Application of Solid-Phase Extraction for the Analysis of Drugs in Biological Matrices

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

by

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To my Grandparents

Margaret and James Barbour

&

Agnes and Frederick Cooper

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The important thing is to

not stop questioning

Albert Einstein

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Summary

Experimental mixed-mode solid-phase extraction columns of differing carbon number and carbon loading were investigated for the efficient extraction of drugs of abuse from biological matrices. Methadone and its two major metabolites (EDDP and EMDP) were chosen due to the increase in methadone drug-related deaths in the West of Scotland. Amphetamine and related compounds (methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA)) were chosen because of the prevalence of use of "speed" and "ecstasy" throughout the United Kingdom and the challenge these volatile drugs pose to the analyst.

Improved methods were developed for the efficient extraction of methadone, EDDP and EMDP from whole blood and for amphetamine and related compounds from whole blood and hair. These methods were successfully applied to the analysis of postmortem samples.

The stability of methadone, EDDP and EMDP in whole blood was investigated using the developed extraction method after storing at various temperatures for a period of six months. Methadone remained stable in blood after six months. This was confirmed by the analysis of postmortem cases samples which when re-analysed within six months of the initial analysis, correlated well.

Postmortem hair samples proved useful for determining antecedent drug use history whether extracted by solid-phase extraction or after screening with a Cozart enzyme immunoassay kit, adapted for hair analysis.

Solid-phase extraction provides a clean and efficient means of extracting drugs of abuse from whole blood and hair. Simple manipulation of the extraction system (sample preteatment, pH, derivatisation) provides a versatile alternative to lengthy liquid-liquid extraction techniques and has the added potential of automation.

1. Introduction

1.1 Drug Use: An Overview

1.1.1 Introduction

The abuse of drugs both legal and illegal have a detrimental effect on society as a whole. The risks from toxicity are compounded by the potential of long-term risks effecting a greater number of the drug using population.

Tobacco smoking is thought to contribute to 100,000 premature deaths in the UK every year and alcohol abuse is prevalent in a society where 90% of the adult population drink.[1] 3,4-methylenedioxymethamphetamine (MDMA), a synthetic stimulant used recreationally, has been associated with psychological and behavioural complications [2]. Drugs are used by many athletes as stimulants to reduce tremor and heart rate, and to alter body composition [3].

The number of deaths caused directly from drug use, e.g. overdose [4] or indirectly, e.g. road traffic accidents [5], have increased throughout the United Kingdom. In the Strathclyde region alone, the number of deaths has increased from 9 in 1985 to 120 in 1998 [6]. Deaths occurring in the late 1980's were attributed to heroin abuse, but at the start of the 1990's the abuse of benzodiazepines featured much more [7].

The drug scene in the latter part of the 1990's has seen a resurgence of heroin use [8]. Heroin is now being used by adolescents from more affluent backgrounds as a

recreational drug and is commonly seen in small towns. This is in sharp contrast to the typical heroin user of the past who almost exclusively lived and bought heroin in the larger cities and was unemployed.

Methadone is often used in the treatment of opiate addiction and was introduced into Scotland at the end of the 1980's. This synthetic opioid analgesic has replaced heroin as the most common cause of drug related death in South-East Scotland [9].

The number of drug related deaths in the Strathclyde Region resulting from methadone alone or in combination with other drugs had increased from 1 in 1991 to 34 in 1996, highlighting the impact of this drug on drug users [10]. Heroin deaths however still contributed a larger proportion of the drug-related deaths in the region.

Drug use impairs psychomotor skills and causes hazardous driving [11-13]. Individuals who are in receipt of methadone are required to inform the authorities of their current drug status. However, many do not and put themselves and other road users at risk [14].

Amphetamines are the most popular illicit stimulants, second only to cannabis [15]. In particular, the ring-substituted amphetamines, e.g. 3,4-methylenedioxy-methamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA) are widely used at organised dance events. "Herbal remedies" were recently introduced into the dance scene and are thought by users to be "natural" and therefore safer. Bateman *et al* [16] highlighted the dangers of these "herbal remedies" which are extremely powerful and potentially lethal. At present there is a lack of regulations governing products like "Khat" and "herbal ecstasy".

1.1.2 Misuse of Drugs Act 1971

The aim of the Misuse of Drugs Act is to prevent the unauthorised use ('misuse') of the drugs regulated under the Act. The Misuse of Drugs Act was introduced as law in the United Kingdom to fulfill its obligation to control drugs in accordance with international agreements.

Under the confines of the law it is illegal to be in possession of a drug with or without the intent to supply to another person. To supply or offer to supply to another person. To allow the use of premises which you occupy or manage to be used for supplying. It is also illegal to produce or cultivate a drug or to import or export any of the drugs regulated under the Act.

The exceptions to the general prohibitions are in the form of 'regulations' made under the Act. The majority of controlled drugs have medical uses or may be of scientific interest. The Act allows the government to authorise medical or scientific organisations to possess, supply, produce and import or export the regulated drugs.

Controlled drugs are divided into five schedules. Schedule 1 includes drugs which are most stringently controlled (e.g. cannabis, cannabis resin and raw opium). Doctors cannot prescribe them or pharmacists dispense them. Schedules 2 through 4 involve the majority of controlled drugs. These drugs are available for medical use and can be supplied or administered by doctors. Heroin is a schedule 2 drug and can still be prescribed by a doctor. Schedule 5 lists preparations of drugs considered to pose minimal risk of abuse. Many of these preparations are sold in pharmacies.

Within the Act controlled drugs are divided up in "classes" according to the maximum penalties for these offences. Class A drugs incur the highest penalties as they are thought to be the most harmful when misused, then class B and C. Class B drugs in injectable form count as class A.

As new drugs are introduced onto the drug scene and require regulation, the Misuse of Drugs Act is amended. A total of thirty-five phenethylamines will be added to Part I of Schedule 2 of the Misuse of Drugs Act to become Class A controlled drugs. They will be listed in Schedule I of the Misuse of Drugs Regulations. A thirty-sixth phenethylamine, α-methylphenethylhydroxylamine will become a Class B controlled drug [17].

1.2 Forensic Toxicology

1.2.1 Introduction

Toxicology is the study of poisons and the term 'poison' is used to describe any substance which has a harmful effect on a living system. The toxicity of a drug does not only depend on its toxic properties but also on the dose administered. However, there is no direct relationship between the dose and the effects that it elicits. 'Forensic Toxicology' is defined as the study of the effects of drugs and poisons on human beings and the investigation of fatal intoxication's for the purpose of a medico-legal enquiry [18].

Sample types analysed include urine, liver, vitreous humour and brain but more commonly, blood. Alternative matrices, e.g. hair, saliva, sweat and nails [13] can also be analysed for the presence of potential poisons. As a consequence of the increase in demand for drug identification, it is the role of the forensic toxicologist to develop methods, which are both rapid and reproducible for the analysis of a wide range of analytes in biological matrices.

Systematic Toxicological Analysis (STA) is defined as the logical chemical - analytical search for an unknown substance of toxicological relevance [11]. The first step is to establish whether the compound can be detected (screening) and secondly, to identify the compound (identification and confirmation).

Biological samples have to undergo a series of sample pre-treatment steps, to remove any endogenous materials and to increase the drug concentration, before being introduced onto a chromatographic column.

Technological advances in chromatographic analytical equipment have afforded the scientist with the means of separating a complex mixture of analytes and confirming their presence. However, sample preparation techniques have not improved as quickly and this remains the limiting factor for a totally automated analysis and is the source of greatest variability [19].

1.2.2 Systematic Toxicological Analysis

The main purpose of 'Systematic Toxicological Analysis' (STA), is to detect and identify unknown substances in a given matrix [20]. Drug screening in systematic toxicological analysis is an extremely important preliminary step in the analysis of biological specimens and involves two main steps;

1. Sample Preparation (clean-up and extraction) and
2. Detection and Confirmation.

Sample pre-treatment produces a cleaner sample and leads to greater selectivity and precision, by eliminating any matrix effects, which may interfere with the analysis. The extraction is optimised through adjustment of the pH, ionic strength and sample concentration.

Traditionally, liquid-liquid extraction (LLE) techniques were used to separate analytes from biological matrices. However, they were found to be tedious, time consuming and were not always sufficiently reproducible.

Solid-phase extraction (SPE) emerged in the mid 1970's [21] as a simpler alternative to liquid-liquid extraction and has since proved to be an extremely effective means of extracting drugs from biological matrices with several distinct advantages over liquid-liquid extraction [20]. These include reduced solvent usage, cleaner extracts, no emulsion formation and considerable reduction in analysis times.

Several analytical techniques have been used for the screening of drugs, these include, Thin Layer Chromatography (TLC), Immunoassay, High Performance Liquid Chromatography (HPLC) and Gas Liquid Chromatography (GLC).
The ideal analytical method, should be specific, statistically defined (accuracy, precision and sensitivity), and routinely reliable [22].

In forensic toxicology, capillary gas chromatography and capillary gas chromatography – mass spectrometry are often the preferred methods for analyte confirmation and quantification [23].

1.3 Solid-Phase Extraction

1.3.1 Introduction

Solid-phase extraction (SPE) was introduced as a simpler alternative to liquid-liquid extraction (LLE) in the mid 1970's, to try and combat the problems encountered with this method [20].

The first bonded phases used were of hydrophobic character, e.g. C18 and XAD resins [24]. These single - phase columns showed great promise for the analysis of biological samples, however problems occurred with co-extraction of endogenous materials.

Ion-exchange columns were introduced in the 1980's and successfully extracted a wide range of compounds of differing chemistries, of both basic and acidic character.

Improvements with sample clean up and recoveries were also reported, however extraction of neutral compounds still remained a problem.

Mixed-mode bonded silicas, consisting of both hydrophobic and ion-exchange properties in one single column e.g., Clean Screen®, first introduced in 1986 [24], enabled the extraction of a range of drugs, including neutral compounds [25].

Solid-phase extraction is a powerful pre-treatment technique for the clinical and toxicological drug analysis of biological samples [26] and over the years has become more and more acceptable in forensic laboratories [20,27], having very distinct advantages over liquid-liquid extraction. These advantages include:

1. High selectivity
2. Cleaner extracts
3. No emulsion formation
4. Better reproducibility
5. Reduced solvent usage
6. Higher throughput due to automation [23,25,28]
7. Less contamination of GC system.

1.3.2 Principles of Solid-Phase Extraction

1.3.2.1 Introduction

The extraction procedure in solid-phase extraction, involves the interaction between a solid and a liquid phase, and is a physical extraction process, similar to liquid

chromatography [29]. However, the aims of solid-phase extraction are to isolate and then concentrate the compounds of interest, while liquid chromatography aims to separate the compounds, with good peak shape and height, and short retention times.

There are many different sorbent materials available, e.g. diatomaceous earth, silica and bonded silica gels. Bonded silicas are the most frequently used sorbent as they can be modified with different functional groups to produce highly stable non-swelling stationary phases which allow for a wider range of extraction capabilities. Secondary interactions also take place where the isolate molecules interact with the silica substrate as opposed to the functional group [29].

The extractions can be separated into three types, depending on the functional groups bonded to the silica; non-polar, polar and ion-exchange.

1.3.2.2 Non-Polar Interactions

Non-polar interactions result between the carbon-hydrogen bonds of the sorbent and of the isolate and are caused by "Van der Waals" or "dispersion forces". The majority of organic molecules have some non-polar character and so are retained on the surface of sorbents containing non-polar functional groups.

Octadecyl silane (C18) is the most frequently used non-polar sorbent and is non-selective, resulting in the retention of a variety of structurally unrelated isolates. Retention of isolates onto non-polar sorbents is facilitated by the use of polar solvents, while elution requires a solvent of sufficient non-polar character to disrupt the non-polar interactions between the isolate and the sorbent.

1.3.2.3 Polar Interactions

Groups with polar character include, hydroxyls, amines, aromatic rings and hetero-atoms, e.g. oxygen and nitrogen. These are retained on sorbents containing polar functional groups due to hydrogen bonding, dipole/dipole, induced dipole/dipole, pi-pi and other polar interactions resulting from the uneven distribution of electrons between individual atoms in the functional group.

Secondary interactions due to the polar character of the silica substrate are present in all bonded silicas. Retention of isolates onto polar sorbents is facilitated by the use of non-polar solvents, while elution requires a polar solvent of sufficiently high ionic strength to disrupt the polar interactions between the isolate and the sorbent.

1.3.2.4 Ionic Interactions

Ion-exchange occurs between groups present on the isolate and the sorbent with opposite charges. There are two classes of these groups: cationic (positively charged), e.g. primary, secondary, tertiary, and quarternary amines, and anionic (negatively charged), e.g. carboxylic and sulphonic acids. Depending on the pH of the solvent environment, these molecules can exhibit cationic or anionic character. Two criteria have to be met before the retention of an isolate by an ion-exchange mechanism can take place:

1. the pH of the system must be such that both isolate and sorbent are charged, and
2. the system must not contain high concentrations of strongly competing ionic species of the same charge as the isolate.

The pKa is defined as the pH at which half of the molecules in solution are charged and the other half are not. Increasing the pH to a value above the pKa of anionic molecules, increases the number of charged anionic groups and decreasing the pH, decreases the number of charged molecules. The opposite is found with cationic groups.

In order to achieve retention of ionic groups, both the sorbent and the isolate have to be charged, i.e. at a pH below the pKa of the cation and at a pH above the pKa of the anion. A pH of at least two pH units below the pKa of the cation and two pH units above the pKa of the anion is required for approximately 99% of the groups to be charged.

Other factors, which are important in ion-exchange, are ionic strength and selectivity. Ionic strength is a measure of the total ionic concentration of ionic species present within the solvent/matrix environment. Ion-exchange is a competitive process and so low ionic strength encourages retention of the isolates while high ionic strength disrupts it. Selectivity describes the preference shown by ion-exchange sorbents for certain types of ionic groups over others. Ionic secondary interactions also occur due to unbonded silanols on the silica substrate.

Retention of an isolate onto a sorbent matrix occurs through the interaction of a combination of the three extraction mechanisms, i.e. non-polar, polar and ion-exchange, these interactions are dependent on the sample matrix and solvent environments.

1.3.3 Procedural Steps in Solid-Phase Extraction

In its simplest form, a solid phase extraction procedure consists of five steps: column pre-conditioning, sample application, column wash, column drying and drug elution.

1.3.3.1 Column Preconditioning

Before applying a pre-treated sample to an extraction column, the active sites on the column have to be activated with a suitable solvent. Bonded silica sorbents are dried and packed and so are not available to interact with the analytes. Application of the sample before solvation would result in low and irreproducible recoveries.

1.3.3.2 Sample Application

Once the column is solvated, the sample is applied onto the column under a light vacuum. The flow rate of the sample through the column is very important, as a low flow rate allows the necessary time for the maximum amount of drugs to interact with the active sites on the column. Normally this would be 1.5 ml/min.

1.3.3.3 Column Wash

The wash step allows the removal of any endogenous components that were retained by the active sites. Water or an appropriate solvent is used to selectively remove any material that could interfere with the analysis. Selection of a solvent that will not remove any of the relevant drugs can be difficult and so a compromise must be reached between acceptable recoveries and removal of interfering compounds.

1.3.3.4 Column Drying

This is an extremely important step, especially when gas chromatography is used for the final analysis, as any residual water may cause damage to the GC column. The water may be removed by applying a vacuum to the column or by the addition of a solvent, e.g. methanol [27].

1.3.3.5 Elution of Relevant Drugs

In order to elute the drugs for analysis, a suitable solvent is chosen which will extract only the drugs of interest and leave any remaining matrix interferents on the column. The solvent should also be strong enough to displace all of the drugs into a small eluent volume. Large eluent volumes have the disadvantage of diluting the extract, extracting more impurities and increasing the extraction time. An evaporation step is usually included at this stage to concentrate the drugs, however care must be taken to prevent the loss of more volatile drugs, e.g. amphetamines.

1.3.4 Development of Solid-Phase Extraction

1.3.4.1 Introduction

The first materials to be used for the Solid-Phase extraction of urine samples were first reported in 1970 with the introduction of Amberlite® XAD-2 resins (cross-linked polystyrene - divinyl benzene by Rohm and Haas; Philadelphia, PA) [20]. Use of these resins included screening for drugs of abuse in urine [30] and more recently, the extraction of cocaine and benzoylecgonine from postmortem blood [21].

The need for large volumes of resin, multiple stepped analysis and the time required to complete the extraction made these phases expensive and difficult to use. Sep-Pak C_{18} cartridges were introduced in 1978 by Waters Associates (Milford, MA) [20]. These smaller columns decreased the analysis time and costs associated with drug analysis and proved useful for the extraction of tricyclic antidepressants from vitreous humour [31] and benzodiazepines from urine [32]. To obtain similar recoveries from plasma, a deproteinization step was required.

New methods reported for solid-phase extraction, outnumber those reported for liquid-liquid extraction, demonstrating the shift from liquid-liquid extraction to solid-phase extraction in both clinical and forensic toxicological laboratories. One other type of solid-phase used, is Extrelut (Merck, France) which has found applications in the analysis of propoxyphene and related compounds [33] and benzodiazepines [34] in blood. Extrelut is available as a prepacked column or in granular form.

Several single-mode columns have been introduced for the analysis of urine and plasma samples including Bond Elut C_2 [35], Bond Elut C_{18} [36] and Detectabuse GC/MS grade columns [37]. These columns contain non-polar sorbent materials (C_2, C_{18}) and methods have been reported for analysis of individual drugs and groups of structurally related drugs. Problems with low recoveries arose when screening samples for a wide range of drugs using a single-mode column. Lillsunde and Korte [38] used a Chem Elut™ (Analytichem International) column to extract 300 drugs from urine and reported recoveries ranging from 10 to 90%.

Cosbey *et al* [39], investigated the use of a two-stage process involving two cartridges with different phases, **1.** Supelclean-ENVIcarb (hydrophobic extraction using non-porous carbon) and **2.** Bondelut PRS cartridge (ionic extraction on a strong cation exchanger), to extract basic drugs from whole blood. Recoveries for the drugs studied were high, e.g. 89.9% for Quinine. However, the problems encountered with single-mode columns remained with this two-stage method as the second extraction can only separate the drugs extracted from the first column.

Logan *et al* [40], reported the use of a Bond Elut strong cation exchange (SCX) column (Analytichem), which contains silica particles with alkyl-bonded benzene-sulphonylpropyl, which combined both cation exchange and non-polar properties. This column was found to extract efficiently, over 100 basic drugs from urine, as well as certain drugs which proved difficult with liquid-liquid extraction e.g. morphine, benzoylecgonine, temazepam and oxazepam.

1.3.4.2 Mixed-Mode Columns

Improvements continued with the introduction of mixed-mode solid-phase extraction columns: Clean Screen™ DAU (Worldwide Monitoring Corporation: Horsham, PA), Bond Elut Certify™ (Varian Sample Preparation Products: Harbor City, CA) and narc2™spe (J.T. Baker: Phillipsburg, PA). These solid-phase extraction columns have provided the analyst with the means of selectively extracting a broad range of drugs from various biological matrices. The sorbent consists of both hydrophobic and cation exchange groups.

Clean Screen™ DAU columns have been used for the extraction of cocaine and its metabolites [41,42] from a variety of matrices and for screening plasma and urine for drugs of different physicochemical properties [28]. Bond Elut Certify™ has been used for the analysis of single or groups of related drugs in plasma [43], whole blood [31,44] and urine [45-46] samples.

The advantages of using a mixed-mode column as opposed to a single-mode column include, cleaner extracts and the ability to extract a wider range of drugs with different physicochemical properties. As these mixed-mode sorbents are contained in just one column, they have the added advantage of requiring less extraction steps and so are easier to use, as well as being more cost effective than the dual column procedure.

Chen *et al* [26], investigated the extraction of a broad range of acidic, neutral and basic drugs from plasma and urine using Bond Elut Certify columns and from whole blood using both Bond Elut Certify and Clean Screen DAU columns [27].

Sample pretreatment techniques are essential for the analysis of whole blood as the presence of red blood cells causes problems with solid-phase extraction. Direct application of whole blood onto an SPE column would result in the column becoming blocked and producing low and irreproducible recoveries. The problem associated with protein binding in plasma and serum, is of greater importance with whole blood. Sonication of the blood was needed to break up the red blood cells, in order to free the drugs which were bound to them, and the dilution step was utilised to reduce the viscosity of the sample and prevent blockage of the column.

1.3.4.3 Automation

Solid-phase extraction has a number of advantages over liquid-liquid extraction outlined previously and one of these is the possibility of automation of the procedure. Automation of present manual solid-phase extraction methods would ideally improve reproducibility, give higher throughput, reduce labour costs, and extractions could be carried out after working hours.

Automated solid-phase extraction systems available include, AASP® (Varian Sample Preparation Products: Harbor City, CA), ASPEC (Gilson: Middleton, WI), Millilab™ (Bedford: Milford, MA), Auto Speed™ (Applied Separations: Allentown, PA) and Zymate™ (Zymark: Hopkinton, MA).

Krogh *et al* [23], described an alternative method for the analysis of opiates in plasma and whole blood using the automated sequential trace enrichment of dialysate (ASTED) system for automated sample preparation. On-line dialysis is used as a purification step prior to chromatographic analysis, and with slight modifications can be used for the analysis of other basic drugs. Approximately 100 plasma or whole blood samples can be analysed in 24 hours.

Automated methods based on the extraction procedures used in manual SPE methods, tend to concentrate on analysis of individual or groups of related drugs and very few methods were reported for screening body fluids [25,28].

Chen *et al* [28], reported a semi-automated SPE procedure for screening using CleanScreen DAU columns using the ASPEC system. The extraction method used was based on the method reported by Chen *et al* [26], and the procedure was semi-

automated, as the collection tubes had to be changed manually to collect the two fractions (A and B).

The recoveries ranged from 73% for methamphetamine to 96.3% for methylphenobarbital, with relative standard deviations of less than 5%. The recoveries were comparable to those achieved by manual SPE methods, and the reproducibilities were better.

Chen *et al* [25], then reported an improved fully automated SPE procedure using the ASPEC system with Bond Elut Certify with recoveries ranging from 80% to 103%, with relative standard deviations less than 4.6%.

Hewlett Packard produce the HP7686 PrepStation allowing automated extraction of biological samples to be linked directly to a gas chromatograph with mass selective detection for analysis [47-48]. Researchers have reported turn around times of between 2.5 and 3 hours from positioning the sample on the PrepStation to the print-out of the search report [49-50].

The benefits of using a PrepStation are reported to include: reduced exposure to hazardous sample and chemicals, better quality results, decrease in analysis costs and unattended operation [51].

1.3.4.4 Solid-Phase Microextraction and Solid-Phase Extraction Discs

Solid-phase extraction discs are produced from the same sorbent phases as conventional solid-phase extraction columns, however they are shaped like thin discs. The larger cross-sectional area of the disc makes them ideal for the high flowrates of larger sample volumes.

Solid-phase microextraction (SPME) involves a coated fused-silica fibre. The fibre is introduced to the sample headspace and elution of the extracted analyte involves either thermal desorption (gas chromatography) or liquid desorption (high performance liquid chromatography).

Degel [52] conducted a comparison of various solid-phase extraction techniques by comparing the recoveries of various drugs from urine. Mixed-mode solid-phase extraction columns, liquid-liquid extraction and multi-modal discs gave the best recoveries with respect to the broad spectrum screening. Both mixed-mode columns, Bond Elut Certify™ LRC and Isolute™ Confirm HCX, performed well, however the HCX columns produced cleaner extracts.

SPME produced excellent recoveries for the more volatile substances, e.g. amphetamines. The extraction of amphetamines from urine and blood using SPME has been reported in various journals [53-55].

1.4 Aims

This project was a continuation of work carried out and completed for a Masters Degree in Forensic Toxicology [56]. Experimental mixed-mode solid-phase extraction columns with varying non-polar character were supplied by International Sorbent Technology to investigate the effect this would have on drug recoveries. A variety of drugs were extracted from whole blood under various conditions and the recoveries noted.

The initial aims of this project involved the development of improved extraction methods for the analysis of methadone and its metabolites from whole blood and for the extraction of amphetamine and related compounds from hair.

Both projects were extended. The aims were to:

Methadone

- develop an optimised extraction method for the analysis of methadone and its two major metabolites 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP) in whole blood,
- investigate the stability of methadone and its two major metabolites EDDP and EMDP in whole blood under various conditions,
- and to investigate the applicability of the Cozart methadone microplate enzyme immunoassay kit for the screening of methadone in hair.

Amphetamines

- To develop an optimised extraction method for the analysis of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylene-dioxymethamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA) in hair,
- adapt the developed method in hair to whole blood,
- and to investigate the effects on analyte recovery with the addition of tartaric acid to the extraction solvent.

The final aim of the project was to apply the developed methods for the analysis of forensic case samples.

2. Methadone

2.1 Introduction

Methadone is an opioid analgesic and although structurally unrelated to morphine, has similar pharmacological properties. Although methadone is often prescribed as a substitute for other opioids, an alarming quantity of illicit methadone is available to individuals not registered on a maintenance programme. Methadone is a class A, schedule 2 drug in the Misuse of Drugs Act 1971 (UK). It is illegal to possess drugs in schedule 2 without a prescription and class A drugs are thought to be the most harmful when misused and as a result, the penalties are the highest.

The number of fatalities confirmed positive for methadone has risen sharply in the West of Scotland since 1991, with the greatest increase coinciding with the introduction of methadone maintenance programmes in 1994 [10]. Of the 173 deaths identified in which methadone was detected, 51% (n = 89) were not enrolled on a methadone programme at the time of their death.

The increase in casework requesting methadone confirmations requires development of a robust routine method. In addition, successful analysis of methadone and its metabolites is important in order to fully understand the mechanisms involved in the metabolism of methadone. This is of particular significance for the analysis of biological samples in forensic and clinical applications.

2.1.1 Chemistry of Methadone

Methadone hydrochloride (6-dimethylamino-4,4-diphenyl-3-heptanone hydrochloride) was discovered by Max Bockmuhl and Gustav Ehrhart in Germany in 1937. A 2- dimensional structure (see figure 2.1), the molecule has two active sites, at the nitrogen atom with hydrochloride bonded to one end, which is thought to act on the peripheral nervous system. The two phenyl rings are thought to be necessary for its opiate-like action on the central nervous system.

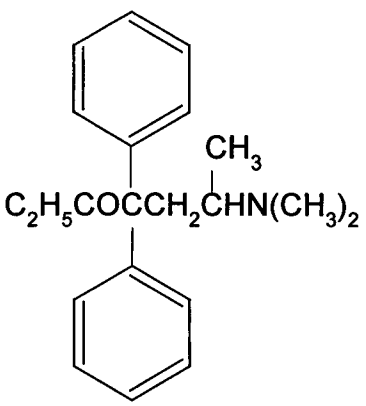


Figure 2.1 Methadone ($C_{21}H_{27}NO$)

Table 2.1 summarises some important physicochemical properties of methadone (a synthetic white or colourless crystalline powder).

Table 2.1 Properties of Methadone Hydrochloride

Property	Methadone
Molecular Weight (Free Base)	345.9 (309.5)
Melting Point (°C)	233 → 236
Solubility:	
in alcohol	1 in 7
in water	1 in 12
in chloroform	1 in 3
in ether	practically insoluble
Dissociation Coefficient	pKa 8.3
Distribution in Blood	
Plasma : Whole blood	1.3
Protein Binding (Plasma)	90%

2.1.2 History of Use and Abuse of Methadone

In 1947, Isbell *et al* [57] carried out experimental work with humans, animals and clinical work with medical patients and concluded that methadone had a high addiction potential.

In 1977, Methadone was described by Thompson *et al* [58] as one of the most commonly used and abused drugs in the United States. The number of addicts receiving methadone through maintenance treatment programmes had increased along with the methadone positive drug screens in medical examiners autopsy cases.

In the UK, the number of known methadone addicts increased from 21 in 1955 to 1687 in 1969, which was partly due to the setting up of clinics [59].

The first opiate addicts became addicted through treatment for pain relief. The early 1960's saw an increase in the number of opiate addicts, who were younger and taking the drugs more for pleasure not as medication. Heroin first overtook morphine as the most notified drug of addiction in 1962 and by 1966 there were 6 times as many heroin addicts as morphine addicts [59]. 1968 saw the introduction of new drug clinics and the number of notified addicts rose to 2881 with 2240 addicted to heroin.

The 1970's saw the importation of illicit heroin for the first time. Drug clinics moved from supplying injectable heroin to oral methadone, which was thought to be more therapeutic, due to prescribing non-injectable drugs. The longer half-life of methadone meant that it could be taken once daily, rather than every few hours. Many of the new addicts at the clinics smoked heroin and therefore the clinics looked on their role as promoting change and increasingly moved towards the use of oral methadone.

The 1980's saw the second increase in heroin use, notified addicts increased slowly through the 1970's from 509 (1973) to 607 (1979) to 1110 (1979), doubled from 1979 to 1982 and doubled again by 1984 [60].

Glasgow introduced methadone maintenance programmes in 1994, almost a decade after Edinburgh [61]. Prior to this, 1986, Scotland had been relatively methadone free [62].

2.1.3 Disposition in the Body

Methadone is soluble in body fats and following oral administration is rapidly absorbed from the gastrointestinal tract into the blood stream. Distribution of methadone occurs throughout various tissues, especially the lungs, kidneys, liver and spleen. There is slow transfer of the drug between the organs and blood, and methadone concentrations in these organs are higher than in blood.

Methadone is primarily broken down in the liver and the main metabolic reaction is mono- and di-N-demethylation, followed by spontaneous cyclization to form two major metabolites, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP) [63]. This metabolic pathway is summarised in figure 2.2.

There are at least eight known metabolites [64], but with the exception of the small amounts of methadol and normethadol produced, none of the metabolites are pharmacologically active [65].

Methadone and its metabolites EDDP and EMDP are eliminated in urine and faeces and are also excreted in saliva and sweat. High concentrations have been detected in the gastric juices and during pregnancy, methadone concentrations in the placental cord blood are approximately half the maternal level [59]. Methadone levels in saliva and gastric juice can be as high as 54 and 100 times those measured in blood [66-67], respectively. Previously, the metabolites could not be detected in whole blood or

plasma [68], however the primary metabolite, EDDP was detected in whole blood and its structure confirmed by GC/MS [66].

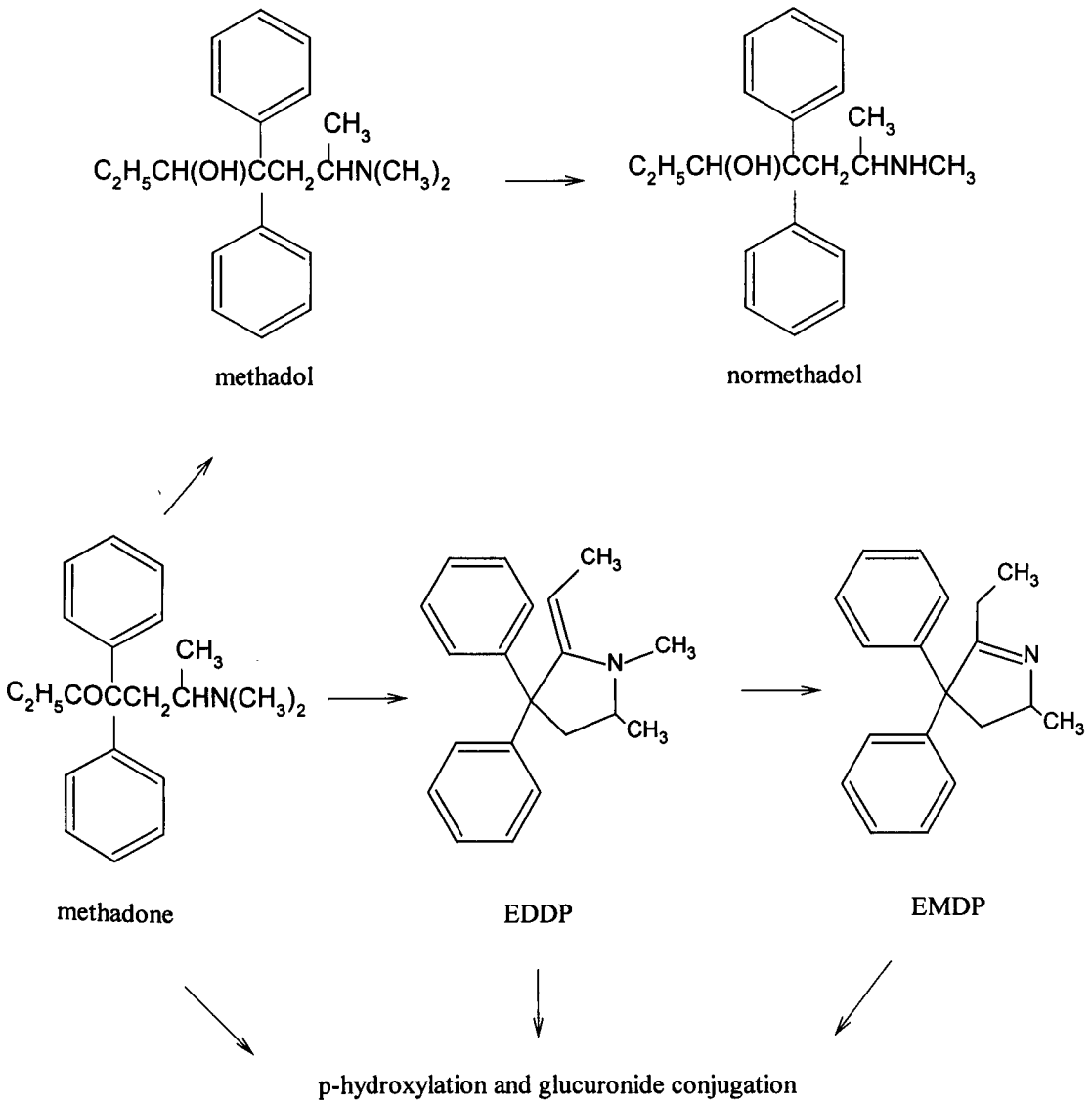


Figure 2.2 Metabolism of Methadone

In patients following a methadone maintenance programme, 20 to 60% of the dose is excreted in urine over 24 hours. Up to 33% as unchanged methadone and up to 43% as EDDP, with EMDP accounting for only 5 to 10% of the dose. When comparing the drug levels of inpatients on a methadone maintenance programme to overdose cases, the ratio of EDDP to unchanged methadone is lower in the simple overdose cases [64]. The concentration of unchanged drug in urine is pH-dependent, being greater in acidic urine.

2.1.4 Toxicity

Methadone is one of the strongest opiates, and the combination of its delayed action and a long half-life, can result in severe respiratory depression which is usually the cause of death. In addition, muscle flaccidity, cold and clammy skin, pupillary constriction, hypotension and coma, are all characteristic symptoms of overdosage with methadone. [63]

The minimum lethal dose in non-tolerant adults is estimated at 50mg, while addicts on a methadone maintenance treatment programme may tolerate doses as high as 200mg or more [64]. The lethal dose decreases in the presence of alcohol.

Nelson and Selkirk [69] gave details of 12 fatal methadone poisonings. Two of the cases had no previous history of methadone abuse and was thought to be the result of naiveté with the injection of narcotic drugs and a lack of tolerance to methadone. Methadone was found to be solely responsible for the deaths in four of the cases, with the remainder being attributed to multiple drug overdoses. Only one case involved an amount of drug other than methadone, consistent with overdose levels.

Tissue concentrations ranged in liver (0.05 - 2.8μg/g), stomach contents (0 - 7.0μg × 10^3), gall bladder (4.5-130 μg total), urine (0.2-25.4μg/ml), and blood (trace - 0.3μg/ml). However, as blood methadone levels were not determined in all the cases, no firm conclusions could be drawn.

A study carried out by Clark *et al* [70], of methadone deaths in Sheffield, in the UK, included details of 18 fatalities between the years of 1991 and 1994. Ten of these had been in receipt of regular methadone, with seven of the ten having only recently started taking methadone. Post-mortem blood levels ranged from 200 – 1863ng/ml, with a mean of 560ng/ml. 16 of the 18 deaths had other drugs present but the concentrations were low, and methadone was more significant.

In Harris County, Texas, 91 deaths were recorded between January 1987 and December 1992 with methadone positive post-mortem drug testing. Blood methadone levels ranged from < 0.02 to ≥ 0.1mg/dl. However, the authors, Barrett *et al* [71], pointed out that there was disagreement as to what constitutes a toxic or fatal blood methadone level. Irey and Froede [72], gave toxic levels ranging from 0.02mg/dl to 0.45mg/dl. The Harris County Medical Examiner regards toxic levels to range from 0.1 to 0.2mg/dl, whereas, McBay considers toxic levels to be 0.3mg/dl or greater [73].

Interpretation of blood methadone levels is very difficult due to drug interaction. Many methadone positive drug screens include positives for other drugs, and a given blood methadone level may or may not be toxic, depending on the presence of these drugs, which may augment or counteract the toxic effects of methadone [71].

Hendra *et al* [74] reported the case of a 22-year-old man who overdosed on 420mg of methadone, 3 hours before admittance to a hospital. Femoral methadone blood concentration was 822ng/ml, stomach contents = 7ng/ml. The following benzodiazepines were also detected at the respective concentrations, diazepam (103ng/ml) and nordiazepam (165ng/ml).

A study of methadone positive fatalities in Strathclyde [10] reported 91% (n = 157) of cases were positive for at least one other drug. Morphine was detected in almost one third of decedents enrolled on a methadone maintenance programme. The most frequently encountered drugs in combination with methadone were diazepam (47%), temazepam (46%), alcohol (40%), morphine (30%) and cannabis (12%).

2.2 Analysis

Various techniques have been used to detect methadone in biological specimens, including, immunoassay [75-78], thin-layer chromatography (TLC) [79], high performance liquid chromatography (HPLC) [80-83], gas liquid chromatography (GLC) [69,84-86], and gas chromatography / mass spectrometry (GC/MS) [75,87-91].

Immunoassay techniques have been used for the determination of methadone in haemolysed whole blood [76] and serum [77], and have proved useful in a clinical setting for quantitative urine drug monitoring [78] and rapid measurement of plasma methadone levels [75] in methadone maintenance programmes. These techniques are very sensitive but are not specific and cannot distinguish between methadone and it's two major metabolites.

Metabolites of Verapamil were reported as causing false-positive results in a methadone enzyme immunoassay [92-93]. Specific immunoassays for EDDP have been developed by CEDIA® [94] and help identify methadone false negatives, i.e. individuals who have a high metabolism (methadone negative, EDDP positive) and cases where samples have been "spiked" with methadone (methadone highly positive, EDDP negative).

Rio *et al* [95], reported a method using high performance liquid chromatography (HPLC) to analyse methadone, propoxyphene and norpropoxyphene in post-mortem blood and tissues, which was linear from 0.1 to 10mg/l. Logan *et al* [40] described a method using the combination of solid-phase extraction (SPE) and a gradient elution HPLC system using diode array detection (DAD). HPLC-DAD increased the range and specificity of HPLC for the screening of 100 basic drugs and metabolites in urine, which included methadone. However, gas chromatographic methods are by far the method of choice.

Hartvig and Naslund [96] developed an improved method, using electron capture detection to quantitate the benzophenone produced by the oxidation of methadone. Several methods have been described for the analysis of urine [68,89,97-99], which did not require an oxidation step. Schmidt *et al* [100] were able to analyse urine, cerebrospinal fluid and plasma using one method, with a limit of quantitation for methadone of 0.5ng/ml.

Early analyses of blood samples involved liquid-liquid extraction (LLE) as sample pre-treatment, prior to injection onto a gas chromatograph [58,66,101-102]. All of these

methods detected either methadone alone or in conjunction with its primary metabolite, EDDP, but did not include EMDP (secondary metabolite). Thompson *et al* [58] determined the recovery of EMDP to be in the range of 30-40%. An explanation for this was, EMDP is a more active substrate for hydroxylation than EDDP, and would therefore be conjugated and not detected by this procedure [103]. The analysis of EMDP was not continued in the above study for the reason stated and because the metabolite was not detected in the medical examiners cases studied.

Several authors have reported the analysis of EMDP in biological samples other than blood [66,88,91,104]. Quantifiable levels were detected in urine, and in one paper, trace levels, below the limit of quantitation, were detected in rat hair [104]. The recoveries of EMDP from hair [104] and whole blood [58] were very low at 53% and 30-40% respectively.

Detection of methadone and its metabolites in hair has great potential as a means of monitoring patients compliance on maintenance programmes [105]. Several papers have reported methods for determining methadone in hair using acidic [106-107] or alkaline digestion [104,108-109], β-glucuronidase [110] and methanol [111-112]. The preferred method of analysis involves gas chromatography/mass spectrometry with various detectors. Radioimmunoassays have been used successfully to detect methadone in hair [109,113-114] however the problem of false positives was greater [108]. No papers identified during the literature search reported the use of enzyme immunoassay (EIA) as a means of detecting methadone in hair, although most EIA kits available for biological fluid analysis, claim to be able to screen for drugs of abuse in hair.

Advances in solid-phase extraction have seen the advent of solid-phase extraction discs. Rudaz *et al* [115] evaluated the efficiencies of discs and mixed-mode cartridges with respect to retention, losses during sample loading, washing and elution using radiolabelled methadone. They concluded that the discs did not perform as well as conventional cartridges caused, in part, by the limited capacity of the discs which resulted in loss of radiolabelled methadone

Several internal standards (IS) have been used when analysing methadone including, SKF-525-A [58,66,68,97,101], dextropropoxyphene [100] and Chirald [102]. Lynn *et al* [66] synthesised SKF-525-A (2-dimethylamino-4,4-diphenyl-5-nonanone), which was described as the ideal internal standard having several advantages over those previously described. SKF-525-A is chemically similar to methadone, and because of the basic centre, could be added at the beginning of the extraction procedure.

2.3 Aims

A great deal of work has been published with respect to the analysis of methadone and its primary metabolite, EDDP in various biological fluids. However, with the increase in deaths involving methadone, the development of a robust routine method for the analysis of methadone and both metabolites (EDDP and EMDP) from whole blood would be advantageous to the forensic toxicologist. In addition, a method optimised for both metabolites would aid in the understanding of the mechanisms involved in the metabolism of methadone.

Following on from previous work [56], the solid-phase extraction method used by Chen *et al* [27], was utilised as it was possible to screen for a variety of drugs with different physicochemical properties. Previous work, using this method, produced methadone recoveries from whole blood between 82.7 and 89.1% using mixed-mode solid-phase extraction cartridges.

The mechanisms involved with the drug retention properties of mixed-mode cartridges was investigated using methadone and its two metabolites extracted from whole blood on cartridges of differing non-polar character.

Chirald was chosen as the internal standard (IS), as it was commercially available and had been used successfully in the past for methadone analysis.

The majority of methods reported for the analysis of methadone in hair involved long and time consuming extraction methods followed by GC/MS confirmation. Initially RIA was used however when compared with GC/MS, RIA identified false positives.

Although enzyme-immunoassay (EIA) kits are available for detecting drugs of abuse in hair, no literature was identified reporting relevant research. A methadone specific EIA kit was obtained from Cozart Bioscience Limited (Oxfordshire, UK) and was investigated as an alternative means of detecting methadone in hair samples. Of particular interest was the detection of methadone in post-mortem hair obtained from known drug users. Obtaining additional information from segmental hair analysis could potentially provide a complimentary technique to post-mortem blood analysis or as an alternative when blood is not available.

2.4 Experimental

2.4.1 Chemicals

Methadone, EDDP and Chirald ((2S,3R)-(+)-4-Dimethylamino-1,2-Diphenyl-3-Methyl-2-Butanol) were obtained from Sigma® Chemical Co., (Dorset, UK). EMDP was obtained from Alltech Associates (State College, PA) and deuterated, d_3-methadone, was obtained from High Standard Products Corporation (Inglewood, CA). HPLC-grade methanol, acetone, chloroform and ethyl acetate were obtained from Lab-Scan Analytical Sciences (Dublin, Ireland). Analytical-grade ammonium hydroxide and glacial acetic acid and HiPerSolv grade potassium-dihydrogen phosphate (KH_2PO_4) were obtained from Merck (Poole, UK).

2.4.2 Stock Standards

Individual stock standard solutions (1mg/ml) of methadone, EDDP and EMDP were prepared in methanol. Combined working solutions of all three drugs were prepared in methanol at various concentrations (100, 10, 1μg/ml) by appropriate dilution of the stock standard. Blood samples were spiked by evaporating 100μL of the working solution to dryness under a stream of nitrogen at room temperature and reconstituting in 1ml of drug free whole blood or buffer before sonicating for 5 minutes. The internal standards, Chirald and d_3-methadone, were prepared in methanol to give stock standard solutions of 1mg/ml. Working solutions of Chirald (3μg/ml) and d_3-methadone (1μg/ml) were prepared by dilution of stock standards with methanol. All stock and working solutions were stored at -20°C.

2.4.3 Instrumentation

2.4.3.1 Gas Chromatography

A Hewlett-Packard model 5890 Series II gas chromatograph equipped with a flame ionisation detector (GC-FID) was used for recovery studies. A Hewlett-Packard, HP-1, cross-linked methyl siloxane capillary cartridge,(30m, 0.25mm i.d., film thickness 0.25μm) was installed. The initial oven temperature of 80°C was held for 2 minutes. The temperature was increased to 215°C at a rate of 20°C/min. and then to a final temperature of 285°C at a rate of 5°C/min. and held for 2 min. at 285°C. The total analysis time was 24.75 min. The injector and detector temperatures were 275°C and 310°C, respectively. Helium was used as the carrier gas. Injections of 2μL were carried out in the splitless mode. Hewlett Packard ChemStation B.02.04 computer software was used for data collection and processing.

2.4.3.2 Gas Chromatography – Mass Spectrometry

A Fisons model GC8000 series fitted with a HP-1 capillary cartridge was used for case sample analysis. The initial column temperature of 100°C was increased to 300° C at a rate of 15°C/min. and held for 5 minutes. The injector temperature and interface temperature were 280°C and 250°C, respectively. The carrier gas was Helium. Injections were performed in the splitless mode.

The GC8000 was interfaced to a Fisons MD800 mass spectrometer and was operated in the electron - ionisation mode at 70eV. The ion source temperature was 250°C and the trap current was 70μA. Selected ion monitoring data was collected using a Lab Base 2, Release 2.11 data system. The ions monitored were m/z 72*, 165, 294 (methadone), m/z 178, 220, 277* (EDDP), m/z 178, 193, 208* (EMDP) and m/z

75(d_3-methadone). The ions labelled "*" were used for quantitation. 1μL injections were carried out using a Fisons AS800 autosampler.

2.4.4 Chirald ((2S,3R)-(+)-4-Dimethylamino-1,2-Diphenyl-3-Methyl-2-Butanol)

(2S,3R)-(+)-4-Dimethylamino-1,2-Diphenyl-3-Methyl-2-Butanol (Chirald) was chosen as the internal standard due to its similar physical and chemical properties to methadone, EDDP and EMDP. Chirald is commercially available and has been used previously as an internal standard for the analysis of methadone in whole blood samples [102]. (see figure 2.3)

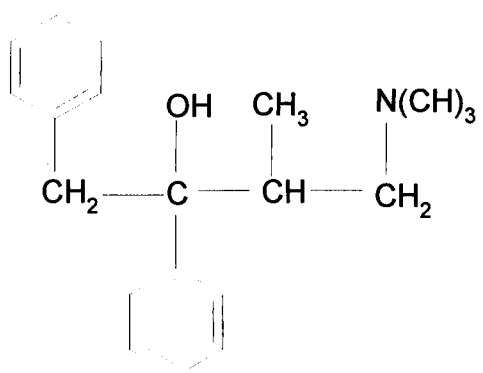


Figure 2.3 (2S,3R)-(+)-4-Dimethylamino-1,2-Diphenyl-3-Methyl-2-Butanol (Chirald)

2.4.5 Optimisation of GLC Drug Separation

2.4.5.1 Experimental

An HP-1 capillary cartridge (30m × 0.25mm i.d., 0.25μm film thickness) was used as it had been shown previously to produce good retention and peak shape for methadone [56].

Three different temperature programmes were investigated to determine the optimum separation of methadone, EDDP, EMDP and Chirald (IS). These programmes are summarised in Table 2.2.

Table 2.2 Temperature Programmes Used for Optimal Drug Separation

Programme	Initial Temp. (°C)	Rate	Final Temp. (°C)	Total Run Time (mins.)
1	100 (2 mins.)	20°C/min.	290(2mins.)	13.5
2	100 (2 mins)	230°C (20°C/min.) → 240°C (2°C/min.) → 290°C (20°C/min.)	290(2mins.)	20
3	80 (2 mins.)	215°C (20°C/min.) → 285°C (5°C/min.)	285(2mins.)	24.75

2.4.5.2 Results and Discussion

The retention times of all four analytes at each of the temperature programmes are summarised in Table 2.3.

Table 2.3 Retention Times of Analytes at Temperature Programmes

Programme	Retention Times (Rt – minutes)			
	EMDP	**EDDP**	**Chirald**	**Methadone**
1	8.946	9.428	9.547	9.993
2	8.978	9.596	9.767	10.521
3	9.049	9.760	9.951	10.745

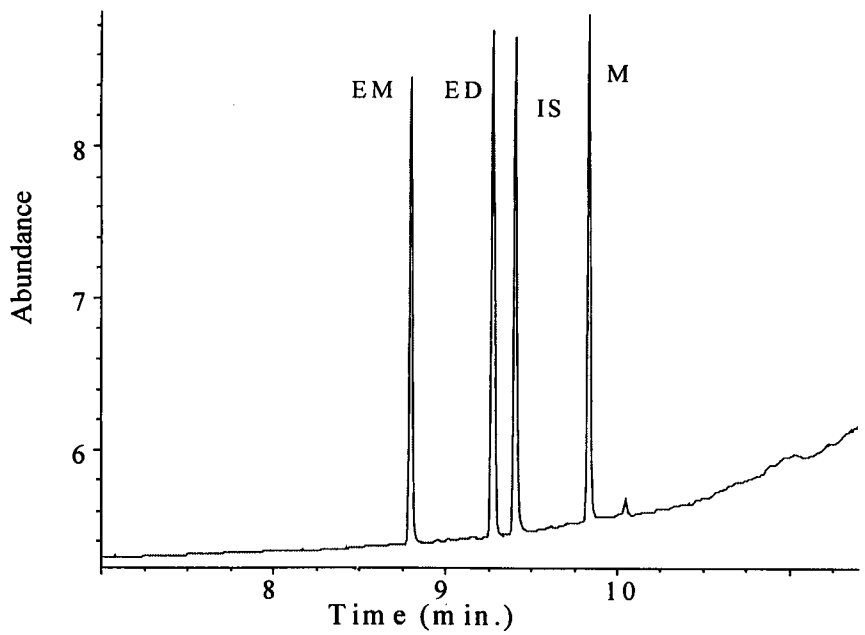


Figure 2.4 Chromatogram showing baseline separation between EMDP, EDDP, Chirald and methadone.

As can be seen more clearly in Figure 2.4, the separation between Chirald (IS) and EDDP (ED) is of greater importance as both EMDP (EM) and methadone (M) show good baseline separation. Although temperature programme 3 has the longest run time, the baseline separation between Chirald (IS) and EDDP (ED) with this programme is better.

A combination of good analyte separation and short run time is ideal. The run time could be shorter, however this programme was utilised in previous work [56] for the screening of several basic drugs from whole blood. The possibility that this method could be used in future work for screening a wide range of basic drugs was more favourable to a shorter analysis time.

2.4.6 Retention Indices

2.4.6.1 Experimental

The retention indices were determined for each of the four analytes using a mixture of seven alkanes (C_{15}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26}) prepared in hexane. The retention index (RI) is related to the retention time, but is more reliable and more useful for comparability and reproducibility of retention data. 1µl was injected onto the GC using the optimised temperature programme.

The retention index RI(A) of a compound A can be calculated using the following equation:

$$RI(A) = [RI(Y) - RI(X)] \frac{\log[t(A) \div t(X)]}{\log[t(Y) \div t(X)]} + RI(X) \quad .....(1)$$

where,

t(A) = net retention time of substance A

t(X) = net retention time of the n-alkane C_xH_{2x+2} eluting immediately before A

t(Y) = net retention time of the n-alkane C_yH_{2y+2} eluting immediately after A

RI(A), RI(X), etc. = retention index of substances A, X, etc...

2.4.6.2 Results and Discussion

The calculated retention indices are summarised in Table 2.4 along with the average retention times from multiple analyses (n = 6). Unfortunately no retention indices are noted in the literature for EMDP, EDDP or Chirald for comparison.

Table 2.4 Retention Indices

Analytes	Retention Index [116]	Calculated RI (Eqn.1)	Average Rt (mins.)
EMDP	-	1944	9.051
EDDP	-	2036	9.763
Chirald	-	2059	9.952
Methadone	2145	2148	10.747

2.4.7 Reproducibility of Retention Times

2.4.7.1 Experimental

An assessment of the reproducibility of retention times was carried out. 1µl of a methanolic standard mixture of EMDP, EDDP, Chirald and methadone (2mg/l) was injected six times.

2.4.7.2 Results and Discussion

The mean retention time, standard deviation (Std. Dev.) and coefficient of variation (%C.V) were calculated for each analyte and are summarised in Table 2.5.

Table 2.5 Reproducibility of Retention Times

Run	EMDP	EDDP	Chirald	Methadone
1	9.049	9.760	9.951	10.745
2	9.050	9.762	9.951	10.746
3	9.051	9.762	9.952	10.747
4	9.050	9.762	9.951	10.746
5	9.052	9.764	9.953	10.747
6	9.053	9.765	9.955	10.750
Mean	9.051	9.763	9.952	10.747
Std Dev.	0.0015	0.0018	0.0016	0.0017
% C.V.	0.016	0.018	0.016	0.016

The coefficients of variation are extremely low in each case indicating excellent reproducibility.

2.4.8 Evaporation Study

2.4.8.1 Experimental

The absolute recoveries of methadone, EDDP and EMDP were determined after evaporating the standard mixture to dryness at 40°C. 100µl of the working solution (1µg/ml) was evaporated to dryness and then reconstituted in 100µl of the internal standard working solution (1µg/ml). The absolute recovery of Chirald (IS) was determined by evaporating 100µl of the working solution (1µg/ml) to dryness and then reconstituted in 100µl of the standard mixture (1µg/ml).

Peak area ratios (P.A.R.'s) were determined for EMDP, EDDP and methadone in relation to the internal standard, Chirald. The P.A.R.'s for each sample were compared to that of the standard to determine the analyte recoveries. The recoveries of Chirald were determined in comparison to EDDP.

In addition, the relative recoveries were determined for each analyte after evaporation of a standard containing methadone, EDDP, EMDP and Chirald at 40°C and 50°C. The samples were reconstituted in 2ml, 2% ammoniated ethyl acetate and were evaporated to dryness at each temperature to determine the extent of analyte loss. The samples were finally reconstituted in 100µl ethyl acetate.

2.4.8.2 Results and Discussion

The absolute recoveries determined at 40°C are summarised in Table 2.6.

Table 2.6 Absolute Recoveries of Methadone, EDDP, EMDP and Chirald (IS)

Sample	EMDP	EDDP	Methadone	Chirald (IS)
1	102	99.6	101	99.2
2	99.8	98.9	102	99.0
3	101	100	100	98.6
Mean	101	99.5	101	98.9
Std. Dev.	1.1	0.6	1.0	0.3
C.V. (%)	1.1	0.1	0.1	0.03

All analytes were stable after evaporation at 40°C.

The relative recoveries obtained at 40°C and 50°C are summarised in Tables 2.7 and 2.8 respectively.

The average recoveries at 40°C were greater than 94.1% with very low coefficients of variation. However, at 50°C the recoveries ranged from 86.0 to 117% with higher coefficients of variation (> 3.23%). At 50°C there was greater loss of both EMDP and Chirald. As a consequence, evaporation of samples containing EMDP, EDDP, Chirald and methadone was conducted at 40°C.

Table 2.7 Analyte Recoveries at 40°C

Sample	EMDP	EDDP	Methadone
1	98.5	93.6	103
2	98.3	94.2	103
3	98.6	93.6	103
4	95.9	94.0	103
5	101	95.0	105
Mean	98.5	94.1	103
Std. Dev.	1.81	0.58	0.89
C.V.(%)	1.83	0.61	0.87

Table 2.8 Analyte Recoveries at 50°C

Sample	EMDP	EDDP	Methadone
1	85.8	115	107
2	85.6	113	109
3	75.7	116	109
4	104	123	130
5	79.0	117	109
Mean	86.0	117	113
Std. Dev.	10.95	3.77	9.65
C.V.(%)	12.7	3.23	8.56

2.5 Whole Blood

2.5.1 Development of Extraction Procedure

The extraction method developed by Chen *et al* [27] was used successfully to extract methadone from whole blood using the experimental mixed-mode solid-phase extraction cartridges which are also investigated in this project [56]. The extraction was carried out using 0.1M phosphate buffer at pH6 and pH7.4. The results are summarised in Table 2.9.

Table 2.9 Percentage Methadone Recoveries from Whole Blood (n = 6) [56]

Cartridge	pH6.0		pH7.4	
	%	Std. Dev.	%	Std. Dev.
HCX	89.1	2.1	98.4	1.2
HCX-2	84.8	3.3	102	1.0
HCX-3	88.3	3.5	84.7	2.4
HCX-4	86.9	1.6	60.4	3.6

These experiments were repeated using whole blood spiked with EMDP, EDDP and methadone (1μg/ml), to determine the optimum extraction conditions.

2.5.2 Solid-Phase Extraction of Whole Blood

2.5.2.1 Experimental

Analyte recoveries were determined for EMDP, EDDP and methadone using the methods developed in previous work [56]. This involved spiking whole blood and

extracting the analytes of interest from the blood using the experimental mixed-mode solid-phase extraction cartridges. 0.1M phosphate buffer was used at pH6 and pH7.4. Chirald was used as the internal standard and was added to the extraction solvent prior to evaporation. The percentage recoveries were determined by comparing the peak area ratios for the spiked samples to the unextracted standard .

2.5.2.2 Results and Discussion

The recoveries of EMDP, EDDP and methadone from whole blood were determined at pH6 and pH7.4 and the results are summarised in Tables 2.10 and 2.11.

Table 2.10 Extraction Recoveries from Whole Blood (pH6)

Cartridge	EMDP	EDDP	Methadone
HCX	38.4	83.1	94.0
HCX-2	36.2	84.5	93.4
HCX-3	40.1	88.5	99.3
HCX-4	39.2	76.9	87.5
Mean	38.5	83.3	93.6
Std. Dev.	1.67	4.81	4.83
C.V.(%)	4.3	5.8	5.2

Table 2.11 Extraction Recoveries from Whole Blood (pH7.4)

Cartridge	EMDP	EDDP	Methadone
HCX	72.7	98.9	107
HCX-2	69.0	86.3	105
HCX-3	68.0	90.0	108
HCX-4	69.5	95.4	105
Mean	69.8	92.7	106
Std. Dev.	2.03	5.60	1.50
C.V.(%)	2.9	6.0	1.4

The increase in average recoveries from using pH6 to pH7.4 phosphate buffer is more pronounced than was evident in previous work (See Table 2.9). Possible explanations for this include:

- Lot-to-lot improvement
- Improvement of extraction technique
- Freshness of buffer

The recommended shelf-life of phosphate buffer is one month. However, significant loss in recoveries with buffers older than three to four weeks was observed. The example summarised in Table 2.12 compares the average recoveries for EMDP, EDDP and methadone for HCX-3 mixed-mode cartridges with fresh buffer and buffer prepared four weeks earlier.

Table 2.12 Comparison of extraction recoveries using fresh and four week old phosphate buffer (pH7.4, 0.1M)

Analyte	Fresh Buffer	4 Week Old Buffer
EMDP	68.0	7.1
EDDP	90.0	73.8
Methadone	108	87.8

Subsequently phosphate buffer was discarded after two weeks and always stored in a refrigerator until the expiry date.

In order to optimise the extraction conditions, the recoveries were noted for EMDP, EDDP and methadone from whole blood using 0.1M phosphate buffer over a range of pH values.

2.5.3 Effect of pH

2.5.3.1 Experimental

Analyte recoveries were determined from spiked whole blood at six different pH values (6, 7.4, 8, 9, 10 and 11). This was carried out using each of the experimental mixed-mode SPE cartridges.

2.5.3.2 Results and Discussion

The average recoveries (n=3) for each analyte are summarised in Tables 2.10, 2.11 and 2.13 to 2.16.

Table 2.13 Extraction Recoveries from Whole Blood (pH8)

Cartridge	EMDP	EDDP	Methadone
HCX	88.2	89.6	100
HCX-2	93.3	93.9	98.0
HCX-3	89.0	82.1	85.4
HCX-4	98.4	91.1	99.8
Mean	92.2	89.2	95.8
Std. Dev.	4.69	5.04	6.99
C.V.(%)	5.1	5.7	7.3

Table 2.14 Extraction Recoveries from Whole Blood (pH9)

Cartridge	EMDP	EDDP	Methadone
HCX	86.7	71.7	102
HCX-2	81.5	60.8	101
HCX-3	75.8	65.2	103
HCX-4	87.3	68.4	102
Mean	82.8	66.5	102
Std. Dev.	5.36	4.65	0.82
C.V.(%)	6,5	7.0	0.8

Table 2.15 Extraction Recoveries from Whole Blood (pH10)

Cartridge	EMDP	EDDP	Methadone
HCX	89.7	55.9	107
HCX-2	77.8	49.3	99.6
HCX-3	72.5	57.9	103
HCX-4	84.2	59.6	99.5
Mean	81.1	55.7	102
Std. Dev.	7.49	4.51	3.55
C.V.(%)	9.2	8.1	3.5

Table 2.16 Extraction Recoveries from Whole Blood (pH11)

Cartridge	EMDP	EDDP	Methadone
HCX	71.3	71.7	102
HCX-2	82.2	60.8	101
HCX-3	62.5	65.2	103
HCX-4	76.2	68.4	102
Mean	73.1	66.5	102
Std. Dev.	8.33	4.65	0.82
C.V.(%)	11.4	7.0	0.8

There is little variation in recoveries between cartridges at each pH, represented by the relatively low standard deviations and coefficients of variation for each analyte summarised in Tables 2.10, 2.11, 2.13-2.16.

Table 2.17 Effect of pH on Mean Extraction Recoveries

pH	EMDP	EDDP	Methadone
6	38.5	83.3	93.6
7.4	69.8	92.7	106
8	92.2	89.2	95.8
9	82.8	66.5	102
10	81.1	55.7	102
11	73.1	34.6	89.1
Mean	72.9	70.3	98.1
Std. Dev.	18.61	22.5	6.31
C.V.(%)	25.5	32.0	6.4

Each of the average recoveries was the result of triplicate analyses. Methadone was least affected by the changes in pH compared with EDDP and EMDP. Table 2.17 summarises the mean recoveries obtained using all four mixed-mode cartridges at each of the pH values.

In order to obtain the optimum recoveries for all three analytes, phosphate buffer at pH8 was utilised in the extraction method. The effects of pH on the recoveries of EMDP, EDDP and methadone from whole blood, is illustrated in Figure 2.5.

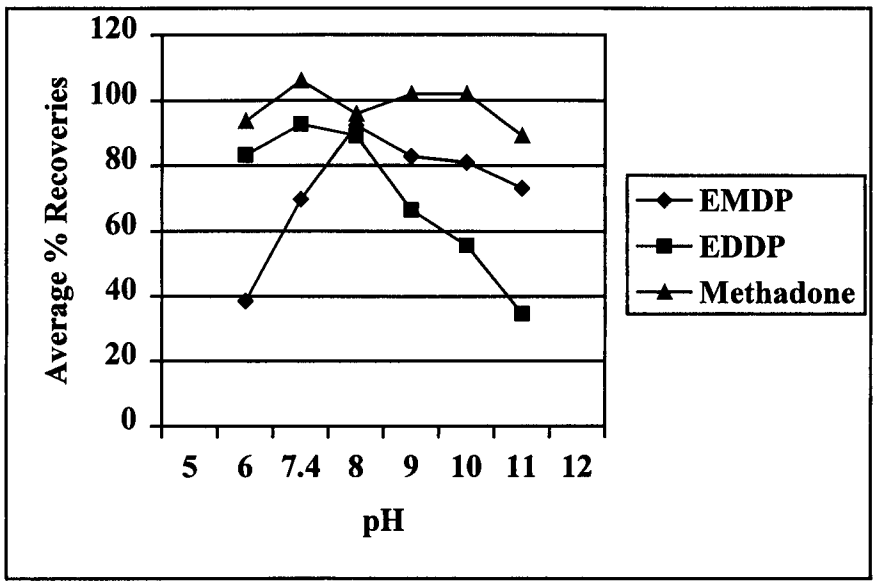


Figure 2.5 Optimisation of extraction pH for EMDP, EDDP and methadone.

2.5.4 Analysis of Fraction B Only

The total analyte recovery was determined by analysing Fractions A and B separately and adding the recoveries. Although this resulted in a lengthy analysis time for each extraction, combining Fractions A and B before evaporation resulted in significantly lower recoveries of all three analytes. Chen *et al* [26] reported similar results when combining the two fractions. When the two fractions were combined after evaporation, that is, reconstituting Fraction B in 50μL of ethyl acetate and then using this volume to reconstitute Fraction A, the recoveries were still unacceptably low.

The total percentage recoveries of EMDP, EDDP and methadone were compared to the % recoveries obtained in Fraction B alone. Table 2.18 summarises the recoveries using HCX-2 cartridges and pH8 phosphate buffer.

Table 2.18 Percentage Recoveries of Methadone, EDDP and EMDP using Isolute Confirm HCX-2* at pH 8.

pH 8	Total (A + B)†	Fraction B Only
Methadone	98.4 ± 4.4	98.4 ± 4.4
EDDP	91.0 ± 5.1	82.0 ± 0.6
EMDP	93.1 ± 0.9	81.0 ± 1.4

* n =3

† Fraction A + Fraction B

The recoveries were greater than 80% for all three analytes in Fraction B alone. Analysing only Fraction B, decreased analysis time by half without jeopardising extraction reproducibility.

2.5.5 HCX-5 and HCX-6 Mixed-Mode Cartridges

2.5.5.1 Experimental

Two additional experimental mixed-mode SPE cartridges were manufactured prior to the completion of this project. The sorbent mixture in these two cartridges contains different non-polar character to the other mixed-mode cartridges investigated in this project. HCX-5 and HCX-6 contain C_4 and C_6 non-polar phases respectively.

Analyte recoveries were determined for EMDP, EDDP and methadone as described under section 2.5.2.1. The extraction was carried out using pH8 phosphate buffer (0.1M) and only Fraction B was analysed.

2.5.5.2 Results and Discussion

The recoveries of EMDP, EDDP and methadone from whole blood were determined at pH8 and the results are summarised in Table 2.19.

The mean, standard deviation (Std. Dev.) and coefficient of variance (% C.V) are also reported for each triplicate analyses.

Table 2.19 Extraction Recoveries from Whole Blood Using HCX-5 and HCX-6

	HCX-5			HCX-6		
Sample	**EMDP**	**EDDP**	**Methadone**	**EMDP**	**EDDP**	**Methadone**
1	81.3	79.1	97.2	65.5	82.4	101
2	89.3	83.8	101	77.8	80.4	101
3	81.7	88.4	105	66.9	80.0	96.0
Mean	84.1	83.8	101	70.1	80.9	99.3
Std. Dev.	4.51	4.65	3.90	6.73	1.29	2.89
C.V (%)	5.4	5.5	3.9	9.6	1.6	2.9

The recoveries obtained for HCX-5 were slightly better than with HCX-6 for each of the analytes. In all cases the coefficients of variance are low (≤ 9.6%) and are acceptable for the present work.

2.5.6 Effect of Carbon Loading on Analyte Recoveries

2.5.6.1 Introduction

Hartley *et al* [117] reported the recoveries of chlormethiazole from plasma using solid-phase extraction. As part of their investigations they noted the different recoveries obtained with Sep-Pak C18 and Bond Elut C_1, C_2, C_4, C_6, C_8 and C_{18} single-phase cartridges. They concluded that the recoveries were not primarily affected by the carbon number, which was secondary to the percentage carbon loading of the bonded phase.

This was investigated using the results obtained during this project for mixed-mode SPE cartridges in comparison to the single phases used by Hartley *et al* [117].

2.5.6.2 Results and Discussion

The recoveries of EMDP, EDDP and methadone using pH 8 phosphate buffer are summarised in Table 2.20. The SPE cartridges have been listed in order of decreasing carbon number in an attempt to assess the relationship between the recoveries obtained for each analyte and the percentage carbon loading of the bonded phase.

With the exception of HCX, as the carbon number decreases the percentage carbon loading also decreases. There does not appear from the data presented, to be any obvious relationship between either factors and the recoveries obtained from whole blood for EMDP, EDDP or methadone.

Table 2.20 Carbon Loading and Analyte Recoveries

Cartridge	Cn	% Loading	EMDP	EDDP	Methadone
HCX-3	18	13.5	87.9	82.1	85.4
HCX-2	12	12.1	81.0	82.0	98.4
HCX-4	8(Std)*	9.7	92.2	85.7	99.8
HCX-6	6	8.1	70.1	80.9	99.3
HCX	8(Non-Std) †	7.5	85.6	87.9	100
HCX-5	4	6.1	84.1	83.8	101

* Std – HCX-4 contains standard C8 non-end capped sorbent

† Non-Std – HCX contains non-standard low carbon loading C8 non-end capped sorbent.

2.5.7. Establishment of Extraction Procedure

The optimised extraction method for the analysis of EMDP, EDDP and methadone in whole blood involves mixed-mode SPE cartridges and pH 8 phosphate buffer (0.1M).

Not all case samples involve toxic to fatal levels therefore an alternative detector with greater sensitivity is required. Gas chromatography – mass spectrometry was employed and the extraction method validated.

2.5.8 GC/MS Analysis

2.5.8.1 Experimental

Individual unextracted standards (1μg/ml) were analysed by GC/MS (1μL) in the full scan mode. Full mass fragment spectra for EMDP, EDDP, methadone and methadone-d_3 was obtained. The object of obtaining the full spectra was to identify the compounds of interest and to determine the optimum fragment ions for monitoring purposes.

Once the correct ions for monitoring were identified, a selected ion monitoring programme was created to increase the sensitivity of the method.

2.5.8.2 Results and Discussion

Figures 2.6 to 2.9 illustrate the full mass fragment spectra for EMDP, EDDP, methadone, and methadone-d_3.

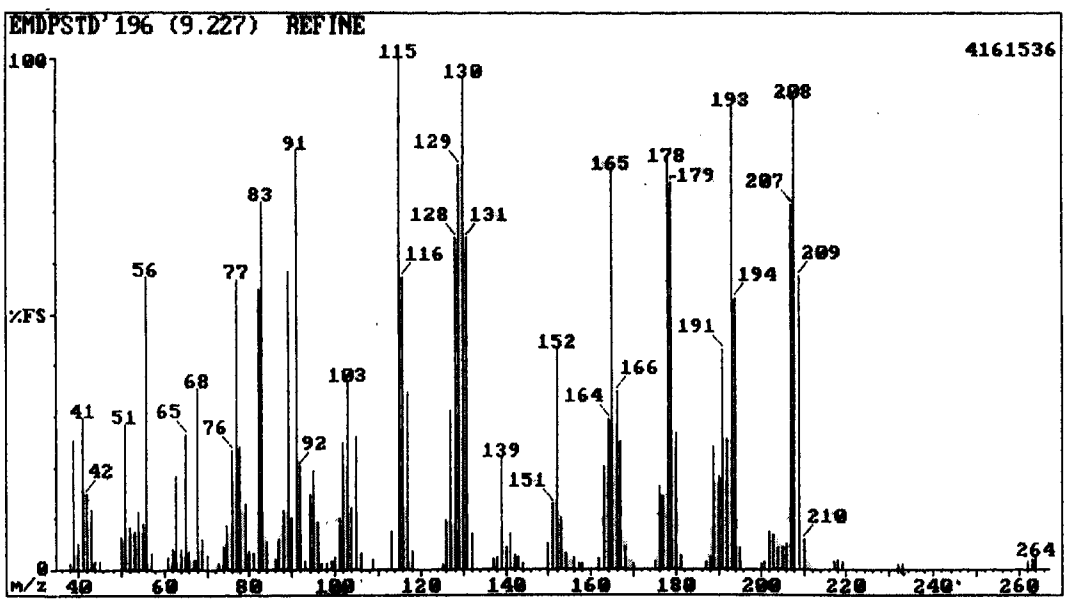


Figure 2.6 Full Mass Fragment Spectrum of EMDP

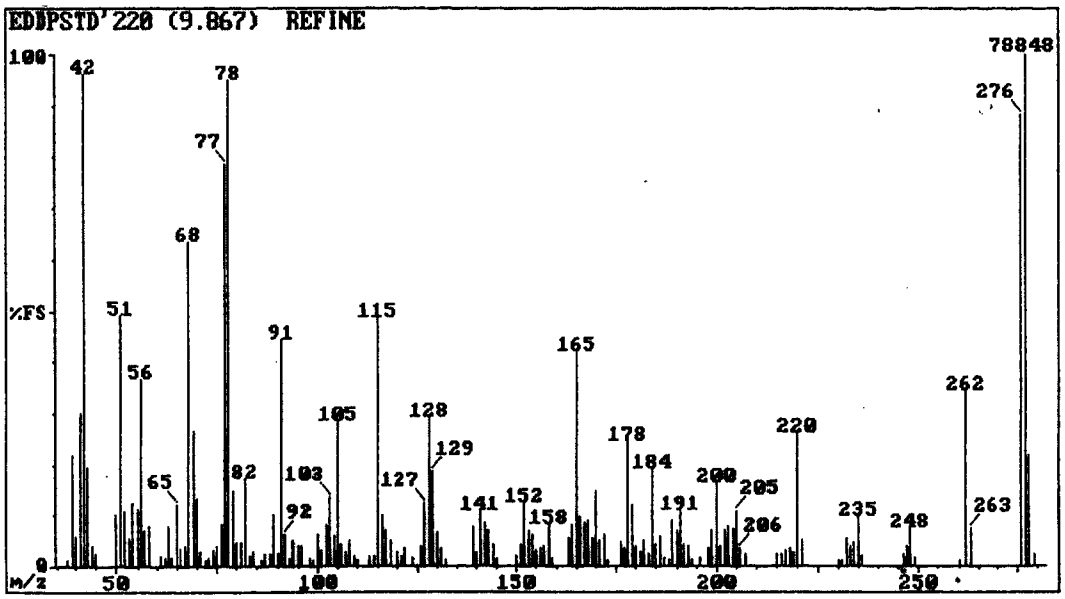


Figure 2.7 Full Mass Fragment Spectrum of EDDP

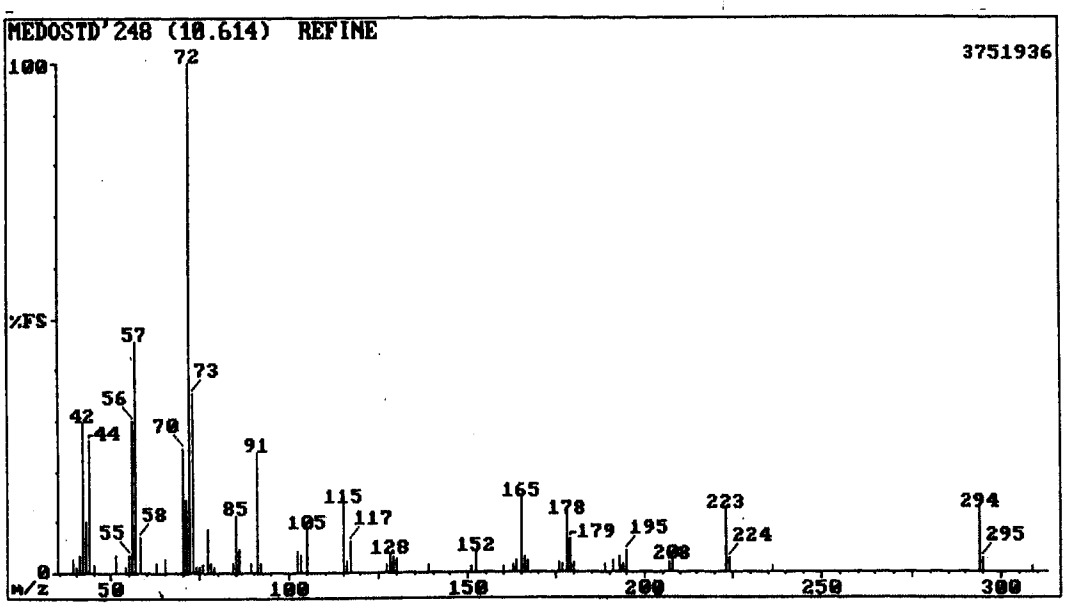


Figure 2.8 Full Mass Fragment Spectrum of methadone

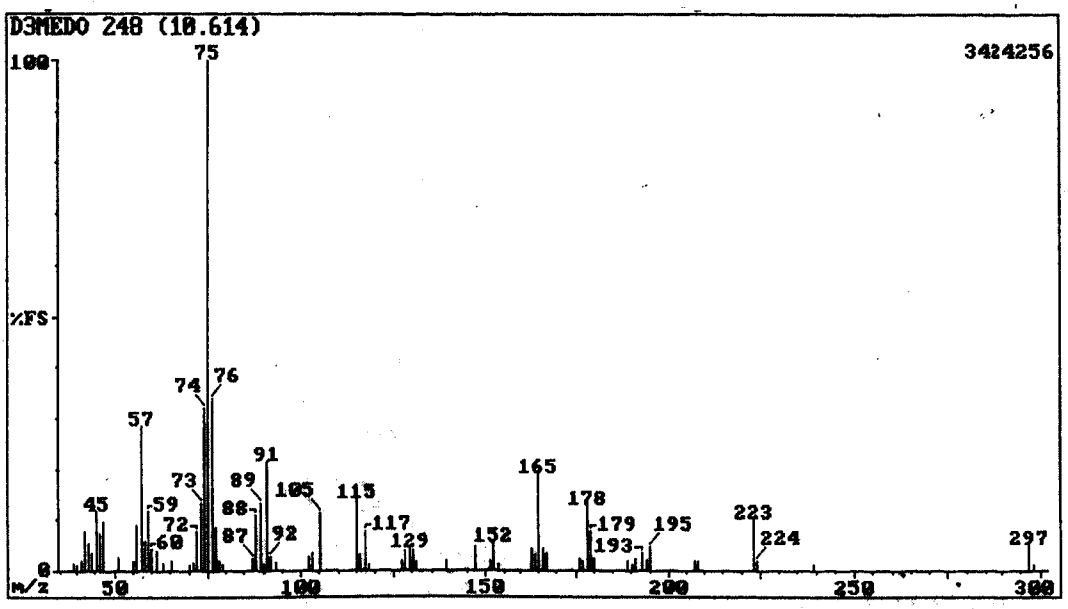


Figure 2.9 Full Mass Fragment Spectrum of methadone-d_3

The ions monitored were m/z 178, 193, 208* for EMDP, m/z 178, 220, 277* for EDDP, m/z 72*, 165, 294 for methadone and m/z 75 for methadone-d_3. The ions labelled with an asterix were used for quantitation.

2.5.9 Validation of Extraction Method

2.5.9.1 Experimental

Validation of the developed method involved carrying out linearity studies over a concentration range of 0 to 0.6mg/l and 0.5 to 5 mg/l. Aliquots of the stock standard solutions were evaporated to dryness under a stream of nitrogen at 40°C and reconstituted in 1ml of whole blood. The resultant samples were spiked at

concentrations of 0, 5, 10, 50, 200, 400 and 600ng/ml and 0.5, 0.75, 1.25, 2.50 and 5mg/l for EMDP, EDDP and methadone in blood.

The internal standard (methadone-d_3) was added to the samples at a concentration of 0.1mg/l (0 to 0.6mg/l) and 0.5mg/l (0.5 to 5mg/l). Peak area ratios (mz 208/75 for EMDP, mz 277/75 for EDDP and mz 72/75 for methadone), obtained at each concentration were plotted as a calibration curve against their respective concentration. Each data point was the mean of triplicate analyses.

2.5.9.2 Results and Discussion

Calibration curves for each of the three analytes were linear over the concentration ranges, 0 to 0.6mg/l and 0.5 to 5mg/l. The limit of detection was 5ng/ml at a signal-to-noise ratio of 3:1.

The results are summarised in Table 2.21. Figures 2.10 and 2.11 illustrate the linear relationship between EMDP, EDDP and methadone over the concentration ranges, 0 to 0.6mg/l and 0.5 to 5mg/l respectively.

Table 2.21 Linearity and Limits of Detection

Analyte	Concentration Range (mg/l)	r	Limits of Detection (ng/ml)
EMDP	0 → 0.6	0.997	5
	0.5 → 5.0		
EDDP	0 → 0.6	0.999	5
	0.5 → 5.0		
Methadone	0 → 0.6	0.994	5
	0.5 → 5.0		

r - correlation coefficient

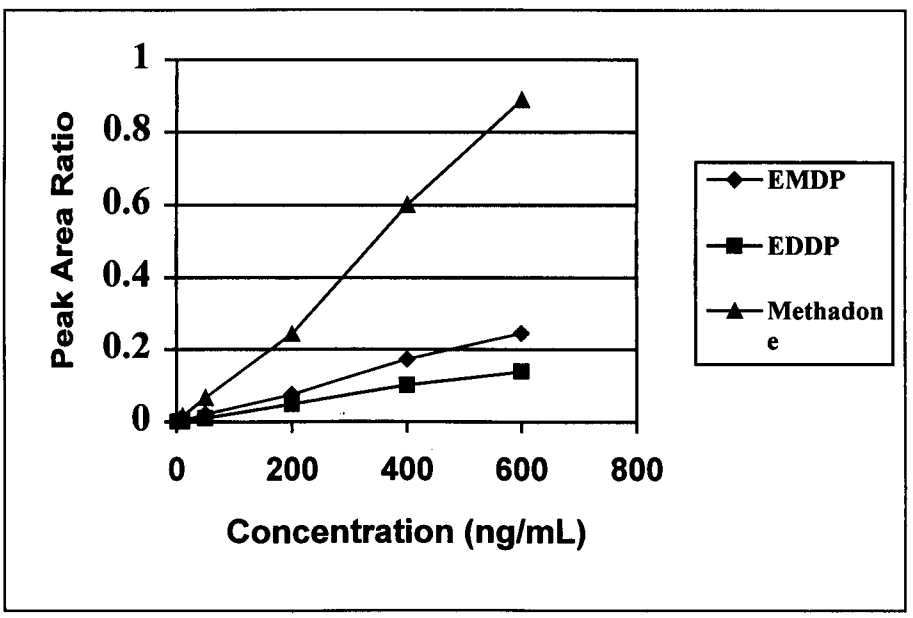


Figure 2.10 Calibration Curves for EMDP, EDDP and methadone (0 to 0.6mg/l)

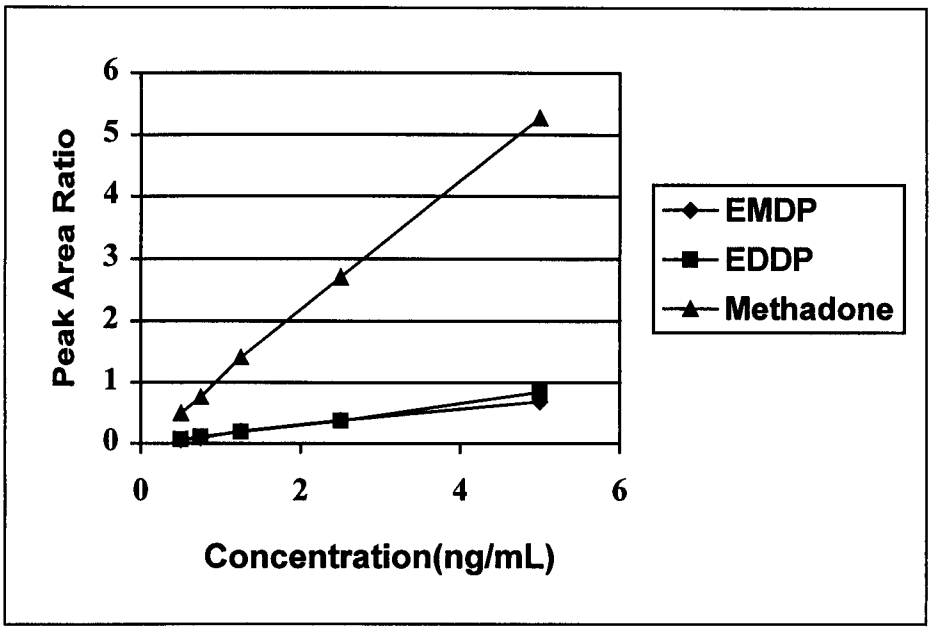


Figure 2.11 Calibration Curves for EMDP, EDDP and methadone (0.5 to 5mg/l)

2.6 Hair Analysis

2.6.1 Introduction

The ability to detect drugs in hair has enormous potential particularly with respect to post-mortem hair samples, an area not commonly investigated.

In many cases the drug use history of the deceased is either not known or not available for the investigation of the death. The drug levels detected in blood provide valuable information regarding the short-term drug use of the decedent prior to death. Segmental analysis of hair could be a possible source of additional information on antecedent drug use history, which would aid in the interpretation of the analytical findings and ultimately the cause of death.

Amphetamine, methamphetamine [118] and codeine [119] were detected in root hairs 30 minutes after administration. Nakahara and Kikura [120] reported MDMA in the root bulb of rat hair five minutes after administration. By collecting post-mortem hair samples pulled from the scalp, it was possible to analyse the root bulb and investigate the relationship between the drug levels detected in blood and those found in the root bulb at the time of death.

Initially methanolic extraction's of hair spiked with methadone were screened using enzyme immunoassay kits supplied by Cozart Bioscience Limited (Oxfordshire, UK), then post-mortem hair samples were analysed by the described method.

2.6.2 Enzyme Immunoassay Screen

2.6.2.1 Introduction

The Cozart methadone microplate used for the EIA screening of hair samples spiked with methadone was intended for use in the "qualitative and semi-quantitative determination of methadone in serum or whole blood". The manufacturers recommend adapting these kits for hair by substituting spiked hair samples at known concentrations for the four protein matrix calibrators included in the kit. In addition they recommend adding 50μL of hair extract to the sample wells as opposed to 25μL added for serum or blood.

2.6.2.2 Immunoassay

The methadone microplate kits supplied by Cozart Bioscience Limited are competitive immunoassays. Horseradish peroxidase labelled drug competes with

unlabelled drug in the sample for antibody sites in wells on a polystyrene plate. Excess enzyme is removed by washing after a 30 minute incubation period and then substrate is added which produces a blue colour. The stop solution is added after a further 30 minutes to produce a yellow colour and the absorbance is measured at 450nm. The absorbance is inversely proportional to the quantity of drug in the sample.

2.6.2.3 Sample Preparation

Blank hair, washed and ground prior to use, was spiked with methadone at a concentration of approximately 100ng/mg. This was achieved by adding 1ml of methadone (10μg/ml in methanol) to 100mg of hair. A sample weight of 10 – 20mg was required and to each hair sample was added 0.5ml methanol. Samples were sonicated for one hour after containers were sealed with parafilm and incubated overnight at 45°C.

The incubated samples were allowed to cool before decanting the methanol extract into a clean vial and evaporating to dryness under a stream of nitrogen at 40°C. The extract was reconstituted in 1ml of phosphate buffer saline (pH7.6).

Experiments were conducted to determine whether or not hair spiked with methadone could be detected by EIA, did not cause false positives and to assess the use of reference hair standards for calibration controls.

2.6.2.4 Experiment A

Nine hair samples of varying concentration and one blank were prepared for EIA screening as previously described. Table 2.22 summarises the hair weights,

concentration of methadone present in the samples corrected for hair weight and the EIA response.

Table 2.22 Experiment A – Summary of Hair Weights, Drug Concentrations and EIA Response.

Sample	Weight (mg)	Concentration (ng)*	EIA Response (ng/ml)
1	10.04	1002	Too high†
2	10.43	100	66.43
3	10.24	10.0	Negative
4	10.24	1041	Too high
5	10.28	104	56.39
6	10.47	10.4	Negative
7	10.49	1022	Too high
8	10.78	102	77.70
9	10.17	10.2	Negative
Blank	10.37	0.0	Negative

* Concentrations (ng) corrected for weight were done so by multiplying the sample weight (mg) by the concentration of the hair spike (ng/mg).

† Too high – Indicated the sample was positive but at a level exceeding the highest calibrator.

The concentrations of methadone detected in hair were calculated with respect to the serum calibrators supplied with the EIA kit (0, 5, 25 and 100ng/ml). The maximum (Samples 1, 4 and 7) and minimum (Samples 3, 6, 9) spiked hair concentrations were above and below the highest and lowest serum calibrators. However, it was possible to detect methadone in hair and the blank sample was negative, tentatively indicating that no interferences were causing a false positive response.

The next step was to repeat experiment A with hair samples spiked over a greater range of methadone concentrations.

2.6.2.5 Experiment B

Eight hair samples of varying concentration and one blank were prepared for EIA screening as previously described. This experiment was conducted in triplicate and the results are summarised in Table 2.23 for each sample.

Only nine samples of concentrations ranging from 5 to 26.3 ng were within the highest and lowest serum calibrators and gave a positive response. All blank samples gave a negative response. Three samples (50, 51.1 and 52.6ng) were positive for methadone but the response was higher than the maximum calibrator.

The experiments thus far have involved hair spiked with methadone. To determine whether or not methadone could be extracted and detected by EIA from drug users hair, two post-mortem case samples were analysed where the deceased were known to have used methadone prior to death.

Table 2.23 Experiment B – Hair Concentrations and Corresponding EIA Response Using Serum Calibrators

Batch A		**Batch B**		**Batch C**	
Concn. (ng)	**Response (ng/ml)**	**Concn. (ng)**	**Response (ng/ml)**	**Concn. (ng)**	**Response (ng/ml)**
51.5	Too High	52.6	Too High	50.0	Too High
25.7	46.9	26.3	87.6	25.0	45.5
10.3	25.7	10.5	39.2	10.0	26.2
5.1	8.1	5.3	12.9	5.0	9.3
2.6	-ve	2.6	-ve	2.5	-ve
1.0	-ve	1.1	-ve	1.0	-ve
0.5	-ve	0.5	-ve	0.5	-ve
0.1	-ve	0.1	-ve	0.1	-ve
Blank A	-ve	Blank B	-ve	Blank C	-ve

2.6.2.6 Experiment C

Two case samples (GC02 and GC03 – refer to Chapter 6 for case information) were cut into segments prior to analysis. In both cases the hair samples were de-rooted. GC02 was analysed in 3 segments: root (R), 1st 3cm (A) from root and in bulk (B). (The bulk sample included the full hair sample with roots still attached). GC03 was analysed in one segment only, the roots (R). Two spiked hair standards and one blank were analysed in addition to the case samples.

Table 2.24 summarises the segments analysed and the results of the EIA screen

Table 2.24 Experiment C – EIA Response Values Using Serum Calibrators

Sample	Weight (mg)	Response (ng/ml)	Concn. (ng/mg)
Standard 1	10.58	412.4	10.8
Standard 2	19.97	146.4	5.4
Blank (A)	16.16	-ve	0.0
GC02 (R)	17.75	Too high	Too high
GC02 (A)	80.62	393.2	10.3
GC02 (B)	132.17	Too high	Too high
GC03 (R)	53.19	48.3	1.3
Blank (B)	18.29	-ve	0.0
GC02 (R) *	17.75	312.7	40.9
GC02 (B) *	132.17	126.1	16.5

* The extracts of samples, GC02 (R) and GC02 (B) were diluted 1:5 with phosphate buffer saline and re-analysed by EIA. The resultant EIA response was multiplied by five prior to calculating the methadone concentration.

Using the supplied serum calibrators for quantitating methadone hair concentrations was too restrictive. Hair spiked with methadone at four different concentrations was investigated as alternative calibrators.

2.6.2.7 Experiment D

Hair spiked calibrators were prepared at four concentrations: 0, 1, 10 and 100ng/mg with equivalent nanogramme concentrations of 0, 24.5, 224 and 2576 nanogrammes. In addition, 13 post-mortem cases were analysed. Table 2.25 summarises the data and results from Experiment D.

The limit of detection for this assay was 24.5ng, based on a spiked hair calibrator (24.03mg; 1.02ng/mg). A large proportion of the cases which were negative had sample weights below 20mg, and may explain the number of false negative results. All blank samples gave negative responses.

Table 2.25 Experiment D – Data and Results Summary

Sample	Segment	Weight(mg)	Response (ng)	Concn. (ng/mg)
GC01	Root	20.75	-ve	-ve
	Bulk	35.16	-ve	-ve
GC04	Bulk (W)*	50.04	113.5	2.3
	Bulk (UW)†	38.55	963.4	25.0
GC05	1st 3cm	3.68	-ve	-ve
	Bulk	5.79	-ve	-ve
GC07	Root	12.39	-ve	-ve
	Bulk	24.73	27.1	1.1

Sample	Segment	Weight(mg)	Response (ng)	Concn. (ng/mg)
GC08	Root	4.01	-ve	-ve
	Bulk	24.58	-ve	-ve
GC11	Root	4.90	-ve	-ve
	1st 6cm	26.99	-ve	-ve
	2nd 6cm	11.78	-ve	-ve
	Bulk A‡	39.73	45.2	1.1
	Bulk B§	48.45	29.5	0.6
GC13	Root	4.80	-ve	-ve
	1st 3cm	45.35	46.7	1.0
	Bulk	38.02	-ve	-ve
GC16	1st 6cm	32.18	-ve	-ve
	2nd 6cm	23.93	30.5	1.3
	Bulk	39.67	34.3	0.9
GC24	Bulk	46.80	418.7	8.9
GC27	Root	6.16	179.1	29.1
	Bulk	27.25	479.8	17.6
GC30	Root	4.84	-ve	-ve
	Bulk	8.79	-ve	-ve
GC35	Root	4.40	-ve	-ve
	Bulk	44.74	-ve	-ve
GC36	Root	3.89	72.17	18.6
	Bulk	43.60	139.5	3.0

2.6.2.8 Discussion

It is difficult to interpret the results from Experiments A to D, as the EIA assay functions primarily as a qualitative determination of methadone in serum or whole blood. However, it can be concluded that the serum/whole blood EIA kit as adapted could detect the presence of methadone in spiked hair and post-mortem case samples.

2.7 Conclusion

The ability to identify and confirm the presence of methadone and its two major metabolites (EDDP and EMDP) in whole blood has been achieved using the combination of solid-phase extraction and gas chromatography – mass spectrometry.

An optimal buffer pH of 8.0 was determined by comparing the percentage recoveries obtained for each analyte following solid-phase extraction at six different pH values (6.0, 7.4, 8.0, 9.0, 10.0, 11.0). Chirald was used as the internal standard. In addition, the analysis time of the extraction was decreased significantly. The original method [27], involved collection of two eluates, however, optimising the pH enabled the developed method to require only one eluate resulting in a faster and more reproducible extraction method.

The resultant method was validated and was linear over a concentration range of 0 to 0.6mg/l and 0.5 to 5 mg/l. The limit of detection for each analyte was 5ng/ml. The concentration range and limit of detection were more than adequate for the routine analysis of methadone, EDDP and EMDP in post-mortem blood samples and was successfully applied to 46 forensic case samples. None of the 46 cases were positive for EMDP.

In general, as the carbon number of the mixed-mode solid-phase extraction cartridges decreases so does the percentage carbon loading. Investigating the results obtained for methadone, EDDP and EMDP, the percentage recoveries obtained do not appear to be affected by either the carbon number or the percentage loading. This is in contrast to the observation reported by Hartley *et al* [117] who concluded that the recoveries of chlormethiazole from plasma using single-mode cartridges were not primarily affected by the carbon number, which was secondary to the percentage carbon loading of the bonded phase.

The importance of the percentage carbon loading and carbon number may depend on the analytes in question and/or the sample matrix, however, in the case of methadone, EDDP and EMDP in whole blood the pH of the extraction system is the main factor determining high recoveries.

Adapting the Cozart methadone microplate for screening hair samples spiked with methadone was achieved and the matrix did not appear to cause any interferences. All blank samples gave a negative response. However, spiking hair results in hair with surface bound drugs, which are easily removed and do not replicate the complex binding involved with drug users hair.

In total, 15 post-mortem hair samples were segmented and screened for methadone following methanolic extraction. Of these, ten samples gave a positive response for methadone, and all blanks a negative response. The majority of the cases which were negative had low sample weights below 20mg, and may have contributed to the number of false negative results.

3. Methadone Stability Study

3.1 Introduction

Drug stability in biological matrices depends on a variety of factors, including physicochemical properties of the drug, the type of matrix and the storage conditions employed. Delays of several days are common place between sampling, blood alcohol analysis and the initial drug screens. Confirmation may not take place for some time or may not be carried out until the case goes to court.

Drug stability is of particular concern in cases where analysis is carried out by more than one institute. Most forensic laboratories are required by law to store blood samples for a minimum of six months to years to enable reanalysis if required.

3.2 Literature

There are a limited number of papers in the literature dealing with drug stability in biological matrices. Forensic toxicologists deal primarily with post-mortem blood and the majority of studies involve whole blood [121-124]. Studies have also been reported on other matrices including urine and plasma [10], and putrefying liver [125].

Al-Hadidi and Oliver investigated the stability of temazepam [122], morphine and buprenorphine [123] in whole blood. They concluded that temazepam, morphine and buprenorphine were reasonably stable after six months and one year respectively, regardless of storage conditions. Skopp *et al* [124] reported a decrease in

concentration of all 13 benzodiazepines studied in blood and plasma during a 240-day interval when stored at 4°C.

Other studies investigating benzodiazepines in whole blood involved diazepam [126], midazolam [127] and bromazepam [128]. Chlordiazepoxide is unstable in whole blood and decomposes rapidly [129].

Giorgi and Meeker [121] conducted a five-year stability study of cocaine, benzoylecgonine, morphine, codeine, methamphetamine, amphetamine and phencyclidine in blood. Cocaine was not detectable after only three months, while methamphetamine and phencyclidine were relatively stable up to five years.

Methadone positive drug related deaths have increased in the West of Scotland from 1991 to 1996 [10]. Commonly used in the treatment of heroin addiction, methadone has also been associated with illicit use. The increase in methadone positive deaths has resulted in an increase in the number of requested methadone confirmations.

No papers were identified in the literature which investigated the stability of methadone in whole blood. Moody *et al* [130] investigated the stability of methadone and its two major metabolites in urine. Neither methadone or its metabolites showed significant decreases indicative of instability when stored at -20°C. Similar conclusions were reported by Alburges *et al* [91], who found methadone and its metabolites to be stable in plasma and urine over a week long study involving two cycles of freezing and thawing.

The present study investigates the stability of methadone and its two metabolites in spiked whole blood and deionised water. This allowed a comparison to be made between the effects of chemical decomposition and putrefactive degradation for each analyte. The spiked and blank samples were stored at -20°C, 5°C and 25°C to reflect common laboratory storage temperatures.

3.3 Aims

The aims of the stability study were to:

- determine the stability of methadone, EDDP and EMDP in whole blood over a period of six months,
- determine the stability of methadone, EDDP and EMDP in whole blood when stored at three different temperatures (-20, 5 and 25 °C) over the six month period,
- investigate the effect of light on the stability of EDDP when stored in whole blood under the above conditions,
- investigate the effect of silanising the vials on the recovery of methadone, EDDP and EMDP in whole blood,
- investigate the contribution of chemical decomposition on the stability of methadone, EDDP and EMDP.

3.4 Experimental

3.4.1 Materials

HPLC-grade methanol, acetone, chloroform and ethyl acetate were obtained from Lab-Scan Analytical Sciences (Dublin, Ireland). Analytical-grade ammonium hydroxide and glacial acetic acid and HiPerSolv grade potassium-dihydrogen phosphate (KH_2PO_4) were obtained from Merck (Poole, U.K.). Methadone, EDDP and Chirald ((2S,3R)-(+)-4-Dimethylamino-1,2-Diphenyl-3-Methyl-2-Butanol) were obtained from Sigma® Chemical Co., (Dorset, U.K.). EMDP was obtained from Alltech Associates (State College, PA).

3.4.2 Sample preparation and storage

Outdated blank whole blood and deionised water were spiked with methadone (1μg/ml), EDDP (1μg/ml) and EMDP (1μg/ml). 0.8mg of each analyte was dissolved in 10ml of methanol, transferred into conical flasks and evaporated to dryness at 40°C under a stream of nitrogen. The analytes were then reconstituted in blank whole blood and deionised water.

Aliquots (15ml) of spiked sample were transferred into 30ml hypovials and were sealed with butyl rubber septa. Standard solutions of methadone, EDDP, EMDP and the internal standard, Chirald were prepared by adding 1mg of each analyte to 100ml of methanol (10μg/ml). The EDDP standard was wrapped in aluminium foil to prevent degradation of the light sensitive analyte.

Three vials were stored for each measurement of whole blood and deionised water spiked with methadone, EDDP and EMDP, and for blank blood and blank deionised water. A further study was conducted for EDDP spiked samples. The samples were prepared as described above but the vials were wrapped in aluminium foil to investigate the effect of light degradation.

The sealed samples were stored at - 20°C, 4°C and 25°C. Three sealed vials were removed from storage at regular time intervals over a six month period (two, four, six and eight weeks and three and six months). In addition sets of hypovials were silanised with dimethylchlorosilane and then washed with methanol and allowed to dry before use. Three silanised vials were stored for six months for each sample type under the three temperature conditions stated above.

3.4.3 Instrumentation

A Hewlett-Packard model 5890 Series II gas chromatograph equipped with a flame ionisation detector (GC-FID) was used with an HP-1, cross-linked methyl siloxane capillary column (30m, 0.25mm i.d, film thickness 0.25μm). The initial oven temperature of 100°C was increased to 300°C at a rate of 10°C/minute. The total analysis time was 20 minutes. The injector and detector temperatures were 285°C and 300°C, respectively. Helium was used as the carrier gas. Injections of 2 μL were carried out in the splitless mode.

3.4.4 Solid-phase extraction

The solid-phase extraction procedure used was developed specifically for the optimised recovery of methadone, EDDP and EMDP [131] from whole blood as detailed in Chapter Two. Spiked samples (1ml) were prepared by sonicating for 15 minutes at room temperature before dilution with 6ml of 0.1M phosphate buffer (pH 8.0). The diluted blood was then vortexed for 30 seconds before centrifuging at 2000 rpm for 15 minutes. The supernatant was collected for analysis and the residual pellet was discarded.

Isolute™ Confirm HCX (130mg) solid-phase extraction columns (International Sorbent Technology LTD (Hengoed, U.K.)) were conditioned with methanol and phosphate buffer (pH 8.0). The diluted samples were applied to the column and then washed with deionised water. The pH was adjusted with 0.01M acetic acid (pH3.3). The columns were air dried under a full vacuum (15in. Hg) for 4 minutes, solvated with methanol and then dried under full vacuum for a further minute.

Acidic, weak basic and neutral drugs were eluted with acetone-chloroform (1:1) and discarded. Methadone, EDDP, EMDP and Chirald were eluted with 2%-ammoniated ethyl acetate, evaporated to dryness at room temperature under a nitrogen stream and reconstituted in 50μL ethyl acetate.

3.5 Results and Discussion

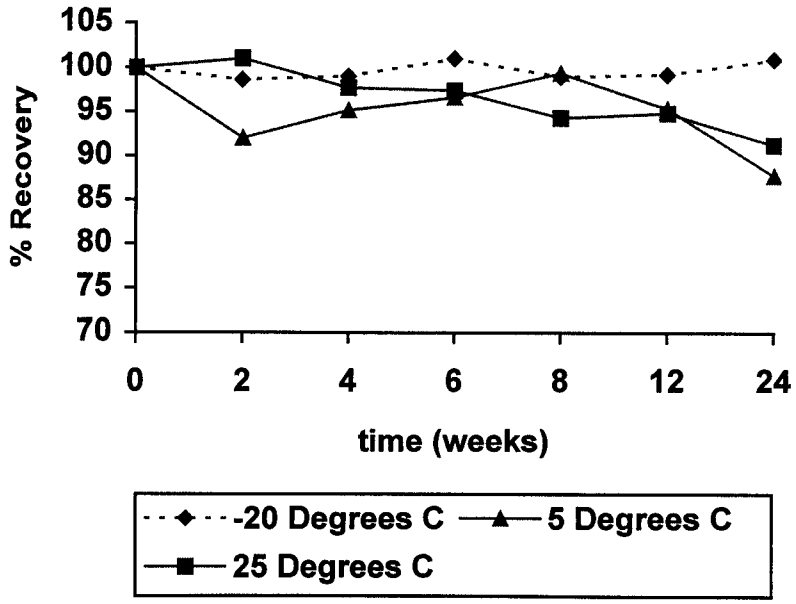
3.5.1 Introduction

Analyte recoveries were determined by comparing the peak area ratios obtained from the extracted stored samples with freshly prepared spiked samples. The recoveries were calculated relative to the initial recovery of 100% obtained on day zero. The mean recovery (n = 3) was determined for each sample type. Standard deviations were low with coefficients of variance ranging from 0 to 11.6% (mean = 5.5%).

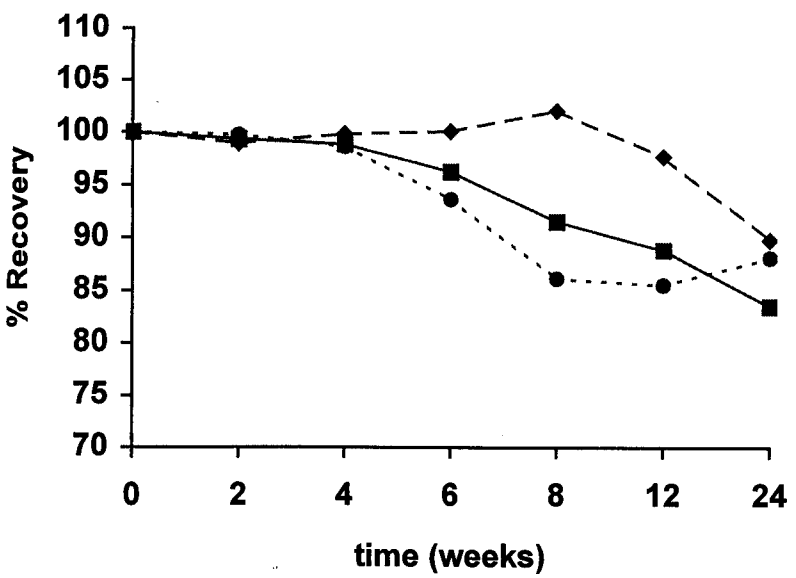
3.5.2 Methadone

Figure 3.1 shows the relative recovery of methadone after six months of storage in spiked blood and deionised water. Methadone is stable in blood when stored at -20°C for up to six months with recovery of 89.8% of the original concentration.

The recoveries decreased to 88.1% at 5°C and 83.5% at 25°C. The decrease in recoveries of methadone from deionised water was less pronounced with recoveries of 101% and 91.3% at -20°C and 25°C respectively. The exception to this being the recovery of methadone from deionised water stored at 5°C with a recovery of 87.8%, similar to that obtained from blood.



3.1.(A)

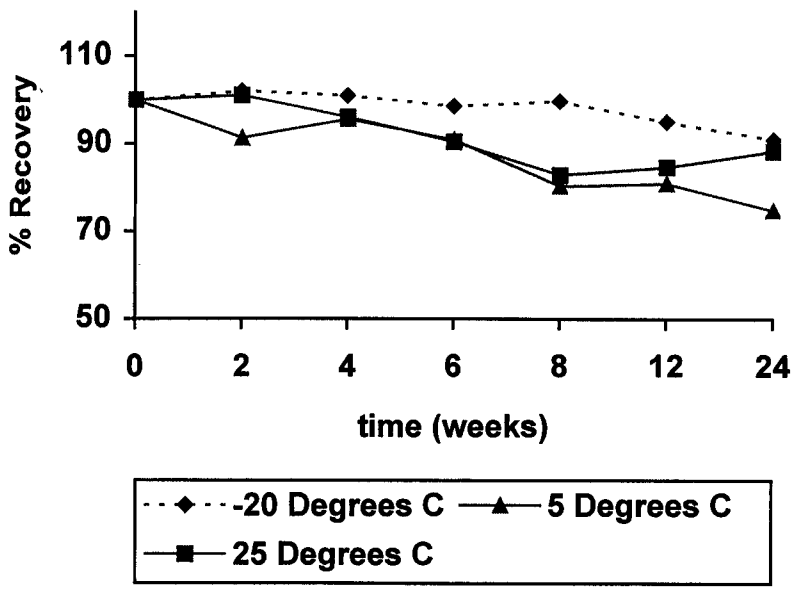


3.1.(B)

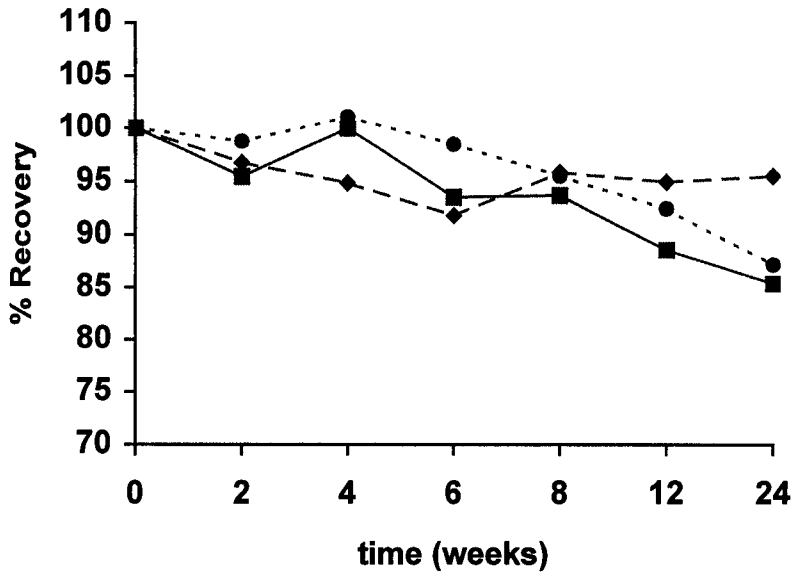
Figure 3.1 Changes in methadone recoveries from (A) deionised water and (B) blood with time.

3.5.3 EDDP

Slightly lower recoveries were obtained for EDDP in comparison to those for methadone. However, in contrast the recoveries of EDDP from deionised water were lower than the recoveries from whole blood (See figure 3.2).



3.2. (A)

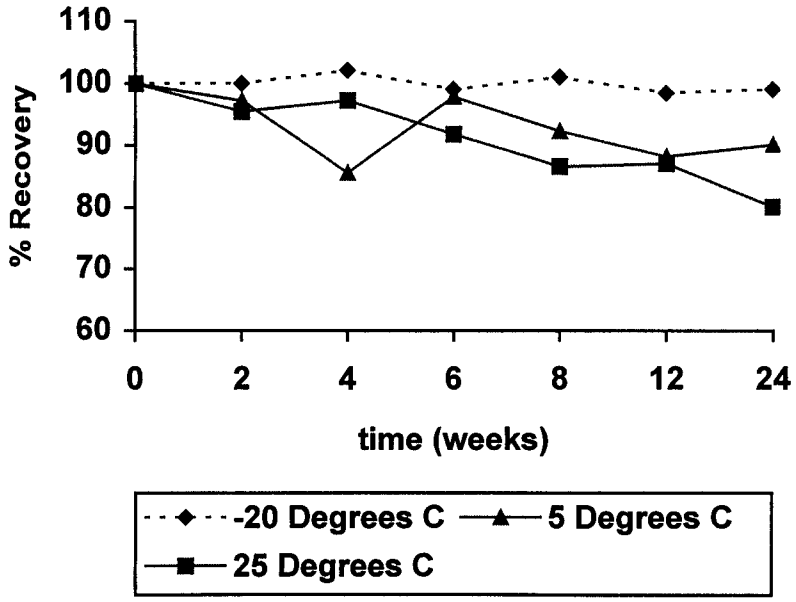


3.2.(B)

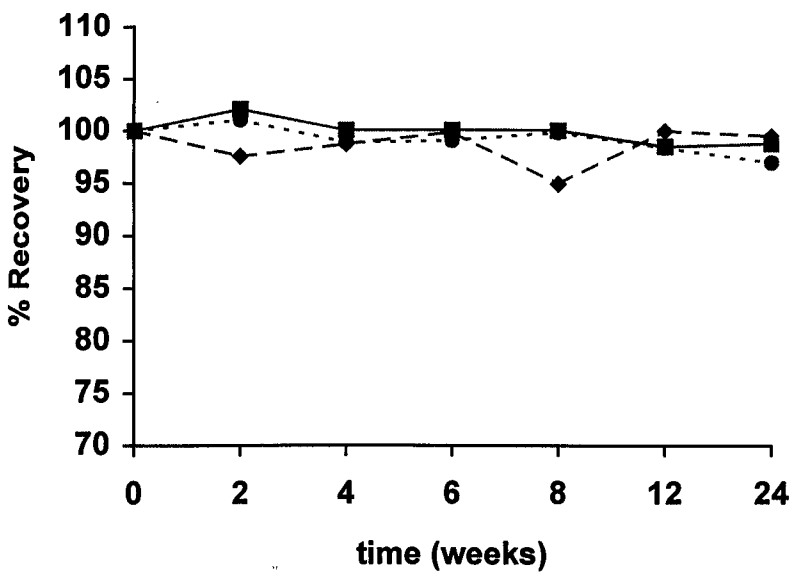
Figure 3.2 Changes in EDDP recoveries from (A) deionised water and (B) blood with time.

3.5.4 EDDP (Foil wrapped)

Foil wrapped hypovials reduced the loss of EDDP from both blood and deionised water samples. The recoveries of EDDP (foil wrapped), see figure 3.3, are greater than 80.1% in deionised water compared with 74.8% in unwrapped vials. Greater than 94.9% of the original EDDP concentration was recovered in whole blood (foil wrapped) compared with 85.4% in unwrapped vials. The difference in recoveries is not significant enough to warrant the expense incurred by using light sensitive containers for all samples taken for toxicological analysis.



3.3.(A)

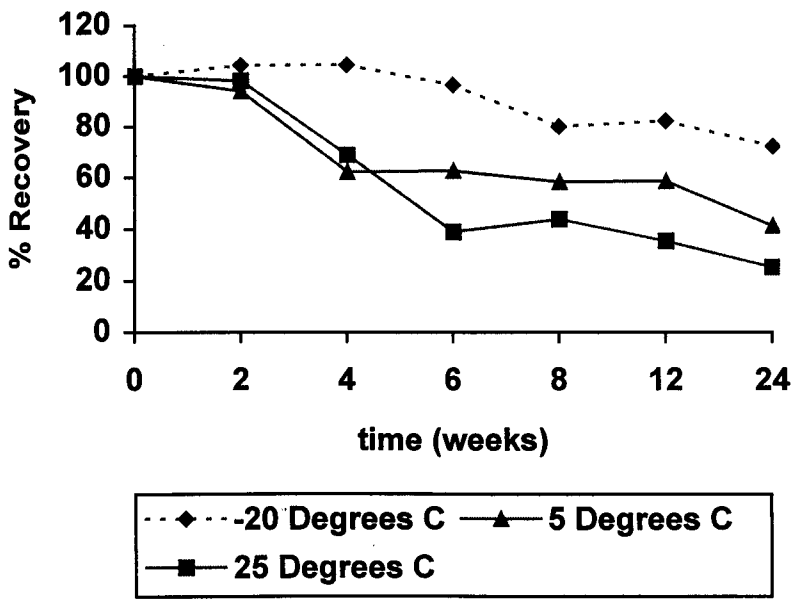


3.3.(B)

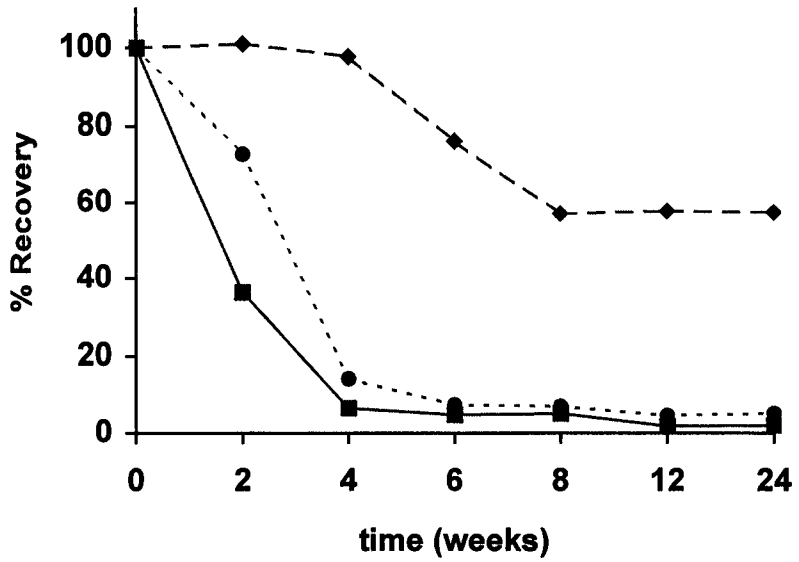
Figure 3.3 Changes in EDDP (foil wrapped) recoveries from (A) deionised water and (B) blood with time.

3.5.5 EMDP

Figure 3.4 shows the rapid decline in EMDP concentration in deionised water and in particular in whole blood, over the six month study period. The storage conditions play a significant role in EMDP stability. In both cases, samples stored at -20°C resulted in the greatest recoveries (72.1% from deionised water and 57.6% from whole blood) after six months of storage. At temperatures of 5 and 25°C, EMDP recoveries of only 5.3 and 2.1% respectively were noted. Corresponding recoveries from deionised water were 41.3 and 25.3% at 5 and 25°C respectively.



3.4.(A)



3.4.(B)

Figure 3.4 Changes in EMDP recoveries from (A) deionised water and (B) blood with time.

EMDP had not been detected in postmortem cases [58,131] during the period of this research. The instability of this analyte in whole blood demonstrated above could explain the lack of EMDP positive cases. In addition, the results presented may confirm the observation by Sullivan and Due [103], that EMDP is a more active substrate for hydroxylation than EDDP.

3.5.6 Silanised Hypovials

Table 3.1 summarises the recoveries of each analyte from whole blood after six months of storage in silanised and non-silanised hypovials. Under each

temperature condition, there was no significant change in analyte recovery between vials, which were silanised, and those, which were not silanised.

Table 3.1 Average Recoveries of Analytes from Whole Blood after Six months Storage in Silanised (S) and Non-Silanised (NS) Hypovials.

	-20°C		5°C		25°C	
Analyte	**S (%)**	**NS (%)**	**S (%)**	**NS (%)**	**S (%)**	**NS (%)**
Methadone	81.6	89.8	93.3	88.1	80.1	83.5
EDDP	88.8	95.5	89.1	87.2	86.0	85.4
EDDP(F)	100	99.5	99.8	97.0	92.2	98.8
EMDP	52.4	57.6	5.8	5.3	2.2	2.1

3.6 Conclusion

Methadone and its primary metabolite, EDDP remain stable in whole blood for up to six months when stored at -20°C. When stored at 25°C, the recoveries were still greater than 83.5 and 85.4% for methadone and EDDP respectively. Foil wrapped vials did not improve recoveries significantly to warrant the use of light sensitive sample containers.

EMDP is extremely unstable in whole blood at storage temperatures of 5 and 25°C. Storage of samples at -20°C only resulted in recovery of 57.6% of the original concentration of EMDP after six months storage.

Silanising the hypovials prior to storage did not result in greater recoveries of methadone, EDDP or EMDP from whole blood.

4. Amphetamines

4.1 Introduction

Amphetamine is a synthetic stimulant, used primarily to treat a number of disorders including catalepsy and obesity. Methamphetamine the N-methyl derivative of amphetamine, has also been used in the treatment of obesity. Both drugs are abused for their stimulant effects, especially in the United States, Sweden and Japan [132]. Amphetamine and methamphetamine are Class B, Schedule 2 drugs in the Misuse of Drugs Act 1971(UK).

The ring-substituted amphetamines, in particular 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxy-ethylamphetamine (MDEA) are abused for their hallucinogenic properties. MDMA along with MDA and MDEA are Class A, Schedule 1 drugs in the Misuse of Drugs Act 1971 (UK) and are most stringently controlled.

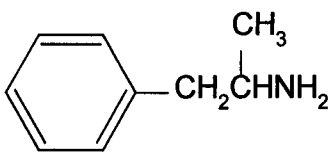
"Ecstasy" is widely used in the United Kingdom and has caused considerable concern following a number of cases of serious toxicity and death, thought to have resulted from use of "ecstasy".

Current information concerning the toxicity of "ecstasy" or the role of adulterants in tablets sold as "ecstasy" is insufficient. "Ecstasy" tablets have reportedly contained amphetamine [133], MDA [134-136], MDEA [135-138] and paramethoxyamphetamine (PMA) [139] in addition to or in place of MDMA.

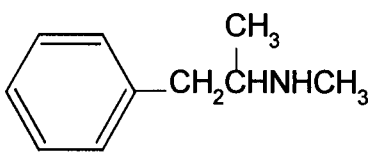
To fully understand the toxic effects of the phenylethylamines and their metabolism, toxicologists require a method, which will successfully extract and confirm the presence of amphetamine and its related compounds in biological matrices.

4.1.1 Chemistry of Amphetamine and Its Related Compounds

Amphetamine is commonly available as the sulphate salt, a white crystalline salt, first synthesized in 1887. The d-isomer of amphetamine has 3-4 times the central activity of the l-form. Methamphetamine hydrochloride was first prepared in 1919 and forms white crystals or crystalline powder. (See Figure 4.1)



Amphetamine



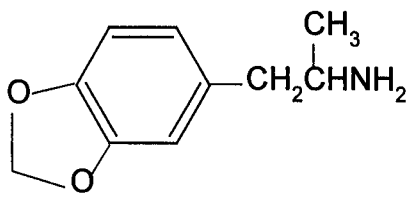
Methamphetamine

Figure 4.1 Amphetamine ($C_9H_{13}N$) and Methamphetamine ($C_{10}H_{15}N$)

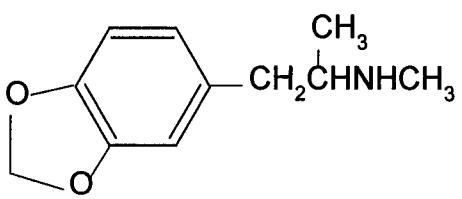
3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA) are ring-substituted amphetamines that have stimulant and hallucinogenic qualities in addition to unique pharmacological and psychological properties. (See figure 4.2)

MDMA was first synthesized in the early part of the 20th century. Similar to amphetamine, the d-isomer of MDMA has higher central activity than the l-isomer.

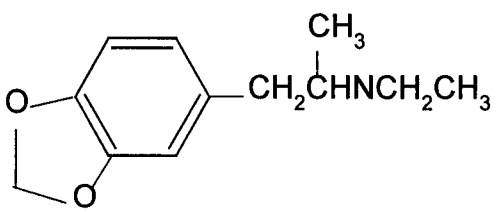
Table 4.1 summarises some important physicochemical properties of the amphetamines.



MDA



MDMA



MDEA

Figure 4.2 MDA ($C_{10}H_{13}NO_2$), MDMA ($C_{11}H_{15}NO_2$) and MDEA ($C_{12}H_{17}NO_2$)

Table 4.1 Properties of Amphetamine, Methamphetamine, MDA, MDMA and MDEA [64]

Drug	Molecular Weight	M.P. (°C)	pKa
AP	135	300	9.9
MA	149	172 – 174	10.1
MDA	179	187 – 188 [140]	-
MDMA	193	148 – 153 [140]	-
MDEA	204	201 – 202 [140]	-

4.1.2 History of Use and Abuse of Amphetamine and Its Related Compounds

Amphetamines are the most popular illicit stimulant in the United Kingdom, second only to cannabis [15]. During the Second World War, amphetamine tablets were given to soldiers to combat the effects of battle-fatigue [141]. In the 1960's amphetamine abuse was widespread especially in London's West End clubs. Young people consumed large quantities of Drinamyl (known as 'purple hearts'). These tablets were withdrawn but amphetamine abuse continued.

Methamphetamine gained popularity during the 1970's, when it was claimed that it gave a better 'high' than amphetamine. Interest in the drug dwindled after it was associated with strange and violent behaviour [141].

A drug misuse survey conducted in 1996 [142], reported that the most significant rise in drug use over the last decade is the 'dance drugs' in particular, amphetamine, LSD and "ecstasy".

"Ecstasy" was patented as an appetite suppressant for soldiers in the First World War [143]. In the United States the drug was used as an adjunct to psychotherapy [144], and in the 1980's became popular among drug users. The late 1980's saw the introduction of "ecstasy" in the United Kingdom where it became popular at dance events or "raves" [145]. In Britain alone, an estimated 500,000 people use "ecstasy" each weekend [146].

Forsyth [147] interviewed 135 participants in the Glasgow rave scene, and concluded that although "ecstasy" was by far the most commonly used drug at raves, poly-drug use was most prevalent. This was further supported by Hammersley *et al* [148] who interviewed 209 individuals who had used "ecstasy" at least once, but found no individuals who used "ecstasy" without using at least one other controlled drugs.

This is not a feature of the rave scene in Glasgow alone, Handy *et al* [149] conducted a study of "ecstasy" use in the Cardiff area of South Wales (n = 389) where poly-drug use and experimentation was prevalent. They concluded from their study that the pattern of drug use had changed from the early 1990's and "ecstasy" was no longer a central feature of the dance.

4.1.3 Disposition in the Body

4.1.3.1 Amphetamine

Amphetamine is readily absorbed after oral administration, followed by deamination to phenylacetone which is oxidised to benzoic acid and excreted as conjugates. A small amount of amphetamine is converted to norephedrine by oxidation. (See figure 4.3)

Approximately 30% of the dose is excreted unchanged in urine in 24 hours, however this is greatly dependent on urinary pH. Acidic urine may increase the amount of unchanged dose to 74% or decrease to 1% in alkaline urine. Amphetamine is also a metabolite of methamphetamine, benzphetamine [150] and selegiline [64].

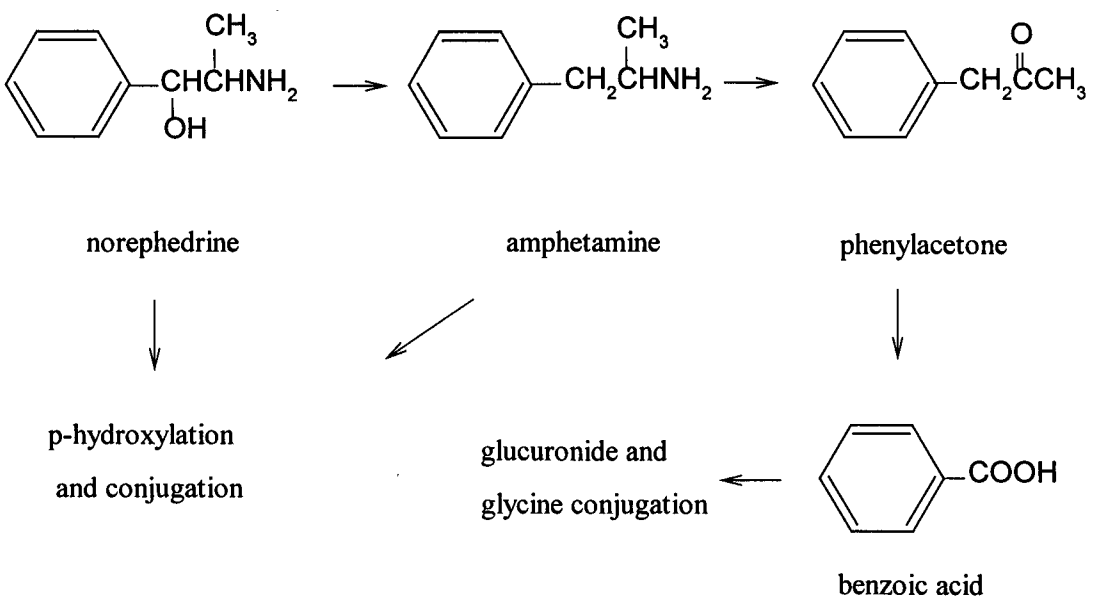


Figure 4.3 The metabolic pathways of amphetamine in the human body

4.1.3.2 Methamphetamine

Similar to amphetamine, methamphetamine is readily absorbed after oral administration and the amount of unchanged drug excreted in urine in 24 hours is pH dependent. Up to 43% of a dose is excreted unchanged as the parent drug, under normal conditions. This can vary from as low as 2% in alkaline conditions to 76% in acidic conditions.

Methamphetamine undergoes N-demethylation to amphetamine. Approximately 4-7% of the dose is excreted as amphetamine (See figure 4.4). Methamphetamine is a metabolite of benzphetamine [150] and selegiline.

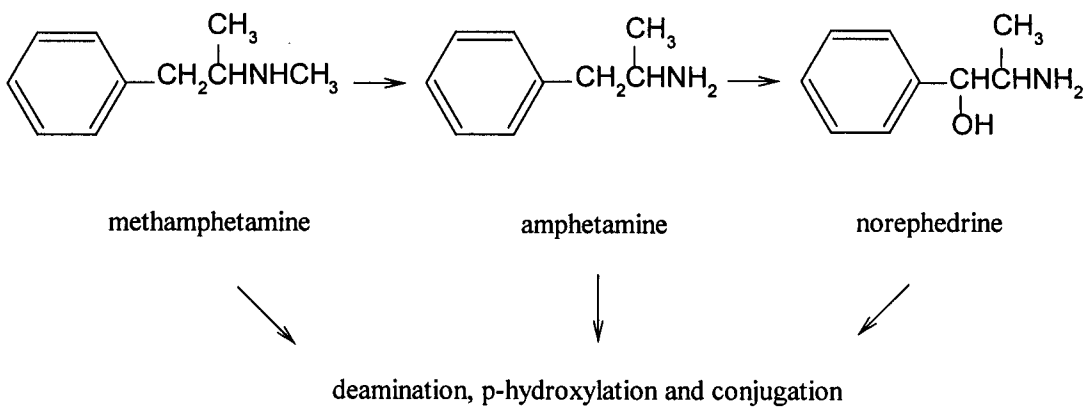
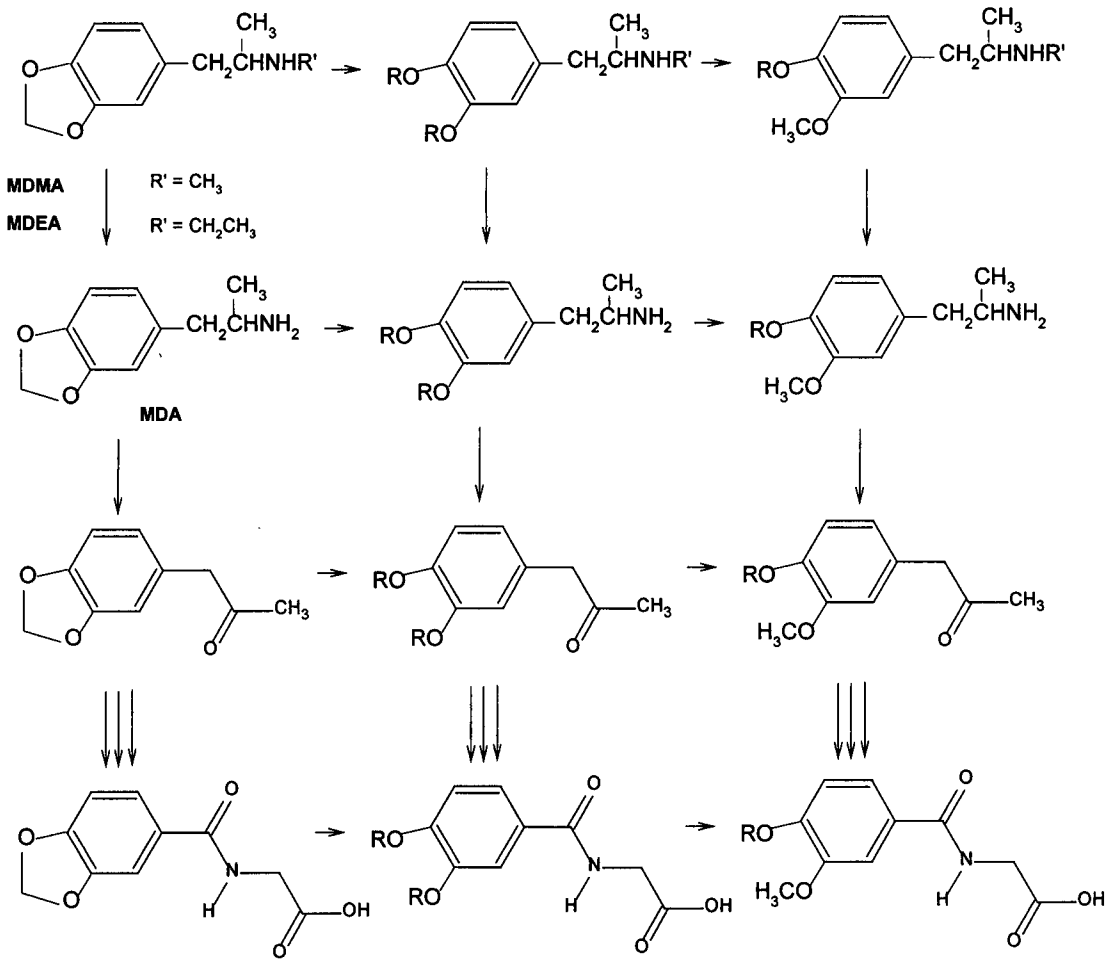


Figure 4.4 The metabolic pathways of methamphetamine in the human body

4.1.3.3 Ring-substituted Amphetamines

Metabolism studies of the ring-substituted amphetamines are few and only limited data can be found in Clarke [64] or Baselt and Cravey [63].

Methylenedioxymethamphetamine (MDMA) is metabolised by N-demethylation to methylenedioxyamphetamine (MDA). 65% of the dose is excreted as the parent drug and 7% as MDA within three days [151].



(phase I: R = H, phase II: R = sulphate or glucuronic acid)

Figure 4.5 The metabolic pathways of MDA, MDMA and MDEA in the human body [152].

Maurer [152] investigated the metabolism of MDA, MDMA, MDEA and their metabolites in urine by GC/MS. The ring-substituted amphetamines undergo two overlapping metabolic pathways, O-dealkylation of the methylene dioxy group to form dihydroxy derivatives which is then followed by methylation of a hydroxy group and degradation of the side chain to N-dealkyl and deaminooxo metabolites [152]. MDA, MDMA and MDEA are further metabolised to glycine conjugates of the corresponding 3,4-disubstituted benzoic acids (hippuric acids) [152] (See figure 4.5).

4.1.4 Toxicity

4.1.4.1 Amphetamine

Amphetamine plasma concentrations are usually below 0.1mg/L after normal therapeutic doses. Chronic usage of amphetamine is characterised by weight loss, hallucinations and paranoid psychosis [153]. In addition, increased heart rate and blood pressure are also symptomatic of amphetamine use.

Deaths resulting from amphetamine overdosage are rare. The minimum lethal dose in non-tolerant adults is estimated at 200mg [64].

Toxic effects may be produced with blood concentrations of 0.2 to 3mg/L, with levels greater than 0.5mg/L resulting from fatalities [64]. Amphetamine tolerant adults who orally consumed 100mg of amphetamine daily [154], maintained a steady-state blood concentration of 2.0 – 3.0 mg/L. Table 4.2 summarises the fatal levels of amphetamine (mg/L or mg/kg) reported in the literature.

Table 4.2 Fatal amphetamine levels (mg/L or mg/kg) detected in various biological matrices.

Reference	Blood	Brain	Liver	Kidney	Urine
63	0.5 – 41 (n = 11)	2.8 – 3.0 (n = 2)	4.3 – 7 (n = 11)	3.2 – 52 (n = 6)	25 – 700 (n = 8)
138	1.54	n/a*	n/a	n/a	n/a
155	2.44	5.50	11.7	3.85	33.4
156	0.25 – 2.6 (n =3)	n/a	n/a	n/a	n/a
157	0.45 – 2.18 (n = 6)	n/a	n/a	n/a	n/a
158	n/a	n/a	n/a	n/a	< 0.5 – 320 (n = 11)

* n/a – matrix not analysed

4.1.4.2 Methamphetamine

Therapeutic doses of methamphetamine result in plasma concentrations in the range 0.01 to 0.05 mg/L. Chronic users may develop paranoid psychosis, while overdosage causes anxiety, hallucinations, cardiac arrhythmias, convulsions and coma.

Similar to amphetamine, deaths resulting from methamphetamine overdosage are rare. The minimum lethal dose in non-tolerant adults is estimated at 1g [64]. Methamphetamine blood concentrations of 0.15 – 0.56 mg/L were reported in seven methamphetamine abusers who exhibited violent and irrational behaviour [159].

Methamphetamine levels of 1.7 and 2.1mg/L were detected in two women found asleep in their car. Table 4.3 summarises the fatal levels of methamphetamine (mg/L or mg/kg) reported in the literature.

Table 4.3 Fatal methamphetamine levels (mg/L or mg/kg) detected in various biological matrices.

Reference	Blood	Brain	Liver	Kidney	Gastric	Urine
63	5.6	n/a	n/a	n/a	n/a	320
63	2.0 0.3 AP	n/a	4.8 0.7 AP	n/a	1.5 0.1 AP	28 2.7 AP
160	1.33	n/a*	n/a	n/a	n/a	n/a
161	43 0.35 AP	102 0.86 AP	174 1.3 AP	75 0.7 AP	1557 2.0 AP	277 10 AP
162	40	n/a	206	n/a	n/a	n/a
163	0 – 0.6 (n = 3) 0 – 0.5 AP (n = 3)	0.2 – 0.8 (n = 3) 0 – 0.8 AP (n = 3)	0.4 – 0.7 (n = 3) 0 – 0.3 AP (n = 3)	0.4 – 0.6 (n = 3) 0 – 0.3 AP (n = 3)	n/a	1.0 4.0 AP

* n/a – matrix not analysed

4.1.4.3 Ring-substituted amphetamines

A literature search revealed very little data on toxicity with respect to 3,4 methylenedioxyamphetamine (MDA), 3, 4 methylenedioxymethamphetamine (MDMA) and 3, 4 methylenedioxyethylamphetamine (MDEA).

The estimated lethal dose of MDA for non-tolerant adults is 0.5g. Symptoms from MDA overdosage include, tachycardia, hyperthermia, muscular rigidity, convulsions and coma.

A non-fatal overdose of MDA resulted in an admission urine concentration of 131mg/L of MDA [63]. The victim, a one year old child presented with muscle rigidity, seizures and was unconscious. Table 4.4 summarises the reported toxic levels of MDA (mg/L or mg/kg) reported in the literature.

Table 4.4 Fatal MDA levels (mg/L or mg/kg) detected in various biological matrices.

References	Blood	Liver	Bile	Urine
164	6 – 26 (n = 5)	8 – 17 (n = 3)	5 – 9 (n = 3)	46 – 160 (n =3)
165	2.3	11	7	175

As "ecstasy" use increases, so the numbers of reports of adverse medical reactions and fatalities increase. Acute clinical toxicity following MDMA ingestion results in hyperthermia and the 'serotonin syndrome' [166-167]. Sudden cardiac death [168], cerebral oedema [169], hyperthermia [170], disseminated intravascular coagulation (DIC) [134],

severe acute hepatitis [171-172] and rhabdomyolysis [173-174] are commonly cited in cases of fatal and non-fatal toxicity following MDMA use.

Unusual cases have also been reported. A 17 year old female collapsed after taking two tablets of 'speckled dove', a combination of MDMA, heroin and cocaine [175]. She was unconscious, unresponsive, and hypothermic with a core temperature of 32.4 °C. No postmortem toxicological results were reported in this case.

Jorens *et al* [176] reported a case of suicidal ingestion of MDEA and heroin. The 25 year old male involved had a history of previous suicide attempts and was thought to have consumed a total of 40 tablets (approximately 4g) of MDEA and 12g of heroin, two hours prior to admission.
High serum levels of both drugs were detected, however he did not present with classical signs and symptoms associated with intoxication from these drugs. Full recovery followed symptomatic treatment. The authors concluded that the opposite pharmacological properties of the two drugs prevented the patients death.

Survival following a massive overdose of MDMA was reported by Ramacharan *et al* [177]. A 30 year old male was admitted to hospital after consuming 50 tablets of "ecstasy", ten tablets of oxazepam and five units of alcohol over a period of four to five hours.

The unpredictable nature of "ecstasy" is reflected in the fact that thousands of people have consumed "ecstasy" yet very few deaths have been reported. Fatalities following the ingestion of just one "ecstasy" tablet, yet survival after consuming 50

"ecstasy" tablets questions the toxicity of the ring-substituted amphetamines. Two possible explanations for the unpredictable responses are contamination of "ecstasy" or genetic predisposition.

In addition to the short-term risks from the toxic effects of MDMA, further research is needed on the long-term effects on the brain, liver and heart [137]. Semple and Johnstone [178] are currently studying the long-term effects of "ecstasy" use on damage to the nerve cells.

MDMA has been implicated with the onset of both psychological and behavioural complications. Researchers have reported an apparent association between MDMA use and serotonergic alterations [179-180]. Other studies have found a correlation between suicide and low levels of cerebrospinal fluid 5 – hydroxyindoleacetic acid, the primary metabolite of serotonin [181-182].

Its is believed that the long-term consequences of neurodegeneration associated with MDMA use is greater then the risk of death from acute toxicity [183]. Evidence of suppressed immune function of rats after a single dose of MDMA has been reported [184].

Amphetamine and related compounds are responsible for a number of deaths, directly (adverse drug reactions, overdoses) or indirectly (traffic accidents, suicides) [156]. Although there is a relatively low number of reported cases of adverse reactions and fatalities in comparison to the vast numbers using "ecstasy", a great deal of cases will go unreported [185].

The use of amphetamines impairs mental function and has resulted in a number of fatalities from road traffic accidents [186-188].

A total of 48 patients were admitted to an Accident and Emergency department over a 15 month period after consuming MDMA at a night club [189]. The mean number of "ecstasy" tablets consumed was two. Polydrug use was common with around 40% having consumed "ecstasy" prior to this occasion. A wide range of adverse effects were described from feeling dizzy or weak (n = 15; 31.3%) to feeling excessively hot, cold, feverish, shivering (n = 7, 15%). There were six episodes, which were classified as severe adverse effects. These included delirium in two cases, seizures in three cases, and unconsciousness (coma) in one case.

No toxicology data was available in this study and the authors, Williams *et al* [189] conceded that this was a major limitation in that confirmation of amphetamine or its related compounds had not been carried out.

The exact number of patients admitted to Accident and Emergency Departments within the United Kingdom with "ecstasy"-related symptoms is not known at this time. Monitoring these cases would present a clearer picture of the toxic effects of "ecstasy". Full toxicological screening of admission urine and blood samples would help rectify the lack of available toxicity data for the ring-substituted amphetamines and would be of great benefit to the toxicologist.

Table 4.5 summarises the reported toxic levels of MDMA and MDEA (mg/L or mg/kg) reported in the literature.

Table 4.5 Fatal MDMA and MDEA levels (mg/L or mg/kg) detected in whole blood.

Reference	MDA	MDMA	MDEA
134	n/d	0.19	1.6
138	0.25	0.43	0.30
156	0.49	4.1	n/d
157	0.04 – 2.4 (n = 3)	2.1 – 5.4 (n = 4)	n/d*
157	0.18 – 2.0 (n = 3)	0.04 – 1.2 (n = 2)	9.0 – 33.0 (n = 3)
190	0.02 – 0.06 (n = 4)	0.06 – 0.53 (n = 7)	≅ 0.09 (n = 1)
191	n/d	1.0	n/d

* n/d – not detected

4.2 Analysis

The analysis of amphetamines in biological matrices reported in the literature deal predominantly with urine [46,152,192-196]. Liquid-liquid extraction (LLE) has long been the preferred method however over the past five years solid-phase extraction (SPE), with its inherent advantages, has become more common place [197-201].

Alternative matrices e.g. serum [190,202-203], plasma [190,204], hair [205-209] and brain [210-211] have also been investigated.

The analysis of amphetamines in biological fluids still presents a challenge to the toxicologist due to their volatility. However, a routine method for the analysis of amphetamines in whole blood is an essential confirmation technique in the forensic toxicology laboratory.

Very few papers published in the literature deal with the analysis of amphetamine, methamphetamine, MDA, MDMA and MDEA in whole blood. Published research involve liquid-liquid extraction (LLE) [212], solid-phase extraction [199,213] and solid-phase microextraction [54]. Gas chromatography – mass spectrometry is the method of choice for confirming the presence of amphetamines in biological matrices.

Hara *et al* [199] reported a method combining solid-phase extraction with Extrelut columns and heptafluoro-n-butyryl derivatisation for the analysis of amphetamine, methamphetamine and their metabolites, 4-hydroxymethamphetamine (HMAMP) and 4-hydroxyamphetamine (HAMP). Recoveries of 79 and 84% were obtained for amphetamine and methamphetamine from whole blood, respectively.

Peschier *et al* [213] developed a solid-phase extraction method for the analysis of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethyl-amphetamine (MDEA) in whole blood. The multi-step method involved evaporating the eluate to 200µl in a vacuum centrifuge prior to derivatisation with HFBA. Recoveries of 60% were reported for amphetamine and MDA, 79% for methamphetamine, 84% for MDMA and 98% for MDEA.

The analysis of amphetamines in hair has been studied extensively, predominately using liquid-liquid extraction (LLE) [214-217]. In general, the majority of methods use either acidic [218-219] or alkaline [220-221] digestion of the hair, but some use enzymatic digestion [222-223] and β-glucuronidase/arylsulphatase [224-225] in order to free the drugs from the tightly bound matrix.

Sachs and Raff [226] compared different extraction methods for the analysis of opiates, cocaine and benzoylecgonine in hair samples. They found that extraction after enzymatic digestion led to higher results, than after treatment with NaOH. In addition, the authors found that although β-glucuronidase/arylsulphatase and methanol did not fully digest the hair, this was not necessary, as the drugs appeared to be extracted sufficiently, with the added advantage that screening for common basic drugs was possible.

Gas chromatography – mass spectrometry is the preferred method for hair analysis, but methods have been published using other techniques. Takahashi [227] described a gas chromatographic method for the detection of methamphetamine and amphetamine in the hairs of monkeys. Nagai *et al* [228], reported a method for the detection of methamphetamine and amphetamine in the hair of addicts, as well as in hair, bones and teeth after animal experiments.

Kintz *et al* [216], developed a method for the simultaneous determination of amphetamine, methamphetamine, MDMA and its primary metabolite MDA. This involved liquid-liquid extraction (LLE) of the drugs, after incubating the hair with 1M NaOH to destroy the matrix whilst in the presence of deuterated internal

standards(IS). Recoveries of greater than 75% were reported for all four drugs. Previously, only one other paper reported the analysis of MDMA in hair [229], but this did not include the analysis of MDA.

Nakahara *et al* [230], developed a solid-phase extraction (SPE) method for the analysis of MDMA and MDA in hair. This method involved the extraction of the hair sample in 2ml of methanol-5N HCl containing the deuterated internal standards for one hour under ultrasonication, followed by storage at room temperature overnight. After filtration of the hair, the filtrate was subjected to solid-phase extraction using Bond Elut Certify mixed-mode columns. Levels were reported in the range of 0.3-1.4ng/mg for MDMA and MDA in human hair samples. Both methods involved the derivatisation of the samples to prevent loss of the volatile drugs during evaporation steps.

Solid-phase extraction (SPE) followed by gas chromatography – mass spectrometry combines improved separation of analytes from biological fluids with greater sensitivity.

Increased analyte stability has been reported with varying degrees of success with derivatisation [216,221,231-235], including on-disc [236] and on-line [197] derivatisation and acidification [216,232,237]. Central to the majority of these techniques, involves derivatising at temperatures greater than 40 °C for extended periods of time (> 20 minutes). These conditions can result in significant loss of the analytes, which leads to lower recoveries and poorer reproducibility.

4.3 Aims

The work completed in this chapter involved two separate projects. The first project involved collaboration with The Scottish Centre for Criminology who investigated the use of "ecstasy" in Glasgow [148]. This study was funded by the Chief Scientist at the Scottish Office (Grant Ref.: K/OPR/2/2/D125). Subjects were questioned on their drug use histories over the previous year and were asked to donate a hair sample.

A method was required which could identify the presence of amphetamine and methamphetamine to confirm "speed" use and 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxy-ethylamphetamine (MDEA) to confirm "ecstasy" use in hair. The developed method was required to analyse 100 hair samples by segmental analysis.

The second project involved a collaboration with the University of Arizona. They had completed an investigation into the addition of tartaric acid as an aid in preventing losses of the small amines, namely, amphetamine and methamphetamine during evaporation and derivatisation. This work was reproduced and then the incorporation of this step into the routine analysis of amphetamines in whole blood and hair was investigated.

4.4 Experimental

4.4.1 Chemicals

Amphetamine (AP), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), d_3-amphetamine (d_3-AP), lauryl sulfate (sodium dodecyl sulphate (SDS)) and β-glucuronidase (activity = 300,000 – 400,000 units/g solid, sulphatase activity = 15,000 – 40,000 units/g solid) were obtained from Sigma® Chemical Co., (Dorset, U.K.). 3,4-methylenedioxy-ethylamphetamine (MDEA) was manufactured by Radian International and supplied by Promochem, (Herts., U.K.). Pentafluoropropionic anhydride (PFPA) was supplied by Fluka Chemicals (Dorset, U.K.). HPLC-grade methanol, acetone, chloroform, deionised water and ethyl acetate chromatographic (HPLC) grade were obtained from Lab-Scan Analytical Sciences (Dublin, Ireland). Analytical-grade ammonium hydroxide and glacial acetic acid and HiPerSolv grade potassium-dihydrogen phosphate (KH_2PO_4) were obtained from Merck (Poole, UK).

4.4.2 Stock Standards

A combined stock standard solution (0.1mg/ml) of AP, MA, MDA, MDMA and MDEA was prepared in methanol. Working solutions were also prepared in methanol (10, 1μg/ml) by appropriate dilution of the stock standard. The internal standard (I.S.), amphetamine - d_3, was prepared in methanol to give a stock standard solution of 0.1mg/ml. Working solutions of AP- d_3 (10, 1μg/ml) were prepared by dilution of the stock standard with methanol. All stock and working solutions were stored in a freezer at -20°C.

4.4.3 Instrumentation (GC/MS)

A Fisons model GC8000 series was used, fitted with a HP-1 capillary column. The initial column temperature of 55°C was held for 2 minutes and then increased to 280°C at a rate of 20°C/min. and held for a further 5 minutes. The carrier gas used was Helium. Injections were made in the splitless mode.

The GC8000 was interfaced to a Fisons MD800 mass spectrometer operated in the electron - ionisation mode at 70eV. The ions monitored were m/z 118, 190* (AP), m/z 118, 160, 204* (MA), m/z 135, 162, 190, 325* (MDA), m/z 135, 162, 204, 339* (MDMA), m/z 135, 162, 190, 218, 353* (MDEA) and m/z 193* (AP- d_3). The ions labelled "*" were used for quantitation.

4.4.4 Derivatisation and GC/MS Analysis

Standard solution or extract was evaporated to dryness under a stream of nitrogen at room temperature. The drugs were then reconstituted using 50µl of PFPA:Ethyl acetate (1:1) sealed and allowed to derivatise at 50°C for 15 minutes. After derivatisation was complete, the samples were evaporated to dryness as before and reconstituted in 50µl of ethyl acetate. 1µl was injected for analysis by GC/MS.

4.4.5 Mass Spectra

4.4.5.1 Experimental

Aliquots of individual stock standards (100µl, 0.1mg/l) were evaporated to dryness and derivatised as described above. 1µl was injected onto the GC/MS. The full mass

fragment spectra were obtained for amphetamine, amphetamine-d_3, methamphetamine, MDA, MDMA and MDEA using the full scan mode.

Once the optimum ions for monitoring the amphetamines were identified, a selected ion monitoring programme was created to increase the sensitivity of the method.

4.4.5.2 Results and Discussion

Figures 4.6 to 4.11 illustrate the full mass fragment spectra for amphetamine, amphetamine-d_3, methamphetamine, MDA, MDMA and MDEA

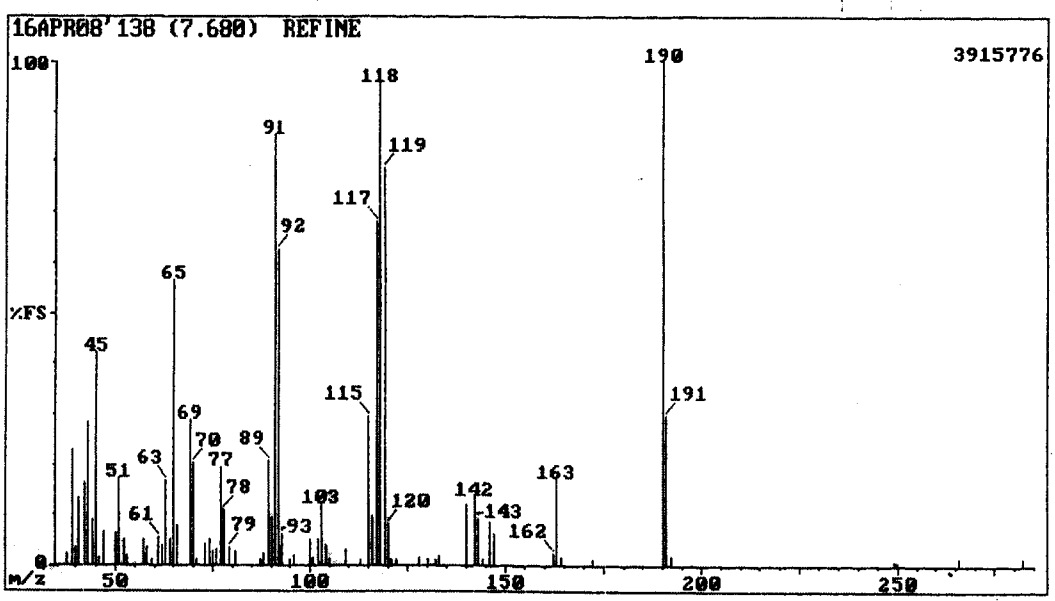


Figure 4.6 Full Mass Fragment Spectrum of amphetamine

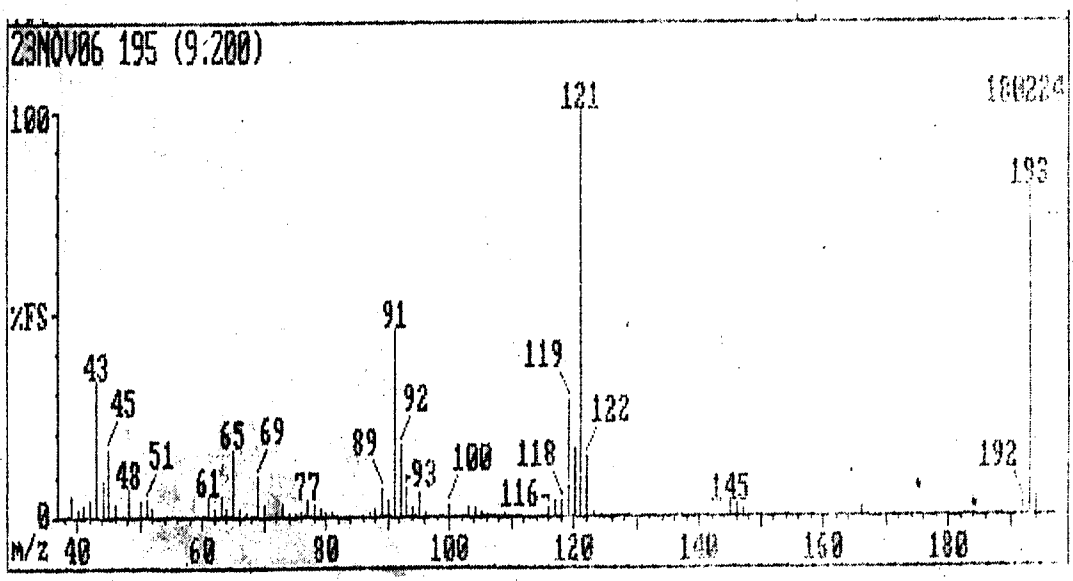


Figure 4.7 Full Mass Fragment Spectrum of amphetamine – d_3

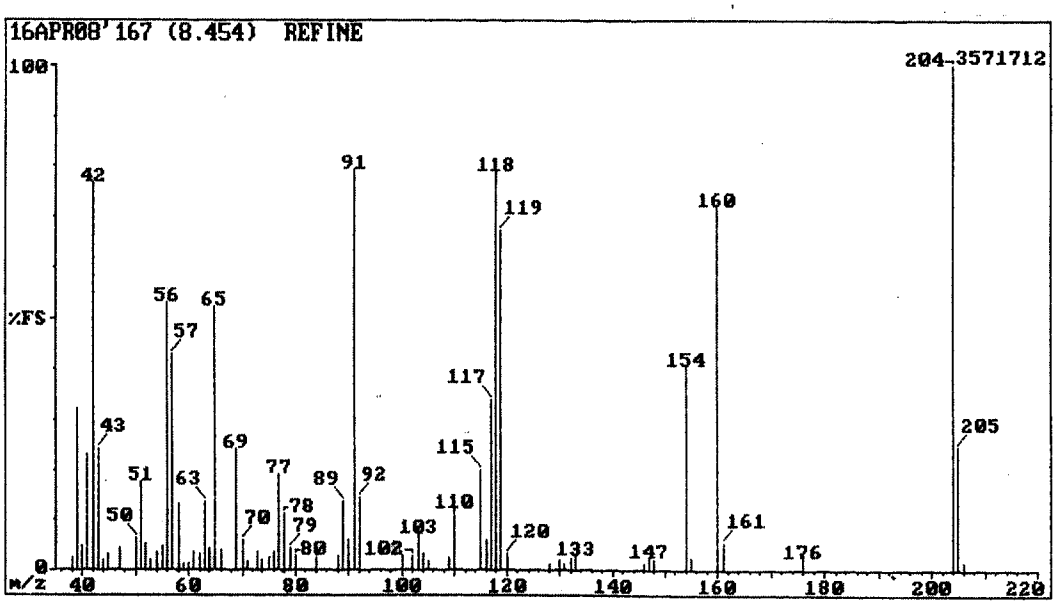


Figure 4.8 Full Mass Fragment Spectrum of methamphetamine

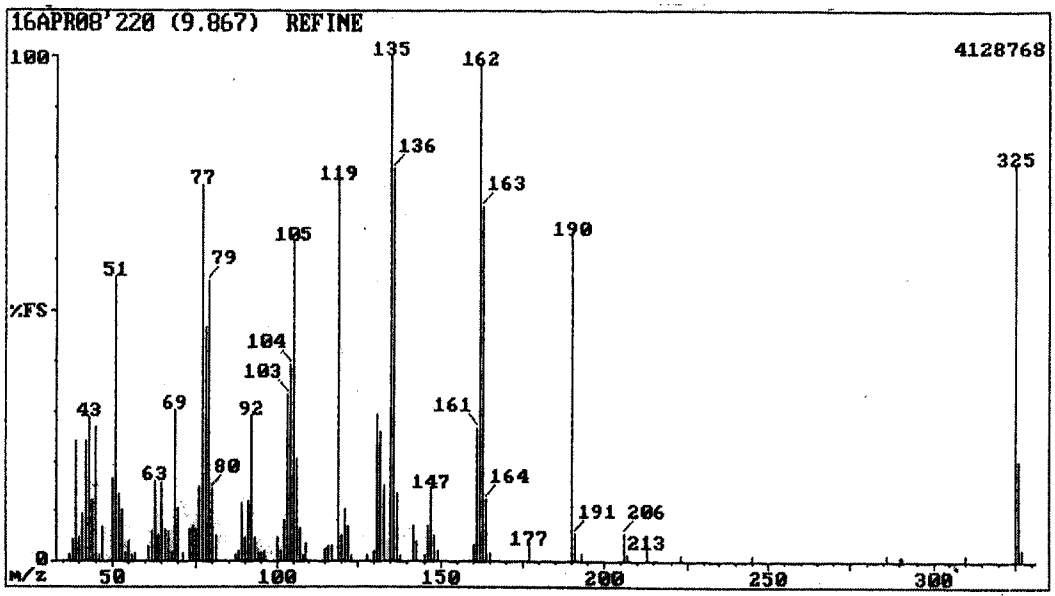


Figure 4.9 Full Mass Fragment Spectrum of MDA

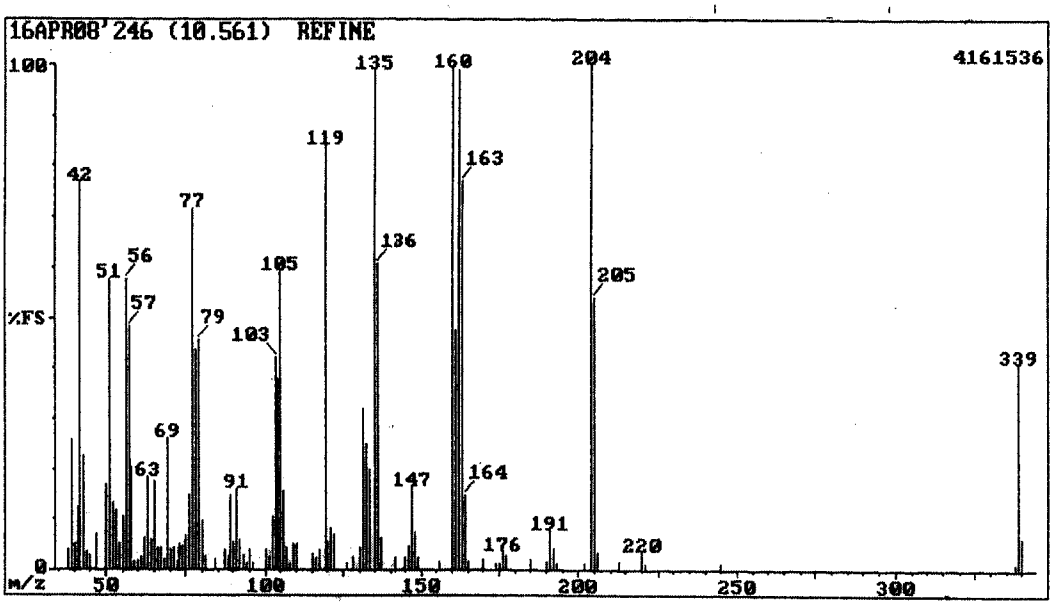


Figure 4.10 Full Mass Fragment Spectrum of MDMA

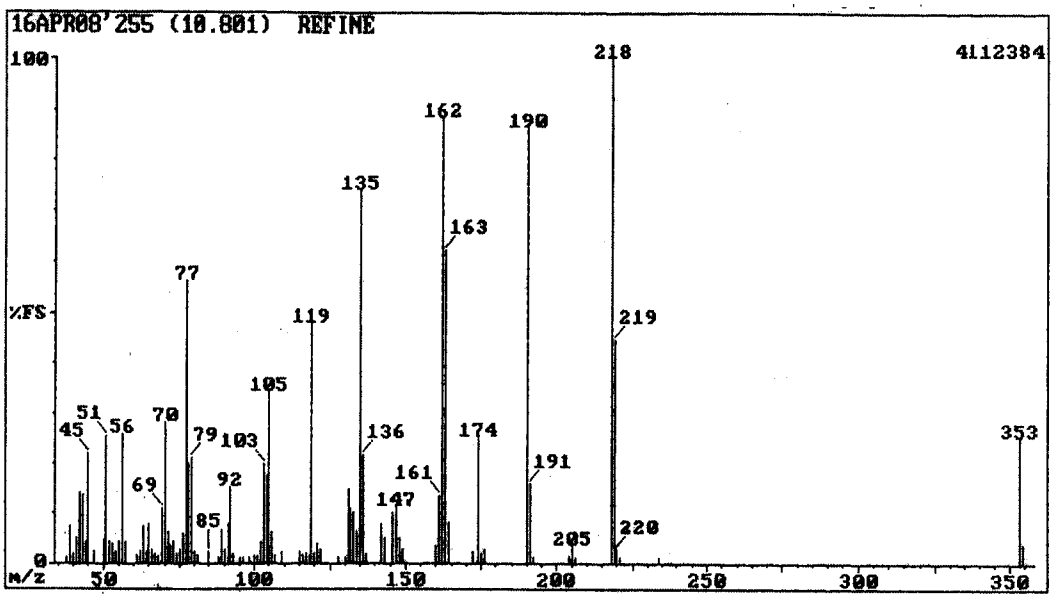


Figure 4.11 Full Mass Fragment Spectrum of MDEA

The ions monitored were m/z 118, 190* (AP), m/z 118, 160, 204* (MA), m/z 135, 162, 190, 325* (MDA), m/z 135, 162, 204, 339* (MDMA), m/z 135, 162, 190, 218, 353* (MDEA) and m/z 193* (d_3-AP). The ions labelled "*" were used for quantitation.

4.4.6 Optimisation of Derivatisation Step

4.4.6.1 Experimental

Pentafluoropropionic anhydride (PFPA) is commonly cited in the literature [193,238-239] as a derivatising agent for amphetamines prior to analysis by GC/MS. The temperature and length of derivatisation used, differs widely from 40 to 70 °C for a total time of between 15 to 45 minutes. In order to assess the effect of different temperatures and derivatisation times, AP, MA, MDA, MDMA and MDEA in the

presence of the internal standard (AP-d_3) were derivatised with PFPA for 15, 30 and 45 minutes at four different temperatures (40, 50, 60 and 70 °C).

4.4.6.2 Results and Discussion

Table 4.6 summarises the results for the optimised derivatisation conditions for amphetamine, amphetamine – d_3, methamphetamine, MDA, MDMA and MDEA. The detector response was noted for the following ions; m/z 190 (AP), m/z 193 (AP-d_3), m/z 325 MDA, m/z 339 (MDMA) and m/z 353 (MDEA).

The difference in detector response was more pronounced with the more volatile amphetamines (amphetamine, amphetamine–d_3 and methamphetamine). In all cases, derivatisation for longer than 15 minutes resulted in lower response values. The optimum temperature to carry out the derivatisation step was at 50 °C. Figures 4.12 to 4.17 illustrate the effects of temperature and derivatisation time on the detector response for amphetamine, amphetamine – d_3, methamphetamine, MDA, MDMA and MDEA respectively.

Table 4.6 Optimisation of the Derivatisation Temperature and Duration for Amphetamines using PFPA.

Analyte	T. (°C)	Peak Area (Detector Response)		
		15 minutes	30 minutes	45 minutes
AP	40	31543942	22737862	12109251
	50	69531288	24425318	16470126
	60	51540912	18037396	11986538
	70	42117208	22828872	12091415
AP-d_3	40	32270562	28011310	14571298
	50	65439980	24834690	16725190
	60	49514524	26144044	15678454
	70	44196360	25963622	12736432
MA	40	48017672	29281044	14978060
	50	78205000	29778634	16746971
	60	55423488	21968266	13434037
	70	55299776	26678398	14502206
MDA	40	1290022	1278518	1095517
	50	1629581	1199926	1077586
	60	1390941	1062945	1087454
	70	1205106	933119	914120
MDMA	40	1135886	1182279	860629
	50	1519512	1106719	931391
	60	1267306	891722	864978
	70	1122389	765132	799930

MDEA	40	311420	322098	229643
	50	384998	284700	236805
	60	362644	261200	234105
	70	308229	216297	204556

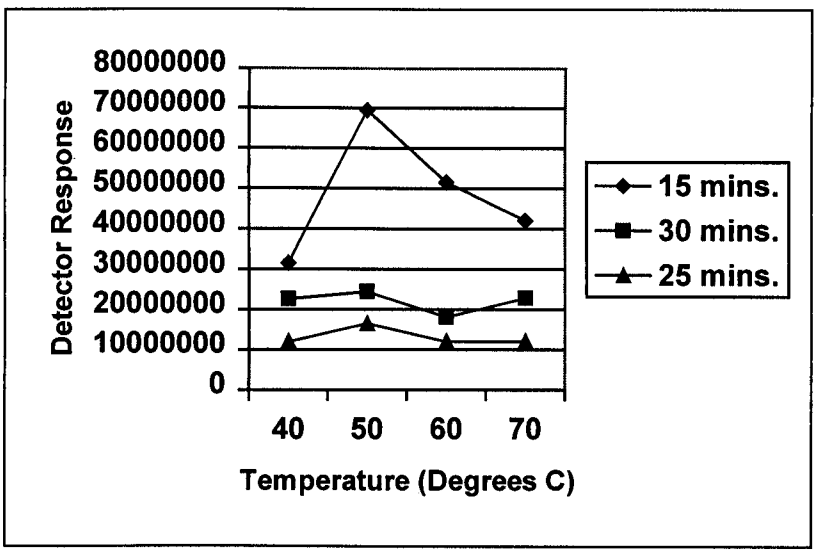


Figure 4.12 Effect of temperature and derivatisation time on the detector response for amphetamine

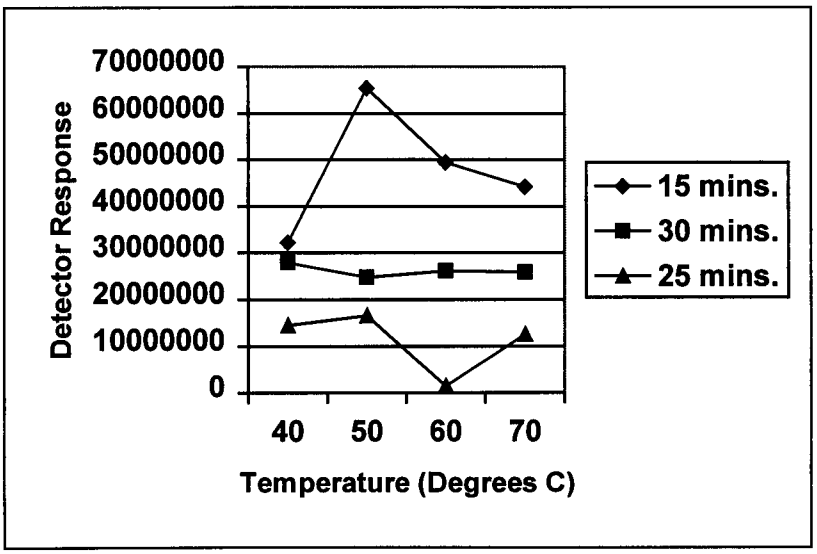


Figure 4.13 Effect of temperature and derivatisation time on the detector response for amphetamine – d_3

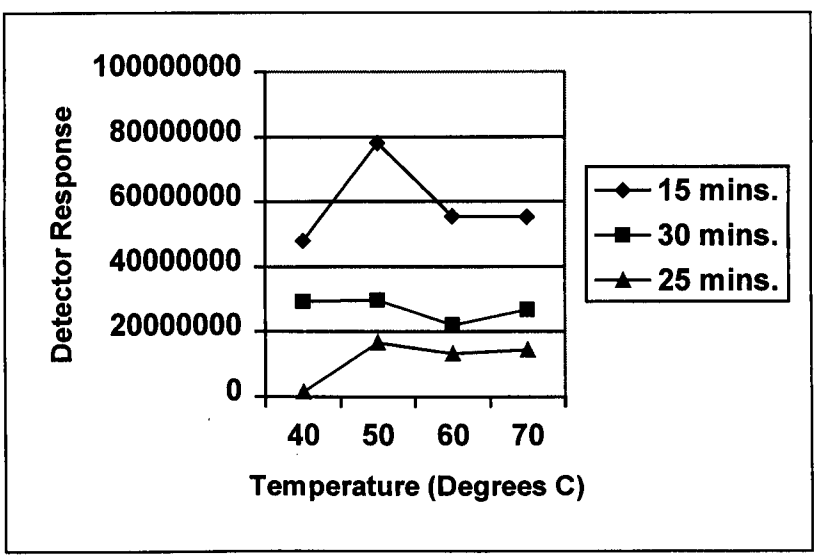


Figure 4.14 Effect of temperature and derivatisation time on the detector response for methamphetamine

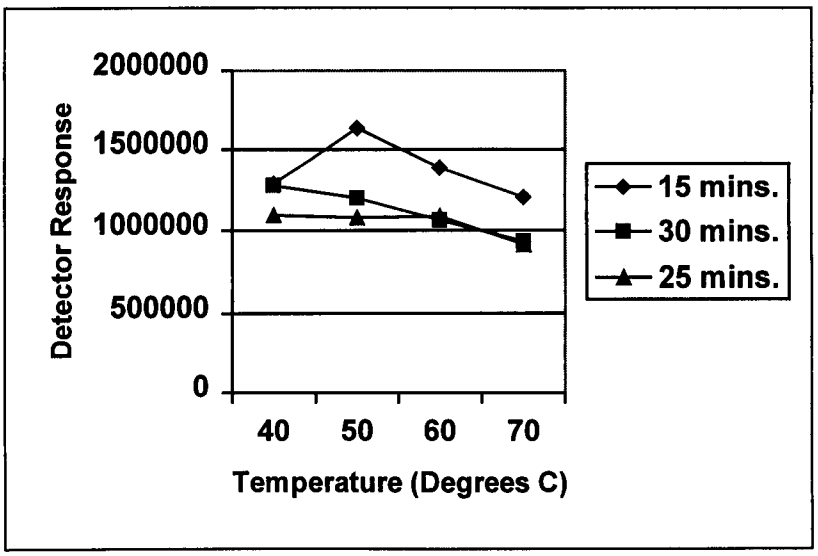


Figure 4.15 Effect of temperature and derivatisation time on the detector response for MDA

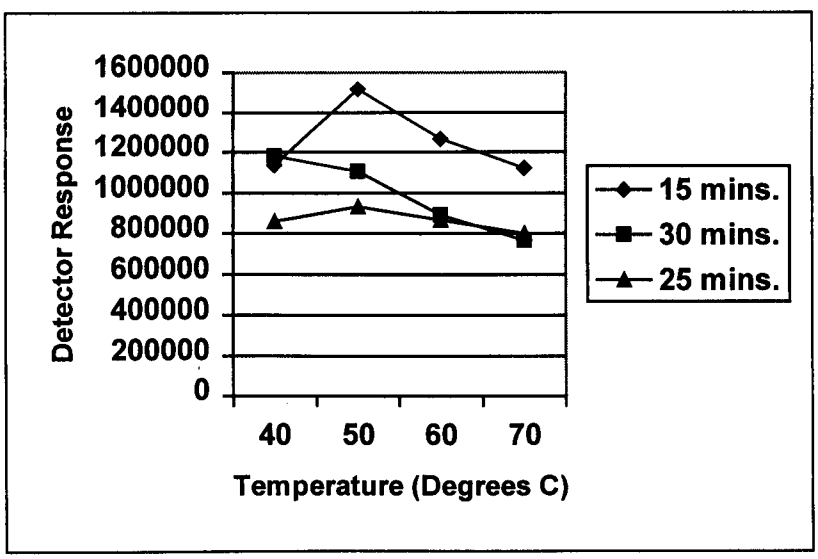


Figure 4.16 Effect of temperature and derivatisation time on the detector response for MDMA

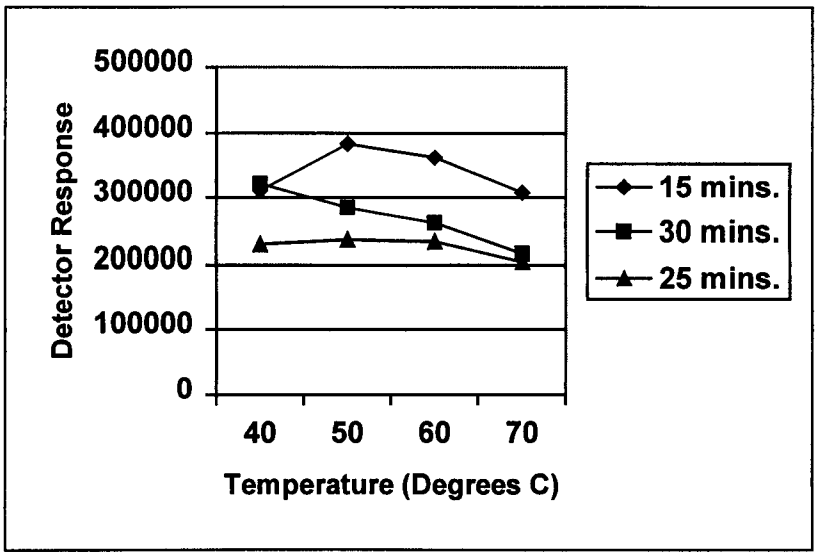


Figure 4.17 Effect of temperature and derivatisation time on the detector response for MDEA

The optimum conditions for derivatisation of the six amphetamines was at 50°C for 15 minutes.

4.4.7 Solid-Phase Extraction of Hair

4.4.7.1 Introduction

Nakahara *et al* [230] developed a solid-phase extraction method to identify MDA and MDMA in hair, along with other hallucinogens (LSD and PCP). This method involved treating the hair with 2ml of methanol – 5N HCl (20:1) in the presence of deuterated internal standards, followed by a standard solid-phase extraction

procedure. β-glucuronidase has been used successfully in the past to extract amphetamines and various other drugs from hair [224-225].

A method combining β-glucuronidase sample pretreatment and a solid-phase extraction procedure, optimised for the analysis of amphetamines in whole blood [56], was investigated as a means of extracting amphetamine, methamphetamine, MDA, MDMA and MDEA from hair.

4.4.7.2 Sample Pre-treatment

Hair samples were washed once with 0.1% SDS in deionised water and then in triplicate with deionised water. Each wash step was carried out by sonicating for 15 minutes. The hair was then rinsed with methanol and allowed to dry overnight in a dessicator.

The dried hair was measured and cut to the appropriate lengths, before being ground to a fine powder under liquid nitrogen using a mortar and pestle. The powdered hair was placed in a clean vial and weighed accurately.

Each hair sample was treated with 50 µl of β-glucuronidase [0.1mg/ml in phosphate buffer (pH7.4, 0.1M)] and 2 ml of phosphate buffer (pH7.4, 0.1M). 100 µl of the internal standard, AP- d_3 (10ng/mg based on a 10mg hair sample) was added and the samples were incubated at 40 °C for two hours. The samples were allowed to cool to room temperature, and then the supernatant was removed following centrifugation (2000 rpm, 5 min.). A further 2 ml of phosphate buffer was then added, centrifuged as before and the two supernatants combined for further analysis.

4.4.7.3 Solid-Phase Extraction

To separate the drugs from the hair matrix, solid-phase extraction was carried out on Isolute® Confirm HCX-3 (130 mg) SPE columns [manufactured by International Sorbent Technology LTD (Hengoed, U.K.) and supplied by Crawford Scientific (Strathaven, U.K.)].

The method used was previously reported for the analysis of methamphetamine and a mixture of different analytes [56]. The SPE columns were conditioned with methanol and phosphate buffer (pH7.4, 0.1M). The supernatant was applied onto the column, which was then washed with deionised water. The pH was adjusted with 0.01M acetic acid pH 3.3 and the column subjected to two drying steps before elution of the drugs. The first fraction (A) was eluted with acetone:chloroform (1:1v/v) and was discarded as no quantifiable levels of the drugs were detected. The analytes of interest were eluted into the second fraction (B) using 2 ml of 2 % ammoniated ethyl acetate.

The extracts were derivatised as described in section 4.4.4.

4.4.8 Validation of the Extraction Procedure

4.4.8.1 Experimental

The developed method was validated using blank hair spiked with the five amphetamines over a concentration range of 0 to 1000ng. In addition, recoveries of each of the five amphetamines from hair were determined by extracting blank hair spiked with three different concentrations (50, 100 and 200 ng), using approximately

10 mg of hair. The internal standard (amphetamine-d_3) was added to the extract following solid-phase extraction. The peak area ratios (P.A.R.'s) of the extracted samples were compared to the peak area ratios of the unextracted standards.

Hair samples were spiked by adding a set volume of the working solution to 100mg of ground blank hair (washed prior to milling). The methanol was allowed to evaporate to dryness overnight at room temperature. The hair was then mixed thoroughly to ensure homogeneity of the sample.

4.4.8.2 Results and Discussion

The average recoveries from the spiked hair were greater than 70 % for all five amphetamines with coefficients of variance ranging from 2.0 to 10.6. The extraction method was linear in the concentration range 0 to 1000 ng with limits of detection (L.O.D.) of 5ng for AP and MA and 1ng for MDA, MDMA and MDEA (signal-to-noise ratio of 3:1). The results are summarised in Table 4.7.

Table 4.7 Recovery and linearity results for the extraction of amphetamines from hair

Analyte*	Concn. (ng) †	Standard P.A.R‡	Sample P.A.R. (n=3)	Recovery/ C.V. §	Linearity (r)**	L.O.D. (ng)
AP	50	1.446	1.021	70.6 / 8.7	0.990	5
	100	3.139	2.342	74.6 / 8.5		
	200	5.824	4.281	73.5 / 2.5		
MA	50	3.705	2.942	79.4 / 6.6	0.993	5
	100	5.194	4.721	90.9 / 10.6		
	200	8.750	7.219	82.5 / 2.7		
MDA	50	0.249	0.205	82.2 / 7.6	0.993	1
	100	0.434	0.313	72.2 / 2.8		
	200	0.826	0.594	71.9 / 2.0		
MDMA	50	0.170	0.148	87.1 / 6.6	0.995	1
	100	0.320	0.264	82.5 / 10.2		
	200	0.660	0.549	83.2 / 5.1		
MDEA	50	0.132	0.113	85.4 / 4.1	0.996	1
	100	0.191	0.182	95.4 5.9		
	200	0.383	0.355	92.7 / 4.0		

* AP: amphetamine, MA: methamphetamine, MDA: 3,4-methylenedioxyamphetamine, MDMA: 3,4-methylenedioxymethamphetamine, MDEA: 3,4-methylenedioxyethyl-amphetamine

† Concentration (nanogrammes)

‡ Peak Area Ratio

§ Coefficient of variance

** Correlation Coefficient

Figure 4.18 illustrates the linear relationship between amphetamine, methamphetamine, MDA, MDMA and MDEA and the peak area ratios over the concentration range 0 to 1000ng, spiked in hair.

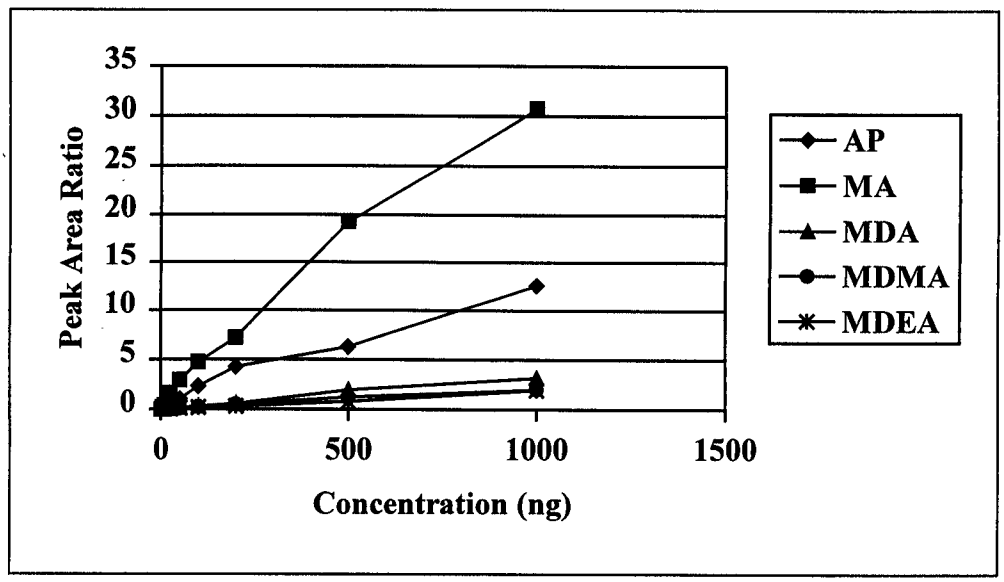


Figure 4.18 Calibration Curves for amphetamine, methamphetamine, MDA, MDMA and MDEA (0 to 1000ng)

This extraction method was used successfully for the analysis of amphetamine, methamphetamine, MDA, MDMA and MDEA in 139 hair segments as part of a joint project with The Scottish Centre for Criminolgy (See section 5.8.1).

4.4.9 Deuterated Internal Standards

4.4.9.1 Experimental

Amphetamine-d_5 (100µg/ml), methamphetamine-d_5 (100µg/ml), MDA-d_5 (1mg/ml), MDMA-d_5 (1mg/ml) and MDEA-d_6 (1mg/ml) standards manufactured by Radian International and supplied by Promochem, (Herts., U.K.), already prepared in 1ml of methanol, were investigated as alternative internal standards.

A working mixture (10µg/ml) of all five deuterated amphetamines was prepared in methanol. 100µl of the working solution was evaporated to dryness (N_2 stream, room temperature) and derivatised with PFPA as described previously. 1µl was injected onto the GC/MS and analysed using the full scan mode.

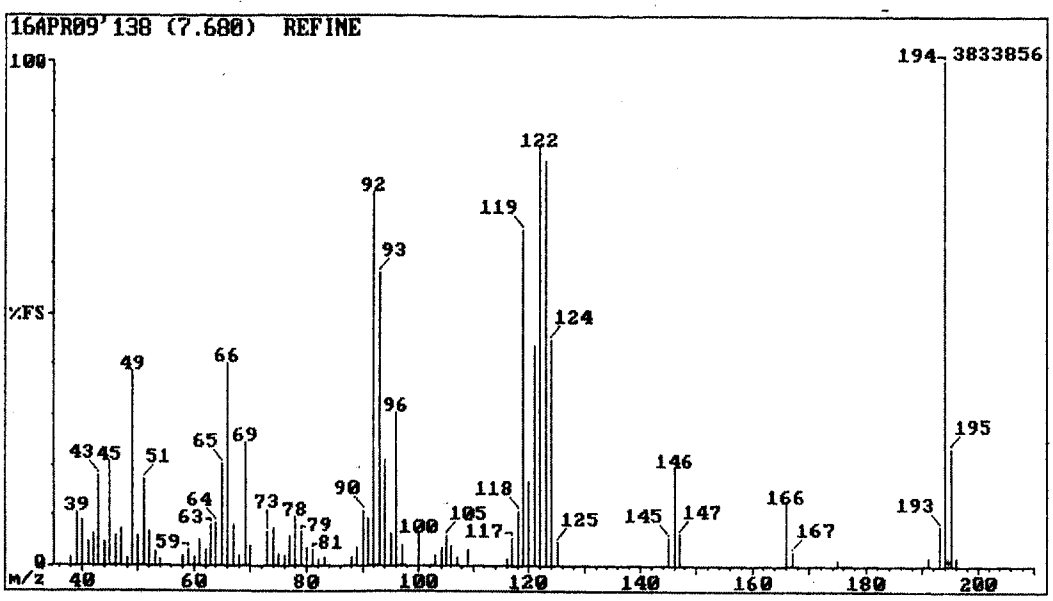


Figure 4.19 Full Mass Fragment Spectrum of amphetamine-d_5

4.4.9.2 Results and Discussion

Figures 4.19 to 4.23 illustrate the full mass fragment spectra of amphetamine-d_5, methamphetamine-d_5, MDA-d_5, MDMA-d_5 and MDEA-d_6

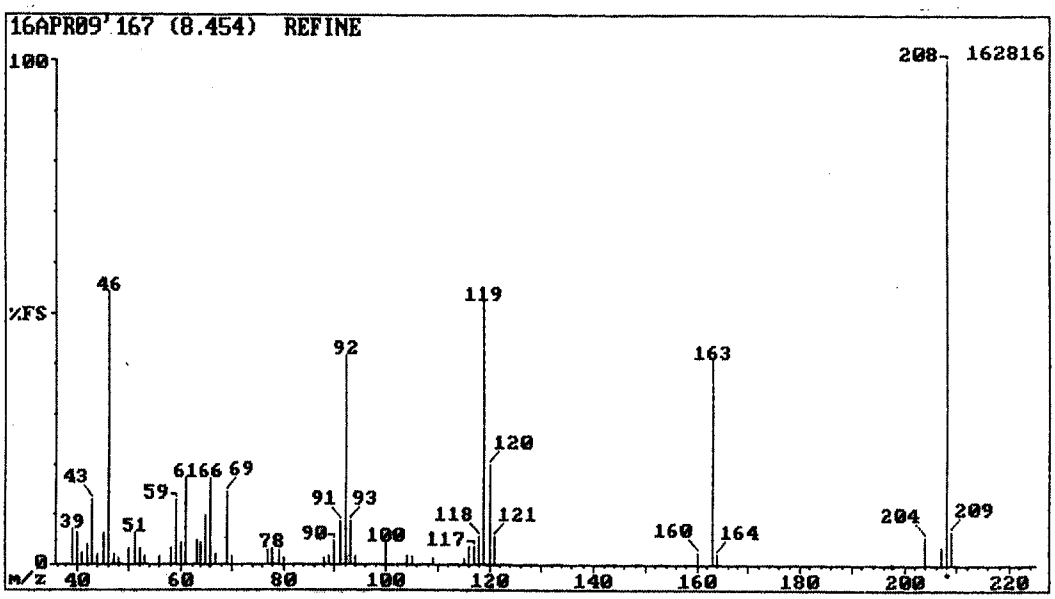


Figure 4.20 Full Mass Fragment Spectrum of methamphetamine-d_5

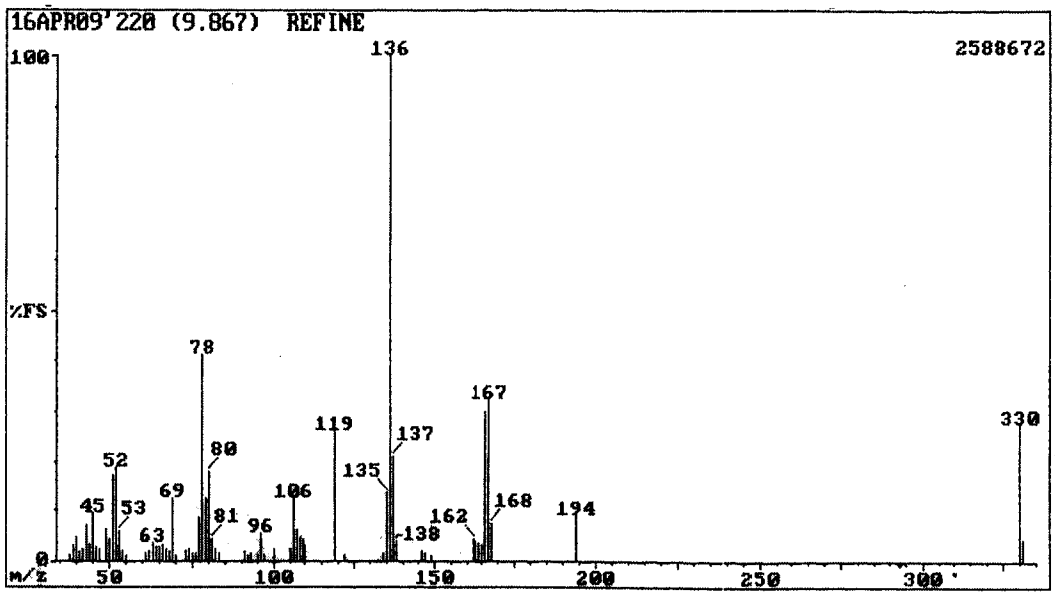


Figure 4.21 Full Mass Fragment Spectrum of MDA-d_5

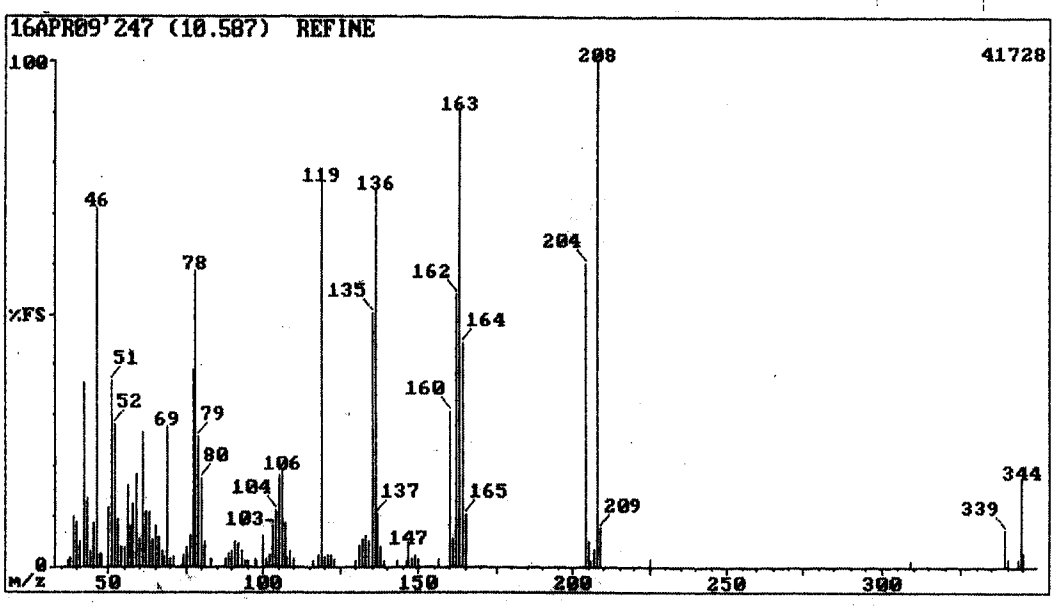


Figure 4.22 Full Mass Fragment Spectrum of MDMA-d_5

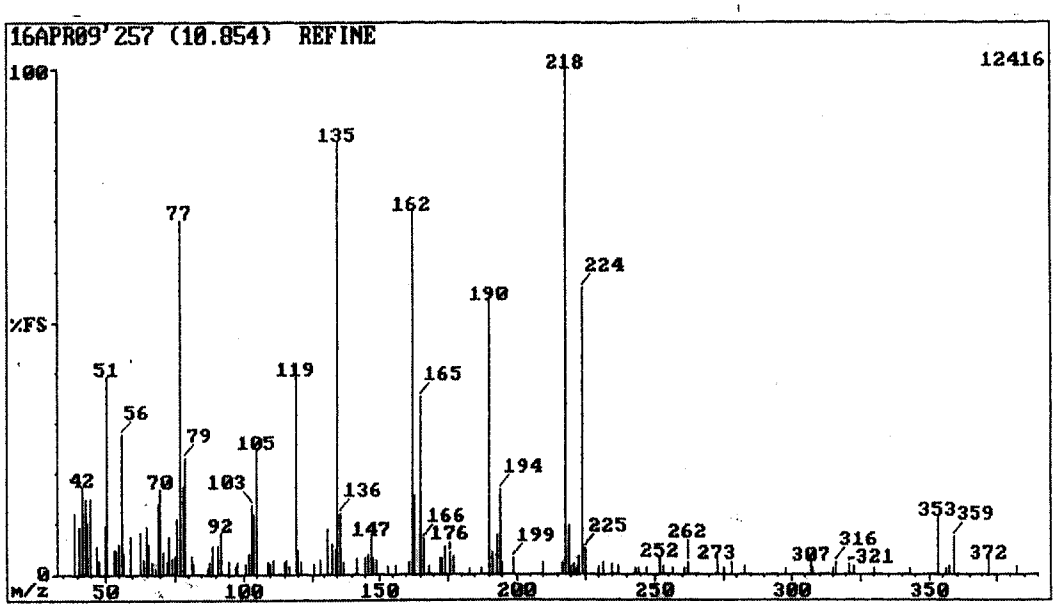


Figure 4.23 Full Mass Fragment Spectrum of MDEA-d_6

Ideally the non-deuterated ion (D_0) used for quantitation would be absent or in negligible quantities in the deuterated standard. This would ensure no false positive results when using deuterated standards as internal standards.

Table 4.8 summarises the ratios (non-deuterated:deuterated; D_0/D_n) of the quantitation ions obtained for each of the analytes. A comparison was made between the ratios obtained in this study and those supplied by the manufacturers.

Table 4.8 Non-deuterated:deuterated (D_0/D_n) ratios of the quantitation ions obtained for each of the deuterated standards.

Analyte	Ratio (D_0/D_n)	Results (D_0/D_n) %	Manufacturer (D_0/D_n) %
Amphetamine	190 / 194	0.10	0.13
Methamphetamine	204 / 208	7.4	0.11
MDA	325 / 330	3.0	0.12
MDMA	339 / 344	49.7	----
MDEA	353 / 359	153.8	0.03

The amount of non-deuterated quantitation ion present in methamphetamine, MDA, MDMA and MDEA was unacceptably high. Only the ratio obtained with amphetamine – d_5 agreed with the manufacturers stated value. Amphetamine – d_5 could be used as an internal standard without causing a false positive response, and was used as a replacement for amphetamine – d_3. The main reason for changing the internal standard being the ease of preparing stock standards from a methanolic solution of known concentration.

4.4.10 Addition of Tartaric Acid Step

4.4.10.1 Introduction

The University of Arizona, Tucson investigated tartaric acid as an aid in preventing losses of the small amines, namely, amphetamine and methamphetamine during evaporation and derivatisation.

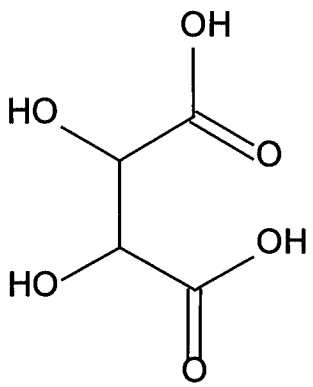


Figure 4.24 Structure of Tartaric Acid $(CHOH.COOH)_2$

The developed method was then reproduced in the laboratories of the Department of Forensic Medicine and Science, University of Glasgow. However, biological fluids are routinely screened for AP, MA, MDA, MDMA and MDEA when determining "speed" and/or "ecstasy" use, so all five amphetamines were included during method development. This also ensured that the addition of tartaric acid did not have a detrimental effect on the methylenedioxyamphetamine derivatives or indeed improve their recoveries. Tartaric acid (dextro-rotatory), [$(CHOH.COOH)_2$, M.W. = 150.09)], see figure 4.24, was obtained from Merck (Poole, U.K.)

4.4.10.2 Experimental

To investigate the usefulness of tartaric acid, derivatisation was carried out at room temperature (Method A) and 70°C (Method B) for 2, 5, 10, 15 and 20 minutes. Derivatisation at 70°C for 20 minutes was used as the standard reaction conditions to represent those commonly cited in the literature for producing PFP-amphetamine derivatives.

Methods A and B, were then compared to the derivatisation conditions used previous to this investigation. Derivatisation was carried out at 50°C (Method C) and did not involve the addition of tartaric acid. To enable a direct comparison, Method C was conducted at each time interval.

The comparison of these three methods involved the preparation of a methanolic standard solution of AP, MA, MDA, MDMA and MDEA (5µg/ml). A three-point calibration curve was obtained using solvent volumes of 20, 50 and 100µl of the standard solution.

To each standard was added 100µl of tartaric acid (1mg/ml) in ethyl acetate, and 50µl of 2% ammoniated methanol (to replicate the basic elution solvent). The samples were then evaporated to dryness (room T, stream N_2). They were derivatised with PFPA:Ethyl acetate (1:1), after which they were evaporated to dryness as before and then reconstituted in 50µl of a 1µg/ml solution of butylated hydroxy toluene (BHT) in ethyl acetate.

Analysis was conducted by GC/MS and quantitation was carried out using the ion ratios of the analytes to BHT (205*, 220). The ion indicated with an * was used for quantitation. These ions were chosen after obtaining the full mass fragment spectrum of BHT (see figure 4.25).

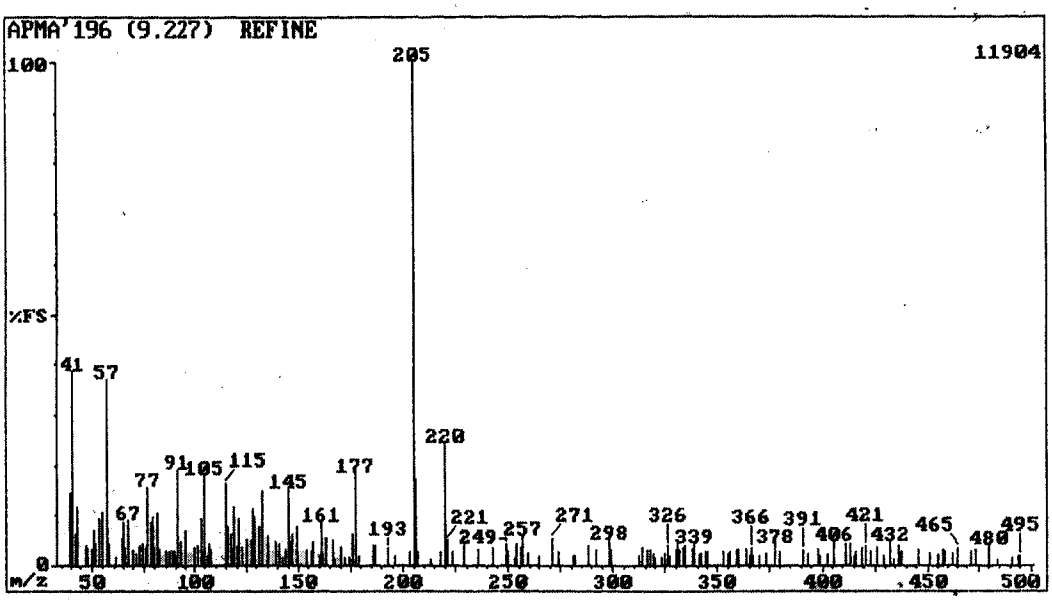


Figure 4.25 Full Mass Fragment Spectrum of BHT

4.4.10.3 Results and Discussion

Figure 4.26 shows the retention time of BHT in relation to the PFP-amphetamine derivatives studied. The chromatogram shows good peak separation with no interfering peaks.

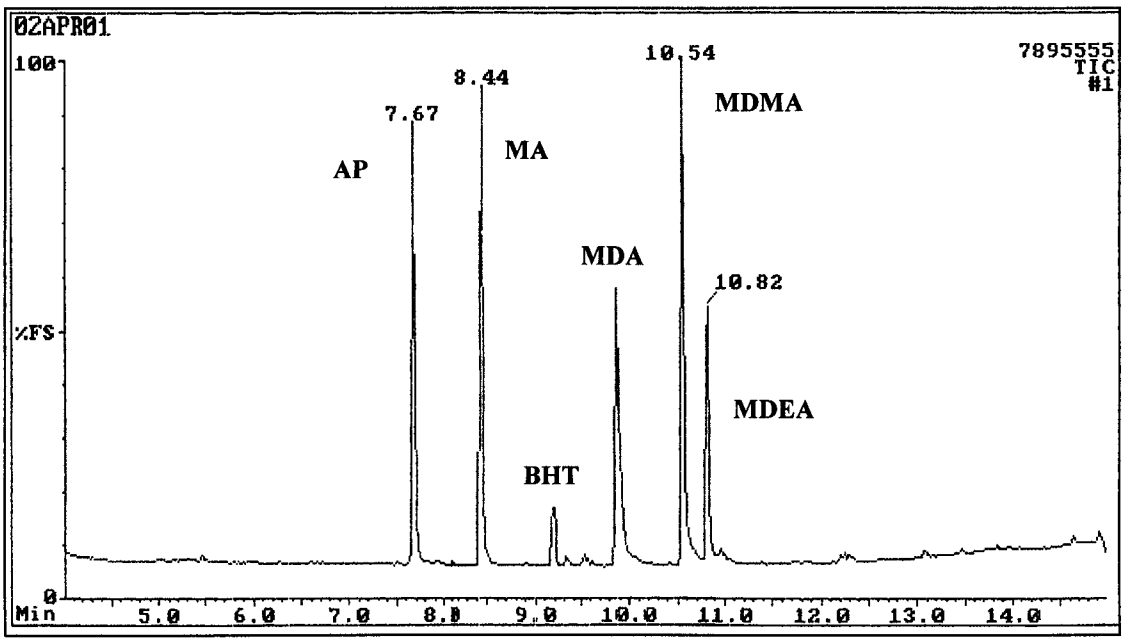


Figure 4.26 Chromatogram showing the retention time of BHT in relation to AP, MA, MDA, MDMA and MDEA.

Derivatisation was conducted at room temperature (Method A) and 70°C (Method B) for 2 to 20 minutes. Methods A and B, were compared to Method C which did not involve the addition of tartaric acid and involved derivatisation at 50°C.

The results of these experiments are summarised in figures 4.27 and 4.28 for the amphetamine derivative (AP-PFP) and methamphetamine derivative (MA-PFP), respectively.

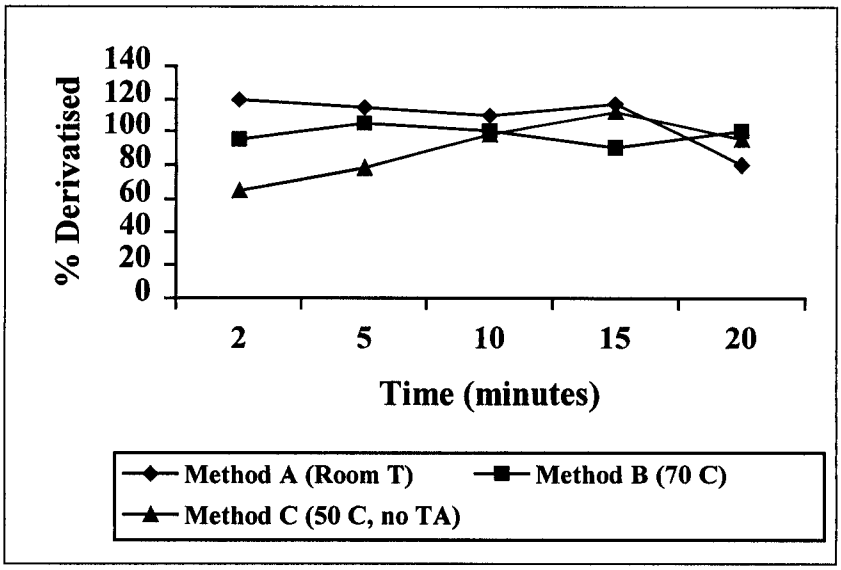


Figure 4.27 Recoveries obtained with Methods A and B compared to Method C which did not involve the addition of tartaric acid for amphetamine

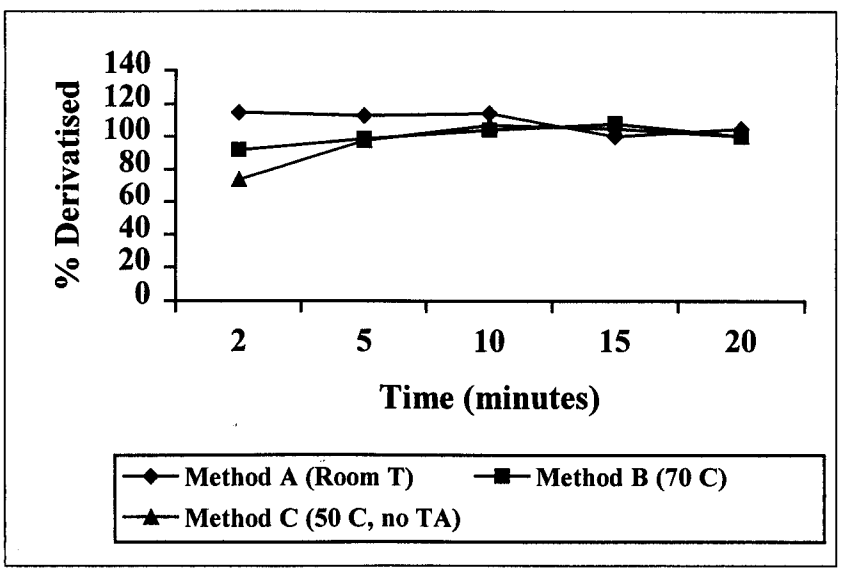


Figure 4.28 Recoveries obtained with Methods A and B compared to Method C which did not involve the addition of tartaric acid for methamphetamine

No appreciable difference in recoveries was seen for MDA, MDMA and MDEA and so the results are not included. With the exception of the reaction time of 20 minutes, derivatisation conducted at room temperature gave consistently better recoveries than at 70°C.

The recoveries resulting from these reaction conditions compared well with those conducted at room temperature. However, when carried out at each of the times shown, the two methods involving tartaric acid gave much better recoveries after only 2 minutes.

From these results it would appear that with the addition of tartaric acid the kinetics of the acetylation reaction are such that the samples do not require elevated temperatures to complete the derivatisation procedure. The developed method is simple and produces no interferences and the analysis time was reduced significantly, with a derivatisation time of just 2 minutes.

4.4.11 Re-validation of Extraction Procedure for Hair and Whole Blood

4.4.11.1 Experimental

The next step was to determine whether or not the addition of tartaric acid would improve the already existing extraction method for blood and hair. Recoveries from blood and hair were determined using spiked samples at three different concentrations. (Blood: 0.1, 0.5 and 1mg/l; hair: 50, 100 and 200ng). Peak area ratios of each analyte to the internal standard (amphetamine – d_5) were determined for each extracted spiked sample and compared to that of the unextracted standards.

Calibration curves were obtained by spiking blank blood and hair over concentration ranges of 0 to 500ng/ml and 0.5 to 5mg/l, and 0 to 1000ng respectively.

4.4.11.2 Results and Discussion

The results of the validation experiments are summarised in Table 4.9.

Table 4.9 Recovery and Linearity Results for the Extraction of Amphetamines from Blood and Hair with the Tartaric Acid Step.

Analyte	Blood		Hair	
	Recovery (%)*	**Linearity (r) 0 → 500ng/ml / 0.5 → 5 mg/l**	**Recovery (%)†**	**Linearity (r) 0 → 1000ng**
AP	87.2	0.995 / 0.997	91.3	0.993
MA	90.2	0.999 / 0.995	94.9	0.996
MDA	93.4	1.000 / 0.996	92.0	0.996
MDMA	95.6	0.998 / 0.997	97.6	0.997
MDEA	97.3	0.996 / 0.998	98.0	0.998

A recovery of greater than 87% was obtained in blood and greater than 91% in hair. In both cases amphetamine (AP) had the lowest recovery. The recoveries of amphetamine from hair increased substantially over the previous method (91% compared to 72.9%), which did not involve the addition of tartaric acid and from

84.3% to 94.9% for methamphetamine. Improvements in recovery was also noted for all three methylenedioxyamphetamine derivatives from hair.

The range of concentrations studied were 0 to 500ng/ml and 0.5 to 5μg/ml in blood and from 0 to 100ng/mg in hair. The methods were linear with r squared values of greater than 0.993 for blood and 0.995 for hair, see figures 4.29 to 4.31.

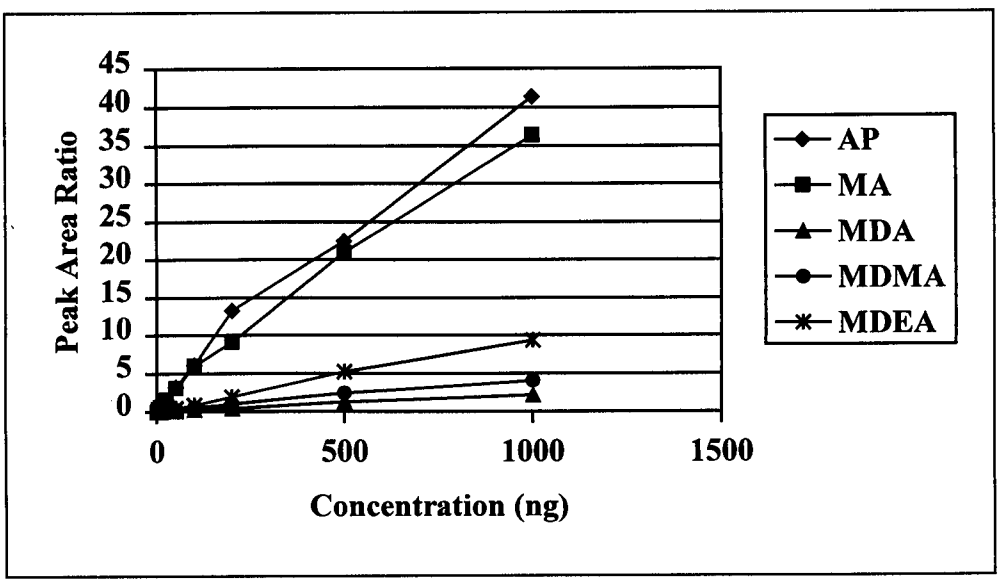


Figure 4.29 Calibration Curves for amphetamine, methamphetamine, MDA, MDMA and MDEA (0 to 1000ng) for hair after addition of tartaric acid

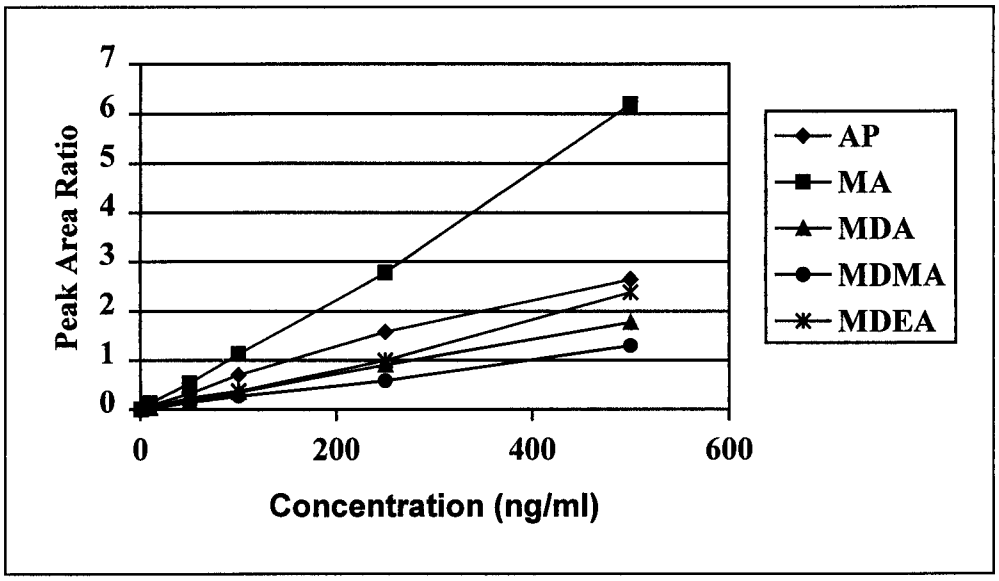


Figure 4.30 Calibration Curves for amphetamine, methamphetamine, MDA, MDMA and MDEA (0 to 500ng/ml) for blood after addition of tartaric acid

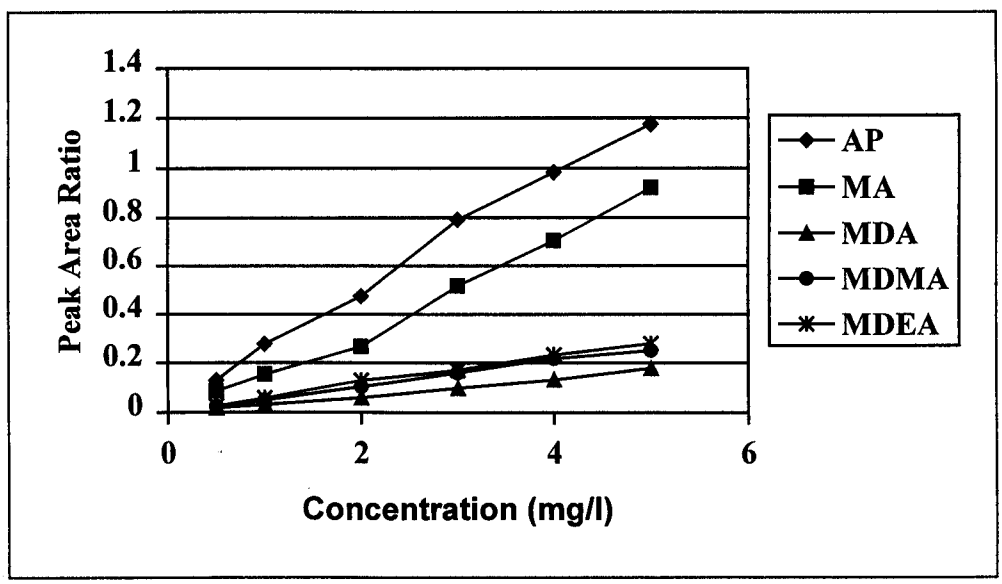


Figure 4.31 Calibration Curves for amphetamine, methamphetamine, MDA, MDMA and MDEA (0.5 to 5mg/l) for blood after addition of tartaric acid

4.5 Conclusion

The initial method combining enzymatic treatment, solid-phase extraction, derivatisation and GC/MS, successfully detected all five amphetamines (AP, MA, MDA, MDMA and MDEA) in hair and was successfully applied to the analysis of over 100 hair segments (see Chapter Five). The method was adapted for the analysis of whole blood, which involved separate sample pretreatment steps but the same solid-phase extraction and derivatisation steps.

This method was further developed with the addition of tartaric acid prior to evaporating an extracted sample or analytical standard to dryness. By the simple addition of tartaric acid prior to evaporation, recovery of the small amines was greatly improved and derivatisation complete in only two minutes at room temperature. The method is simple and produces no interferences.

In addition to the significant reduction in the total time of analysis, the recoveries from blood and hair improved and were greater than 87% for all five amphetamines. The method was successfully applied to the analysis of postmortem samples (see Chapter Six).

5. Hair Analysis

5.1 Introduction

Over the past decade hair testing for drugs of abuse has developed into an important tool with both forensic and clinical applications. In the United States, Japan and several European countries, hair testing is routinely used as an alternative or complementary technique to urinalysis and as evidence in court cases. This is not the case in the United Kingdom. Only two private companies offer hair testing for drugs of abuse and to date the detection of these drugs in hair has not been accepted as evidence in British courts.

Hair analysis offers several advantages over urinalysis:

- collection of a hair sample is less intrusive and causes less embarrassment,
- hair is a strong, stable tissue which results in a test unaffected by adulterants or short-term abstinence
- hair does not require refrigeration and can be stored indefinitely.
- hair grows at approximately 1cm per month and it is proposed that utilising segmental analysis can provide an individuals drug use history,
- if required a second representative hair sample can be analysed

The importance of hair analysis is in its ability to confirm long-term drug use, while biological fluids yield short-term information. The two techniques compliment each other, aiding the toxicologist to interpret the role if any, of drugs in each case.

5.2 Hair Anatomy and Physiology

5.2.1 Introduction

Although hair testing has great potential as a technique for determining drug use, there is still little understood as to how drugs are incorporated into hair, including the influence of individual variations and the role of contamination.

In order to fully understand and interpret the results of hair tests, its is important to have some basic understanding of the biology of hair.

5.2.2 Hair Structure

Hair is a complex tissue of cylindrical shafts of tightly packed cells (Figure 5.1). The cuticle is the outermost layer, which serves to protect the interior of the hair shaft. The cuticle consists of a single layer of flat, overlapping cells, which can be damaged or destroyed by heat, light and chemical treatments.

The cortex constitutes the bulk of the hair and contains long keratinised cells that form long fibres. The cortical cells contain a variety of chemicals including melanin, the principal pigment of hair. The colour of human hair varies with the quantity, distribution and type of pigments present.

The medulla is the innermost region of hair and is made up of a framework of spongy keratin and air spaces. In human hair the medulla may be discontinuous or absent.

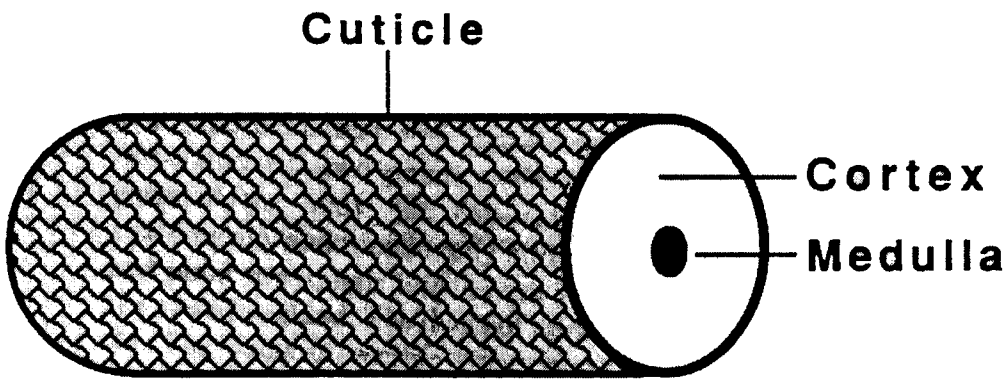


Figure 5.1 Cross-section of a Hair Shaft (Copyright © Elsevier Scientific Publishers, 1993)

Hair contains sulphur rich proteins called keratin, which accounts for approximately 65 to 95% of hair content. Lipids, trace elements, heavy metals and water are also present.

5.2.3 The Hair Follicle

Hair follicles are extremely complex structures embedded in the epidermal epithelium of the skin. There are estimated to be between 80,000 and 100,000 in the human head alone [240]. A simplified structure of the hair follicle is shown in Figure 5.2.

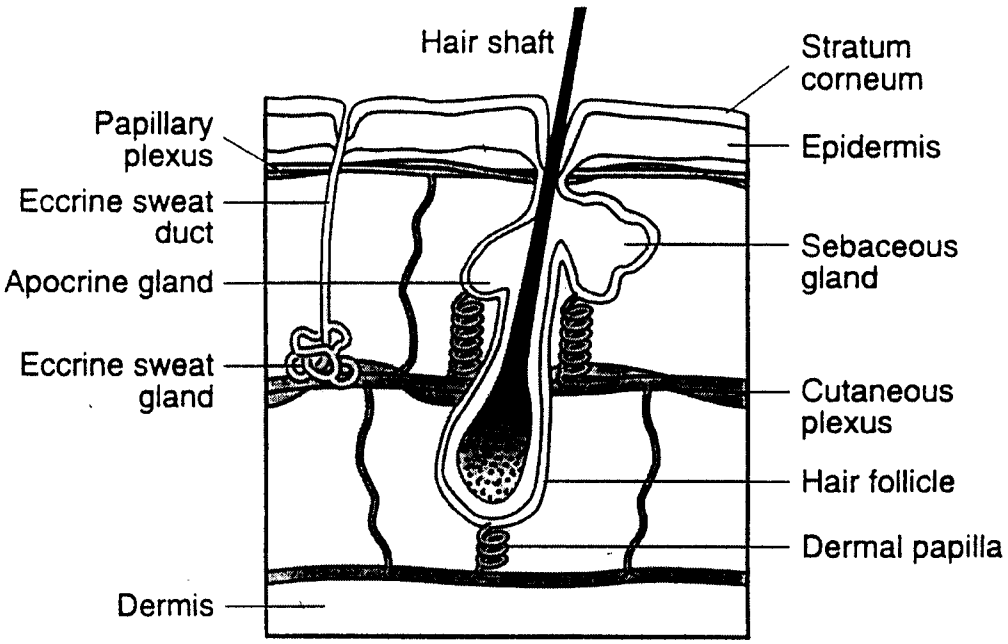


Figure 5.2 The Hair Follicle and Surrounding Structures (Copyright © Elsevier Scientific Publishers, 1993)

The sebaceous and apocrine glands secrete directly into the hair follicle, while the eccrine sweat glands secrete near the exit of the hair follicle [241]. Both the sebaceous and eccrine glands are located over nearly the entire surface of the body. The apocrine glands are localised in the eyelids, axilla, the external auditory meatus and the perineal region.

In addition to the secretory glands, a dense capillary network at the base of the bulb and the cutaneous plexus may nourish the hair follicle. It is possible that secretory glands and capillaries are sources of drugs present in hair [242].

Biological synthesis of hair cells is located within the hair bulb. The keratogenous zone is located above the hair bulb and is the site of keratinization, where hair cells differentiate, lose water and harden to form the hair shaft.

5.2.4 The Hair Growth Cycle

There are three recognised stages in hair growth, the anagen, catagen and telogen phases. During the anagen phase, there is an increase in metabolic activity, cell division and growth. It is at this time that drugs and trace elements are thought to be incorporated into hair [243].

Following this period of active growth, cell division stops, the hair shaft becomes fully keratinised and the bulb begins to degenerate. This is termed the catagen phase, which is a short transitional stage, the length of which varies with the hair type.

The telogen phase is a period when there is no hair growth, the follicle is short and can be easily removed by pulling. The length of time that hair is in this phase is dependent on the hair type and increases with age.

Other factors affecting hair growth include pregnancy, metabolic and genetic disorders, nutrition, endocrine dysfunction and seasonal changes [244]. Routinely, an average growth rate of 1cm/month is assumed, however this is an oversimplification with reported growth rates ranging from 0.75 – 1.35cm/month [245] and 0.6 – 3.36cm/month [242].

This wide window of variation makes interpretation of hair analysis very difficult. It has been suggested that individual hair growth rates should be calculated by bleaching or colouring a hair strand directly above the scalp. The growth rate can then be estimated by examining the hair 4 weeks later. Hair cut from the scalp does not represent the drug use over the previous month because it has been estimated to take approximately 3 weeks for the growing hair follicle to reach the surface of the scalp.

On average 85% of the hairs on the human scalp are in the anagen phase, It is important when interpreting results of hair tests to keep in mind the different rates of growth. Hair sampled from the vertex region of the scalp is preferred because it has the fastest growth rate and the largest percentage of follicles in the anagen phase.

5.2.5 Types of Hair

There are three basic types of hair on the human body. Vellus hair is fine, short, non-pigmented hair found on the eyelids, forehead and bald scalp. Intermediate hair is intermediate in length and diameter and is found on the arms and legs of adults. Terminal hair is coarse, long and pigmented with a large cross-sectional area and is found on the scalp, beard, eyebrow, eyelash, armpit and pubic areas.

These three different hair types are formed due to differences in hair follicles. Intermediate hair is produced by non-sexual hair follicles, which are not influenced by hormones and do not change after puberty. Ambisexual hair follicles are influenced by hormones and change during puberty, e.g. pubic areas, axilla and temple of scalp.

When it comes to analysis, scalp hair is the easiest to collect and causes less embarrassment. However, scalp hair is also subject to contaminants from the environment, sweat and cosmetic modifications. Beard hair is thick, has the slowest growth rate (approximately 0.27mm/day) and is subject to the same environmental contamination as scalp hair. Although sebaceous secretions may not contaminate beard hair, the specimen is likely to contain pieces of epidermis when obtained by shaving.

The advantages of using pubic hair include, availability of sample and not being subject to environmental or cosmetic effects. However, urine and gland secretions are still potential sources of contamination. Pubic hair grows slower than scalp hair and has a longer telogen phase.

5.3 Drug Incorporation into Hair

To accurately interpret hair test results requires knowledge of the mechanisms by which drugs are incorporated into hair, and the factors which affect their binding. Although a great deal of research is on-going in this key area, the exact mechanisms by which drugs are incorporated into hair are unknown. Several routes have been proposed and are illustrated in the following two models:

- Passive Diffusion Model - drugs enter from the blood supply at the base of the hair follicle [246]

- Multi-Pool Model - drugs are incorporated from sweat, sebum, blood and the environment [247].

Passive diffusion of drugs from blood into hair would in theory result in a drug concentration in hair proportional to the drug concentration in blood at the time of hair synthesis [248]. Controlled dosage studies with animals support the concept of a linear relationship for cocaine [249] and/or benzoylecgonine [246,249-250].

However, this model does not explain the different metabolic profiles in hair and blood. Cocaine, heroin and 6-mono-acetylmorphine (6-MAM) are rarely detected in blood but are the primary analyte found in hair. In addition, drug concentrations in hair can vary substantially in subjects receiving the same dose [251].

The multi-pool model encompasses all major routes by which drugs can enter the hair. If drugs are incorporated via many pathways, hair tests should be interpreted with caution until the exact role of external contamination can be assessed [248]. Of particular concern is the exogenous-endogenous shunt [252-253], where drugs can gain access to systemic circulation by respiration or transdermal adsorption. The determination of metabolites is of great importance to help distinguish between passive contamination and ingestion [254].

Binding studies suggest that hair has different affinities and binding capacities for various drugs and the binding mechanisms may be unique for each drug [231,255]. Factors affecting the incorporation and binding of drugs include, the pKa value, non-protein bound drug molecules, size, lipophilicity [256], structure [255] and melanin affinity [245,257]. Methadone, buprenorphine, L-α-acetylmethadol (LAAM) and their metabolites were detected in higher concentrations in pigmented hair than in non-pigmented hair [258].

Potsch *et al* [259], proposed the 'biochemical concept' to explain the endogenous incorporation of drug molecules into growing hair. This concept tentatively explains the high parent drug to metabolite ratio in hair, the dependence of incorporation on the physico-chemical properties of the drug, the incorporation of drugs into non-pigmented hair and the dependence of drug content on hair pigmentation. Further work is currently underway to further prove the theories postulated.

Studies to investigate the incorporation of drugs into hair, which involve animal models do provide useful information when interpreting human hair tests [260]. However, animal and human hair differs with respect to growth cycles and animal hair is more hollow, which may result in different drug distribution. Alternatives to animal models include in vitro human hair growth [260] and transplantation of human hair onto nude mice [261-262]. Human studies provide useful information regarding the stability of drugs in hair but are of limited value for studying the mechanisms of drug incorporation due to ethical considerations.

The stability of drugs in hair is dependent on the morphology and phsyicochemical properties of hair. Skopp *et al* [263], investigated the existence of pathways for drug molecules to diffuse into and out of the hair fibre. Diffusion of morphine, codeine and dihydrocodeine did occur. They concluded that the non-keratinous regions in human hair could provide channels for diffusion of small molecules in the presence of water. Therefore, the diffusion process can result in external contamination or loss of drug concentration. This was also illustrated by Potsch and Moeller [264] who used fluorescence microscopy to exam Rhodamine B stained hair fibres.

Several researchers have investigated the effect of cosmetic treatments on hair [265-270]. Although the studies carried out varied with respect to the drugs and the cosmetic treatments investigated, all were in agreement that dyeing, perming and bleaching have a deleterious effect on the drug concentration detected in hair. Daily shampooing does not significantly affect drug concentration [246].

Jurado *et al* [267], focused on the effects of bleaching and dyeing on the concentrations of opiates, cocaine, cannabinoids and nicotine detected in hair. The decrease in drug concentrations was more prominent for bleached hair than dyed. Damaged hair showed significantly lower levels.

Donahue *et al* [270] investigated five shampoos which individuals or manufacturers claimed would remove drugs from hair. In general, the treatments did reduce drug concentrations, however, this was not significant and did not cause negative screening results.

5.4 New Developments in Hair Testing Methodology

The historical development of hair testing techniques for drugs of abuse was described by Sachs [271] as having progressed through different phases beginning with the 'Pre-MSD' period where techniques used were predominantly immunoassay based, e.g. radioimmunoassay [272-273]. Following this was the 'uncritical' use of mass selective detectors (MSD's) and then the 'critical evaluation' of methods involving MSD's from 1993 to 1996. 1997 onwards will hopefully see an increase in papers

dealing with the areas where a greater understanding is required to further develop the usefulness of hair testing.

The majority of present day hair testing methods for drugs of abuse involve five steps:

1. The hair sample is washed to remove external contamination [HG9,10], and is then measured and cut to the appropriate lengths required.
2. Individual hair samples are then cut into smaller pieces or ground to a powder.
3. The drugs are then extracted from the hair matrix using one of a variety of techniques. These include methanol and β-glucuronidase which remove the drugs without damaging the hair and are preferentially used for screening for a range of different drugs [226]. Acidic or alkaline hydrolysis are commonly used [254] while supercritical fluid extraction has been used lately as an alternative method [275].
4. Once the drugs are separated from the hair they can be extracted by the same techniques used for blood or urine.
5. Gas chromatography – mass spectrometry (GC/MS) is the method of choice for confirming the presence of drugs in hair, however GC-MS-MS and LC-MS are used in several laboratories as alternatives for greater sensitivity [276-277]. Capillary electrophoresis (CE) has also been used to detect drugs in hair [209,278].

Research has centred predominantly on the analysis of opiates [279-281], cocainics [256,282-283], amphetamines [121,284-285] and cannabinoids [217,246] in hair. Heroin and 6-monoacetylmorphine have been detected in the hair of heroin users, providing better alternative markers to morphine for confirming heroin use [286].

Henderson *et al* [287] carried out work with isotopically labelled cocaine and contradicted previous work by claiming that the detection of cocaine in hair does not provide an accurate record of either amount, time or duration of drug use.

Areas requiring future research include:

- Developing standard procedures for hair testing [288]. Welch *et al* [219] compared the results from an interlaboratory study involving the analysis of drugs users hair and blank hair fortified with drugs. Qualitatively the results were good and comparative for both drug users and fortified hair, however, quantitatively, the results from fortified hair was better. Analysts had greater difficulty extracting drugs from drug users hair.
- A greater understanding of how to interpret test results.
- Determining the influence of sweat on the levels of drugs incorporated into hair.
- Conducting tightly controlled human studies to determine dose-response relationships.

5.5 Applications of Hair Testing

Interpretation of hair test results is at present extremely difficult. Hair testing is generally agreed to be useful for determining the general exposure of the population to certain drugs [289]. Although there remains many unanswered questions regarding reliability, hair testing is used for a variety of applications including:

- Proving drug use in drug-related fatalities [229].
- Determining physical fitness to obtain a driving license [290].

- Criminal responsibility [291-292].
- Prenatal drug exposure [286,293].
- Offenses of drug laws [294].
- Monitoring compliance with medication or abstinence [295].

5.6 Conclusion

Hair testing has enormous potential, however additional studies will have to be carried out before hair achieves the accuracy and reliability of urine testing [296]. There exists several problematic issues which require resolution. These include contradictory findings reported for the effectiveness of washing techniques [223,274], the exact role of external contamination including the effects of cosmetic treatments, the possible bias in hair testing for drugs due to hair colour, ethnic origin and sex and unknown mechanisms by which drugs are incorporated into hair.

Many researchers believe that hair testing will become a routine complimentary technique to urinalysis once the methods have been refined and are made more amenable to larger-scale applications [297]. At present hair tests are expensive with little or no quality controls [298].

Although hair testing has proved useful for some substances [299] until these issues have been resolved hair testing will continue to be received with caution.

5.7 Experimental

5.7.1 The Scottish Centre for Criminology

5.7.1.1 Introduction

The following project was part of a larger study investigating the use of "speed" and "ecstasy" in Glasgow [300] and involved individuals from multiple disciplines[†].

Subjects claiming use of "speed" and "ecstasy" were recruited from the "dance scene" by chain referral (i.e. subjects nominated other subjects). They were asked to complete a detailed questionnaire of their drug use over the previous 12 months and to donate a hair sample for analysis. Subjects were compensated for samples.

The aims of the project were to investigate the accuracy of hair analysis in confirming self-reported use of "speed" and "ecstasy" and to determine whether or not a correlation exists between the amount of drug ingested and the levels detected in hair. Amphetamine (AP) and methamphetamine (MA) were identified to confirm "speed" use and MDA, MDMA and MDEA for "ecstasy" use.

[†] Desiree L. Allen,[1] P.D.F.S.; Karen S. Scott,[1] Ph.D.; John S. Oliver, [1] Ph.D.; Jason Ditton,[2] Ph.D. and Iain D. Smith, [3] M.R.C.Psych. [1]Department of Forensic Medicine and Science, University of Glasgow, Glasgow, Scotland. [2]Faculty of Law, The University of Sheffield and The Scottish Centre for Criminology, Charing Cross Clinic, 8 Woodside Crescent, Glasgow, Scotland. [3]Consultant Psychiatrist, Gartnavel Royal Hospital, Glasgow, Scotland.

5.7.1.2 Hair Analysis and Self-Reported Drug Use

Several independent laboratories in Europe, Japan and the US have confirmed the accumulation of drugs such as cocaine [219,283,301], heroin and other opiates [279-281,301], amphetamines [206-207,216,221] and phencyclidine [230,302] in the hair of drug users. Other studies published have reported a dose-response relationship in hair for a variety of drugs in controlled animal studies [214,249,303-304]. Most of the studies involving human subjects compared self-reported drug use to hair or urinalysis. A linear relationship was observed between hair cotinine levels and daily nicotine intake [305], however, this is an exception to what is generally reported. A relationship between low and high use and the levels of drugs detected in hair has been observed for cocaine [306], heroin [307] and buprenorphine [220]. The lack of a linear dose-response relationship is not surprising due to the number of unknown variables, such as the exact dose or purity of drug consumed, the accuracy of the self-report data, individual metabolism variations and the types and frequency of hair treatments.

5.7.1.3 Sample Collection

100 subjects were interviewed (male:female; 51:49). 95% of the participants were Caucasian. Ages ranged from 15-44 years, with a mean age of 24 years. "Ecstasy" use was recorded as the number of tablets consumed per month for each of the previous 12 months. "Speed" use was recorded as "ever consumed" and then broken down into how often they had consumed it in the previous week, month and year.

The hair samples were cut close to the scalp in the vertex region, wrapped in aluminium foil and sealed in labelled plastic bags. Where possible, two 6 cm segments were analysed. The proximal 6 cm was labelled the "root" sample and the

distal 6cm the "tip" sample. In total, 139 hair segments were analysed without prior knowledge of the self-report data.

A summary of the segments analysed is shown in Table 5.1. The hair lengths ranged from 1.5-12 cm. A total of 56 samples were analysed in full.

Table 5.1 Lengths of hair segments analysed

Sample Type	Length (cm)	Total
Roots	< 3	4
	3 ≤ 6	10
	6	40
Tips	6	29
Full	6.1 ≤ 12	10
	12	46
Total		139

5.7.1.4 Summary of Drug Use History

Of the 100 subjects questioned, 90 admitted taking "ecstasy" during the year prior to interview. Consumption of "ecstasy" was primarily at one of four locations (night-clubs, licensed raves, illegal parties and private parties).

The total number of "ecstasy" tablets consumed within these two groups ranged from 1 – 144 (mean = 19, median = 10) over the previous year. In this study, the heaviest user consumed 144 tablets in the previous year, which translates to more than weekly

but less than daily consumption of "ecstasy". This is in sharp contrast to heavy heroin users, who use heroin at least once a day. The control group (n = 9) consisted of individuals who participated in the dance scene, but denied ever using "speed" or "ecstasy". This number is low due to the difficulty in recruiting individuals who satisfied this criteria.

5.7.1.5 Results and Discussion

Hair segments were analysed as detailed in section 4.4.7. Each hair segment (n = 139) was analysed once by the described method. Of the 139 segments, 73 (52.5%) tested positive for at least one of the five amphetamines, the remaining 66 segments were negative for all five. In general, the levels of AP, MA, MDA, MDMA and MDEA detected in the hair samples are in the same range as those reported elsewhere [206,218,221,231,308].

Table 5.2 Drug concentrations detected in hair segments

Drug	No. (Range) ng/mg	Median ng/mg	Mean ng/mg
AP	11 (0.7 – 97.7)	2.5	12.5
MA	27 (0.6 – 32.3)	2.6	5.2
MDA	20 (0.1 – 8.4)	1.0	1.9
MDMA	56 (0.1 – 82.9)	0.7	4.6
MDEA	23 (0.1 – 12.0)	0.5	2.8

Some exceptions to this were found where levels detected were higher than those previously reported. These cases were few and the median of the results was found to lie within the reported ranges. The results are summarised in Table 5.2.

The ratio of metabolite to parent drug levels in hair has been used to determine whether a positive result is due to contamination or from ingestion [309-310]. This ratio is expected to be lower than 1.0 due to the preferential incorporation of the parent drug to the metabolite. Therefore, a value greater than one is thought to result from contamination. The Society of Hair Testing recognises set ratios for cocaine and heroin and their metabolites, but not as yet for amphetamines [310].

The metabolite to parent drug ratios are summarised in Table 5.3. With the exception of the ratio MDA: MDMA, the ranges of the others were higher than those previously reported. This can be explained in the case of AP and MA. "Speed" can contain both AP and MA as the parent drug, which will effect the metabolite to parent drug ratio. Combining the levels of MDMA and MDEA found in individual hair samples was carried out to determine if a relationship exists between the combined levels of these parent drugs in hair and the amount of drug consumed. Results obtained for the ratio MDA: (MDMA+MDEA) ranged from 0.03 to 6.5 in contrast to those found by Rothe *et al* [308] (0.03 to 0.2, n=67). However, they too reported cases with ratios > 1. A possible explanation for this would be the consumption of MDA as a parent drug as well as its metabolic contribution from MDMA and MDEA.

Table 5.3 Metabolite to parent drug ratios

Ratio	No. (Range)	Median	Mean
AP : MA	9 (0.15 – 44.40)	0.85	7.26
MDA : MDMA	12 (0.01 – 6.28)	0.40	0.54
MDA : MDEA	11 (0.10 – 6.50)	0.67	1.59
MDA : (MDMA + MDEA)	14 (0.03 – 6.50)	0.33	0.95

Concordance between the self-reported data and the levels detected in hair was investigated for all 139 segments. A total of 73 segments tested positive for at least one of the five amphetamines with 71 of these agreeing with the self-report data. 66 were found to be negative, with only six agreeing with the self-report data. Overall, agreement or concordance was greater than 50% with a low number of false positives, consistent with other studies. The results of which are summarised in figure 5.3.

A large number of false negatives were found. There are a number of possible explanations for this including the method lacking sensitivity, over reporting of drug use by the individual and the influence of cosmetic treatment on the hair [265-267]. Unfortunately, no information was available on previous hair treatments (e.g. perms, bleaching etc.) and so the contribution of this to the results could not be assessed.

		SELF: Yes	SELF: No
LAB	Yes	71 (51.1%)	2 (1.4%)
	No	60 (43.2%)	6 (4.3%)

Figure 5.3 Overall concordance between self-report and hair analysis (n = 139)

Comparing the different types of sample analysed, it was expected that root samples would be more accurate than tip samples as they have been subject to fewer environmental and cosmetic influences, which have been shown to affect the drugs stability in hair. In addition, the self-report data would be expected to be more reliable due to better recollection of drug use in recent months as opposed to 12 months prior to interview.

The root samples have slightly better concordance (59.3%) than the tips (51.7%) and full (53.6%) samples. Figure 5.4 summarises the concordance between self-report and hair analysis for each of the segment groups. These consist of roots (proximal sample ≤ 6cm), tips (distal 6cm) and full samples (> 6cm).

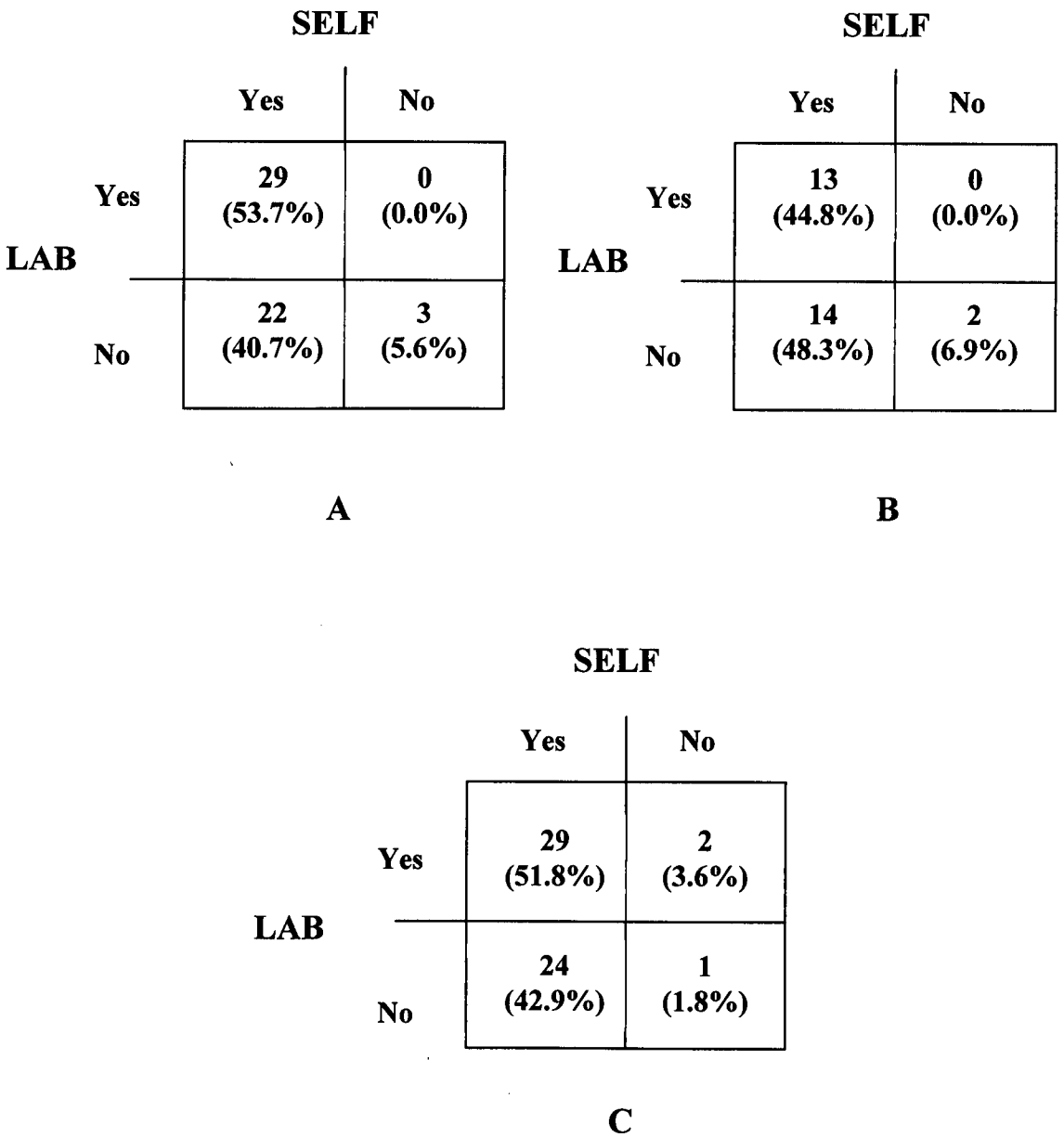


Figure 5.4 Concordance between self-report and hair analysis for each segment type; A. Roots (n = 54), B. Tips (n = 29) and C. Full (n = 56).

When comparing the self-report data for the use of "speed" only or "ecstasy" only, the concordance is very low for "speed" (28.3%) but higher for "ecstasy" (57.5%). The results are illustrated in figure 5.5. Unlike the "ecstasy" data, the "speed" data was not recorded for each month of the previous year and in addition, higher limits of detection were employed. The combination of these factors will decrease the number of potentially positive results.

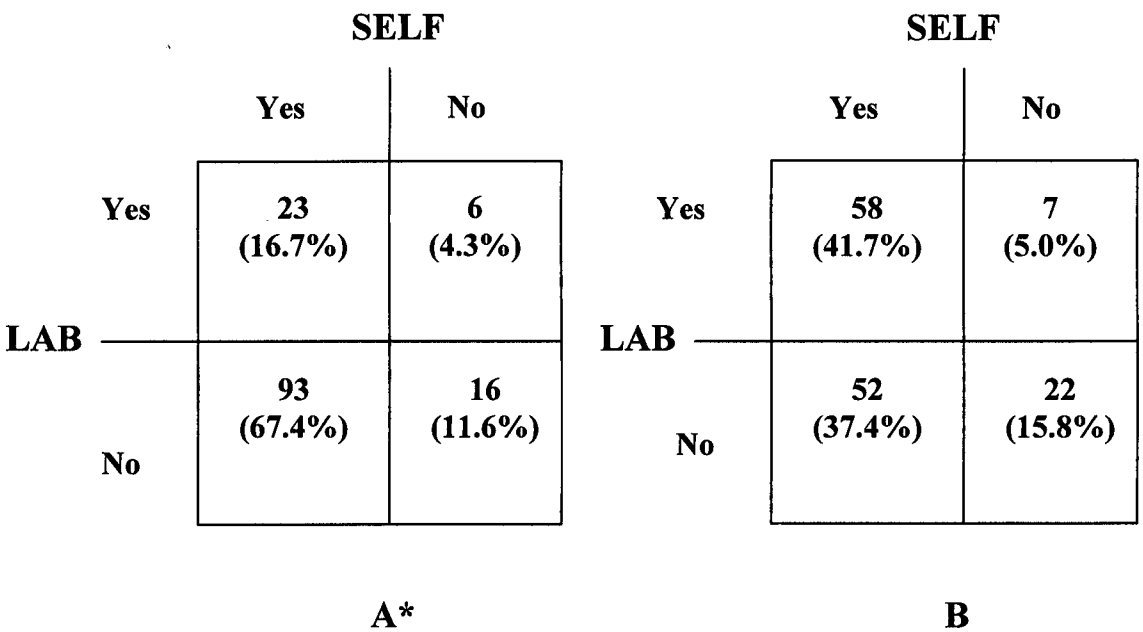


Figure 5.5 Concordance between self-report data and hair analysis for "speed" only (A) and "ecstasy" only (B). [One subject did not answer question on "speed"].

Of the six false positive segments where individuals denied "speed" use but tested positive for amphetamine and/or methamphetamine, five admitted "ecstasy" use, and were also positive for MDA, MDMA or MDEA. Six of the seven false positives for "ecstasy" admitted taking "speed" but only one case was positive for amphetamine. It is certainly plausible that the subjects were unaware whether they were consuming "speed" or "ecstasy" and this would account for some of the false positive or negatives.

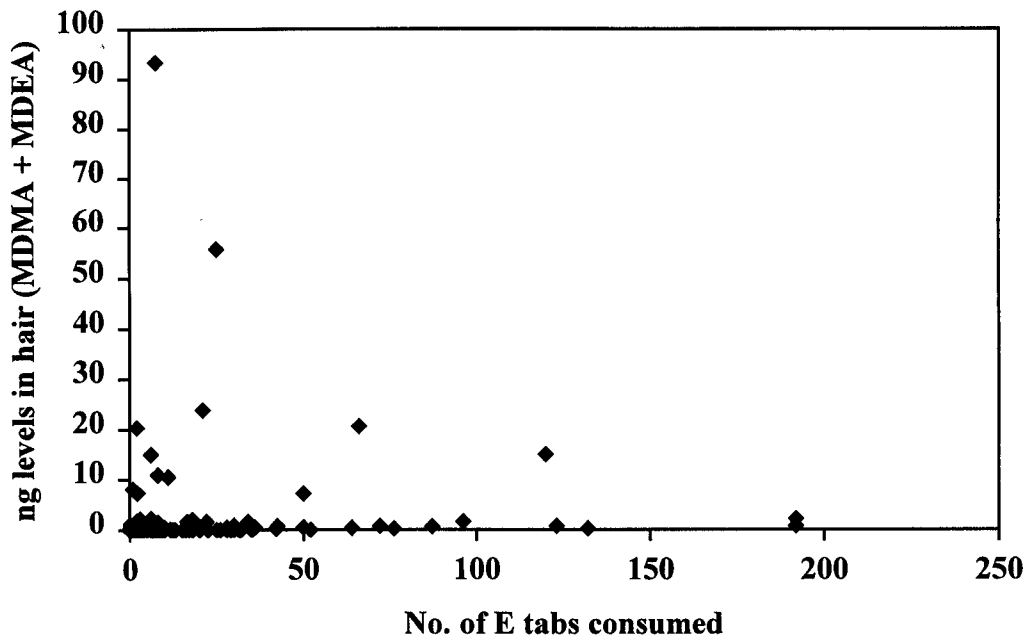


Figure 5.6 Correlation between the amount of ecstasy consumed and the total concentration of (MDMA + MDEA) in hair (n = 139).

Figure 5.6 illustrates the correlation between the total number of "ecstasy" tablets consumed and the total concentration of "ecstasy" (MDMA + MDEA) detected in each hair segment. There is a large scatter of results with the majority congregated along the axes. No linear dose-response relationship was observed ($r = 0.0484$, $p = 0.572$). This was also the case when comparing the amount of "ecstasy" consumed and the total concentrations of MDMA or (MDA + MDMA + MDEA) measured.

The results for each hair segment were separated into five groups depending on the average number of "ecstasy" tablets consumed each month. Each group consisted of approximately 20% of the segments analysed. The mean, median and range are noted for each group and summarised in Table 5.4.

Table 5.4 Correlation between the number of "ecstasy" tablets consumed/month and the total concentration of (MDMA + MDEA) detected in hair

No. E Tablets per month *	Frequency	Mean ng/mg	Median ng/mg	Range ng/mg
None	29	0.086	0.00	0 – 1.00
< 0.5	29	1.493	0.10	0 – 20.3
0.5 – 1.25	28	4.285	0.00	0 – 93.4
> 1.25 – 3.5	27	1.615	0.00	0 – 23.9
> 3.5	26	4.285	0.55	0 – 55.7
Total	**139**	**1.942**	**2.00**	**0 – 93.4**

* The average number of ecstasy tablets consumed each month.

An increase in the mean and the median values for the total concentration of MDMA + MDEA is observed from low to high frequency of use. The one exception to this is the group where between 0.5 to 1.25 tablets were consumed each month. The mean value is higher due to one case which had a total concentration of 93.4 ng/mg. Another important point here is the high number of false negatives which are prevalent throughout each of the four groups claiming "ecstasy" use. However, the number of false negatives did decrease from low to high use.

The data was evaluated with respect to sex and hair colour. There was no significant difference between the sexes [300]. 95% of the participants were Caucasian with the vast majority with hair of various shades of brown. The lack of a relationship with hair colour is probably indicative of this.

5.8 Conclusion

Although concordance between self-reported data and laboratory findings was approximately 50%, there were a high number of false negatives (43.2%) which may highlight the methods lack of sensitivity in confirming low use of "speed" and/or "ecstasy". No correlation was observed between the amount of "ecstasy" consumed and the levels detected in hair. However, there was evidence of a relationship existing between the level of use (measured in average "ecstasy" tablets consumed per month) and the levels detected in hair from low to high use.

The data was evaluated with respect to sex and race, however no significant relationship was observed in either case. The results of this study have given rise to a number of key questions which need to be answered to fully realise the potential of

hair analysis, in particular, for the analysis of "ecstasy" in hair. A combination of low concordance between self-report data and hair analysis results, and the high number of false negatives, raises questions regarding both the method sensitivity and the reliability of the self-report data.

The method of collection of hair samples is also of utmost importance. As highlighted earlier in this paper, the samples obtained for this study were too small to be analysed more than once. In some cases, this one analysis yielded a false negative result as the sample size was too small to give a significant level by GC/MS. It is acknowledged that obtaining larger samples on a voluntary basis could be problematic due to the sampling area (vertex region). However it is practical to be able to analyse samples in triplicate in order to decrease experimental errors and thus increase the validity of analytical results.

The large number of false negatives and low drug levels detected is likely to be a reflection on low drug consumption, with the heaviest users consuming approximately one "ecstasy" tablet per week of unknown purity. Comparing this to heavy heroin or cocaine users (> one per day), approximately 80% of "ecstasy" users in this study consumed less than one tablet per week.

The parent drug to metabolite ratio is unusual in comparison to the studies carried out with heroin and cocaine, where ratios of less than one are expected. Tablets seized from within the UK prior to the hair samples being collected, had high quantities of MDA present [136-137]. With this in mind the ratios are not unusual due to the unknown contribution of MDA as either a parent drug and/or a metabolite.

Large discrepancies between cases are reflected in the lack of a dose-response relationship for the segments analysed. Two examples are given which clearly illustrate this.

One subject reported use of 52 "ecstasy" tablets over a six month period, however the hair was negative for all five amphetamines. Conversely, another subject denied use of "ecstasy" but 0.6 ng/mg of MDMA were found in the corresponding 2 cm hair segment.

Determining the smallest dose detectable in hair, in combination with dose-response studies, would certainly help explain why in one case, where only five "ecstasy" tablets were consumed, a level of 1.1 ng/mg of MDMA was detected. However, when 50 tablets were consumed by another subject, this only gave rise to a level of 0.5 ng/mg of MDMA.

The influence of various factors, i.e. cosmetic treatments, individual metabolism variations, and the uncertainty of the content and dose of the "ecstasy" tablets, are currently being investigated further. A wealth of information can be gained from studies involving consumption of known doses and subsequent hair analysis. One additional factor, highlighted by Rothe *et al* [308], is the influence of drug incorporation via sweat. This is of particular relevance with "ecstasy" use, where participants in the dance scene are known to dance for several hours in hot, humid conditions.

The relationship observed between the average number of "ecstasy" tablets consumed per month and the total concentration of (MDMA + MDEA) detected in hair, gives some indication of the potential of hair analysis as an indicator of "ecstasy" use. This in addition to the previous points, will aid greatly with future interpretations of amphetamine levels in hair.

6. Case Studies

6.1 Introduction

The methods developed for the analysis of both blood and hair were applied to forensic case samples. Blood and hair samples were collected at postmortem, in addition to routine biological samples. Hair samples were pulled from the vertex region of the scalp and blood was collected from peripheral veins, usually the sub clavea. If this was not available, blood was collected from the femoral vein. This was done with the permission of the Procurator Fiscal who is in overall charge of sudden death investigations in Scotland.

Blood samples were analysed prior to this project by the technical staff at the Department of Forensic Medicine and Science. The samples were then re-analysed using the developed methods and a comparison made between the levels detected. In some cases the blood samples were re-analysed more than 2 years after the initial analysis, enabling a study of the analytes stability in postmortem blood over a period of 6 months to 2 years.

Postmortem hair samples were pulled from the scalp to free the root bulbs. The hair was de-rooted and then segmented depending on the length of the deceased's hair. The root samples were not washed, but the segments were, to remove any external contamination. Hair washes were analysed for the analytes of interest in addition to the hair segments.

In addition to determining the presence of the analytes, a preliminary investigation was conducted to determine whether or not a relationship existed between levels of analytes in blood and the corresponding levels detected in the root bulb. Segmental analysis of the hair samples was also carried out in an attempt to confirm antecedent drug use history.

A summary of the relevant case information was noted from the police sudden death report, including relevant information on antecedent history, medical notes and the circumstances surrounding the death.

In total, 22 hair samples were analysed and 54 blood samples re-analysed. The relationship between postmortem analyte levels detected in blood and the root bulb was investigated in 13 cases. The results of all cases are discussed in the following chapter.

6.2 Case Information

Reference: GC01 **Sex:** Female **Age:** ?

Circumstances: No police report

Cause of Death: 1.a No postmortem report.

Toxicology: 1.44mg methadone/L blood, 0.76mg morphine/L blood.

Samples Re-analysed: Blood + hair

Reference: GC02 **Sex:** Male **Age:** 36

Circumstances: Evidence of old healing needle puncture marks. IVDA for 15 years. Past 12 years attempted a number of drug rehabilitation programmes. Due to uplift methadone prescription 12 days prior to being found dead, but never did. Last seen alive 16 days prior to being found dead.

Cause of Death: 1.a Pneumonia with Multiple lung abscesses. 2. Intravenous Drug Abuse.

Toxicology: 0.12mg methadone/L blood.

Samples Re-analysed: Hair

Reference: GC03 **Sex:** Male **Age:** 22

Circumstances: No history of IVDA, but certified unfit for work due to smoking heroin and cannabis. Administered course of dihydrocodeine and nitrazepam. Day prior to death, 2100hrs – smoked 2 joints. 2200hrs – fell asleep. 0830hrs – found dead.

Cause of Death: 1.a Inhalation of gastric contents, b. Drugs overdose.

Toxicology: 0.18mg methadone/L blood.

Samples Re-analysed: Hair roots only.

Reference: GC04 **Sex:** Male **Age:** 39

Circumstances: Possible needle puncture mark in right groin. Evidence of chronic IVDA. Last seen by Doctor 8 days prior to death, collected weekly prescription of methadone and diazepam. (Methadone = 50mLs/day + diazepam (15mLs/day). 3 days prior to death complained of feeling unwell. 2 days prior to death, 1000hrs –

feverish. Admitted to being assaulted and condition deteriorated, transferred to ICU. Suffering from cellulitis of the abdominal wall.

Cause of Death: 1.a Sepsis syndrome due to, b. cellulitis anterior abdominal wall, due to c. chronic IVDA.

Toxicology: 0.36mg methadone/L blood, 0.44mg desmethyldiazepam/L blood, 0.25mg diazepam/L blood.

Samples Re-analysed: Blood + Hair

Reference: GC05 **Sex:** Male **Age:** 23

Circumstances: Known heroin user for 3 years. Last seen by Doctor 4 days prior to death. Repeat prescription (methadone, diazepam and temazepam). Released from prison 1 month previous after serving 13 months. 2330hrs – arrived home under the influence, 0100hrs – sleeping soundly. 0430hrs – heard coughing, 0955hrs – found dead.

Cause of Death: 1.a Overdose of morphine.

Toxicology: 0.14mg methadone/L blood, 0.23mg morphine/L blood.

Samples Re-analysed: Hair

Reference: GC06 **Sex:** Male **Age:** 16

Circumstances: No medical history of note. Under the influence (1750hrs). Sleeping but still alive (2145hrs), found lying face down (1125hrs, next day). Informed that decedent had consumed 30mg methadone before midday the previous day.

Cause of Death: 1.a Inhalation of gastric contents, b. Drugs overdose.

Toxicology: 0.07mg methadone/L blood, 0.33mg desmethyldiazepam/L blood, 0.33mg diazepam/L blood.

Samples Re-analysed: Blood

Reference: GC07 **Sex:** Male **Age:** 25

Circumstances: Evidence of chronic IVDA. Last seen by Drug Clinic day prior to death. 1745hrs – felt unwell, 1815hrs – struggling to breathe, administered oxygen. 2157hrs – condition deteriorated and died.

Cause of Death: 1.a Unascertained.

Toxicology: 19mg alcohol/100mL blood, 0.055mg methadone/L blood, 2.4mg lignocaine/L blood, 0.21mg desmethyldiazepam/L blood, 0.06mg diazepam/L blood.

Samples Re-analysed: Blood + hair.

Reference: GC08 **Sex:** Female **Age:** 35

Circumstances: Old needle puncture marks. Known drug user for 8 years. Last seen by Doctor 2 months prior to death. Released from prison day prior to death. 1300hrs – overdosed on methadone, treated with Narcan, signed self out (1430hrs). 2000hrs – consumed alcohol, smoked two joints, went to bed 2200hrs. Found dead (0900hrs).

Cause of Death: 1.a Methadone Intoxication.

Toxicology: 25mg alcohol/100mL blood, 15mg paracetamol/L blood, 0.68mg methadone/L blood, 31mg alcohol/100mL urine, 0.49mg desmethyldiazepam/L blood, 0.27mg diazepam/L blood.

Samples Re-analysed: Hair

Reference: GC09 **Sex:** Male **Age:** ?

Circumstances: No police report.

Cause of Death: No postmortem report.

Toxicology: 0.56mg methadone/L blood, 0.46mg amphetamine/L blood, 0.44mg desmethyldiazepam/L blood, 0.25mg diazepam/L blood.

Samples Re-analysed: Blood

Reference: GC10 **Sex:** Male **Age:** 33

Circumstances: Evidence of recent needle puncture marks and old scars. Known drug addict for 7 years, suffered from deep vein thrombosis. Prescribed methadone 5 months prior to death. Ate (0230hrs) and fell asleep. Found dead (1515hrs). Drug paraphernalia found at scene.

Cause of Death: 1.a Overdose of Morphine.

Toxicology: 0.09mg methadone/L blood, 0.28mg diazepam/L blood, 0.29mg temazepam/L blood, 0.70mg morphine/L blood.

Samples Re-analysed: Blood

Reference: GC11 **Sex:** Female **Age:** 29

Circumstances: Multiple healing needle puncture marks. Long history of drug use, involved in various detoxification programmes. Last seen by Doctor 6 days prior to death, requested methadone detoxification (40mL/day). Last seen alive 1700hrs day prior to death. Drug paraphernalia found at scene.

Cause of Death: 1.a Heroin intoxication.

Toxicology: 27.4mg paracetamol/L blood, 0.32mg methadone/L blood, 1.1mg desmethyldiazepam/L blood, 1.6mg diazepam/L blood, 0.7mg morphine/L blood.

Samples Re-analysed: Blood + Hair.

Reference: GC12 **Sex:** Male **Age:** 28

Circumstances: Evidence of recent needle puncture marks and old scars. Known heroin addict for a number of years. Last seen by Doctor 10 days prior to death and was prescribed methadone (35mg methadone/day), first prescribed 20 months prior to death. Consumed methadone (1200hrs), returned home, seen to shake violently and then collapsed unconscious (1640hrs).

Cause of Death: 1.a Inhalation of gastric contents, b. Methadone and Trichloroethanol Intoxication.

Toxicology: 0.41mg methadone/L blood, 33.2mg trichloroethanol/L.

Samples Re-analysed: Blood

Reference: GC13 **Sex:** Female **Age:** 27

Circumstances: Old and fresh needle puncture marks. Known drug user for 9 years. On methadone programme for 2 years. Last seen by Doctor 3 weeks prior to death to collect methadone (70mLs/day). Last seen 0930hrs, didn't collect methadone, Found dead (1215hrs) with needle in situ.

Cause of Death: 1.a Mixed heroin and temazepam intoxication.

Toxicology: 0.12mg methadone/L blood, 1.29mg desmethyldiazepam/L blood, 2.79mg temazepam/L blood, 0.94mg morphine/L blood.

Samples Re-analysed: Blood + Hair

Reference: GC14 **Sex:** Male **Age:** 28

Circumstances: Recently developed a drug habit. Diagnosed schizophrenic 6 years prior to death. Known to use heroin for past 3 years. Witness described decedent as being agitated and drunk. Demanded medication, but never received anti-depressants. Injuries consistent with falling from a height.

Cause of Death: 1.a Multiple injuries due to, b. Fall from a height.

Toxicology: 115mg alcohol/100mL blood, 0.1mg methadone/L blood, 161mg alcohol/100mL urine.

Samples Re-analysed: Blood

Reference: GC15 **Sex:** Male **Age:** 24

Circumstances: Numerous scars and injuries to body. Known solvent abuser and agoraphobic after several assaults. Day prior to death, under the influence, + 29 valium and 4 stronger valium. 0430hrs – half a tin of glue, witness stated decedent smelled of glue (1000hrs), went to bed, sleeping at 1300hrs and found dead at 1500hrs.

Cause of Death: 1.a Solvent and Drug Related Death, conditions contributing to death: 2. Cardiomegaly and coronary artery atheroma.

Toxicology: 0.34mg methadone/L blood, 0.16mg desmethyldiazepam/L blood, 0.46mg diazepam/L blood, 1.04mg toluene/L blood and 4.08mg toluene/kg brain.

Samples Re-analysed: Blood

Reference: GC16 **Sex:** Female **Age:** 20

Circumstances: Old healing scars. Known drug user, suffered from anxiety and panic attacks. Last seen by Doctor 6 months prior to death. Week prior to death

heavily under the influence of valium, temazepam and heroin (smoked). Day prior to death complained of stomach pains and headaches (withdrawal symptoms). 2100hrs – watched TV in bed until 0300hrs. Found dead at 0940hrs.

Cause of Death: 1.a Drug related death due to, b. methadone, diazepam and temazepam intoxication.

Toxicology: 0.36mg methadone/L blood, 0.35mg desmethyldiazepam/L blood, 0.14mg diazepam/L blood, 0.31mg temazepam/L blood.

Samples Re-analysed: Hair.

Reference: GC17 **Sex:** Male **Age:** 21

Circumstances: Fresh needle puncture marks and old scars. History of drug abuse. Methadone prescription stopped 2 months prior to death. Using heroin and temazepam for 2 years. Last seen by witnesses at midnight going to bathroom. Found dead next morning in bathroom with needle and tourniquet in situ.

Cause of Death: 1.a Drug Related Death, due to, b. Morphine (Heroin) and Temazepam Intoxication.

Toxicology: 33mg alcohol/100mL blood, 0.036mg methadone/L blood, 1.36mg temazepam/L blood, 0.38mg morphine/L blood.

Samples Re-analysed: Blood

Reference: GC18 **Sex:** Male **Age:** 17

Circumstances: Evidence of old scars. Known to abuse temazepam for 2 years. Diazepam and alcohol overdose 7 months prior to death. Evening – consumed 5 double strength dihydrocodeine + 7 yellow diazepam, excess alcohol and smoking

cannabis. Took dog for a walk (0430hrs), ate and fell asleep. Found dead in sitting position(1030hrs).

Cause of Death: 1.a Inhalation of gastric contents, due to, b. methadone, diazepam, cannabis and alcohol intoxication.

Toxicology: 103mg alcohol/10mL blood, 0.23mg methadone/L blood, 153mg alcohol/100mL urine, 0.19mg desmethyldiazepam/L blood, 0.40mg diazepam/L blood, 26ng THC-COOH/ml blood.

Samples Re-analysed: Blood

Reference: GC19 **Sex:** Female **Age:** 25

Circumstances: IVDA, treated for depression, Last seen by Doctor 5 days prior to death for repeat methadone prescription. (85mL methadone, diazepam, antidepressants), Decedent smoking hash alone (2200hrs), witness awoken by smell of smoke but could not rescue decedent due to fire. Extensive charring of body, no hair remaining.

Cause of Death: 1.a Inhalation of smoke and fire gases due to, b. house fire. Conditions contributing to death: 2. Drug Intoxication (temazepam, methadone, morphine, diazepam and prothiaden.

Toxicology: 59.9% carboxyhaemoglobin in blood, 0.10mg methadone/L blood, 3.08mg prothiaden/L blood, 0.41mg desmethyldiazepam/L blood, 0.04mg diazepam/L blood, 4.04mg temazepam/L blood and 0.11mg morphine/L blood.

Samples Re-analysed: Blood

Reference: GC20 **Sex:** Male **Age:** 36

Circumstances: Known IVDA for 8 – 10 years. Prescribed methadone on/off basis for past 3 years. Last prescribed 50mg/day methadone one week prior to death. Last seen 3 days prior to death under the influence of alcohol. Found dead slumped in a sitting position. Changes of putrefaction most marked in face.

Cause of Death: 1.a Morphine and Methadone Intoxication.

Toxicology: 55mg alcohol/10mL blood, 1.6mg methadone/L blood, 110mg alcohol/100mL urine, 0.34mg desmethyldiazepam/L blood, 0.19mg diazepam/L blood, 0.19mg temazepam/L blood, 0.26mg morphine/L blood.

Samples Re-analysed: Blood

Reference: GC21 **Sex:** Female **Age:** 24

Circumstances: IVDA. Prescribed diazepam daily, also treated for asthma. Suicidal, treated for overdoses in the 18 months prior to death and attempted to slit wrists. Evening prior to death, unconscious, roused by slapping appeared drowsy for a couple of hours. Visited drug centre next morning, no cause for concern. Bought £10 bag of heroin + 3 cans of super lager (1900hrs). Gargling and lips turned blue (2130hrs). Drug paraphernalia found at scene.

Cause of Death: 1.a Overdose of drugs.

Toxicology: 282mg alcohol/10mL blood, 0.07mg methadone/L blood, 357mg alcohol/100mL urine, 0.32mg morphine/L.

Samples Re-analysed: Blood

Reference: GC22 **Sex:** Female **Age:** 19

Circumstances: Known drug user (dihydrocodeine and diazepam). Treated for overdoses in previous 2 years. Last seen by Doctor 2 weeks prior to death, known to be abusing valium, temazepam and heroin. Last seen at midnight and found dead at 1515hrs the following day.

Cause of Death: 1.a Acute pulmonary oedema and congestion due to, b. intoxication with combined drugs (methadone, amphetamine and ecstasy).

Toxicology: 0.75mg methadone/L blood, 9mg alcohol/100mL urine, 0.47mg amphetamine/L blood, 0.84mg MDMA/L blood, 0.40mg desmethyldiazepam/L blood, 0.16mg diazepam/L blood, 99ng THC-COOH/ml blood.

Samples Re-analysed: Blood

Reference: GC23 **Sex:** Male **Age:** 21

Circumstances: Possible recent needle puncture marks. Known to use alcohol and drugs (heroin, temazepam). History of depression. Overdosed on prescription drugs 10 months prior to death. Prescribed anti-depressants. Day prior to death, from 1530hrs consumed vast quantity of alcohol. After 1830hrs, he ate and stated concern over court case, mentioned suicide. Fell asleep, 10:45pm, 0145hrs – snoring loudly, 0430hrs – still breathing, 0800hrs – aspirating, choking – signs of vomit/blood from mouth.

Cause of Death: 1.a Methadone Intoxication

Toxicology: 30mg alcohol/100mL blood, 1.1mg methadone/L blood, 104mg alcohol/100mL urine, 0.48mg fluoxetine/L blood, 0. 94mg Norfluoxetine/L blood, 0.17mg desmethyldiazepam/L blood, 0.11mg diazepam/L blood.

Samples Re-analysed: Blood

Reference: GC24 **Sex:** Male **Age:** 37

Circumstances: Known IVDA. Dependent on alcohol and heroin. Last seen by Doctor 2 weeks prior to death for methadone prescription (20mL/day). Last seen alive, 1700hrs heavily under the influence. Found dead 3 days later,

Cause of Death: 1.a Gastro-intestinal haemorrhage due to, b. oesophageal varices due to c. cirrhosis of the liver. Conditions contributing to death. 2. Methadone Intoxication.

Toxicology: 0.07mg methadone/L blood, 0.33mg desmethyldiazepam/L blood, 0.33mg diazepam/L blood.

Samples Re-analysed: Hair.

Reference: G25 **Sex:** Male **Age:** 21

Circumstances: Known drug user, suffered withdrawal seizure. Last seen by Doctor 16 months prior to death. Evening prior to death, smoked 4 joints and consumed beer (1900hrs). Ate breakfast (0700hrs), returned to bed. 0900hrs – sleeping, 1400hrs – sick but snoring. 1500hrs – 1645hrs – snoring/sleeping. 1700hrs – found dead.

Cause of Death: 1.a Inhalation of gastric contents

Toxicology: 27mg alcohol/100mL blood, 3.84mg methadone/L blood, 87mg alcohol/100mL urine, 2.35mg cocaine/L urine, 0.47mg temazepam/L blood, 26.5mg BZE/L urine, 0.44mg EME/L urine.

Samples Re-analysed: Blood

Reference: GC26 **Sex:** Male **Age:** 35

Circumstances: Fresh needle puncture mark. Long history of drug use (15 years). Prescribed methadone and diazepam. Depressed after recent death of husband. Last seen day prior to death at 2200hrs drowsy. 1430hrs found dead.

Cause of Death: 1.a Drug related death due to, b. methadone, heroin, temazepam and diazepam intoxication.

Toxicology: 0.92mg methadone/L blood, 0.44mg desmethyldiazepam/L blood, 0.31mg diazepam/L blood, 0.70mg temazepam/L blood, 0.35mg morphine/L blood.

Samples Re-analysed: Blood.

Reference: GC27 **Sex:** Male **Age:** 23

Circumstances: Fresh needle puncture marks. Drug user for 2 to 3 years. Prescribed methadone and valium. 1910hrs – under the influence, went to bathroom and then very sleepy. Drank lager then left. 2000hrs found sleeping on ground, laboured breathing, mouth to mouth unsuccessful.

Cause of Death: 1.a Inhalation of gastric contents, due to b. Methadone and trichloroethanol intoxication.

Toxicology: 1.27mg methadone/L blood, 96.3mg trichloroethanol/L blood.

Samples Re-analysed: Blood + Hair.

Reference: GC28 **Sex:** Male **Age:** 21

Circumstances: Abrasions and old scars. Early putrefactive changes. Known to have drank excessive amounts of alcohol, smoked half of a joint and taken 1 ecstasy tab the previous day. Lethargic, grey colour and lips blue. Lapsing in and out of

consciousness. Witness heard gurgling noises night prior to death. 0300hrs, found dead.

Cause of Death: 1.a Pulmonary oedema and congestion due to, b. amphetamine and methadone intoxication.

Toxicology: 0.31mg methadone/L blood, 1.81mg amphetamine/L blood, 0.22mg desmethyldiazepam/L blood, 0.49mg diazepam/L blood, 0.14mg temazepam/L blood.

Samples Re-analysed: Blood

Reference: GC29 **Sex:** Male **Age:** 24

Circumstances: Evidence of chronic drug use. History of asthma and smoking heroin. Last seen by Doctor 2 days prior to death with acute back strain. Last seen alive, 1600hrs day prior to death. Witnesses heard TV playing loudly but could get no answer at door the following day (1200hrs and 1350hrs). Entered house to turn down TV and found decedent lying on bed (2330hrs).

Cause of Death: 1.Heroin Intoxication.

Toxicology: 0.23mg morphine/L blood.

Samples Re-analysed: Blood.

Reference: GC30 **Sex:** Male **Age:** 29

Circumstances: Advanced signs of decomposition. Registered drug user. Heroin overdose 3 months prior to death. Released from prison 1 month prior to death, last seen alive 3 days prior to death, found with needle at scene.

Cause of Death: 1.a Unascertained.

Toxicology: 102mg alcohol/100mL blood, 56mg alcohol/10mL urine, 1.28mg chlordiazepoxide/L urine, 0.09mg desmethyldiazepam/L urine, 0.15mg diazepam/L urine, 2.24mg temazepam/L urine, 1.1mg morphine/L urine.

Samples Re-analysed: Hair

Reference: GC31 **Sex:** Female **Age:** 32

Circumstances: Fresh needle puncture mark. Known IVDA for 12 years and heavy drinker. Released from prison at 0800hrs and took train to city station. Train arrived at 0902hrs. Found dead at 1350hrs with a needle and tourniquet in situ.

Cause of Death: 1.a Heroin and temazepam intoxication.

Toxicology: 121mg alcohol/100mL blood, 205mg alcohol/100mL urine, 6.85mg temazepam/L blood, 0.42mg morphine/L blood.

Samples Re-analysed: Blood.

Reference: GC32 **Sex:** Male **Age:** 18

Circumstances: Unremarkable medical history. Complained of feeling unwell 8 days prior to death – bloody mucus and sore stomach. Drank evening prior to death. 1300hrs – smoked a joint. 1520hrs – in good spirits. 1700hrs – retired to bed, tired, flu, heavy stitch in side, had difficulty breathing, choking. Blood from mouth.

Cause of Death: 1.a Acute pulmonary oedema and congestion due to, b. Ecstasy ingestion.

Toxicology: 0.02mg MDMA/L blood, 1.25mg desmethyldiazepam/L blood, 0.62mg diazepam/L blood.

Samples Re-analysed: Blood

Reference: GC33 **Sex:** Male **Age:** 30

Circumstances: Drug user (cannabis, cocaine, temazepam) for 5 years. 0630hrs – under influence (alcohol), 1530hrs – deep sleep, 1850hrs – still thought to be sleeping. Dead (2030hrs).

Cause of Death: 1.a Drug related death due to, b. Heroin, cocaine, amphetamine, chlordiazepoxide, temazepam and cannabis intoxication.

Toxicology: 0.5mg temazepam/L blood, 1.06mg chlordiazepoxide /L blood, 0.044mg amphetamine/L blood, 1.54mg MDA/L blood, 8.0mg MDMA/L blood, 0.99mg cocaine/L blood, 10.28mg MEC/L blood, 14.33mg BZE/L blood, 0.76mg morphine/L blood.

Samples Re-analysed: Blood + Hair

Reference: GC34 **Sex:** Female **Age:** 17

Circumstances: Evidence of old scars. Known heroin addict, recently prescribed methadone. Asthmatic and kidney problems. Attempted suicide 5 years previous to death. Prior to death witness noticed decedents breathing was laboured, believed to have consumed 2 days prescription of methadone.

Cause of Death: 1.a Methadone intoxication.

Toxicology: 0.51mg methadone/L blood.

Samples Re-analysed: Blood.

Reference: GC35 **Sex:** Male **Age:** 29

Circumstances: Drug user for 10 to 15 years. Seen by consultant as addicted to methadone. Last seen by Doctor about heroin addiction. Witness left house at

0800hrs, returned at 1740hrs, found decedent lying face down on the bedroom floor. Syringe nearby.

Cause of Death: 1.a Heroin (morphine), diazepam and alcohol intoxication.

Toxicology: 0.44mg morphine/L blood, 0.53mg desmethyldiazepam/L blood, 1.21mg diazepam/L blood, 166mg alcohol/100mL blood, 26mg alcohol/100mL urine.

Samples Re-analysed: Blood + Hair.

Reference: GC36 **Sex:** Male **Age:** 18

Circumstances: Mild asthmatic. Known to smoke heroin, on methadone programme for two months. Methadone prescription increased from 50 to 60mgs 4 days prior to death. 1245hrs not well, 1545hrs thought to be asleep, 2200hrs checked again but thought to be sleeping, 0930hrs found dead.

Cause of Death: 1.a Inhalation of gastric contents.

Toxicology: 27mg alcohol/100mL blood, 0.21mg methadone/L blood, 12mg alcohol/100mL urine, 2.26mg carbamazepine/L blood.

Samples Re-analysed: Blood + hair.

Reference: GC37 **Sex:** Male **Age:** 36

Circumstances: Evidence of chronic IVDA. Last seen by Doctor 5 months prior to death, treated for heroin addiction (13 years). 1100hrs – decedent left house. Witness returned home and found decedent lying face down. Syringe still in situ.

Cause of Death: 1.a Drug related Death due to, b. Heroin and methadone intoxication.

Toxicology: 0.30mg methadone/L blood, 0.53mg morphine/L blood.

Samples Re-analysed: Blood

Reference: GC38 **Sex:** Male **Age:** 18

Circumstances: No evidence of IVDA. Social drug user, mildly asthmatic. Evening prior to death, 1800hrs – left house, returned 0030hrs – claimed to have taken speed. 0400hrs – watching TV, 0815hrs – left house, 1230hrs – feeling unwell, pains in legs, thought to be under the influence. Went to bed. 1630hrs –found dead. Bottle of green fluid ceased from scene.

Cause of Death: 1.a Drug related death due to, b. Methadone intoxication.

Toxicology: 2.11mg methadone/L blood.

Samples Re-analysed: Blood

Reference: GC39 **Sex:** Female **Age:** 24

Circumstances: Evidence of old needle puncture marks. Known drug user who used heroin, temazepam and temgesic. Developed septicemia 2 years prior to death and developed cardiac complications. Chronic renal failure and hepatitis. Prescribed methadone. Last seen alive 2100hrs, had taken methadone and 10 sleeping tablets. 0820hrs found dead.

Cause of Death: 1.a Bacterial endocarditis involving the aortic valve, b. IVDA.

Toxicology: 16mg alcohol/100mL blood, 0.34mg methadone/L blood, 0.20mg desmethyldiazepam/L blood, 0089mg diazepam/L blood, 0.083mg morphine/L blood.

Samples Re-analysed: Blood.

Reference: GC40 **Sex:** Male **Age:** 36

Circumstances: Ligature around neck with superficial incised wound on wrist. Known to abuse alcohol and diazepam. 3 years prior to death diagnosed with depression. Suffered from acute pancreatitis 2 months prior to death. On several medications (Zimobane, dihydrocodeine, omeprazole, metroclepromide, Ventalin inhaler).

Cause of Death: 1.a Hanging.

Toxicology: All analyses were negative.

Samples Re-analysed: Blood.

Reference: GC41 **Sex:** Male **Age:** 29

Circumstances: Evidence of IVDA. Known to use heroin. Prescribed methadone (60mL/day) on the day prior to death, consumed dose in two halves at 1630hrs and at 2200hrs. Consumed whole dose at pharmacy next day. Fell asleep (1430hrs), woken at 1445hrs but still tired, found dead at 1800hrs.

Cause of Death: 1.a Acute pulmonary oedema and congestion due to, b. methadone intoxication.

Toxicology: 0.63mg methadone/L blood, 0.024mg EDDP/L blood, 0.10mg desmethyldiazepam/L blood, 0.11mg diazepam/L blood.

Samples Re-analysed: Blood.

Reference: GC42 **Sex:** Male **Age:** 23

Circumstances: Ligature around neck. Recent IVDA. Treated in a psychiatric hospital 5 years prior to death. Started on a methadone programme (20mg) 4 years

prior to death, described as suicidal. 1700hrs – last seen alive, following day found dead at 1100hrs.

Cause of Death: 1.a Hanging associated with heroin overdose.

Toxicology: 69mg alcohol/100mL blood, 0.28mg methadone/L blood, 0.12mg EDDP/L blood, 0.11mg desmethyldiazepam/L blood, 0.11mg diazepam/L blood, 0.12mg codeine/L blood, 0.66mg morphine/L blood.

Samples Re-analysed: Blood.

Reference: GC43 **Sex:** Male **Age:** 27

Circumstances: Known to inhale heroin on a daily basis. Regularly used others' methadone script. 1040hrs – consumed methadone and seen to swagger and act strangely. Gargling and collapsed unconscious.

Cause of Death: 1.a Temazepam, morphine and trichloroethanol intoxication.

Toxicology: 0.10mg methadone/L blood, 0.04mg EDDP/L blood, 5.9mg lidocaine/L blood, 0.75mg desmethyldiazepam/L blood, 0.45mg diazepam/L blood, 1.39mg temazepam/L blood, 0.06mg codeine/L blood, 0.26mg morphine/L blood, 90mg trichloroethanol/L blood.

Samples Re-analysed: Blood.

Reference: GC44 **Sex:** Female **Age:** 23

Circumstances: Recent needle puncture mark. History of depression and anxiety – prescribed anti-depressants. Admitted addiction to diazepam 3 months prior to death. 0100hrs admitted taking 2 ecstasy tablets. Difficulty walking, collapsed unconscious (0130hrs). Pronounced dead (0300hrs), high core body temperature (107.6F).

Cause of Death: 1.a Trimipramine and ecstasy intoxication.

Toxicology: 43mg alcohol/100mL blood, 72mg alcohol/100mL urine, 0.065mg MDA/L blood, 3.0mg MDMA/L blood, 0.96mg trimipramine/L blood.

Samples Re-analysed: Blood + hair.

Reference: GC45 **Sex:** Female **Age:** 28

Circumstances: Evidence of IVDA and head injuries. Believed to be on methadone programme. Under the influence (2030hrs), found lying on ground with laceration to head (2100hrs).

Cause of Death: 1.a Head injury.

Toxicology: All analyses were negative.

Samples Re-analysed: Blood.

Reference: GC46 **Sex:** Male **Age:** 28

Circumstances: Ligature mark. Evidence of IVDA. Known drug user for 6 years. Prescribed 22mLs methadone/day. Last seen by Doctor one week prior to death for repeat prescription. Last seen by family 6 days prior to death.

Cause of Death: 1.a Hanging.

Toxicology: 30mg alcohol/100mL blood, 8mg alcohol/100mL urine.

Samples Re-analysed: Blood.

Reference: GC47 **Sex:** Female **Age:** 28

Circumstances: Possible needle puncture marks. Intermittent drug user for 15 years (heroin). On methadone programme (80mLs) for 2 months. 8 months pregnant, didn't look well and complained of sore stomach. Found dead, drug paraphernalia found at scene.

Cause of Death: 1.a Acute and chronic IVDA.

Toxicology: 0.06mg morphine/L blood.

Samples Re-analysed: Blood.

Reference: GC48 **Sex:** Male **Age:** 28

Circumstances: Ligature mark. Possible old needle puncture marks. Known drug user, on methadone programme for 3 months. In prison, confided to coming off methadone and feeling shaky. No signs that deceased was depressed prior to hanging.

Cause of Death: 1.a Hanging.

Toxicology: 9mg alcohol/L blood.

Samples Re-analysed: Blood.

Reference: GC49 **Sex:** Female **Age:** 29

Circumstances: Evidence of chronic IVDA. History of infection and abscess on left valve of heart. Thought to consume £20 heroin/day. Finished dihydrocodeine detoxification 1 week prior to death.

Cause of Death: 1.a Pulmonary thromboembolism. 2. Chronic IVDA.

Toxicology: 12mg alcohol/100mL blood, 0.74mg propoxyphene/L blood, 12ng THC-COOH/ml blood.

Samples Re-analysed: Blood.

Reference: GC50 **Sex:** Female **Age:** 18

Circumstances: Evidence of old and fresh needle puncture marks. Known heroin user. Year prior to death attempted dihydrocodeine and methadone detoxification

programme. 2000hrs consumed vodka and heroin. 1250hrs the following day convulsing and stopped breathing.

Cause of Death: 1.a Heroin and temazepam intoxication.

Toxicology: 0.30mg morphine/L blood, 0.08mg temazepam/L blood.

Samples Re-analysed: Blood.

Reference: GC51 **Sex:** Male **Age:** 20

Circumstances: Recent needle puncture marks. Known drug abuser (heroin, temazepam, cannabis). Victim of several serious assaults. On a methadone programme for 4 months, ending 2 months prior to death. Suspected heroin overdose 3 days prior to death. Found with tourniquet and needle in situ.

Cause of Death: 1a. Morphine intoxication.

Toxicology: 0.27mg desmethyldiazepam/L blood, 0.07mg diazepam/L blood, 0.03mg temazepam/L blood, 0.098mg codeine/L blood, 0.43mg dihydrocodeine/L blood, 0.44mg morphine/L blood.

Samples Re-analysed: Blood.

Reference: GC52 **Sex:** Male **Age:** 19

Circumstances: Possible fresh and healing needle puncture marks. Known drug user. On methadone programme for 3 months. Last seen by Doctor 25 days prior to death (prescribed 700mL methadone and 18 diazepam tablets). Evening prior to death consumed methadone and diazepam, police found decedent 2114hrs the following day.

Cause of Death: 1a. Methadone intoxication.

Toxicology: 0.28mg methadone/L blood, 0.53mg EDDP/L blood, 0.14mg desmethyldiazepam/L blood, 0.12mg diazepam/L blood.

Samples Re-analysed: Blood.

Reference: GC53 **Sex:** Male **Age:** 30

Circumstances: Recent and healing needle puncture marks. Known heroin IVDA. Evening – returned to room at 2000hrs. Found dead at 0935hrs with tourniquet and syringe in situ.

Cause of Death: 1.a Unascertained.

Toxicology: 183mg alcohol/100mL blood, 199mg alcohol/100mL urine, 0.78mg temazepam/L blood.

Samples Re-analysed: Blood.

Reference: GC54 **Sex:** Male **Age:** 28

Circumstances: History of heroin use. On a methadone maintenance programme, suffering from depression. Believed to have taken 25 to 28 diazepam tablets. Last seen by Doctor 3 days prior to death, prescribed 7-day course of methadone. Heron overdose 16 days prior to death. Consumed 2 methadone scripts (1230hrs and 1430hrs). 2000hrs looked "high". 2300hrs – went to bed, 0100hrs – breathing heavily, found dead 0755hrs.

Cause of Death: 1.a Acute pulmonary oedema and congestion due to, b. Methadone intoxication.

Toxicology: 0.36mg methadone/L blood, 0.017mg EDDP/L blood, 0.28mg desmethyldiazepam/L blood, 0.40mg diazepam/L blood.

Samples Re-analysed: Blood.

Reference: GC55 **Sex:** Male **Age:** 23

Circumstances: Suffering from depression. Attempted on two occasions to commit suicide. Claimed to have cocaine habit (£300/week). Overdosed on amitriptyline and valium. Developed respiratory failure in hospital and septic shock. Placed on ventilator, condition deteriorated.

Cause of Death: 1.a Bronchopneumonia.

Toxicology: Trace of Fluconazole in blood, 0.21mg desmethyldiazepam/L blood, 0.17mg diazepam/L blood, 0.21mg temazepam/L blood.

Samples Re-analysed: Blood.

Reference: GC56 **Sex:** Male **Age:** 26

Circumstances: Evidence of IVDA. History of drug use (heroin). Last seen by Doctor who prescribed Amitriptyline, decedent seemed agitated. Personality disorder, history of self-poisoning and over last four years has either been in prison or attending Forensic Psychiatrist. Last seen alive six days prior to death.

Cause of Death: 1.a Acute pulmonary oedema and congestion due to, b. Amitriptyline overdose.

Toxicology: 3.12mg amitriptyline/L blood, 1.42mg nortriptyline/L blood.

Samples Re-analysed: Blood.

Reference: GC57 **Sex:** Male **Age:** 33

Circumstances: Injuries consistent with fall. Known drug user, not on medication at time of death. Snorted cocaine, became agitated and jumped from window.

Cause of Death: 1.a Multiple injuries due to, b. fall from a height.

Toxicology: 51mg alcohol/100mL blood, 3.37mg BZE/L blood, 0.05mg cocaine/L blood, 2.35mg MEC/L blood, 67mg alcohol/L urine.

Samples Re-analysed: Blood.

Reference: GC58 **Sex:** Male **Age:** 32

Circumstances: History of IVDA problems. On methadone programme 3 days before death but did not collect script. Last seen alive 2100hrs, found dead the following day (1000hrs) clutching a syringe.

Cause of Death: 1.a Acute pulmonary oedema and congestion due to, b. morphine (heroin) intoxication.

Toxicology: 0.01mg EDDP/L blood, 0.07mg methadone/L blood, 0.01mg 6-MAM/L blood, 0.02mg codeine/L blood, 0.14mg morphine/L blood.

Samples Re-analysed: Blood.

Reference: GC59 **Sex:** Male **Age:** 23

Circumstances: Fresh needle puncture mark, antecubital fossa. Suffered from schizophrenia and depression. Known IVDA. History of overdoses. Evening prior to death, consumed excessive amounts of alcohol, plus ecstasy and heroin. 2200hrs – involved in assault, sustained injuries to eyes. Smoked hash. 1130hrs the following day, was administered prescribed drugs for schizophrenia. Consumed more alcohol. 2000hrs – unable to stand, sleepy. 2300hrs – found dead.

Cause of Death: 1.a Unascertained.

Toxicology: 53mg alcohol/100mL blood, 0.15mg amphetamine/L blood, 90.9mg carbamazepine/L blood, 81ng THC-COOH/ml blood, 94.6mg paracetamol/L blood, 108mg alcohol/100mL urine.

Samples Re-analysed: Blood

Reference: GC60 **Sex:** Female **Age:** 44

Circumstances: History of alcoholism. Had attempted suicide on several occasions. Suffered from depression and epilepsy. History of a fall prior to sudden death.

Cause of Death: 1.a Multiple injuries due to, b. fall from a height.

Toxicology: 0.02mg codeine/L blood, 0.07mg dihydrocodeine/L blood, 0.05mg morphine/L blood.

Samples Re-analysed: Blood.

Reference: GC61 **Sex:** Male **Age:** 25

Circumstances: History of chronic alcoholism and epileptic. Last seen under the influence of alcohol and was aggressive. Found dead in a derelict building. Medication (Eiplin, diazepam, Rantidine)

Cause of Death: 1.a Hypothermia due to, b. chronic alcoholism.

Toxicology: 297mg/100mL blood, 493mg alcohol/100mL urine.

Samples Re-analysed: Blood.

6.3 Analysis

Postmortem blood samples were analysed in triplicate where the sample volume allowed. The majority of hair samples were too small to analyse more than once, the exception being when bulk hair was available.

Blood samples were re-screened by EIA for the target analyte (methadone or amphetamines) and were then confirmed positive or negative by the developed GC/MS method.

6.4 Methadone

6.4.1 Postmortem Blood

A total of 47 samples were analysed using the optimised extraction method for methadone and its two major metabolites (EDDP and EMDP) in whole blood. Of these 29 samples were confirmed positive and 17 confirmed negative. Only one blood sample GC17 was not confirmed positive for methadone when re-analysed. The initial concentration was 0.036mg/L. The sample was re-analysed 8 months after the initial analysis and the condition of the blood had deteriorated which could explain the negative result when re-analysed.

Fourteen case samples were re-analysed within six months of the initial analysis. The in use liquid-liquid extraction (LLE) method was not optimised for the detection of EDDP or EMDP, therefore a comparison could only be made between the methadone levels determined by both methods.

The results are summarised in Table 6.1.

Table 6.1 Levels of methadone and EDDP (mg/L) found in case studies by LLE and SPE (Analysed and re-analysed within 6 months).

Case	*LLE (mg/L)	†Time (months)	‡SPE (mg/L)
1	0.32	4	0.29 (0.07)
2	0.23	6	0.18 (0.04)
3	0.09	6	0.07 (0.15)
4	0.41	6	0.45 (0.12)
5	0.12	6	0.13 (0.12)
6	0.10	6	0.08 (---)§
7	0.32	6	0.26 (0.29)
8	---	5	---
9	---	5	---
10	---	5	---
11	0.36 (0.017)	5	0.50 (0.06)
12	---	5	---
13	---	5	---
14	0.07 (0.01)	5	0.07 (0.01)

* Levels of methadone detected by the in-use liquid-liquid extraction method. Levels of EDDP detected are noted in (brackets). EDDP was not screened for routinely.

† The time in months, elapsed between the initial analysis carried out by LLE and the 2nd analysis carried out by the developed solid-phase extraction method.

‡ Levels of methadone detected by the developed solid-phase extraction method. Levels of EDDP detected are noted in (brackets).

§ Negative for the analyte analysed.

Methadone levels in the fourteen blood samples ranged from 0 to 0.41 mg/L when analysed by the in use LLE method and from 0 to 0.50 mg/L using the described SPE method. As seen in figure 6.1, results obtained by SPE correlate well with those found by the LLE method (r = 0.96, m = 1.07).

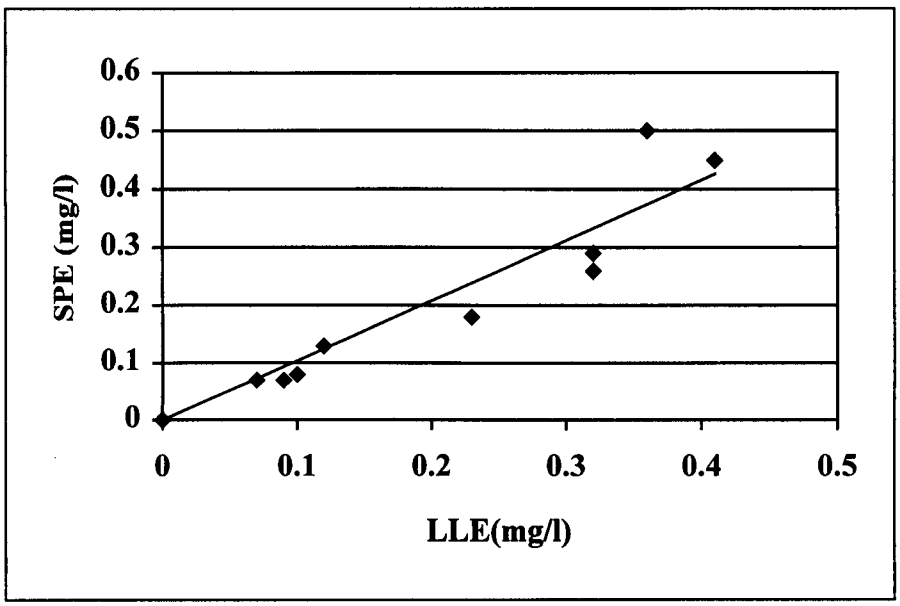


Figure 6.1 Correlation of case results between the in use LLE method and the developed SPE method (Analysed and reanalysed within 6 months).

EDDP was detected in eight of the fourteen cases. Figure 6.2 shows the GC/MS trace obtained for Case 5 which was positive for methadone and EDDP. EMDP was not detected in any of the cases by either extraction method.

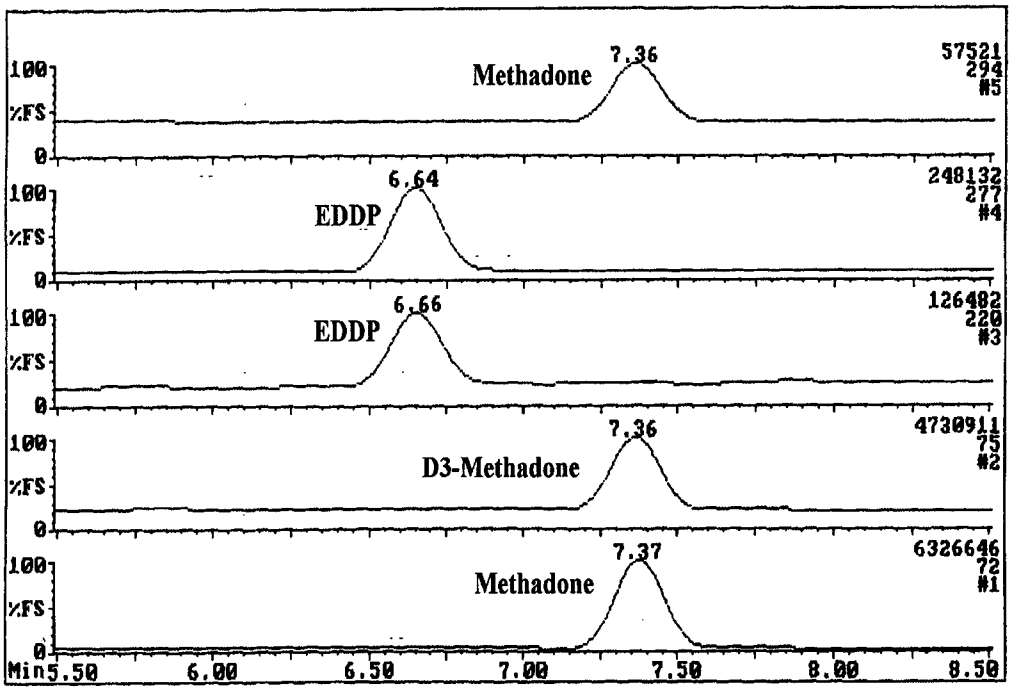


Figure 6.2 GC/MS selected ion chromatogram of methadone, EDDP and methadone-d_3 (Case 5).

Table 6.2 summarises the results for all blood samples analysed for methadone and its two major metabolites by LLE and the developed extraction method. The samples were stored for longer than 6 months prior to reanalysis by SPE, but less than one year.

Methadone levels in the sixteen blood samples ranged from 0 to 1.60 mg/L when analysed by the in use LLE method and from 0 to 0.57 mg/L using the described solid-phase extraction method. As seen in figure 6.3, results obtained by SPE correlate with those found by the LLE method ($r = 0.94$, $m = 0.36$). However, it

would appear that storage for greater than 6 months does have a detrimental effect on the analyte concentrations in whole blood.

Table 6.2 Levels of methadone and EDDP (mg/L) found in case studies by LLE and SPE (Analysed and re-analysed after 6 months of storage but less than one year).

Case	*LLE (mg/L)	†Time (months)	‡SPE (mg/L)
1	0.06	8	0.21 (0.16)
2	0.036	8	---
3	1.60	7	0.57 (0.99)
4	---	10	---
5	0.63 (0.024)	10	0.27 (0.05)
6	0.28 (0.12)	9	0.23 (---)
7	0.10 (0.04)	9	0.04 (---)
8	---	11	---
9	---	10	---
10	---	10	---
11	---	10	---
12	---	9	---
13	---	9	---
14	---	9	---
15	0.28 (0.53)	9	0.12 (0.25)
16	---	9	---

* Levels of methadone detected by the in-use liquid-liquid extraction method. Levels of EDDP detected are noted in (brackets). EDDP was not screened for routinely.

† The time in months, elapsed between the initial analysis carried out by LLE and the 2nd analysis carried out by the developed solid-phase extraction method.

‡ Levels of methadone detected by the developed solid-phase extraction method. Levels of EDDP detected are noted in (brackets).

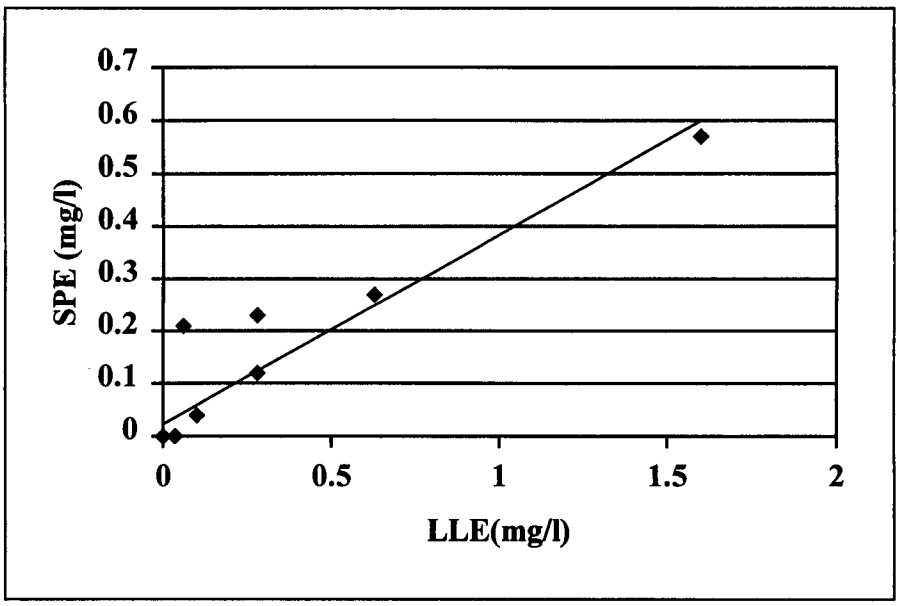


Figure 6.3 Correlation of case results between the in use LLE method and the developed SPE method (Analysed and reanalysed within 6 to 12 months).

Table 6.3 summarises the results for all blood samples analysed for methadone and its two major metabolites by LLE and the developed extraction method where the samples were stored for longer than 12 months prior to reanalysis by SPE.

Table 6.3 Levels of methadone and EDDP (mg/L) found in case studies by LLE and SPE (Analysed and re-analysed after 12 months of storage.

Case	*LLE (mg/L)	†Time (months)	‡SPE (mg/L)
1	1.44	36	0.39 (0.17)
2	0.36	36	0.41 (0.16)
3	0.10	29	0.07 (0.01)
4	0.07	27	0.03 (---)
5	1.10	27	0.25 (0.46)
6	3.84	27	0.56 (0.06)
7	0.92	27	0.28 (0.11)
8	1.27	27	0.2 (0.06)
9	---	26	---
10	---	25	---
11	0.51	2	0.53 (---)
12	---	23	---
13	0.21	22	0.40 (---)
14	0.30	21	0.21 (---)
15	2.11	21	0.43 (0.06)
16	0.34	21	0.17 (0.19)
17	0.07	13	0.21 (0.16)

* Levels of methadone detected by the in-use liquid-liquid extraction method. Levels of EDDP detected are noted in (brackets). EDDP was not screened for routinely.

† The time in months, elapsed between the initial analysis carried out by LLE and the 2nd analysis carried out by the developed solid-phase extraction method.

‡ Levels of methadone detected by the developed solid-phase extraction method. Levels of EDDP detected are noted in (brackets).

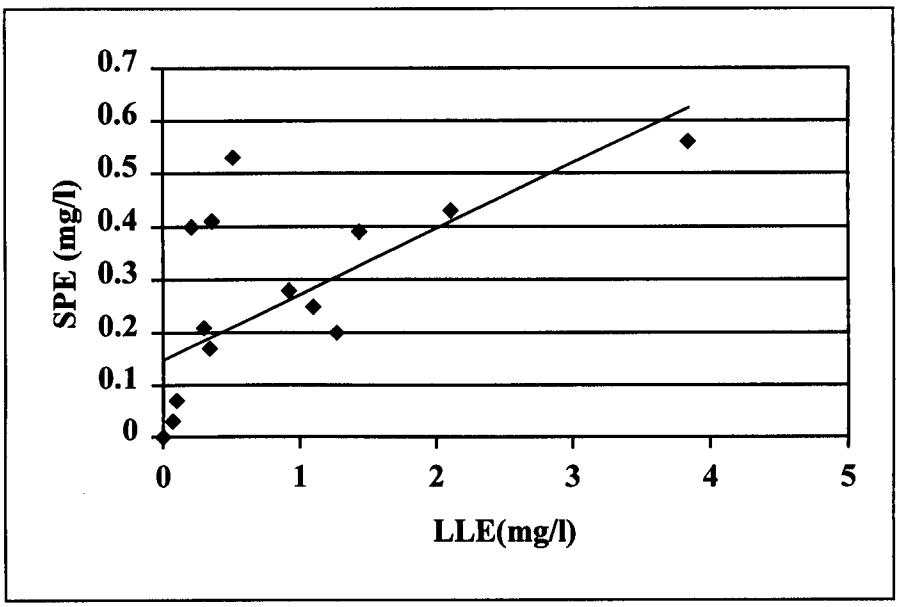


Figure 6.4 Correlation of case results between the in use LLE method and the developed SPE method (Analysed and reanalysed within 12 to 36 months).

Methadone levels in the seventeen blood samples ranged from 0 to 3.84 mg/L when analysed by the in use LLE method and from 0 to 0.56 mg/L using the described SPE method. As seen in figure 6.4, results obtained by SPE show a slight correlation with those found by the LLE method ($r = 0.66$, $m = 0.12$). The decrease in the analyte

concentrations in whole blood are greater in this group where storage of the whole blood was greater than 12 months between analysis and reanalysis.

6.4.2 Postmortem Hair

In total, 15 post-mortem hair samples were segmented (n = 34) and screened for methadone following methanolic extraction. The results are summarised in Table 6.4 along with any antecedent history mentioned in the police report and the toxicology results pertaining to the consumption of methadone in the six months prior to death. A comparison is made between the detection of methadone and whether or not this agrees (A) or disagrees (D) with the police report.

Where no police report was not available the comparison was not applicable (N/A).

Out of the 37 segments analysed, five were omitted due to a lack of antecedent history. An agreement of 65.5% was obtained for the segments which tested positive and confirmed the antecedent history. The majority of the cases which were negative and disagreed (34.5%, n =10) with the antecedent history had low sample weights below 20mg, and this may have contributed to the number of false negative results.

It is important to note the difference in methadone levels detected in Case GC04. When the hair sample was washed prior to analysis, the concentration of methadone was 2.3ng/mg in comparison to 25.0ng/mg when the sample was not washed. The loss of methadone during washing could be removal of external contamination or from within the hair shaft.

Table 6.4 Comparison of Methadone Detected in Hair and Antecedent History.

Sample		Weight (mg)	Concentration (ng/mg)	Methadone History	Agree or Disagree
GC01	Roots	20.75	-ve	No Report	D
	Bulk	35.16	-ve	No Report	N/A
GC02	Roots	17.75	40.9	Yes	A
	1st 3cm	80.62	10.3	Yes	A
	Bulk	132.17	16.5	Yes	A
GC03	Roots	53.19	1.1	No	A
GC04	Bulk (W)*	50.04	2.3	Yes	A
	Bulk (UW)†	38.55	25.0	Yes	A
GC05	1st 3cm	3.68	-ve	Yes	D
	Bulk	5.79	-ve	Yes	D
GC07	Roots	12.39	-ve	No Report	D
	Bulk	24.73	1.1	No Report	N/A
GC08	Roots	4.01	-ve	Yes	D
	Bulk	24.58	-ve	No	A
GC11	Roots	4.90	-ve	Yes	D
	1st 6cm	26.99	-ve	Yes	D
	2nd 6cm	11.78	-ve	Yes	D
	Bulk A	39.73	1.1	Yes	A
	Bulk B	48.45	0.6	Yes	A

Sample		Weight (mg)	Concentration (ng/mg)	Methadone History	Agree or Disagree
GC13	Roots	4.80	-ve	Yes	D
	1st 3cm	45.35	1.0	Yes	A
	Bulk	38.02	-ve	Yes	D
GC16	1st 6cm	32.18	-ve	No Report	N/A
	2nd 6cm	23.93	1.3	No Report	N/A
	Bulk	39.67	0.9	No Report	N/A
GC24	Bulk	46.80	8.9	Yes	A
GC27	Roots	6.16	29.1	Yes	A
	Bulk	27.25	17.6	Yes	A
GC30	Roots	4.84	-ve	No	A
	Bulk	8.79	-ve	No	A
GC35	Roots	4.40	-ve	No	A
	Bulk	44.74	-ve	No	A
GC36	Roots	3.89	18.6	Yes	A
	Bulk	43.60	3.0	Yes	A

* Bulk (W) – sample washed prior to analysis

† Bulk (UW) – sample not washed prior to analysis

Too many of the root samples analysed were of low weight and produced a negative response. The results are summarised in Table 6.5 along with the postmortem blood

level quantified during the initial analysis and the level quantified when re-analysed by the optimised solid-phase extraction method.

Table 6.5 Comparison of Methadone Detected in Postmortem Hair Roots and Blood.

Sample	Concentration (ng/mg)	Initial [Blood] mg/L	Re-analysis mg/L
GC01	-ve	1.44	0.39
GC02	40.9	0.12	n/a
GC03	1.3	0.18	n/a
GC07	-ve	0.06	0.21
GC08	-ve	0.68	n/a
GC11	-ve	0.32	0.26
GC13	-ve	0.12	0.13
GC27	29.1	1.27	0.20
GC30	-ve	-ve	n/a
GC35	-ve	-ve	-ve
GC36	18.6	0.21	0.40

Of the four cases where the root sample was positive for methadone (GC02, GC03, GC27 and GC36) only case reference GC27 died as a result of methadone intoxication. It is not possible to draw any firm conclusions from these results due to the limited sample size with respect to the number of root segments analysed and the small sample weights.

Methanolic extraction followed by enzyme immunoassay correctly identified methadone use in almost 66% of the hair segments analysed. No false positives were identified however there were a high number of false negatives (n = 10). The combination of small sample weights and high calibrator weights would effect the sensitivity of this assay!

6.5 Amphetamines

6.5.1 Postmortem Blood

A total of seven samples were analysed using the optimised solid-phase extraction method for amphetamines in whole blood. Of these, six were confirmed positive and one confirmed negative. The results are summarised in Table 6.6.

Table 6.6 Comparison of amphetamines detected in postmortem blood by the Initial Analysis and when Re-analysed by the Optimised Extraction Method.

Sample	Initial Analysis (mg/L)	Re-Analysis (mg/L)
GC09	0.46 Amphetamine	1.34 Amphetamine
GC22	0.47 Amphetamine 0.84 MDMA	1.45 Amphetamine 0.36 MDMA
GC32	0.02 MDMA	0.16 MDMA
GC33	+ve Amphetamine MDA MDMA (urine only)	0.02 Amphetamine 0.11 MDA 0.84 MDMA 0.08 MDEA
GC44	0.07 MDA 3.00 MDMA	0.11 MDA 3.99 MDMA 0.05 MDEA
GC57	-ve	-ve
GC59	0.15 Amphetamine	0.42 Amphetamine

In general the specific amphetamines detected in the initial analysis were identified when re-analysed by the optimised extraction method. MDEA was detected in two cases (GC33 and GC44) in addition to the amphetamines detected in the initial analysis.

In the majority of cases, the concentrations of analytes detected in blood was greater when re-analysed by the developed method. This may highlight the greater efficiency of the developed method at disrupting the protein binding effects compared with the initial in-use extraction method.

6.5.2 Postmortem Hair

Two cases were analysed by the optimised solid-phase extraction method for the analysis of amphetamines in hair.

Case One (GC33)

The first case involved a 30 year old male, who was a known drug abuser, predominantly using cannabis, cocaine and temazepam. The cause of death was established as a drug related death due to heroin, cocaine, amphetamine, chlordiazepoxide, temazepam and cannabis intoxication. The deceased was witnessed to be under the influence, and was checked throughout the day and was thought to be sleeping. He was later found dead in bed.

Case Two (GC44)

The second case involved a 23 year old female who had a history of depression and was addicted to diazepam. The cause of death was established as trimipramine and

ecstasy intoxication. The deceased admitted taking two ecstasy tablets at a dance event and complained of feeling unwell. She had difficulty walking and collapsed. Resuscitation was unsuccessful and she was pronounced dead some two hours after she first complained of feeling unwell. Her core temperature was noted as 42°C shortly after admission to hospital.

The results of the analysis of blood and hair from case one are shown in Table 6.7. In this case, the hair sample was separated into the root sample and a one cm segment. The blood was negative (-ve) for MA but positive for the other four amphetamines. Unfortunately, the sudden death report only contains details of the events leading up to the deceased's death with only a brief medical history. From this we were not able to determine whether or not the positive results in the one cm segment from the scalp confirmed use of ecstasy by the deceased in the month prior to their death.

Its interesting to note that the drugs detected in the postmortem blood sample correspond to those detected in the root sample.

Table 6.7 Amphetamines Detected in Postmortem Blood and Hair Samples (Case One)

Analyte	Blood	Hair (ng/mg)	
	(mg/L)	Roots	1st 1cm
AP	0.04	1.5	-ve
MA	-ve	-ve	-ve
MDA	1.54	3.5	1.5
MDMA	8.00	16.0	5.2
MDEA	0.07	7.6	-ve

Table 6.8 Amphetamines Detected in Postmortem Blood and Hair Samples (Case Two)

Analyte	Blood	Hair (ng/mg)		
	(mg/L)	Roots	1st 3cm	2nd 3cm
AP	-ve	-ve	-ve	-ve
MA	-ve	-ve	-ve	-ve
MDA	0.11	1.5	-ve	-ve
MDMA	3.99	42.1	25.2	6.0
MDEA	0.05	18.2	2.4	-ve

Table 6.8 summarises the results from case two. The hair was analysed in three segments, the root sample, the first 3cm segment from the scalp and then a second

3cm segment. The blood sample was positive for all three methylenedioxyamphetamines with corresponding positives in the root sample. Again, it wasn't possible to confirm use of ecstasy in the six months prior to the deceased's death.

6.6 Conclusion

Postmortem blood and hair samples were analysed for the presence of methadone, its two metabolites (EDDP and EMDP) and amphetamines (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-methamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA)), using the developed solid-phase extraction methods detailed in Chapters Two and Four.

Both SPE methods successfully extracted the drugs from the matrices involved. The results obtained were comparable to the in-use liquid-liquid extraction methods even though many samples were re-analysed more than six months after the initial analysis.

The Cozart enzyme immunoassay kit was readily adapted to screen for methadone in hair. Antecedent drug use history was confirmed in 65.5% of all cases There were no false positives identified. The limiting factor of this assay was sensitivity due to a combination of small sample weights and high calibrator weights.

7. Conclusion

The first aim of this project involved the development of improved extraction methods for the analysis of methadone and its metabolites and for the analysis of amphetamine and related compounds from whole blood and hair.

Solid-phase extraction methods were developed for the successful extraction of all analytes from whole blood by manipulating the pH of the extraction system. The recoveries of methadone, EDDP and EMDP were not affected by the carbon number or carbon loading of the solid-phase extraction columns investigated.

Improvements were made with respect to recoveries, linear ranges, extraction efficiency and analysis time for all analytes studied in both whole blood and hair. In particular, the improvements with stability of the volatile amines by addition of tartaric acid has resulted in a robust and reproducible extraction method.

The stability of methadone and its two major metabolites in whole blood was investigated under various conditions. Methadone and EDDP remained stable for up to six months when stored at all three temperatures (-20, 5 and 25°C). EDDP was slightly more stable when stored in foil wrapped vials, but not sufficiently to warrant the expense of light sensitive vials as routine pathology specimen containers.

EMDP was extremely unstable under all conditions with a recovery of only 57.6% of the original concentration of EMDP after six months storage at -20°C. Silanising the

hypovials prior to storage did not result in greater recoveries of methadone, EDDP or EMDP from whole blood.

A total of 61 postmortem cases were analysed by the developed methods. Methadone levels detected in blood samples which were re-analysed within six months correlated well with the initial levels. After six months the correlation decreased.

It has been shown that solid-phase extraction has successfully extracted several analytes of differing physico-chemical properties from whole blood and hair using the same method by manipulating the sample pretreatment steps and pH of the extraction system. The same basic extraction method was used to extract methadone, EDDP and EMDP from whole blood and amphetamine, methamphetamine, MDA, MDMA and MDEA from whole blood and hair.

In addition, 15 postmortem hair samples were successfully screened and semi-quantitated for methadone using the Cozart methadone microplate enzyme immunoassay kit which was easily adapted for hair. This technique could prove useful for the initial screen prior to confirmation using GC/MS.

7.1 Further Work

The initial work completed on the adaptation of the Cozart methadone microplate kit for hair samples proved useful for screening spiked hair samples and postmortem hair samples. To fully realise the potential of this technique the procedure requires validation with a view to adapting and validating other drug specific immunoassays for hair analysis.

A solid-phase extraction method was developed for the analysis of amphetamines in hair. This method successfully removed the analytes of interest from the endogeneous material and produced a clean extract. The applicability of this extraction method for the analysis of other drugs of abuse would be an interesting subject for future work.

In addition to improving extraction methods for the analysis of drugs in hair, a greater understanding is required of the mechanisms by which drugs are incorporated into the hair. The analysis of postmortem hair and blood samples during this study gave strong indications that a relationship could exist between drug levels detected in blood and hair roots at the time of death. This could potentially provide more information on the cause of death and antecedent drug use history of the deceased, as well as aiding researchers in understanding drug incorporation into hair.

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Appendix One: Papers in Support of this Thesis

Full Paper

- G.A.A. Cooper and J.S. Oliver. Improved Solid-Phase Extraction of Methadone and its Two Major Metabolites from Whole Blood. *J. Anal. Toxicol.*, **22**:389-392 (1998).
- G.A.A. Cooper, A.S., M.T. Cassidy, J.S. Oliver. A Study of Methadone in Fatalities in the Strathclyde Region, 1991-1996. *Med., Sci. and the Law*, **39**:233-242 (1999).

Abstract Only

- G.A.A. Cooper, D.L. Allen, K.S. Scott, J.S. Oliver, J. Ditton and I.D. Smith. Hair Analysis: Self-Reported Use of "speed" and "ecstasy" Compared with Laboratory Findings. *Journal of Forensic Science*, **In press**.
- J. Ditton, G.A.A. Cooper, K.S. Scott, D.L. Allen, J.S. Oliver and I.D. Smith. Forensic hair testing for "ecstasy" in a large volunteer sample of Scottish drug users. *Addiction,* **In press**.
- G.A.A. Cooper, T.D. Mann, M.F. Burke, J.S. Oliver. An Improved Solid-Phase Extraction Procedure for the Recovery and Quantitation of Amphetamine and Related Compounds from Blood and Hair. *Proceedings of the SOFT-TIAFT 1998 Meeting, Albuquerque, NM, USA,* (1998). **In press**.

IAFS Emerging Scientist Award

- G.A.A. Cooper, J.S. Oliver. The Stability of Methadone and its Two Major Metabolites in Whole Blood. *Proceedings of the 15th Triennial Meeting of the International Association of Forensic Sciences, Los Angeles, C.A.*, (1999).

Hair Analysis: Self-Reported Use of "speed" and "ecstasy" Compared with Laboratory Findings*

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Abstract

Drug use histories were collected from 100 subjects recruited from the "dance scene" in and around Glasgow, Scotland. In addition, each subject donated a hair sample which was analysed by gas chromatography – mass spectrometry (GC/MS) for amphetamine (AP), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA).

The hair samples were analysed in two 6cm segments or in full, ranging from 1.5 - 12cm depending on the length of the hair. Approximately 10mg of hair was ground to a fine powder before treatment with β-glucuronidase/aryl sulphatase. A solid-phase extraction procedure was carried out followed by derivatisation with pentafluoropropionic anhydride (PFPA). All extracts were analysed by gas chromatography/mass spectrometry (GC/MS).

Of the 139 segments analysed, 77 (52.5%) were positive for at least one of the five amphetamines. The drug concentrations found in the hair were compared with the

self-reported drug histories. A concordance of greater than 50% was found between the self-report data and levels detected in hair. However no correlation was found between the reported number of "ecstasy" tablets consumed and the drug levels detected in hair.

An increase in the average drug levels measured was observed from low to high use (number of "ecstasy" tablets/month). A large number of false negatives and a low number of false positives were observed.

Keywords: forensic science, hair analysis, self-report, "speed", "ecstasy", solid-phase extraction

Forensic hair testing for "ecstasy" in a large volunteer sample of Scottish drug users

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Abstract

Aims. To compare self reported "ecstasy" use with the results of the analysis of hair harvested from the same users. **Design.** A survey using a detailed questionnaire with subjects recruited by multi-site chain-referral sampling. Hair donated after questionnaire administration. **Setting.** The "dance scene" in Glasgow. **Participants.** One hundred and forty nine subjects. **Findings.** Overall gross concordance between self reported "ecstasy" use, and discovery of MDMA (or related compounds) in analysed hair did not surpass 65 per cent, and no relationship had a Cohen's Kappa of more than 0.19. Within the positive concordant data set (n=83), scatter was considerable, with no correlation being significant, and none more strongly positive than -0.0444. **Conclusions.** It is elsewhere acknowledged that analysis of hair for the the presence of drugs does not reach a level of sophistication or accuracy that would permit its use as a criminal justice (or other) individual sanction. The results presented here indicate that, as far as MDMA is concerned, hair does not reach a level of apparent accuracy that would permit its use as a general population estimator. Large scale inter- and intra-laboratory comparative research is recommended.

An Improved Solid-Phase Extraction Procedure for the Recovery and Quantitation of Amphetamine and Related Compounds from Blood and Hair.

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Abstract

Solid-phase extraction (SPE) has become the standard approach to sample preparation for the GC or GC/MS analysis of drugs in biological fluids. While this technology has proven to be applicable to a wide variety of compounds, the determination of amphetamines has been especially challenging because of their volatility. Significant loss of analyte occurs both when the elution solvent is evaporated and under the thermal conditions employed during thederivatisation procedure. A SPE method, which addresses both these limitations, is described for the improved recovery and quantitation of amphetamine (AP), methamphetamine (MA), 3, 4-methylenedioxyamphetamine (MDA), 3, 4-methylenedioxy-methamphetamine (MDMA) and 3, 4-methylenedioxyethylamphetamine (MDEA) in blood and hair.

The initial extraction was achieved using ISOLUTE® CONFIRM HCX mixed-mode SPE columns. The drugs were determined using GC/MS

with d_5 - amphetamine (d_5 – AP) as the internal standard. The problems with volatility were addressed by simple addition of 100µl of tartaric acid (1mg/ml in ethyl acetate) to each standard and analytical sample prior to evaporating to dryness. All of the amphetamine compounds demonstrated significant increases in recovery.

Data is also presented on the kinetics of the derivatisation of these compounds using pentafluoropropionic anhydride. In contrast to many reports in the literature which call for elevated temperatures (> 40 °C) for extended times (> 20 minutes) our data shows that the reaction is complete for all species in less than 2 minutes at room temperature. This represents a significant reduction in the total time of analysis. Recoveries were greater than 87% for all five amphetamines from both blood and hair and were linear over the concentration ranges studied ($r^2 > 0.993$).

Keywords: Extraction, Amphetamines, Derivatisation

The Stability of Methadone and its Two Major Metabolites in Whole Blood.

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Abstract

The accurate interpretation of drug levels detected in postmortem blood is an important aspect of forensic toxicological analysis. The stability of the analytes of interest depends not only on their physicochemical properties but also on the storage conditions employed.

Methadone, a synthetic morphine substitute, is commonly used in the treatment of heroin addiction and has been associated with illicit use and fatalities. In humans, methadone is metabolized by mono- and di-N-demethylation, with spontaneous cyclization to form 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP). The determination of these analytes in whole blood is important for studying the toxicity of methadone, and for investigating factors affecting its metabolism.

The stability of methadone, EDDP and EMDP was investigated in whole blood at various temperatures (- 20°C, 4°C, 25°C), over a six month period. Whole blood was spiked for each analyte at a concentration of 1μg/ml. Deionised water was also spiked for each analyte (1μg/ml) to allow a comparison to be made between the amount of chemical decomposition and the degree of putrefactive degradation for each analyte. In addition to this, a comparison was made between EDDP stored in foil wrapped vials and EDDP stored in unwrapped vials. Methanolic EDDP

standards are routinely stored in foil wrapped containers to prevent exposure to light, which degrades EDDP.

Three vials for each measurement of spiked blood, spiked deionised water and blank blood were stored at different temperatures before reanalysis. The time interval of storage between each analysis was two, four, six and eight weeks and three and six months. The storage temperatures were monitored throughout the experiment. A solid-phase extraction procedure, developed specifically for the optimised recovery of methadone, EDDP and EMDP (1) from whole blood was used in conjunction with gas chromatography – flame ionisation detection. The recoveries were greater then 80% for all three analytes.

Methadone and EDDP were found to be stable when stored under these conditions, where at least 83.5% and 85.4% of the original concentration were recovered for methadone and EDDP respectively. EMDP was found to be very unstable, with a loss of approximately 98% of the original concentration after six months when stored at 4°C. In all cases, blood samples stored at - 20°C were more stable (methadone = 89.8%, EDDP = 91.8%, EMDP = 57.6%).

The EDDP spiked samples stored in foil wrapped containers resulted in higher recoveries than those stored in unwrapped containers, 94.9% and 85.4% respectively. However, the difference in recoveries isn't significantly large enough to warrant the expense incurred by using light sensitive containers for all samples taken for toxicological analysis.

The results presented here may give a possible explanation as to why EMDP has not been detected in postmortem cases (1,2) to date, and confirms the observation that EMDP is a more active substrate for hydroxylation than EDDP (3). Methadone and its main metabolite, EDDP remain relatively stable under the conditions employed in this study for up to six months.

Keywords: Stability, methadone, metabolites