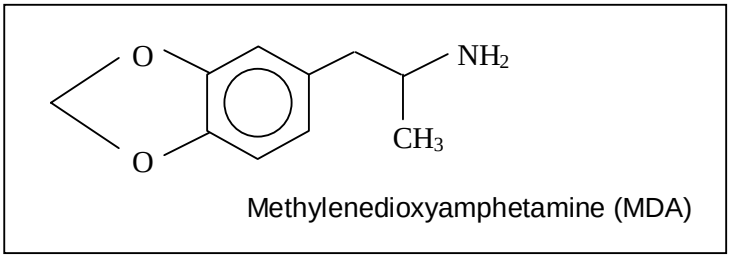


Figure 1-10: Structure of Methylenedioxyamphetamine (MDA)

In the 1960s, amphetamine abuse was associated with psychosis and central nervous system disorders. Amphetamine is known to induce monoamine release by disruption of vesicular storage and transporter reversal. MA causes

dose-dependent releases of DA, NA and 5-HT, with it having a less potent effect on the latter.[79] The release of DA by DAT and also a considerable release of 5-HT via SERT are responsible for the behaviour and locomotor effects of MA.[80] Research in rats, primates and humans has shown that multiple doses of MA cause long-lasting depletions of DA and 5-HT, marked decreases in the activity of tyrosine hydroxylase and tryptophan hydroxylase (enzymes responsible for the biosynthesis of DA and NA and the regulation of 5-HT respectively), the concentration of DA and 5-HT metabolites, and the number of DA and 5-HT transporters.[81,82] In rats, multiple administration of MA provoked impaired memory function.[81]

Similarly MDMA induces both dopamine and serotonin release but with reversed priority, with the role of DAT in the behaviour of MDMA being questionable.[80] MA causes neurotoxicity within the terminal fields of both dopaminergic and serotonergic neurons. MDMA induces a similar neurotoxic effect but exclusively in serotonergic neurons.[80]

A survey carried out in New Zealand by SHORE[83] showed that one in ten New Zealanders aged 18-29 had used an amphetamine type stimulant drug in the last year. Amphetamines were the most commonly tried drug (10%) while 5% had tried ecstasy and 1% had tried crystal MA. The average price of ecstasy in New Zealand is $60- $80 per pill, and amphetamine $100-$150 per gram. Findings from another study carried out by SHORE[75] comparing household surveys from various years showed that in 1998, 2.9% of people reported that they had used amphetamine in the last year compared with 5% in 2001 and 4% in 2003. This was said to indicate a levelling off of amphetamine use in New Zealand, although this may not be the view of the police and other communities. However other countries have reported waves of amphetamine epidemics.

1.3.2.2. Opiates

Opiates are alkaloids derived from the seed pods of the opium poppy. The main opiates include heroin, morphine, codeine and thebaine. In New Zealand approximately 4% of the population admitted to having tried opiates (1998 National Drug Survey).[72] Opiates, costing around NZ$1000 per gram,[84] are relatively expensive compared with the amphetamines.

Morphine is a narcotic analgesic used to treat moderate to severe pain especially in cancer patients. It effectively numbs pain and hence is widely used as a prescription medicine especially in hospitals. It also alters mood and can induce sleep. Side effects include nausea, vomiting, constipation and at high doses it can also cause respiratory failure. Morphine is a Class B drug in the United Kingdom and New Zealand (Misuse of Drugs Act) and a Schedule II drug on the CSA in the United States, as it has medicinal uses.

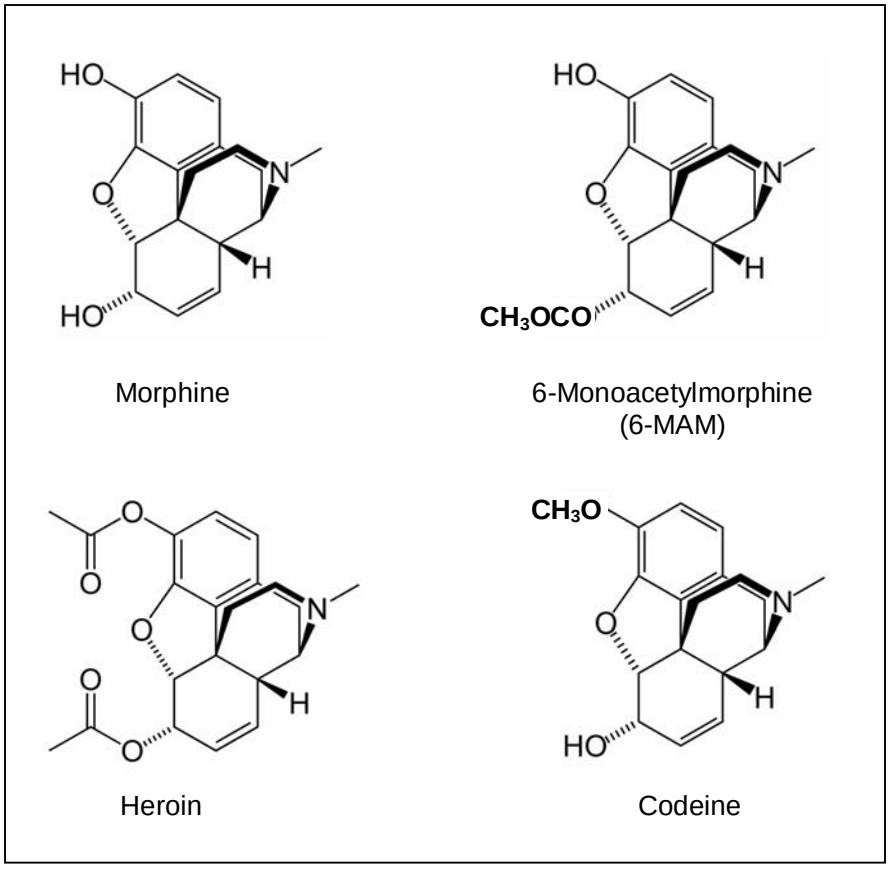


Figure 1-11: Structures of Morphine, 6-MAM, Heroin and Codeine

Codeine (methyl morphine) can be extracted directly from the opium poppy but is most commonly synthesised from morphine by *O*-methylation. It is used in analgesic, cough suppressant and antidiarrheal medications. In the liver it is converted to morphine by the CYP-450 enzyme CYP2D6.[85] Therefore it has a similar mechanism to morphine and has a weak affinity for the μ-opioid receptor (see below) however is much less potent as only approximately 10% is converted.[86] In the United States, codeine is a Schedule 2 drug on the CSA when sold in formulations by itself, however when sold in preparations with paracetamol and aspirin (for pain-relief) it is a Schedule 3 drug. In New Zealand it is classified as Class C (pharmacy only medicine) and is available without a prescription in combination analgesics containing up to 15 mg/tablet usually of a codeine salt such as codeine hydrochloride or codeine phosphate. It is commonly used as a recreational drug, most likely due to its ease of availability.

Heroin is a semi-synthetic lipophilic morphine derivative formed by the acetylation of morphine. The structures of heroin, morphine, codeine and 6-monoacetylmorphine (6-MAM) are shown in Figure 1-11. In plasma, heroin itself is unstable, and it rapidly hydrolyses to 6-MAM and morphine.[87] Hence the detection of both of these molecules can be used to indicate the presence of heroin. Heroin is a Schedule I drug (CSA) in the United States and a Class A drug in the United Kingdom, and New Zealand (Misuse of Drugs Act).

Morphine, codeine and heroin work by binding to specific opioid receptors in the central nervous system. When activated, the opioid receptors regulate responses to pain, stress and emotions. The three main opioid receptors are μ-, δ- and κ-, which are activated by endogenous peptides. Each of the opioid receptors exist in different parts of the body and are responsible for different functions. Research has shown that the μ-receptor agonists display the best analgesic activity but also the highest abuse liability, the δ-receptor agonists possibly exhibit less addictive potential, while being poor pain-relievers and the use of κ-receptor agonists for pain treatment should be restricted to the periphery because of the strong distressing properties of these compounds.[88] Many studies have shown that morphine binds to all three opioid receptors,

but has a particularly high affinity for the μ-receptor. It appears that the biological actions (analgesic properties and side-effects as well as addiction potential) of morphine are activated by the interaction of the molecule with the μ-receptor.[88]

Chapter 2: Experimental Procedures

2.1. Methods for Detection and Quantitation of BZP and TFMPP in Blood

The LC-MS method was based on a method developed by Tsutsumi *et al.* for the testing of BZP and TFMPP,[55] and originally used for the detection of amphetamines in urine.[89] It was modified to ensure better sensitivity and shorter run times for the particular equipment used in this study.

2.1.1. Materials And General Procedures

2.1.1.1.Drug Supplies and Reagents

BZP and TFMPP drug standards were obtained from ESR drug stores, and made up to concentrated stock standards (see 2.1.1.3 below). All reagents used were of analytical grade, except those used for the LC which were of HPLC-grade.

2.1.1.2. 0.1M Phosphate Buffer

0.1 M Phosphate buffer is used in the solid phase extraction, described below. The buffer was made by ESR technicians on a regular basis and was always less than 1 month old). 13.61 g of KH_2PO_4 (potassium dihydrogenphosphate) in 900 mL of distilled water, is adjusted to pH 6 with 1 M KOH (potassium hydroxide). Distilled water is added to make a final volume of 1000 mL.

2.1.1.3.Stock Standards

For initial validation, a stock standard containing a desired concentration of 1 mg/mL of BZP and TFMPP in ethanol was prepared. Stock standards for BZP and TFMPP were made up to desired concentrations 1 mg/mL and 0.5 mg/mL respectively, for standards used in quantitation of subjects.

2.1.1.4.Internal Standards

Biological matrices from different sources/individuals may have varying effects on the drug specimen leading to inconsistent quantitative results. This can be accounted for by using deuterated analogues of the drugs, as they have almost identical physico-chemical properties to the drugs being identified. Therefore, by adding the internal standard to the sample before analysis, it will be present throughout the entire analytical process, compensating for any loss of the drugs during the process.[90] It was desired that both deuterated analogues of BZP and TFMPP be used. The deuterated compounds obtained were BZP-*d7* and TFMPP-*d4*. Stock standard solutions of 1 mg/mL were made up and then diluted to reach desired internal standard concentrations of 25 ng/mL in 0.2 mL of blood for TFMPP and 200 ng/mL in 0.2 mL of blood for BZP.

2.1.2. Sample preparation - Solid Phase Extraction

50 µL of internal standard was added to 200 µL of sample, the samples were briefly vortexed and equilibrated for 10 minutes. After addition of 1.5 mL of 0.1 M phosphate buffer (pH 6) the samples were sonicated for 15 minutes and centrifuged at 2700 rpm for 10 minutes. Solid Phase Extraction was carried out on 60 mg/3 mL Phenomenex Strata-X 33 µm polymeric sorbent columns, on an automated Gilson ASPEC XL4 Robot system. The cartridges were pre-conditioned with 2 mL of methanol and 2 mL of 0.1 M phosphate buffer (pH 6). 1.5 mL of sample was loaded on to the column which was then washed successively with water (2 mL), 0.01 M acetic acid (2 mL), and 5% aqueous acetonitrile (2.1 mL). The column was dried for 3 minutes and the analytes eluted with 0.7 mL acetonitrile. 200 µL of 100 mM ammonium formate with 0.03% formic acid (pH 3.5) was added to the collected fraction and 10 µL was injected into the LC-MS/MS system.

2.1.3. Liquid Chromatography-Mass Spectrometry

The LC system consisted of a Shimadzu autosampler, pump, and triple quadrupole LC-MS/MS mass spectrometer (API 300, Perkin-Elmer Sciex

Instruments) with a TurboIonspray interface. An Apple Macintosh System 8.1 with Sample control 1.4 software controlled the system. The chromatography was carried out on a SCX (strong cation exchange) column (2.0 mm i.d. x 150 mm, Luna 5µ SCX 100Å, Phenomenex) with column temperature set at 35 ºC. The mobile phase was 100 mM ammonium formate with 0.03% formic acid (pH 3.5) – acetonitrile (25:75), with a flow rate of 200 µL/min, and a run time of 11 minutes per sample. AutoTune software was used to optimise state file MS parameters for target compounds. However it does not affect the ion spray voltage, the values for the gases or temperature - these have to be adjusted manually. The parameters for the MS were as displayed in Table 2-1.

Table 2-1: State File Parameters for API 300 LC-MS/MS

Gases	
Nebuliser Gas (NEB)	12
Curtain Gas (CUR)	11
CAD Gas (CAD)	3
Controls	
Ion Spray Voltage (IS)	5500
TEM	450
Orifice Voltage (OR)	36
Focusing Ring Voltage (RNG)	160
High Pressure Entrance Quadrupole (Q0)	-6
Inter-Quadrupole Lens 1 (IQ1)	-7
Prefilters (ST)	-11
First Resolving Quadrupole (RO1)	-7
Inter-Quadrupole Lens 2 (IQ2)	-32
Collision Cell Quadrupole (RO2)	-37
Inter-Quadrupole Lens 3 (IQ3)	-63
Last Resolving Quadrupole (RO3)	-42
Deflector (DF)	-150
Channel Electron Multiplier (CEM)	2400

2.1.3.1.Mass Reaction Monitoring

The MS was carried out in MRM (mass reaction monitoring) mode. This is a common scan type in MS/MS, which looks at molecular ion and product ion fragments (fragmentation patterns can be found in Appendix B). The following transitions were monitored (Table 2-2).

Table 2-2: LC Retention Times and MRM Transitions Monitored for Quantitation

Compound	Retention time (min)	Transition Monitored (*m/z*)
BZP	9.33	177.4 → 91.0
BZP-*d7*	9.80	184.4 → 98.0
TFMPP	5.39	231.4 → 188.0
TFMPP-*d4*	5.37	235.0 → 190.0

2.1.4. Validation

It is essential to ensure that a method works as intended, precisely and accurately. This is achieved by measuring a variety of parameters: selectivity (specificity), precision (repeatability and reproducibility), accuracy, linearity, range, limit of detection, limit of quantification, and stability.[91]

A calibration curve was constructed using a series of points and plotting concentration versus area ratio of drug to internal standard. Good linearity is an indication of accuracy and demonstrates that the results are directly proportional to the concentrations.[91] Also, an intercept not significantly different from zero is important to ensure that there is no significant interference from co-extractants. All calibration curves were forced through zero, as a blank (zero) standard was used for the analysis.

The reproducibility was demonstrated by running the experiment on three different days. The repeatability was determined by carrying out a series of five replicate standards each day at a low concentration of BZP and TFMPP (52.3 ng/mL and 7.2 ng/mL respectively). Comparing calculated and known concentration values and showing good agreement between them, assessed accuracy. Accuracy was calculated by the following equation, expressed as a percentage where 100% represents excellent accuracy:

$$\text{Accuracy (\%)} = \frac{\bar{x}Di}{S} \times 100$$

Where: $\bar{x}Di$ = mean of the determined concentration

S = nominal concentration

The recovery of the analytes (extraction efficiency) was measured by comparing drug concentrations in samples spiked before extraction compared with the concentrations of drugs spiked into the reconstituted extract. A series of five extraction samples was prepared by adding 35 μL of BZP and TFMPP standard ([BZP] = 52.3 ng/mL and [TFMPP] = 7.2 ng/mL) and 50 μL of internal standard to 0.2 mL of blank blood then extracting. A series of five control samples was prepared by extracting 0.2 mL blank blood and adding the same quantity as for the extraction samples, after extraction. The extraction efficiency was calculated by the following equation:

$$\text{Extraction Efficiency (\%)} = \frac{\text{Area of extracted drug}}{\text{Area of unextracted control samples}} \times 100$$

Stability is important as the drugs can decompose during sample preparation, extraction, quantitation and storage. Stability was determined by LC-MS/MS analysis of a prepared standard sample (extracted and left at room temperature in LC-MS solvent) over a period of one month.

Selectivity is extremely important in forensic applications, as the identity of the drug must be ensured. LC-MS/MS not only gives a valid retention time but also a mass spectral "fingerprint", and hence is highly specific.

The coefficient of variation (CV) indicates the variability around the mean in relation to the size of the mean, and is defined as:

$$\text{CV (\%)} = \frac{100\% \times \text{Standard deviation}}{\text{Mean}}$$

By determining the within (intra-) and between (inter-) day CVs, which should not exceed 20%, precision, accuracy, and sensitivity is demonstrated and the analytical method can be deemed acceptable.[91]

2.1.5. Samples

A trial was carried out by MRINZ on the effects of BZP and TFMPP in combination with and without alcohol. Subjects were given two doses of BZP/TFMPP or a placebo, either with alcohol (6 units) or orange juice. The subjects were assessed on their ability to drive (using a STISM driving simulator). Systolic and diastolic blood pressure, heart rate and temperature were constantly measured.

Blood samples taken at baseline, 3.5, 6.5 and 10 hours were collected by Dr. Imogen Thompson (MRINZ), and sent to ESR for analysis. An anti-coagulant (EDTA, ethylenediaminetetraacetic acid) was added to the blood and the samples were stored in the refrigerator for up to one week prior to testing then frozen at -80ºC.

2.2. Methods for Detection and Quantitation of Recreational Drugs in Hair

The current method employed at ESR for the detection of amphetamine and methamphetamine in hair is GC-MS. The preparation and washing of the hair used in the original method was kept the same, however the method for extraction of drugs from hair was developed based on a published method for the extraction of opiates from hair.[92] The LC-MS/MS method used was the same as the method described in section 2.1.3 for BZP and TFMPP, and was modified to accommodate multiple drugs.

2.2.1. Materials and General Procedures

2.2.1.1.Drug Supplies and Reagents

All drugs standards (except MDMA, which is supplied in solution) were obtained from ESR drug stores as powders (salts), and made up in 1 mg/mL solutions by ESR technicians (stored for up to one year at 4˚C).

All solvents were of analytical grade except acetonitrile used in the chromatography (HPLC grade).

Acid solutions and base solutions were stored at room temperature for up to 6 months, except NaOH, which was kept for up to a month.

2.2.1.2.Stock Standards

A 10 µg/mL stock solution (stored for one month) was made up by diluting 1 mg/mL solutions (prepared by ESR technicians) of methamphetamine, amphetamine, MDMA, MDA, BZP, morphine, 6-MAM and codeine. Diluted solutions in methanol were made for each extraction and used for spiking of blank hair for the standard curve. A 1 mg/mL stock solution of phentermine, made up by ESR technicians, was diluted in methanol, and used to check for interference.

2.2.1.3.Check Solution

1mg/mL stock solutions of all drugs were diluted to 1 µg/mL and used as the check solution for all three validation runs.

2.2.1.4.Internal Standards

A 1 µg/mL solution in methanol was used, containing the following deuterated internal standards: methamphetamine-*d5*, amphetamine-*d5*, MDMA-*d5*, MDA-*d5*, BZP-*d7*, morphine-*d3*, codeine-*d3* and 6-MAM-*d3*. 50 µL of the stock standard was added to each sample.

2.2.2. Extraction of Drugs from Hair

2.2.2.1.Sample Preparation

A 8 mm (diameter) tuft of hair was removed from the vertex posterior (rear of crown) as close as possible to the scalp and placed in a bag with the scalp end correctly labelled. Samples were stored at room temperature until analysis.

Hair samples were tightly wrapped in Glad Wrap, ensuring scalp ends were tightly aligned with the edge of the film. Hair was divided into 2 cm sections up to 10 cm (from scalp end), and cut using sharp scissors. Cut sections were transferred into labelled glass jars, and were then chopped into one millimetre (or smaller) pieces with sharp pointed scissors.

2.2.2.2.Decontamination

Finely chopped hair (20 mg) was placed into extraction tubes with methanol (1 mL). Tubes were sonicated briefly, left for 5 minutes, then centrifuged at 2700 rpm for 5 minutes. The methanol washings were removed with a Pasteur pipette and either discarded or retained for analysis (washings from at least one section were retained, in a test-tube, dried under nitrogen and reconstituted as in 2.2.2.6 below). The washing process was repeated twice more with methanol.

2.2.2.3.Standards

A series of six standards was prepared by spiking blank hair with the drugs of interest at the following concentrations (ng/mg): 0, 0.2, 0.5, 1, 10, 50. Tubes were left to equilibrate for 15 minutes.

2.2.2.4.Extraction

0.1 M HCl (1 mL) was added to each tube (excluding the tubes containing the washings). The tubes were tightly capped, vortexed briefly and then set aside overnight at 37˚C.

After centrifugation (2700 rpm, 10 minutes), the extract was adjusted to pH 7 using 1 M NaOH (100 µL). 1 mL of 0.1 M phosphate buffer (pH 7) was added to the tubes, which were then vortexed briefly and centrifuged at 2700 rpm for 5 minutes.

2.2.2.5.Sample Clean-up: Solid Phase Extraction

Solid Phase Extraction was carried out on Varian Bond Certify columns (130 mg sorbent mass, 120 µm particle size) on an automated Gilson ASPEC XL4 Robot system. The cartridges were pre-conditioned with methanol (2 mL) and 0.1 M phosphate buffer (2 mL, pH 7). Sample (2 mL) was loaded on to the column which was then washed successively with water (2 mL), 0.1 M acetic acid (1 mL), and methanol (2 mL). The column was dried for 5 minutes and the analytes eluted with dichloromethane, propan-2-ol and ammonium hydroxide (80:20:2 v/v, (2 x 1 mL)).

50 µL of acid methanol (15 µL of HCl in 25 mL methanol) was added to the collected fraction before it was evaporated to dryness under a gentle stream of nitrogen.

2.2.2.6.Reconstitution

Samples were reconstituted in 100 µl of 75:25 acetonitrile:100 mM ammonium formate buffer with 0.03% formic acid, and 10 µL was injected into the LC-MS/MS.

2.2.3. Detection and Quantitation by LC-MS/MS

The LC system consisted of a Shimadzu autosampler, pump, and triple quadrupole LC-MS/MS mass spectrometer (API 300, Perkin-Elmer Sciex Instruments) with a TurboIonspray and a HSID interface. The chromatography was carried out on a SCX column (Luna 5µ SCX 100Å 15.0 x 2.0 mm, Phenomenex) with column temperature set at 40ºC. The mobile phase was 100 mM ammonium formate with 0.03 % formic acid and acetonitrile (25:75) with a flow rate of 200 µL/min, and a run time of 11 minutes per sample. Analyst 1.4.1 and Dionex Chromatography MS Link were used to control the system. The parameters for the MS were as displayed in Table 2-3. The declustering potential, collision energy and collision cell entrance potential were set differently for each drug as the instrument showed more sensitivity towards the amphetamines than the opiates. Final optimised settings can be found in Appendix D.

Table 2-3: State File Parameters for API 300 LC-MS/MS

Gases	
Nebuliser Gas (NEB)	13.0
Curtain Gas (CUR)	10.0
CAD Gas (CAD)	3.0
Controls	
Ion Spray Voltage (IS)	5200.0
TEM	500.0

2.2.3.1.Mass Reaction Monitoring

The MS was carried out in MRM (mass reaction monitoring) mode. This is a common scan type in MS/MS and looks at molecular ion and product ion fragments (fragmentation patterns can be found in Appendix B). The

following transitions were monitored (Table 2-4). Three transitions for each drug were monitored to ensure valid identification. Quantitation was based on the first fragment ion, and the second and third transitions were used to confirm drug presence, measured as a ratio of first transition to the second and third transitions. This gives valid identification if the ratio does not deviate more than 20%.

Table 2-4: LC Retention Times and MRM Transitions Monitored for Quantitation of Multiple Drugs in Hair

Compound	Retention time (min)	Parent Ion (m/z)	Fragment Ions (*m/z*)
Methamphetamine	4.89	150.01	91.10, 119.30, 65.20
Methamphetamine-d_5	4.84	155.16	92.10
Amphetamine	4.88	136.06	91.09, 119.1, 65.07
Amphetamine-d_5	4.86	141.10	93.1
MDMA	4.70	194.10	163.2, 135.1, 105.1
MDMA-d_5	4.66	199.11	165.1
MDA	4.72	180.05	163.20, 133.0, 105.10
MDA-d_5	4.68	185.12	168.08
BZP	8.02	177.12	91.0, 85.20, 65.10
BZP-d_7	8.34	184.20	98.20
Morphine	8.39	286.12	152.10, 165.20, 183.20
Morphine-d_3	8.38	289.18	152.10
6-MAM	5.40	328.15	165.11, 211.11, 193.10
6-MAM-d_3	5.39	331.15	165.11
Codeine	7.63	300.11	165.18, 152.16, 128.16
Codeine-d_3	7.61	303.20	165.10
Phentermine	4.80	150.01	133.16, 91.12, 65.09

2.2.4. Validation

A series of five standards at the following concentrations: 0.2, 0.5, 1, 10, 50 ng/mL; a blank and a standard containing only internal standard, were used to create a calibration curve for each run. The calibration curve was constructed

by plotting the ratio of actual analyte concentration:internal standard against the ratio of measured analyte area:internal standard.

Five samples containing 25 μL of the check solution described above, were used to check accuracy and precision. The inter- and intra- day CVs and the accuracy of the method were calculated using the equations shown in 2.1.4 above. Limits of Detection (LOD) are evaluated as the lowest concentration giving a chromatographic peak with the signal to noise ratio S/N = 3. These were estimated using the lowest standard (0.2 ng/mg) and baseline noise.

The extraction efficiency was determined by running a series of five unextracted control samples at the same concentration as the check samples and comparing values. The control samples were prepared by extracting five blank hair samples and adding 25 μL of check solution to the samples after SPE, drying under nitrogen and reconstituting as above. The extraction efficiency was calculated using the equation in 2.1.4 above.

Phentermine has the same molecular weight as methamphetamine and two similar transitions. The potential of phentermine to interfere with the methamphetamine analysis was therefore assessed by spiking two extracted standards at concentrations of 5 ng/mg and 0.5 ng/mg of phentermine (after SPE).

2.2.5. Samples

Three samples from methamphetamine cases previously reported by ESR were tested using the above method. Originally the three samples had been tested by a GC-MS method, regularly used by ESR for the detection of methamphetamine and amphetamine in hair. The concentrations of methamphetamine in the three samples as detected and quantitated by GC-MS are shown in Table 2-5. Sample one had a very low level of methamphetamine detected, which was close to cut-off. Two analyses of sample two were carried out, showing very close agreement of results with an average of 0.14 ng/mg. The third sample contained no traces of methamphetamine.

Table 2-5: Concentration of methamphetamine in hair samples as detected (GC-MS) previously by ESR

Sample ID (ESR ID)	Concentration determined by GC-MS
1 (845)	0.06 ng/mg
2 (159)	0.15, 0.13 ng/mg
3 (665E)	No Drugs Detected

Chapter 3: Results

3.1. Results for BZP and TFMPP Blood Analysis for Driving Study

3.1.1. Extraction Efficiency – Solid Phase Extraction

The drugs were extracted from blood using solid phase extraction. A series of extracted samples (internal standard and spikes added to blood and extracted) and unextracted control samples (internal standard and spike added after extraction) were prepared. The extraction efficiencies are shown in Table 3-1 and Table 3-3. The recovery of TFMPP (109 %) was higher than that of BZP (63 %), however any loss was compensated by using internal standards. Calculated concentrations for extracted and unextracted samples are within very close correlation to each other, this is shown in Table 3-2 and Table 3-4.

Table 3-1: BZP Extraction Efficiency

	Peak Area	
BZP (52.3 ng/mL)	**Extracted**	**Control**
	30960	45682
	27342	43638
	24291	45010
	31767	44257
	24827	43148
Average	27837.40	44347.00
Extraction Efficiency		**63%**

Table 3-2: Calculated Concentrations of BZP (using internal standard ratio) in Extracted and Control Samples

BZP (52.3 ng/mL)	**Extracted**	**Control**
	58.112	52.964
	59.399	51.109
	54.152	55.257
	57.051	48.408
	59.273	56.398
Average	57.60	52.83

Table 3-3: TFMPP Extraction Efficiency

	Peak Area	
TFMPP (7.2 ng/mL)	**Extracted**	**Control**
	3241	3381
	3079	2943
	3220	2925
	4045	2639
	2586	2889
Average	3234.20	2955.40
Extraction Efficiency		**109%**

Table 3-4: Calculated Concentration (using internal standard ratio) of TFMPP in Extracted and Control Samples

TFMPP (7.2 ng/mL)	Extracted	Control
	6.334	8.527
	7.063	6.423
	8.182	6.991
	7.007	7.230
	6.995	7.075
Average	7.12	7.25

3.1.2. Liquid Chromatography – Mass Spectrometry

The chromatography gave good peak shapes and consistent retention times, with a total run time of 11 minutes per sample. Chromatograms from standard and actual blood samples are shown in Appendix E, Figures E1-5.

3.1.3. Validation

Calibration curves were constructed with seven points (and a blank) by plotting concentration versus area ratio of drug to internal standard. All calibration curves were forced through zero, as a blank (zero) standard was used for the analysis.

Calibration curves (reproduced using actual data) for BZP and TFMPP are shown in Figure 3-1 and Figure 3-2. The standard curves were very reproducible and accurate, with correlation coefficients of ≥ 0.999. The

method was found to be linear over a range of 7 - 7000 ng/mL and 10 - 10000 ng/mL of BZP and TFMPP respectively. However, after analysis of some preliminary blood samples at similar concentrations to that expected in the BZP/TFMPP study by MRINZ, a smaller validated range of approximately 30 – 1500 ng/mL of BZP and 5 – 200 ng/mL of TFMPP was used as the standards during sample analysis.

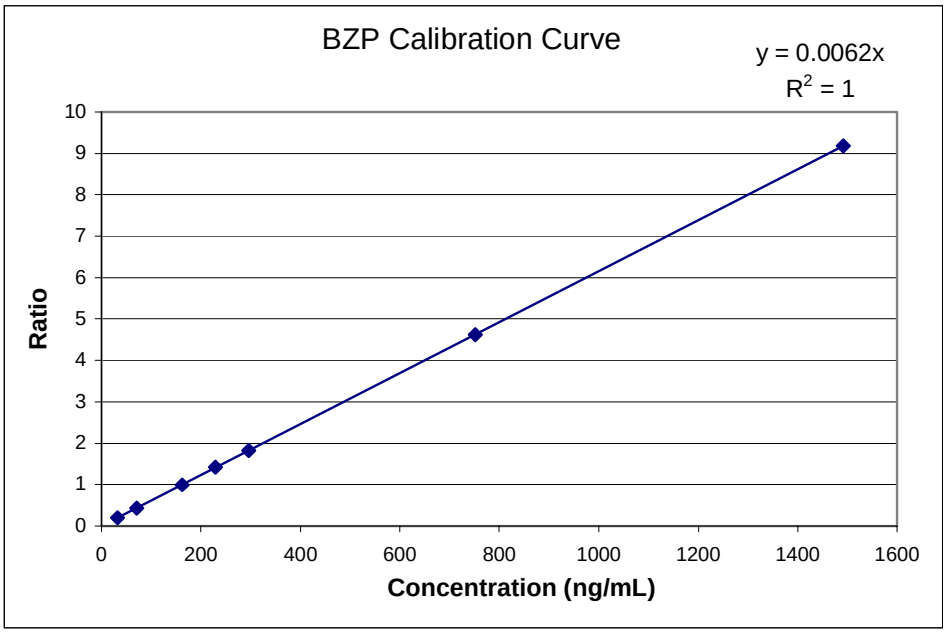


Figure 3-1: LC-MS/MS Calibration Curve BZP

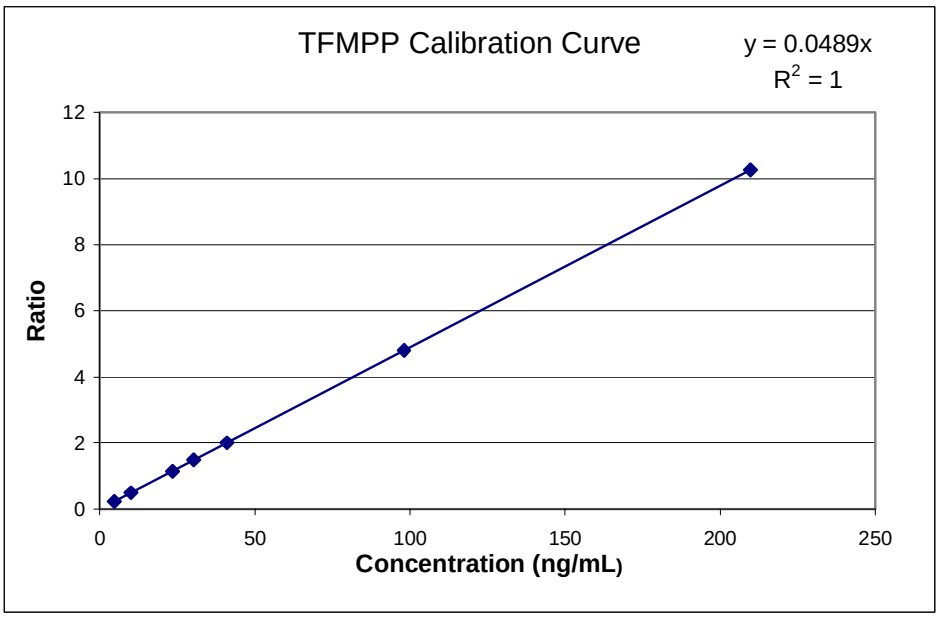


Figure 3-2: LC-MS/MS Calibration Curve TFMPP

Validation was carried out on three different days, using a standard curve (BZP 7-7000 ng/mL, TFMPP 10-10000 ng/mL) and five replicate standards (BZP 17.7 ng/mL, TFMPP 28.3 ng/mL) each day. Summarised inter- and intra-day results are shown in Table 3-5 and Table 3-6 below. For full validation data see Appendix C. All inter- and intra- day CVs were below 10%. The accuracy was greater than 96% for both BZP and TFMPP with BZP giving slightly higher results. The smaller linear range (BZP 30-1495 ng/mL, TFMPP 4-207 ng/mL) was also validated with CVs below 10% and accuracy greater than 90%.

Table 3-5: Summary of Validation Results
Range: BZP 7 - 7000 ng/mL, TFMPP 10 - 10000 ng/mL

Compound	Mean	Std Dev	CV	Accuracy
Intraday results				
BZP 1 (17.7ng/mL)	20.7	1.2	5.8%	116.7%
BZP 2 (17.7ng/mL)	19.5	1.9	9.5%	110.0%
BZP 3 (17.7ng/mL)	19.8	1.3	6.8%	111.6%
TFMPP 1 (28.3ng/mL)	27.7	2.1	7.5%	98.0%
TFMPP 2 (28.3ng/mL)	30.8	2.7	8.6%	108.9%
TFMPP 3 (28.3ng/mL)	27.3	2.5	9.0%	96.6%
Interday results				
BZP	20.0	1.5	7.4%	112.8%
TFMPP	28.6	2.8	9.6%	101.2%

Table 3-6: Summary of Validation Results
Range: BZP 30-1495 ng/mL, TFMPP 4-207 ng/mL

Compound	Mean	Std Dev	CV	Accuracy
Intraday results				
BZP 1 (53.3ng/mL)	51.8	1.2	2.4%	99.1%
BZP 2 (53.3ng/mL)	58.7	0.7	1.1%	112.2%
BZP 3 (53.3ng/mL)	53.6	1.8	3.3%	102.5%
TFMPP 1 (7.2ng/mL)	7.1	0.6	7.8%	99.2%
TFMPP 2 (7.2ng/mL)	6.8	0.4	5.5%	94.4%
TFMPP 3 (7.2ng/mL)	6.5	0.6	9.4%	90.2%
Interday results				
BZP	54.7	3.2	5.9%	104.6%
TFMPP	6.8	0.6	8.2%	94.6%

The samples in solution (analysis by LC-MS/MS) proved to be stable within analytical variation, with little change observed over a period of one week. The internal standard should compensate for the loss and samples prepared for LC-MS/MS analysis were always analysed within three days of preparation.

3.1.4. Results from subjects

A randomised double-blind placebo controlled study of the effects of BZP and TFMPP alone and in combination with alcohol was conducted by MRINZ. The primary investigator for the BZP/TFMPP Ethanol Safety Study, conducted by MRINZ, was Dr. Imogen Thompson. The study assessed cardiovascular and psychological effects, driving performance, and effects on sleep and mood. A total of 64 subjects were to participate in the study, however the trial ceased halfway through due to adverse effects experienced by 41 % of those administered BZP and TFMPP.

Of the 35 of the 64 subjects who participated; six subjects received the placebo, twelve subjects received alcohol, ten subjects received the BZP/TFMPP combination and seven subjects received the BZP/TFMPP/alcohol combination. 140 Samples were tested for BZP and TFMPP (however one subject, who received placebo combination, was removed from the study at the discretion of MRINZ). Of these, 48 samples contained BZP and TFMPP, the rest were baseline samples or subjects whom received the placebo.

The subjects had a median age of 24 (range 20-38) and 22 (62.9 %) were male. Each subject who received party pills was given 150 mg of BZP.2HCl and 36 mg of TFMPP.2HCl at time zero, then a further 150 mg of BZP.2HCl and 36 mg of TFMPP.2HCl at time two hours (resulting in a total dose of 300 mg BZP.2HCl and 72 mg TFMPP.2HCl). An initial blood sample was taken (baseline), then blood samples were taken at 3.5 hours after the first dose (1.5 hours after the second dose), 6.5 and 10 hours. An actual chromatogram of the blood samples (baseline, 3.5, 6.5 and 10 hours) is shown in Appendix E. The final results from the 16 subjects who received BZP and TFMPP (17 subjects

received BZP/TFMPP however blood samples could not be taken for one subject who had a severe reaction) are shown in Table 3-7.

Table 3-7: Results from 16 subjects testing positive to BZP and TFMPP

BZP, TFMPP and Alcohol Results - BESS1 Study

Sex	Age	ESR Sample ID	Time taken	Sample Type	BZP (ng/mL)	TFMPP (ng/mL)	Blood Alcohol (mg/100mL)
M	29	1634/11	8:41	Baseline	0	0	0
		1634/12	12:37	3.5 Hours	346	16	28
		1634/13	15:42	6.5 Hours	581	27	0
		1634/14	19:26	10 Hours	458	17	0
F	29	1634/23	8:42	Baseline	0	0	0
		1634/24	13:44	3.5 Hours	659	42	0
		1634/25	16:51	6.5 Hours	635	43	0
		1634/26	20:36	10 Hours	418	25	0
F	26	1634/27	8:53	Baseline	0	0	0
		1634/28	13:04	3.5 Hours	394	27	0
		1634/29	16:01	6.5 Hours	583	34	0
		1634/30	19:30	10 Hours	580	28	0
M	29	1634/31	9:56	Baseline	0	0	0
		1634/32	13:48	3.5 Hours	721	77	30
		1634/33	16:57	6.5 Hours	776	63	0
		1634/34	20:33	10 Hours	546	45	0

Sex	Age	ESR Sample ID	Time taken	Sample Type	BZP (ng/mL)	TFMPP (ng/mL)	Blood Alcohol (mg/100mL)
F	32	1634/35	7:49	Baseline	0	0	0
		1634/37	12:40	3.5 Hours	443	28	27
		1634/36	15:44	6.5 Hours	464	26	0
		1634/38	19:12	10 Hours	338	18	0
M	21	1634/43	9:27	Baseline	0	0	0
		1634/44	14:22	3.5 Hours	319	22	0
		1634/45	16:25	6.5 Hours	395	30	0
		1634/46	20:53	10 Hours	291	19	0
M	28	1634/51	8:35	Baseline	0	0	0
		1634/52	13:29	3.5 Hours	423	33	17
		1634/53	16:34	6.5 Hours	522	40	0
		1634/54	19:58	10 Hours	306	20	0
F	29	1634/55	9:38	Baseline	0	0	0
		1634/56	14:31	3.5 Hours	613	65	0
		1634/57	17:26	6.5 Hours	652	72	0
		1634/58	20:53	10 Hours	484	51	0
M	32	1634/59	7:45	Baseline	0	0	0
		1634/60	12:36	3.5 Hours	340	19	25
		1634/61	15:42	6.5 Hours	426	21	0
		1634/62	19:06	10 Hours	377	16	0

Sex	Age	ESR Sample ID	Time taken	Sample Type	BZP (ng/mL)	TFMPP (ng/mL)	Blood Alcohol (mg/100mL)
M	21	1634/63	8:31	Baseline	0	0	0
		1634/64	13:32	3.5 Hours	410	31	0
		1634/65	16:35	6.5 Hours	681	46	0
		1634/66	19:59	10 Hours	455	28	0
M	27	1634/79	7:44	Baseline	0	0	0
		1634/80	12:40	3.5 Hours	618	47	0
		1634/81	15:37	6.5 Hours	549	33	0
		1634/82	19:15	10 Hours	319	16	0
M	22	1634/87	8:32	Baseline	0	0	0
		1634/88	13:29	3.5 Hours	393	19	33
		1634/89	16:32	6.5 Hours	438	22	0
		1634/90	20:01	10 Hours	339	12	0
M	29	1634/95	9:36	Baseline	0	0	0
		1634/96	14:22	3.5 Hours	388	18	0
		1634/97	17:25	6.5 Hours	538	18	0
		1634/98	20:51	10 Hours	359	10	0
F	23	1634/103	7:48	Baseline	0	0	0
		1634/104	12:38	3.5 Hours	576	70	54
		1634/105	15:42	6.5 Hours	750	105	17
		1634/106	19:13	10 Hours	446	54	0

Sex	Age	ESR Sample ID	Time taken	Sample Type	BZP (ng/mL)	TFMPP (ng/mL)	Blood Alcohol (mg/100mL)
F	22	1634/115	7:44	Baseline	0	0	0
		1634/116	12:36	3.5 Hours	477	33	0
		1634/117	15:42	6.5 Hours	801	50	0
		1634/118	19:09	10 Hours	665	37	0
F	21	1634/143	7:47	Baseline	0	0	0
		1634/144	12:37	3.5 Hours	553	31	0
		1634/145	15:46	6.5 Hours	560	29	0
		1634/146	19:12	10 Hours	331	14	0

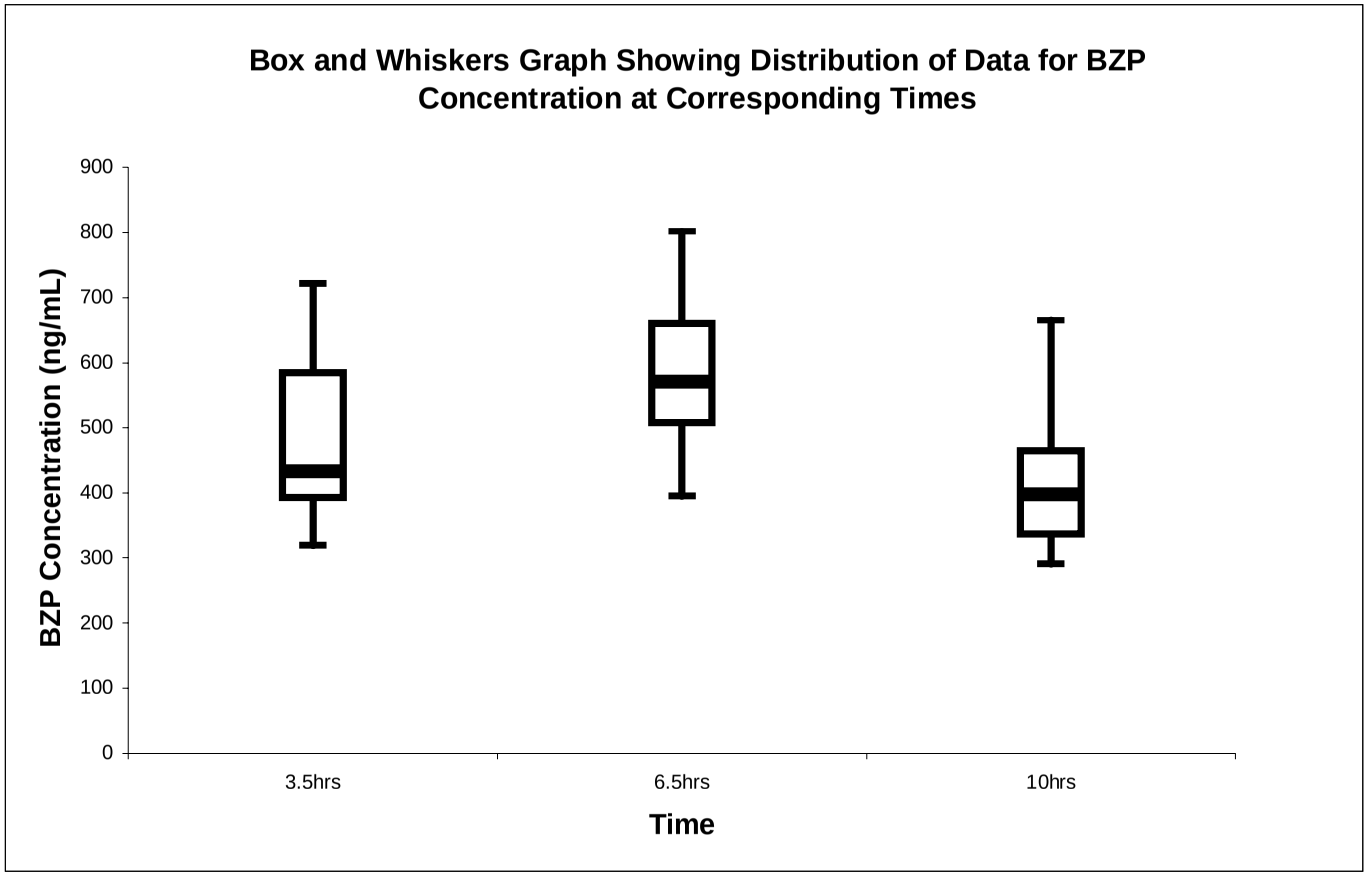


Figure 3-3: Box and Whiskers Plot of Concentration of BZP in Blood Over Time.
Plot shows the highest and lowest points as well as minimum, maximum and median points.

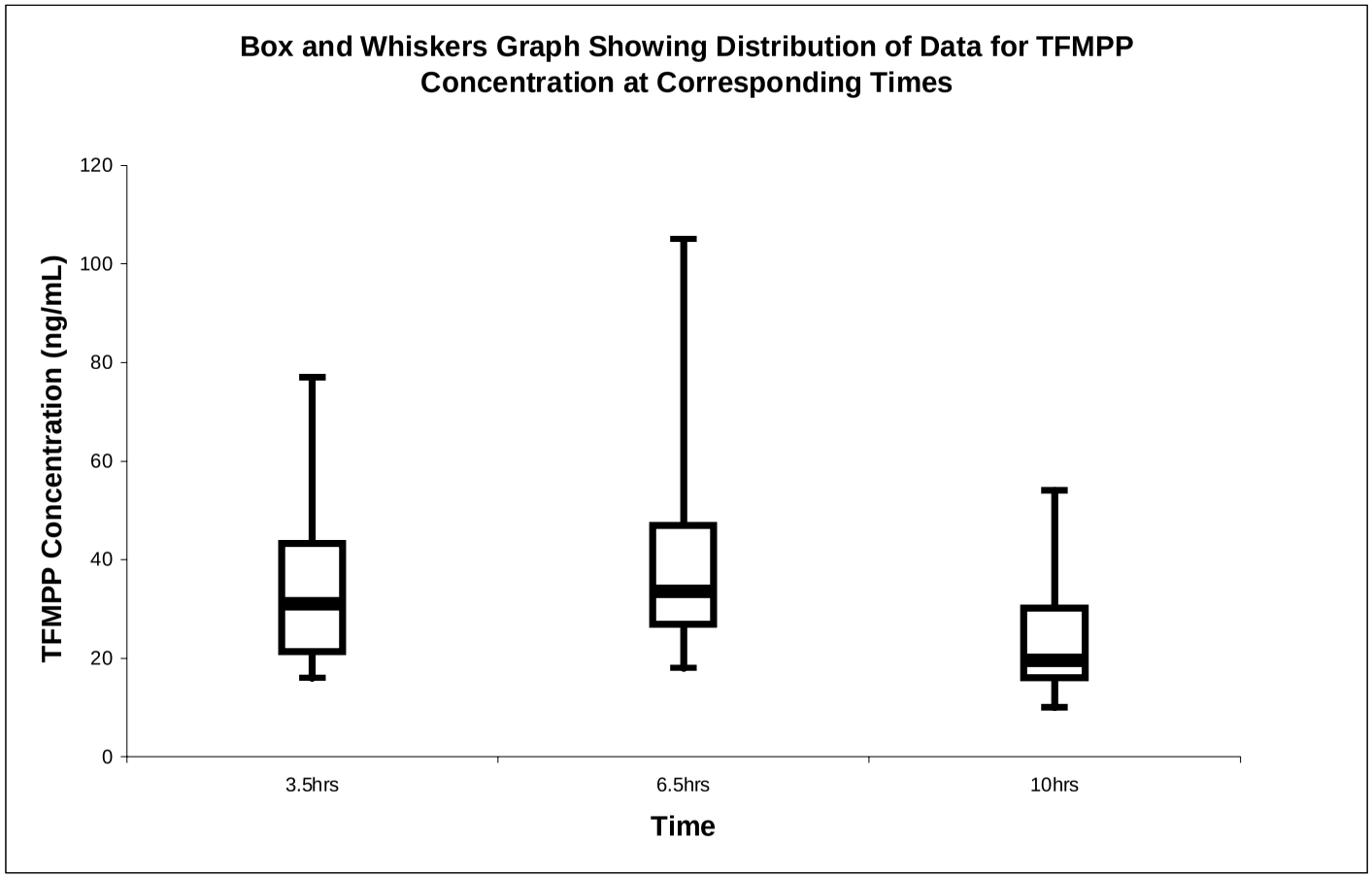


Figure 3-4: Box and Whiskers Plot of Concentration of TFMPP in Blood Over Time.
Plot shows the highest and lowest points as well as minimum, maximum and median points.

The box and whiskers graphs shown in Figure 3-3 (BZP) and Figure 3-4 (TFMPP), show the concentration of the drugs in blood over time. The distribution of data is shown clearly, as the graph shows the maximum and minimum points, the upper and lower quartiles and the median. 50 % of the data is distributed within the box, and trends in the data are clearly shown. At 3.5 hours the drugs are still not fully absorbed, the concentrations continue to increase, with the highest concentrations recorded at 6.5 hours. At 10 hours the concentrations have dropped off, but maximum points are still fairly high for both drugs.

Figure 3-5 and Figure 3-6 depict the concentrations of BZP and TFMPP in the blood at each time period. The error bars represent the standard error of the mean calculated by the following equation:

$$S.E = \frac{\sigma}{\sqrt{\eta}}$$

Where: σ = Standard Deviation and η = Number of samples/data points

The standard error of the mean provides an estimate of how close the sample mean is to the population mean. The error greatly depends on sample size and due to the relatively small sample size, the associated errors are fairly large.

The graphs do not demonstrate a true curve. As the subjects received two doses of the pills, it is diffcult to determine absorption rates and pharmacokinetics, as it becomes quite complicated. One would expect the graph to have a small platform between baseline and 3.5 hours (this is where the concentrations peak for the first dose). Between 3.5 and 6.5 hours there would also be a peak, this is where the drugs reach maximum concentration. The drug concentration most likely peaks at around 4-5 hours which is at least 2 hours after the second dose.

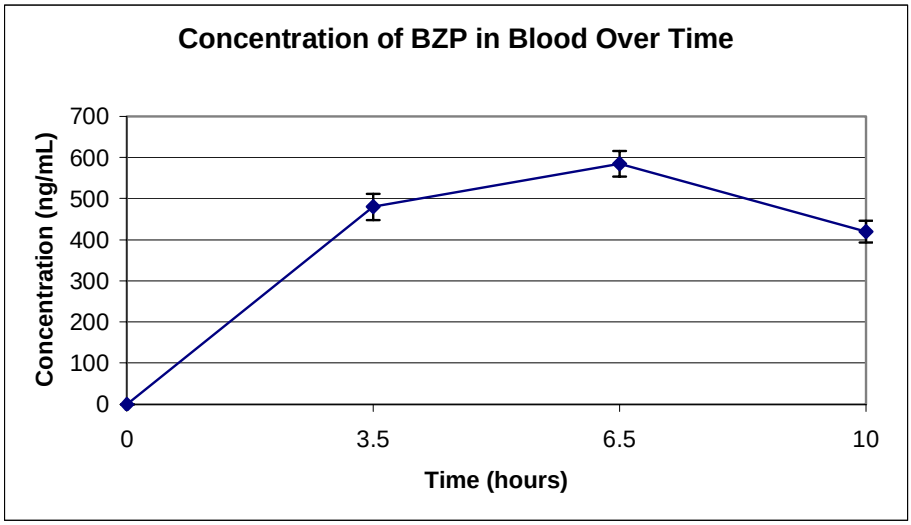


Figure 3-5: Concentration of BZP (ng/mL) in Blood over Time (hours).
Error bars represent the standard error of the mean for 16 individuals.

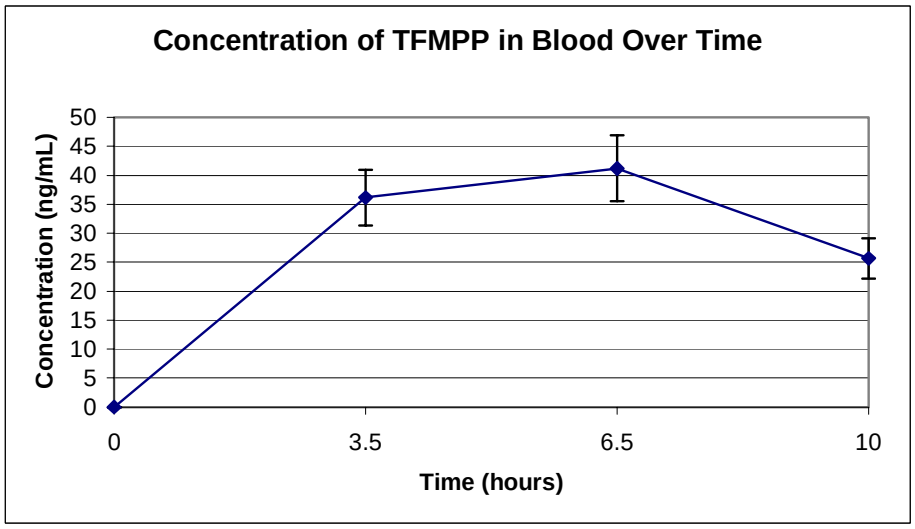


Figure 3-6: Concentration of TFMPP (ng/mL) in Blood over Time (hours).
Error bars represent the standard error of the mean for 16 individuals.

Driving performance was assessed on a STISIM Driving Simulator. The subjects had to undergo a number of real-life situations which assessed their ability to follow other cars at a safe distance, maintain a constant speed (and the speed limit) and tested their attention to the scenario. The primary outcome variable was the Standard Deviation of Lateral Position (SDLP). The less the SDLP, the better the driving performance. The results from the driving simulator tested at 6.5 hours are shown in Table 3-8. MRINZ reported a statistical analysis on the data (using SAS version 9.1, based on a general

linear model), which showed that in the baseline model, the BZP/TFMPP combination improved driving performance by giving a significantly smaller SDLP. Alcohol caused a weak effect, however there was no statistically significant interaction between the BZP/TFMPP/juice and the BZP/TFMPP/alcohol combinations.[93] It is important to note that of the 19 subjects who received alcohol (either in combination with the party pills or alone), only 3 had a small concentration left in the blood at 6.5 hours.

Table 3-8: Treatment effects on the Standard Deviation of Lateral Position (SDLP, cm) at 6.5 hours.[93]

Treatment	N	Mean (SD)	Median (Inter-quartile range)	Range
Alcohol/Placebo	12	30.8 (4.4)	32.6 (27.3 to 34.0)	22.9 to 35.7
Placebo/Placebo	6	28.9 (9.1)	26.5 (22.3 to 31.5)	30.0 to 45.9
Alcohol/BZP/TFMPP	7	24.8 (6.4)	26.0 (19.3 to 30.1)	15.8 to 34.0
Placebo/BZP/TFMPP	9	24.9 (5.1)	25.1 (22.5 to 29.1)	16.8 to 31.6

Adverse effects were experienced in seven of the seventeen subjects that were administered BZP and TFMPP, compared with none experienced in those that received the placebo. Four of the subjects with adverse effects received the BZP/TFMPP/juice combination and three received the BZP/TFMPP/alcohol combination. Effects experienced were severe agitation, anxiety, panic attack (which required treatment), hallucinations, vomiting, headaches/migraines, fatigue, and confusion. A few of the subjects experienced some mild effects, which included headaches, exhaustion and fatigue. The drug concentration in the blood bore no relationship to the symptoms experienced.[93]

Subjects that received the BZP/TFMPP combination (with or without alcohol) showed significant increases in their systolic and diastolic blood pressure. Heart rate and temperature were slightly elevated in the BZP/TFMPP subjects.

The BZP/TFMPP combination caused subjects to have greater difficulty getting to sleep on the night of the test day.[93]

3.2. Results for Recreational Drugs in Hair

3.2.1. Extraction Efficiency

Extraction efficiency was assessed by preparing five unextracted control samples, at the same concentration as the check samples, and comparing peak area of the unextracted samples with the extracted check samples (see Section 2.2.4). The extraction efficiencies of the drugs was greater than 68 % except for that of 6-MAM which was 36 %. Quantitation of these drugs using the deuterium-labelled internal standards adequately compensates for such losses. Low recoveries will of course adversely affect limits of detection. A summary of extraction efficiencies is shown in Table 3-9.

Table 3-9: Detection of Drugs in Hair Extraction Efficiencies for SPE

Extraction Efficiency			
	Mean Area		Extraction
	Extracted	Unextracted	Efficiency
MDMA	1954000	2634000	**74.2%**
Amphetamine	306000	401600	**76.2%**
Methamphetamine	378600	505400	**74.9%**
BZP	959800	1407600	**68.2%**
MDA	637000	833800	**76.4%**
Codeine	351400	476200	**73.8%**
Morphine	576600	617400	**93.4%**
6-MAM	220800	607200	**36.4%**

3.2.2. Liquid Chromatography – Mass Spectrometry

The chromatography gave good peak shapes and consistent retention times, with a total run time of 11 minutes per sample. A total ion chromatograph is shown in Figure 3-7.

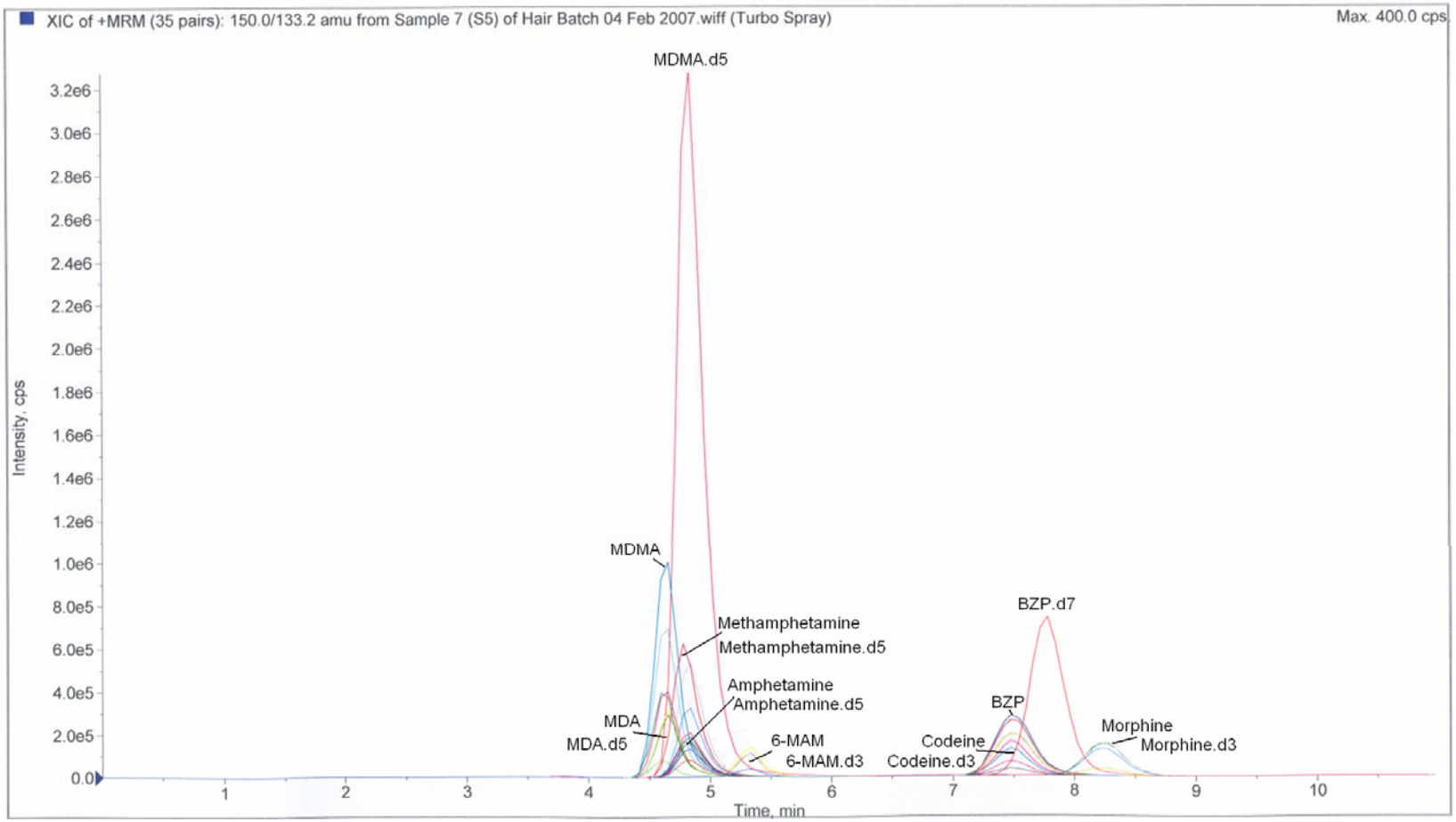


Figure 3-7: Total Ion Chromatogram for LC-MS/MS Hair Analysis

3.2.3. Validation

Calibration curves were constructed with five points (and a blank) by plotting concentration vs. area ratio of drug to internal standard. All calibration curves were forced through zero, as a blank (zero) standard was used for the analysis. All analytes had genuine blanks of close to zero (with added internal standard). All calibration curves were linear with correlation efficiencies ≥0.9997, except BZP which was a quadratic (correlation efficiency 0.9999) with the highest concentration (50 ng/mg) curving off on all three validation runs. The following six figures (Figure 3-8 - Figure 3-15) show example calibration curves for all drugs.

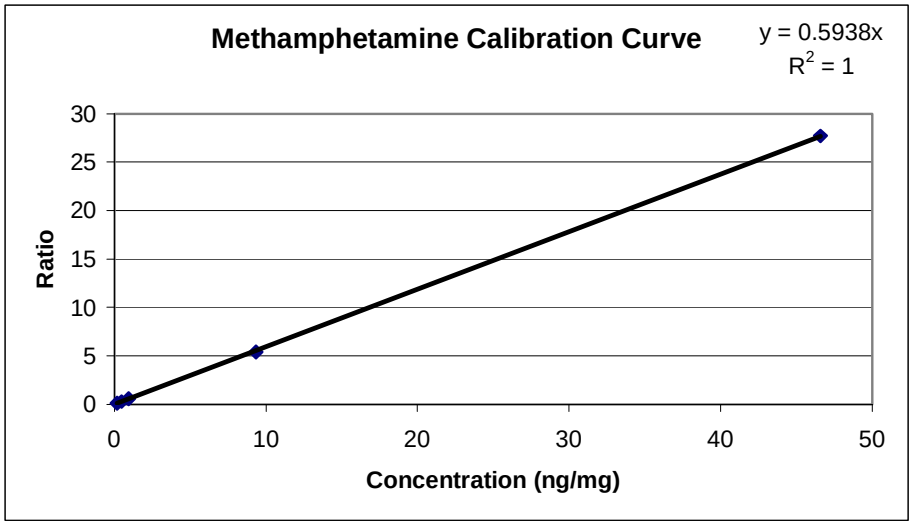


Figure 3-8: Hair Analysis Calibration Curve for Methamphetamine

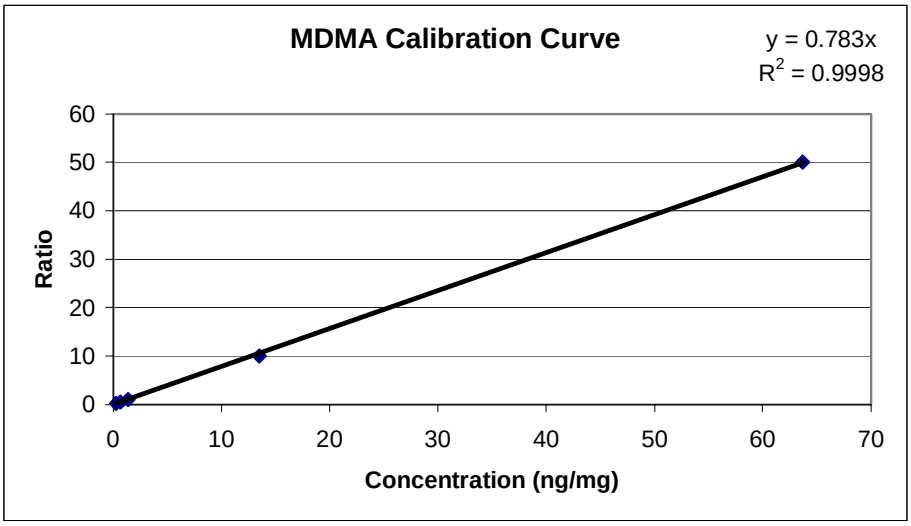


Figure 3-9: Hair Analysis Calibration Curve for MDMA

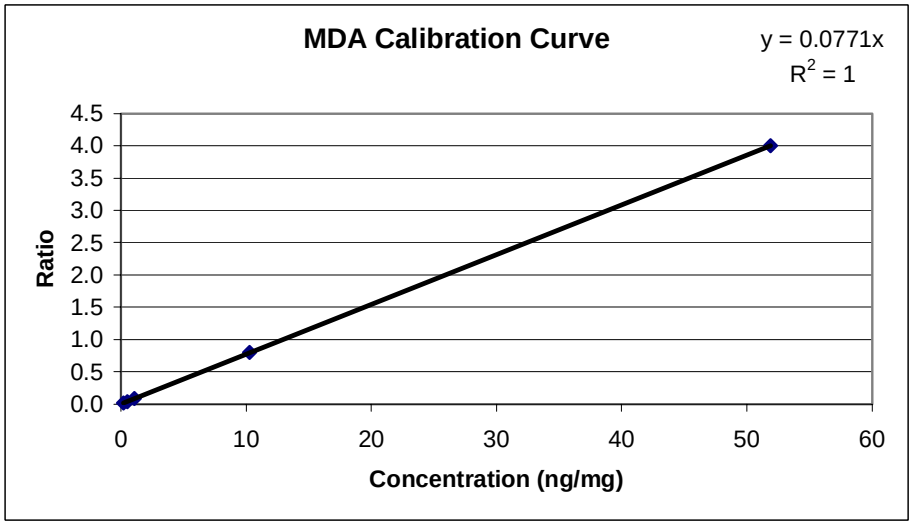


Figure 3-10: Hair Analysis Calibration Curve for MDA

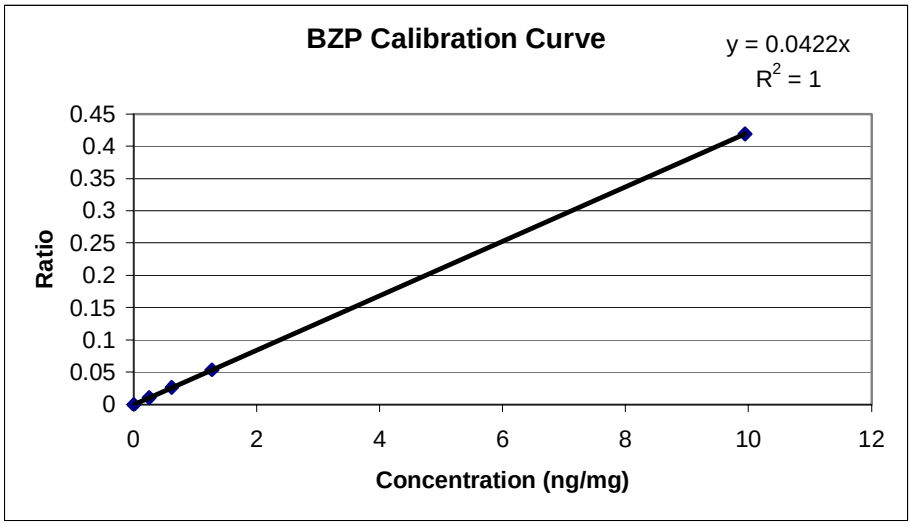


Figure 3-11: Hair Analysis Calibration Curve for BZP

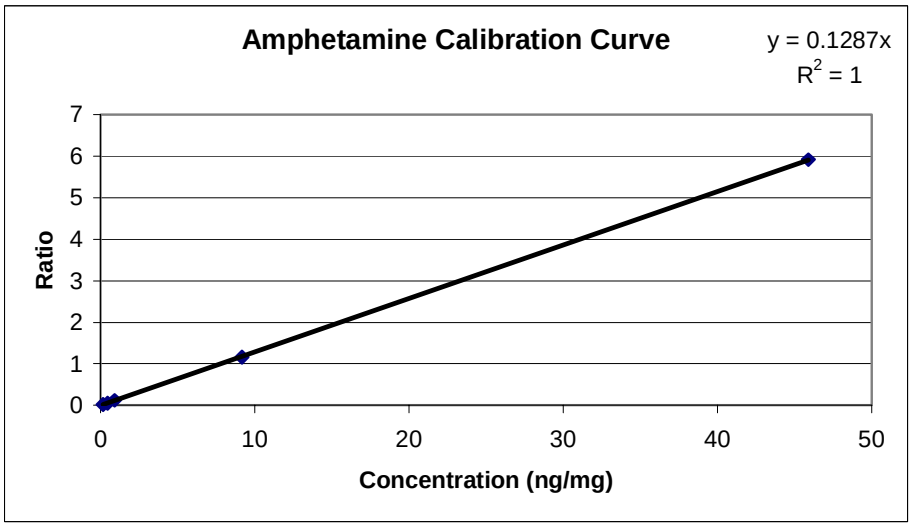


Figure 3-12: Hair Analysis Calibration Curve for Amphetamine

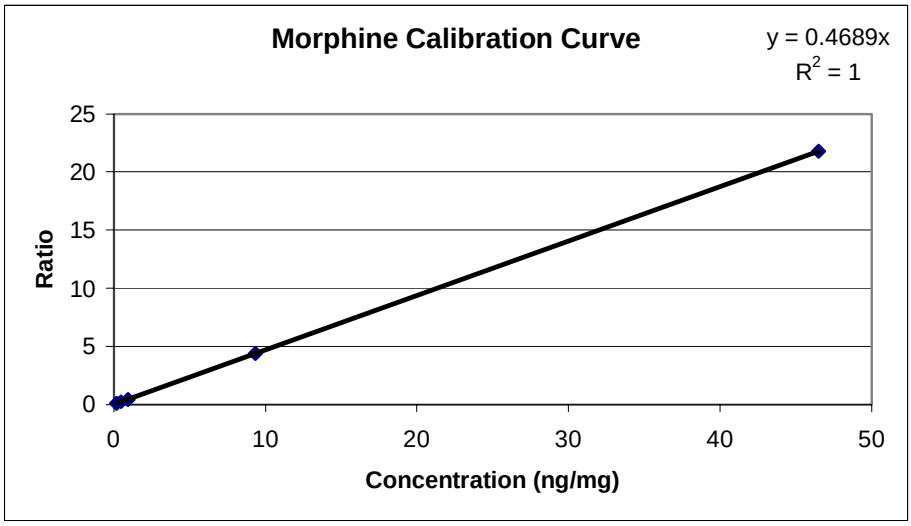


Figure 3-13: Hair Analysis Calibration Curve for Morphine

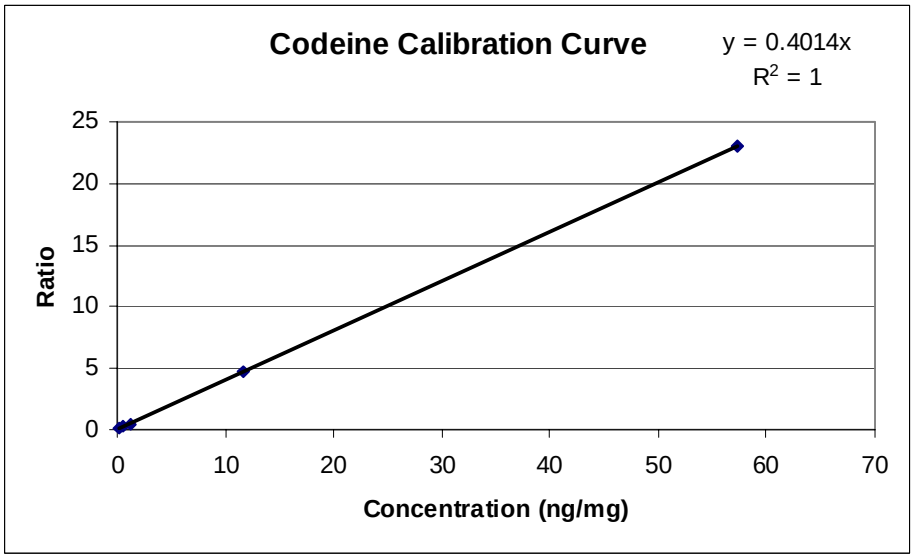


Figure 3-14: Hair Analysis Calibration Curve for Codeine

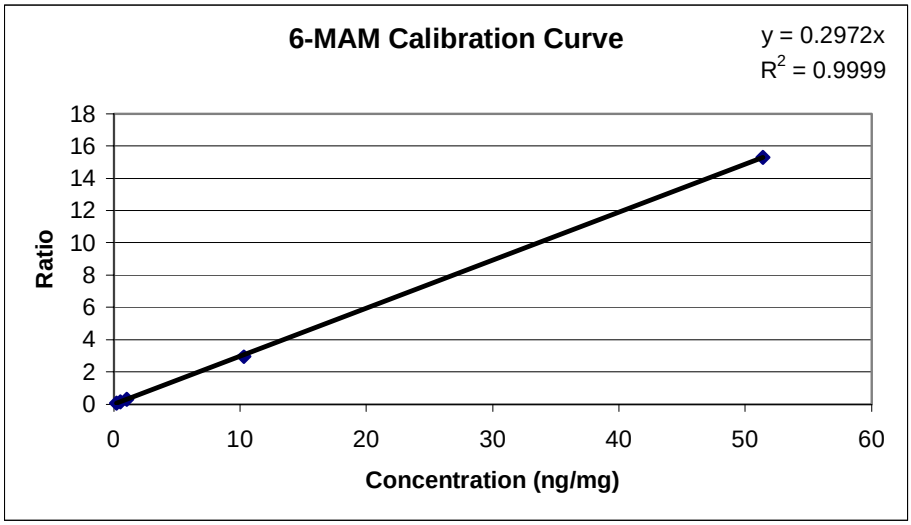


Figure 3-15: Hair Analysis Calibration Curve for 6-MAM

Valid identification of the drugs, measured using the ratios of the second and third transitions monitored by MRM, was ensured, with all ratios consistent. The average ratio (all three validation runs) for the second and third transitions are shown in Table 3-10, and did not deviate more than 12 %. In reality, the transition ratios would be compared to standard samples used that day, however this demonstrates that the method is stable for postitive identification.

Table 3-10: Transition Ion Ratios (Detection of Drugs in Hair)

	Average for 2nd Transtion	Standard Deviation	Average for 3rd Transition	Standard Deviation
Methamphetamine	10.61	1.25	15.43	1.73
MDMA	2.55	0.10	1.29	0.19
MDA	1.06	0.03	1.05	0.04
BZP	1.20	0.05	1.42	0.08
Amphetamine	1.24	0.09	1.96	0.11
Morphine	1.09	0.04	1.27	0.08
Codeine	1.26	0.06	2.35	0.12
6-MAM	0.78	0.06	1.05	0.09

Intra-day CVs were all below 12 % and inter-day CVs less than 11 %. Accuracy was acceptable for all drugs although for amphetamine, it was only 80 %. A summary of intra-day validation results can be seen in Table 3-11, and inter-day validation results are displayed in Table 3-12. Full validation results can be found in Appendix C.

Table 3-11: Detection of Drugs in Hair Intra-day Validation Results Summary

Hair Validation Intra-day Summary							
	Day	r	Mean	Std Dev	CV	Actual Conc.	Accuracy
MDMA	1	0.9998	1.22	0.02	1.6%	1.25	97.8 %
	2	0.9999	1.31	0.03	2.4%	1.25	104.5 %
	3	0.9997	1.47	0.07	4.8%	1.25	117.8 %
Amphetamine	1	0.9999	0.82	0.07	9.1%	1.15	71.3 %
	2	1.0000	0.93	0.02	2.2%	1.15	81.1 %
	3	1.0000	0.99	0.03	2.7%	1.15	86.0 %
Methamphetamine	1	1.0000	1.11	0.01	1.3%	1.17	94.9 %
	2	0.9998	1.30	0.01	1.0%	1.17	111.5 %
	3	0.9999	1.32	0.04	3.1%	1.17	113.0 %
BZP	1	1.0000	1.35	0.07	5.2%	1.25	108.0 %
	2	1.0000	1.23	0.02	1.5%	1.25	98.7 %
	3	0.9999	1.47	0.05	3.3%	1.25	117.9 %
MDA	1	0.9999	1.02	0.04	3.6%	1.30	78.7 %
	2	1.0000	1.25	0.04	2.9%	1.30	96.3 %
	3	1.0000	1.28	0.04	3.2%	1.30	98.3 %
Codeine	1	0.9999	0.99	0.12	12.3%	1.06	93.5 %
	2	1.0000	1.09	0.04	3.4%	1.06	102.6 %
	3	1.0000	1.11	0.03	3.1%	1.06	104.5 %
Morphine	1	1.0000	1.30	0.08	6.5%	1.44	90.3 %
	2	1.0000	1.38	0.03	2.3%	1.44	95.6 %
	3	1.0000	1.38	0.03	2.4%	1.44	95.7 %
6-MAM	1	0.9999	1.41	0.12	8.6%	1.33	106.0 %
	2	0.9999	1.54	0.08	4.9%	1.33	115.5 %
	3	0.9998	1.64	0.12	7.4%	1.33	123.3 %

Table 3-12: Detection of Drugs in Hair Inter-day Validation Results Summary

Hair Validation Inter-day Summary					
	Mean	Std Dev	CV	Actual Conc.	Accuracy
MDMA	1.33	0.12	8.7%	1.25	106.7 %
Amphetamine	0.91	0.08	9.3%	1.15	79.4 %
Methamphetamine	1.25	0.10	8.2%	1.17	106.4 %
BZP	1.35	0.11	8.3%	1.25	108.2 %
MDA	1.18	0.12	10.5%	1.3	91.1 %
Codeine	1.06	0.09	8.3%	1.06	100.2 %
Morphine	1.35	0.06	4.7%	1.44	93.8 %
6-MAM	1.53	0.14	9.1%	1.33	114.9 %

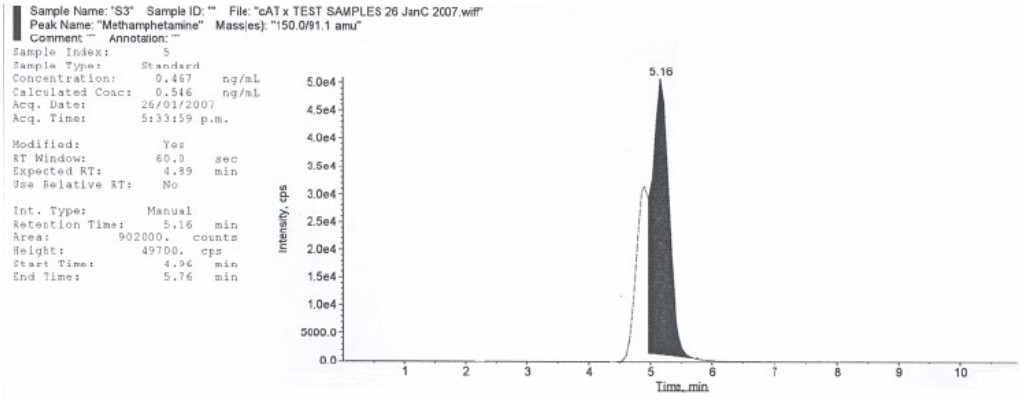
LOD were all very low and fitted within concentrations found in casual to abuser drug users hair. This is shown in Table 3-14.

Table 3-13: LOD and Common Drug Levels Found in Hair of Casual Users/Abusers

	Approximate LOD (ng/mg)	Common Levels Found in Casual Users - Abusers (ng/mg)	References
Methamphetamine	0.02	0.30 - 22.7 ng/mg	64, 94
MDMA	0.001	0.56 - 64.42 ng/mg	64, 95
MDA	0.04	0.05 - 1.06 ng/mg	64, 95
BZP	0.03	Unknown	
Amphetamine	0.06	0.1 - 4.8 ng/mg	94, 95
Morphine	0.01	0.1 (cut off) - 54 ng/mg	64, 9
Codeine	0.08	0.52 - 1.89 ng/mg	64
6-MAM	0.01	0.1 (cut off) - 65 ng/mg	64, 9

The potential of phentermine to interfere with the quantitation of methamphetamine was assessed by spiking known concentrations of phentermine into two standards during a validation run. Phentermine has the same mass as methamphetamine and a similar retention time, however the main transition of phentermine (150.1/133) differs from that of methamphetamine (150.1/91.1). Figure 3-16 shows the distinctive peak of phentermine next to methamphetamine in a spiked sample, monitoring the transition of 150/91 shared by both drugs. To correctly identify methamphetamine, the ratios of the transition ions need to stay constant. If phentermine is present it can be identified by monitoring the transition 150/133.

Figure 3-16: Methamphetamine Peak LC-MS/MS Showing Phentermine Interference at 0.5 ng/mg



3.2.4. Samples

Three samples previously analysed for methamphetamine and amphetamine by GC-MS were analysed using the above method. The results can be seen in Table 3-14. LC-MS/MS results for two samples, previously reported to contain methamphetamine, were similar to the GC-MS concentrations. Sample 2 also showed a low level of MDMA (which was not detected in the GC-MS method). The results for the third sample also agreed with previous results with no drugs detected. The washings of each hair sample were retained and were negative for all drugs.

Table 3-14: Results of Actual Hair Samples Analysed by LC-MS/MS

Sample	Drug/s Detected	Concentration	Concentration of Methamphetamine Measured by GC-MS (LOD 0.5 ng/mg)
1 (845)	Methamphetamine	0.0913 ng/mg	0.06 ng/mg
2 (159)	Methamphetamine MDMA	0.169 ng/mg 0.130 ng/mg	0.15, 0.13 ng/mg Not Detected
3 (665E)	None	N/A	No Drugs Detected

Chapter 4: Discussion/Conclusion

4.1. Discussion Of Results

4.1.1. The effects on driving performance of the legal party drugs BZP and TFMPP alone and in combination with alcohol

Suppliers state on packaging and websites, that users should take one dose (this could be 1-2 tablets/capsules) then take another dose 1-2 hours later if required. However the time taken for BZP and TFMPP to absorb into the bloodstream appears to be a lot longer than expected. From the results it is evident that the drugs are not completely absorbed until approximately 4-5 hours after the initial dose, which is 2-3 hours after the final dose. This could explain why users are being admitted to hospital emergency wards, as it would be easy for a user to take too many pills under the assumption that they are not working.

During the testing of tablets and capsules for uniformity (see Appendix A) a few different kinds of over-the-counter party pills were tested. It appeared that the package labelling could be misleading and hard to follow. The ingredients on the package were listed per serving size, which differed between formulations (e.g. for Jet and Bliss this was two capsules and for E-Formula it was one tablet). Without reading the package cautiously it could be hard for the user to establish what dose of BZP or TFMPP they would be receiving. Results from the prevalence study in Hamilton showed that only 64 % of users read the packet specifications before using the pills and 32.8 % self-reported that they had taken more than the recommended amount.[47] It would appear that this could also contribute to the small percentage of users that have ended up in the emergency wards at hospitals around New Zealand.

There is nothing in the literature that suggests a safe dosage level for BZP and TFMPP, however manufacturers recommendations and package labelling suggest the recommended dose of BZP containing party pills is somewhere

around 200-300 mg, a safe level for TFMPP has not been mentioned. The amount given to the subjects in this study, models the higher end of the recommended dose sold at retail outlets and specified on the packaging. The autopsy case in Sweden found a level of 1.78 μg/mL,[29] which is over twice the highest levels seen here. This level, however, was said only to have contributed to the case and the significance of the contribution is unknown.

According to the house-hold survey carried out by SHORE,[48] 15.9 % of those who took legal party pills, self-reported they completed at least some of their driving under the influence of the pills. The effect of stimulants on driving has not been conclusively determined. Methamphetamine has been shown to improve alertness, and psychomotor abilities, however epidemiological studies indicate that it may cause impairment in drivers.[96] Amphetamine has been shown to improve driving performance in small doses,[97,98] although the effects of large quantities (taken by drug abusers) has not been assesed.[96] There have been no driving performance studies conducted on MDMA, however it has been shown to impair cognitive abilities in users,[96] so may have negative effects on driving. BZP has very close similarities to amphetamine, therefore it is not entirely surprising that BZP enhanced driving performance. However it is often at the end of the night that users will be driving, and they are often "coming down" off the drugs, possibly having been awake for several hours. In this situation users can suffer from fatigue, which is a well-known cause of fatal road crashes. Some of the volunteers experienced fatigue and exhaustion after administration of BZP and TFMPP, this could be a potential risk associated with taking the party pills and driving.

A preliminary estimation of half-life can be calculated using two points (t_1 = 6.5 hours and t_2 = 10 hours) by the following formulae:

$$K_{e\lim} = \frac{In(C_{t_1}) - In(C_{t_2})}{t_2 - t_1}$$

$$t_{1/2} = \frac{0.693}{K_{e\lim}}$$

Where: t_1 = Point obtained after drug has been fully distributed
t_2 = Point obtained at some time later than t_1

Calculated for each individual who received party pills, the average half-life for BZP was 8 hours, compared with 6 hours for TFMPP. The highest and lowest points are shown in Table 4-1. This excludes one individual for BZP as there was no significant change from 6.5 to 10 hours.

Table 4-1: Estimated Half-life of BZP and TFMPP

	$T_{1/2}$ (hours) BZP	**$T_{1/2}$ (hours) TFMPP**
Maximum	20	12
Minimum	4	3
Average	**8**	**6**
Standard Deviation	4	3

Any calculation of half-lives from two points (6.5 and 10 hours) is unreliable however, the concentrations and estimated half-life, give a good indication of concentration and time range for planning a rigorous pharmacokinetics study.

The study was stopped halfway through due to adverse reactions to the pills suffered by 41 % of those who where given the party pills (compared to no adverse effects observed in the placebo group). The effects observed were similar to those seen in the emergency ward at Christchurch Hospital.[25] It can be concluded that these pills, when taken in dosages as suggested by manufacturers, can still cause severe adverse reactions and marked cardiovascular effects.

The findings from this study have been reported by MRINZ to the EACD who in turn has made a recommendation to the government based on these findings, and results from other groups undertaking study on BZP in New Zealand. The EACD has recommended that the BZP pills should be made illegal and suggested they be placed in a similar class to cannabis (Class C, Misuse of Drugs Act). Communities in New Zealand have varying opinions however on the future status of these drugs. As the drugs are marketed for harm minimisation, it is possible that users of the drugs may turn to harder drugs such as methamphetamine, if the party drugs are made illegal. Making the drugs illegal could push them underground, possibly falling into a similar position to methamphetamine or MDMA. The harms of combining drugs are unknown, and a mixture of BZP and, for example, MDMA could be potentially dangerous, or lethal. By placing tighter restrictions on the drugs, and providing education to the public, they would fall into a category similar to that of alcohol. Research shows that the pills have some adverse reactions in users. Although there have been no known deaths attributed to the sole use of BZP and TFMPP containing party pills, safe dose and lethal dose levels are still unknown. By making the pills illegal, theoretically less of the population will be exposed to them, thereby reducing associated risks.

The LC-MS/MS method for the detection of BZP and TFMPP in blood, has great linearity over a wide range (10-10,000 ng/mL), with CVs under 10 %, and accuracy greater than 90 % at low concentrations (BZP 17.7 and 53.3 ng/mL, TFMPP 7.2 and 28.3 ng/mL) therefore falling within requirements suggested by validation guidelines. The method is quick, with limited preparation required by the analyst, especially using automated SPE. Also time consuming evaporation of samples after SPE, was eliminated by eluting samples off the column in pure acetonitrile, requiring only a dilution, before LC-MS/MS.

The method could be improved by monitoring a second transition of BZP and TFMPP. However as we were only looking for known drugs for this study, it was not crucial to have more than one transition. In the case of forensic testing however, it would be necessary, as the monitoring of two (or more) transition

ions would ensure absolute certainty of the identity of the drug. Specificity of identification is essential to forensic cases, as often the fate of an individual relies on the final result given by a forensic scientist. Forensic methods are subjected to scrutiny so there needs to be a high level of reliability, and extensive quality assurance and quality control programs in place.[99] LC-MS is currently the standard for a lot of forensic analysis and this project is a good example of how reliable it can be and the potential applications it has for the future.

4.1.1. Detection of Multiple Recreational Drugs in Hair by LC-MS/MS

The aim of the project was to detect and quantify levels of different recreational drugs in hair. The method was successful in detecting methamphetamine, amphetamine, MDMA, MDA, BZP, morphine, codeine and 6-MAM. All CVs were below 12 % and the accuracies were acceptable for all drugs.

The method proved useful for analysing drugs in hair. The accuracy of the method is not optimum for amphetamine, but the need for accurate quantitation is not an essential requirement. The method also gave valid identification for all drugs, which is fundamental in any forensic method. It monitored three transitions for each drug, giving consistent ratios of the first transition ion to the second and third transtions.

The validation for this method was carried out with blank hair samples, spiked with drugs. This does not quite represent a true hair sample, as the drugs are not incorporated within the hair, like that of a drug user. By testing three samples from previous cases, the reliability of the method was further tested. The results proved very successful with agreement between the developed method and the previously used GC-MS method. It also showed great specificity, as there was no interference seen in the samples. This is especially important in hair samples as due to the complexitiy of the matrix, interference can be a problem.

The ability of the method to detect multiple drugs saves time and reduces costs. By using an automated SPE method for sample clean-up, the time the analyst spends preparing the samples is also reduced.

The SPE method had good extraction efficiencies for all drugs except 6-MAM. Table 3-14 shows the approximate detection limit for 6-MAM calculated at 0.01 ng/mg. The concentration ranges detected for 6-MAM in drug users and abusers were well within this limit, so even though a large loss is occurring, 6-MAM would still be detected by the LC-MS/MS. The possibility of hydrolysis of 6-MAM to morphine during the extraction in acid overnight could explain a lower recovery and is mentioned in other articles.[64,92] For future development of this method, trying different SPE cartridges and changing the elution solvent (perhaps using a different solvent or doing more elutions) may improve recoveries for 6-MAM and the other drugs.

4.2. Conclusion

There still remains a lot of research to be done on BZP and TFMPP (as with many other drugs of abuse). Depending on the legal status of the drug and the ability of scientists to carry out research, many different aspects of the drug could be investigated. Testing of the drug in hair and nails could prove valuable in forensic cases as well as the testing of oral fluid for roadside drug analysis. More knowledge on its mechanisms of action and pharmacokinetics would also prove beneficial.

The LC-MS/MS method developed for this study proved reproducible, reliable and accurate, specific and was easily adapted to test for other drugs of abuse. The method could be modified to include other legal recreational piperazines such as MeOPP, mCPP, Flipiperazine (*p*-fluorophenylpiperazine, PFPP) which are also beginning to appear on the market in New Zealand.

Solid phase extraction was an important part of both methods, and provided a fast automated clean-up of both blood and hair samples. For further work in this area, the extraction efficiency of the different columns (Strata X and Certify Bond Elut) could be determined. It may even be possible to develop one SPE method which can be used for multiple biological matrices.

The overall findings of the BZP and TFMPP safety study show that the dosage (300 mg BZP.2HCl and 72 mg TFMPP.2HCl taken in two doses at two hourly intervals), which represents the recommended intake (as stated by manufacturers) can cause serious adverse reactions in humans. BZP has very close similarities to amphetamine, as demonstrated by its stimulant properties in this report and supported by other studies.[31,33,34,39,44] It is also said to have a similar abuse liability to amphetamine,[31] however the undesirable side effects have also been said to deter users. The legal status of these piperazines remains in question, and their future lies in the hands of the government.

The method for the testing of multiple drugs in hair proved fairly simple, quick, and robust. It achieved lower limits of detection (0.02 ng/mg methamphetamine) than GC-MS (0.05 ng/mg methamphetamine) and similar results to samples previously reported.

Overall, the LC-MS/MS method using a strong cation exchange column proved very versatile with all drugs measured, and could probably be easily adapted to accommodate other, or new similar designer drugs of abuse in different biological matrices.

Appendix A: Tablet Uniformity

A.1 Method for Quantitation of Tablets

It is important that the capsules that the volunteers received contained (within analytical variation) the same amount of BZP and TFMPP. Three different types of party pill obtained over-the-counter from Cosmic Corner (E-Formula, Jet, Bliss) were analysed. Ten of each tablet/capsule were diluted 1000-fold (in 50 mL ethanol (to dissolve) and deionised water), 50 μL of internal standard was added, then the tablets were analysed by LC-MS-MS using the method described in section 2.1.3. The results were compared to the normal extracted curve, and concentrations of the drugs were determined. Following this, ten capsules formulated by the hospital pharmacy (combination of Jet and Bliss) for MRINZ were analysed in the same manner as above.

A.2 Results for Tablet Uniformity

Three types of over-the-counter tablets/capsules (ten of each brand) were analysed. As the labelling did not indicate what form of BZP/TFMPP the capsules contained, this had to be worked out by comparing the amount indicated with the actual amount found. It was concluded that the amount contained in the pills was in the form of the dihydrochloride salt (BZP.2HCl). A summary of this can be seen in Table A-1.

The party pills bought from Cosmic Corner contained varying amounts of the drugs, as demonstrated by the high standard deviation (S.D). The final capsules (the ones administered) were formulated by homogeneously combining two types of capsules (bought over the counter) into new capsules (this was done by the hospital pharmacy, and sent to ESR for analysis). The final formulation contained 75 mg of BZP.2HCl and 18 mg of TFMPP.2HCl and varied only within analytical measures.

Table A-1: Capsule Analysis of Over-the-Counter Party Pills (adapted from reference 93)

Brand	BZP.2HCl (mg)		TFMPP.2HCl (mg)	
	Amount as indicated on packaging (S.D)	Amount as found by analysis (S.D)	Amount as indicated on packaging (S.D)	Amount as found by analysis (S.D)
E-Formula	160 Both	129 (4.4)	160 Both	39 (1.7)
Jet	85	91 (5.8)	10	10 (0.8)
Bliss	50	55 (4.8)	25	25 (2.5)
Pharmacy Capsules	N/A	75 (2.2)	N/A	18 (0.7)

The capsules also contain (as stated on packaging); L-glutamine, D,L-phenylalanine, L-tyrosine, *Ginko biloba* extract, ginger extract, *Guarana* extract, antioxidants, minerals, vitamin B complex and vitamin C. However analysis for quantities/presence of these ingredients was not carried out. These ingredients are commonly found in most formulations sold at various retail outlets.

Appendix B: Fragmentation Patterns

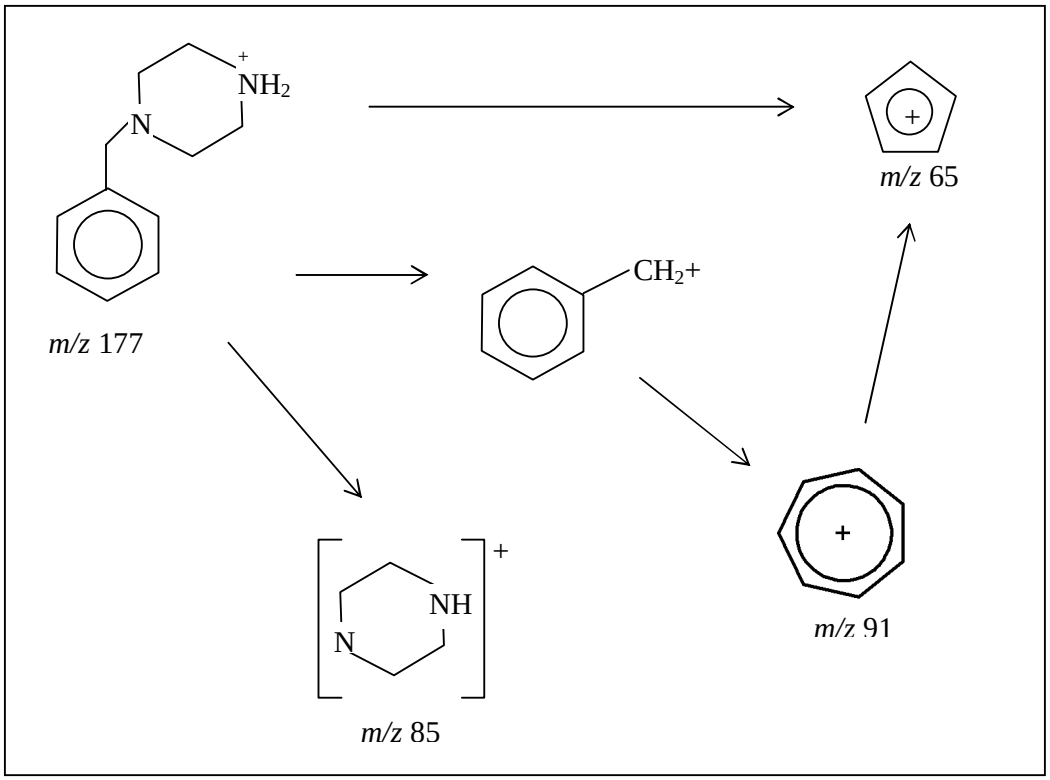


Figure B-1: Proposed Fragmentation of BZP

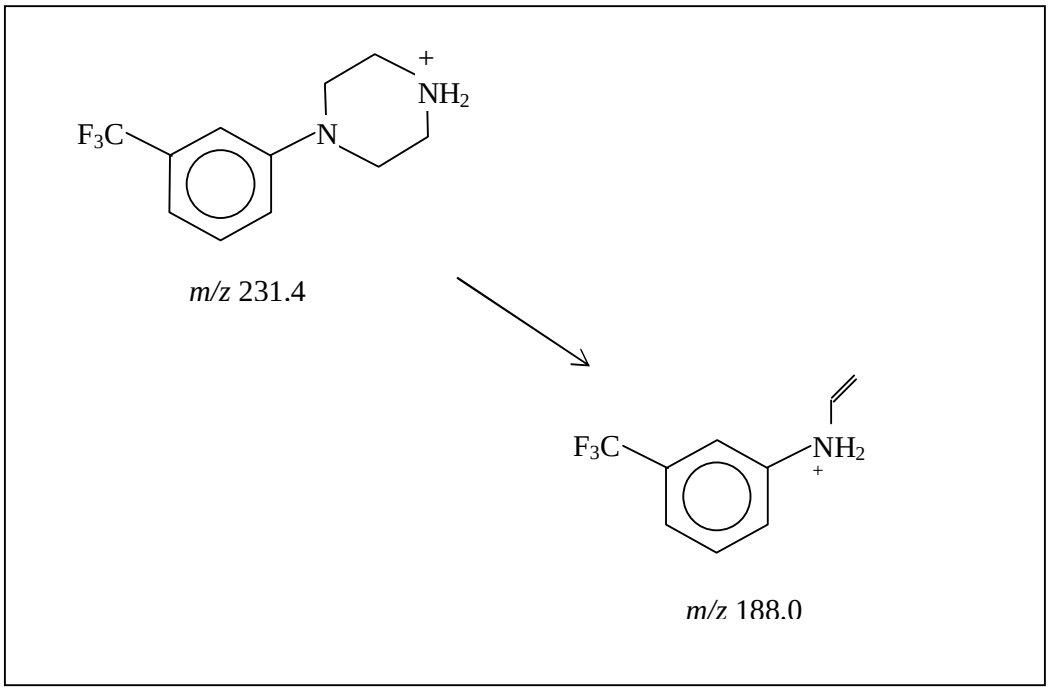


Figure B-2: Proposed Fragmentation of TFMPP

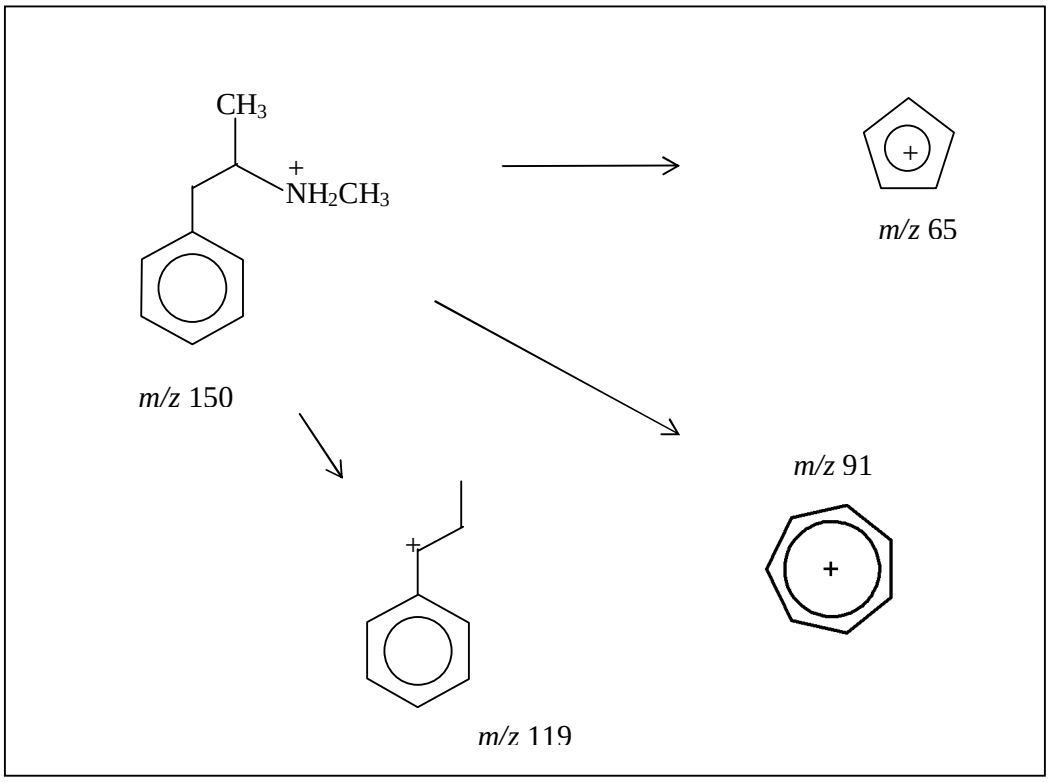


Figure B-3: Proposed Fragmentation of Methamphetamine

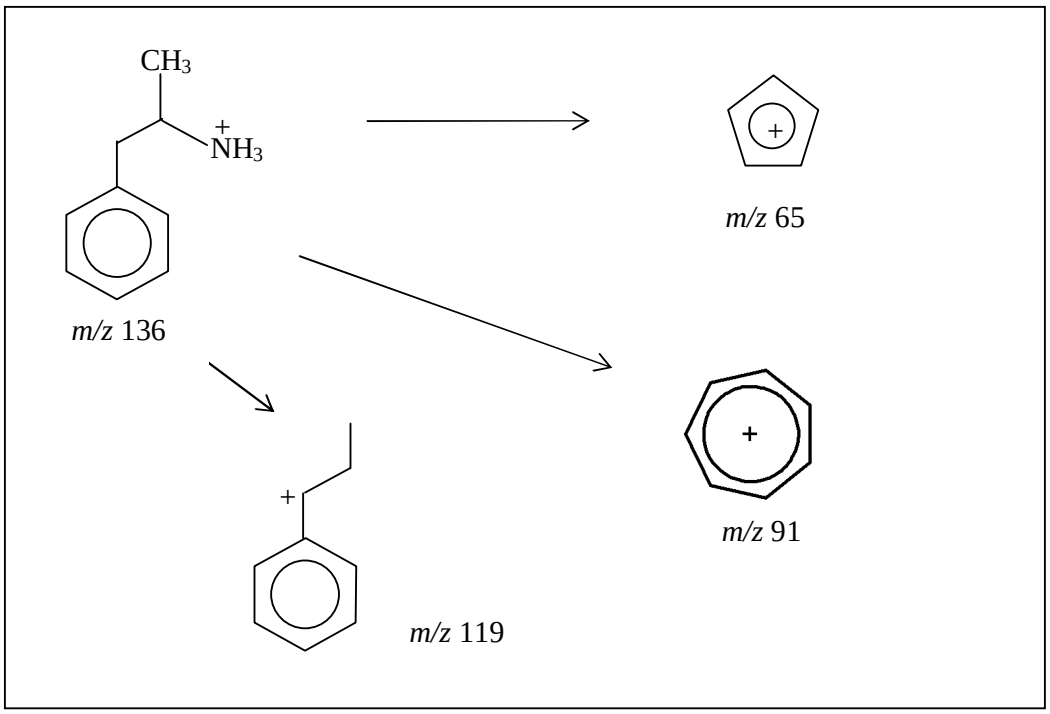


Figure B-4: Proposed Fragmentation of Amphetamine

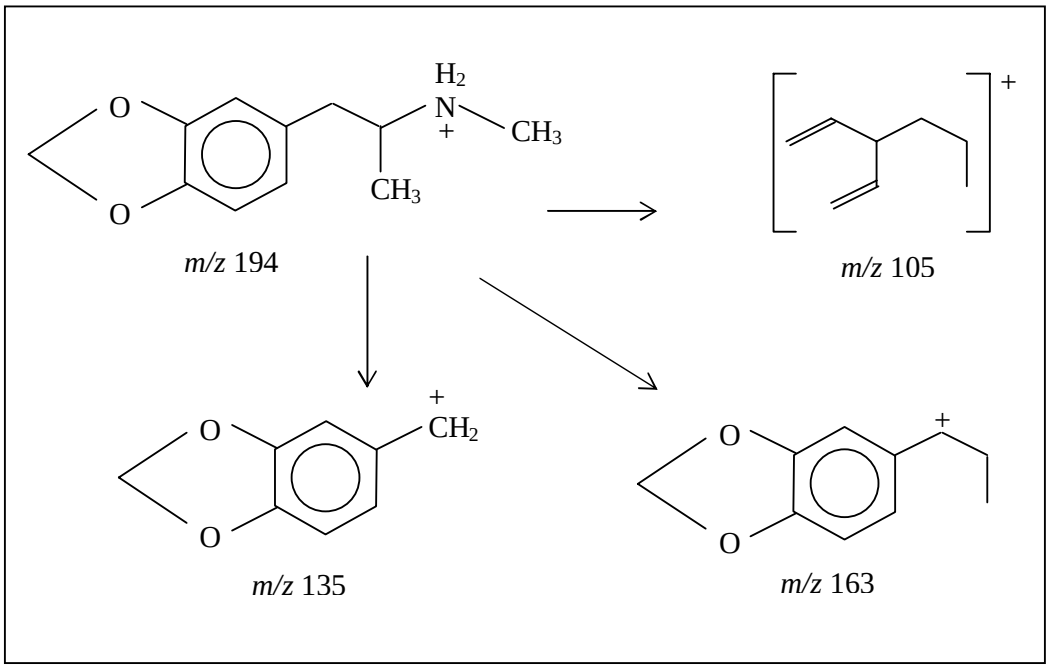


Figure B-5: Proposed Fragmentation of MDMA

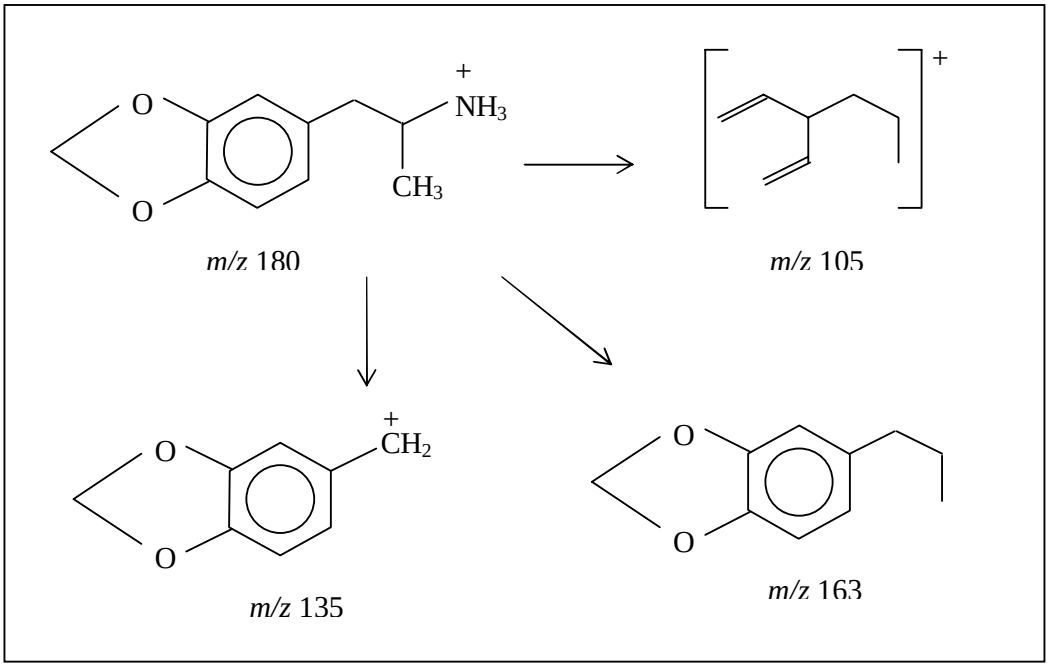


Figure B-6: Proposed Fragmentation of MDA

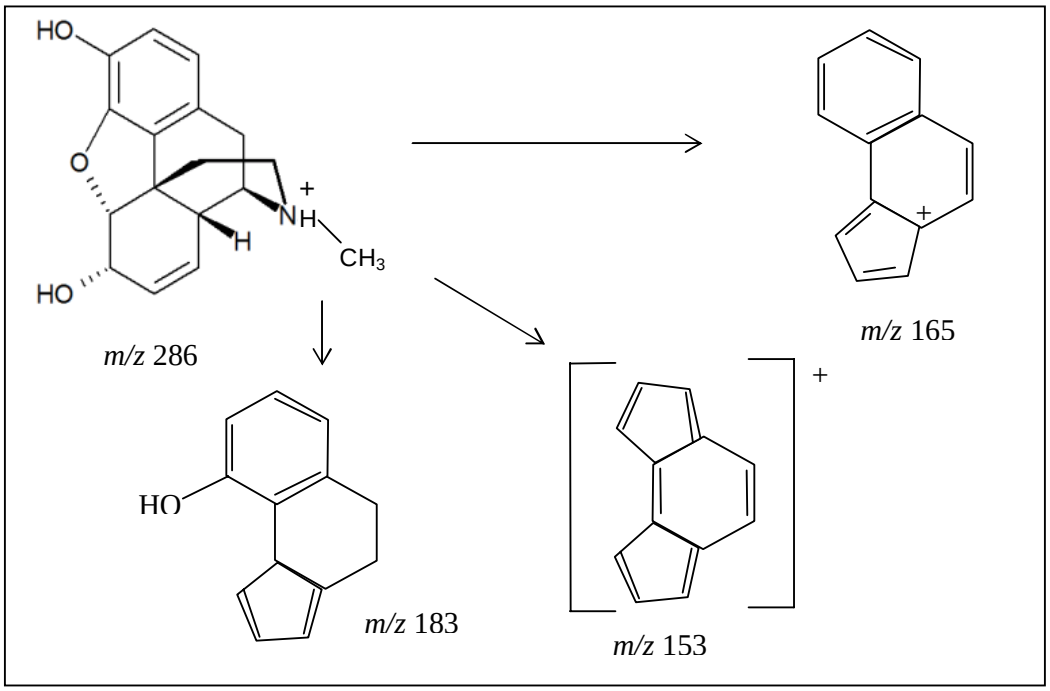


Figure B-7: Proposed Fragmentation of Morphine (adapted from reference 100)

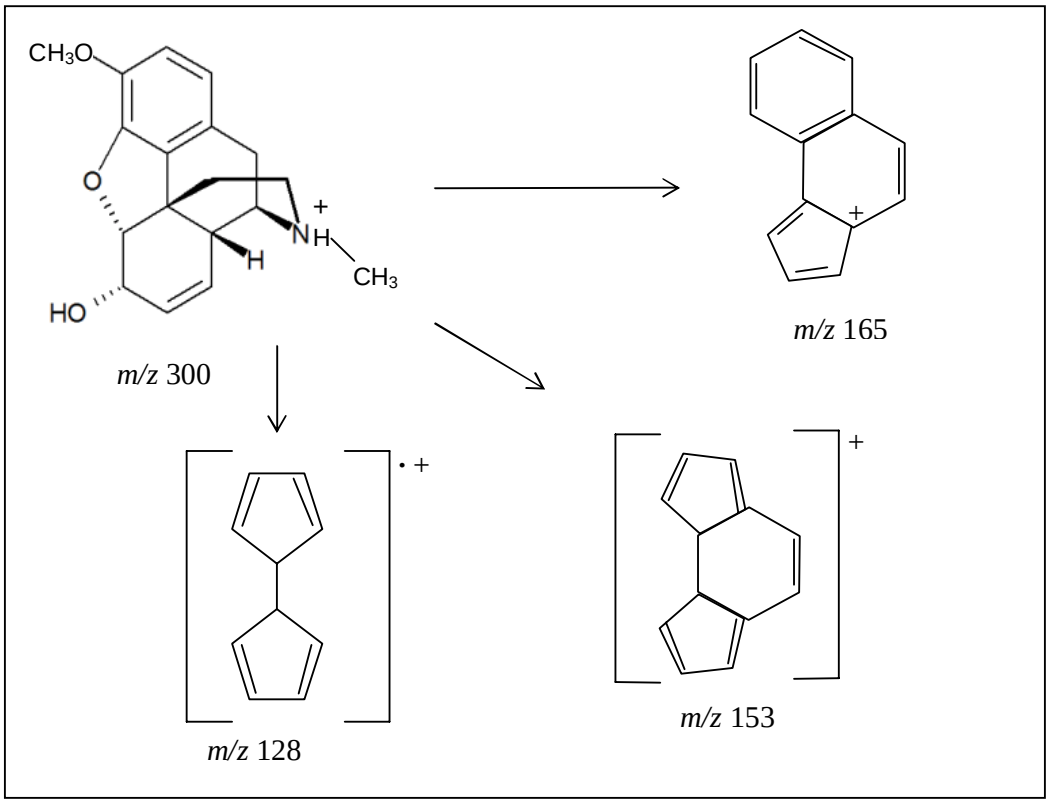


Figure B-8: Proposed Fragmentation of Codeine (adapted from reference 100)

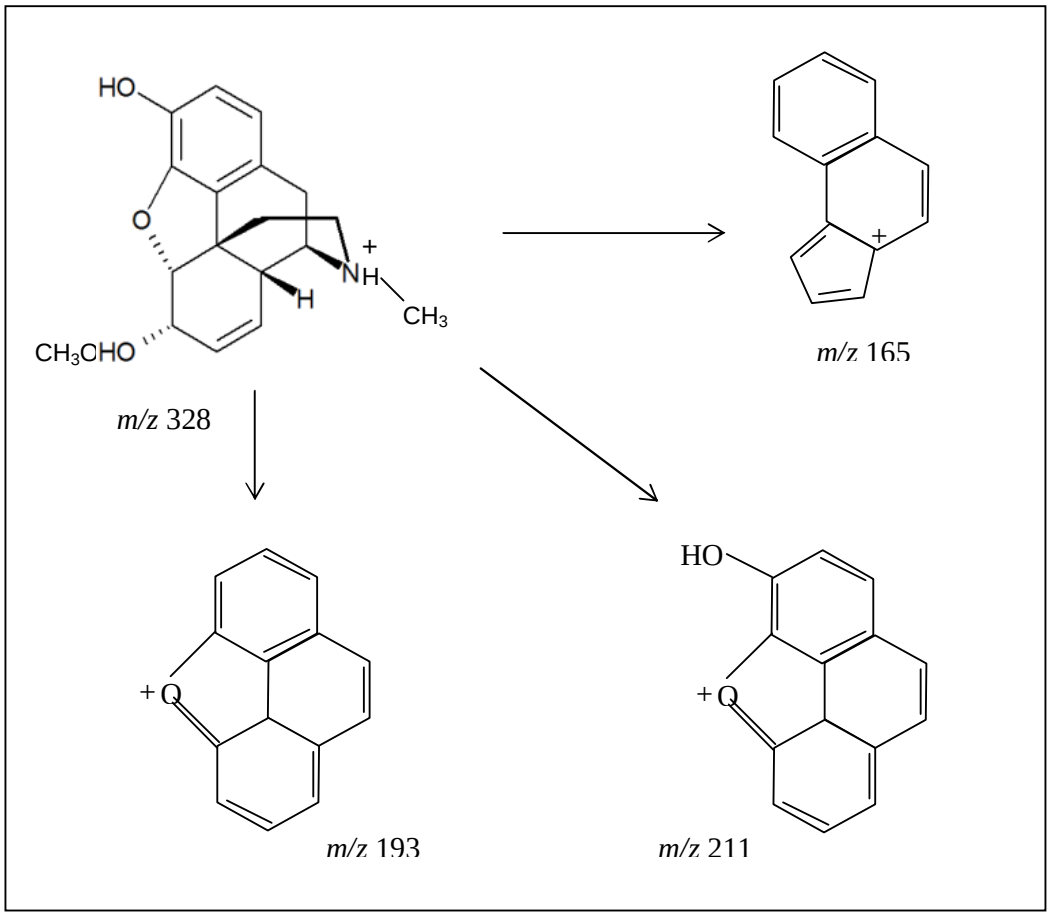


Figure B-9: Proposed Fragmentation of 6-MAM (adapted from reference 100)

Appendix C: Validation Results

Table C-1: BZP/TFMPP Validation Data

Range:	BZP	7-7000 ng/mL	Calibration Curve:	BZP	0.002
	TFMPP	10-10000 ng/mL		TFMPP	0.01
			Correlation Coeff:	BZP	0.999
				TFMPP	0.999

Intra-day Results

BZP	(17.7 ng/mL)		14/06/2006
22.203			
21.154			
20.889			
20.021			
19.023			
Mean	**Std Dev**	**CV**	**Accuracy**
20.7	1.2	5.8%	116.7%

BZP	(17.7 ng/mL)		19/06/2006
22.537			
18.623			
19.346			
19.264			
17.587			
Mean	**Std Dev**	**CV**	**Accuracy**
19.5	1.9	9.5%	110.0%

BZP	(17.7 ng/mL)		22/06/2006
20.483			
18.961			
21.783			
18.730			
18.819			
Mean	**Std Dev**	**CV**	**Accuracy**
19.8	1.3	6.8%	111.6%

TFMPP	(28.3 ng/mL)		14/06/2006
31.154			
25.615			
27.952			
27.019			
26.946			
Mean	**Std Dev**	**CV**	**Accuracy**
27.7	2.1	7.5%	98.0%

TFMPP	(28.3 ng/mL)		19/06/2006
33.749			
32.588			
30.690			
30.249			
26.791			
Mean	**Std Dev**	**CV**	**Accuracy**
30.8	2.7	8.6%	108.9%

TFMPP	(28.3 ng/mL)		22/06/2006
29.170			
28.347			
28.798			
23.136			
27.209			
Mean	**Std Dev**	**CV**	**Accuracy**
27.3	2.5	9.0%	96.6%

Inter-day Results

BZP				
22.203	22.537	20.483		
21.154	18.623	18.961		
20.889	19.346	21.783		
20.021	19.264	18.730		
19.023	17.587	18.819		
Mean	**Std Dev**	**CV**	**Accuracy**	
20.0	1.5	7.40%	112.8%	

TFMPP				
31.154	33.749	29.170		
25.615	32.588	28.347		
27.952	30.690	28.798		
27.019	30.249	23.136		
26.946	26.791	27.209		
Mean	**Std Dev**	**CV**	**Accuracy**	
28.6	2.8	9.6%	101.2%	

Table C-2: BZP/TFMPP Validation Data

Range:	BZP	30-1495 ng/mL	Cal. Curve:	BZP	0.006
	TFMPP	4-207 ng/mL		TFMPP	0.044
			Correlation Coeff:	BZP	0.999
				TFMPP	0.999

Intra-day Results

BZP	(52.3 ng/mL)		19/07/2006
52.112			
51.178			
53.625			
50.312			
51.864			
Mean	**Std Dev**	**CV**	**Accuracy**
51.8	1.2	2.4%	99.1%

BZP	(52.3 ng/mL)		1/09/2006
59.241			
59.548			
58.493			
58.190			
58.050			
Mean	**Std Dev**	**CV**	**Accuracy**
58.7	0.7	1.1%	112.2%

BZP	(52.3 ng/mL)		12/09/2006
56.422			
53.424			
52.355			
51.955			
53.951			
Mean	**Std Dev**	**CV**	**Accuracy**
53.6	1.8	3.3%	102.5%

TFMPP	(7.2 ng/mL)		19/07/2006
7.645			
6.440			
7.309			
7.646			
6.680			
Mean	**Std Dev**	**CV**	**Accuracy**
7.1	0.6	7.8%	99.2%

TFMPP	(7.2 ng/mL)		1/09/2006
7.141			
6.550			
6.296			
7.135			
6.874			
Mean	**Std Dev**	**CV**	**Accuracy**
6.8	0.4	5.5%	94.4%

TFMPP	(7.2 ng/mL)		12/09/2006
7.565			
6.331			
5.998			
6.319			
6.272			
Mean	**Std Dev**	**CV**	**Accuracy**
6.5	0.6	9.4%	90.2%

Inter-day Results

BZP			
52.112	59.241	56.422	
51.178	59.548	53.424	
53.625	58.493	52.355	
50.312	58.190	51.955	
51.864	58.050	53.951	
Mean	**Std Dev**	**CV**	**Accuracy**
54.7	3.2	5.9%	104.6%

TFMPP			
7.645	7.141	7.565	
6.440	6.550	6.331	
7.309	6.296	5.998	
7.646	7.135	6.319	
6.680	6.874	6.272	
Mean	**Std Dev**	**CV**	**Accuracy**
6.8	0.6	8.2%	94.6%

Table C-3: Intra-day Validation Results Hair Analysis Day 1

Hair Validation

Run: 1 **Date:** 22/01/2007

	MDMA	Amph.	Methamp.	BZP	MDA	Codeine	Morphine	6-MAM
	1.23	0.773	1.12	1.23	0.978	1.07	1.23	1.47
	1.24	0.815	1.1	1.4	1.03	0.862	1.35	1.2
	1.23	0.855	1.1	1.35	1.07	0.992	1.28	1.41
	1.22	0.731	1.1	1.38	0.996	1.15	1.22	1.5
	1.19	0.924	1.13	1.39	1.04	0.883	1.42	1.47
r	0.9998	0.9999	1.0000	1.0000	0.9999	0.9999	1.0000	0.9999
Mean	1.222	0.8196	1.11	1.35	1.0228	0.9914	1.3	1.41
Std. Dev.	0.02	0.07	0.01	0.07	0.04	0.12	0.08	0.12
CV	1.6%	9.1%	1.3%	5.2%	3.6%	12.3%	6.5%	8.6%
Actual Conc.	1.25	1.15	1.17	1.25	1.3	1.06	1.44	1.33
Accuracy	97.8%	71.3%	94.9%	108.0%	78.7%	93.5%	90.3%	106.0%

Table C-4: Intra-day Validation Results Hair Analysis Day 2

Hair Validation

Run: 2 **Date:** 26/01/2007

	MDMA	Amph.	Methamp.	BZP	MDA	Codeine	Morphine	6-MAM
	1.36	0.954	1.32	1.25	1.3	1.14	1.41	1.6
	1.29	0.91	1.29	1.22	1.24	1.04	1.33	1.51
	1.3	0.923	1.31	1.21	1.26	1.09	1.36	1.55
	1.3	0.955	1.31	1.25	1.26	1.1	1.38	1.6
	1.28	0.919	1.29	1.24	1.2	1.07	1.4	1.42
r	0.9999	1.0000	0.9998	1.0000	1.0000	1.0000	1.0000	0.9999
Mean	1.306	0.9322	1.304	1.234	1.252	1.088	1.376	1.536
Std. Dev.	0.03	0.02	0.01	0.02	0.04	0.04	0.03	0.08
CV	2.4%	2.2%	1.0%	1.5%	2.9%	3.4%	2.3%	4.9%
Actual Conc.	1.25	1.15	1.17	1.25	1.3	1.06	1.44	1.33
Accuracy	104.5%	81.1%	111.5%	98.7%	96.3%	102.6%	95.6%	115.5%

Table C-5: Intra-day Validation Results Hair Analysis Day 3

Hair Validation

Run: 3 **Date:** 1/02/2007

	MDMA	Amph.	Methamp.	BZP	MDA	Codeine	Morphine	6-MAM
	1.5	0.974	1.3	1.48	1.27	1.11	1.37	1.65
	1.48	0.995	1.33	1.44	1.27	1.12	1.39	1.69
	1.5	1.03	1.39	1.51	1.33	1.14	1.42	1.73
	1.35	0.961	1.29	1.41	1.22	1.05	1.33	1.43
	1.53	0.983	1.3	1.53	1.3	1.12	1.38	1.7
r	0.9997	1.0000	0.9999	0.9999	1.0000	1.0000	1.0000	0.9998
Mean	1.472	0.9886	1.322	1.474	1.278	1.108	1.378	1.64
Std. Dev.	0.07	0.03	0.04	0.05	0.04	0.03	0.03	0.12
CV	4.8%	2.7%	3.1%	3.3%	3.2%	3.1%	2.4%	7.4%
Actual Conc.	1.25	1.15	1.17	1.25	1.3	1.06	1.44	1.33
Accuracy	117.8%	86.0%	113.0%	117.9%	98.3%	104.5%	95.7%	123.3%

Table C-6: Intra-day Validation Results Hair Analysis

Hair Validation Inter-day Results

	MDMA	Amph.	Methamp.	BZP	MDA	Codeine	Morphine	6-MAM
	1.23	0.773	1.12	1.23	0.978	1.07	1.23	1.47
	1.24	0.815	1.1	1.4	1.03	0.862	1.35	1.2
	1.23	0.855	1.1	1.35	1.07	0.992	1.28	1.41
	1.22	0.731	1.1	1.38	0.996	1.15	1.22	1.5
	1.19	0.924	1.13	1.39	1.04	0.883	1.42	1.47
	1.36	0.954	1.32	1.25	1.3	1.14	1.41	1.6
	1.29	0.91	1.29	1.22	1.24	1.04	1.33	1.51
	1.3	0.923	1.31	1.21	1.26	1.09	1.36	1.55
	1.3	0.955	1.31	1.25	1.26	1.1	1.38	1.6
	1.28	0.919	1.29	1.24	1.2	1.07	1.4	1.42
	1.5	0.974	1.3	1.48	1.27	1.11	1.37	1.65
	1.48	0.995	1.33	1.44	1.27	1.12	1.39	1.69
	1.5	1.03	1.39	1.51	1.33	1.14	1.42	1.73
	1.35	0.961	1.29	1.41	1.22	1.05	1.33	1.43
	1.53	0.983	1.3	1.53	1.3	1.12	1.38	1.7
Mean	1.33	0.91	1.25	1.35	1.18	1.06	1.35	1.53
Std. Dev.	0.12	0.08	0.10	0.11	0.12	0.09	0.06	0.14
CV	8.7%	9.3%	8.2%	8.3%	10.5%	8.3%	4.7%	9.1%
Actual Conc.	1.25	1.15	1.17	1.25	1.3	1.06	1.44	1.33
Accuracy	106.7%	79.4%	106.4%	108.2%	91.1%	100.2%	93.8%	114.9%

Appendix D: MS Parameters for Hair Analysis

Table D-1: Collision Cell Parameters for Various Drugs Tested In Hair.

Drug	M+	Fragment Ion	DP (V)	CEP (V)	CE (V)
Methamphetamine	150.1	91.1	34.0	4.65	54.0
		119.30	34.0	4.65	23.00
		65.2	34.0	4.65	56.0
Amphetamine	136.06	91.09	26.0	4.25	49.0
		119.1	26.0	4.25	21.0
		65.07	26.0	4.25	51.0
MDMA	194.10	163	38.0	5.93	28.0
		135.1	38.0	5.93	43.0
		105.10	38.0	5.93	43.0
MDA	180.05	163.2	28.0	5.53	25.0
		133.0	28.0	5.53	28.0
		105.1	28.0	5.53	36.0
BZP	177.12	91.10	41.0	5.44	63.0
		85.20	41.0	5.44	22.0
		65.10	41.0	5.44	62.0
Morphine	286.12	152.1	45.0	8.60	76.0
		165.2	45.0	8.60	52.0
		183.2	45.0	8.60	37.0
Codeine	303.20	165.18	61.0	9.01	51.0
		152.16	45.0	9.01	81.0
		128.16	45.0	9.01	73.0
6-MAM	328.12	165.11	73.0	9.82	65.0
		211.11	73.0	9.82	34.0
		193.10	73.0	9.82	37.0

NB: DP – declustering potential, CE – collision energy, CEP – collision entrance potential.

Appendix E: LC-MS/MS Raw Data (BZP and TFMPP in Blood)

Figure E-1: LC-MS/MS S6 Standard (BZP 177 ng/mL; TFMPP 283 ng/mL)

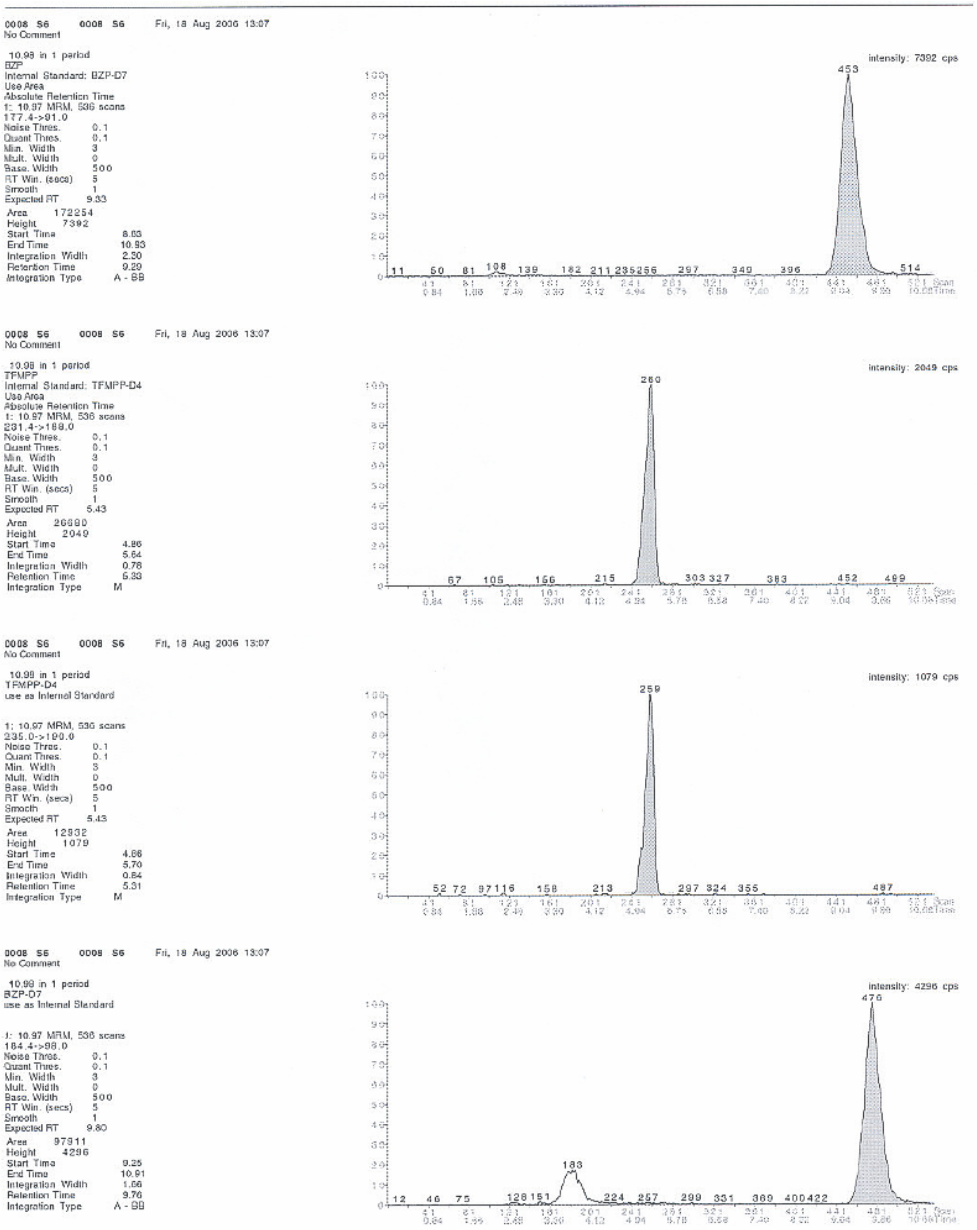


Figure E-2: LC-MS/MS Sample 1634/35 - 0 Hours (Contains no BZP or TFMPP, but shows BZP.d7 and TFMPP.d4 Internal Standards)

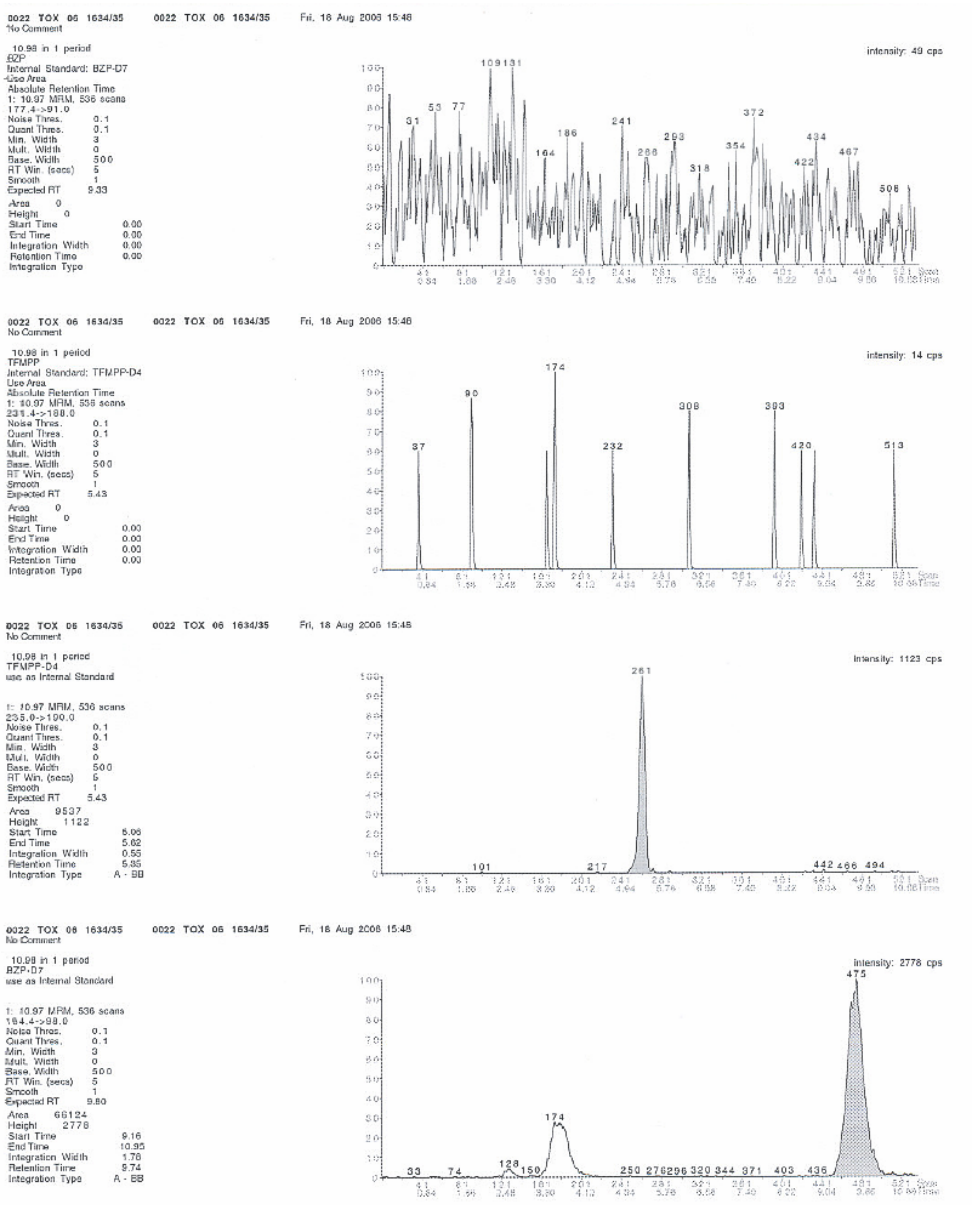


Figure E-3: LC-MS/MS Sample 1634/37 - 3.5 Hours (BZP 433 ng/mL; TFMPP 28 ng/mL)

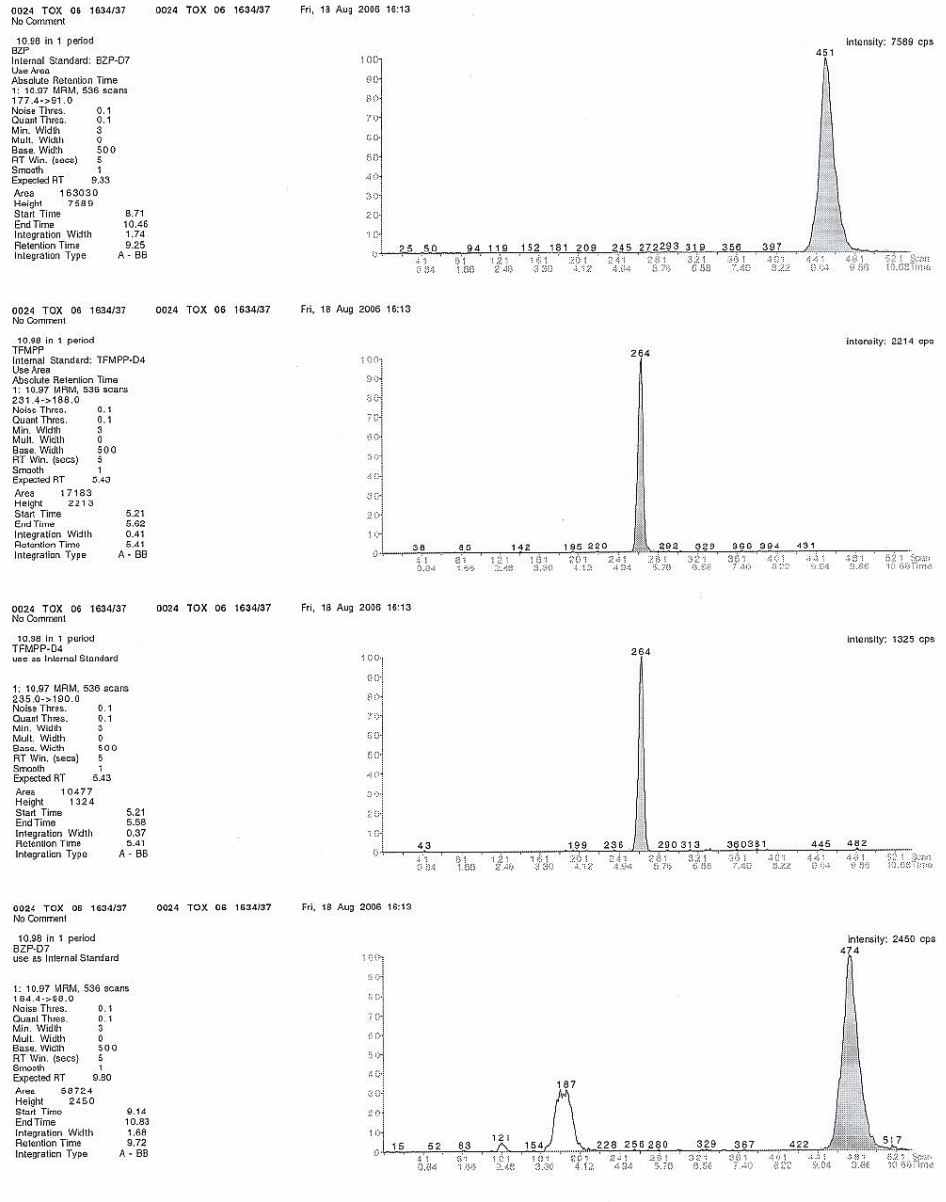


Figure E-4: LC-MS/MS Sample 1634/36 - 6.5 Hours (BZP 464 ng/mL; TFMPP 26 ng/mL)

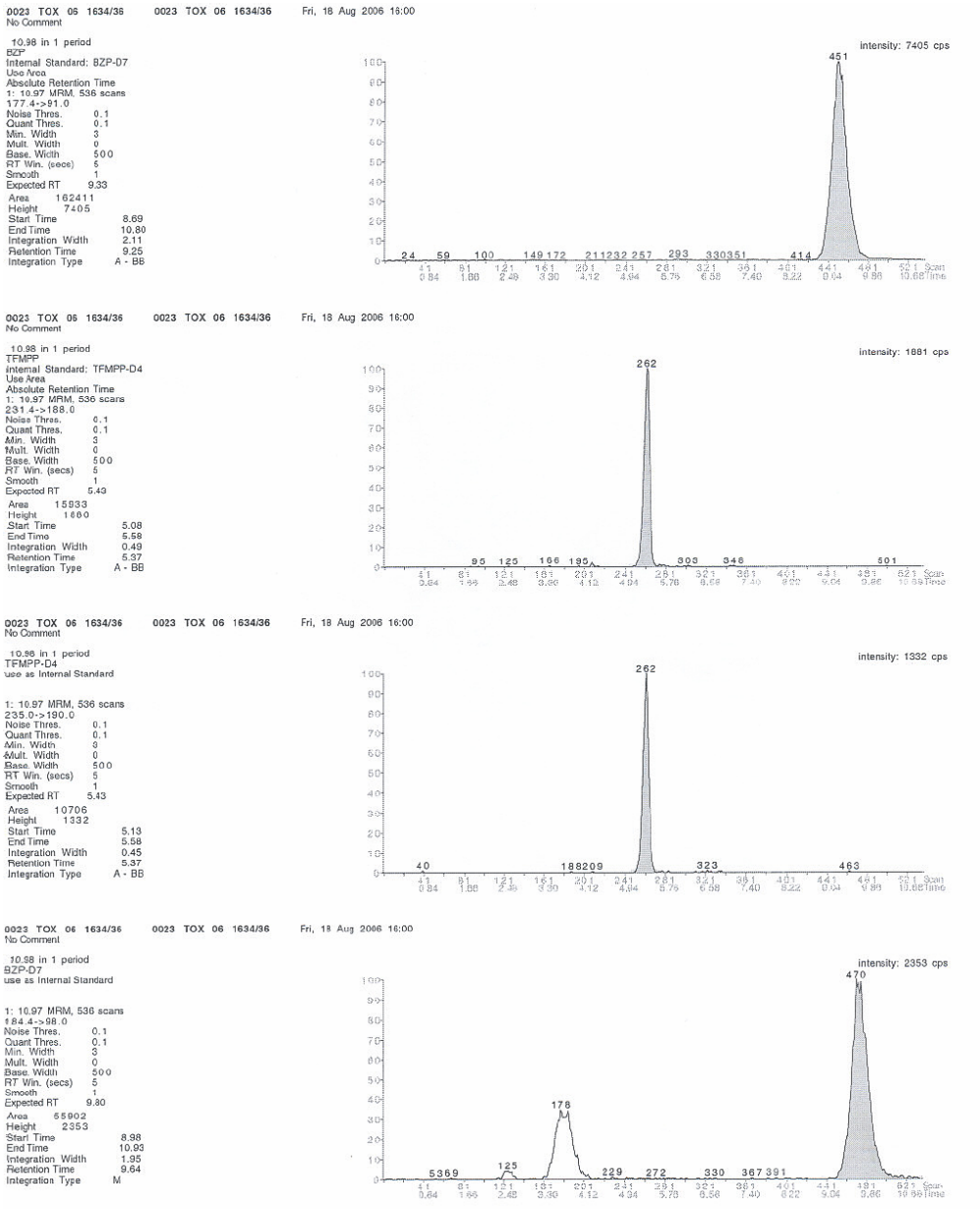
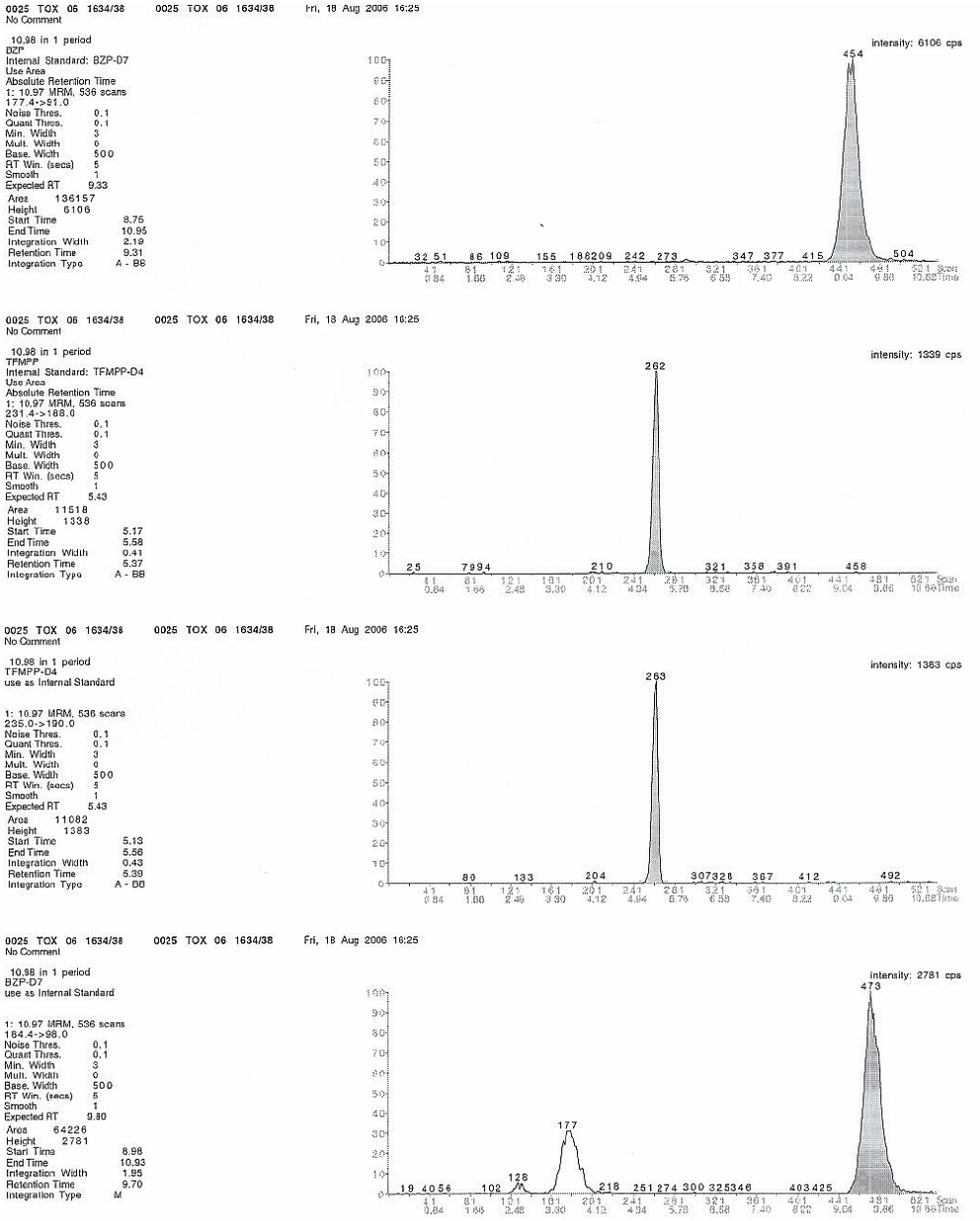


Figure E-5: LC-MS/MS Sample 1634/38 - 10 Hours (BZP 338 ng/mL; TFMPP 18 ng/mL)



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