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THE DETECTION OF DRUGS OF ABUSE IN BIOLOGICAL MATRICES USING ENZYME-LINKED IMMUNOSORBENT ASSAY AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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

Sam and Isabella Miller

&

To My Parents

Bobby and Linda Miller

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Summary

Enzyme linked immunosorbent assay (ELISA) is a useful "initial screening test" for the detection of a particular drug class for example opiates. It is a rapid, highly sensitive and in most laboratories automated procedure. This is highly beneficial in a busy toxicology laboratory with high sample throughput as a means of eliminating negative samples. Appropriate cross reactivity of the ELISA kits with a range of drugs or drug metabolites within a drug class is essential to the success of this screening method.

Liquid chromatography-tandem mass spectrometry (LC-MS-MS) combines selective chromatographic and mass selective detection of analytes. It has established itself as a complementary technique to gas chromatography-mass spectrometry (GC-MS) in the analysis of less volatile compounds and/or high molecular weight compounds and an increasing number of laboratories are using it. A major advantage of LC is that unlike GC, time-consuming derivatisation steps are not generally required.

The aim of this study was to investigate the potential use of ELISA and LC-MS-MS in combination and as individual techniques, for the detection of drugs of abuse in biological matrices.

An ELISA method was validated for the detection of buprenorphine in urine samples and the results were compared with an in-house LC-MS-MS method. 69 urine samples were received into the laboratory as routine samples and from volunteers on the Subutex® treatment programme. The limit of detection (LOD) was calculated as 0.5 ng/mL buprenorphine. The intra-day (n=20) and inter-day precision (n=100) were good (6.4 and 12.9 % respectively). The kit demonstrated appropriate cross reactivity with norbuprenorphine at low and high concentrations. 21 urine samples were confirmed positive and 48 samples were confirmed negative by LC-MS-MS. Using a cut-off value of 0.5 ng/mL buprenorphine, the immunoassay demonstrated a sensitivity and specificity of 100 %.

An ELISA method was validated for the detection of amphetamines, cocaine, benzodiazepines and opiates in hair. The kits for each drug class demonstrated

high sensitivity with LOD values ≤ 0.1 ng/mg using a 10 mg hair sample, good precision (intra-day < 9 %, inter-day < 18 %) and appropriate cross reactivity with analytes found in hair. The method was applied successfully for the screening of post-mortem samples as discussed in chapters 6-8. However more data is required to determine if the cut-off values recommended by the Society of Hair Testing for amphetamines, cocaine and opiates are suitable for screening hair samples.

This ELISA method was also validated for the detection of methamphetamine in hair and the results compared to GC-MS as part of collaboration with the National Institute of Scientific Investigation in South Korea. 29 scalp hair samples were obtained as routine cases submitted to the forensic toxicology laboratory in South Korea by the police. 28 of these samples were screened and confirmed as positive using a cut-off concentration of 0.5 ng/mg *d*-methamphetamine. The ELISA demonstrated a sensitivity and specificity of 97 % and 100 % respectively using this cut-off concentration. The ELISA kit demonstrated appropriate cross-reactivity with *d*-methamphetamine and did not cross-react to any significant extent with the licit *l*-methamphetamine isomer. The intra-day and inter-day assay precisions were good (7.4 % and 7.6 % respectively). The LOD was calculated as 0.05 ng/mg *d*-methamphetamine using a 10 mg hair sample.

The study in chapter 7 was designed to compare the ELISA method with a validated LC-MS-MS method for the detection of 9 benzodiazepines in hair. The LC-MS-MS method provided the sensitivity necessary to quantify benzodiazepine levels in post-mortem hair samples. Mild alkaline extraction conditions and an incubation time of 16 hours were demonstrated to be suitable for the extraction of benzodiazepines in the LC-MS-MS method. 13 out of 16 hair samples were screened and confirmed as positive. The benzodiazepines detected included diazepam, nordiazepam, temazepam, oxazepam, nitrazepam, and lorazepam. Using a cut-off concentration of 0.1 ng/mg oxazepam, the ELISA method demonstrated a sensitivity and specificity of 100 % and 81 % respectively, compared with LC-MS-MS results.

Also in chapter 7, a preliminary study into the use of a diazepam molecularly imprinted polymer (MIP) is described for the successful detection of

benzodiazepines in post-mortem hair samples. The molecularly imprinted solid-phase extraction (MISPE) method was compared to the classical solid-phase extraction (SPE) method for 10 hair samples. The MISPE method detected a higher number of diazepam positive case samples than the classial SPE method because of the higher extraction recovery of the MISPE method and the excellent molecular recognition of the template molecule (diazepam) imparted by the imprinting process. The MISPE method also demonstrated selectivity for other benzodiazepine analogues. MISPE could be used as a complementary method to classical SPE for the detection of benzodiazepines in post-mortem hair samples.

An LC-MS-MS method was developed and validated for the simultaneous identification and quantification of opiates, cocaine and its metabolites, amphetamines and diazepam and its metabolites from one hair sample without roots (8-30 mg) and root samples (in some cases < 1 mg) as described in chapter 8. The method was applied to 17 post-mortem hair samples, detecting at least one drug class in 16 out of 17 samples. The results of the analysis were compared to GC-MS results obtained by in-house methods.

Overall, the LC-MS-MS method showed good correlation results for the opiates compared to the GC-MS method. 6-MAM was however detected in more root segments and segments excluding roots by LC-MS-MS. Morphine was detected in a greater number of root segments by LC-MS-MS compared to GC-MS. However morphine was detected in a greater number of segments excluding roots by GC-MS. Codeine and dihydrocodeine were also detected in a greater number of root segments and segments excluding roots by GC-MS. The cocaine results showed excellent qualitative correlation between the LC-MS-MS and GC-MS methods for cocaine and benzoylecgonine. The GC-MS method did however extract greater concentrations of cocaine and its metabolites compared to LC-MS-MS due to the higher recovery of the drug group specific GC-MS method. Cocaethylene and EME were detected in some samples by LC-MS-MS where GC-MS detected none. As a result of the lower recovery of the LC-MS-MS method for opiates and cocaine and its metabolites compared to the GC-MS method, there may be some cases where the GC-MS method would detect the analytes where the LC-MS-MS method would not. This has been demonstrated in 3 samples for morphine and in 6 samples for codeine.

The LC-MS-MS method analysed for and detected amphetamines in samples that were not tested for amphetamines by GC-MS. In 1 sample that was tested by both methods, amphetamine was detected in the root sample by LC-MS-MS where GC-MS failed to detect it. Also a greater concentration of amphetamine was extracted using the LC-MS-MS method in the segment without roots.

The LC-MS-MS method was useful for the analysis of 17 drugs of abuse in post-mortem hair samples in forensic toxicology cases. Using this method, it is possible to obtain maximum information from 1 hair sample which is extremely useful when the sample weight is limited. The ability of the LC-MS-MS method to extract and analyse a greater number of drug groups from one hair sample highlights the advantages of using this method over GC-MS which targets individual drug groups and requires splitting of the sample. This method is particularly applicable for implementation in the forensic toxicology laboratory at the University of Glasgow where currently GC-MS methods that target individual drug groups are used for routine hair screening and confirmation.

List of Abbreviations

AAFS	American Academy of Forensic Sciences
7-AF	7-Aminoflunitrazepam
7-AF-d_7	7-Aminoflunitrazepam-d_7
APCI	Atmospheric Pressure Chemical Ionisation
API	Atmospheric Pressure Ionisation
APPI	Atmospheric Pressure Photoionisation
APLI	Atmospheric Pressure Laser Ionisation
BSA	Bovine Serum Albumin
BSTFA	*N,O*-Bis (Trimethylsilyl) trifluoroacetamide
DA	Dark agouti
DAD	Diode Array Detector
DESI	Desorption Electrospray Ionisation
DNA	Deoxyribonucleic Acid
EDDP	2-Ethylidene-1,5-Dimethyl-3,3-Diphenylpyrrolidine
EGDMA	Ethylene Glycol Dimethacrylate
ELISA	Enzyme Linked Immunosorbent Assay
EMIT	Enzyme Multiplied Immunoassay Technique
ESI	Electrospray Ionisation
FAB	Fast Atom Bombardment
FN	False Negative
FP	False Positive
FPIA	Fluorescence Polarisation Immunoassay
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High performance Liquid Chromatography

HRP	Horseradish Peroxidase
Igs	Immunoglobulins
IRS	Inner Root Sheath
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS-MS	Liquid Chromatography-Tandem Mass Spectrometry
LLE	Liquid Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
MAA	Methacrylic acid
MALDI	Matrix Assisted Laser Desorption Ionisation
6-MAM	6-Monoacetylmorphine
MDA	3,4-Methylenedioxyamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MDEA	3,4-Methylenedioxyethamphetamine
ME	Matrix Effect
MIP	Molecularly Imprinted Polymer
MISPE	Molecularly Imprinted Solid Phase Extraction
NCI	Negative Chemical Ionisation
NPC	Normal Phase Chromatography
ORS	Outer Root Sheath
PBI	Particle Beam Ionisation
PCC	Pyridium Chlorochromate
PFPA	Pentafluoropropionic anhydride
PFPOH	Pentafluoro-1-propanol
RF	Radiofrequency
RIA	Radioimmunoassay

RME	Relative Matrix effect
RPC	Reverse Phase Chromatography
RSD	Relative Standard Deviation
SAMHSA	Substance Abuse and Mental Health Services Administration
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
SIM	Selective Ion Monitoring
SOFT	Society of Forensic Toxicologists
SoHT	Society of Hair Testing
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
SSI	Sonic Spray Ionisation
TIC	Total Ion Count
THC	Δ^9-Tetrahydrocannibinol
THC-COOH	11-Nor- Δ^9-Tetrahydrocannibinol-9-Carboxylic Acid
TMB	3,3',5,5'-Tetramethylbenzidine
TMCS	Trimethylchlorosilane
TN	True Negative
TP	True Positive
TSI	Thermospray Ionisation
UV	Ultraviolet

1 Introduction

Enzyme linked immunosorbent assay (ELISA) is well established and routinely practiced in forensic and toxicological analyses as an "initial screening test" for drugs of abuse in blood and urine samples. ELISAs have more recently been adapted to detect and semi-quantify a wide range of drugs or drug metabolites in a variety of less conventional biological matrices such as hair and oral fluid.

ELISA is a highly sensitive method for determining the amount of drug present in a sample by means of an enzyme-catalysed change in absorbance signal. In drugs of abuse screening, ELISA serves as a useful means of eliminating negative samples from further confirmatory tests and also identifying any drug class that requires confirmation by a second step test such as liquid chromatography-tandem mass spectrometry (LC-MS-MS).

The use of liquid chromatography-mass spectrometry (LC-MS) in all areas of analytical chemistry has increased exponentially from 1990. The development of a suitable interface for coupling LC to MS in the form of atmospheric pressure ionisation (API) is responsible for the success of the technique. LC-MS has many attractive features for analytical toxicologists including the direct analysis of polar, less volatile and high molecular weight compounds. Prior to its availability, LC was used with an ultraviolet (UV) detection system for compounds that were not suitable for gas chromatography-mass spectrometry (GC-MS). Unlike GC-MS, time-consuming derivatisation steps are generally not required in LC-MS. The combination of selective LC with mass-selective detection provides two pieces of information gained from chromatography and MS data, which is highly specific to a particular analyte. LC-MS also provides the flexibility required for screening or quantification.

Hair testing for drugs of abuse has been an area of interest, particularly over the last decade. It is a useful sample for establishing drug use over the weeks or even months prior to collection, depending on the length of the hair sample and the stability of the drug within the hair. This has led to its international application in many areas of forensic and clinical toxicology. Hair results are currently used to complement blood or urine analysis.

An investigation into the potential use of ELISA and LC-MS-MS for the complementary detection of drugs of abuse in urine and hair is described in this thesis. An LC-MS-MS method was developed and validated as described in chapter 8 for the identification and quantification of a wide range of drugs of abuse in hair. This method will be implemented in the forensic toxicology laboratory at the University of Glasgow initially as a complementary technique to the drug group specific GC-MS methods currently being used for routine hair screening and confirmation.

2 Hair

2.1 Introduction

Testing human hair for the presence of drugs is an extremely useful tool in forensic and clinical toxicology and has been applied internationally. It has been used to investigate drug-related deaths[1], in employee screening[2], to assess physical fitness to obtain a driving license[3], to monitor compliance or abstinence in drug treatment programmes[4], in child custody[5] and criminal cases[6], in military court-martials[7], in sports testing[8] and to monitor pre-natal drug exposure.[9]

Hair analysis is useful for establishing drug use over the weeks or even months prior to collection, depending on the length of the hair sample and the stability of the drug within the hair. In contrast, blood and urine samples only provide information on drug use related to time intervals of hours to a few days prior to collection. Hair analysis is particularly useful in cases where proof of abstinence from drug use is required for example in workplace testing. It is well known that both parent drugs and metabolites incorporate into hair where often only metabolites are detected in blood and urine.

Hair testing offers several advantages over more conventional blood and urine testing. It is difficult to adulterate a hair sample and hair is unaffected by short-term abstinence. It is a stable and biologically inert material and it can retain drugs for extended time periods. The first forensic case involving the detection of a poison in human scalp hair was the detection of arsenic in 1857. The hair had been collected from a body exhumed eleven years after burial.[10]

Hair is a readily accessible and non-invasive choice of sample which can be collected under close supervision. This excludes the embarrassment and lack of privacy associated with urine collection. In addition, it is usually possible to collect a second representative sample for analysis if the sample integrity is in question.

Hair stability is such that it can be stored at ambient temperature indefinitely, without refrigeration. Storage and transportation of hair samples is easier and the analysis does not require to be carried out immediately.

Although hair testing is extremely useful, there are still many controversial issues surrounding it which need to be addressed. These include conflicting data on the exact contribution of external drug contamination and the effectiveness of decontamination procedures. There is the possibility of bias from cosmetically treated hair and also hair from individuals of different cultures. In addition, the exact mechanism of drug incorporation is not yet known. Until these issues are resolved, hair testing should not be used as a single source of evidence in a toxicological investigation but should be used in combination with blood and urine analysis, to provide information on both acute and chronic drug use or to rule out the role of drugs in a case. The consensus opinion of the Society of Forensic Toxicologists (SOFT) in 1990 was that "Hair may be a useful specimen in forensic investigations when supported by other evidence of drug use."[11] Since then the Society has not changed its opinion on hair testing. The issues contributing to a lack of a firm scientific basis are discussed in subsequent sections of this chapter and are partly responsible for the current opinion of the SOFT.

2.2 Hair Physiology

2.2.1 Introduction

Despite the lack of conclusive scientific knowledge on drug incorporation mechanisms, the contribution of external contamination and inter-individual variations, an integral part of the accurate interpretation of hair results is to have an understanding of hair structure and growth. Therefore this is discussed in sections 2.2.2-2.4.4.

2.2.2 Structure

Hair tissue is a complex cross-linked polymeric network, which is synthesised in the hair follicle. It contains functional chemical groups such as acidic, basic and peptide groups which can potentially bind to small drug molecules. Hair is composed of 65-95 % protein, 1-9 % lipids, 0.1-5 % pigments (melanin) and small quantities of water, polysaccharides and trace elements.[12]

The hair shaft consists of three distinct layers namely the cuticle, cortex and medulla (Figure 2-1). Pigment granules are present in the cortex and medulla and their presence or absence can affect drug incorporation. These structural components are discussed in more detail in sections 2.2.2.1-2.2.2.4.

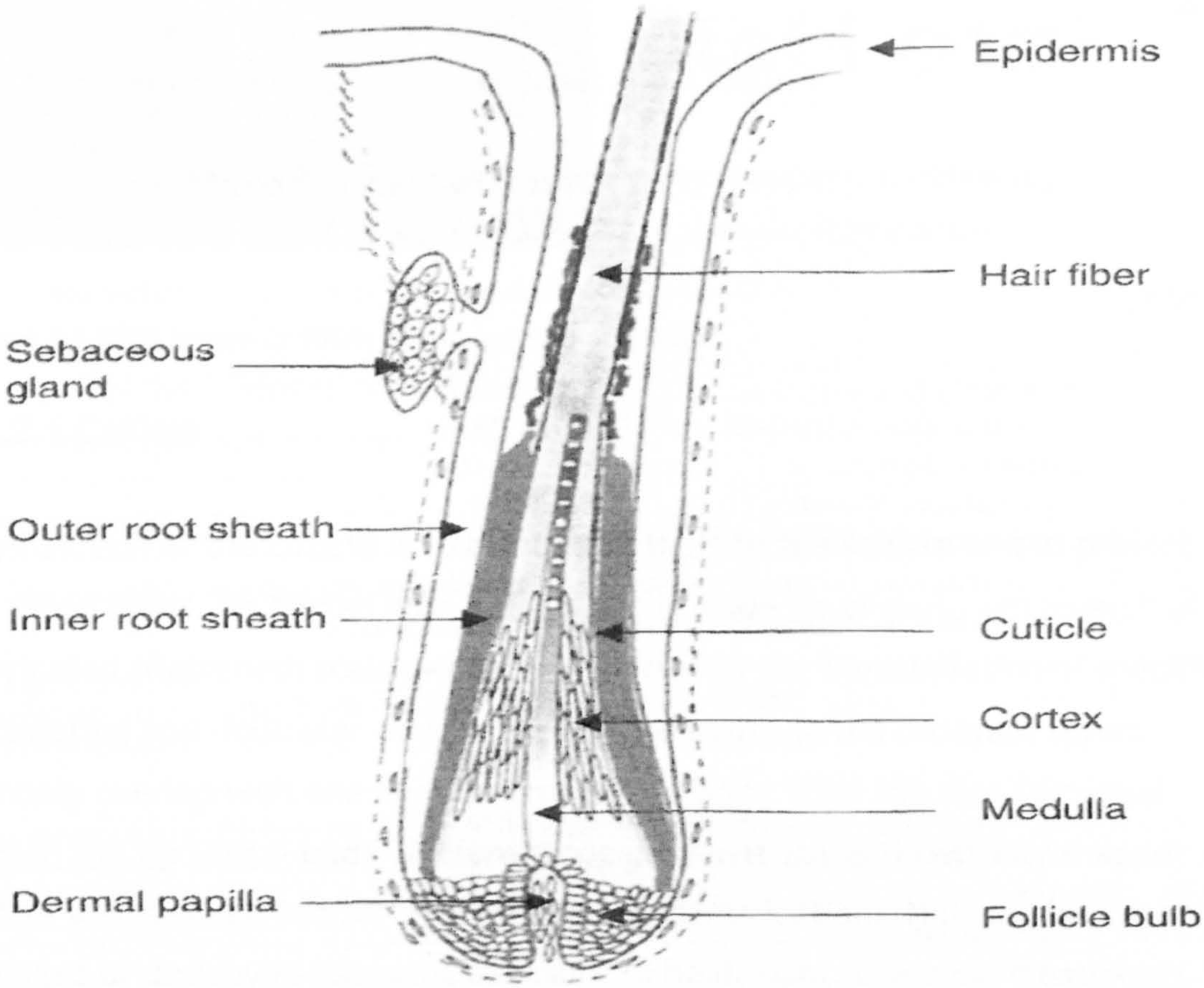


Figure 2-1 Schematic diagram of the hair follicle[13] (© 2007 by Taylor & Francis Group, LLC)

The layers are distinguishable by microscopy. Figure 2-2 shows a scanning electron microscope (SEM) image of the surface of a hair taken in the University of Glasgow. The flattened cuticle scales are visible. The raised cuticle scales are typical of damage resulting from everyday haircare such as brushing.

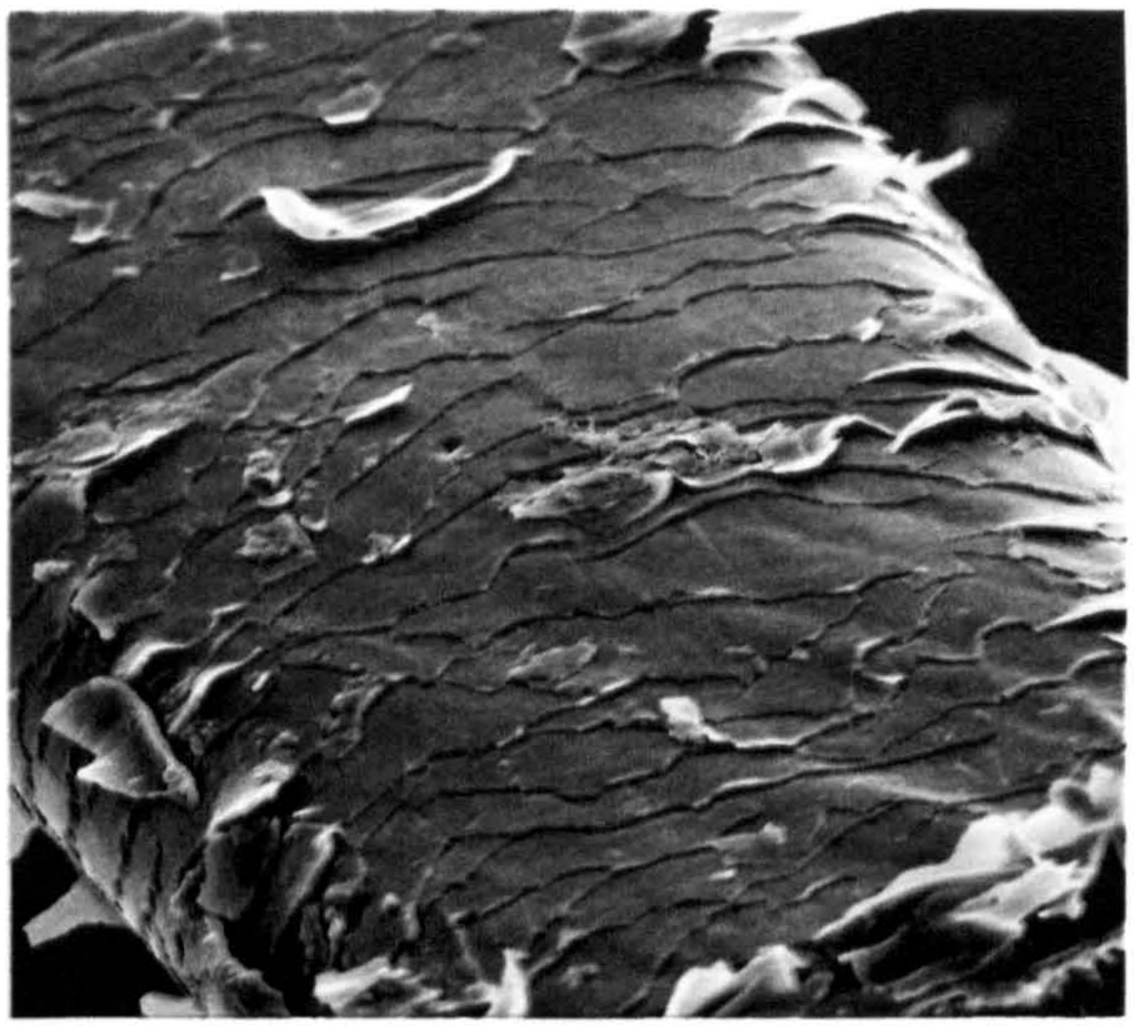


Figure 2-2 SEM image (x 1000) of the surface of a hair

2.2.2.1 Cuticle

The function of the cuticle is to attach the shaft to the follicle and to protect the inner fibres. In human hair, the cuticle is made up of six to ten layers[14] of imbricated (flattened) scales which are formed by the keratinisation of specific cells during post-follicular development. The scales in the different layers narrowly overlap with one another and always point from the root (proximal end) to the tip (distal end). Different species have characteristically shaped cuticle scales which can be used for species identification. Hair can become damaged or destroyed following exposure to heat, light, chemical treatments and mechanical abrasion. If the cuticle gets damaged, drugs can be removed more readily from hair.[12,15]

2.2.2.2 Cortex

The cortex constitutes the bulk of the hair shaft and is made up of long fibres of sulfur rich keratin. It is mainly responsible for the stability and retention of structural features in hair and contributes most to the mechanical strength of the hair fibre.[14] Fusi can be present between the fibres in the cortex. These are small areas filled with fluid in the living section of the hair root but as the hair grows and dehydrates, they become air spaces which can be distributed throughout the hair shaft.

2.2.2.3 Medulla

The medulla may be present in the core of the cortex. In human hair, it can be present in various forms, or absent for example in fine vellus hair. If present, it can be continuous or interspersed with air spaces throughout the hair shaft. In human hair, the medulla is usually present in the fragmented form or is absent; it is rarely in the continuous form. Mongoloid hair is the exception to this as it usually possesses continuous medullae. The medulla can be complex in beard hair and may even possess a double medulla.[12] It is usually amorphous in appearance but different species demonstrate differently shaped medullae.

The medullar cells at the follicle are loosely packed together. These cells become dehydrated to form vacuoles further away from the follicle. Generally, the number of medullar cells increases with hair shaft diameter. Fine hairs may for example possess only a cuticle and cortex while thicker hairs may possess a relatively high number of medullar cells.

2.2.2.4 Pigment Granules

Pigment granules are present mainly in the cortex, to a lesser extent in the medulla and rarely in the cuticle. Melanin is the main pigment in hair and is made in melanosomes, which are specialist organelles contained in melanocytes within the apex of the dermal papilla. It is transferred into the cells migrating from the hair follicle bulb and distributed throughout the body of the hair shaft.

Pigment formation (follicular melanogenesis) occurs in four stages. Tyrosinase and protein are the basic structural components in the first stage, followed by the formation of an inner membrane in which melanin is synthesised and accumulates. The melanosome is finally transformed into a melanin particle and transferred into cortical and medulla keratinocytes which then distribute the pigment through the shaft. This whole process is regulated by enzymes, structural and regulatory proteins, transporters and receptors and their ligands during the anagen phase of the hair growth cycle.

Hair colour is genetically controlled and there is a diverse range of pigmentation phenotypes. The type, quantity, size and distribution of pigment granules are responsible for imparting hair colour. There is now thought to be four types of

pigment responsible for the diversity in hair colour namely pheomelanin, oxypheomelanin, eumelanin and oxyeumelanin.[16] Oxypheomelanin and oxyeumelanin are the oxidative products of pheomelanin and eumelanin pigments respectively, induced by the presence of hydrogen peroxide.

A four class system has been proposed for defining hair colour according to its melanin content.[16] The study demonstrated that black to dark brown hair contains virtually intact eumelanin whereas brown hair contains more of the oxidative breakdown product, oxyeumelanin. Furthermore, blonde hair contains large quantities of oxyeumelanin. The study also showed that the traditional view of mixed-type melanins in brown/chestnut hair produced from the same melanocyte is incorrect since only eumelanin and oxyeumelanin were found. The author concluded that the diversity of hair colour results from eumelanin and pheomelanin at different degrees of structural integrity.

2.3 Hair Follicle

Hair follicles are small sacs surrounding the hair root, embedded in the skin's epidermal epithelium and lie approximately 3-4 mm below the surface of the skin. They are connected with the sebaceous and apocrine glands and also eccrine sweat glands in the axillary and pubic areas.

The hair follicle consists of several layers of cells. The outer root sheath (ORS) is part of the epidermis and surrounds the other layers. The inner root sheath (IRS) region encases the growing hair fibre. Hair cells are synthesised in the hair follicle bulb by extensive mitotic activity and move upward to form the body of the hair fibre and the IRS. The adjacent region (keratogenous zone) is where the hair keratinises, hardens and solidifies. The final region is the permanent hair where the hair cells have dehydrated to form the hair shaft.

2.4 Hair Growth

Hair grows out of the epidermis and is synthesised in the hair follicle. Hair growth begins in cells present in a germination centre (matrix) which is found in the base of the follicle. As cell division occurs, the cells become larger and elongate out of the matrix into the keratogenous zone. They produce melanin

pigment in this zone and keratinise into long fibres through the cross-linking of amino acid functional groups such as the sulfhydryl group in cysteine. The cells gradually die and their decomposition involves the release of cell nuclei and the elimination of water.

Human head hair can be found in one of three growth stages; anagen, catagen or telogen (Figure 2-3). The size and the shape of the hair roots differ depending on the particular growth stage that the hair is in. Hair growth is an intermittent cyclic process which alternates between periods of growth and dormancy. The hair growth process and growth rate is different between body regions and even within a single region.[12] The different stages and rates of growth are described in more detail in sections 2.4.1-2.4.4.

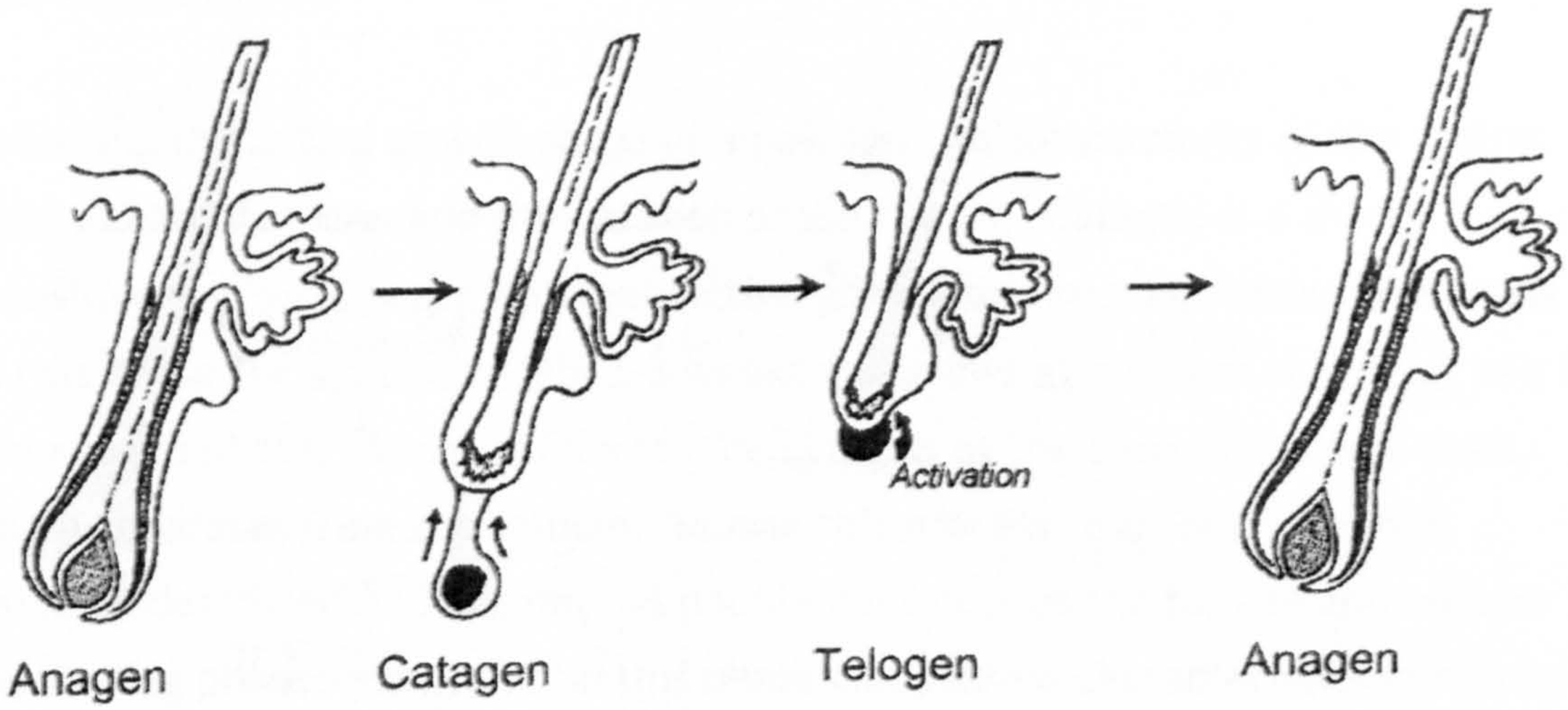


Figure 2-3 Different stages of the hair growth cycle[13] (© 2007 by Taylor & Francis Group, LLC)

2.4.1 Anagen

Anagen is the first growth phase in which the follicle develops and the hair is produced. Hair grows for varying amounts of time depending on the anatomical region, usually continuing between 7 to 94 weeks[11] but potentially lasting several years. In the anagen phase, there is an increase in mitotic activity of the matrix cells at the base of the hair follicle. Newly divided cells elongate to form thin filaments which grow upwards into the follicular canal. The cells differentiate into the cortex, cuticle and medulla and the hair begins to keratinise to produce a new hair. After the follicle is fully grown, it is elongated to at least six times

its original length in the resting phase.[17] Nutrients and drugs, supplied by a blood capillary, are incorporated into the hair shaft during this phase.

Approximately 85 % of hairs in the posterior vertex region of the scalp are in the anagen phase at any one time, while the other 15 % are in the resting (telogen) phase.[12] Hair in the anagen phase has characteristic dark roots as it contains pigment granules which extend into the root bulb. It also possesses a follicular tag, which is a clear piece of tissue surrounding the hair shaft near the root. The presence of the follicular tag can indicate if a hair has been removed by unnatural means, for example during a violent attack. As the follicular tag is made up of cells, it can provide a source of deoxyribonucleic acid (DNA) for testing purposes and can be used to determine the sex of the hair.

2.4.2 Catagen

Following the active growth stage of a new hair, mitotic activity of the matrix cells suddenly ceases and the catagen phase begins. Catagen is a short transitional growth phase between active growth and resting. Hair can remain in this phase for approximately 2-3 weeks and grows at a slower rate than hair in the anagen phase. A mass of keratin cells forms at the base of the hair shaft, which separates from the follicle. During this process, the follicle bulb is partially destroyed and the dermal papilla contracts as the follicle approaches the resting phase. Hair roots in this phase can possess characteristics of hair in either anagen or telogen.

2.4.3 Telogen

Telogen is the final phase in the hair growth cycle, in which follicular activity ceases and the follicle rests. The hair becomes completely detached from the bulb and is retained in the follicular canal. Consequently, the hair can be easily pulled out or plucked from the scalp. Roots of telogen hairs are either clear or white as they are lacking pigment granules. Telogen hairs do not have follicular tags but instead have root sheaths which are pieces of tissue attached to the root.

The length of time the hair cells spend in the telogen phase depends on the body area and age of the individual. Scalp hair can be in the resting phase for about

3-4 months however this can last 2-6 years, depending on anatomical region.[12] The old hair is forced out through the scalp surface as a new hair shaft grows. After the telogen phase, another growth cycle begins.

2.4.4 Growth Rate

Hair growth rate is not clearly defined and it can vary greatly between individuals and in different and also in the same body regions on a particular individual. This is because not all hair is growing at any one given time and the percentage of follicles in the anagen phase varies. Human scalp hair is generally assumed to grow at a rate of 0.35 mm/day for males and females. However a range of 0.07-0.78 mm/day was observed in one study where 82 % of the test population had a growth rate in the range of 0.32-0.46 mm/day.[13]

Hair type and anatomical location are most influential in determining growth rate however gender, race and age can also affect growth rate. Scalp hair has a faster growth rate than axillary and pubic hair, while beard hair has the slowest growth rate. Scalp hair growth rate is faster in females than males, is faster in Caucasians than Asians and tends to decrease with age.[12] Growth rate is also influenced by seasonal changes, nutrition, genetic make-up, endocrine dysfunction and pregnancy.[17] These factors affecting growth rate must be considered in the interpretation of hair results.

2.5 Drug Incorporation Mechanisms into Hair

Several studies have been carried out in an attempt to explain the factors that influence drug incorporation into hair from the bloodstream.[18,19,20,21] However an exact mechanism has not yet been established. This is partly due to the limited number of controlled drug administration studies in humans that involve the collection of plasma, hair and sweat samples.[22]

There are three proposed models for incorporation of drugs entering into the hair (Figure 2-4). These are (1) active or passive diffusion from the bloodstream into the dermal papilla, (2) diffusion from sweat and other secretions surrounding the growing or mature hair fibre, or (3) external drug contamination from vapours or powders that diffuse into the mature hair fibre. The most likely

model is probably a combination of all of these routes of incorporation however the importance of the different routes has not been determined and will vary depending on the drug and also between individuals. For interpretation purposes, the incorporation route via the bloodstream is the most significant when information on for example dose taken or time of drug intake is required.

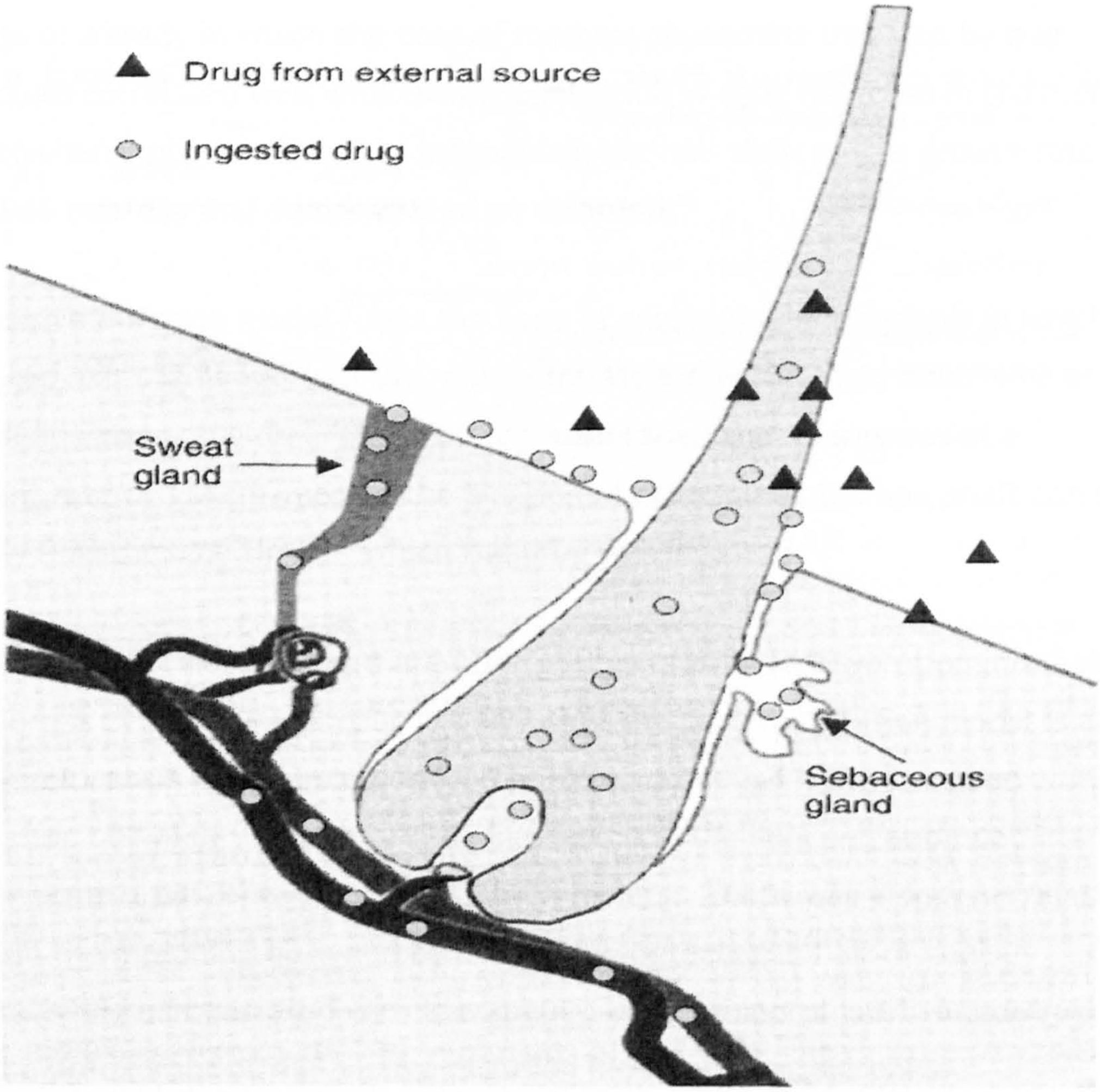


Figure 2-4 Three models of drug incorporation into hair[13] (© 2007 by Taylor & Francis Group, LLC)

2.5.1 Incorporation from the Bloodstream

The hair follicle is provided with a good blood supply. Any drugs circulating in the bloodstream will also be passed to the hair follicle. Drugs passively diffuse through the cell membrane from the bloodstream into matrix cells of the growing hair positioned at the base of the follicle. The diffusion rate across the

membrane is related to drug lipophilicity and the pH gradient between the plasma and the hair cell. This will be discussed later in sections 2.6.1 and 2.6.2.

This incorporation model, also known as the passive diffusion model, proposes that as the hair grows out from the scalp, the drugs form bands in the hair that are directly proportional to the concentration present in the bloodstream at the time the hair was formed. The passive diffusion model is supported by the findings of a study in which the dose of methoxyphenamine ingested by five individuals correlated well with the concentration of drug detected in the hair. Methoxyphenamine was found to move along the hair shaft with a growth rate of 0.40-0.46 mm/day and demonstrated no diffusion.[23]

The passive diffusion model forms the basis of segmental hair analysis in which measured hair segments from the root to the tip are analysed to determine an individual's prior drug use. Based on the assumption that hair grows at a constant rate of 1.0 cm/month, the position of drugs along the hair shaft can be directly linked to the time at which the drug was ingested.

As mentioned previously, there are other routes of drug incorporation into hair and there is evidence supporting the fact that the passive diffusion model is an oversimplification of the incorporation process. Although there is a good correlation between the dose of drug ingested and the concentration detected in hair for some drugs, this is not the case for others. There was a poor correlation between the morphine dose and concentration detected in hair of cancer patients being administered with therapeutic doses of morphine.[24] In contrast with the predictions of the passive diffusion model, higher morphine concentrations were detected in the hair of patients exposed to the lowest doses using radioimmunoassay (RIA). Drug concentrations in hair have also been shown to vary significantly in individuals receiving the same dose.[25] In addition; there is also some evidence to suggest that the position of the drug along the hair shaft does not correlate with time of exposure.[25]

A further discrepancy with this model is that parent drug to metabolite ratios in blood do not correlate with those in hair. Cocaine is for example predominantly found in hair, despite its short half-life in blood (0.7-1.5 hr). Although hair, in particular the bulb, contains cytochrome P450 enzymes capable of drug metabolism, it does so less readily than other organs.[26]

As a result of the discrepancies associated with the passive diffusion model and the apparent complexity involved with drug incorporation into hair, a multi-compartment model has been proposed to include incorporation from sweat and other secretions and also incorporation from external contamination.

2.5.2 Incorporation from Sweat, Other Secretions and External Contamination

The multi-compartment model shown in Figure 2-4 includes the incorporation route via the bloodstream into the dermal papilla as well as by sweat and sebum bathing the mature hair fibre. The model also considers external drug contamination through vapours or powders as a route for potential incorporation into the mature hair fibre.

It is widely accepted that drugs and their metabolites are excreted in sweat[27,28] and there are some studies which investigate this in relation to drug incorporation into hair. As hair is very porous and can increase its weight by 18 % by absorption of liquids, drugs can be easily transferred into hair from sweat.[29] Deuterated cocaine was detected in multiple segments after a single dose, supporting the evidence that sweat or other secretions are a possible route for drug incorporation into hair.[30] It has also been proposed that differences in drug concentrations found in hair could be attributed to secretion variability between individuals. Different concentrations of cocaine were detected in the sweat of individuals receiving the same dose.[25] Sebum was shown to deposit insignificant quantities of cocaine, flunitrazepam and nicotine onto the hair.[31]

Both in vitro and in vivo experiments have been conducted in an attempt to differentiate between drugs deposited in hair from external sources and drugs which have been ingested, with markedly different results. Rhodamine dye was found to penetrate the hair at the cuticle scale junctions and further along the non-keratinous cell membrane complex after soaking in a solution.[32] In contrast an in vivo experiment using mice found that fluorescein and rhodamine were deposited mainly in the cortex and the medulla.[33] The dyes, whether incorporated in vitro or in vivo, could not be removed by extensive wash procedures making interpretation of analytical results difficult.

Several decontamination procedures have been recommended recently for differentiation between external drug contamination and ingested drug. These will be discussed in section 2.10. Although drugs deposited on the hair from the external environment can be removed by a decontamination procedure, it has been shown that a small fraction of these drugs are able to penetrate the cuticle of the hair shaft, therefore affecting the true results of hair testing.

2.5.3 Binding Mechanisms

The mechanisms of drug binding in hair are not fully understood but melanin and keratin hair components have been suggested as potential sites of interaction. Drug binding to melanin was identified more than four decades ago[13] and there have since been many studies in this area which have investigated the binding of a variety of drugs with different physicochemical properties.

Melanin pigment is a polyanionic indolequinone based polymer that will potentially interact with positively charged (cationic) molecules.[19] This interaction may explain the preferential incorporation of some basic drugs into pigmented hair over non-pigmented hair.[19,34,35,36] Basic drugs exist as cationic molecules at physiological pH and could preferentially interact with the melanin in pigmented hair by electrostatic forces between the cationic functional groups and the anionic carboxylic groups on the surface of the melanin polymer. Depending on the structure of the bound drug, the electrostatic binding can be strengthened by van der Waals forces, hydrophobic interactions, charge transfer reactions and covalent bonding.

It has been suggested that the most important association of drugs with melanin occurs during melanin synthesis at which time the drug molecules become entrapped within the melanin polymer.[18] Therefore drug incorporation into hair may relate to melanin content in the melanocyte.

The in vitro binding of cocaine, benzoylecgonine, amphetamine and N-acetylamphetamine to synthetic melanin subtypes has been studied. The results indicated that the more basic drugs (cocaine and amphetamine) bind to eumelanins and mixed eumelanin/pheomelanins to different degrees but do not bind to pure pheomelanin. The net neutral molecules (benzoylecgonine and N-acetylamphetamine) did not bind to any melanin subtype.[35]

A number of in vivo experiments using dark-agouti (DA) rats (which have no sweat glands) were carried out in Japan and the results demonstrated that the plasma concentration was not the major factor affecting drug incorporation into hair but rather the physicochemical properties of the drugs.[37,38] Although the conclusions of these experiments cannot be directly applied to human hair as it is more complex than DA rat hair which only contains eumelanin, the relationship between incorporation rate and drug basicity and melanin affinity is relevant. Hence these relationships are discussed in section 2.7.

There are few studies which address the issue of drug binding to keratin in hair. Human studies in subjects with grey hair have shown that various drugs can be detected in the pigmented and non-pigmented hair shafts of these individuals. This supports the proposition that keratin has an important role in drug binding to hair. Various studies have detected higher concentrations in the pigmented hair shafts compared to the non-pigmented (white) hair shafts.[39,40] This could be explained by the presence of a strong ionic interaction between the positively charged drugs and the negatively charged melanin polymer which is absent in non-pigmented hair.

2.6 Drug Properties Influencing Incorporation

There are a number of physicochemical properties which influence drug incorporation into hair, the main ones being lipophilicity, basicity and melanin affinity. Hair structure and cosmetic treatments also have a significant effect on drug binding in hair (discussed in section 2.7). Other factors affecting incorporation are bioavailability, the pK_a value, plasma elimination half-life, volume of distribution and cell membrane permeability.[1]

2.6.1 Lipophilicity

Due to the chemical nature of the cell membrane, non-polar, more lipophilic parent drugs are more likely to pass through from the bloodstream to the hair forming cells than polar, more hydrophilic drug metabolites.[41] This would explain why parent drug concentrations in hair are mostly higher than their respective metabolites. Greater incorporation of parent drugs can be advantageous when trying to distinguish heroin use from prescribed morphine or

codeine use for example. A positive correlation of 0.770 was found to exist between lipophilicity and incorporation rate into rat hair for nineteen basic drugs of abuse.[37]

2.6.2 Basicity

Drug ionisation at physiological pH influences the extent of incorporation into hair. Basic drugs exist as cationic molecules at physiological pH and should preferentially bind to melanin in pigmented hair. Codeine which is a weak base and cationic at physiological pH was incorporated more into rat hair to a greater extent than phenobarbital, which is a weak acid and is not cationic at physiological pH.[26] The conclusion from this study was that at a given plasma concentration, there is a greater concentration of cationic drugs incorporated into hair than anionic drugs.

Basic drugs were found to have higher incorporation rates into hair compared to more acidic and neutral drugs in a study examining the incorporation rate of twenty acidic, basic and neutral drugs of abuse into rat hair.[37] Cocaine had the highest incorporation rate and THCA had the lowest. There was a 3600 fold difference observed between their incorporation rates.

Amphetamine incorporates into both pigmented and non-pigmented hair to a greater extent than its non-basic analogue N-acetylamphetamine.[19] Two basic benzodiazepines, flurazepam and medazepam, were shown to have relatively high incorporation rates in a study into the rate of incorporation of eight benzodiazepines into rat hair.[42]

2.6.3 Melanin Affinity

Drug incorporation into hair can be influenced by pigmentation i.e. melanin content of hair. As discussed in section 2.5.3, melanin polymers possess many negatively charged carboxylic acid groups in their structure which attract positively charged basic drug molecules under physiological conditions. Melanin affinity is therefore associated with drug basicity.

Experiments were carried out to test the melanin affinity of twenty drugs of abuse. Each drug displayed a different affinity for melanin binding, however

cocaine had the highest affinity, followed by benzphetamine and phencyclidine. The correlation coefficient for a graph of melanin affinity versus cocaine incorporation rate into hair was 0.947 and this direct relationship supports the evidence that melanin content in hair is strongly related to drug incorporation into hair.[37] In controlled oral codeine administration studies, a significantly positive relationship ($R^2 = 0.73$) was found to exist between codeine concentrations in hair and the total melanin concentration present.[43] This contrasts with findings for more neutral and acidic drugs such as 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) and N-acetylamphetamine where a lack of correlation between concentrations of these drugs in hair to hair melanin content has been observed.[19,37]

In an animal study involving Long Evans rats, six phenethylamines were detected at relatively high concentrations in black hair however none were detected in white hair collected from the same rats. These findings indicate that the presence of melanin strongly influences the retention and incorporation of basic drugs.[44]

2.6.4 Chemical Structure

Drug-melanin binding is affected by the presence of different para-substituents on the benzene ring of methamphetamine. Nitro-, amino-, methylenedioxy-, methoxy- and bromo- groups all showed relatively higher melanin affinities than the unsubstituted methamphetamine.[45]

A study into the incorporation rates of seven phenethylamine analogues in rat hair roots also found different incorporation rates for each analogue, supporting the evidence that changes in the drug functional groups greatly affect drug incorporation into hair.[44]

2.7 Bias in Hair Testing

Bias in drug testing can be defined as an increased chance of detecting drugs in one group of individuals over another when both groups have been exposed to or used the same quantity of drugs. In hair testing, bias can appear in two main situations: (1) Two individuals ingest the same quantity of drug but due to

biological or genetic reasons, one individual does not incorporate as much drug into the hair as the other, producing a false negative result; (2) Two individuals who are not drug users are exposed to drugs from their surrounding environment. Due to biological, genetic or cultural reasons one individual incorporates the drugs more readily into the hair from the environment and retains them longer than the other individual, producing a false positive result.

The effect of hair bias remains an unresolved issue with hair testing. Cosmetically treated hair incorporates drugs more readily than untreated hair as it has structural damage and residual chemicals. When a drug penetrates the hair it can either bind or be removed through normal hygiene practices or decontamination as part of the hair testing procedure in the laboratory. Bias is associated with melanin content which facilitates drug binding. Different hair treatments associated with Africans and other ethnic groups are more prone to environmental contamination leading to false positive results.

Cosmetic treatments such as dyeing, bleaching, chemical straightening and permanent waving reduce the binding of drugs in hair as these processes damage the hair structure. After these treatments, drug concentrations can decrease by 50-80 % of their original concentration.[29] This is an important consideration for interpretation of hair analysis results since a change in drug concentration is induced by cosmetic treatments. Products used for these treatments are strong bases which can cause hair damage. Commonly used bleaching and dyeing formulas are combinations of hydrogen peroxide and an agent containing ammonium hydroxide. Ethanol and natural pigments are also used for bleaching and dyeing respectively.[46] In bleaching formulas, ammonium hydroxide opens the cuticle scales which aid the entry of drugs. Hydrogen peroxide is also a strong oxidant which attacks the melanin pigment resulting in decolourisation of hair. In dyeing formulas, natural pigments dye the hair to a desired colour. Chemical straightening and permanent waving causes the hair to become porous and permeable as the cuticle is disrupted.[47] In addition, some of the cosmetic products that are applied to hair to add shine after chemical straightening can aid transfer and binding of drugs.[13]

All of the above mentioned cosmetic treatments can change the hair ultrastructure, resulting in drugs being lost from the hair matrix, or they can

become incorporated more easily into the hair matrix following exposure to external contamination.[48] The increased risk of obtaining false positive hair analysis results, due to drug uptake by sweat or sebum, has been demonstrated for bleached and permanently waved hair but not to a great extent.[49]

Hair collected from a female drug addict who had brown hair with strands of bleached hair was tested.[50] 3-fold higher concentrations of cocaine and codeine were found in the brown hair compared with the bleached hair. A decrease in benzodiazepine, cocaine and opiate concentrations was found in hair that had been bleached in vitro in another study.[51] The effect of bleaching and dyeing hair on the concentrations of opiates, cocaine, cannabinoids and nicotine has also been investigated.[46] Bleaching was found to produce lower drug concentrations than dyeing. In this study, the degree of hair damage was also identified as a factor affecting drug concentrations; the more damaged the hair, the greater the loss of drug.

Several studies have shown that some drugs incorporate into hair with higher melanin content and therefore there may be a bias in drug testing in hair from different races. Generally, Caucasian hair incorporates smaller quantities of drugs compared to African hair. Significantly more cocaine was detected in male African American hair than male and female black/brown Caucasian hair.[52] An animal study found that in cases of high codeine intake by guinea pigs, black pigmented hair incorporated the highest concentration compared with white non-pigmented hair.[34] Another animal study noted that black pigmented rat hair incorporated a higher methadone concentration (21-fold) than white non-pigmented hair.[53]

Morphological differences have been identified between black African hair and hair from Caucasians and Asians. The African hair samples contained a tightly interwoven mat of hair shafts and many hair shafts were knotted, broken and inter-locked in comparison with Caucasian and Asian hair samples.[54] This damage to hair structure through twisting in African hair could explain the differential drug binding observed between races since drugs have to first penetrate the cuticle which is made easier by the damage.[13]

It is important to consider any wash procedures carried out in the laboratory which may affect the concentration of drug that is detected in the sample. It is

good laboratory practice, on receiving a sample, to note the physical condition of the hair and the type of cosmetic treatment, if any.

An attempt was made to correct for racial bias observed in hair samples, using dyes, which can provide additional information on the permeability and binding capacity of hair.[55] The authors concluded that although the method was useful, it would be difficult to implement this process as part of a production system.

Other studies have found no bias and have proposed that differences in drug levels observed in hair from various races are probably due to different rates of drug use.[56,57] A statistical analysis has been conducted on reported drug levels in hair where the hair colour was indicated.[58] There were differences in the drug levels detected in the differently coloured hair samples; however, these were not significant. The authors of the study propose that although hair colour does contribute to drug incorporation in hair, its contribution is yet to be detected statistically.

2.8 Collection of Hair Samples

It is necessary to consider a number of important factors when analysing hair samples. These include the method of collection used: whether the sample was collected from a living or deceased subject; any potential contamination which may result during the collection of, for example post-mortem samples; the sample size; and the anatomical location of the hair.

2.8.1 Methods of Collection

In post-mortem cases, hair samples should be collected prior to the post-mortem examination to prevent them from getting wet or contaminated with blood. Head hair is collected from the posterior vertex region and is either plucked from the scalp (in which case the roots will be present) or cut as closely as possible to the scalp. General Practitioners or solicitors normally collect hair samples from living individuals and in these cases the hair is cut as closely as possible to the scalp.

The results of segmental analysis are meaningless if the collection and handling of the sample is inappropriate. Therefore, it is essential to obtain a sample that

has been correctly aligned with the root and tip ends clearly labelled. If this is not done, each segment will contain different portions of the hair strand therefore representing longer and overlapping time periods.

Plucked hair may be advantageous over cut hair in some instances for example in an acute fatal poisoning case resulting in a delayed death. In this situation, a period of 2-3 days in a coma may yield negative blood and urine results due to elimination and cut hair may also be negative.[13]

Plucked or cut hair samples are collected and stored inside hair collection kits at ambient temperature. The kits contain a circular piece of aluminium foil which has an area labelled "root end". After the "root ends" are aligned, the sample is wrapped in the foil and placed in an envelope clearly labelled with the case details. The envelope is then placed in a plastic evidence bag and sealed with adhesive tape. Figure 2-5 shows a typical hair collection kit.

Figure 2-5 Typical hair collection kit

2.8.2 Sample Size

The sample size required for analysis varies between laboratories. It depends on the number of analyses to be carried out using different extraction methodologies and also on the sensitivity of the analytical method. A bundle of hair from the posterior vertex of approximately "pencil thickness" is normally sufficient for testing, but this depends on the amount of hair a particular individual possesses. In thin-haired or bald subjects for example, it may be necessary to collect the hair further down the head from the posterior vertex.

Sample sizes ranging from a single hair to 200 mg hair have been reported in the literature.[29]

It is generally best to have as large a sample size as possible to ensure the detection of low drug concentrations for example in date-rape cases where the subject has potentially ingested a single dose of drug.

2.8.3 Sample Selection

There are three basic hair types on the human body namely vellus, intermediate and terminal hair. Vellus hair is fine, short and non-pigmented and is found on eyelids and forehead. Intermediate hair is of medium length and diameter and is found on the arms and legs of adults. Terminal hair is coarse, pigmented and has a large diameter and is found on the scalp, beard, armpit and pubic region. The different hair types form due to differences in the hair follicles. Ambosexual hair follicles are influenced by hormones and change during puberty e.g. pubic and armpit regions.

For the purpose of hair analysis, head hair from the posterior vertex region of the scalp is normally chosen as it does not grow at such a variable rate compared to hair in other body regions and is less affected by differences in age and sex. In addition, there is a greater and more constant percentage of hair in the anagen growth phase in this region. Beard, axillary and pubic hair have also been used for drug analysis[59,60] but does not offer any significant advantages over head hair testing as hair from these regions are also exposed to sources of contamination. The drug concentrations in these hair types vary for an individual depending on the location of the hair sample. This has been attributed to a number of factors such as the presence of apocrine glands, blood supply, telogen/anagen ratios and hair growth rate in different body regions.[29]

The location of the hair sample is an important consideration as the biology of hair from different body regions and sources of contamination vary, influencing the interpretation of results.

2.8.3.1 Scalp Hair

Scalp hair is the easiest, most accessible sample to obtain but as there are hairs in various growth phases on the head, the sampling area is crucial. The growth rate is highest in scalp hair therefore it displays the greatest variability. The ducts of the sebaceous gland empty directly into the hair follicle and so the hair shaft gets exposed to sebum before it grows out from the skin. Scalp hair can also be contaminated by sweat gland secretions, cosmetic treatments and contaminants from the air, water or dust.

2.8.3.2 Beard Hair

Beard hair is thick; the follicles are relatively large and grow at the slowest rate. Beard follicles are different from other follicles as the ducts of the sebaceous glands do not discharge into the follicle but onto the surface of the skin. Consequently, beard hair may be less contaminated by sebum than scalp hair and hence may be the sample of choice a few days after drug use. Beard hair can however become contaminated with pieces of epidermis through shaving and by environmental exposure.

2.8.3.3 Pubic Hair

Analysis of pubic hair may be necessary when there is no scalp hair available. It may be less contaminated from environmental exposure or cosmetic treatment but it may however be contaminated with urine and sebaceous, apocrine and eccrine gland secretions and is not a convenient sample to collect.[12,15] Segmental analysis would not be appropriate for the analysis of pubic hair as interpretation of results would not be possible due to variable growth rates.

2.9 Drug Stability in Hair

The stability of drugs in hair depends on the morphological and physicochemical properties of the hair. The concept of diffusion bridges in hair fibres was investigated as a potential means of drug molecules entering into and out of the hair fibre.[61] Aqueous solutions of codeine, dihydrocodeine and morphine were shown to diffuse. A hypothesis was formed that organic substances may be

linked to the non-keratinous (morphological) hair regions and that these regions provide diffusion bridges for small molecules when water is present. These diffusion bridges could be a route for external contamination to enter the hair or loss of endogenous drug from the hair. This phenomenon was further supported in another study which used fluorescence microscopy to investigate the diffusion of Rhodamine B dye by staining the hair fibres.[32]

Various studies on drug stability in hair have investigated the effect of cosmetic treatments, such as dyeing, bleaching and permanent waving.[49,51,62] The results are in agreement that drug concentrations decrease in hair that has been exposed to these treatments. Repeated shampooing does not have a significant effect on the drug concentration of hair.[63]

Opiate concentrations in hair were found to decrease dramatically after bleaching, permanent waving and UV exposure.[62] UV damages hair as free radicals are formed when UV light is absorbed by amino acids such as cysteine, phenylalanine, tyrosine and tryptophan and this causes disulfide bonds within the hair to break.[14]

Bleaching and exposure to water or soil for six months, as well as exposure to tap water for 4 weeks, was found to result in formerly positive hair samples producing negative results.[64]

2.10 Decontamination

As hair grows out of the epidermis, it is exposed to the environment, including any potential drug contamination which may be present in the surroundings. Sweat and other body secretions could also be regarded as contamination although it is not from an external source. The “bathing” of a mature hair in sweat could result in the broadening of the drug band previously incorporated through the bloodstream. Decontamination or “washing” of hair samples is therefore used to allow an accurate interpretation of results, by removing any loosely bound external drug contamination as well as gels, mousses, oils and everyday dirt which may be present on the hair. This step can help to make the hair extract cleaner, reducing potential interferents which may be present. In addition to this, it can minimise the number of false positive results.

All decontamination procedures are based on the assumption that exogenous drugs deposited in hair from the environment are loosely bound to the surface of the hair. It is a necessary step in the hair testing procedure since the amount of drug present in hair through ingestion is low compared with that in the environment. Several authors have however reached the conclusion that no washing procedure would be 100 % effective in removing external contamination in any contamination situation.[13,63,64] There are still many more decontamination experiments which need to be performed in order to determine the exact set of conditions in which a procedure succeeds or fails. An attempt should be made in these studies to mimic real-life contamination scenarios but the design of these will be based on preconceptions.

There is currently no standard decontamination procedure for hair. It is difficult to standardise a decontamination procedure since it is not known if all of the external contamination has been removed or if drug incorporated into the hair shaft through ingestion is being removed. Several authors have shown that some decontamination procedures remove drug present in the hair shaft through systemic exposure.[65,66] In addition, passive drug contamination has been found within the hair and not just on the surface.[67] Furthermore it has been suggested that the extent of hair damage may affect the quantity of drug which is lost from the hair shaft. Highly damaged hair is more porous and hence is more likely to lose a greater quantity of drug present in the shaft through systemic exposure.[65]

In general the less polar solvents remove less externally bound drug as the solvent does not swell the hair as much as more polar solvents.[68] Among the agents used for decontaminating hair are aqueous media such as phosphate buffer[64] and detergents[69] as well as organic solvents such as, ethanol[70], acetone[71], dichloromethane[72] and methanol.[73] Decontamination with organic solvents is currently preferred over washing with aqueous media as it does not result in hair swelling however it may not remove all externally incorporated drugs.[13] Dichloromethane has been shown to only remove the loosely adhering cocaine in hair.[74] Furthermore it was shown to be as equally effective as phosphate buffer in removing loosely bound cocaine, which in contrast was found to remove some of the more tightly bound drug.[72] A washing procedure should be a compromise

where sufficient external contamination is removed but incorporated drugs are not.

The identification of unique drug metabolites could be used to distinguish passive contamination and actual drug ingestion. Careful consideration must be given to the interpretation of metabolite data however as cocaine and heroin metabolites can be produced during the parent drug degradation.

Benzoylecgonine has been detected in hair contaminated once with cocaine. Hydrolysis of cocaine to form benzoylecgonine can occur in the presence of sweat or during personal hygiene practices and also due to the presence of enzymes in matrix cells.[77] Another study found that heroin contaminants can enter into the hair matrix and become transformed into significant concentrations of 6-monoacetylmorphine (6-MAM), morphine and codeine metabolites.[78] In these hydrolysis situations, cut-off values and metabolite to drug ratios are of limited use for evaluating passive contamination. It is ideal to detect unique drug metabolites which are produced by the body only as a result of pharmacological processing of the drugs as these could only be present as a result of actual drug use. A negative hair testing result can be used as a means of ruling out either drug use or contact with drugs however a positive hair testing result cannot be used as a definitive sign of drug use and should be confirmed with blood and/or urine results.

2.11 Sample Preparation

Sample preparation is one of the most important steps in hair analysis yet this varies between laboratories, with some selecting to cut the hair while others select to grind it into powder form.

Powdered hair is thought to be more homogeneous and suitable for comparative studies.[75] Hair that was ground using a traditional ball mill at room temperature produced better qualitative and quantitative results for drugs of abuse compared to cut hair due to the greater surface area in contact with the extraction medium.[79] Another study which used a cryogenic grinding device confirmed these findings when ground hair produced better quantitative results. However cut hair was shown to be the better preparation method for qualitative results

for drugs of abuse.[80] A common finding with the hair grinding method is that there is a significant loss of hair fragments retained in the ball mill. One laboratory noted that the differences between cutting and grinding were not very large and recommended a fast cutting procedure with a razor, scalpel or scissors.[13]

2.12 Extraction Methods

Several different extraction procedures have been reported for the extraction of drugs and their metabolites from the hair matrix. Some of these include acidic hydrolysis[81], alkaline hydrolysis[82], enzymatic hydrolysis[73], direct solvent[83] or buffer extraction[84] and other less conventional methods such as supercritical fluid extraction[85] and subcritical fluid extraction.[86]

Following the removal of the drugs and their metabolites from the hair matrix, the extraction procedures used are very similar to those used for blood and urine testing.[87] Solid-phase extraction (SPE) using different kinds of columns[51,73,88,89,82] and liquid-liquid extraction (LLE)[81,90,91] are commonly used but less conventional methods such as solid-phase micro-extraction (SPME)[92,93] and molecularly imprinted solid phase extraction (MISPE)[94,95] have also been reported.

The two main types of extraction involve digestion of the hair matrix and extraction of drug from the undigested hair matrix with subsequent extraction using SPE or LLE. Extraction conditions cited in the literature differ in pH, temperature and duration of extraction. The extraction method chosen for a particular drug depends on its chemical properties, such as pK_a value, as well as its chemical stability. For example, sodium hydroxide is a good solvent for drug extraction mainly because of the destruction of the protein hair matrix however it is not suitable for 6-MAM which is chemically unstable in sodium hydroxide.

Enzymatic and direct solvent extractions using methanol for example, are less harsh than alkaline hydrolysis methods, which can destroy the drug as mentioned above. Extractions using β-glucuronidase and methanol do not completely dissolve the hair matrix and are advantageous in drug screening.[96] Alkaline and acidic hydrolysis extractions are however very efficient as they completely solubilise the hair matrix, producing better drug recoveries than direct

extraction with organic solvents. Therefore if a drug is chemically stable, hydrolysis extraction methods are preferred.

A potential problem associated with hydrolysis extraction is that drug metabolites may be formed, which could result in false results. An example of this is 6-MAM, which can degrade during acidic or alkaline hydrolysis to form morphine. The choice of extraction is also influenced by the analytical method to be used in the subsequent analysis.

2.13 Analytical Methods

Radioimunoassay (RIA) was predominately used for hair testing for drugs of abuse before the introduction of mass selective detectors in GC-MS systems.[87] Opiates were first detected using RIA in the hair of heroin abusers in 1979 and it initiated many investigations into drugs of abuse in hair. Since then, various methods including other immunoassays such as enzyme linked immunosorbent assay (ELISA) or chromatographic techniques have been reported. Immunoassays are useful as screening tests due to their sensitivity, speed and simple use. However immunoassays are usually semi-quantitative since the kits are specific to a group of target drugs or drug metabolites rather than a single target drug. Identification and quantification is carried out using chromatographic techniques which have been coupled with MS for excellent sensitivity and selectivity.

Gas chromatography (GC) coupled with mass spectrometry (MS) is the most commonly used chromatographic method and has been widely applied in the detection of drugs of abuse in hair.[19,21,35,37,38,46,51,50] Ion spray liquid chromatography-mass spectrometry (LC-MS) is also used now and is becoming more popular due to its high sensitivity and shorter analysis time as unlike GC-MS, there is no derivatisation step required.[97] Capillary zone electrophoresis has also been applied in quantitative hair analysis.[98,99]

2.14 Current International Hair Testing Guidelines

The role of hair testing in toxicological applications has been widely discussed among various scientific bodies such as the Society of Hair Testing (SoHT),

Society of Forensic Toxicologists (SOFT) and the Substance Abuse and Mental Health Services Administration (SAMHSA).

In 2004, international representatives of the SoHT published a set of recommendations for laboratories carrying out hair testing in forensic cases.[100] The areas agreed upon include; sampling, shipping and storage; decontamination; hair disintegration and extraction; screening test; criteria for mass spectrometric analysis; analysis of specific drug classes such as opiates, cocaine, amphetamines and cannabinoids; cut-off values; and internal and external quality control protocols. Higher cut-off values than those recommended by the SoHT have been proposed by SAMHSA for amphetamines and marijuana after consulting mainly with commercial hair testing companies.[101]

3 Immunoassay

3.1 Introduction

The application of immunoassays as "initial screening tests" for drugs of abuse in urine specimens is well established and routinely practiced. Immunoassays have also been adapted as "initial screening tests" for detecting the presence of drugs and drug metabolites in a variety of other biological matrices.

Immunoassays are generally defined as analytical systems that depend on specific antigen-antibody reactions for detecting target analyte(s) in different sample matrices. In drugs of abuse screening, immunoassay serves as a useful means of eliminating negative specimens from further confirmatory tests and also identifying any drug class that requires confirmation by a second step test.

International guidelines for substance abuse management, including scientific protocols for initial drug screening and confirmatory analysis, have been developed by a number of government agencies, professional organisations and forensic or clinical societies. Immunoassay systems have mainly been designed to meet the guidelines stipulated by the Substance Abuse and Mental Health Services Administration (SAMHSA). SAMHSA has provided cut-off values and quality control requirements for initial drug screening in workplace testing which were published in the Federal Register in 2004.[98] A sample containing drugs below the cut-off is reported as negative for screening. However, if a sample screens in the initial test as positive, a confirmatory test that exploits a different chemical principle to immunoassay is used to specifically identify and quantify the presence of a drug and/or metabolite. The joint *Forensic Toxicology Laboratory Guidelines* provided by the Society of Forensic Toxicologists and American Academy of Forensic Sciences (SOFT/AAFS) specifies a minimal requirement for laboratories to perform immunoassay.[11] A second immunoassay can be used to support the initial screening result prior to confirmation but cannot be used to confirm the results of another immunoassay result.

3.2 General Overview

In the last 35 years, analytical methods that rely on specific analyte recognition by high affinity binding proteins have been applied in many diverse areas of biochemical analysis to the extent that they are now regarded as representing a standard analytical principle. Although there are various kinds of naturally occurring high affinity specific binding proteins such as hormone receptors, it is the unique properties of antibodies including their high affinity, specificity and range of antibody-antigen binding reactions that have resulted in their widespread use in protein binding assays in routine analyses and also in research.

Antibodies have some advantages compared with other binding proteins. They are stable, soluble and abundant. There are standard procedures for antibody preparation, selection, isolation, coupling and immobilisation. They are suitable for the detection of antigens and non-antigenic small molecules (molecular weight > 150 amu). They are available as a polyclonal mixture (with related binding specificities for a variety of analytes) or as a monoclonal mixture (with a single binding specificity for a particular analyte). They can be modified and systematically improved with recombinant DNA methods.[102]

There are many different immunoassay systems however all commonly rely on the specific molecular recognition of antigen-antibody binding interactions to detect minute drug quantities in complex biological matrices. The antigen-antibody binding pair has been described as "a form of ligand and receptor binding pair".[103] Most immunoassays use a label attached to either the antigen (ligand) or the antibody (receptor) which is fundamental to the measurement of the binding interactions between the pair. Immunoassays are named according to the label used in the particular system. A label is any substance which can be chemically attached to either an antigen or antibody to enable a measurable property necessary for the specific immunoassay. Some examples of the types of label chosen for adequate sensitivity include radioisotopes, fluorescence and enzymes.

Immunoassays for small molecules such as drugs of abuse operate as a competitive immunoassay. Free drug in the sample competes with drug-derivative reagent for the binding sites on a specified amount of antibody.

Drugs of abuse molecules are known as 'haptens' since they are not naturally immunogenic and have to be coupled to an antigenic carrier molecule to induce antibody formation. Central to the development of a successful competitive immunoassay system is the design and synthesis of two suitable haptens, namely one for "immunogen" and the other for drug derivative.[103] The immunogen is designed to produce a selective antibody with the required reactivity characteristics when introduced into a suitable host animal. The "activated drug derivative" possesses a suitable molecular linker to allow coupling to a carrier or labelling molecule. The drug derivative has a similar chemical structure and immunogenic properties to the analyte(s) of interest and this enables successful competition between the free drug in the sample and the drug derivative reagent for antibody binding.

There are two types of competitive immunoassay, heterogeneous and homogeneous, depending if there are additional steps in the process to separate the "antibody bound" complexes from the "free" drug in the sample and/or "free" drug derivative in the reaction mixture. Heterogeneous immunoassays such as enzyme linked immunosorbent assay (ELISA) use washing for example to remove the unbound labels, leading to an increase in the signal to noise ratio as the background noise from the labels has been removed as well as potential sample matrix effects. Heterogeneous assays can therefore achieve lower sensitivity levels compared with homogeneous assays.

3.3 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA has become the most widely used immunoassay technology since its development in 1971-1972.[103] An array of commercially available ELISA kits has been developed specifically for the purposes of drug testing in the forensic toxicology field. These have been applied in the detection of drugs of abuse in a variety of biological matrices including blood[104,105], urine[106,107,108,109], serum, hair[84,108,109,110,111,112,113], oral fluid[108,109,114,115], meconium, sweat and vitreous humor.[103,116]

ELISA is a solid phase, heterogeneous, competitive immunoassay system used for the highly sensitive detection of small drug molecules. The amount of bound

antigen or antibody is measured by a signal change produced by enzymatic catalysis.

The ELISA tests are performed using microtitre plates coated with antibodies with a high affinity to the target drug. ELISA kits are supplied as microtitre plates (normally supplied as 12 x 8 well strips), enzyme labelled drug derivative, substrate and stop reagent (Figure 3-1).

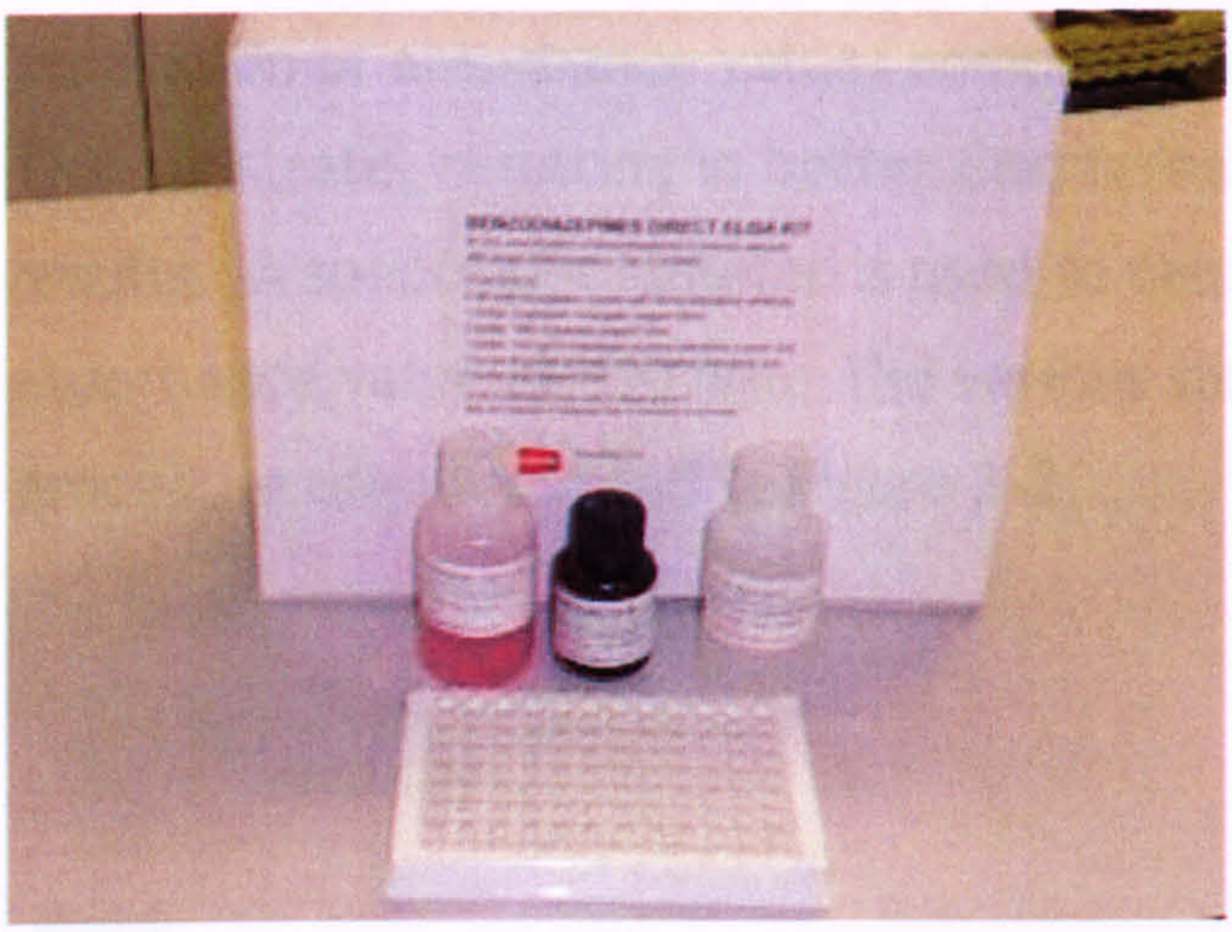


Figure 3-1 Typical contents of an ELISA kit as supplied by manufacturer

A suitable aliquot of sample, standard or control is added to the corresponding well, usually in duplicate, followed by the enzyme conjugate. The reaction mixture is then left to incubate for typically 45-60 minutes to allow adequate time for drug binding, enhancing assay sensitivity. During the incubation period, the enzyme conjugate competes with the drug in the sample for the antibody binding sites on the wells. The most commonly used enzyme for drug ELISA is horseradish peroxidase (HRP). Azide buffer preservatives should be avoided as they affect ELISA performance by inhibiting the HRP enzyme.

The wells are washed with deionised water or phosphate buffer in order to remove any unbound drug that may be present in the sample or any residual enzyme conjugate remaining in the wells. Blood samples with high haemoglobin levels can react with 3,3',5,5'-tetramethylbenzidine (TMB) substrate used for the final colour development, resulting in high background noise. This washing step removes any background noise, effectively improving the assay sensitivity.

TMB, a chromogenic substrate containing hydrogen peroxide, is then added to the wells and develops a blue colour with any bound enzyme conjugate. The colour development is a result of the enzyme label catalysing the oxidation of TMB in the presence of hydrogen peroxide. The darker the blue colour, the greater the amount of bound enzyme conjugate present and the lesser the amount of bound drug. The addition of dilute acid, such as hydrochloric acid, ends the catalytic reaction and hence the blue colour development. The well contents are turned yellow which allows greater sensitivity and a better separation of absorbance values compared with the blue colour produced by the TMB substrate, resulting in better discrimination between positive and negative results. A spectrophotometer is used to calculate the result by measuring the absorbance value at 450 nm. The various steps forming part of the ELISA procedure are illustrated in Figure 3-2.

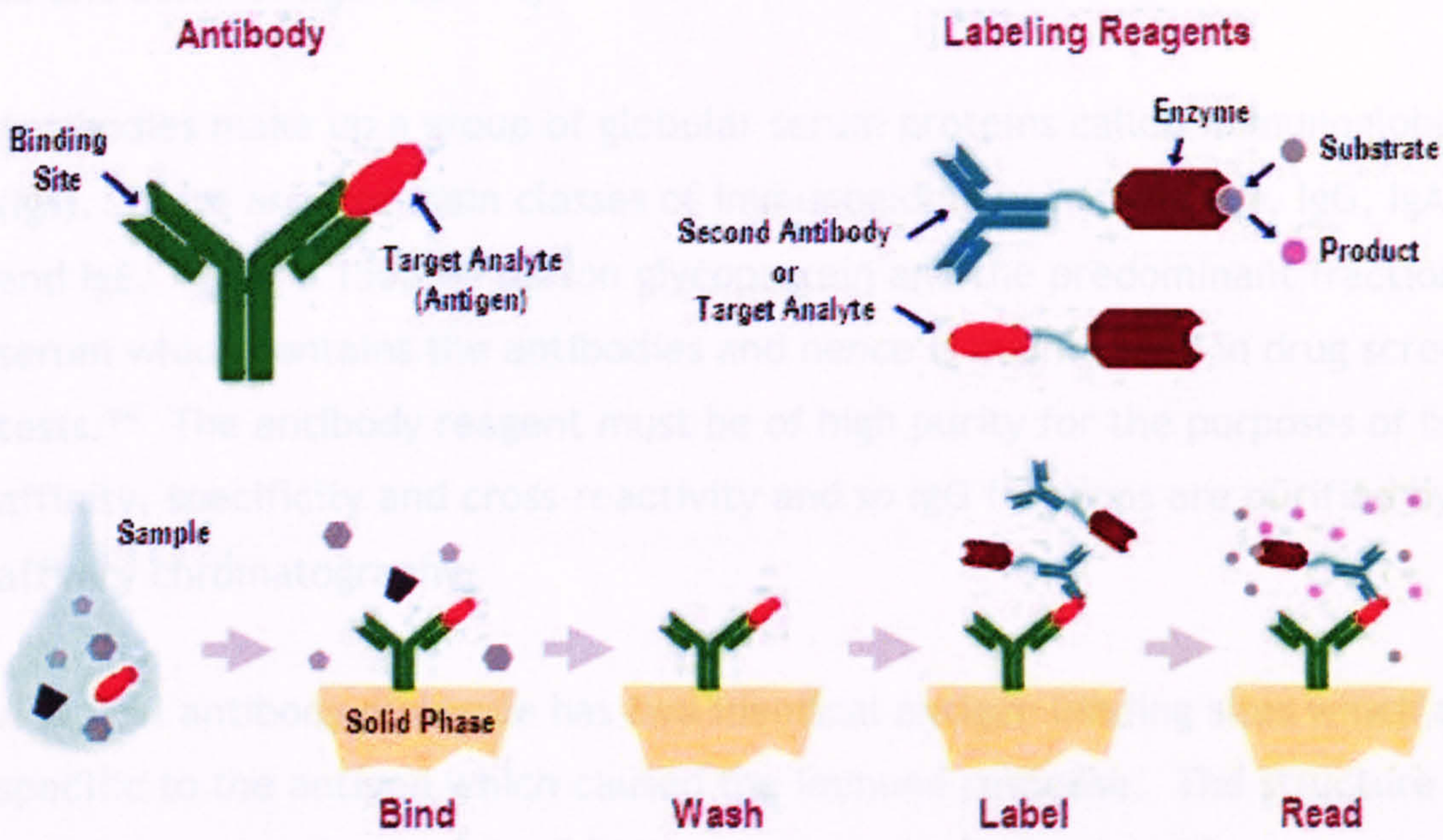


Figure 3-2 General schematic of ELISA procedure[117]

The absorbance value is inversely proportional to the concentration of drug in the sample. A sample producing an absorbance value less than that produced by the relevant cut-off standard is considered a presumptive positive whereas a sample producing an absorbance value greater than or equal to the cut-off standard is considered negative. The result is regarded as "qualitative" or "semi-quantitative" and the quantity of drug and/or metabolite in any sample

cannot be determined by immunoassay. The assay is "semi-quantitative" since the result represents the total contribution of all the compounds in the sample that can compete for antibody binding sites and is normally not a definitive measurement resulting from binding of the target analyte.

3.4 Antibodies

3.4.1 Action and Structure

In immunoassay systems, the antibodies are the active components derived from animal blood. Antigens are the specific part of the immunogen that are recognised by and react with antibodies. Antibodies are proteins which are produced by the B-lymphocytes of the immune system. The body manufactures antibodies in an attempt to destroy the antigen molecule. The antibodies bind to and block antigen activity.

Antibodies make up a group of globular serum proteins called immunoglobulins (Igs). There are five main classes of immunoglobulins namely IgM, IgG, IgA, IgD and IgE. IgG is a 150,000 Dalton glycoprotein and the predominant fraction in serum which contains the antibodies and hence is mainly used in drug screening tests.[102] The antibody reagent must be of high purity for the purposes of binding affinity, specificity and cross-reactivity and so IgG fractions are purified by affinity chromatography.

A typical antibody molecule has two identical antigen binding sites which are specific to the antigen which caused the immune response. The structure of an antibody molecule consists of four protein chains, two of which are heavy (H) identical chains and two of which are light (L) identical chains. The H and L chains in IgG for example are 420 and 215 residues respectively.[102] They are held together by disulfide bridges to form a Y-shape. Each antibody molecule can bind 2 molecules at one time, on each 'arm' of the Y-shape. There are variable (V) regions of the H and L chains positioned at the tips of the Y 'arms'. The amino acid sequence in these regions varies significantly between antibodies. An H chain variable region and an L chain variable region together form a unique binding site, which accounts for the high antibody specificity. The "tail" of the Y-shaped antibody molecule is formed by the constant (C) regions of the H

chains which are responsible for distribution in the body and for the mechanisms involved in antigen disposal, show very little variation in amino acid sequence between antibodies.[119] Immunoglobulin molecules demonstrate flexibility, particularly at the "hinge regions" of the H chains as this aids their binding to adjacent specific antigens and also allows the aggregation of soluble antigens.[102] A typical two-dimensional structure of an antibody is illustrated in Figure 3.3. The H chains are represented in blue while the L chains are in red.

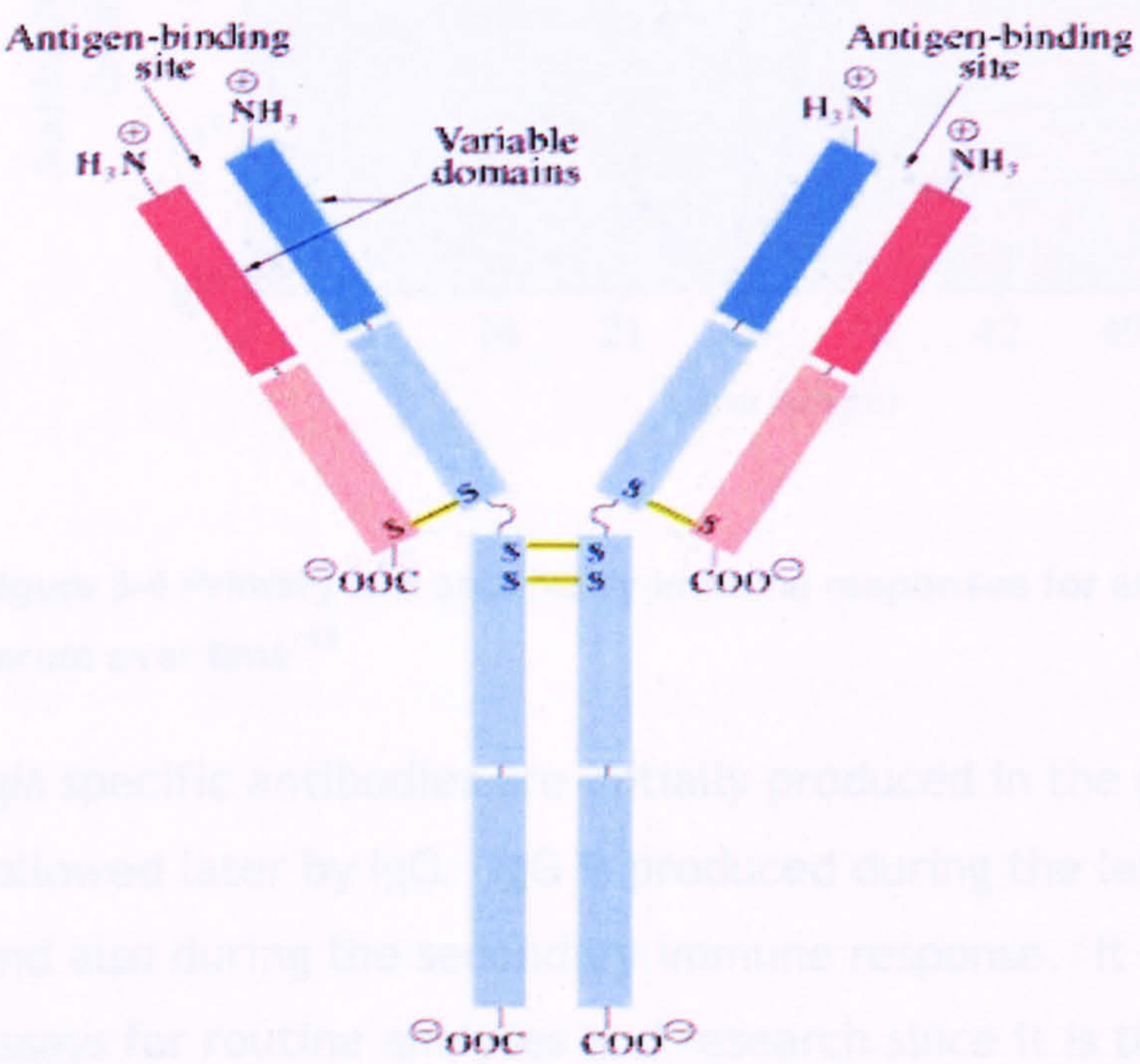


Figure 3-3 Typical two-dimensional antibody structure[118]

3.4.2 Immune Response

Antibody production is stimulated by introducing an antigen into a host animal such as a rabbit. The primary immune response is the selective proliferation and differentiation of B and T lymphocyte cells that occurs the first time the body is exposed to a given antigen. During this phase, there is a time lag of about 7 days before antibody-secreting cells are produced. It takes about 10-17 days following the initial exposure to antigen to generate the maximum response to antibody production, which is relatively small compared to the secondary response.[119] The secondary immune response is specific and occurs when the body has had at least one prior exposure to the same antigen. A faster, larger and more prolonged response is produced due to the presence of long-lived memory cells. Figure 3-4 shows the differences between the primary and

secondary immune responses for antibody concentrations in blood serum over time.

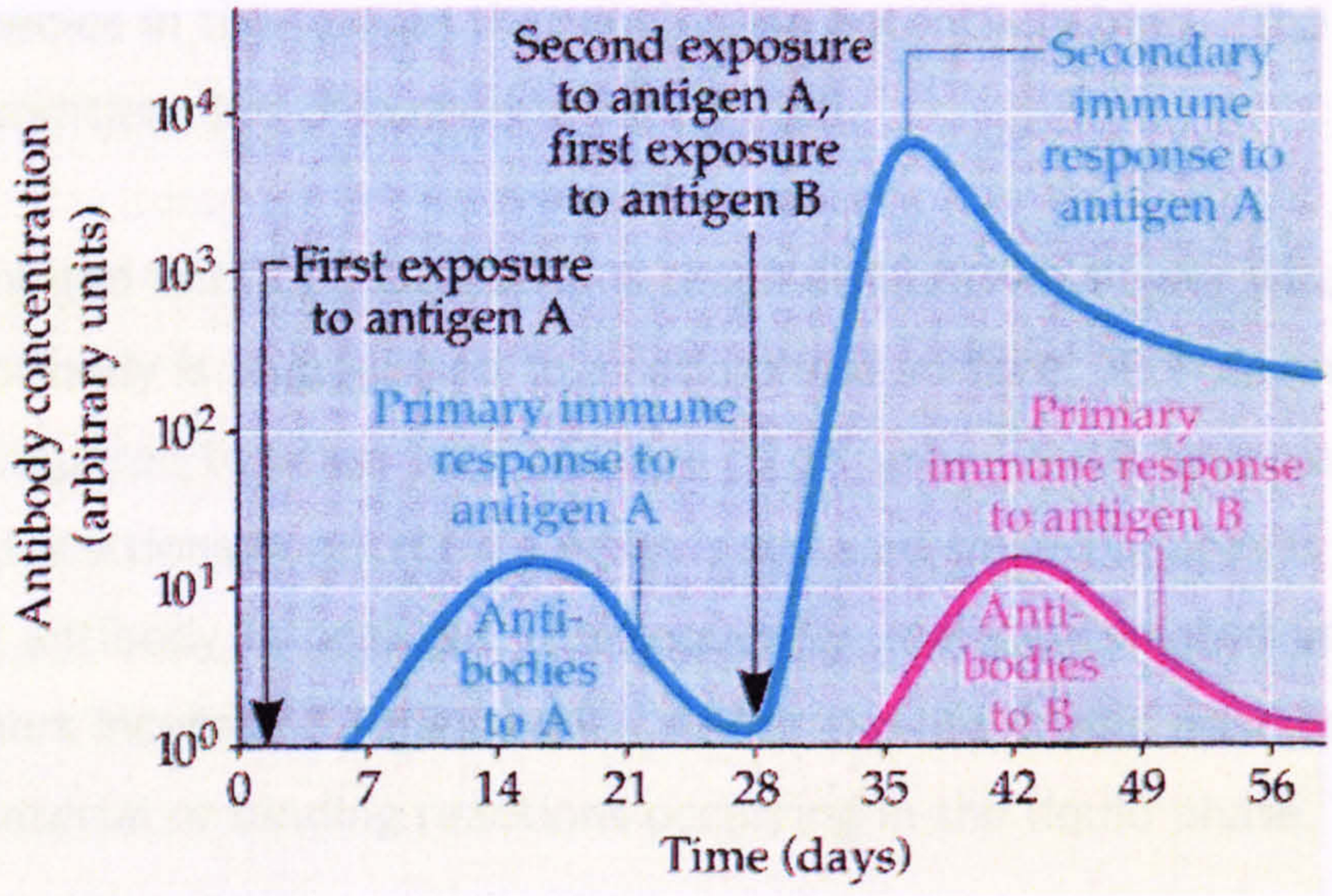


Figure 3-4 Primary and secondary immune responses for antibody concentrations in blood serum over time[119]

IgM specific antibodies are initially produced in the primary immune response followed later by IgG. IgG is produced during the late primary immune response and also during the secondary immune response. It is used in protein binding assays for routine analyses and research since it is the predominant fraction in serum and also because IgG antibodies bind to antigen with a higher affinity than IgM antibodies.[120]

3.4.3 Antigen-Antibody Binding

Antigen-antibody binding is a reversible process. In high-affinity binding interactions, the ease of association outweighs the ease of dissociation. During association, repulsive forces between atoms on the antibody-binding site (paratope) and the antigenic determinant region (epitope) of the specific protein antigen are overcome so that subsequently, attractive interactions such as hydrogen bonds, ionic bonds and van der Waals forces can occur.

A collision between the antigen and antibody must take place before an interaction is established. The collision rate depends on the diffusion rate, which, in turn, is affected by molecular shape and mass, reactant mobility and temperature. Hence, haptens generally associate more readily with their

specific antibodies than large antigens. As well as temperature, other physicochemical conditions which influence antigen-antibody binding in an aqueous environment include pH, ionic strength and the presence of other ionic species in the sample that could also potentially bind. The effects of these conditions are discussed in section 3.5.

Antigen-antibody interactions reach equilibrium slower when the antigen or antibody is immobilised to a solid phase surface, such as microtitre wells, compared to when both are free to diffuse. This is because the potential for interactions in microtitre wells is reduced since the area is small and the antigen or antibody is localised. Consequently, many automated immunoassays requiring short incubation times exploit either the use of microparticulate solid phase material or binding reactions occurring in the liquid phase.

Antibodies bind strongly to antigens in a similar way to enzyme-substrate binding, via a "lock and key" mechanism (Figure 3-5). They do not bind via covalent chemical bonds but via electrostatic forces, van der Waals forces, hydrogen bonds and hydrophobic bonds. Binding therefore depends upon short range factors hence the need for a highly specific association between antibody and antigen. Antigen binding can be reversed by high salt concentration, low pH, denaturing agents such as urea, organic solvents, heat and competitive antigens.

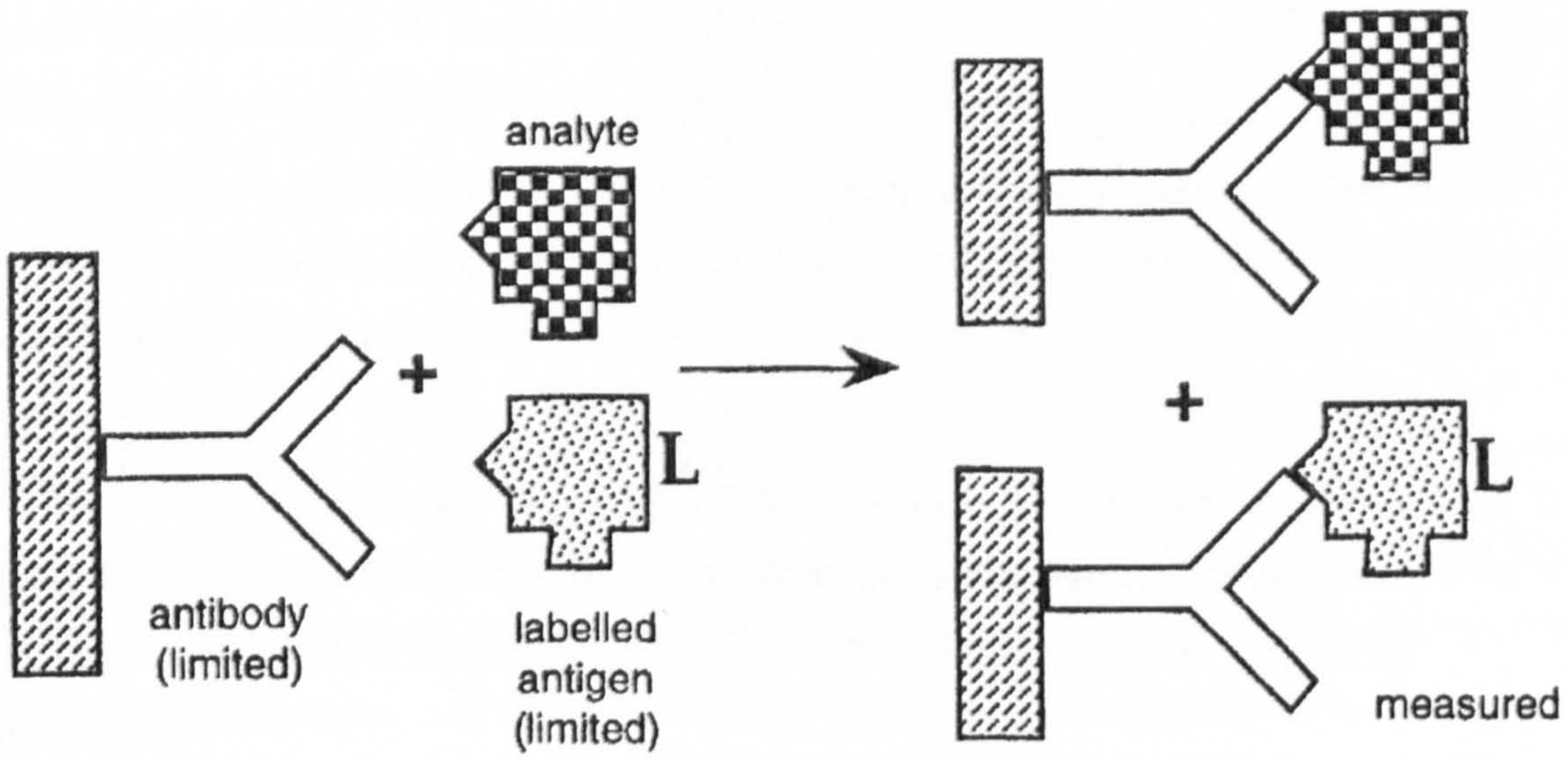


Figure 3-5 Antigen-antibody binding mechanism[102]

Immunoassays can be designed using polyclonal or monoclonal antibodies depending on how the antibodies are manufactured. Polyclonal antibodies bind

less specifically and so are useful for screening for a group of related drugs such as opiates. Monoclonal antibodies on the other hand are highly specific for a particular drug such as morphine.

3.5 Variables Influencing Evaluation of Results

3.5.1 Inter-Individual Variations

Inter-individual variations, diet and medication are some of the contributing variables that can influence the outcome and interpretation of immunoassay results. Pharmacogenetic and physiological variations exist between every individual and these ultimately affect the drug metabolism and excretion profile. Ingestion of certain foods or medication can result in the need for more complex interpretation of drug screening results.[121,122]

3.5.2 Sample Adulteration

There are many readily available adulterants and urine substitutes that are designed to defeat drugs tests. Some of these are produced specifically for "passing" a urine drug test, while others are common household products. These can be easily researched or purchased via the Internet. A recent search using the Internet and psychiatric and medical literature identified a comprehensive list of products, compounds and tampering methods, including data on their efficacy.[123] Methods for tampering with a sample can be associated with three basic categories: in vivo adulteration which is the ingestion of a chemical prior to sample collection such as sample dilution by excessive water consumption; in vitro adulteration which is the addition of a foreign substance to a sample after collection; and urine substitution which occurs when a sample negative for drugs is substituted for a drug positive sample during sampling. SAMHSA (2004) guidelines recommend that a number of validity checks are carried out for urine drug screens, including temperature, pH, creatinine, specific gravity (when creatinine < 20 mg/100 mL) and tests for oxidising adulterants.[101]

In vitro adulteration can pose a problem in immunoassay; particularly in urine testing since it can potentially interfere with the immunoassay detection system

by decreasing absorbance rates[124] or convert a target drug into a compound that is not detected by screening or confirmation methods.[125] The commercially available in vitro products are marketed under a variety of brand names but are essentially limited to the same active ingredients including: glutaraldehyde, sodium or potassium nitrate, pyridinium chlorochromate (PCC) and peroxide/peroxidase. There is conflicting results in terms of the tampering effectiveness of these adulterants in producing false negative immunoassay results. The discrepancy in effectiveness is probably due to differences in the urine drug concentrations and/or the concentration of adulterant.[123] Both AdultaCheck 6 and Intect 7 urine test strips were shown to successfully detect the presence of glutaraldehyde, nitrite and PCC in urine and could be used as a means of checking sample integrity.[126] The presence of some in vitro household products such as sodium chloride, bleach, vinegar, Visine™ eye drops, detergent/soap, ammonia, aspirin, ibuprofen, riboflavin (vitamin B_2) and golden seal tea have been shown to interfere with immunoassay by affecting drug binding.[121,122,123,124,125,127,128]

3.5.3 Cross Reactants

Antibodies developed for a particular drug can cross-react with other structurally related or unrelated drugs or even endogenous compounds present in the sample matrix. Cross reactivity is a measurement of antibody response to substances that are not the target analyte. An understanding of assay cross reactivity and potential interference is crucial in the interpretation of immunoassay results. Some structurally unrelated medications can possess three dimensional conformations that have a weak but sufficient binding affinity for some particular antibodies.[103]

The presence of over the counter sympathomimetic amines, pseudoephedrine and diphenhydramine, in blood samples taken from non-decomposed bodies, produced a large proportion of false positive screening results using an amphetamines ELISA system.[127] It was proposed that ingestion of over the counter medication, pholcodine, was the cause of four false positive hair results for samples that had been screened using an opiates ELISA system.[110] This finding was supported in another study where pholcodine, rifampicin and ofloxacin were found to cross react with an opiates ELISA system.[128] Some

examples of other published cases include the cross reactivity of oxaprozin with a benzodiazepines assay and ranitidine with a methamphetamine assay.[129, 130] Some sample pretreatment procedures have been proposed to reduce the effects of immunoassay cross reactivity such as the use of glucuronidase hydrolysis to enhance the sensitivity of the benzodiazepines assay.[131]

As well as the potential presence of drugs and/or adulterants in a sample, there may be cross reactive endogenous sample components present such as putrefactive amines. Phenethylamine could be present in decomposed blood and it has been reported to contribute to a large proportion of false positive results due to its cross-reactivity with an amphetamines ELISA system.[127] Phenethylamine is structurally very similar to amphetamine, differing only by a methyl group (Figure 3-6). Furthermore, the sensitivity of the ELISA screening was shown to increase from 87.2 % to 93.6 % when the known decomposed blood samples were removed from the analysis.[127]

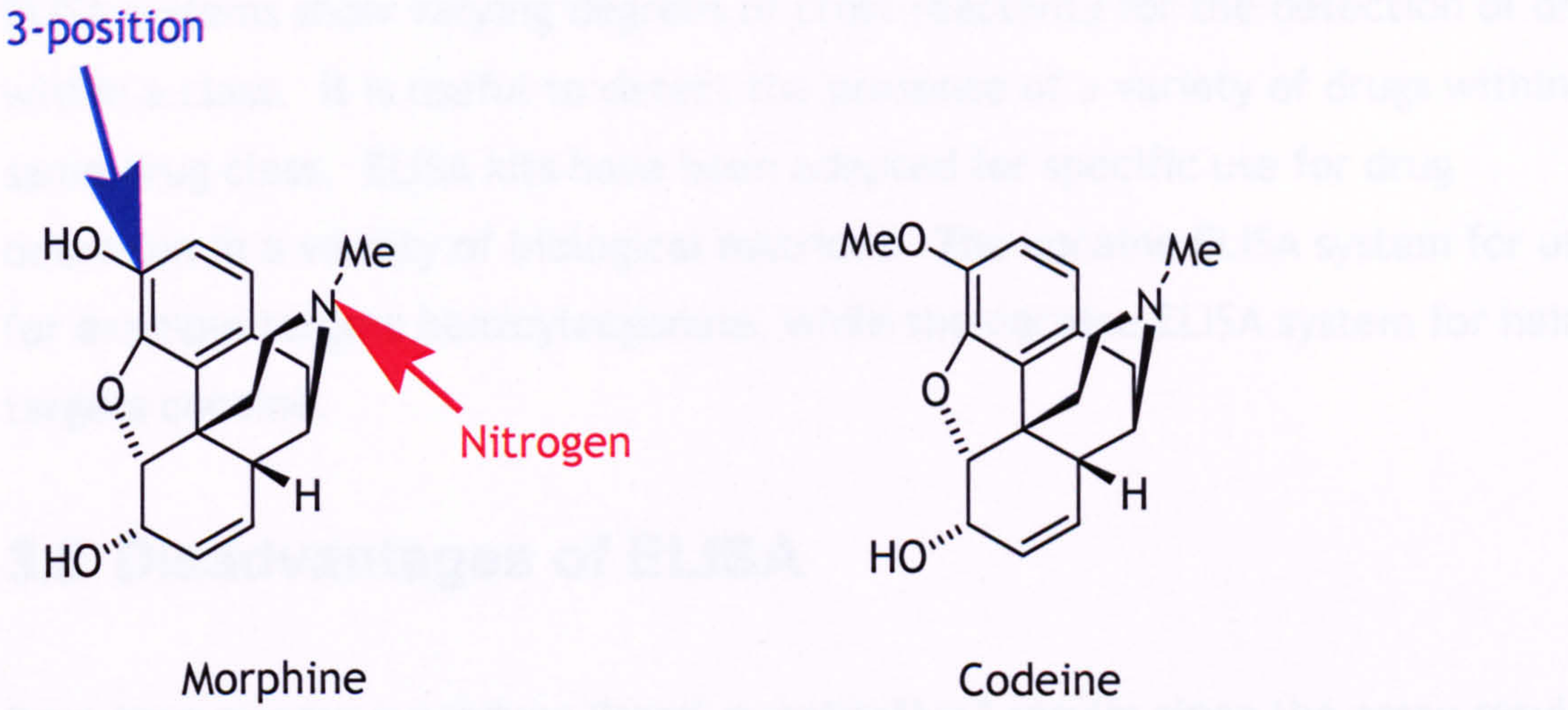
Amphetamine Phenethylamine

Figure 3-6 Molecular structures of amphetamine and phenethylamine

3.6 Coupling

In order to be immunogenic, molecules have to have a molecular weight > 5000 amu. Drugs of abuse molecules are known as 'haptens' since they have relatively low molecular weights of 100-500 amu and are not naturally immunogenic. Haptens are transformed to immunogens by coupling to a macromolecule such as bovine serum albumin (BSA), for example 20-30 hapten molecules per BSA molecule.[102,132,133] Coupling produces an immunogen capable of manufacturing antibodies specific to free hapten which binds with high affinity. When a particular hapten complexes with an antibody-binding site, or paratope, it is generally located in a cavity which possesses functional groups of the peptide residues and backbone that are complementary in structure to the binding regions of the hapten molecule. High affinity paratopes often produce complexes in which the hapten is buried by 80-90 % or more.[102]

The site of coupling is a crucial consideration as it determines the antibody specificity. The coupling site affects the cross-reactivity of the antibody and can be altered to increase or decrease the cross-reactivity as required. An example is the coupling site on morphine. When the morphine molecule is coupled through the 3-position, the antibody demonstrates a higher cross-reactivity with codeine than morphine. This is because codeine has a greater structural similarity with the immunising group coupled to the 3-position compared to morphine itself. In contrast, if the morphine is coupled through the nitrogen, the cross reactivity of morphine is higher than codeine as morphine has a greater structural similarity to the molecule coupled through the nitrogen compared to codeine. Figure 3.7 shows the molecular structure of morphine and codeine with the two potential coupling sites indicated on morphine.



Morphine Codeine

Figure 3.7 Molecular structures of morphine and codeine with potential coupling sites indicated on morphine

3.7 Advantages of ELISA

Radioimmunoassay (RIA) has been used in the past as a screening technique due to its reliability and its ability to detect low drug levels in a wide range of biological samples whilst remaining relatively unaffected by sample matrix effects. The use of radioactively labelled material does however prevent its performance out with safe areas and consequently a non-radioactive technique such as ELISA is a safer and highly sensitive alternative. ELISA and RIA were evaluated as screening procedures for post-mortem samples for nine major drug classes and ELISA was found to be a suitable alternative to RIA.[134]

From a practical viewpoint, the reagents used in ELISA have a longer shelf life than RIA reagents which are limited due to radioactive decay. ELISA lends itself well to automation and high throughput while RIA does not. In addition, RIA screening takes a longer time than ELISA screening, is more labour intensive and presents a higher risk of carry-over.[135]

ELISA is a fast and sensitive technique which can be used to screen large numbers of unknown samples. There is no need to extract blood and urine samples, which only require dilution with phosphate buffer. Although an extraction is required for hair screening, this takes a relatively short time. Small sample sizes are used in ELISA so matrix effects are minimised as there is less background interference with the developing colour.

ELISA systems show varying degrees of cross-reactivity for the detection of drugs within a class. It is useful to detect the presence of a variety of drugs within the same drug class. ELISA kits have been adapted for specific use for drug detection in a variety of biological matrices. The cocaine ELISA system for urine for example targets benzoylecgonine, while the cocaine ELISA system for hair targets cocaine.

3.8 Disadvantages of ELISA

Drug immunoassays produce "semi-quantitative" results since the assay result represents the summative contribution of all drugs from a particular drug class in a sample that can compete for antibody binding and is generally not representative of a single target analyte.

Absolute specificity, based solely on a binding reaction, is not achievable and this might be interpreted as a limitation of ELISA. Irrespective of the type of binding protein assay, there is a possibility that structurally related or unrelated drugs or even endogenous compounds present in the sample matrix could cross-react with the binding protein in the assay. Due to the increasing availability of many new over the counter and prescription medications, it is essential that the manufacturers of ELISA kits continually update the cross reactivity studies to allow accurate evaluation of immunoassay results.

A major limitation of using immunoassay in forensic toxicology is that antibodies are commercially available for a relatively small number of drugs, mainly for drugs of abuse as there is a large market for these kits. New kits are being manufactured to try to meet requirements however this is a huge task and is continually changing with the availability of new drugs. ELISA assays for new drugs of abuse such as methylphenidate (Ritalin®), fluoxetine (Prozac®), zolpidem (Ambien®), sertraline (Zoloft®) as well as other drugs such as paracetamol have recently been introduced onto the market.[136]

4 Liquid Chromatography-Mass Spectrometry

4.1 Introduction

A combination of liquid chromatography and mass spectrometry (LC-MS) is a valuable analytical tool in various fields. The development of a suitable LC-MS interface that allows the LC mobile phase to pass into the mass spectrometer, which is held under vacuum, is central to the increasing number of published LC-MS applications over the last few decades. LC-MS has established itself as a complementary technique to gas chromatography-mass spectrometry (GC-MS) in the analysis of less volatile compounds and/or high molecular weight compounds.[133] It has been estimated that almost 70 % of routine samples in the toxicology laboratory can be analysed by LC.[133] A clear advantage of LC is that unlike GC, time-consuming derivatisation steps are not generally required. An important practical advantage of LC-MS over GC-MS is that the optimisation of ionisation and MS parameters can be performed by the continuous infusion of a dilute solution of the analyte into the ion source, without the involvement of chromatography.

Since the amount of energy transferred to the molecule during the ionisation process is small, electrospray ionisation (ESI) is known as a soft ionisation technique. As a result, pseudo molecular ions $[M + H]^+$ or $[M - H]^-$ are readily formed, generally with little or no molecular fragmentation resulting in limited structural information. It is however suitable for quantitative analysis and benefits the sensitivity of MS-MS analysis as most of the total ion count (TIC) relates to one precursor ion in MS-MS mode. The formation of other charged species such as clusters with solvents such as acetonitrile ACN $[M + 42]^+$ and methanol $[M + 32]^+$ or adducts with sodium $[M + 23]^+$, ammonium $[M + 18]^+$ or potassium $[M + 39]^+$ have been described for some compounds under the soft ionisation ESI technique. The use of cluster ions or adducts as precursor ions can lead to poor reproducibility so the protonated or deprotonated pseudo molecular ions should be used where possible.

LC-ESI-MS/MS has been used to analyse various drugs of abuse including opioids[134,135,136,137,138,139,140], benzodiazepines[141,142,143,144,145,146,147], amphetamines[2,137,148,149] and cocaine and its metabolites[134, 135, 137, 139, 150, 151] as in this thesis, as well as

other drugs of abuse such as cannabinoids[139,157,158] and LSD[159,160] in a variety of biological matrices. Some studies have developed ESI methods for screening and quantifying drugs from multiple drug classes.[97,141,143,161,162]

In this thesis, a Thermo Finnigan LCQ™ Deca XP Plus instrument was used for method development and validation. The instrument has three main components namely an LC system, ESI interface and a mass spectrometer detection system; hence these will be discussed in subsequent sections of this chapter.

4.1.1 Liquid Chromatography

LC is a powerful separation technique that can be operated in both normal-phase (NP) and reverse-phase (RP) mode. Both modes of chromatography rely on two phases, namely the stationary phase and mobile phase. Generally, the separation is achieved by differential migration of analytes through a microparticulate solid stationary phase. A liquid mobile phase consisting of a single solvent or buffer/solvent mixture is degassed, filtered and blended (if a mixture is required) before flowing through the stationary phase to elute the analytes. All analytes differ in polarity and are therefore retained on the stationary phase for different amounts of time. Furthermore, analytes will respond differently depending on the type of stationary phase and mobile phase system used.

In NP chromatography (NPC), the stationary phase is of higher polarity than that of the mobile phase hence is commonly used for the analysis of relatively non-polar analytes. Silica is used as the stationary phase in combination with non-polar solvents such as dichloromethane and hexane. Since these solvents are hazardous to health and the environment, NPC is avoided in most laboratories.[163]

In LC-MS applications however, RP chromatography (RPC) using aqueous mobile phases has been the most widely applied. RPC is suitable for the analysis of polar analytes since the polarity of the mobile phase is higher than that of the stationary phase. There is a range of non-polar silica-bonded stationary phases available, with C_{18} being the most popular. The solvents typically used for the mobile phase, in combination with an aqueous buffer are acetonitrile and methanol, which are less hazardous and easier to handle compared with the

solvents used in NPC. NPC may however be more suited in the analysis of drug enantiomers, for example, which require chiral stationary phases.

4.2 Liquid Chromatography-Mass Spectrometry

4.2.1 Mobile Phase Composition and Flow Rate

The composition of the mobile phase, type and concentration of buffer and organic modifier are extremely significant as they affect the separation and ionisation of analytes.[164] One study demonstrated that formic acid produced a response that was 10 times higher than trifluoroacetic acid using a low buffer strength of 2-3 mmol/L.[165] The separation ability however decreases as the buffer strength decreases. A high organic percentage of the mobile phase favours ion formation as solvent evaporation efficiency is improved and hence the ion yield passing into the mass analyser is increased.[163] Therefore, a compromise between chromatographic separation and ion signal intensity has to be reached.

Usually, the LC-MS mobile phase is comprised of an aqueous buffer and an organic solvent. Buffer selection is important not only to achieve and control the pH of the LC mobile phase but also because of some practical considerations. In LC-MS, non-volatile buffers should be avoided as they will precipitate in the ion source, resulting in clogging and impaired instrument performance i.e. reduction in signal intensity due to competition between analyte ions and electrolyte ions for conversion to gas phase ions. Therefore volatile buffers such as ammonium formate, ammonium acetate, acetic acid and ammonia must be used. Low buffer concentrations should be used to prevent saturation of the surface of the droplet, preventing the analyte from passing into the gaseous phase and ultimately lowering its response. At high buffer concentrations (> 10 mM), the ionisation efficiency is affected and the relationship between detector response and concentration is not linear.[166]

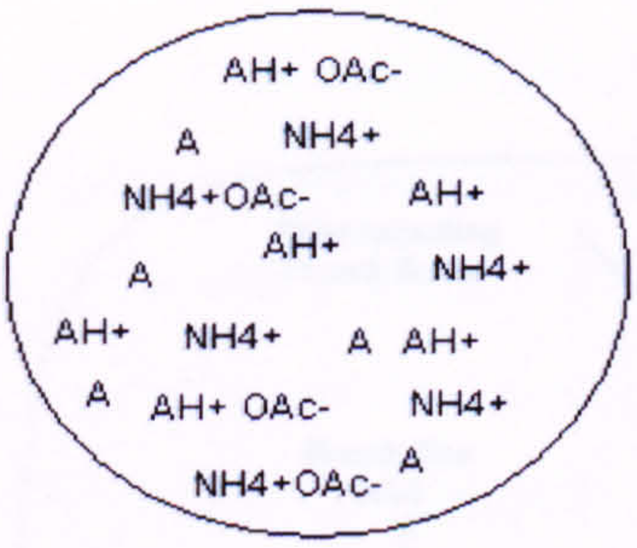


Figure 4-1 Surface competition between analyte and electrolytes

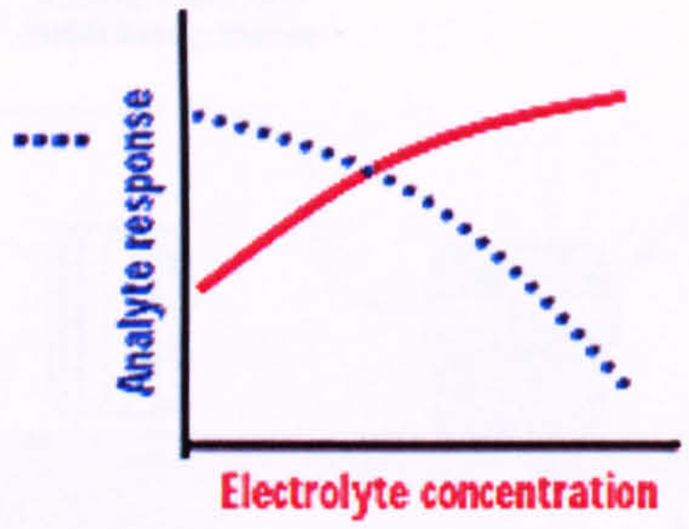


Figure 4-2 Decrease in analyte response with increasing electrolytes[167]

A pre-requisite of ESI is that the analyte exists as an ion in solution therefore the pH of the mobile phase should be carefully selected depending on the pK_a of the analytes of interest and usually contains a small amount of volatile acid or base. A general rule of thumb is 'basic analyte, acidic mobile phase and vice versa'.[163] This is because an acidic mobile phase contains an excess of H^+ ions which would ionise basic analytes by the addition of a proton. Conversely, a basic mobile phase contains an excess of OH^- ions which would cause deprotonation of acidic analytes. The mobile phase carrying the analytes of interest is passed into the atmospheric pressure ionisation (API) source (typically in µL/min) through a fused silica inner capillary which is inserted through the sample inlet and ESI needle (Figure 4-3).

As well as the composition of the mobile phase, the flow rate also affects ionisation. Higher signal to noise (S/N) ratios are achieved at lower flow rates since solvent evaporation is more efficient and ultimately the transmission of gas phase ions into the vacuum region is improved.[163] This explains why a drying gas (nitrogen) and a high probe temperature e.g. 300 °C are used to assist droplet formation and solvent evaporation.

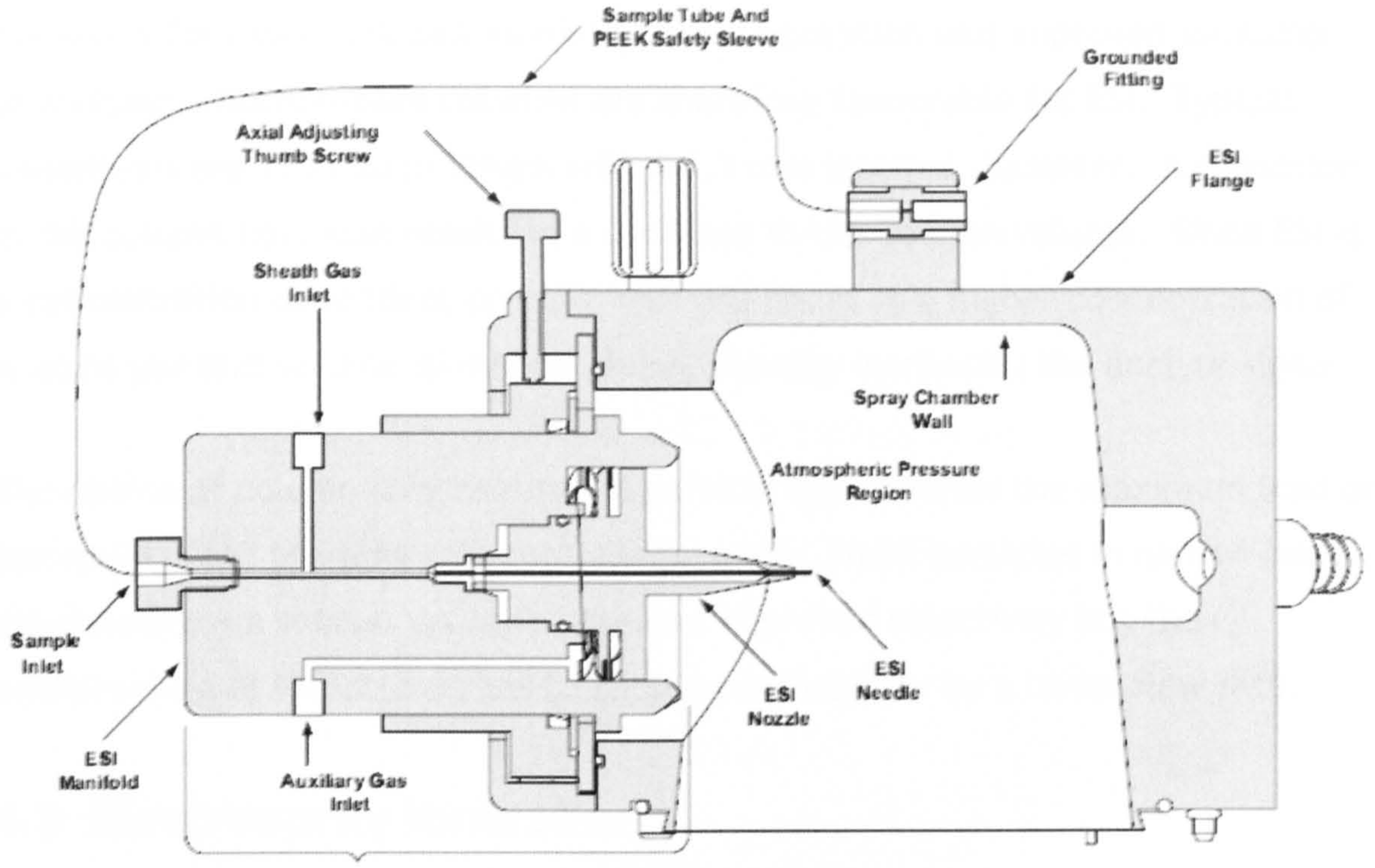


Figure 4-3 Cross-sectional view of ESI probe assembly[168]

4.2.2 Column

There are a wide variety of LC columns available to the analyst but one important consideration in selecting an appropriate column is that a higher organic concentration in the mobile phase improves the ionisation efficiency. If a more retentive column is used, the organic content can be increased in the mobile phase without losing chromatographic retention.[166] Analyte retention on a column depends on the polarity of the analyte and stationary phase. The most widely used columns contain a chemically modified silica stationary phase, the most popular stationary phase being one in which a C_{18} alkyl group is bonded to the silica surface.

The two basic types of stationary phase have been discussed in section 4.1.1, however, the binding sites on the stationary phase can be end-capped to improve chromatographic performance and increase column stability. An example is the use of polar end-capping with bonding of C_{18} alkyl chains, is a successful approach for the reproducible retention of polar analytes such as quaternary ammonium compounds under highly aqueous conditions.[169]

The nature of the ESI interface is such that lower mobile phase flow rates are necessary for more efficient mobile phase evaporation and improved ionisation of analytes. Narrow-bore columns are therefore favourable for ESI. Typical dimensions are 150 mm in length with a 2.1 mm internal diameter. A reduction in the column bore size results in a decrease in the column volume. Since ESI is a concentration dependent process, this will result in a higher concentration of analyte per unit volume of mobile phase, thereby increasing the analyte signal. The choice of column dimensions and particle size dictates the maximum load on the column and the flow rate that can be used. Small particles in narrow-bore columns allow a shorter analysis time and improved selectivity but the backpressure of the column has to be compensated for by a lower flow rate.

4.3 Electrospray Ionisation

ESI and atmospheric pressure chemical ionisation (APCI) are regarded as the golden standards of LC-MS.[170] They are complementary techniques, with most analytes being sufficiently ionised by either or both ion sources.[163] Furthermore, the two ion sources are usually interchangeable within the same instrument. In recent years however the family of API sources has expanded to include atmospheric pressure photo-ionisation (APPI), atmospheric pressure laser ionisation (APLI) and sonic spray ionisation (SSI), which have all contributed to the analytical potential of LC-MS. Other ion sources include thermospray ionisation (TSI), particle beam ionisation (PBI), fast atom bombardment (FAB), desorption electrospray ionisation (DESI) and matrix-assisted laser desorption ionisation (MALDI).

Electrospray ionisation (ESI) was the first developed API source and to date is the most frequently used ionisation mode used in analytical toxicology.[163] A literature review of metabolism studies for drugs of abuse and prescription drugs published in 2000 reported that ESI was used in 73 % of methods, with APCI being used in 23 % of methods.[171] ESI allows the determination of thermolabile, high molecular weight compounds such as proteins[172] and/or highly polar compounds such as quaternary ammonium compounds and herbicides.[169]

The general ESI process involves four stages; droplet formation from the LC mobile phase, charging of droplets, desolvation of droplets and formation of analyte ions (Figure 4-4). A high voltage (typically 3-5 kV) is applied to the ESI needle which is sufficient to nebulise the liquid into highly charged droplets on exiting the needle. Depending on the voltage polarity, nebulised droplets containing the ionised analytes can be positively or negatively charged. For example, if a positive voltage is used then the droplets will be positively charged. These charged droplets are subsequently desolvated before passing through the ion sweep cone and into the analyser. Analyte ions are obtained directly from the liquid mobile phase droplets so thermally labile compounds do not suffer from degradation.

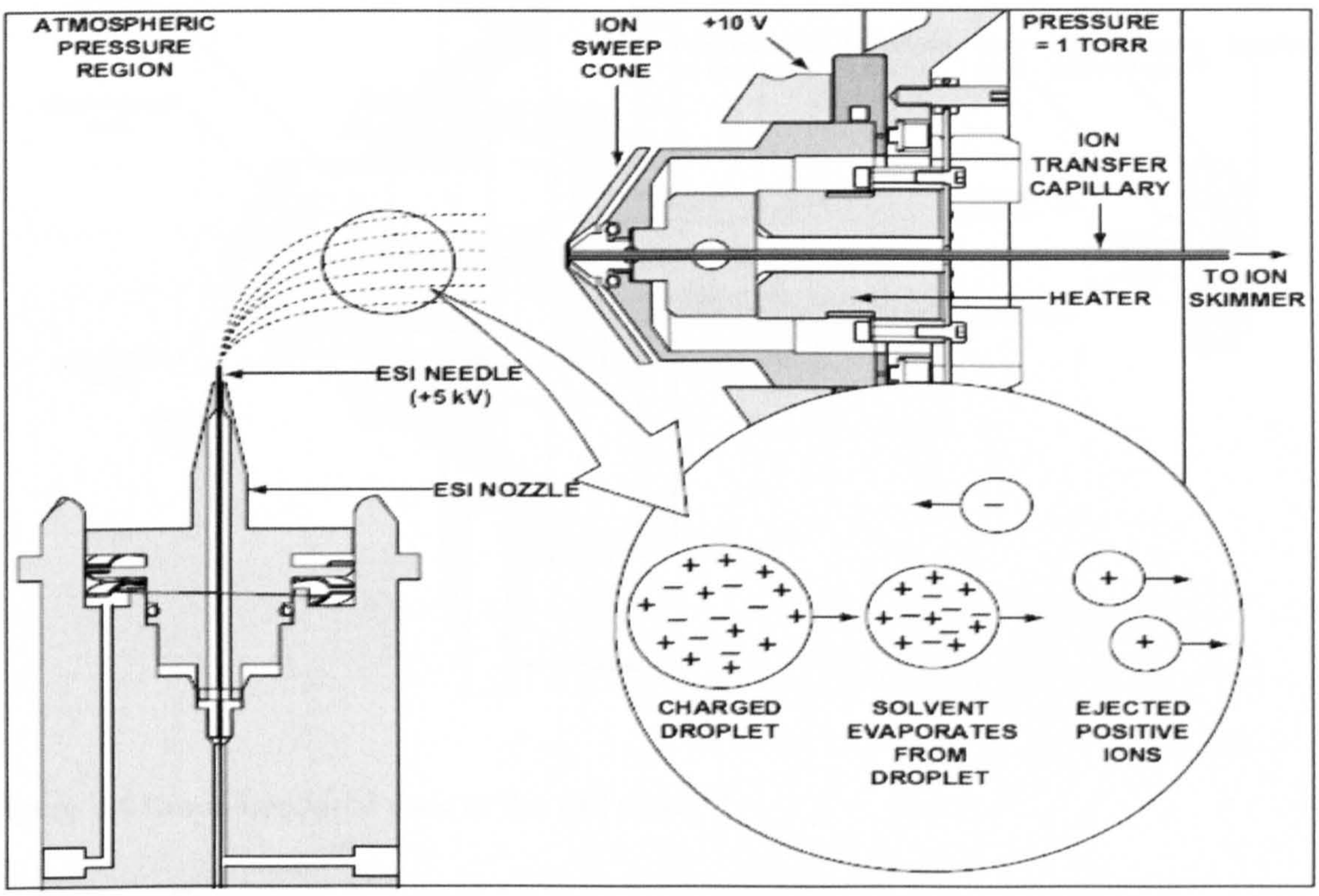


Figure 4-4 ESI process in positive ion mode[168]

As droplet size decreases, the charged species move closer together and are repulsed. When this repulsion on the droplet surface is overcome by the cohesive forces of the surface tension, a coulombic explosion results where micro droplets with radii of ~10 % those of the parent droplets are formed.[166] A series of these explosions occur until gas phase ions are produced from direct emission of the micro droplets then the sample ions enter into the API stack via an ion transfer capillary (Figure 4-5).

The ESI needle is orthogonal to the axis of the ion transfer capillary that carries the ions to the mass analyser. The reason for this is to keep the ion transfer capillary clean. The ion sweep cone is a mechanical barrier that prevents large droplets and particulates from entering the ion transfer capillary.

Ions from the ion transfer capillary pass through the tube lens which has a voltage applied to it to focus the ions towards the opening of the skimmer. From the skimmer, ions move towards the ion optics assembly which focuses ions from the API source to the mass analyser.

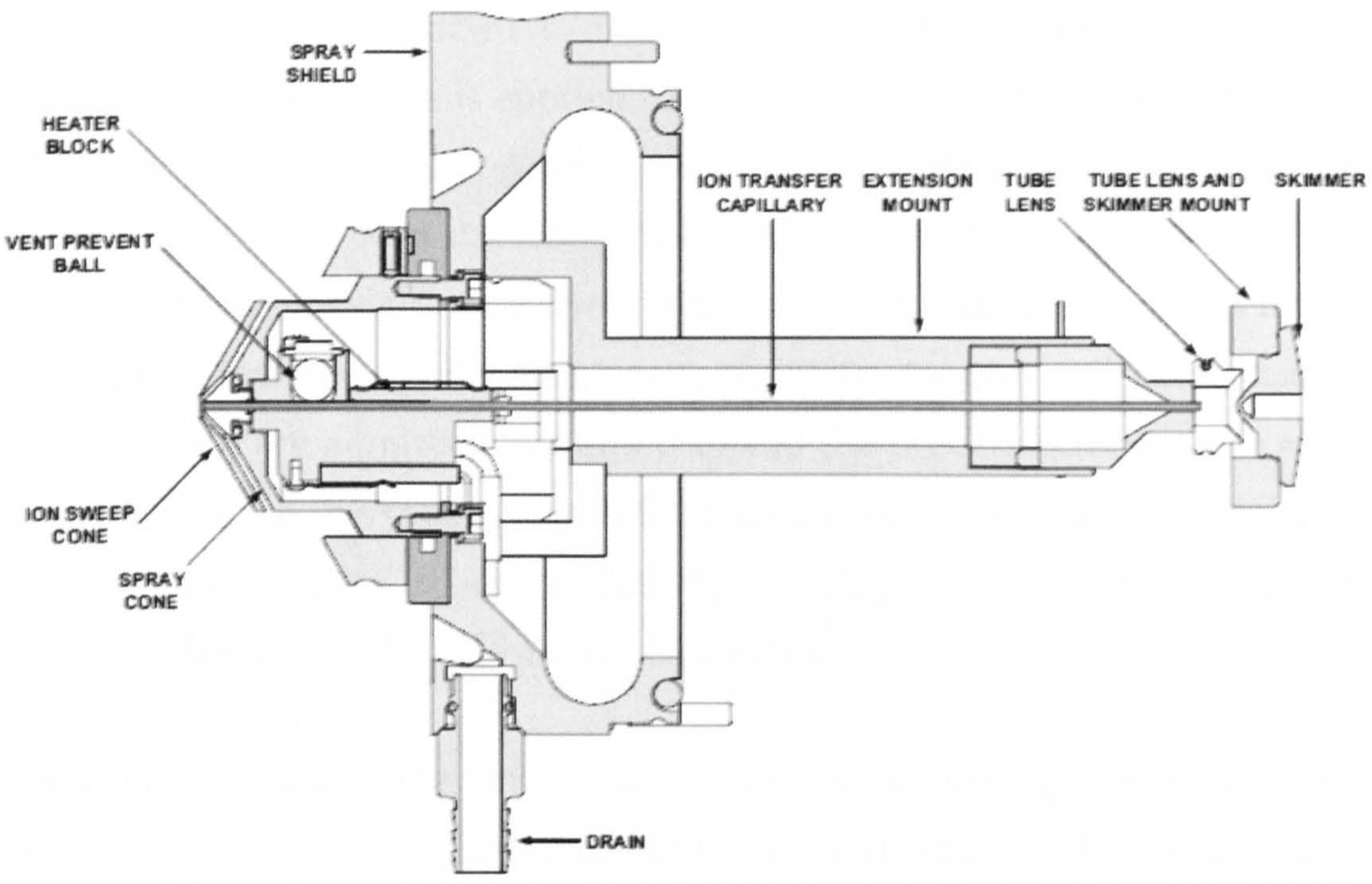


Figure 4-5 Cross-sectional view of the API stack[168]

4.4 Ion Optics

In the LCQ™ Deca XP Plus system, the ion optics consists of one quadrupole, one octapole, separated by an interoctapole lens which helps with ion focusing. The quadrupole and octapole are an array of square and cylindrical rods respectively, which act as ion transmission devices. An RF voltage and DC offset voltage is applied to the rods to create an electric field that guides the selected ions of particular m/z along the axis of the quadrupole/octapole into the mass analyser.

4.5 Ion Trap Mass Analyser

The ion trap mass analyser is also known as the quadrupole ion trap mass analyser and has been described as a three-dimensional quadrupole.[166] It consists of three stainless steel electrodes: the entrance endcap electrode, the exit endcap electrode; and the ring or 'doughnut' electrode (Figure 4-6). The two endcap electrodes are hemispherical and are positioned above and below the ring electrode, to form a hyperbolic cavity. They each have a small hole in their centres to permit the passage of ions into and out of the ion trap.

When sample ions of a specified m/z are held in a static position or 'trapped', a radiofrequency (RF) voltage is applied to the endcap electrodes and the amplitude of the RF voltage applied to the ring electrode is ramped in order to facilitate ion ejection from the trap. As the voltage on the ring electrode is increased, trapped ions are selectively ejected out through the exit lens by increasing m/z into the ion detection system. Following the completed scan cycle, other ions are admitted into the trap and the process is repeated. As the ion trap operates in this mode, it is defined as a pulsed mass analyser.[166] Ion trap mass analysers allow MS-MS analysis by isolating specific product ions and fragmenting them by collision induced dissociation.

Helium is present within the ion trap and is used as a damping gas and collision activation partner to greatly enhance sensitivity and mass spectral resolution. When sample ions enter the trap, they collide with helium atoms therefore reducing their kinetic energy and the amplitude of their oscillations. As a result, the ions migrate to the centre of the cavity where they are tightly focused in a long, narrow cloud. Helium atoms are also used in tandem mass spectrometry (MS-MS) for collision induced dissociation of precursor ions to product ions.

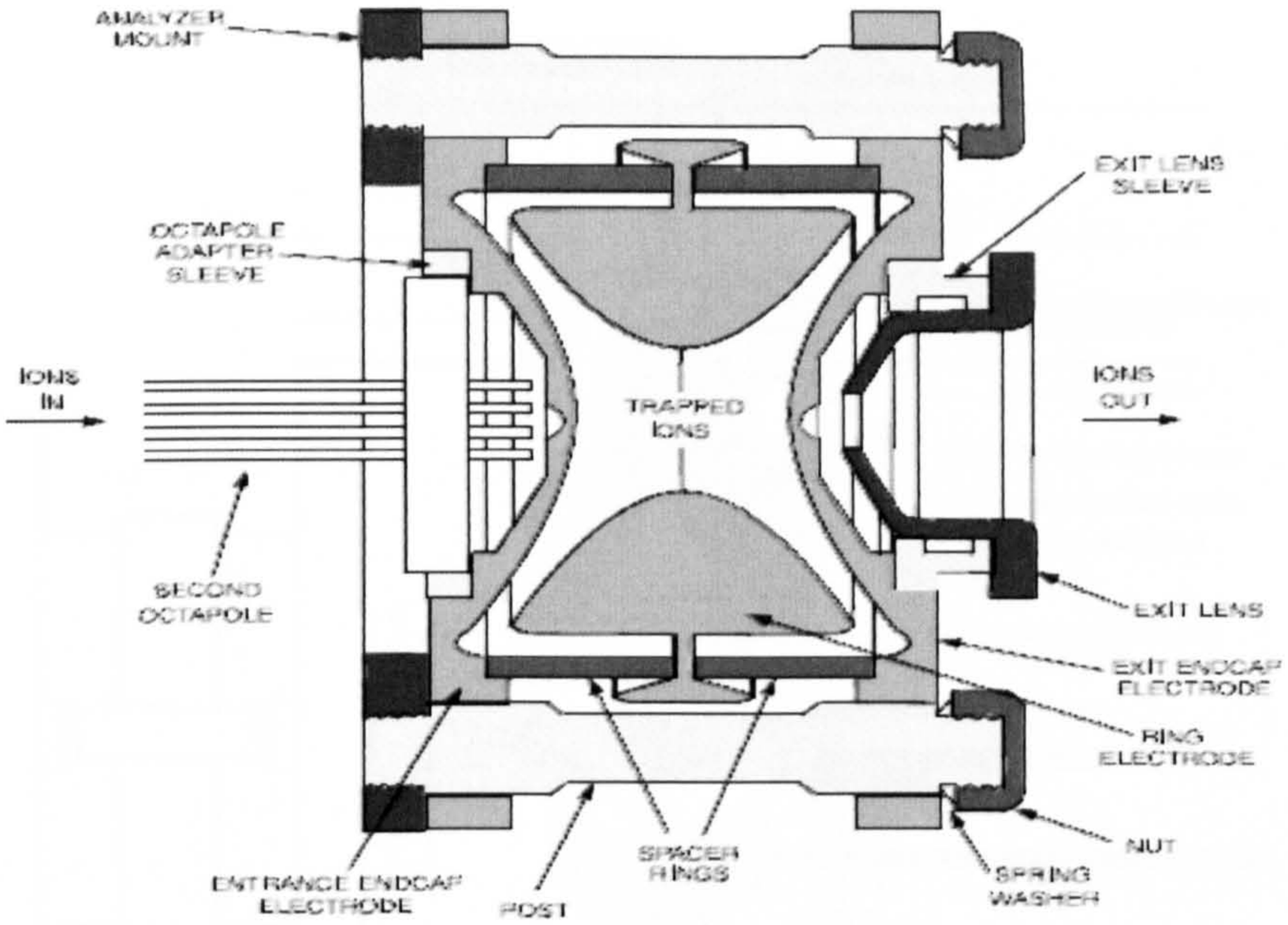


Figure 4-6 Cross-sectional view of the mass analyser[168]

4.6 Detector

Selected ions are passed from the ion trap mass analyser to the detector system. This system contains a conversion dynode and an electron multiplier. An ion strikes the surface of the conversion dynode, readily emitting secondary particles (Figure 4-7). These secondary particles are then focused by the curved surface of the conversion dynode and are accelerated into the electron multiplier by means of a voltage gradient. The electron multiplier contains a cathode and an anode. If the secondary particles from the conversion dynode strike the inner walls of the electron multiplier cathode with sufficient energy, electrons will be ejected. These electrons are accelerated further into the cathode by a potential gradient. As the cathode is funnel-shaped, the electrons continue to strike the inner walls of the cathode, creating an electron cascade which results in a measurable electric signal (current) and typically a signal gain of 10^6-10^7.[166] The current is then converted to a voltage by an electrometer circuit for processing and recorded by the MS data system.

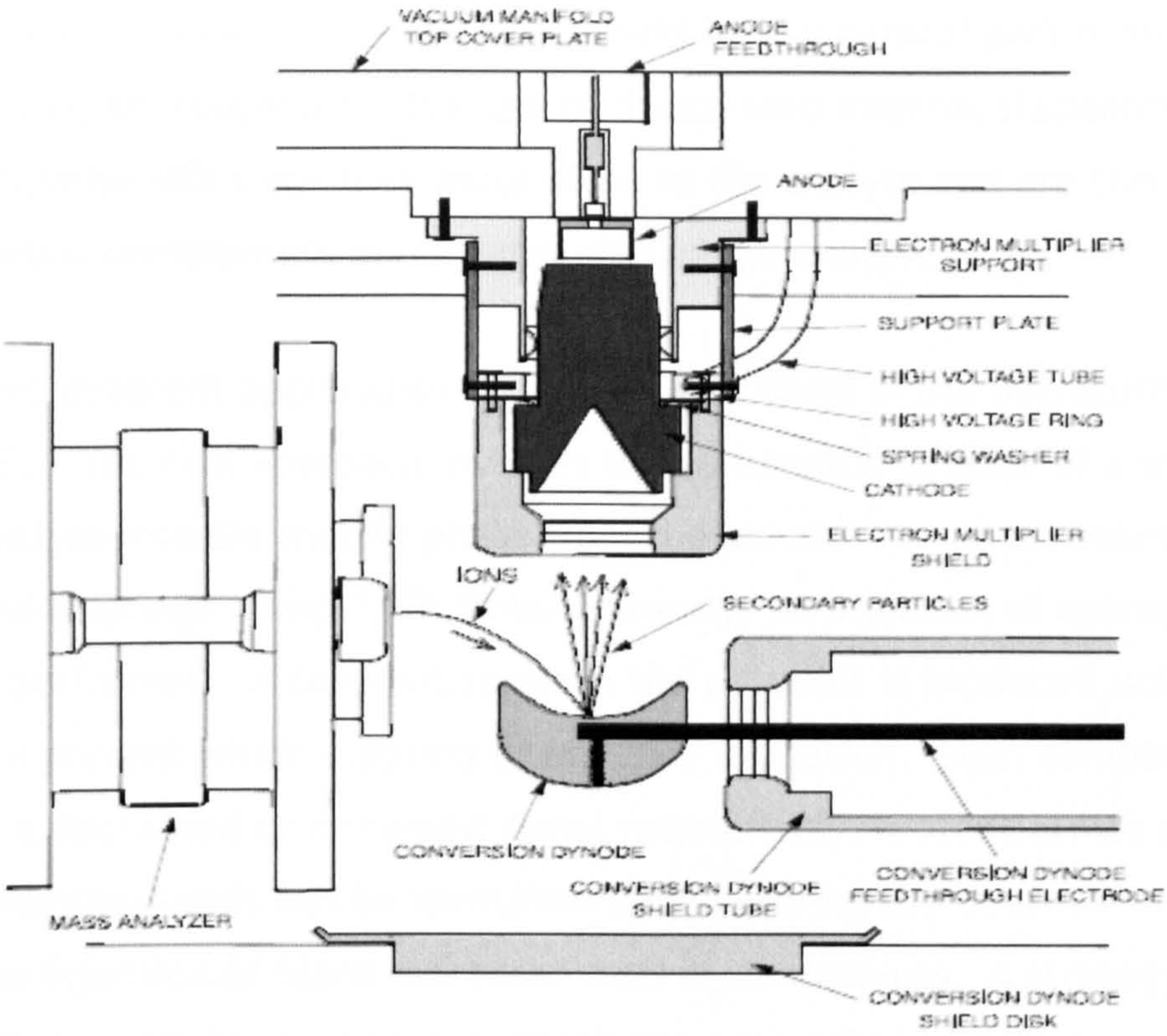


Figure 4-7 Cross sectional view of the ion detection system showing the electron multiplier and conversion dynode[168]

4.7 Matrix Effects

A major problem with generating ions using API techniques is matrix effects (MEs) from biological matrices such as blood, urine and hair. Ion suppression or enhancement of analyte ionisation by co-eluting compounds is known as matrix effects. MEs depend mainly on the sample matrix, sample preparation method, quality of chromatographic separation, mobile phase additives and type of ionisation used. The exact mechanism of MEs is yet to be established but it is generally believed to originate from the competition between an analyte and the co-eluting matrix component in the ion source. ESI is particularly prone to matrix effects, more so than APCI. This is because of the presence of excess reagent ions which produce charged species in APCI; hence the APCI ionisation process is less susceptible to MEs.[163]

Since analyte ionisation depends on the matrix constituents present, this can pose problems when the matrix is not consistent between individuals. As a result, validation parameters such as limit of detection (LOD), limit of

quantitation (LOQ), linearity, precision and accuracy will be affected. Therefore an evaluation of MEs should form a central part of the validation of any LC-MS procedure. The use of deuterated internal standards helps to minimise MEs since they elute close to the analyte and are therefore affected by matrix constituents in the same way as the analyte.

Two different approaches have been described in the literature for assessing MEs. The first approach involves the continuous infusion of a solution of the analyte into the mobile phase from the column via a post-column T-connection and a syringe pump.[173,174] Simultaneously, an injection of extracted blank matrix is performed. A constant signal in the detector is produced unless compounds are present which suppress or enhance ionisation. Such compounds would result in a decreased or increased signal respectively. Potential MEs and their retention times can be identified by monitoring the detector response following the injection of blank extracted matrix components. A strategy for assessing the MEs quantitatively is to determine the peak areas of analyte in two different sets of samples. The first set consists of standards prepared in neat mobile phase (set A). The second set are extracts using blank matrix from different sources which are spiked after extraction (set B). From this data, the ME (%) can be calculated by dividing set B by set A and multiplying by 100.[175] Values greater than 100 % are indicative of ion enhancement while values less than 100 % are indicative of ion suppression.

5 Buprenorphine in Urine

5.1 Buprenorphine

Buprenorphine is a semi-synthetic opioid derivative of thebaine. It has a chemical structure which is closely related to morphine (Figure 5-1) and at low doses (typically 0.3-0.6 mg intravenous or intramuscular) is a powerful analgesic, with 25 - 40 times the potency of morphine.[171] The binding potential of opioids to brain receptors is related to analgesia therefore buprenorphine binds tightly to the μ- and κ-opioid brain receptors, acting as a partial agonist and antagonist when bound to the respective receptor types.[172] In naïve users, buprenorphine demonstrates agonist activity, similar to morphine while in addicts it acts as an antagonist, producing withdrawal symptoms. The kinetics of the opioid receptors is slow; hence the duration of action is long-lived.[173]

Buprenorphine was developed by Reckitt & Colman Products with a view to producing a useful analgesic with greater safety and a lower chance of abuse compared with other opiate agonists.[173] Buprenorphine has been prescribed in the United States since the early 1980s under the tradename Buprenex® and in most European countries as Temgesic® as a treatment for pain relief (especially for surgical purposes) and in anaesthesiology. More recently, it has been identified as an alternative medication to methadone for the treatment of heroin addiction. Under the tradename Subutex®, high dose tablets (0.4, 2 and 8 mg) are administered sublingually for this purpose.

In Scotland from 2006-2007, the number of buprenorphine prescriptions for use in drug dependence was 9,100 compared to 457,045 methadone prescriptions.[174] Buprenorphine has not been identified as a contributor in either drug-related deaths or impaired driving in the Strathclyde region of Scotland in 1995-2007.[175,176,177] Another study into drug-related deaths in Edinburgh and Glasgow in 1990-1992 found buprenorphine to be present in one case.[178] The authors concluded that the small number of positive samples implied that the drug is very unlikely to cause death. However it is more likely that buprenorphine was not commonly prescribed at that time for the treatment of heroin addiction.

In contrast, buprenorphine is used widely in France for the treatment of 90,000 heroin addicts[184] where, in fact, a black market has developed with frequent misuse by intravenous injection.[185] Furthermore, buprenorphine abuse by intravenous injection of crushed tablets meant for sublingual use contributed to the deaths of 20 heroin addicts.[185] A study in the Nord-Pas-de-Calais region of France in 2003-2004 which tested for drugs of abuse in 1000 urine samples collected from truck drivers confirmed the presence of buprenorphine in 1.8 % of positive samples.[186]

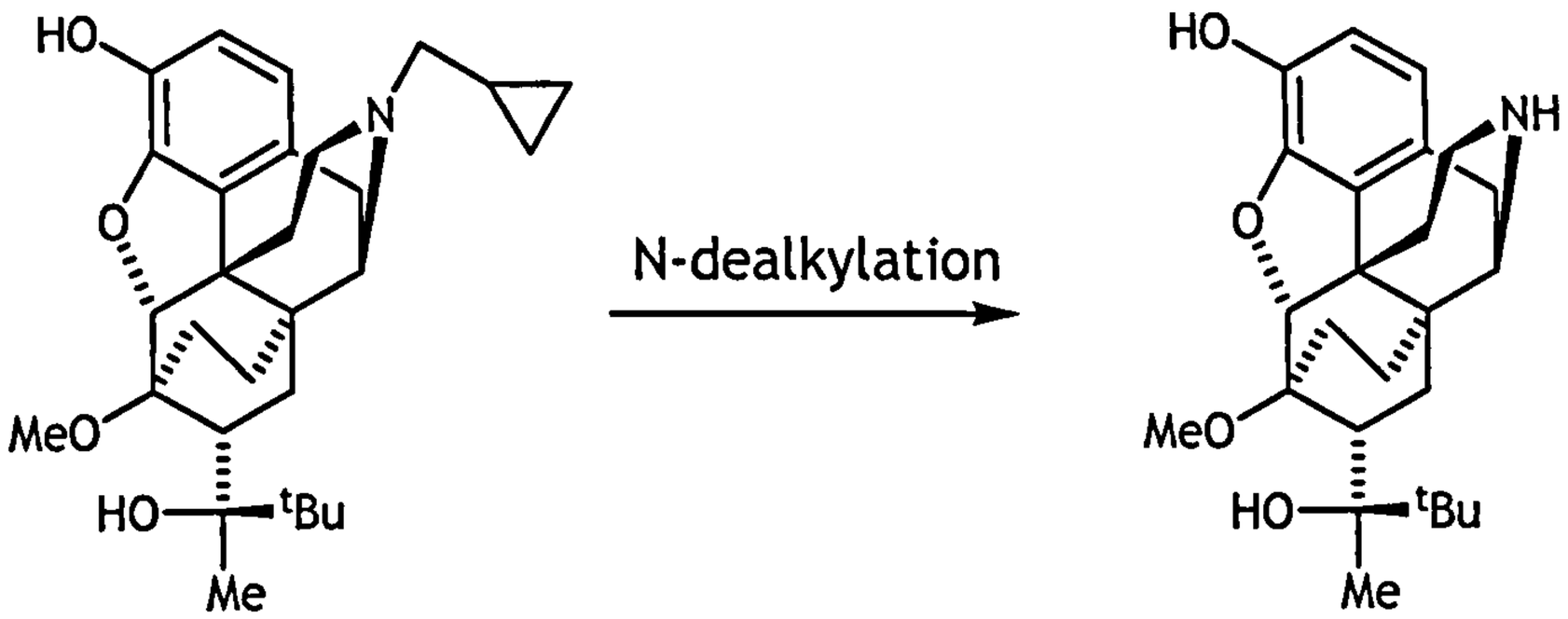
5.2 Pharmacokinetics, Metabolism and Excretion

The pharmacokinetic and physicochemical data for buprenorphine are shown in Table 5-1.

Table 5-1 Pharmacokinetic and physicochemical data for buprenorphine[176]

	Half Life (hr)	Vd (L/kg)	Protein Binding (%)	pK_a
Buprenorphine	2-4	2.5	96	8.5, 10.0

Buprenorphine is rapidly metabolised in the liver by the cytochrome P450 CYP3A4 system. During metabolism, it becomes *N*-dealkylated to form norbuprenorphine, which is also pharmacologically active (Figure 5-1). Buprenorphine is predominantly cleared from plasma by biliary excretion following conjugation with glucuronic acid or *N*-dealkylation and conjugation. However a small amount of drug and metabolites is cleared slowly by the kidney.[178] Within 144 hours of a single intramuscular dose of radiolabelled buprenorphine, 95 % is eliminated with 68 % radioactivity measured in the faeces and 27 % in the urine.[176]



Buprenorphine Norbuprenorphine

Figure 5-1 Buprenorphine metabolism

Blood concentrations of buprenorphine can be very low since only low doses are required in the treatment of pain due to its high potency. A 2 mg sublingual dose of buprenorphine administered to 6 healthy adults produced an average peak plasma concentration of 1.6 ng/mL after 1.3 hours.[176] Opiate-dependent subjects taking an 8 mg sublingual dose of buprenorphine each day as part of their maintenance therapy were shown to have a plasma drug concentration range of 1-8 ng/mL.[187] Therapeutic concentrations of free buprenorphine in urine can also be low (less than 1 ng/mL), requiring sensitive methods of detection.[106,176,184] In contrast, free buprenorphine and norbuprenorphine concentrations greater than 20 ng/mL urine have been observed in cases of abuse.[106,184,188]

5.3 Toxicity

Symptoms associated with buprenorphine overdose include confusion, dizziness, pinpoint pupils, hallucinations, hypotension, respiratory difficulty, seizures and coma. Concentrations found in the blood and urine of a drug addict who had died following intravenous injection of buprenorphine were 3.0 and 616 ng/mL buprenorphine and 3.1 and 48 ng/mL norbuprenorphine respectively.[189] Average post-mortem concentrations found in the blood and urine of 20 heroin addicts who had intravenously injected buprenorphine meant for sublingual use were 8.4 and 172 ng/mL buprenorphine and 2.6 and 67 ng/mL norbuprenorphine.[185]

More than 150 fatal overdoses involving the combined use of buprenorphine and psychotropic drugs (in particular benzodiazepines and neuroleptics) have been reported in France.[190] A study in Singapore also highlighted that concurrent

administration of buprenorphine and benzodiazepines was potentially fatal.[191] Interestingly, an Australian study reported a greater number of opioid toxicity symptoms in relation to methadone consumption compared to buprenorphine in concurrent benzodiazepine users.[192]

5.4 Previously Reported Methods of Analysis

Buprenorphine detection in urine has been reported using immunoassay[106,107,184,193,194], thin layer chromatography[195], high performance liquid chromatography (HPLC)[193,196], GC-MS[194,197,198] and LC-MS.[106,107,184,186,191,199]

ELISA has been widely used for the determination of buprenorphine in urine for a number of different purposes. It has proved useful in the rapid screening of large numbers of post-mortem samples[184,191] and also in a clinical setting for the rapid measurement of urine concentrations in maintainence programmes.[107,184,194] ELISA has also been applied in the urine testing of truck drivers in France in the interests of road safety.[186]

As discussed in chapter 3, this method of detection is highly sensitive but is not specific due to the potential antibody cross reactivity with drug metabolites and other structurally similar compounds other than the target drug. A buprenorphine ELISA system by the International Diagnostics Systems Corporation showed no cross-reactivity with dihydrocodeine, ethylmorphine, 6-monoacetylmorphine, pholcodine, propoxyphene, dextromoramide, dextrometorphan, codeine, morphine, methadone and EDDP.[184] Another buprenorphine ELISA system by the Immunalysis Corporation demonstrated no cross-reactivity with dihydrocodeine, codeine, morphine, methadone, EDDP, tramadol and propoxyphene.[107] Standard buprenorphine immunoassays cross-react with the major metabolite, norbuprenorphine to a relatively high extent depending on the concentration of norbuprenorphine that is present. This is an advantage since norbuprenorphine concentrations are generally higher than buprenorphine concentrations.[106,107,197,199]

A recently published HPLC method describes the combined use of solid-phase extraction and an HPLC system using diode array detection (DAD) to detect buprenorphine and norbuprenorphine in urine samples obtained from drug

addicts on a therapy programme.[197] The advantages of using this method include the use of small sample volumes, high extraction efficiencies for both compounds (≥ 97 %) as well as good levels of precision (≤ 4.9 %) and highly purified extracts. The LOD was determined to be 0.5 ng/mL and 0.2 ng/mL for buprenorphine and norbuprenorphine respectively. One urine sample taken from a patient who was receiving 16 mg/day buprenorphine contained 51.7 ng/mL buprenorphine and 50.4 ng/mL norbuprenorphine. A disadvantage of using this method is that the linearity range for both compounds was 10.0-1500 ng/mL which is acceptable for quantification of the sample concentrations in this study but would need to be extended to include lower concentrations if lower doses were being administered. As mentioned previously, therapeutic urine concentrations as low as 1 ng/mL free buprenorphine have been observed.[106]

The first HPLC method with electrochemical detection for the detection of buprenorphine and norbuprenorphine in urine samples was published 15 years ago.[193] The extraction involved a three-step alkaline toluene extraction and reported limits of detection were 0.2 ng/mL buprenorphine and 0.15 ng/mL norbuprenorphine. The HPLC results in this study were compared to RIA which was only able to detect buprenorphine concentrations higher than 1 ng/mL.

A GC-MS method involving solid-phase extraction has been reported to analyse buprenorphine and norbuprenorphine in urine after therapeutic doses, which was linear from 1-100 ng/mL.[198] Urine samples were hydrolysed using β-glucuronidase and subsequently converted to their methyl derivatives by extractive alkylation. The LOD for both compounds was determined to be 0.2 ng/mL, with high recovery values of 92 % and 104 % for buprenorphine and norbuprenorphine respectively. Free norbuprenorphine was detected in urine in the range of 0.4-1.7 ng/mL following a single 0.2 mg sublingual dose of Temgesic®. Buprenorphine levels were however below the method's detection limit. A more recent GC-MS method involving liquid-liquid extraction of urine was developed for the purposes of monitoring buprenorphine withdrawal protocols, which was linear up to 2000 ng/mL for buprenorphine and norbuprenorphine.[197] Like the previously mentioned GC-MS method, urine samples were hydrolysed using β-glucuronidase to cleave the conjugates but a different derivatisation method involving bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing trimethylchlorosilane (TMCS) was used. A higher LOD was

found for buprenorphine and norbuprenorphine (1 ng/mL for both) compared to the other method mentioned, with lower recovery values of 59 % and 58 % respectively. Buprenorphine and norbuprenorphine concentration ranges were 28-1458 ng/mL and 28-1843 ng/mL respectively.

There have been several LC-MS methods published over the last decade for the determination of buprenorphine and norbuprenorphine in urine. This is mainly because of easier sample pre-treatment required for LC-MS compared to GC-MS which requires a derivatisation step.

LC-ESI-MS with a single quadrupole mass spectrometer was used to detect both compounds using a linear LC gradient system in a variety of matrices including urine, blood and hair.[184] Urine samples were hydrolysed overnight with ß-glucuronidase and the analytes isolated using a single liquid-liquid extraction step with a mixture of chloroform/propan-2-ol/heptane (25:10:65, v/v/v). Extraction recovery for buprenorphine was greater than 80 % and the LOQ was 0.2 ng/mL, which is more sensitive than the GC-MS methods discussed previously. The LC-ESI-MS method was used for confirmation after ELISA screening and was shown to be more sensitive than ELISA, detecting buprenorphine in 7 urine samples which screened as negative using ELISA at a cut-off value of 0.5 ng/mL.

Another reported LC-ESI-MS method for buprenorphine and norbuprenorphine detection in urine used a single quadrupole mass spectrometer supplied from a different manufacturer. Both compounds were separated by gradient elution following liquid-liquid extraction with 1-chlorobutane and pH alteration to alkaline conditions.[191] The LOQ value for buprenorphine was higher (0.5 ng/mL) and extraction recovery similar (average 87 %) using this method compared to the LC-ESI-MS method described previously.

An LC-ESI-MS-MS method has been developed for the rapid screening and quantification of buprenorphine and its metabolites.[199] The authors evaluated three approaches in the study including: the direct injection of diluted urine to measure conjugated metabolites; the direct injection of diluted urine after enzymatic hydrolysis for quantification of buprenorphine and norbuprenorphine; and the quantification of buprenorphine and norbuprenorphine after enzymatic hydrolysis and SPE clean-up. It was concluded that the direct analysis of

glucuronide conjugates was an acceptable screening method but that the direct analysis of free drug sometimes required SPE clean-up to improve the LC-MS sensitivity 20-fold, especially in cases where both buprenorphine and norbuprenorphine were below the cut-off value.

5.5 Aim of Study

At the time of this study, the Immunalysis® ELISA kit had just become commercially available for the detection of buprenorphine in urine. The aim of the work was to validate the ELISA test and to apply it in the analysis of real samples. The screening results were compared with an in-house LC-MS-MS method.

5.6 Experimental

5.6.1 Samples

A total of 69 urine samples were tested in the study. 7 of these samples were obtained from volunteers on the Subutex® treatment programme at the Turning Point Drugs Crisis Centre, Glasgow while the other 62 samples were routine samples which had been submitted to the University laboratory for buprenorphine testing.

5.6.2 Chemicals

Buprenorphine, norbuprenorphine, buprenorphine-d_4 and norbuprenorphine-d_3, dihydrocodeine, codeine, morphine, tramadol, propoxyphene, methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) were obtained from LGC Promochem (Teddington, UK). Buprenorphine Direct ELISA kits were obtained from the Immunalysis Corporation (Pomona, CA, USA). The kits contained a 96-well antibody-coated microplate, buprenorphine conjugate labelled with horseradish peroxidase (HRP), substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide and stop solution containing 1 N hydrochloric acid. Phosphate buffer saline (0.1 M, pH 7.0) was used to dilute urine samples in the ELISA test and this was also obtained from the Immunalysis Corporation. Disposable 75 x 12 mm borosilicate glass culture

tubes for ELISA were obtained from VWR International, UK. HPLC grade methanol, acetonitrile, acetone, dichloromethane, glacial acetic acid, ammonium hydroxide, formic acid and potassium dihydrogen phosphate were obtained from BDH (Poole, UK). Ammonium formate and β-glucuronidase crude solution were obtained from Sigma-Aldrich (Dorset, UK). Bond Elut Certify® LRC SPE columns (130 mg, 10 mL) were obtained from Crawford Scientific (Strathaven, UK).

5.6.3 Standard Solutions

Individual drug stock standard solutions and deuterated drug standards were obtained as 100 µg/mL prepared in methanol. A combined working drug solution of buprenorphine and norbuprenorphine was prepared at 1 µg/mL by 100-fold dilution with methanol. This was achieved by adding 25 µL of 100 µg/mL drug solution into a 25 mL volumetric flask and making up the volume to the 25 mL mark with methanol. A combined working deuterated standard solution of the buprenorphine-d_4 and norbuprenorphine-d_3 standards was also prepared at 1 µg/mL in the same way as the working drug solution.

5.6.4 Extraction Solutions and Mobile Phase

5.6.4.1 Preparation of 1 M, pH 5 Sodium Acetate Buffer

42.9 g of sodium acetate trihydrate and 10.4 mL of concentrated (17 M) glacial acetic acid was dissolved in 400 mL of deionised water, the pH was adjusted to 5.0 with 1 M acetic acid and the solution made up to 500 mL with deionised water.

5.6.4.2 Preparation of 0.01 M, pH 3.0 Acetic Acid

286 µL of concentrated (17 M) glacial acetic acid was placed in a 500 mL volumetric flask and this was made up to volume with deionised water.

5.6.4.3 Preparation of 0.1 M, pH 6.0 Phosphate Buffer

1.70 g of potassium hydrogen phosphate and 12.14 g of potassium dihydrogen phosphate were weighed into a 1 L beaker. 800 mL deionised water was added

and the solid was dissolved by stirring. After the solid had dissolved, the pH of the solution was adjusted to 6.0 using 1 M potassium hydroxide solution. The solution was then transferred into a 1 L volumetric flask and was made up to the 1 L mark with deionised water.

5.6.4.4 Preparation of Mobile Phase

The mobile phase consisted of a mixture of aqueous buffer and acetonitrile. To prepare the 3 mM ammonium formate + 0.001 % formic acid aqueous buffer, 0.189 g of ammonium formate and 10 μL of concentrated formic acid were added to a 1 L volumetric flask and made up to the mark with deionised water.

5.6.5 Instrumentation

ELISA analysis of urine samples was carried out using a MiniPrep 75 automatic pipettor purchased from Tecan (San Jose, CA). The microplate wells were washed and read using a Columbus Plus washer system and a Sunrise Remote EIA autoreader from Tecan (Grödlg, Austria).

LC-MS-MS analysis was carried out using a Surveyor HPLC system with an LCQ Advantage™ ion trap mass spectrometer (Thermo Finnigan, San José, CA, USA). Chromatographic separation was performed using a Gemini C_{18} column (150 mm x 2.0 mm ID, 5 μm particle size), fitted with a guard column with identical packing material (4 mm x 2.0 mm, 5 μm particle size) (Phenomenex, Torrance, CA, USA).

5.6.6 ELISA and LC-MS-MS Methods

5.6.6.1 ELISA

Each ELISA run contained a set of calibrators consisting of a blank and spiked urine samples at a concentration of 0.5, 1 and 5 ng/mL buprenorphine (cut-off value 0.5 ng/mL). This cut-off value was also used in another ELISA study which screened 93 buprenorphine positive urine samples and produced no FN results when compared to a validated LC-ESI-MS method.[184]

A high positive control (10 ng/mL) and a negative control provided with the kit were used to check kit performance. These controls were distributed to wells at the beginning and end of the plate to check instrument drift. Each calibrator, control and sample was vortex mixed after 10-fold dilution with phosphate buffer. The on-line dilution was achieved by adding 100 µL of urine to 900 µL phosphate buffer saline (pH 7.0).

20 µL of diluted calibrator, control or sample was added to the wells in duplicate followed by 100 µL of enzyme conjugate. The plate was then left in the dark at room temperature for an incubation period of 1 hour. After incubation, the wells were washed 6 times with deionised water in order to remove any unbound sample or residual enzyme conjugate which may be left in the wells. 100 µL of TMB substrate reagent was then added and the plate was left to incubate in the dark at room temperature for a further 30 minutes. After 30 minutes, the catalytic reaction was stopped by adding 100 µL of stop reagent (containing 1 N hydrochloric acid) to each well. The contents of the wells turned yellow following addition of the stop reagent to enable the chromophore to be detected at 450 nm. A reference wavelength of 620 nm was used. The absorbance readings were inversely proportional to the concentration of buprenorphine and/or norbuprenorphine present in the sample.

5.6.6.2 LC-MS-MS

The LC-MS-MS method was an in-house method that had already been developed and validated by Dr Hazel Torrance within the forensic toxicology laboratory at the University of Glasgow.

5.6.6.2.1 Sample Preparation

The calibration graph was constructed from spiked urine samples in the concentration range 5-200 ng/mL for buprenorphine and norbuprenorphine. To achieve this, the appropriate amount of working drug solution at 1 µg/mL (5, 10, 25, 50, 100 and 200 µL) was added to 1 mL blank urine. 100 ng (100 µL) of working deuterated standard solution at 1 µg/mL was also added to each test tube. A blank urine sample was prepared without any drugs or deuterated standards, as well as another blank urine sample with only deuterated standards to assess any contamination which may be present. 1 mL of acetate buffer

(pH 5.0, 1 M) was added to each test tube along with 40 µL of β-glucuronidase crude solution (helix pomatia). The samples were placed in an oven for 3 hours at 60 °C and after cooling, 3 mL of phosphate buffer (pH 6.0, 0.1 M) was added to each sample, followed by vortex mixing. The pH of each sample was adjusted to pH 6.0 using 1 M potassium hydroxide. The samples were then centrifuged at 2500 rpm for 10 minutes.

5.6.6.2.2 Extraction Procedure

Bond Elut Certify® LRC cartridges were conditioned with 2 mL methanol, followed by 2 mL phosphate buffer (0.1 M, pH 6.0). The samples were then applied to the cartridges and allowed to drip through without a vacuum to allow maximum time for analyte-sorbent interactions. The cartridges were washed with 1 mL deionised water followed by 0.5 mL acetic acid (0.01 M, pH 3.3) and dried under full vacuum for 10 minutes. 50 µL of methanol was added to each cartridge and the cartridges dried under full vacuum for 2 minutes. 4 mL acetone/dichloromethane (1:1 v/v) was then added to further wash the cartridges. The basic analytes were eluted with 2 x 1.5 mL ethyl acetate with 2 % ammonia. The extracts were blown down under nitrogen at 30 °C and reconstituted in 80 µL mobile phase (initial conditions 3 mM ammonium formate + 0.001 % formic acid: acetonitrile (75:25 v/v)).

5.6.6.2.3 Analysis

The column temperature was maintained at 35 °C. LC was carried out using a mixture of 3 mM ammonium formate + 0.001% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 mL/min. The gradient elution programme was 75 %-20 % solvent A within 15 minutes. The % of solvent A was decreased to 10 % between 15-17 minutes and was held at 10 % solvent A for 17-22 minutes. After this time, initial mobile phase conditions were restored for 5 minutes. A divert valve taking LC flow to waste was used for the first 3 minutes and for the last 7 minutes of the programme in order to preserve the MS source.

All MS data was collected in electrospray positive ion mode. The capillary temperature, sheath and auxiliary gas flow rates and collision energies were optimised for each analyte. The following settings were applied throughout the analysis: capillary temperature 280 °C; sheath and auxiliary gas flow rates were set to 20 and 15 units on the data system arbitrary scale and collision energies

were 36 % for buprenorphine and 32 % for norbuprenorphine. The probe voltage used was 4.5 kV. Internal standard data was collected in selected ion monitoring (SIM) mode and analytes were identified on the basis of their full MS-MS spectra. Buprenorphine produced a pseudo-molecular ion of m/z 468, with a major product ion of m/z 414 while norbuprenorphine produced a pseudo-molecular ion of m/z 414, with a major daughter ion of m/z 396. Quantification was carried out using the major product ion to deuterated drug standard ratio.

5.7 ELISA Method Validation

In the method validation, all spiked samples were analysed according to the ELISA procedure described in section 5.6.6.1.

5.7.1 Dose Response Curve

A dose response curve was produced over the concentration range 0.5 ng/mL-100 ng/mL buprenorphine. This was achieved by spiking 1 mL blank urine with the appropriate volume of buprenorphine solution at 0.1 µg/mL (5, 10, 100 µL) for the lower concentrations and 1 µg/mL (25, 50, 100 µL) for the higher concentrations. The spiked urine standards were 0.5, 1, 10, 25, 50 and 100 ng/mL buprenorphine.

5.7.2 LOD and Assay Precision

The LOD was calculated from the mean B/B_0 (%) value (A_0) and standard deviation (s) of the B/B_0 (%) values for 48 confirmed negative post-mortem urine samples.[102] The use of post-mortem urine negatives in the determination of the assay LOD is more accurate than using blank urine collected from a living individual. The possible presence of interfering compounds in post-mortem urine may affect the assay LOD. Spiked urine standards were prepared at low concentrations (0.25, 0.5 and 1 ng/mL buprenorphine) and the B/B_0 (%) value calculated for the LOD was matched to the B/B_0 (%) value of the spiked calibrator to determine the LOD in ng/mL.

Equation 5-1: $$LOD = A_0 - 3s$$

Assay precision was tested at the cut-off value of 0.5 ng/mL buprenorphine. To achieve this concentration, 5 µL of 0.1 µg/mL buprenorphine solution was added to 1 mL blank urine.

Within-sample precision (n=10) was calculated from 10 readings of the same spiked urine sample. Intra-day precision (n=20) of the absorbance readings was calculated from duplicate readings of 10 spiked urine samples. The intra-day precision experiment was carried out on five separate days to calculate the inter-day precision (n=100).

5.7.3 Cross Reactivity

A buprenorphine and norbuprenorphine dose response graph was produced to calculate norbuprenorphine cross reactivity. The buprenorphine dose response curve consisted of 4 spiked urine samples at 0.5, 1, 5 and 10 ng/mL buprenorphine. This was achieved by spiking 1 mL blank urine with 5, 10, 50 and 100 µL of 0.1 µg/mL buprenorphine solution. The norbuprenorphine dose response graph consisted of 3 spiked urine samples at 1, 5 and 10 ng/mL norbuprenorphine. This was achieved by spiking 1 mL blank urine with 10, 50 and 100 µL of 0.1 µg/mL norbuprenorphine solution.

Graphs of B/B_0 (%) vs Concentration (ng/mL) were plotted manually on log-log graph paper. The best fit line was drawn through the points for the buprenorphine and norbuprenorphine graphs. The concentration of buprenorphine producing the same B/B_0 (%) value as 1 ng/mL and 10 ng/mL norbuprenorphine was identified from reading off the graphs. Cross reactivity was then calculated by dividing this buprenorphine concentration by the norbuprenorphine concentration and multiplying by 100 to express as a percentage. Cross reactivity was calculated at 1 ng/mL and 10 ng/mL norbuprenorphine for comparison with the manufacturer's results which were provided at these concentrations.

5.8 ELISA Results and Discussion

5.8.1 Dose Response Curve

A dose response curve was generated for blank urine spiked at buprenorphine concentrations of 0.5, 1, 10, 25, 50 and 100 ng/mL (Figure 5-2). The B/B_0 (%) values were calculated where B is the absorbance value of the bound calibrator and B_0 is the absorbance value of the blank calibrator. The results were obtained in duplicate and an average B/B_0 (%) value was calculated. The optical density values showed a hyperbolic decrease with increasing buprenorphine concentration. This is because at higher concentrations, more buprenorphine is bound to the antibody sites compared to enzyme conjugate, producing lower optical density values.

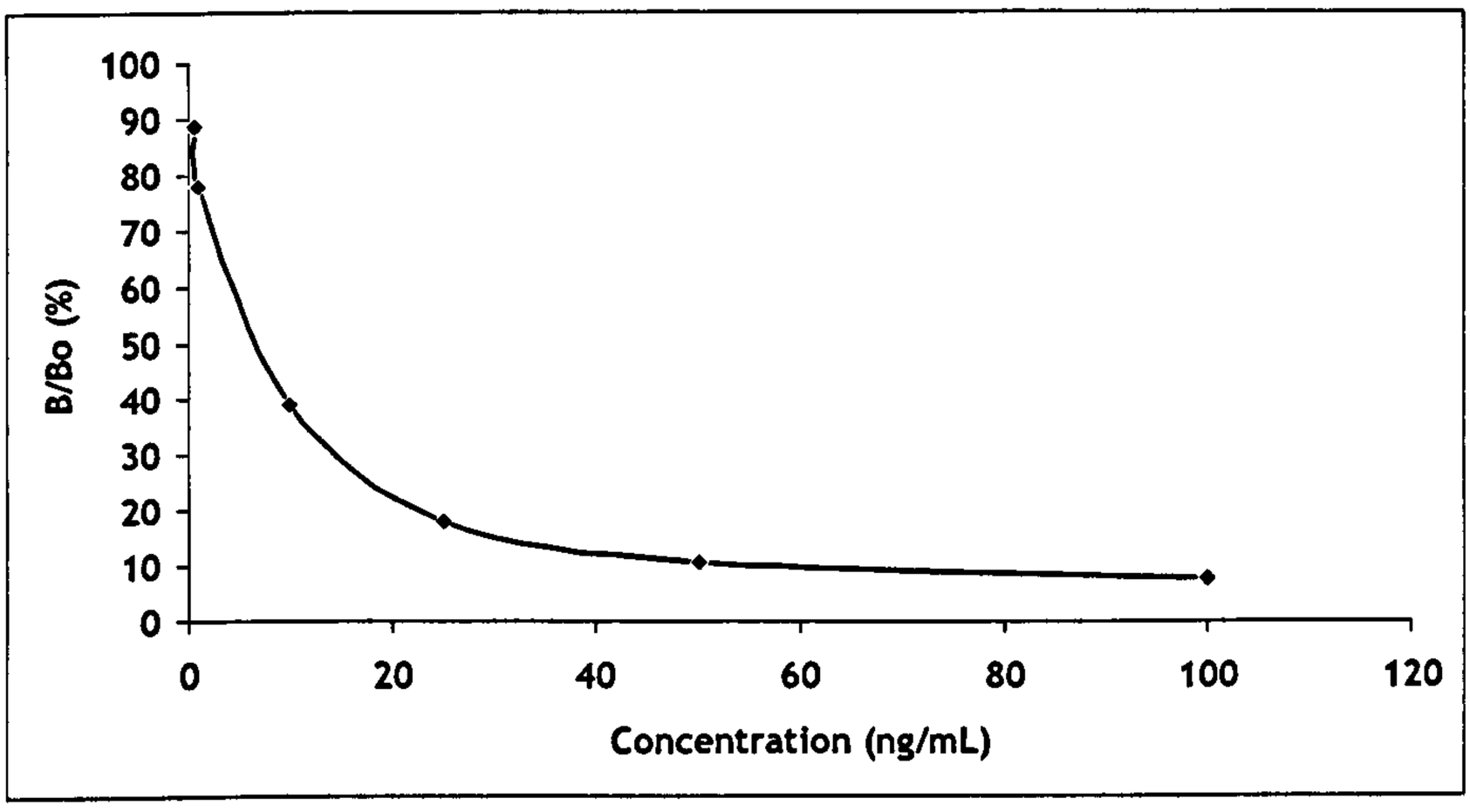


Figure 5-2 Buprenorphine dose response curve (linear scale)

The same set of data is shown in Figure 5-3 only plotted on log scale for both axes. The graph is an S-shaped ligand reaction curve.[200] As seen from this graph, the immunoassay has a short linearity range and low precision at both ends of the range. Usually a cut-off value is chosen in the linear range, which would be 10-50 ng/mL in this graph. This is the optimum region for discriminating between true positive (TP) and true negative (TN) samples. However a cut-off < 10 ng/mL is required for buprenorphine screening due to its potentially low levels and therefore 0.5 ng/mL was chosen as the cut-off value.

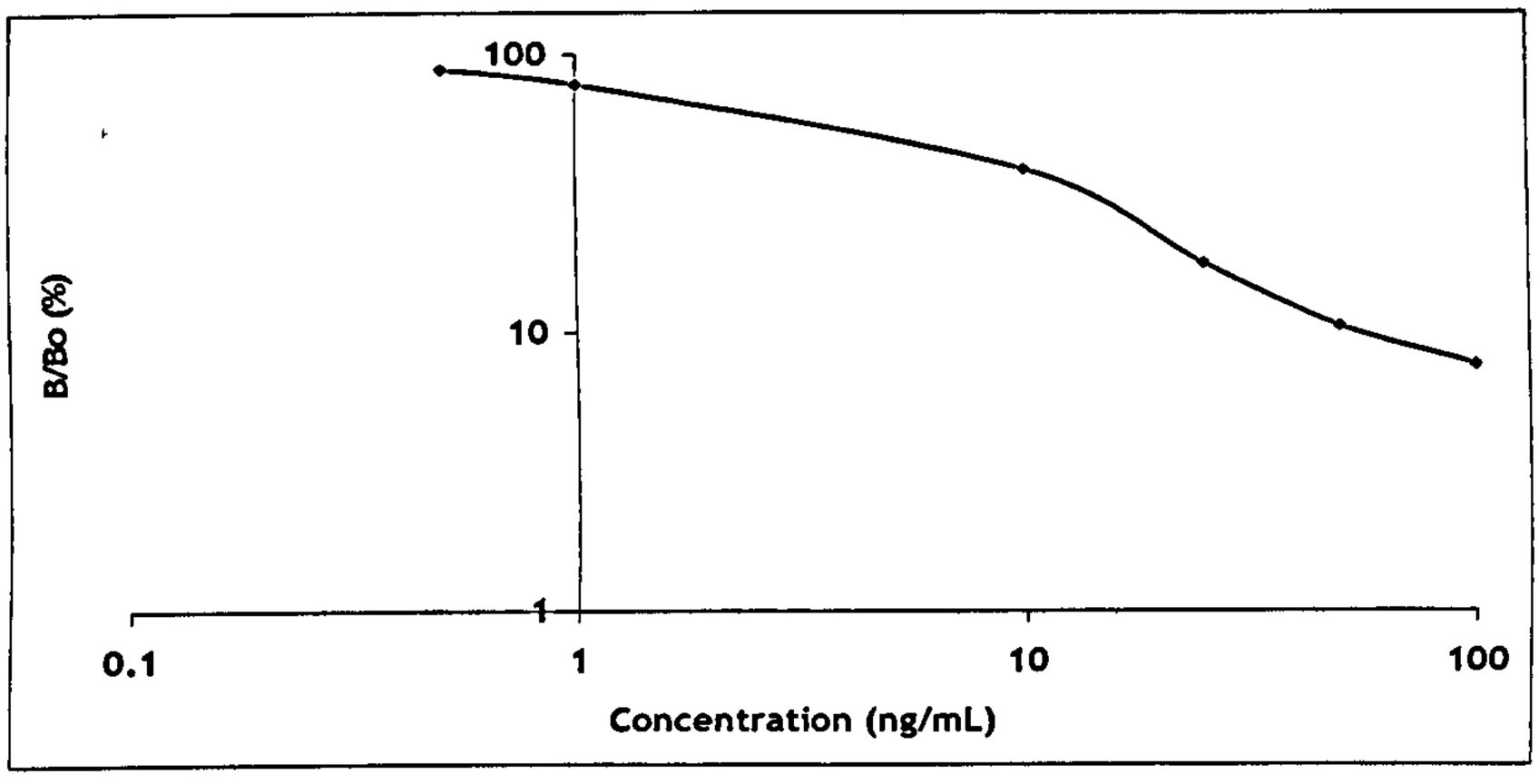


Figure 5-3 Buprenorphine dose response curve (log-log scale)

5.8.2 LOD and Assay Precision

The LOD was calculated as 0.5 ng/mL buprenorphine using Equation 5-1.

Within-sample precision (n=10) was calculated as 5.3 % from 10 readings of the same spiked urine sample. Intra-day precision (n=20) of the absorbance readings was calculated as 6.4 % from duplicate readings of 10 spiked urine samples. The intra-day precision experiment was carried out on five separate days to calculate the inter-day precision (n=100) of 12.9 %.

5.8.3 Cross Reactivity

A high degree of cross reactivity was observed for norbuprenorphine at low and high concentrations, although this was greater at the higher concentration. The results were comparable to the manufacturer's values (Table 5-2). As mentioned previously in section 5.4, this high degree of norbuprenorphine cross reactivity is advantageous in an ELISA screening assay as norbuprenorphine concentrations detected in urine are generally higher than buprenorphine concentrations.

Table 5-2 Cross reactivity of buprenorphine ELISA

Analyte	Concentration (ng/mL)	% Cross Reactivity Found	% Cross Reactivity Quoted by Manufacturer
Norbuprenorphine	1	78	80
	10	116	120

A high concentration of dihydrocodeine, codeine, morphine, propoxyphene, tramadol, methadone and EDDP (10,000 ng/mL) did not cross react with the assay. This concentration was also tested by the kit manufacturers. The average absorbance values observed for these drugs are given in Table 5-3. None of these were equal to or less than the average absorbance value of 1.58 observed for the assay sensitivity level of 0.5 ng/mL buprenorphine. Although the average values are relatively close to the average value for the cut-off, the concentration of drugs tested would not usually be as high in actual case samples.

Table 5-3 Absorbance values for opiate drugs at 10,000 ng/mL

Analyte	Absorbance Value (n=2)
Dihydrocodeine	1.67
Codeine	1.75
Morphine	1.66
6-MAM	1.67
Propoxyphene	1.71
Tramadol	1.74
Methadone	1.63
EDDP	1.76

5.8.4 Sensitivity and Specificity

The number of true positive (TP), true negative (TN), false positive (FP) and false negative (FN) results are usually tallied and applied to Equation 5-2 and Equation 5-3 to determine the sensitivity and specificity of an analytical system. A TP result is defined as one where both the screening and confirmation result are positive. Conversely, a TN result is one in which the screening and confirmation result are both negative. The greatest number of TP and TN results can be obtained by choosing the optimum cut-off concentration for a particular system. Although there were no FP or FN results found in this particular study, a FP result is one in which screening is positive and confirmation negative and a FN is where screening is negative and confirmation is positive. It is essential that cut-off levels are low enough to eliminate FN screening results. Furthermore, this is more important than the elimination of FP screening results.

Equation 5-2 $$Sensitivity = (TP \times 100)/(TP + FN)$$

Equation 5-3 $$Specificity = (TN \times 100)/(TN + FP)$$

Of the 69 urine samples screened as part of this study, there were 21 TP and 48 TN results. Therefore the sensitivity and specificity of the ELISA system as calculated using Equation 5-2 and Equation 5-3 were both 100 % using a cut-off value of 0.5 ng/mL buprenorphine. This cut-off value was recommended by the manufacturers and was found in this study to be the optimum concentration for achieving the highest number of TP and TN results compared to higher cut-off concentrations.

In a review on hair analysis by immunological methods up until the year 2000, it was recommended that the cut-off is not set at the limit of detection due to the very large incidence of false positive results.[200] It was also pointed out that the precision at both ends of the dose response curve is low due to the S-shaped graph. In this study the assay sensitivity level of 0.5 ng/mL buprenorphine was selected as the cut-off as it produced 100 % TP and TN results, it was recommended as the cut-off by the manufacturer and the precision of the system was well within the published acceptance criteria of 10 % for intra-day precision and 20 % for inter-day precision.[201]

5.8.5 Case Samples

The ELISA and LC-MS-MS results for the case samples are provided in Table 5-4.

5.8.5.1 ELISA

14 out of 21 samples screened as positive at levels greater than the highest calibrator (> 5 ng/mL buprenorphine). However the ELISA concentration detected in 5 out of 21 samples were in the range 0.61-0.93 ng/mL buprenorphine equivalents. These samples contained low levels of buprenorphine in some cases levels < LOQ of the LC-MS-MS method and did not contain norbuprenorphine. This finding supports the use of a low cut-off level of 0.5 ng/mL buprenorphine as the samples would have screened as negative at higher cut-off levels.

The buprenorphine equivalent concentrations obtained by the ELISA test for samples 5, 7, 17 and 18 were lower than those obtained by LC-MS-MS. Apart from ELISA being a semi-quantitative test, a possible reason for this could be that the ELISA kit does not cross-react with buprenorphine and norbuprenorphine glucuronide conjugates which may have been present in these particular urine samples. The ELISA extraction, unlike the LC-MS-MS extraction did not involve a hydrolysis step in order to reduce the time required for sample preparation prior to screening.

5.8.5.2 LC-MS-MS

Buprenorphine and norbuprenorphine concentrations in the urine case samples, as determined by LC-MS-MS, ranged from 1 - 1931 ng/mL and 7 - 7550 ng/mL respectively. Most concentrations detected were in the ranges reported in the literature.[184,199] However the concentrations determined for sample 20 are higher than reported concentrations. This sample was collected from a patient on a Subutex® treatment programme.

Buprenorphine concentrations in samples 2, 6, 9 and 11 could not be quantified by LC-MS-MS. The LC-MS-MS concentrations found for these samples were higher than the LOD (0.4 ng/mL) but lower than the LOQ (1.3 ng/mL). As buprenorphine was detected in these samples, they were counted as TPs.

5.8.5.3 ELISA vs LC-MS-MS

The excellent qualitative agreement between the ELISA and LC-MS-MS results is demonstrated in Table 5-4. As mentioned in section 5.8.4, the sensitivity and specificity of the ELISA kit tested in this study were both found to be 100 %. Another study which tested 138 urine samples using the same cut-off value but a different ELISA kit (One Step™) to the one used in this work detected 26.5 % FP results.[184] The One Step™ ELISA kit demonstrated a low cross reactivity (< 1 %) with norbuprenorphine at 50 ng/mL buprenorphine. This cannot be compared directly to the cross reactivity observed with the Immunalysis® kit tested in this study as the cross reactivity varies with concentration. No cross reactivity was observed with the drugs shown in Table 5-3 using this kit.

5.9 Conclusion

The Buprenorphine Direct ELISA kit from Immunalysis® is a highly sensitive and specific screening procedure for detecting buprenorphine in urine using a cut-off value of 0.5 ng/mL buprenorphine. The method has been validated and applied successfully in the semi-quantitative analysis of 21 TP case samples. The assay is capable of detecting buprenorphine concentrations in both therapeutic and abuse situations. The assay also demonstrated a high degree of cross reactivity with norbuprenorphine which is advantageous as it is usually present at higher levels than buprenorphine.

Table 5-4 ELISA vs LC-MS-MS results for positive buprenorphine case samples

ELISA		LC-MS-MS		
Sample Number	BUP Equivalents (ng/mL)	BUP (ng/mL)	NBUP (ng/mL)	Source
1	> 5	40	48	Routine
2	> 5	< LOQ	7	Routine
3	> 5	12	125	Routine
4	>5	8	34	Routine
5	2.50	1	22	Routine
6	0.61	< LOQ	NEG	Routine
7	0.93	8	NEG	Routine
8	> 5	31	131	Routine
9	0.63	<LOQ	NEG	Routine
10	> 5	12	NEG	Routine
11	0.77	<LOQ	NEG	Routine
12	> 5	179	97	Routine
13	> 5	246	2288	Crisis Centre
14	> 5	130	1001	Crisis Centre
15	> 5	146	2195	Crisis Centre
16	> 5	123	1835	Crisis Centre
17	0.63	6	NEG	Routine
18	2.15	21	82	Routine
19	> 5	531	5730	Crisis Centre
20	> 5	1931	7550	Crisis Centre
21	> 5	167	2255	Crisis Centre

"BUP"=Buprenorphine, "NBUP"=Norbuprenorphine, "NEG"=Negative

6 Drugs of Abuse in Hair by ELISA

6.1 Introduction

Historically radioimmunoassay (RIA) was used for screening drugs of abuse in hair. The first report was the detection of opiates in 1979[10] and since this initial study, there has been many more publications in this area.[61,197,198,199] In the last decade, several groups have used RIA to detect opiates and cocaine in hair for the purposes of proving the illegal consumption of heroin and cocaine.[200,201] Another more recent publication used RIA to screen for amphetamines in the hair of known users and workplace subjects.[2] Benzodiazepine detection in hair by RIA has also been reported.[202] Although RIA is a sensitive and robust method, the use of radioactive materials has imposed strict safety regulations and has limited its application out with safe areas. It is therefore only cost effective when used for a large number of samples. For these reasons, there is currently an increasing trend towards non-radioactive alternatives such as ELISA.

ELISA is now being routinely applied as a screening method for the detection of drugs of abuse in hair[81,105,106,107,108,109,110,179,203] as it is safe, simple, highly sensitive, uses relatively small sample sizes and can be automated. Other immunoassay systems including enzyme multiplied immunoassay technique (EMIT)[204,205] and fluorescence polarisation immunoassays (FPIA)[205,206] have also been used for this purpose. One group developed an LC-MS-MS screening method for drugs of abuse as an alternative to immunoassay before confirming any positive screens with GC-MS.[94]

ELISA kits have been specifically designed for detecting drugs of abuse in hair. As mentioned previously, conventional urinary immunoassays are targeted towards the detection of hydrophilic metabolites whereas it is usually the parent drug which is found in higher concentrations in hair. A hair immunoassay test should have a sensitivity and specificity greater than 90 % at the selected cut-off.[195] Some urinary ELISA kits, when applied for the purposes of hair testing, have been shown to lack the required specificity.[81,108] This is in contrast to other reports which found excellent ELISA sensitivity and specificity of greater than 90 %.[107,109]

There are three main requirements for immunoassays used for hair testing: they cross react with the drugs actually present in hair; they are not affected by sample matrix interference; and they must be capable of detecting the drug concentrations in hair.[200]

ELISA kits for hair usually demonstrate cross reactivity with the parent drug and lipophilic metabolites actually found in hair. A comparison of EIA and FPIA for the detection of cocaine in hair found that EIA was more sensitive, specific and efficient.[212] The authors proposed that the differences in results were due to the cross reactivity towards the parent drug, cocaine. The EIA kit was designed for oral fluid testing and had a high cross reactivity with cocaine of 100 %, while the FPIA was designed for urinary analysis and had a very low cocaine cross reactivity of 1 %. Only kits with relatively high cross reactivity with the parent drug should be validated for hair screening. However the exception is the ELISA screening test for cannabis in hair. This kit cross reacts 100 % with the 11-nor-delta(9)-tetrahydrocannabinolcarboxylic acid (Δ^9THC-COOH) metabolite instead of the delta(9)-tetrahydrocannibinol (Δ^9THC) parent drug to avoid screening false positive hair samples that have been contaminated through exposure to cannabis smoke.

ELISA demonstrates minimal matrix interference because of the wash step after the competitive incubation. This removes the sample matrix and any unbound label before the addition of the substrate and subsequent colour development. The use of an enzyme label amplifies the signal during catalysis and this reduces the limits of detection to the extremely low levels (pg/mg range) required for hair testing. The sensitivity required for the immunoassay screening test for opiates, cocaine and amphetamines has been stipulated in the most recent publication by the SoHT.[100] The following cut-off concentrations must result in a positive screening result: 0.2 ng/mg morphine or 6-MAM; 0.5 ng/mg cocaine; 0.2 ng/mg MDMA, MDEA, MDA, methamphetamine or amphetamine; and 0.1 ng/mg THC.

6.2 Aim

The aim of this to study was to validate an ELISA method for the detection of drugs of abuse in hair and apply it in the analysis of case samples.

6.3 Experimental

6.3.1 Samples

The post-mortem scalp hair samples screened for amphetamine, benzodiazepines, cocaine, opiates and methadone were routine samples submitted to the laboratory at the University of Glasgow. They were tested for diagnostic purposes and the results were reported to the Procurator Fiscal.

The hair samples screened for methamphetamine as part of a collaboration with the National Institute of Scientific Investigation in Seoul, South Korea were routine samples submitted to the Korean lab which had been collected from living individuals suspected of drug abuse.

6.3.2 Chemicals

Amphetamine (AMP), benzodiazepine, cocaine, methamphetamine (MAMP) and opiate direct ELISA kits were obtained from the Immunalysis Corporation (Pomona, CA, USA). The kits contained a 96-well antibody-coated microplate, enzyme conjugate labelled with horseradish peroxidase (HRP), substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide and stop solution containing 1 N hydrochloric acid. Hair extraction buffer (0.025 M monobasic phosphate buffer pH 2.7), neutralising buffer (0.5 M dibasic phosphate buffer pH 9.0) and phosphate buffer saline (0.1 M, pH 7.0) used to dilute hair extracts in the ELISA test were also obtained from the Immunalysis Corporation. Disposable borosilicate glass culture tubes (75 x 12 mm) were obtained from VWR International, UK. The drug standards used to assess kit cross reactivity were purchased from LGC Promochem (Teddington, UK).

6.3.3 Instrumentation

A MiniPrep 75 automatic pipettor from Tecan (San Jose, CA) was used. The microplate wells were washed and read using a Columbus Plus washer system and a Sunrise Remote EIA autoreader also from Tecan (Grödlg, Austria).

6.4 ELISA Method

The post-mortem hair samples were firstly analysed by LC-MS-MS as described in chapters 7 and 8. Only samples which had a sufficient amount of sample left were analysed by ELISA.

6.4.1 Sample Preparation

The root-0.5 cm segment was removed from each hair sample and the remaining hair segmented depending on the length. The decontamination procedure used was the same as the LC-MS-MS procedure for the purposes of method comparison. Each section was washed once with 0.1 % sodium dodecyl sulfate with a 10 minute ultrasonication, twice with deionised water with a 10 minute ultrasonication and twice with dichloromethane with a 10 minute ultrasonication. Dichloromethane was chosen for the organic washes since it was demonstrated that no more than 10 % of cocaine, opiates and benzodiazepines were extracted when dichloromethane was used to wash real hair samples.[208] It was not however known if the drugs detected in the dichloromethane washes were incorporated through use or passive exposure.

After decontamination, the hair segments were cut into smaller segments of 2-3 mm in length using a pair of clean scissors. A previously published aqueous extraction method was used which had been applied in the ELISA screening of cocaine and amphetamine in hair.[108,109] Spiked hair calibrators were prepared for each assay at the cut-off value, 2 x cut-off value and 5 x cut-off value by adding the appropriate concentration of target drug to 10 mg blank decontaminated hair. The concentrations used for the calibrators are given in Table 6-1. A blank calibrator was also prepared. Negative and positive spiked controls were prepared in-house and these were distributed across the plate to ensure consistent system performance.

The cut-off values used for cocaine and opiates were recommended by the Society of Hair Testing (SoHT) and SAMHSA.[100,101] The cut-off values used for the amphetamines was the GC-MS cut-off used by the toxicology laboratory in Seoul[59] and had to be the same for the purposes of comparing the ELISA and GC-MS methods as discussed in section 6.6.4.4. The same screening cut-off was

proposed for amphetamines by SAMHSA.[101] ELISA cut-off values have not yet been recommended for methadone or benzodiazepine screening in hair by the SoHT or SAMHSA.

The methadone cut-off selected was therefore the same as the opiates (0.2 ng/mg). This concentration produced a B/B_0 (%) value just above 70 % but was still in the linear portion of the dose response curve. The benzodiazepine cut-off was 0.1 ng/mg as this concentration produced the optimum sensitivity and specificity for 13 positive hair samples compared with a validated LC-MS-MS reference method as discussed later in section 7.6.4.[111]

Table 6-1 ELISA cut-off values

ELISA kit	Cut-off (ng/mg)	2 x Cut-off (ng/mg)	5 x Cut-off (ng/mg)
Amphetamines	0.5	1	2.5
Benzodiazepines	0.1	0.2	0.5
Cocaine	0.5	1	2.5
Methamphetamine	0.5	1	2.5
Methadone	0.2	0.4	1.0
Opiates	0.2	0.4	1.0

0.5 mL of monobasic phosphate buffer was added to each sample, calibrator and control then the vials were capped and incubated at 60 °C for 1 hour in an oven. After cooling, 50 µL of dibasic phosphate buffer was added to neutralise the acidic buffer and the extracts were transferred to labelled EIA glass culture tubes. All extracts were then diluted 1:5 on-line by adding 800 µL phosphate buffer saline to 200 µL hair extract. The diluted extracts were then vortex mixed.

6.4.2 ELISA Assay

An aliquot of the diluted extract was added to the individual micro-plate well coated with the target drug specific antibody, depending on the ELISA kit used. The aliquot volume used was dependent on the ELISA test for a particular drug class and the sensitivity required. The sample volumes used for each drug class (Table 6-2) gave good separation of absorbance values between the blank calibrator and the spiked cut-off calibrator.

After adding the aliquots of diluted extract, 100 µL of HRP enzyme labelled target drug derivative (specific to the ELISA test) was added to each well. After a 1 hour incubation period at room temperature, the micro-plate wells were washed 6 times with deionised water (6 x 300 µL). 100 µL TMB was added to each well and the plate incubated in the dark at room temperature for a further 30 minutes. The reaction was stopped using 100 µL 1 N hydrochloric acid and the plate read at 450 nm with a reference wavelength of 650 nm. The case samples were considered to be presumptively positive if the B/B_0 (%) value was lower than the B/B_0 (%) value of the cut-off calibrator.

Table 6-2 Aliquot volumes used in each ELISA test

ELISA Test	Aliquot volume (µL)
AMP	25
Benzodiazepines	50
Cocaine	20
Methadone	50
MAMP	25
Opiates	25

6.5 ELISA Method Validation

6.5.1 Dose Response Graph

Dose response curves were generated for all drugs of abuse assays over the concentration range 0.05-5 ng/mg target drug (dependent on the assay). The target drug demonstrated 100 % cross reactivity with a particular assay according to the ELISA kit manufacturers and these are named in Table 6-3 for each kit. A blank was also prepared as a reference for calculating the B/B_0 (%) values. The B/B_0 (%) values were calculated where B_0 is the absorbance value of the blank calibrator and B is the absorbance value of the bound calibrator. Although the methadone assay cross reacts 100 % with *l*-methadone, *d,l*-methadone was used to spike calibrators since methadone is usually consumed in the racemic form. As discussed in section 6.6.2, an acceptable sensitivity level was achieved using *d,l*-methadone.

The curves were generated by spiking 10 mg of blank decontaminated hair with the appropriate volume of target drug solution at 0.1 µg/mL (5, 10, 25, 50 µL) for the lower concentrations and 1 µg/mL (10, 25, 50 µL) for the higher

concentrations. The spiked hair standards were 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 ng/mg target drug.

Table 6-3 Target drugs for particular ELISA kits

ELISA kit	Target drug
AMP	*d*-Amphetamine
Benzodiazepine	Oxazepam
Cocaine	Cocaine
Methadone	*l*-Methadone
MAMP	*d*-Methamphetamine
Opiate	Morphine

6.5.2 LOD and Assay Precision

The LOD was calculated from the mean B/B_0 (%) value (A_0) and standard deviation(s) of the B/B_0 (%) values for 10 x 10 mg confirmed negative hair samples. This has been recommended as the minimum number of blank wells that should be run when estimating the LOD.[102] 10 mg spiked blank hair samples were prepared at low concentrations (0.025, 0.05, 0.075 and 0.1 ng/mg target drug) and the B/B_0 (%) value calculated for the LOD for each assay was matched with the B/B_0 (%) value of the spiked calibrator to determine the LOD in ng/mg.

The precision was tested at ½ x cut-off, the cut-off and 2 x cut-off as the SOFT guidelines state that the precision of a screening assay must be demonstrated at and around the cut-off value.[11] The cut-off concentrations are provided in Table 6-1. Within-sample precision (n=10) was calculated from 10 B/B_0 (%) readings of the same spiked hair sample. Intra-day precision (n=20) of the B/B_0 (%) was calculated from duplicate readings of 10 spiked hair samples. The intra-day precision experiment was carried out on five separate days to calculate the inter-day precision (n=100).

6.5.3 Cross Reactivity

The cross-reactivity of the ELISA kits was tested and the results compared with those provided by the manufacturers. The drugs tested were considered analogues of the target drug and were included in the validated LC-MS-MS method described in chapter 8. Other common drugs of abuse with no obvious structural similarity to the target drug were also tested. The list of drug analogues tested for each assay is given in Table 6-7-6-12.

Cross reactivity was calculated by constructing a target drug dose response curve and a dose response curve for the drug to be tested. The drugs were spiked into phosphate buffer saline (pH 7.0) for testing. The concentration of target drug producing the same B/B_0 (%) value as the drug being tested was determined at a given concentration. The cross reactivity was then calculated by dividing this target drug concentration by the concentration of drug being tested and multiplying by 100 to express as a percentage.

6.6 ELISA Validation Results and Discussion

6.6.1 Dose Response Curves

Dose response curves were generated for all drugs of abuse assays (Figure 6-1- Figure 6-6). The results were obtained in duplicate for each concentration and an average B/B_0 (%) value was calculated. The B/B_0 (%) values showed a hyperbolic decrease for all assays with increasing target drug concentration. As discussed in chapter 5, this is because of the inverse relationship that exists between absorbance and drug concentration when using ELISA. From the dose response curves, the B/B_0 (%) values for the cut-off calibrators were all below 70 % and were in the linear portion of the curve. The ELISA kit manufacturers recommend that the selected cut-off concentration produces a B/B_0 (%) below 70 % to ensure the highest number of true results.

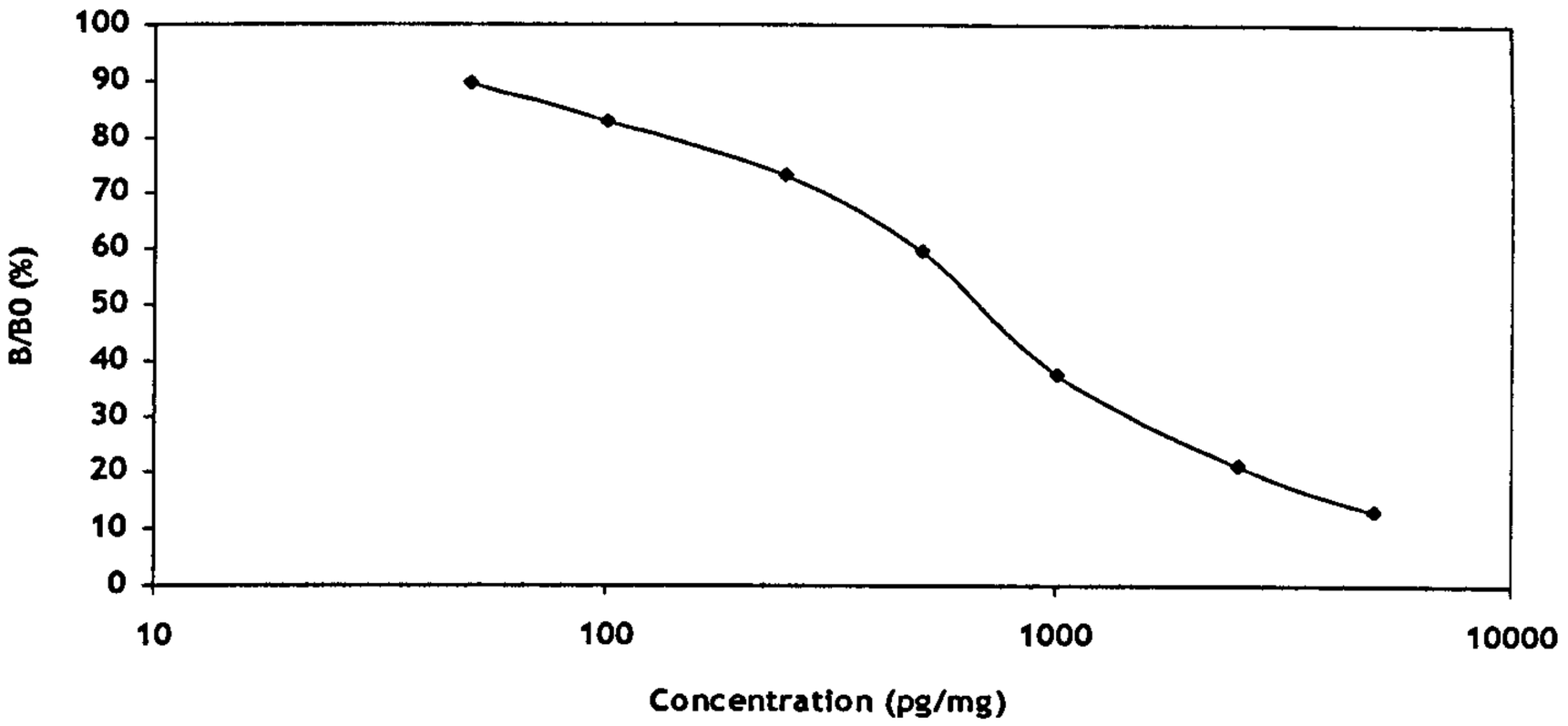


Figure 6-1 *d*-Amphetamine dose response curve

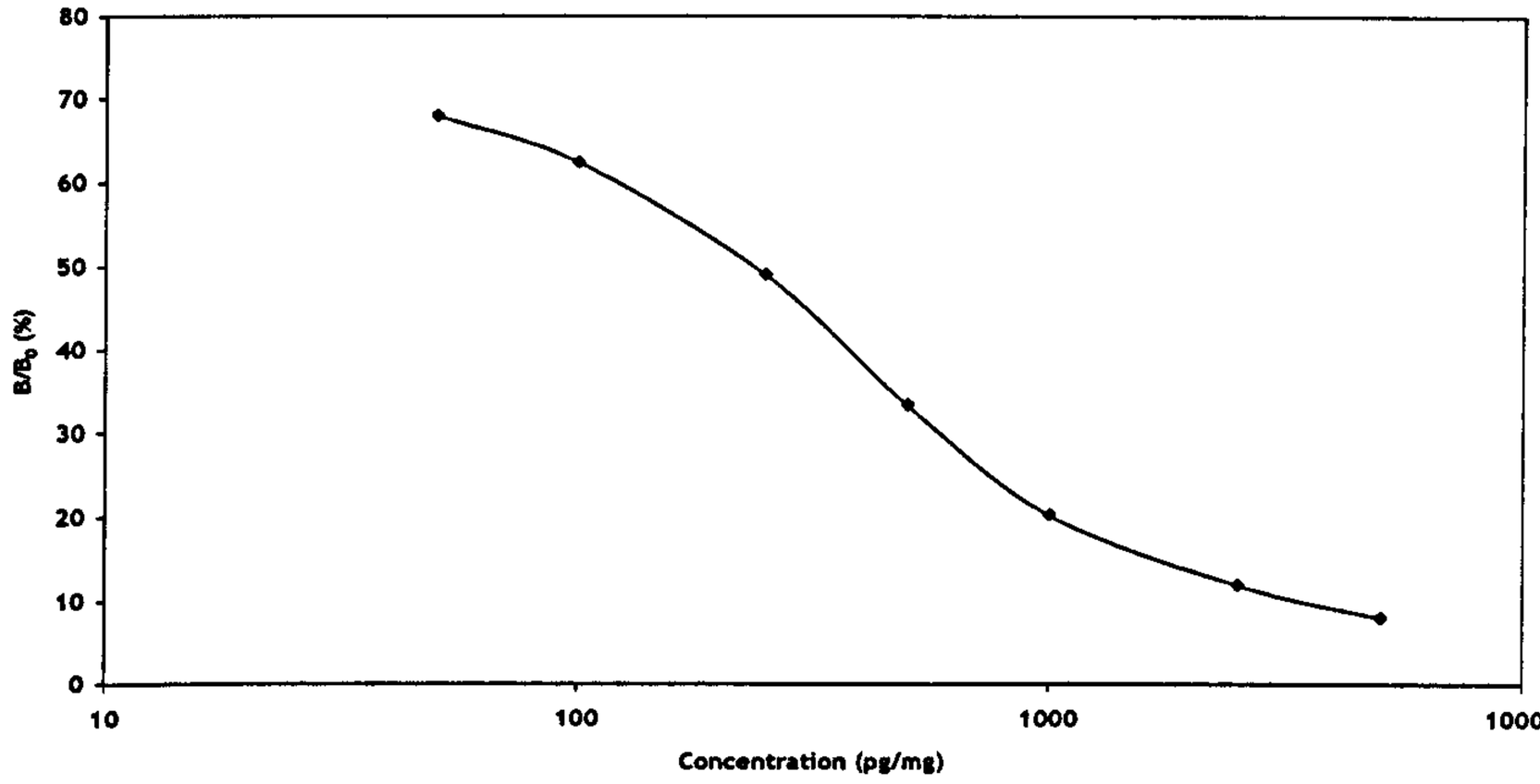


Figure 6-2 Oxazepam dose response curve

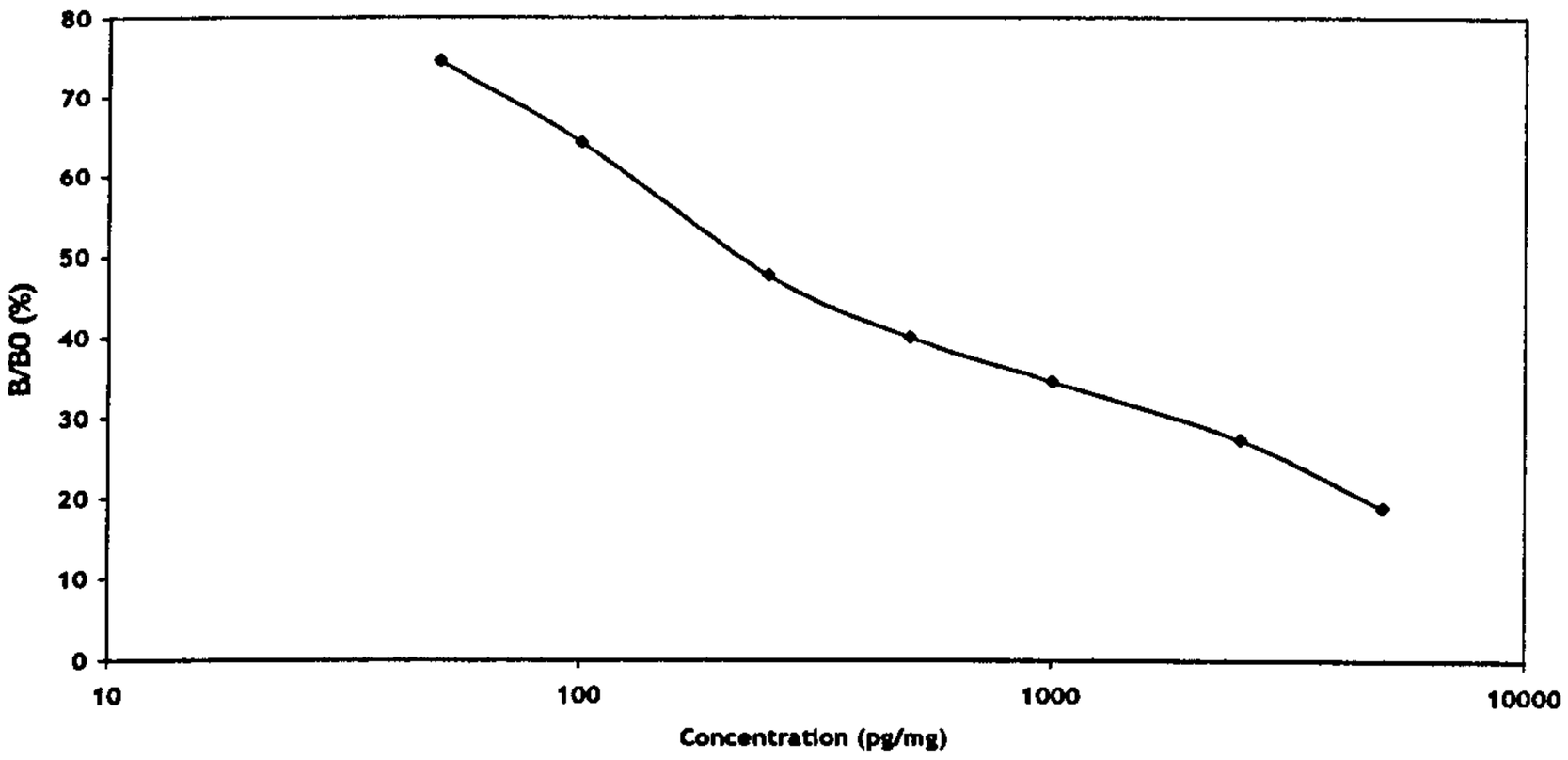


Figure 6-3 Cocaine dose response curve

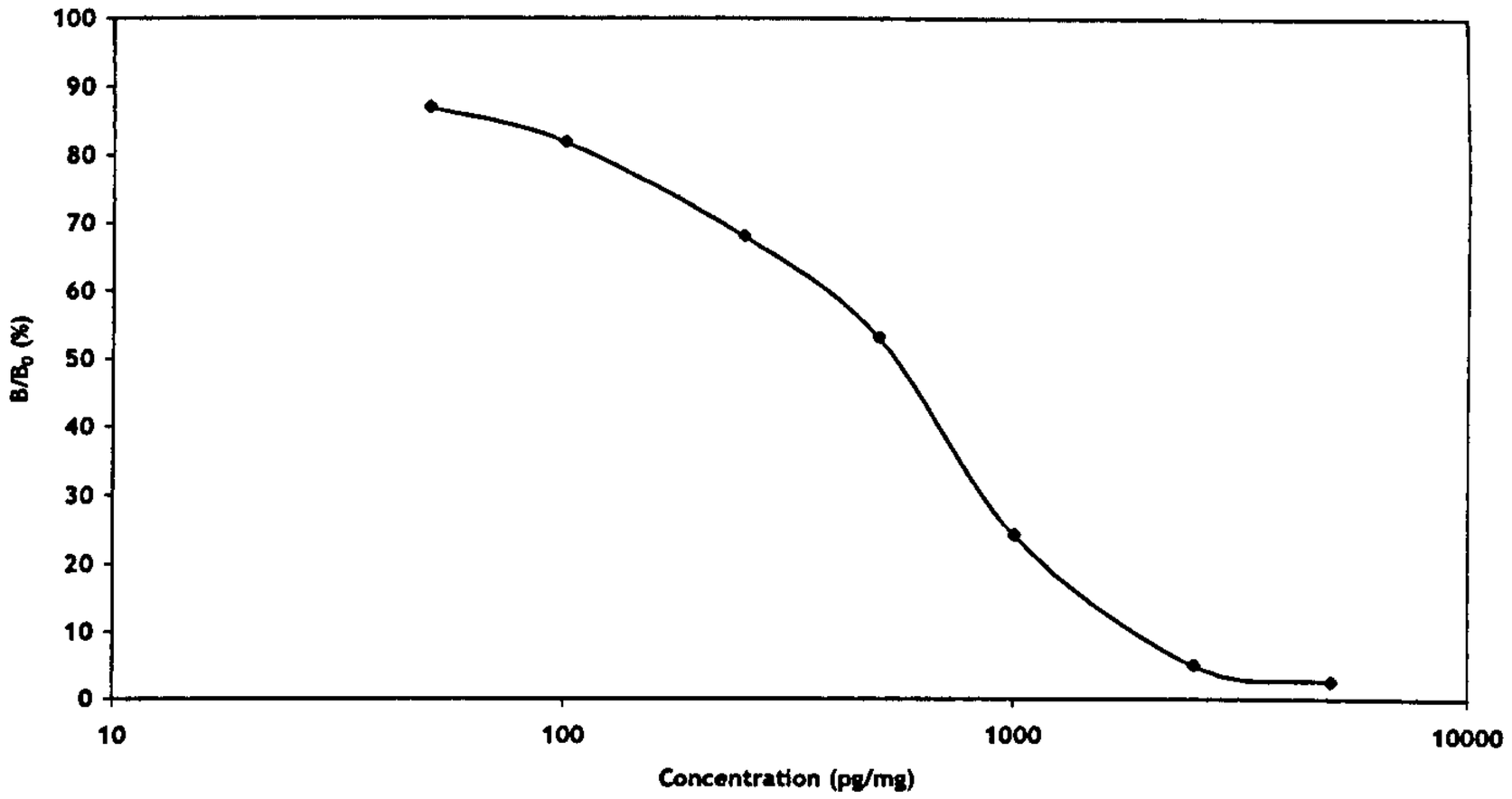


Figure 6-4 *d,l*-Methadone dose response curve

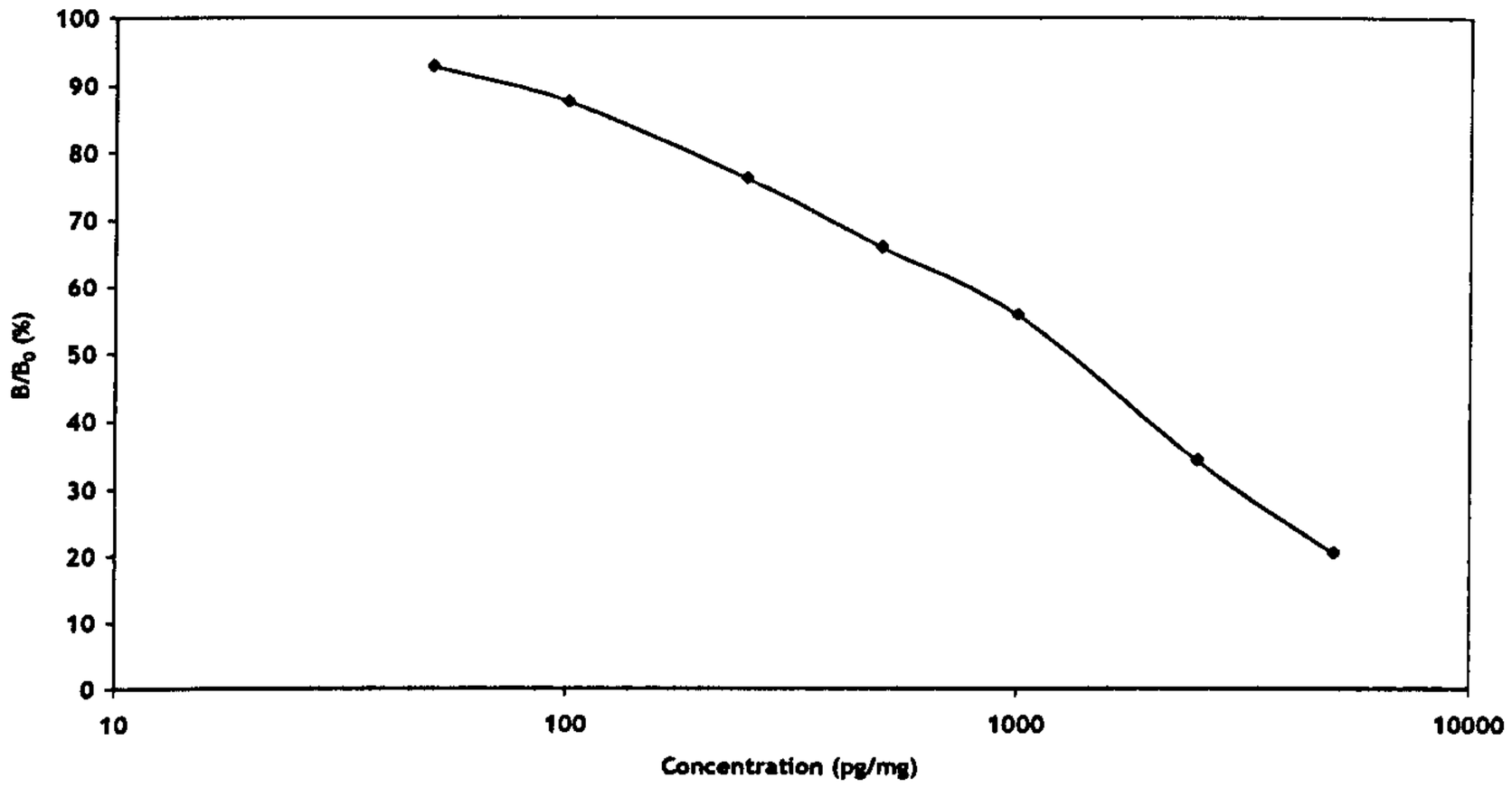


Figure 6-5 *d*-MAMP dose response curve

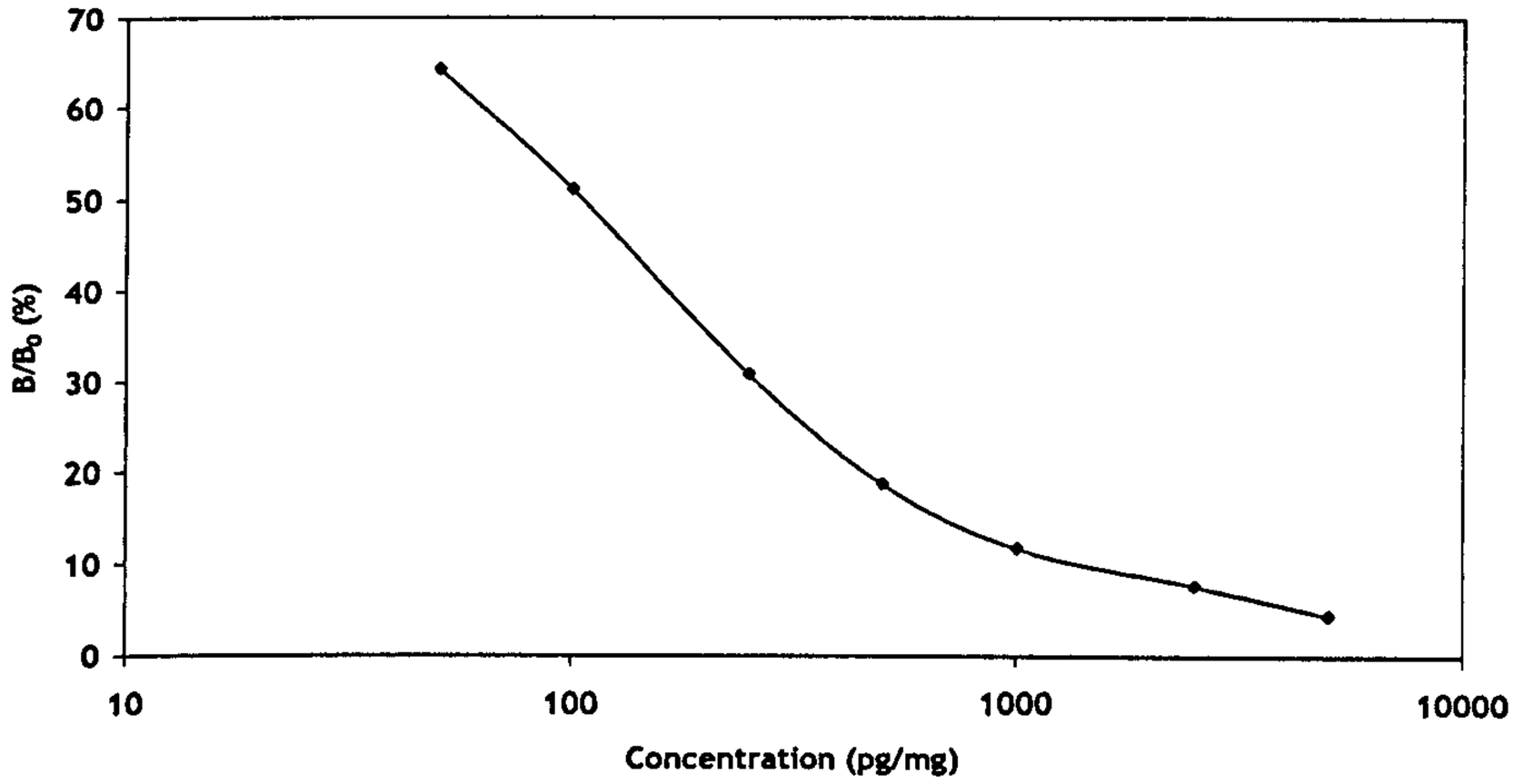


Figure 6-6 Morphine dose response curve

6.6.2 LOD and Assay Precision

The LOD values for the drugs of abuse assays are shown in Table 6-4. With the exception of the benzodiazepines assay, the LOD values were lower than the cut-off concentrations used for screening. Although the cut-off concentration used for the benzodiazepine assay was the same as the LOD, the B/B_0 (%) was 70 %. Furthermore this cut-off concentration was found to produce the greatest number of true results when compared to a validated LC-MS-MS method as discussed later in section 7.6.4.[111]

Precision data is shown in Table 6-5 and Table 6-6. There are currently no published guidelines on acceptable levels of precision for drug screening by ELISA however all within sample and intra-day data was < 9 % at all levels tested, which is accepted as good precision in chromatographic methods according to SOFT guidelines.[11] Inter-day precision was higher as expected due to the greater number of results and the fact that the tests were carried out on five separate days.

Table 6-4 ELISA LOD values

ELISA Assay	LOD (ng/mg)
AMP	0.05
Benzodiazepine	0.1
Cocaine	0.05
Methadone	0.1*
MAMP	0.05
Opiate	0.05

*n=8 for blank samples

Table 6-5 ELISA within-sample precision

ELISA Assay	Within-sample Precision (% RSD for n=10)		
	½ x Cut-Off	Cut-Off	2 x Cut-Off
AMP	4.9	3.1	4.1
Benzodiazepine	4.1	3.9	3.1
Cocaine	6.5	5.5	4.3
Methadone	6.0	4.0	6.9
MAMP	3.0	4.0	3.6
Opiate	6.4	6.9	6.1

Table 6-6 ELISA intra-day and inter-day Precision

ELISA Assay	Intra-day Precision (n=20)			Inter-day Precision (n=100)		
	½ x Cut-Off	Cut-Off	2 x Cut-Off	½ x Cut-Off	Cut-Off	2 x Cut-Off
AMP	8.0	8.3	7.4	8.7	9.7	11.9
Benzodiazepine	6.9	4.6	2.3	8.6	8.2	5.9
Cocaine	7.7	6.9	5.9	10.5	11.9	13.5
Methadone	8.3	7.3	6.5	10.9	13.2	17.4
MAMP	8.8	7.4	5.7	6.6	7.6	6.7
Opiate	8.7	8.4	7.6	11.9	10.9	14.1

6.6.3 Cross Reactivity

Table 6-7 Cross reactivity of amphetamine analogues with amphetamine ELISA kit

Compound	Approx. ng/mL equivalent to 25 ng d-AMP	% Cross Reactivity quoted by Immunalysis®	% Cross Reactivity Found
l-AMP	1700	2.5	1.5
d-MAMP	> 1000	6.5	< 1.0
l-MAMP	> 2000	2.0	< 1.0
d,l-MDA	11	250	263
d,l-MDMA	> 2500	< 1.0	< 1.0
d,l-MDEA	> 2500	< 1.0	< 1.0

Overall, the cross reactivity values determined for the amphetamine specific kit were comparable to the values quoted by the manufacturers. A relatively high cross reactivity was demonstrated for MDA however the kit had less than 1 % cross reactivity with *d*- and *l*-methamphetamine, *d,l*-MDMA and *d,l*-MDEA. The kit manufacturers have two different ELISA kits to accommodate regional differences in amphetamine abuse which are the amphetamine specific and the methamphetamine specific kits. As mentioned previously, the amphetamine specific ELISA is 100 % cross reactive with *d*-amphetamine, which is the illicit form. As the cross reactivity of the licit *l*-AMP enantiomer is very low, this ELISA test is an excellent means of distinguishing between illegal amphetamine use and medication. Furthermore it could eliminate the need for chiral separation of the amphetamine enantiomers in the confirmation method. The methamphetamine specific ELISA kit which is 100 % cross reactive with the illicit *d*-methamphetamine should be used in tandem with the *d*-amphetamine specific kit.

Table 6-8 Cross reactivity of oxazepam analogues with benzodiazepine ELISA kit

Compound	Approx. ng/mL equivalent to 100 ng oxazepam	% Cross Reactivity quoted by Immunalysis®	% Cross Reactivity Found
7-aminoflunitrazepam (7-AF)	1300	Not given	8
Chlordiazepoxide	750	17	13
Diazepam	125	91	80
Flunitrazepam	310	31	32
Lorazepam	860	13	12
Nitrazepam	320	33	31
Nordiazepam	70	150	143
Temazepam	130	83	77

The experimental cross reactivity values were comparable with the manufacturer's values for the benzodiazepine ELISA kit. As shown in chapters 7 and 8, the majority of hair samples tested positive for diazepam and its metabolites by LC-MS-MS so this kit is ideally suited for screening.

The kit may not be suitable for screening hair samples positive for chlordiazepoxide, flunitrazepam and its metabolite, lorazepam or nitrazepam as they cross-react to a relatively lower extent than diazepam and its metabolites. This study did not investigate this potential problem due to a lack of positive samples for these drugs alone.

There is a commercially available flunitrazepam ELISA kit which should ideally be used in tandem with the benzodiazepine ELISA kit for screening. These kits cross react 100 % with the 7-aminoflunitrazepam (7-AF) metabolite which has generally been found in higher concentrations in hair, due to its higher basicity. The kit was not tested for the purposes of this study since there were no LC-MS-MS positives for flunitrazepam or 7-AF as shown in chapters 7 and 8.

Table 6-9 Cross reactivity of cocaine metabolites with cocaine ELISA kit

Compound	Approx. ng/mL equivalent to 50 ng cocaine	% Cross Reactivity quoted by Immunalysis®	% Cross Reactivity Found
Benzoylecgonine	960	7.6	5.2
Cocaethylene	40	110	125
Ecgonine methyl ester (EME)	> 5000	< 1.0	< 1.0

This cocaine specific ELISA kit was developed for the detection of cocaine in hair. It is well known that parent drug concentrations are higher than metabolite concentrations in hair so it is more appropriate for a screening test to be calibrated towards the parent drug. Cocaine and cocaethylene are the primary analytes found in hair after cocaine ingestion hence the high % cross reactivity for cocaethylene. Benzoylecgonine is present at lower levels but has been shown to be an artefact of some sample treatment and extraction procedures involving acidic and alkaline hydrolysis. Cocaethylene is only present when the individual has used both cocaine and alcohol at the same time and is a useful marker of cocaine use. EME levels have not been widely reported in hair. In this study as discussed in chapter 8, it was detected in a very small number of samples at low levels.

Table 6-10 Cross reactivity of methadone analogue with methadone ELISA kit

Compound	Approx. ng/mL equivalent to 300 ng methadone	% Cross Reactivity quoted by Immunalysis®	% Cross Reactivity Found
EDDP	13,000	< 5.0	2.3

Like other drugs of abuse, methadone is found in hair at higher levels in hair than EDDP. In fact, EDDP is not detected in some cases where methadone is present.[208] Reported concentration ratios of the EDDP metabolite and methadone parent drug are 0.05-0.67.[90]

Table 6-11 Cross reactivity of MAMP analogues with MAMP ELISA kit

Compound	Approx. ng/mL equivalent to 50 ng *d*-MAMP	% Cross Reactivity quoted by Immunalysis®	% Cross Reactivity Found
d,l-MAMP	80	65	63
l-MAMP	> 2000	8.0	<1.0
d,l-MDMA	38	135	132
d-AMP	> 10,000	2.0	< 1.0
l-AMP	> 2000	3.4	< 1.0
d,l-MDA	> 10,000	< 1.0	< 1.0
d,l-MDEA	1500	10	3.3

As shown in Table 6-11, the methamphetamine specific ELISA kits cross reacts with the racemic mixture of methamphetamine and MDMA to a relatively high extent but to a low extent with the other designer drugs MDA and MDEA and amphetamine enantiomers. The experimental values were mostly comparable to those provided by the manufacturer. However there was a greater difference in the value obtained for *l*-methamphetamine. The cross reactivity determined experimentally was lower than the cross reactivity quoted by the manufacturers. This is an advantage when screening samples for illicit methamphetamine as it is unlikely that the licit form would be detected in the screen due to its low cross reactivity.

Table 6-12 Cross reactivity of morphine analogues with opiate ELISA kit

Compound	Approx. ng/mL equivalent to 25 ng morphine	% Cross Reactivity quoted by Immunalysis®	% Cross Reactivity Found
Codeine	11	200	227
Dihydrocodeine	58	Not given	43
6-MAM	320	83	69

After heroin use, hair often contains primarily 6-MAM, with smaller quantities of diamorphine and morphine. Therefore this opiates ELISA is suited to hair

screening due to its relatively high cross reactivity with 6-MAM. The kit is also highly cross reactive with codeine which can be present in the hair of heroin abusers. Morphine and codeine which are extracted from the Papaver Somniferum poppy are acetylated during the production of illicit heroin. Acetylcodeine is metabolised to codeine after heroin use.

Dihydrocodeine was tested as it is commonly prescribed as a narcotic analgesic in Scotland and is frequently detected in blood and urine samples submitted to the laboratory for routine testing.

6.6.3.1 Cross Reactivity of Structurally Unrelated Drugs

Various drugs of abuse, which are not structurally related to the target drug in a particular assay, were tested at 10,000 ng/mL. This included amitriptyline, *d*-amphetamine, buprenorphine, cocaine, codeine, 6-MAM, MDMA, methadone, morphine, Δ^9THC-COOH and tramadol. All of the compounds tested for a particular assay were tested in duplicate and did not produce a B/B_0 (%) value that was less than that produced from the assay sensitivity levels shown in Table 6-4.

6.6.4 Case Samples

ELISA results are provided for post-mortem hair samples submitted to the laboratory for opiates, cocaine and methadone testing. These were initially compared to GC-MS results obtained by in-house methods used within the Forensic Medicine and Science Section at the University of Glasgow. In brief, this involved incubating the hair in methanol overnight at 45 °C, removing the methanol then incubating the hair overnight in 0.1 M hydrochloric acid at 45 °C. The combined extracts were cleaned up using Bond Elut Certify™ SPE cartridges. Opiates were derivatised using *N,O*-Bis (Trimethylsilyl) trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane (TMCS). Cocaine and its metabolites were derivatised using pentafluoropropionic anhydride (PFPA) and pentafluoro-1-propanol (PFPOH) prior to GC-MS analysis.

Methamphetamine ELISA results for the collaboration with the Korean laboratory are provided in section 6.6.4.4. Results for other post-mortem samples screened for amphetamines, benzodiazepines, cocaine and opiates are provided in

chapters 7 and 8 respectively as these were compared to LC-MS-MS methods which were developed and validated as part of the work for this thesis.

6.6.4.1 Opiates

The results for opiate screening in 20 post-mortem hair samples are provided in Table 6-13. 10 drug-free samples collected from volunteers working in the laboratory were also screened. The root-0.5 cm section was removed in each case and was not analysed as the sample weight was generally less than 5 mg. The remaining hair sample was decontaminated using the procedure described in section 6.4.1, cut up into 2-3 mm segments, extracted and analysed by ELISA.

Table 6-13 ELISA vs in-house GC-MS method for opiates

ELISA			GC-MS				
Sample Number	Weight (mg)	MOR Equivalents (ng/mg)	Weight (mg)	MOR	6-MAM	COD	DHC
1	7.50	> 1.0	17.89	17.9	39.9	3.2	0.2
2	5.21	< 0.2	39.71	-	-	-	0.4
3	13.98	0.25	34.20	0.5	1.1	-	-
4	15.60	> 1.0	31.00	0.7	0.4	3.4	4.6
5	2.93	> 1.0	15.42	5.2	7.3	1.8	-
6	10.40	> 1.0	82.96	3.8	6.5	7.6	4.2
7	10.46	> 1.0	19.62	18.8	18.8	5.0	4.0
8	6.04	> 1.0	14.52	13.5	23.3	2.5	-
9	8.83	< 0.2	10.04	-	-	-	-
10	18.66	> 1.0	38.42	3.8	9.2	0.8	-
11	7.18	0.38	58.08	0.7	1.7	-	-
12	1.98	> 1.0	5.99	6.8	7.5	1.8	1.7
13	10.03	> 1.0	13.83	0.2	-	-	0.6
14	6.36	> 1.0	12.07	0.3	1.4	0.8	1.6
15	10.45	> 1.0	47.66	35.4	38.5	17.1	0.4
16	20.74	> 1.0	67.73	10.9	23.3	4.7	0.2
17	10.23	> 1.0	25.43	-	-	4.55	-
18	6.68	0.37	10.55	-	-	-	0.6
19	5.51	> 1.0	9.56	0.7	1.9	-	-
20	9.05	0.24	9.52	1.5	2.7	0.4	-

"MOR"=Morphine,"6-MAM"=6-Monoacetylmorphine,"COD"=Codeine,"DHC"= Dihydrocodeine

The ELISA sensitivity and specificity were 95 % and 100 % respectively using the SoHT cut-off of 0.2 ng/mg morphine. Sample 2 produced a FN ELISA result however the sample weight and the dihydrocodeine level detected by GC-MS were both low. The cross reactivity of dihydrocodeine with the opiates kit was found to be 43 %, further explaining this result.

6.6.4.2 Cocaine

The results for cocaine screening of 16 post-mortem hair samples tested in section 6.6.4.1 are presented in Table 6-14. The sample numbers correspond to the ones given in Table 6-13.

Table 6-14 ELISA vs in-house GC-MS method for cocaine

ELISA			GC-MS-MS			
Sample Number	Weight (mg)	COC Equivalents (ng/mg)	Weight (mg)	COC	BZE	COCAETH
1	7.50	< 0.5	17.89	-	-	-
2	5.21	< 0.5	39.71	-	-	-
4	15.60	0.57	31.00	0.7	-	-
5	2.93	< 0.5	15.42	-	-	-
6	10.40	> 2.5	82.96	32.5	14.1	-
7	10.46	< 0.5	19.62	0.2	0.03	-
8	6.04	> 2.5	14.52	172.0	17.1	-
10	18.66	< 0.5	38.42	-	-	-
11	7.18	< 0.5	58.08	-	-	-
12	1.98	< 0.5	5.99	-	-	-
13	10.03	> 2.5	13.83	1.2	-	0.2
14	6.36	> 2.5	12.07	0.5	-	-
15	10.45	< 0.5	47.66	0.4	-	-
17	10.23	> 2.5	25.43	2.0	-	0.1
18	6.68	< 0.5	10.55	-	-	-
19	5.51	< 0.5	9.56	-	-	-

"COC"=Cocaine, "BZE"=Benzoylecgonine, "COCAETH"=Cocaethylene

There were a smaller number of cocaine positive samples (n=8) compared to the number of opiate positive samples (n=19). All of the cocaine positive samples were also positive for opiates. The ELISA sensitivity and specificity for this small number of positive cocaine samples compared to the in-house validated GC-MS method was 75 % and 100 % using the recommended SoHT and SAMHSA cut-off of 0.5 ng/mg cocaine. A greater number of positive cocaine samples need to be screened to determine if a lower cut-off value is required for future screenings. However from the GC-MS results for the 2 FN samples (samples 7 and 15), it would appear that this was necessary.

6.6.4.3 Methadone

The results for methadone screening for 20 post-mortem hair samples tested in 6.6.4.1 and 6.6.4.2 are presented in Table 6-15. The sample numbers correspond to the ones given in Table 6-13 and Table 6-14.

Table 6-15 ELISA vs in-house GC-MS method for methadone

ELISA			GC-MS-MS		
Sample Number	Weight (mg)	*d,l*-MD Equivalents (ng/mg)	Weight (mg)	MD	EDDP
1	7.50	> 1.0	17.89	70.6	10.9
2	5.21	< 0.2	39.71	-	-
3	13.98	< 0.2	34.20	-	-
4	15.60	< 0.2	31.00	-	-
6	9.89	0.27	82.96	2.8	0.6
7	10.46	0.82	19.62	11.5	2.2
8	1.03	< 0.2	14.52	3.7	0.4
9	8.83	< 0.2	10.04	-	-
10	18.66	0.51	38.42	7.7	0.7
11	7.18	< 0.2	58.08	-	-
14	6.36	< 0.2	12.07	-	-
15	10.45	< 0.2	47.66	-	-
16	20.74	> 1.0	67.73	25.6	6.4
17	10.23	< 0.2	25.43	-	-
18	6.68	< 0.2	10.55	-	-
19	5.51	< 0.2	9.56	-	-
20	9.05	< 0.2	9.52	-	-

"MD"=Methadone, "EDDP"=2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine

There was a small number of hair samples which tested positive for methadone (n=6). 5 out of 6 of these tested positive by ELISA and GC-MS to give an ELISA sensitivity of 83 % at a cut-off of 0.2 ng/mg *d,l*-methadone. The FN result produced by sample 8 is due to an insufficient sample weight for ELISA. All of the methadone positive samples also tested positive for opiates.

6.6.4.4 Methamphetamine: Korean Collaboration

29 hair samples were screened for methamphetamine using ELISA as part of a collaboration with the National Institute of Scientific Investigation in Seoul, South Korea. The samples had been submitted to the laboratory in Seoul by the police force. The reason for the collaboration was that there is currently an insufficient number of methamphetamine positive hair samples in Scotland as it

is not a common drug of abuse. In addition, 9 drug free samples were collected from laboratory personnel and these were all screened and confirmed as negative.

The ELISA results obtained within the Forensic Medicine and Science Section at the University of Glasgow were compared with a validated GC-MS method that is currently used in the in the National Institute of Scientific Investigation in Seoul (Table 6-16).[112]

Table 6-16 ELISA vs GC-MS methamphetamine positive hair results

ELISA			GC-MS		
Sample Number	Weight (mg)	*d*-MAMP Equivalents (ng/mg)	Weight (mg)	*d,l*-MAMP (ng/mg)	*d,l*-AMP (ng/mg)
1	7.01	> 5.0	4.6	19.5	0.9
2	9.00	< 0.5	12.7	1.2	0.1
3	7.08	0.7	7.0	2.2	0.0
4	8.10	> 5.0	12.7	9.4	1.1
5	7.04	> 5.0	2.0	22.7	0.9
6	9.57	> 5.0	17.2	12.7	0.3
7	9.51	> 5.0	19.0	2.4	0.2
8	9.37	1.6	6.5	20.4	0.8
9	9.50	2.7	18.5	3.3	0.2
10	7.73	> 5.0	4.4	8.0	0.4
11	9.86	2.9	19.3	6.6	1.0
12	8.05	> 5.0	10.2	11.9	0.8
13	7.18	> 5.0	9.9	45.3	2.5
14	8.13	1.6	12.2	4.1	0.3
15	9.28	3.0	19.2	7.2	0.5
16	8.51	2.8	14.5	4.4	0.2
17	8.10	> 5.0	7.8	9.3	0.9
18	9.09	> 5.0	12.8	10.6	0.4
19	9.73	3.0	19.0	12.7	0.4
20	9.06	> 5.0	19.8	27.5	2.4
21	10.40	2.4	20.1	2.7	0.2
22	9.83	1.5	19.4	8.1	1.0
23	7.11	> 5.0	16.6	12.5	0.4
24	10.15	> 5.0	16.5	10.2	0.3
25	9.27	> 5.0	16.6	11.7	0.6
26	10.21	> 5.0	18.8	20.4	0.9
27	6.68	> 5.0	14.0	8.1	0.60
28	10.77	0.6	19.0	1.8	0.1
29	10.89	> 5.0	19.5	6.9	1.1

"MAMP"=Metamphetamine, "AMP"=Amphetamine

The ELISA method is described previously in section 6.4.1 and section 6.4.2. The same decontamination method was used by the Korean lab to enable accurate ELISA and GC-MS comparison. In brief for the GC-MS analysis, the samples were incubated in methanol containing 1 % v/v hydrochloric acid for 20 hours at 45 °C. The methanol extracts were blown down under nitrogen and derivatised with trifluoroacetic acid (TFA) for 15 minutes at 65 °C.

The ELISA sensitivity and specificity were 97 % and 100 % respectively, which are in the accepted range of values[200] however the FN sample (sample 2) would have screened as positive at a lower cut-off value and this supports the recommendation by the SoHT. 28 out of 29 samples were screened and confirmed as positive using the cut-off in place in the Korean lab of 0.5 ng/mg *d,l*-methamphetamine. GC-MS analysis found that this sample contained the lowest quantity of methamphetamine of all the samples tested (1.2 ng/mg). Average ELISA absorbance values for this sample and the cut-off calibrator were very close to each other (1.30 and 1.25 respectively). Furthermore, one of the duplicate sample absorbance values was 1.22 (a positive result) but the average absorbance value meant the screening result was negative. The sample was re-run on another day with similar results. Samples that screen at or near the ELISA cut-off should be further tested by confirmation.[2] The FN result may be a result of using different ELISA and GC-MS extraction methods.

A study published by the National Institute of Scientific Investigation in 2006 determined criteria for obtaining positive results using the metabolite-to- parent drug ratios for methamphetamine in scalp hair.[215] 2389 positive scalp hair samples collected from methamphetamine abusers were examined in the study. Amphetamine to methamphetamine drug ratios ranged from 0.04-1.16 (mean 0.09). The hair samples were washed twice with water for 5 minutes and twice with methanol for 5 minutes. The authors propose that the detection of metabolites and parent drug within this ratio range is a useful means of distinguishing between external contamination and drug incorporation through ingestion.

In the collaborative work presented in this thesis, the amphetamine to methamphetamine ratios ranged from 0.02-0.159 (mean 0.07). As the same GC-MS extraction method was used, the difference in the ratio range is probably due

to the different decontamination procedure used. 1 sample only contained methamphetamine.

SAMHSA guidelines stipulate that a hair sample which has been confirmed as positive for methamphetamine using a cut-off concentration of 0.3 ng/mg should also contain amphetamine levels ≥ LOD of the method.[101]

6.6.5 Conclusion

An aqueous extraction method was validated for the purposes of ELISA screening of amphetamine, benzodiazepines, cocaine, methadone, methamphetamine and opiates in hair. The assays demonstrated high sensitivity, good precision and appropriate cross reactivity with the analytes found in hair. The ELISA method was applied successfully for the screening of post-mortem samples for cocaine, methadone and opiates in this chapter. There was an excellent correlation observed between the ELISA and in-house GC-MS method for opiates, with a sensitivity and specificity of 95 % and 100 % respectively. There was a small number of cocaine (n=8) and methadone (n=6) positive samples. This preliminary study suggested that a lower cut-off value may be required for cocaine screening as 2 FN results were produced using the SoHT and SAMHSA currently recommended cut-off of 0.5 ng/mg. A cut-off value of 0.2 ng/mg appears to be suitable for methadone screening. The 1 FN result was probably a result of a very low sample weight of approximately 1 mg. However more data is required to determine if the cut-off values recommended by the SoHT and SAMHSA for opiates and cocaine are suitable for screening hair samples.

Hair samples collected from living methamphetamine abusers were also screened successfully using this ELISA method. Excellent correlation was observed between the ELISA and GC-MS methods, with a sensitivity and specificity of 97 % and 100 % respectively. The amphetamine to methamphetamine ratios ranged from 0.02-0.159 (mean 0.07).

7 Benzodiazepines in Hair

7.1 Benzodiazepines

Benzodiazepines are the most frequently prescribed drugs for the treatment of anxiety and insomnia.[210] They are also used as sedatives, muscle relaxants and anticonvulsants prior to surgery and other medical procedures. Drug abusers take benzodiazepines as illicit replacement drugs, sometimes simultaneously with alcohol and other drugs such as opiates, sedatives, antidepressants and neuroleptics[211] to either calm themselves down or amplify the drug-induced high.

Benzodiazepines are highly addictive, cheap and widely available and are therefore widely abused.[141] One study in the west of Scotland in 1995-1998 found that 45 % of drug related deaths tested positive for diazepam while 33 % of these cases tested positive for temazepam.[175] An assessment of drug prevalence in the same region during the same time period found benzodiazepines to be present in 82 % of cases.[176] A more recent evaluation of drug-related deaths in the Greater Glasgow National Health Board Area in 2006 found benzodiazepines to be present in 79 % of cases[212], a figure which is similar to the findings of the earlier study in the west of Scotland in 1995-1998. A study in Denmark, Iceland, Finland, Norway and Sweden in 2002 frequently detected benzodiazepines in addition to the main intoxicant.[213] Diazepam and flunitrazepam were most commonly associated with drug-related deaths.

Chlordiazepoxide is the prototype of the benzodiazepine drug class and was the first commercially available benzodiazepine which was approved for human use in 1960.[171] The Scottish Health Statistics for 2006-2007 show that diazepam is currently the most commonly prescribed benzodiazepine in Scotland.[174] Newer and shorter-acting benzodiazepine drugs included in this study such as temazepam, oxazepam, lorazepam, nitrazepam and flunitrazepam are also prescribed in the UK and in many other countries worldwide.

7.2 Pharmacokinetics, Metabolism and Excretion

The molecular structures are given for the nine benzodiazepines included in this study in Figure 7-1. The pharmacokinetic and physicochemical data are also

provided for these benzodiazepines. No data is available for 7-AF however the data can be assumed to be similar to that for flunitrazepam.

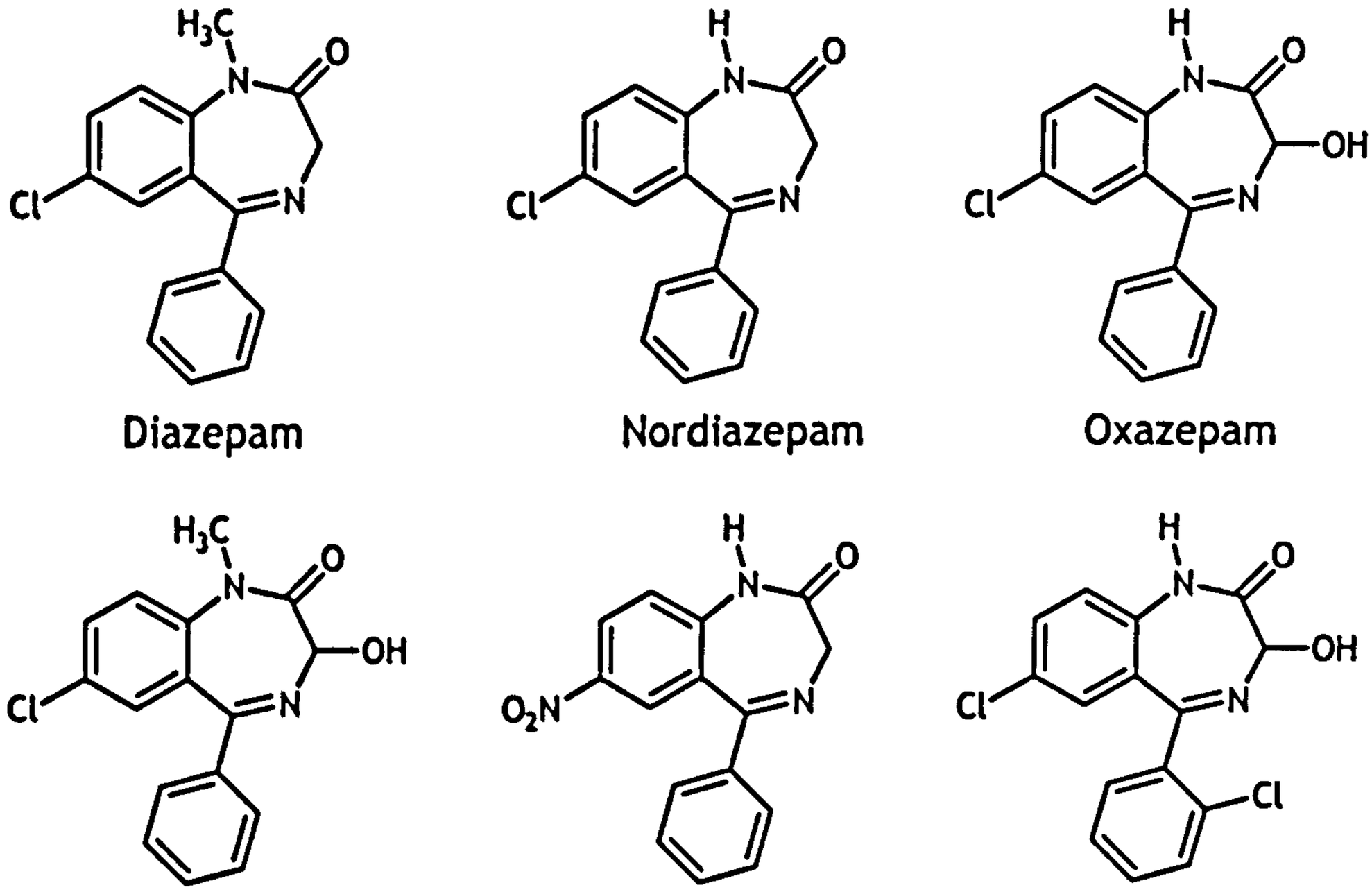


Figure 7-1 Molecular structures of benzodiazepines

Table 7-1 Pharmacokinetic and physicochemical data for benzodiazepines[176]

	Half Life (hr)	Vd (L/kg)	Protein Binding (%)	pK_a
Chlordiazepoxide	6-27	0.3-0.5	94	4.8
Diazepam	21-37	0.7-2.6	96	3.4
Flunitrazepam	9-25	3.4-5.5	78	1.8
Lorazepam	9-16	0.9-1.3	80	1.3, 11.5
Nitrazepam	17-48	2-5	87	3.2, 10.8
Nordiazepam	31-97	-	97	3.5, 12.0
Oxazepam	4-11	0.7-1.6	87-94	1.7, 11.6
Temazepam	3-13	0.8-1.0	97	1.3

Chlordiazepoxide is metabolised by N-demethylation to form norchlordiazepoxide, which is then further metabolised to demoxepam by deamination. These 2 major metabolites are as pharmacologically active as the parent drug. The reduction of demoxepam will produce nordiazepam which is also an active metabolite and is found in the plasma of patients undergoing long-term therapy treatment. Oxazepam is produced by the hydroxylation of nordiazepam but has not been detected in plasma. Less than 1 % of a dose is excreted as the parent drug; about 6 % is excreted as demoxepam while most is excreted as glucuronide conjugates of oxazepam.[176]

Diazepam is converted to nordiazepam by N-demethylation in the liver. Both of these compounds are further converted to oxazepam and temazepam by hydroxylation. The 3-hydroxy derivatives oxazepam and temazepam do not accumulate in plasma or blood to any significant extent. Trace amounts of diazepam and nordiazepam are found in urine; about 33 % of the dose is excreted as oxazepam glucuronide conjugates and about 20 % as conjugates of nordiazepam, 4'-hydroxydiazepam and temazepam.[176]

Flunitrazepam and nitrazepam are both metabolised by reduction of the nitro group to form 7-amino metabolites. A single 2 mg dose of flunitrazepam produces a 7-AF concentration of 50-500 ng/mL within 6-24 hours.[176]

Lorazepam is rapidly conjugated with glucuronic acid following ingestion to form an inactive metabolite. This accumulates in plasma to a greater extent than the parent drug.[176] Trace amounts of unchanged lorazepam are found in urine. Most

of the dose is eliminated as lorazepam glucuronide with the rest being conjugates of minor hydroxylation metabolites.

Oxazepam conjugates are also rapidly formed following ingestion but is found to a limited extent in serum. Most of the excreted drug in urine after 48 hours is oxazepam glucuronide. Temazepam is converted to oxazepam during metabolism. Both compounds form glucuronide conjugates. The majority of excreted drug in urine is temazepam glucuronide.

7.3 Previously Reported Methods of Analysis

The detection of benzodiazepines in hair is relatively difficult due to the low levels which incorporate in the hair. Sramek first reported the detection of diazepam in hair by RIA in 1992.[202] This work was followed by the analysis of benzodiazepines in hair using highly sensitive analytical methods. Benzodiazepines have been detected in the hair of living and deceased users by GC-MS[214,215,216,217,218], HPLC[219,220], and LC-MS-MS.[91,94,108,142,145,147,221] Various extraction methods have been reported including alkaline digestion[222], acid hydrolysis[223], enzymatic treatments[40,214] and Soerensens buffer.[224,217,218]

There have been a number of studies using gas chromatography-negative ion chemical ionisation-mass spectrometry (GC-NCI-MS) for the detection of benzodiazepines in hair as it is more sensitive than positive-ion chemical ionisation (PCI).[218,222,224,225,226] These were published in the mid to late 1990s prior to the increasing trend towards using LC-MS-MS for this purpose. One group achieved very low LOD values in the range 1-20 pg/mg for alprazolam, bromazepam, diazepam, flunitrazepam, lorazepam, nordiazepam, oxazepam and triazolam using a Soerensens buffer extraction.[218] A study by another group investigating alprazolam in rat hair also used a very sensitive method achieving an LOD of 25 pg/mg.[222] An overnight alkaline digestion with sodium hydroxide at 40 °C was used to extract the alprazolam.

A GC-MS method was reported for the detection of benzodiazepines and other psychotropic drugs in hair.[214] The LOD values ranged from 0.01-2 ng/mg and the authors proposed that this method could be used for screening.

The use of an on-line restricted access extraction column coupled with an HPLC-diode array detector (DAD) has been reported.[226] Ammoniated methanol was found to produce optimum extraction recoveries from hair spiked with clonazepam, diazepam, flunitrazepam, midazolam and oxazepam. The LOD was 0.2 ng/mg for all benzodiazepines in the study and acceptable recovery values (> 90 %) were achieved using spiked hair. The validated method was applied to 2 case samples. In one of the cases, flunitrazepam (Rohypnol®) was detected in 3 hair segments with the concentration ranging from 3.2-3.9 ng/mg. The other case sample was collected from an individual who sometimes used oxazepam (Semesta®). Two segments were analysed in this case and no oxazepam was detected. It was proposed that this was because oxazepam has a shorter half life compared to other benzodiazepines and is a polar compound which would not readily incorporate into hair. The simultaneous determination of these benzodiazepines using this HPLC method was rapid and therefore suitable for routine analyses. It was proposed that the use of a MS detector would improve the results. Seven extraction methods were compared for the extraction of incorporated benzodiazepines from rat hair by HPLC and GC-MS.[42] The method which produced the highest recovery for diazepam, flurazepam and medazepam was an overnight extraction in methanol-TFA (50:1 v/v) followed by LLE with dichloromethane.

Another HPLC method for the detection of psychotropic drugs in hair (including benzodiazepines) found that the method was not sensitive enough to detect diazepam, nitrazepam and oxazepam in post-mortem samples collected from individuals who were known to be taking psychotropic drugs prior to death.[227] This study compared extraction techniques from post-mortem hair samples and found that alkaline digestion with sodium hydroxide was more effective at recovering benzodiazepines from case hair than acidic (dilute hydrochloric acid), methanolic or enzymatic (Subtilisin) treatments. This is in agreement with the findings of another study.[226] The authors did however recommend the use of alternative procedures to sodium hydroxide digestion for the optimal detection of benzodiazepines in hair since all benzodiazepines were degraded under alkaline conditions.

As discussed in chapter 4, LC-MS is becoming increasingly popular due to simpler sample preparation requirements, its ability to analyse compounds not suitable

for GC and its excellent sensitivity and specificity. Reports on benzodiazepine detection in hair using LC-MS have recently become more common than GC-MS.

An LC-MS-MS method was reported for the detection of sedatives (including benzodiazepines) in hair and nail samples. Dichloromethane was used as the organic solvent in the LLE. The hair samples were initially incubated overnight in methanol/TFA (50:1 v/v) at room temperature. The LOD values for the selected benzodiazepines were very low; in the range of 0.20-6.00 pg/mg.[227] A mixture of diethyl ether/dichloromethane (10:90 v/v) was used by another group as the organic phase in a LLE for 16 benzodiazepines following incubation of the hair in Soerensens buffer.[147] The LOD values in this study were in the range of 0.5-5 pg/mg. It was proposed that this method would be suitable for screening purposes and is capable of monitoring single dose exposures.

SPE methods have also been reported for extraction of benzodiazepines from hair.[91,92,108,141] One group reported an extraction method using Bond Elut Certify™ SPE columns for sample clean-up after enzymatic digestion with proteinase K.[141] The method was applied to hair samples collected from forensic and clinical psychiatric patients. The LOD values ranged from 0.025-0.125 ng/mg. Recent work carried out within the Forensic Medicine and Science Section at the University of Glasgow has involved the use of molecularly imprinted solid phase extraction (MISPE) for extracting benzodiazepines from hair.[91,92] The results were compared with a classical SPE method as discussed in section 7.6.6 of this chapter.

7.4 Aim

There were two aims of this study. The first was to develop and validate an ELISA and LC-MS-MS method to screen and confirm 9 benzodiazepines in post-mortem hair samples. The second aim was to compare this validated SPE method with a novel MISPE method for the detection of benzodiazepines in 10 post-mortem samples. The MISPE method was developed and validated by Dr Marinah Ariffin, a colleague within the Forensic Medicine and Science Section at the University of Glasgow at the time of the study.[91]

7.5 Experimental

7.5.1 Samples

All samples were post-mortem scalp hair samples submitted to the toxicology laboratory of the Forensic Medicine and Science Section at the University of Glasgow for routine testing. The samples were tested for diagnostic purposes and the results were reported to the Procurator Fiscal.

7.5.2 Chemicals

Methanol, acetonitrile, acetic acid, ammonium hydroxide, cyclohexane, ethyl acetate, formic acid, dichloromethane, toluene and propan-2-ol were purchased from BDH (Poole, UK) and were of analytical grade. Ammonium formate and sodium dodecyl sulfate were purchased from Sigma-Aldrich (Dorset, UK). 7-AF, flunitrazepam, oxazepam, lorazepam, chlordiazepoxide, temazepam, diazepam, nordiazepam and nitrazepam, 7-AF-d_7, flunitrazepam-d_7, oxazepam-d_5, lorazepam-d_4, temazepam-d_5, diazepam-d_5 and nordiazepam-d_5 used in the LC-MS-MS confirmation were obtained from Promochem (Teddington, England). Clean Screen® (ZSDAU 020) mixed mode SPE columns were purchased from United Chemical Technologies Incorporation (Pennsylvania, US).

Benzodiazepine Direct ELISA kits were purchased from Immunalysis (Pomona, CA, USA). The kits contained a 96-well antibody-coated microplate, benzodiazepine conjugate labelled with horseradish peroxidase (HRP), substrate solution containing 3, 3', 5, 5'-tetramethylbenzidine (TMB) and stop solution containing 1 N hydrochloric acid. Hair extraction buffer (0.025 M monobasic phosphate buffer pH 2.7), neutralising buffer (0.5 M dibasic phosphate buffer pH 9.0) and phosphate buffer saline (0.1 M, pH 7.0) used to dilute hair extracts in the ELISA test were also obtained from the Immunalysis Corporation. Disposable borosilicate glass culture tubes (75 x 12 mm) were obtained from VWR International, UK.

Diazepam for polymer synthesis in the MISPE work was obtained from Roche (Hertfordshire, UK). Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were from Aldrich (Steinheim, Germany), chloroform was from

Rathburn (Peebles, UK) and 2,2'-azobisisobutyronitrile (AIBN) was from Acros Organics (Geel, Belgium). 1 mL polyethylene SPE cartridges without sorbent and frits (20 µm mean pore size) for diazepam MISPE were purchased from International Sorbent Technology Limited (Mid Glamorgan, UK).

7.5.3 Standard Solutions

Individual drug stock standard solutions and deuterated drug standards were obtained as 100 µg/mL prepared in methanol. For LC-MS-MS confirmation, a combined working drug solution of 7-AF, chlordiazepoxide, diazepam, flunitrazepam, lorazepam, nitrazepam, nordiazepam, oxazepam and temazepam was prepared at 1 µg/mL by 100-fold dilution with methanol. This was achieved by adding 25 µL of each 100 µg/mL drug solution into a 25 mL volumetric flask and making up the volume to the 25 mL mark with methanol. A combined working deuterated standard solution of the 7-AF-d_7, diazepam-d_5, flunitrazepam-d_7, lorazepam-d_4, nordiazepam-d_5, oxazepam-d_5 and temazepam-d_5 was also prepared at 1 µg/mL in the same way as the working drug solution. It was not financially viable to use chlordiazepoxide-d_5 therefore oxazepam-d_5 was selected for chlordiazepoxide quantitation since its retention time (t_R) was close to the t_R for chlordiazepoxide (5.1 and 6.2 minutes respectively). The *m/z* of the nitrazepam-d_5 precursor ion is the same *m/z* of the oxazepam precursor ion (*m/z* = 287) and both have a similar t_R of 5.8 minutes, resulting in co-elution. Therefore oxazepam-d_5 was used for nitrazepam quantitation.

7.5.4 Extraction Solutions and Mobile Phase

7.5.4.1 Preparation of 0.1 M, pH 6.0 Phosphate Buffer

The solution was prepared as described in section 5.6.4.3.

7.5.4.2 Preparation of Mobile Phase

The mobile phase consisted of a mixture of aqueous buffer and acetonitrile. To prepare the 3 mM ammonium formate + 0.001 % formic acid aqueous buffer, 0.189 g of ammonium formate and 10 µL of concentrated formic acid were added to a 1 L volumetric flask and made up to the mark with deionised water.

7.5.5 Instrumentation

7.5.5.1 ELISA

A MiniPrep 75 automatic pipettor from Tecan (San Jose, CA) was used. The microplate wells were washed using a Columbus Plus washer system and read with a Sunrise Remote EIA autoreader also from Tecan (Grödlg, Austria).

7.5.5.2 LC-MS-MS

LC-MS-MS analysis was carried out using a Surveyor HPLC system with an LCQ Deca XP Plus™ ion trap mass spectrometer (Thermo Finnigan, San José, CA, USA). Chromatographic separation was performed using a Gemini C_{18} column (150 mm x 2.0 mm ID, 5 μm particle size), fitted with a guard column with identical packing material (4 mm x 2.0 mm, 5 μm particle size) (Phenomenex, Torrance, CA, USA).

7.5.6 ELISA and LC-MS-MS Methods

7.5.6.1 ELISA Development

As there are generally low levels of benzodiazepines detected in hair, the benzodiazepines assay was optimised to achieve maximum sensitivity. Sample volumes (25, 50 μL) and pre-incubation times (30, 60, 120 minutes) were optimised. The parameters which produced the best separation of absorbance values between blank hair and the cut-off calibrator of 0.1 ng/mg oxazepam were used for analysis (Table 7-2).

Table 7-2 Sample volume and pre-incubation optimisation results

Sample Volume (μL)	Pre-incubation time (min)	Average B/B0 (%) for n=5
25	30	88.6
25	60	86.9
25	120	82.0
50	30	74.3
50	60	71.9
50	120	70.8

As shown in Table 7-2, 50 µL sample volume with a pre-incubation time of 2 hours produced the best separation of absorbance values between the blank and cut-off calibrator. However 50 µL sample volume with a pre-incubation time of 1 hour produced a marginally smaller difference and this was selected for validation due to the shorter analysis time. The optimised ELISA procedure for benzodiazepine screening in hair is described below. This method was used to validate the assay and was applied in the screening of post-mortem hair samples. The sample preparation method was described in section 6.4.1.

50 µL of diluted extract of calibrator, control or sample was added to the microplate wells in duplicate and left to infiltrate the antibodies at room temperature for 1 hour. Subsequently, 100 µL of enzyme conjugate was added to each well and the plate was left to incubate for a further hour at room temperature. After this time, the wells were washed 6 times with deionised water (6 x 300 µL). 100 µL TMB was added to each well and the plate incubated in the dark at room temperature for 30 minutes. The reaction was stopped using 100 µL 1 N hydrochloric acid and the plate was read at 450 nm with a reference wavelength of 650 nm.

The case samples were considered to be presumptively positive if the B/B_0 (%) value was lower than the B/B_0 (%) value of the 0.1 ng/mg oxazepam cut-off calibrator.

7.5.6.2 LC-MS-MS

7.5.6.2.1 Sample Preparation

The decontamination, segmentation and cutting up of the hair samples was described previously in section 6.4.1. After cutting, each sample was split into 3 separate vials for ELISA, SPE and MISPE procedures. As the likelihood of benzodiazepine contamination is low, the dichloromethane washings were not analysed.

An alkaline incubation using methanol with 25 % ammonium hydroxide was used prior to benzodiazepine extraction by solid phase.[42] Furthermore, ammoniated methanol was found to produce slightly higher recoveries than just methanol for diazepam, oxazepam and flunitrazepam in another study.[226] 1.5 mL of methanol: 25 % ammonium hydroxide solution (20:1 v/v) was added to each calibrator and

sample, left to sonicate for one hour and stored at room temperature overnight. The solvent was removed from the vials and transferred to a test tube. The hair was washed twice with 0.75 mL solvent and the washings removed and transferred to a test tube. The contents of the test tubes were evaporated to dryness under nitrogen and reconstituted in 1 mL of phosphate buffer.

7.5.6.2.2 SPE

The solid phase extraction method for this study operates by a mixed-mode cationic exchange mechanism. The sorbent is composed of C_8 chains and benzene sulfonic acid. The benzodiazepines adsorb onto the column via both hydrophobic and ionic attraction.

Clean Screen® columns (ZSDAU 020) were conditioned sequentially with 3 mL methanol, 3 mL distilled water and 1 mL of phosphate buffer. The vortexed samples were loaded onto the columns and allowed to drip through without the presence of a vacuum. The columns were washed sequentially with 2 mL distilled water, 2 mL phosphate buffer: acetonitrile (80:20 v/v), 2 mL cyclohexane and 2 mL distilled water. The columns were dried under full vacuum for 5 minutes after the second wash step, for 1 minute after the third wash step and for 5 minutes after the final wash step. The benzodiazepines were eluted using 1.5 mL 2 % ammoniated ethyl acetate (followed by a 2 minute drying step under full vacuum) and 1.5 mL dichloromethane: isopropanol: ammonium hydroxide (78:20:2 v/v). The eluted samples were blown down to dryness under a stream of nitrogen and reconstituted in 100 µL of the mobile phase (initial conditions 3 mM ammonium formate (65 %) and acetonitrile (35 %)). 20 µL of the reconstituted extract was injected for analysis.

7.5.6.2.3 MISPE

The molecularly imprinted polymer (MIP) is synthesised in the presence of a template molecule (in this study diazepam) and consists of a highly cross-linked porous polymer network. After the polymerisation, the template is removed, leaving a polymer network with strategically positioned functional groups in binding sites that are complementary in size and shape to the template molecule. These binding sites have the potential to re-bind with the template molecule or other molecules, which have a similar molecular structure to the template molecule, in a strong and selective way.

Diazepam MIP cartridges were prepared as detailed in another study.[94] The cartridges were conditioned using 0.5 mL toluene. The vortexed samples were added to the columns and allowed to drip through without the application of a vacuum. The columns were washed with 0.3 mL toluene and eluted using 0.5 mL 15 % (v/v) acetic acid in acetonitrile. The eluted samples were blown down under nitrogen and reconstituted with 100 µL of the mobile phase (initial conditions 3 mM ammonium formate (65 %) and acetonitrile (35 %)). 20 µL of the reconstituted extract was injected for analysis.

7.5.7 LC-MS-MS Analysis

The C_{18} column was maintained at 25 °C. The mobile phase consisted of 3 mM ammonium formate + 0.001 % formic acid (A) and acetonitrile (B) at a flow rate of 0.3 mL/min. The initial gradient conditions were 65 % A, decreasing to 20 % A after 13 minutes. The mobile phase composition was 10 % A from 13.5 to 16.5 minutes. Initial gradient conditions were restored from 16.5 to 20 minutes as this was sufficient time to allow the system to equilibrate. The total run time was 20 minutes. A divert valve taking LC flow to waste was used for the last 7 minutes of the run to preserve the MS source.

All mass spectral data was acquired in electrospray positive ion mode. The capillary temperature, sheath and auxiliary gas flow rates and collision energies were optimised for each analyte. The probe voltage used was 4.5 kV. Internal standard data was acquired in selective ion monitoring (SIM) mode and analytes were identified on the basis of their full MS-MS spectra. 2 product ions were monitored where possible, to gain qualitative identification data. The major product ion was used for quantitation. Optimum tuning parameters, precursor and product ions are shown in Table 7-3.

Table 7-3 Optimum tuning parameters, precursor and product ions

Analyte	Sheath Gas (AU)	Auxiliary Gas (AU)	Capillary Temp (°C)	Collision Energy (%)	Precursor *m/z*	Product *m/z*
7-AF	20	20	280	40	284	264*, 256
Chlordiazepoxide	20	20	300	29	300	283*, 241
Diazepam	20	20	300	42	285	257*, 222
Flunitrazepam	20	20	280	43	314	286*, 268
Lorazepam	30	20	290	30	321	303*, 275
Nitrazepam	30	20	300	42	282	254, 236*
Nordiazepam	20	15	300	41	271	243*, 140
Oxazepam	20	20	300	29	287	269*, 241
Temazepam	20	20	300	29	301	283*, 255

* = quantitation ion

LC-MS-MS chromatograms of the product quantitation ions are shown in Figure 7-2 for the lowest standard concentration (2 ng/ 30 mg blank hair).

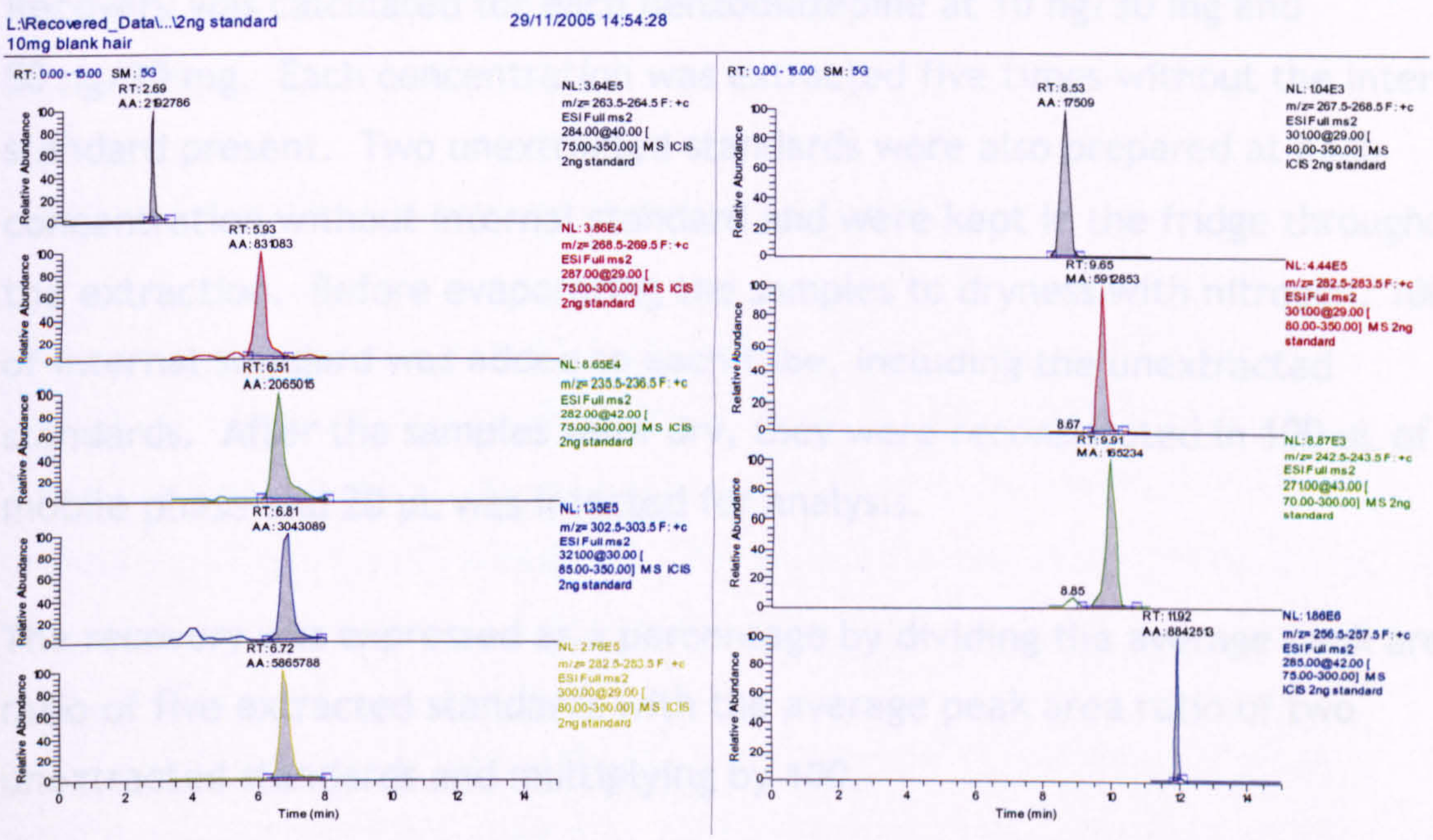


Figure 7-2 Chromatograms of product quantitation ions for 2 ng/ 30 mg standard

7.6 Method Validation

7.6.1 ELISA

The ELISA method was validated for the benzodiazepines assay as described in section 6.5.

7.6.2 LC-MS-MS

7.6.2.1 Linearity

Linearity was established over the concentration range of 2-100 ng/30 mg of blank hair to produce a regression line for each analyte. This was achieved by adding 2, 5, 10, 25, 50 and 100 µL of 1 µg/mL working drug solution to 30 mg blank decontaminated hair. 100 ng of deuterated internal standard was also added by adding 100 µL of 1 µg/mL working deuterated standard solution. A blank containing only internal standard and a blank containing no standards were also extracted and analysed.

7.6.2.2 Recovery

Recovery was calculated for each benzodiazepine at 10 ng/30 mg and 50 ng/30 mg. Each concentration was extracted five times without the internal standard present. Two unextracted standards were also prepared at each concentration without internal standard and were kept in the fridge throughout the extraction. Before evaporating the samples to dryness with nitrogen, 100 ng of internal standard was added to each tube, including the unextracted standards. After the samples were dry, they were reconstituted in 100 µL of mobile phase and 20 µL was injected for analysis.

The recovery was expressed as a percentage by dividing the average peak area ratio of five extracted standards with the average peak area ratio of two unextracted standards and multiplying by 100.

An additional recovery experiment was carried out for one post-mortem hair sample to determine the efficiency of the extraction. The sample was cut up into four portions. Each portion was left to incubate in the alkaline extraction medium for 1, 2, 4 and 16 hours and subsequently extracted using the SPE method described in section 7.5.6.2.2.

7.6.2.3 Stability

The stability of the benzodiazepines was assessed during the alkaline overnight incubation by comparing the average peak area ratio of four extracted samples at 50 ng/30 mg with the average peak area ratio of two unextracted samples.

The stability during evaporation was also determined. The total extraction recovery of benzodiazepine amino metabolites was found to be at least 25 % lower than the extraction recovery of their respective parent drugs.[235] This was attributed to loss of the 7-amino metabolites during evaporation and the authors added tartaric acid to prevent this. In this current study, evaporation of the SPE eluant was investigated as a potential means of lowering analyte recovery. Four tubes containing SPE eluant were spiked at 50 ng and evaporated under nitrogen at 40 °C. The peak area ratio results were compared with two samples spiked at the same concentration which were not prepared in eluant and were left to evaporate at room temperature.

7.6.2.4 Intra-Day Precision between Extracts

Five extracted standards were prepared at 10 ng/30 mg and 50 ng/30 mg. Standards were prepared at 2, 5, 10, 25 50 and 100 ng/30 mg to produce linearity data. A blank was prepared containing no standards as well as a blank containing only internal standard. The extracts were reconstituted in 100 µL of mobile phase and 20 µL was injected for analysis.

Intra-day precision for each extracted standard (n=5) was calculated by substituting the average peak area ratio value into the regression equation for a particular drug. The mean concentration and % RSD were calculated.

7.6.2.5 Inter-Day Precision

The inter-day precision was calculated at 10 ng/30 mg and 50 ng/30 mg using five sets of linearity data acquired on five different days (n=25). The average peak area ratio value for the five days was substituted into the regression equation for a particular drug. The mean concentration and % RSD was calculated.

7.6.2.6 LOD and LOQ

The LOD for each drug was calculated statistically as the intercept of the calibration graph plus three times the standard error of the line as shown in Equation 7-1 and Equation 7-2.[236] The LOQ was calculated statistically as the intercept of the calibration graph plus five times the standard error of the line as shown in Equation 7-3 and Equation 7-4.[236] The values were calculated where y_B is the intercept, s_B is the standard error of the regression line and m is the gradient. 30 mg blank hair portions were spiked with 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 2 and 5 ng to produce the regression lines used to calculate the LOD and LOQ.

Equation 7-1
$$y_{LOD} = y_B + 3s$$

Equation 7-2
$$LOD = \frac{(y_{LOD} - y_B)}{m}$$

Equation 7-3
$$y_{LOQ} = y_B + 5s$$

Equation 7-4
$$LOQ = \frac{(y_{LOQ} - y_B)}{m}$$

7.6.2.7 Matrix Effect

A previously published quantitative assessment of the hair matrix effect (ME) was carried out for the LC-MS-MS method.[175] The aim of the experiment was to obtain more accurate data on the extent of the hair ME between individuals. Drug free hair was collected from five individuals. Each sample was decontaminated according to the procedure described in section 6.4.1.

An unextracted set of five 50 ng standards containing 100 ng internal standards was prepared to assess the MS-MS response for neat standards of the analytes of interest (set 1). The contents of the vials were evaporated to dryness using nitrogen and reconstituted in 100 µL of mobile phase initial conditions. An extracted set of five 50 ng standards containing 100 ng internal standards was also prepared in hair extracts obtained from five individuals (set 2). For set 2, approximately 30 mg of blank, decontaminated hair was weighed out and taken through the extraction process. The analytes and internal standards were spiked

into these after the SPE. The eluants were evaporated to dryness using nitrogen and reconstituted in 100 µL of mobile phase (initial conditions 3 mM ammonium formate (65 %) and acetonitrile (35 %)). 20 µL of the reconstituted extract was injected for analysis.

The average peak area ratio was compared between the two data sets to assess the absence or presence of ME. The absolute ME (% ME) was calculated using Equation 7-5 where A is the standard response obtained from neat solutions in set 1 and B is the corresponding response for standards spiked into hair extracts after SPE.

Equation 7-5

$$ME(\%) = \frac{B}{A} \times 100$$

The absolute ME calculated in this way takes into account ion suppression and ion enhancement. It is described as the "absolute" ME as it compares the response of standard present in the hair extract to the response of a standard prepared in neat solutions without the presence of the matrix. A value of 100 % indicates that the standard response from the neat solution was the same as the standard response in the hair extracts and no absolute ME was observed. A value <100 % indicates ion suppression while a value >100 % indicates ion enhancement.

The relative ME (% RME) was also calculated as it is very important to compare ME values between hairs collected from different individuals to assess any inter-individual variations that may exist. Hair collected from different individuals may contain different endogenous compounds that may not necessarily have been present in the hair used to validate the method. If these compounds are undetected and co-elute with the analytes of interest, the efficiency of analyte ionisation could be affected, resulting in a decreased or increased MS-MS response.

7.6.3 Results

7.6.3.1 Linearity

The gradient and intercept values are shown in Table 7-4 along with the correlation coefficient (R^2) values for the regression lines. All regression lines had an $R^2 > 0.99$.

Table 7-4 Gradient, intercept and R^2 values for regression lines

Analyte	Equation of line	R^2
7-AF	y=0.0017x-0.0028	0.9981
Chlordiazepoxide	y=0.0129x-0.0156	0.9994
Diazepam	y=0.0016x-0.0032	0.9984
Flunitrazepam	y=0.0019x-0.0011	0.9992
Lorazepam	y=0.0092+0.0106	0.9974
Nitrazepam	y=0.0036x+0.0082	0.9977
Nordiazepam	y=0.0012x-0.0009	0.9995
Oxazepam	y=0.0093x-0.0122	0.9996
Temazepam	y=0.0113x-0.0323	0.9985

7.6.3.2 Recovery

The recovery for each analyte is given in Table 7-5 for 10 ng/30 mg and 50 ng/30 mg and ranged from 53-98 %. Generally the analytes had recoveries > 70 % at both concentrations. 7-AF and chlordiazepoxide recoveries were lower than the other analytes.

Table 7-5 Recovery in spiked hair

Analyte	Average % Recovery for n=5 (% RSD)	
	10 ng/30 mg	50 ng/30 mg
7-AF	53 (13)	55 (10)
Chlordiazepoxide	59 (19)	63 (5)
Diazepam	77 (13)	69 (2)
Flunitrazepam	90 (11)	89 (6)
Lorazepam	76 (11)	94 (10)
Nitrazepam	98 (10)	91 (9)
Nordiazepam	88 (12)	82 (5)
Oxazepam	71 (10)	83 (8)
Temazepam	73 (16)	88 (8)

The experiment carried out on the post-mortem hair sample to assess the efficiency of the extraction found that the greatest concentration of diazepam and nordiazepam was detected following 16 hour incubation (4.26 and 1.67 ng/mg respectively). The diazepam and nordiazepam concentrations detected after 1, 2 and 4 hour incubation were similar and were within a small range (2.40-2.70 ng/mg and 0.90-1.16 ng/mg respectively). This incubation time of 16 hours was selected for method validation.

7.6.3.3 Stability

The recovery for all benzodiazepines following the alkaline overnight extraction ranged from 89-103 % as shown in Table 7-6. These results show that the milder alkaline extraction using methanol/25 % ammonium hydroxide (20:1) is suitable for benzodiazepine extraction. The lower 7-AF and chlordiazepoxide recoveries were not a result of alkaline hydrolysis. Lorazepam was hydrolysed to a greater extent than temazepam or oxazepam, which have a similar molecular structure to lorazepam. It is possible that the presence of an electron withdrawing chlorine atom on both aromatic rings of the lorazepam structure (Figure 7-1) increases the lability of the ring C=N to attack by the base used in the extraction, promoting hydrolysis. Temazepam and oxazepam only have one electron withdrawing chlorine atom and are, therefore, less susceptible to hydrolysis.

For the evaporation experiment, all benzodiazepines with the exception of 7-AF and chlordiazepoxide had a recovery ≥ 95 %. 7-AF recovery was 73 % and chlordiazepoxide recovery was 87 %, which partly explains the lower extraction recoveries found for these analytes. The recovery values for the evaporation step are shown in Table 7-6.

Table 7-6 Benzodiazepine stability during alkaline incubation and evaporation step

Analyte	Average % Recovery (% RSD) for n=4	
	Alkaline Incubation	Evaporation Step
7-AF	100 (2)	73 (6)
Chlordiazepoxide	98 (2)	87 (8)
Diazepam	96 (4)	100 (5)
Flunitrazepam	99 (4)	107 (1)
Lorazepam	89 (9)	104 (5)
Nitrazepam	103 (6)	102 (4)
Nordiazepam	98 (12)	95 (12)
Oxazepam	98 (6)	99 (4)
Temazepam	91 (3)	104 (7)

7.6.3.4 Intra-Day and Inter-Day Precision

As shown in Table 7-7 and Table 7-8, the intra-day and inter-day precision were all generally acceptable within SOFT regulations of ± 20 %.[11] The % RSD values were generally lower at the higher concentrations. As expected, the intra-day precision was lower than the inter-day precision.

Table 7-7 Intra-day precision between extracts

Analyte	Intra-Day Precision (% RSD) for n=5	
	10 ng/30 mg	50 ng/30 mg
7-AF	10.9 (5.2)	50.1 (1.4)
Chlordiazepoxide	9.6 (9.7)	45.3 (6.1)
Diazepam	9.6 (4.5)	51.4 (4.0)
Flunitrazepam	8.5 (3.0)	49.3 (2.1)
Lorazepam	9.5 (7.1)	49.1 (5.8)
Nitrazepam	10.0 (9.0)	52.4 (4.1)
Nordiazepam	9.7 (8.8)	45.9 (3.1)
Oxazepam	7.0 (10.8)	58.6 (7.1)
Temazepam	9.1 (12.3)	53.3 (4.3)

Table 7-8 Inter-day precision between extracts

Analyte	Inter-Day Precision (% RSD) for n=25	
	10 ng/30 mg	50 ng/30 mg
7-AF	10.3 (9.9)	50.7 (4.5)
Chlordiazepoxide	8.0 (19.9)*	53.3 (16.5)
Diazepam	9.2 (21.1)*	45.9 (11.8)
Flunitrazepam	9.1 (9.8)	48.8 (6.5)
Lorazepam	7.9 (21.2)	50.6 (13.5)
Nitrazepam	8.9 (7.7)	47.9 (11.1)
Nordiazepam	9.2 (9.3)	48.0 (5.5)
Oxazepam	6.3 (13.0)	49.2 (19.6)
Temazepam	7.8 (18.6)	48.2 (10.0)

*n=24

7.6.3.5 LOD and LOQ

The LOD and LOQ for all the benzodiazepines tested ranged from 0.03-0.62 ng/30 mg and 0.05-1.02 ng/30 mg respectively. The values are shown in Table 7-9.

Table 7-9 LOD and LOQ

Analyte	LOD (ng/30 mg)	LOQ (ng/30 mg)
7-AF	0.14	0.23
Chlordiazepoxide	0.07	0.13
Diazepam	0.13	0.22
Flunitrazepam	0.30	0.50
Lorazepam	0.62	1.02
Nitrazepam	0.03	0.05
Nordiazepam	0.24	0.41
Oxazepam	0.11	0.19
Temazepam	0.09	0.16

7.6.3.6 Matrix Effect

The % ME and % RME are shown in Table 7-10. % RME was calculated as the % RSD of the responses for the hair from 5 individuals. The % RSD for five unextracted standards is also shown in Table 7-10 to compare against the % RME.

Table 7-10 Hair matrix effect

Analyte	% ME	% RME	% RSD for n=5 unextracted standards
7-AF	94	1.6	3.4
Chlordiazepoxide	77	10.8	12.7
Diazepam	84	10.5	3.4
Flunitrazepam	88	5.7	2.1
Lorazepam	105	6.5	5.1
Nitrazepam	79	9.3	8.7
Nordiazepam	88	5.2	2.0
Oxazepam	93	9.3	3.9
Temazepam	96	10.5	1.6

The % ME results show that there is some ion suppression for most of the benzodiazepines. In contrast, the lorazepam response shows slight enhancement. The % RME values were within the range of 1.6- 10.8 %. These values show that the method allows an acceptable level of precision between hair samples collected from five different individuals according to the intra-day and inter-day precision guidelines recommended by SOFT.[11] However acceptable % ME and % RME ranges have not been specified for LC-MS-MS yet as there is currently not enough data to provide these guidelines. Small differences between the values for the standards spiked into hair extracts after SPE (B values according to Equation 7-5) indicate that the MS-MS response from the

same quantity of analyte is similar in different hair samples. The difference in variability was higher for chlordiazepoxide, diazepam and temazepam. This indicates that the interferent from the hair matrix that co-elutes at the same time as these benzodiazepines is present in different quantities between individuals.

7.6.4 ELISA vs LC-MS-MS Results

The validated ELISA and LC-MS-MS methods for the detection of benzodiazepines in hair were successfully applied to 13 post-mortem positive hair samples.[111] Overall, the ELISA and LC-MS-MS results correlated well. 13 of the 16 hair samples were screened and confirmed as TP using a cut-off value of 0.1 ng/mg oxazepam. This cut-off value was used as it produced no FN results. Higher cut-off values of 0.2 and 0.5 ng/mg oxazepam produced 1 FN and 2 FN results respectively. Using a 0.1 ng/mg cut-off, the sensitivity and specificity of the ELISA method were 100 % and 81 % respectively versus LC-MS-MS. The results are shown in Table 7-11.

Table 7-11 ELISA vs LC-MS-MS results for benzodiazepine positive hair samples

ELISA			LC-MS-MS (ng/mg)						
Sample Number	Weight (mg)	OXAZ Equivalents (ng/mg)	Weight (mg)	DZ	NDZ	TMZ	OXAZ	NTZ	LOR
1	8.96	> 0.5	13.23	-	-	0.35	-	-	-
2	10.18	> 0.5	9.88	<LOD	0.28	0.22	-	-	-
3	8.19	> 0.5	9.29	0.65	1.20	0.24	-	0.24	-
4	4.30	> 0.5	7.13	<LOD	0.36	0.31	0.89	-	0.38
5	10.03	> 0.5	9.90	<LOQ	0.27	-	-	-	-
6	6.36	> 0.5	5.94	0.32	0.47	0.36	-	-	-
7	10.33	> 0.5	9.86	0.46	-	-	-	-	-
8	10.45	> 0.5	6.47	2.86	1.79	0.73	0.35	-	-
9	20.74	> 0.5	40.56	0.39	1.07	0.30	-	-	-
10	10.23	> 0.5	9.95	< LOQ	0.31	0.22	-	-	-
11	9.94	> 0.5	10.02	0.03	0.43	-	-	-	-
12	9.89	> 0.5	9.80	0.06	0.28	0.23	0.59	-	-
13	5.51	0.48	7.96	< LOQ	-	0.27	-	-	-

DZ=diazepam, NDZ=nordiazepam, TMZ=temazepam, OXAZ=oxazepam, NTZ=nitrazepam, LOR=lorazepam

The benzodiazepines detected by LC-MS-MS included diazepam (10 samples), nordiazepam (10 samples), oxazepam (3 samples), temazepam (10 samples), lorazepam (1 sample) and nitrazepam (1 sample). The diazepam levels detected

in 3 of the 10 positive samples were below the LOQ therefore only 7 samples were reported as positive. Concentrations ranged from 0.01-2.86 ng/mg diazepam, 0.27-1.79 ng/mg nordiazepam, 0.35-0.89 ng/mg oxazepam and 0.22-0.73 ng/mg temazepam. One sample tested positive for lorazepam at 0.38 ng/mg while another sample tested positive for nitrazepam at 0.24 ng/mg. Chromatograms are shown for a blank hair sample spiked with temazepam at the lowest standard concentration (Figure 7-3), a negative sample (Figure 7-4), a temazepam positive case sample (Figure 7-5) and a temazepam-d_5 internal standard (Figure 7-6).

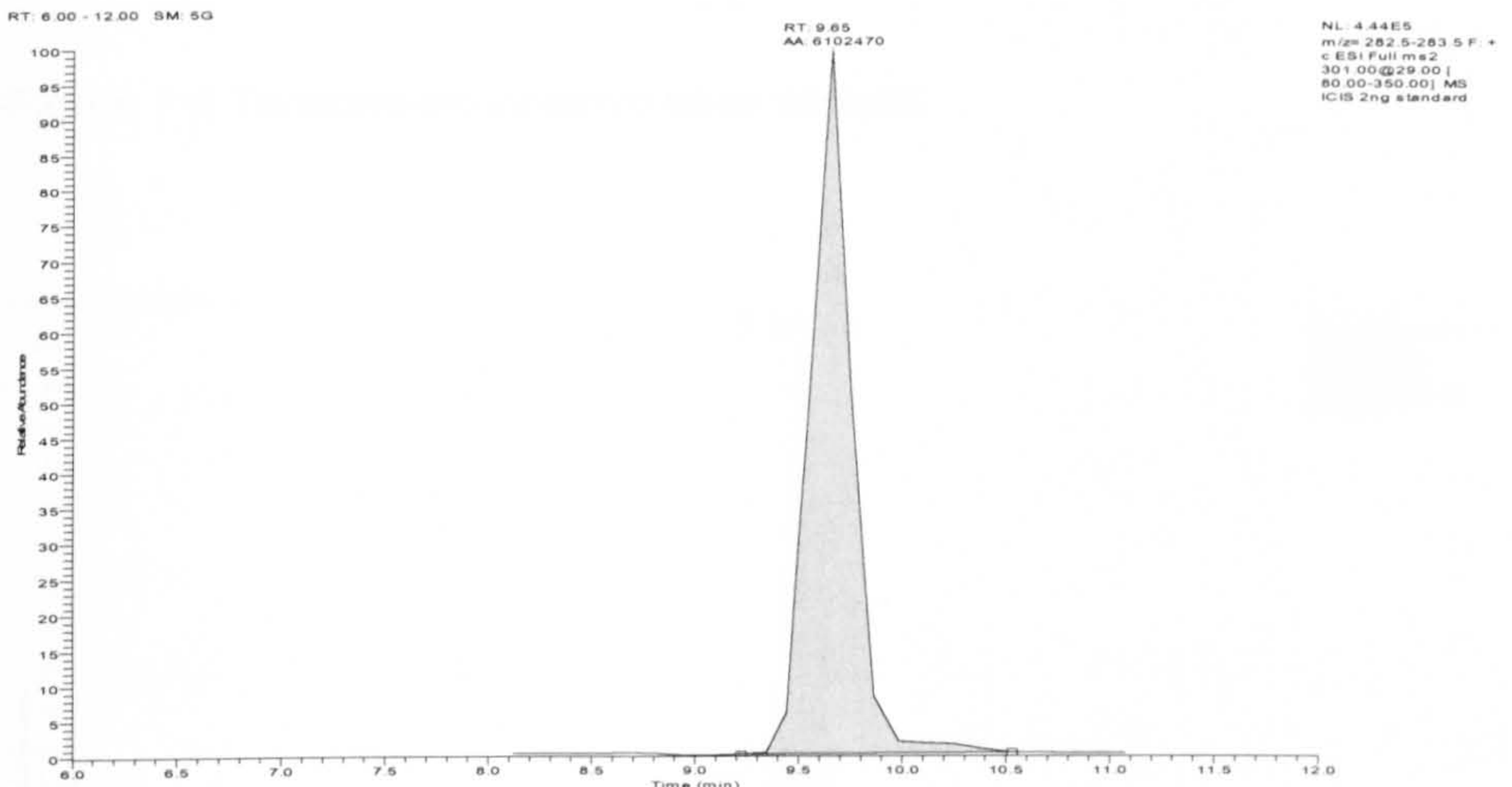


Figure 7-3 Blank hair sample spiked with temazepam at 2 ng/ 30 mg

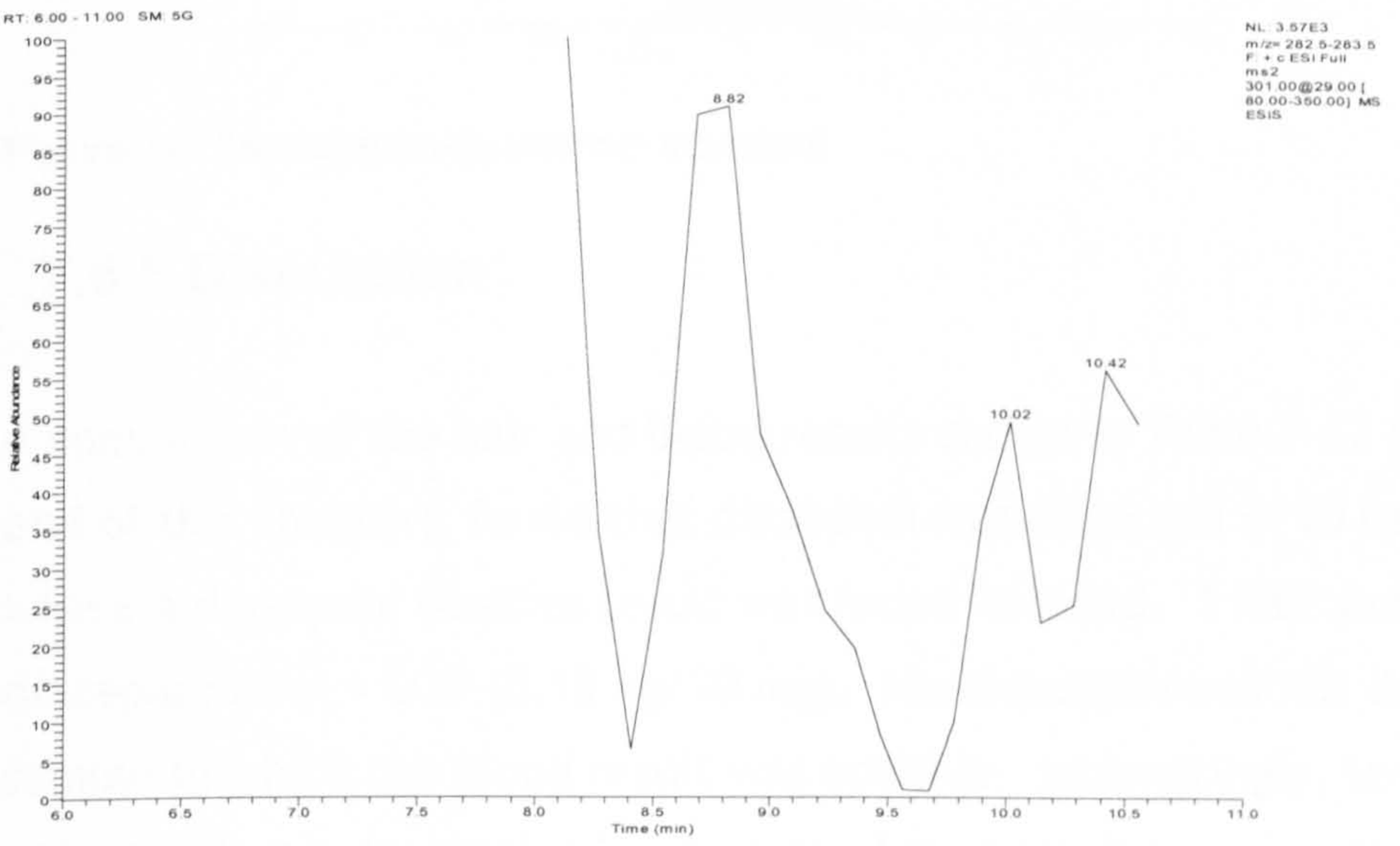


Figure 7-4 Negative hair sample

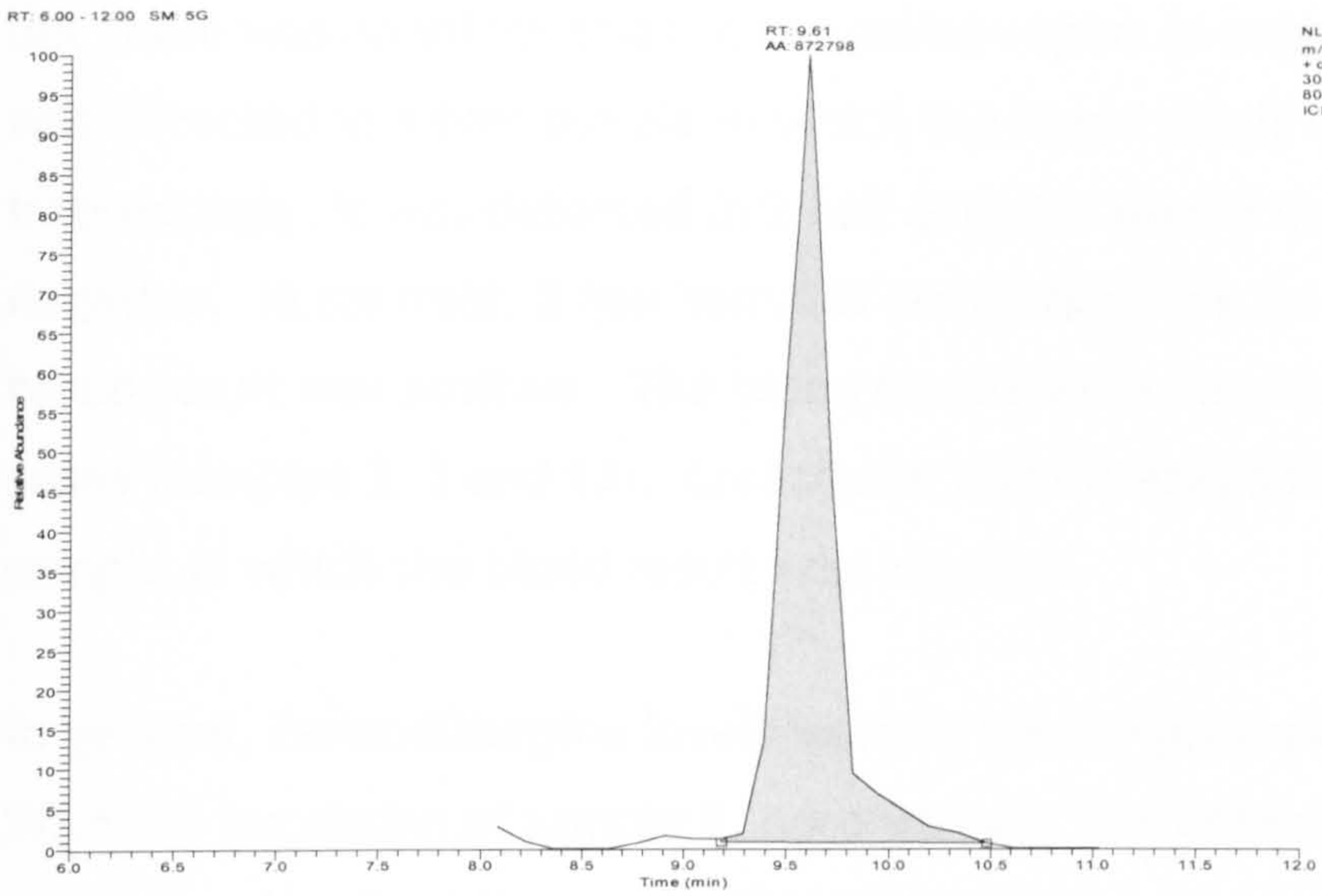


Figure 7-5 Temazepam positive case sample

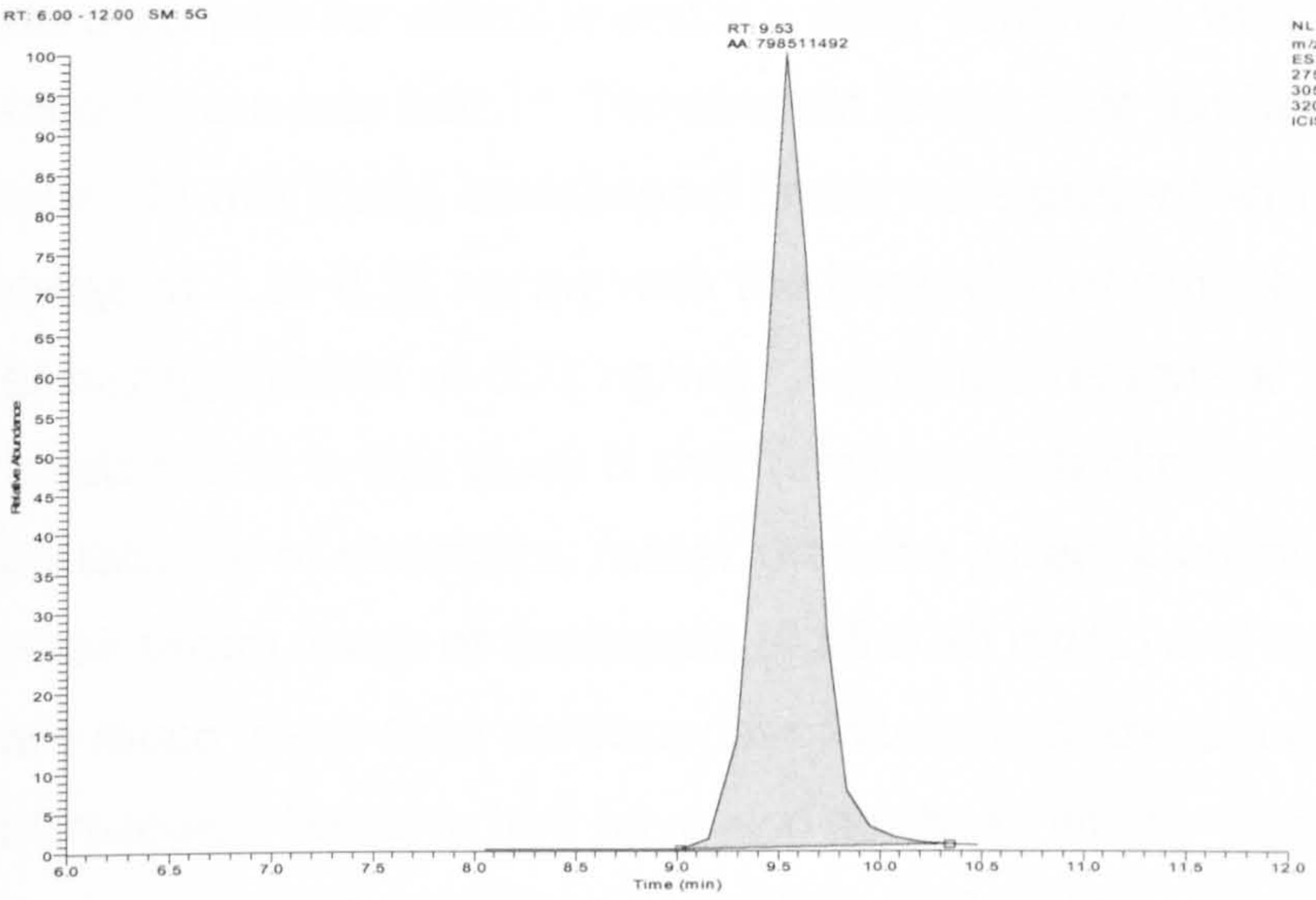


Figure 7-6 Temazepam-d_5 internal standard

7.6.5 Discussion

A comparison of the hair and blood results shown in Table 7-12 (provided at the end of this chapter), found that diazepam was detected in 10 hair samples where a diazepam positive result was found in blood. 1 hair sample contained a diazepam level < LOD (0.13 ng/30 mg). Nordiazepam was not detected in 1 sample in which the blood result was positive. Interestingly, temazepam was detected in 4 hair samples in which the blood result was negative. There was however 1 sample where the hair tested negative for temazepam in which the blood results were positive. The levels of temazepam in blood were very low in these cases (0.02 mg/L). These blood levels could have been from a naïve user

but there was no information in the police report to support this. Oxazepam was detected in 1 hair sample in which the blood result was positive. Interestingly, it was detected in 2 hair samples where the blood result was negative. In contrast, 5 hair samples tested negative for oxazepam where the blood result was positive. The blood results were very low in three of these cases (samples 3, 5 and 13). Lorazepam and nitrazepam were detected in 1 hair sample in which the blood result was negative.

In general, benzodiazepine levels were in the ranges reported in the literature. With the exception of sample 8, nordiazepam was detected at a higher concentration than diazepam. This finding is consistent with other studies.[221,225] Oxazepam was only detected in 3 samples. A possible reason for this low detection rate may be that oxazepam has a relatively short half life compared to nordiazepam for example and is a polar compound which may not readily incorporate into hair.[226] Temazepam levels have not been widely reported in hair. In this study, temazepam levels were present within a relatively small range of 0.22-0.36 ng/mg with the exception of sample 8 which had a higher temazepam level of 0.73 ng/mg. A possible reason for the lower temazepam levels found in this study is that temazepam is likely to be present as a metabolite of diazepam, rather than the parent compound. In addition steady-state serum levels of oxazepam (0.05-0.40 mg/L) and temazepam (0.1-0.6 mg/L) are much lower than nordiazepam following diazepam use.[176] Lorazepam and nitrazepam levels in hair have also not been widely reported. The lorazepam positive sample was found to contain 0.38 ng/mg, which is slightly higher than the reported range of 31-300 pg/mg.[224,237] The nitrazepam level detected in sample 3 (0.24 ng/mg) was also higher than the range found in another study (0.05-0.13 ng/mg).[235]

Overall, it is difficult to directly compare the benzodiazepine levels detected in this study with those reported by other authors because of the range of extraction and analytical methods used which will have varying recoveries of the drugs from hair.

It is also difficult to interpret the hair results since the amount and type of drug taken over the months prior to death is not known in the majority of cases. The individual from which sample 8 was collected was being prescribed 4x5 mg

diazepam tablets a day and 5 mg nitrazepam at night. It is not known how long the individual had been under this treatment. The highest diazepam level of all the hair samples tested was found in this particular sample however no nitrazepam was detected. The individual from which sample 12 was collected had been prescribed diazepam 6 months prior to death. From the background report, it is not known if the individually had been prescribed diazepam for chronic or single use.

Another problem with the interpretation of results is that diazepam is metabolised to nordiazepam and temazepam which can both be further metabolised to oxazepam. As well as potentially being present in hair samples due to diazepam metabolism, nordiazepam, temazepam and oxazepam can also be ingested as parent drugs or could be present as an association with both metabolism and parent drug use.

7.6.6 MISPE and SPE Case Results

The MIP produced was selective for not only diazepam (the template) but also for nordiazepam, oxazepam, temazepam and nitrazepam because they are close structural analogues of diazepam.

10 post-mortem hair samples were tested using MISPE to compare with the SPE method. The other samples did not have sufficient sample weight to test using MISPE as well. The same incubation method as described in section 7.5.6.2.1 was used prior to extraction. Benzodiazepines were detected in 9 samples using the MISPE method and in 7 samples using the classial SPE method. No benzodiazepines were detected by either extraction method in sample 3 where a very low level of nordiazepam was detected in the blood (0.01 mg/L).

Diazepam was detected in 4 samples using MISPE where none was detected using SPE. Nordiazepam and oxazepam were also detected in 1 sample (samples 4 and 2 respectively) at low levels using MISPE where none was detected using SPE. The results for sample 10 did, however, show a significantly lower oxazepam concentration using MISPE compared to SPE. Temazepam was detected in 2 samples by SPE where none was detected by MISPE. The nitrazepam results for the two methods showed good correlation.

The MISPE and SPE results are provided in Table 7-13 (at the end of this chapter) along with the corresponding blood concentrations. The sample numbers in Table 7-13 do not correspond to the same samples provided in Table 7-12. In general, the hair results are in good qualitative agreement using both extraction methods.

7.6.7 MISPE vs SPE Discussion

The MISPE demonstrated higher extraction recoveries for 6 out of 9 analytes, whereas the SPE method achieved lower LOD values for 6 out of 9 analytes. The recovery, LOD and LOQ values for both extractions are given in Table 7-14.

Table 7-14 Spiked recovery, LOD and LOQ of benzodiazepines using MISPE and SPE

Analyte	Mean % Recovery (% RSD) for n=5 at 50 ng/30 mg		LOD (ng/30 mg)		LOQ (ng/30 mg)	
	MISPE	SPE	MISPE	SPE	MISPE	SPE
7-AF	92 (2)	55 (10)	0.03	0.14	0.06	0.23
Chlordiazepoxide	62 (14)	63 (5)	0.33	0.07	0.57	0.13
Diazepam	93 (2)	69 (2)	0.09	0.13	0.14	0.22
Flunitrazepam	39 (5)	89 (6)	0.78	0.30	1.32	0.50
Lorazepam	97 (17)	94 (10)	0.66	0.62	1.11	1.02
Nitrazepam	92 (5)	91 (9)	0.06	0.03	0.11	0.05
Nordiazepam	103 (10)	82 (5)	0.21	0.24	0.33	0.41
Oxazepam	73 (9)	83 (8)	0.13	0.11	0.21	0.19
Temazepam	90 (13)	88 (8)	0.39	0.09	0.63	0.16

In this study, benzodiazepine concentrations were measured in 9 post-mortem hair samples using MISPE and SPE sample preparation methods. The results show that diazepam was most frequently detected, followed by its metabolite nordiazepam.

Both the MISPE and SPE methods were validated for 9 benzodiazepines.[94,111] However, only diazepam, its metabolites and nitrazepam were detected in the samples. The greater number of diazepam and nordiazepam positive samples detected using MISPE could be attributed to the higher extraction recovery and lower LOD values obtained when using this protocol compared to SPE. Conversely, temazepam was detected in less samples using MISPE compared to SPE due to the higher LOD value using MISPE. Temazepam recoveries for the two methods were comparable.

In the samples which tested positive for diazepam using both extraction methods, the concentration detected using MISPE was generally similar to those detected using SPE. The excellent recognition of the template molecule by the MIP resulting in a higher extraction recovery and lower LOD meant that diazepam was detected in 4 samples at low levels where SPE detected none. For the samples where both extraction methods tested positive for nordiazepam, temazepam and oxazepam, the concentrations were always higher for SPE. Interestingly, oxazepam was detected in 1 sample using MISPE where none was detected using SPE despite its slightly lower LOD. Nitrazepam levels detected by the MISPE and SPE methods were comparable.

Although the MISPE and SPE methods are in good qualitative agreement, there are some quantitative differences between the two extraction methods, particularly for diazepam metabolites. Concentration differences of this type can be due to systematic bias or else they may reflect some degree of selectivity by the MIP between the analyte and internal standard during the extraction process. Taking the classical SPE method as the Gold Standard procedure, the new MISPE method shows in most cases a negative bias, which, in percentage terms, is high in some cases. The bias is not completely systematic as the % bias varies with concentration. Most of the large differences relate to cases in which the concentrations measured are low and close to the LOQ.

The most likely explanation is that the MIP procedure results in extracts with less interference than the SPE procedure (Figure 7-7) and that these interferences augment the peak areas in the product ion chromatograms obtained for classical SPE extracts. This implies that the classical procedure may show lower selectivity than the MIP procedure.

The results show that the MISPE method is selective for diazepam metabolites and other benzodiazepine analogues. It detected diazepam and oxazepam in samples which tested negative using the classical SPE approach. Overall, the MISPE method appears to be significantly more streamlined than the SPE method, providing a simpler and more time efficient procedure.

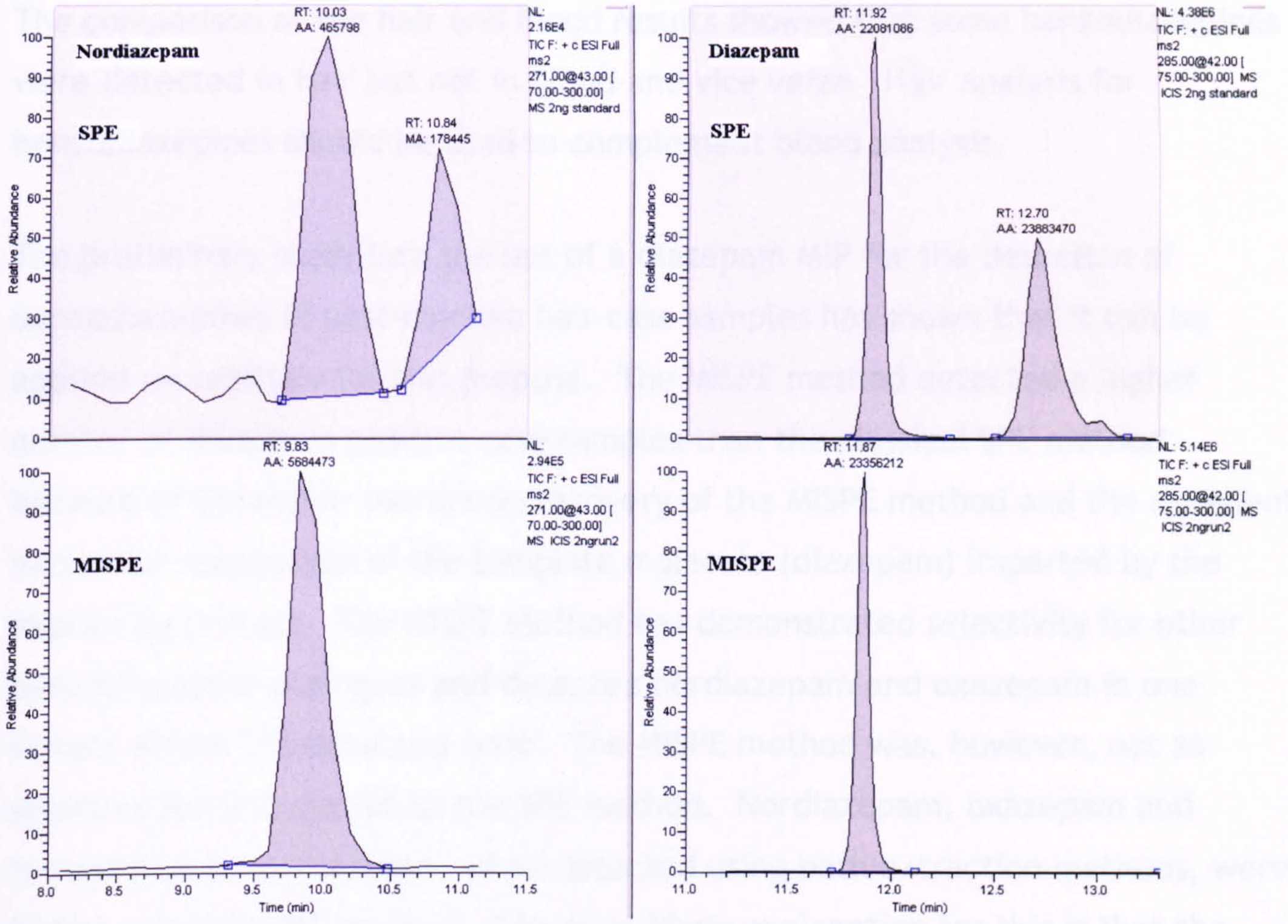


Figure 7-7 Chromatograms of the SPE and MISPE extracts showing LC-MS-MS interference in the nordiazepam and diazepam scan segment for the SPE extract only

7.7 Conclusion

The validated ELISA and LC-MS-MS methods detected 6 therapeutic benzodiazepines in hair. 13 samples screened as TP using the ELISA versus LC-MS-MS method. The ELISA method showed 100 % sensitivity and 81 % specificity using a cut-off value of 0.1 ng/mg oxazepam.

The LC-MS-MS method provided the sensitivity necessary for quantitation of benzodiazepines in post-mortem hair samples. It appears that mild alkaline extraction conditions are suitable for benzodiazepines and do not result in any significant hydrolysis. An incubation time of 16 hours in the alkaline medium produced the greatest diazepam and nordiazepam recoveries compared to shorter incubation times for one case sample. The evaporation step in the SPE method resulted in a relatively high loss of 7-AF and chlordiazepoxide, which is partly responsible for the lower recoveries for these analytes.

The comparison of the hair and blood results showed that some benzodiazepines were detected in hair but not in blood and vice versa. Hair analysis for benzodiazepines should be used to complement blood analysis.

The preliminary study into the use of a diazepam MIP for the detection of benzodiazepines in post-mortem hair case samples has shown that it can be applied successfully for this purpose. The MISPE method detected a higher number of diazepam positive case samples than the classical SPE method because of the higher extraction recovery of the MISPE method and the excellent molecular recognition of the template molecule (diazepam) imparted by the imprinting process. The MISPE method has demonstrated selectivity for other benzodiazepine analogues and detected nordiazepam and oxazepam in one sample where SPE detected none. The MISPE method was, however, not as selective for temazepam as the SPE method. Nordiazepam, oxazepam and temazepam concentrations, when detected using both extraction methods, were higher using the SPE method. The most likely explanation for this is that the MISPE method has a higher selectivity than the SPE method, with fewer matrix interferences. Further work is required to investigate the possibility of mixing two or more MISPE sorbents in one cartridge to detect a particular drug class and also the ability of the MISPE to detect benzodiazepines in single dose hair samples.

Table 7-12 Comparison of hair vs blood benzodiazepine results

Sample Number	DZ		NDZ		TMZ		OXAZ		NTZ		LOR	
	Hair (ng/mg)	Blood (mg/L)	Hair (ng/mg)	Blood (mg/L)	Hair (ng/mg)	Blood (mg/L)	Hair (ng/mg)	Blood (mg/L)	Hair (ng/mg)	Blood (mg/L)	Hair (ng/mg)	Blood (mg/L)
1	-	-	-	-	0.35	2.81	-	0.16	-	-	-	-
2	<LOD	0.03	0.28	0.01	0.22	-	-	-	-	-	-	-
3	0.65	0.72	1.20	1.42	0.24	0.13	-	0.08	0.24	-	-	-
4	-	0.10	0.36	0.04	0.31	0.01	0.89	-	-	-	0.38	-
5	<LOQ	0.15	0.27	0.20	-	0.02	-	0.02	-	-	-	-
6	0.32	0.38	0.47	0.44	0.36	0.09	-	0.21	-	-	-	-
7	0.46	0.11	-	-	-	-	-	-	-	-	-	-
8	2.86	0.31	1.79	0.54	0.73	0.05	0.35	0.12	-	-	-	-
9	0.39	0.07	1.07	0.06	0.30	-	-	-	-	-	-	-
10	<LOQ	0.04	0.31	0.08	0.22	-	-	-	-	-	-	-
11	0.03	0.02	0.43	0.04	-	-	-	-	-	-	-	-
12	0.06	0.06	0.28	0.11	0.23	-	0.59	-	-	-	-	-
13	<LOQ	0.51	-	0.25	0.27	0.12	-	0.03	-	-	-	-

DZ=diazepam, NDZ=nordiazepam, TMZ=temazepam, OXAZ=oxazepam, NTZ=nitrazepam, LOR=lorazepam

Table 7-13 MISPE vs SPE hair benzodiazepine results with corresponding blood results

Sample Number	Diazepam			Nordiazepam			Oxazepam			Temazepam			Nitrazepam		
	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)
	MISPE	SPE		MISPE	SPE		MISPE	SPE		MISPE	SPE		MISPE	SPE	
1	0.06	-	0.03	0.09	0.28	0.01	-	-	-	-	0.22	-	-	-	-
2	0.68	0.65	0.72	0.97	1.20	1.42	0.06	-	0.08	0.17	0.24	0.13	0.23	0.24	-
3	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-
4	-	-	0.03	0.08	-	0.05	-	-	-	-	-	-	-	-	-
5	0.03	-	0.15	0.08	0.27	0.2	-	-	0.02	-	-	0.02	-	-	-
6	0.46	0.46	0.11	-	-	-	-	-	-	-	-	-	-	-	-
7	0.06	-	0.04	0.19	0.31	0.08	-	-	-	0.16	0.22	-	-	-	-
8	0.08	0.03	0.02	0.12	0.43	0.04	-	-	-	-	-	-	-	-	-
9	0.02	-	0.15	-	-	0.07	-	-	-	-	-	0.01	-	-	-
10	0.50	0.06	0.06	0.12	0.28	0.16	0.09	0.59	-	-	0.23	-	-	-	-

8 Drugs of Abuse in Hair

8.1 Introduction

Reports on hair testing in literature generally restrict analysis to one or a few drug groups. One of the reasons for this is that with hair analysis the possible number of examinations is limited due to a lack of sample. In addition, the reproducibility of quantitative analysis of a hair sample is limited by the amount of sample and the unknown recovery of the extraction procedure. Therefore, semi-quantitative estimations of drug concentrations based on single injections are more typical than validated high throughput assays.[13,230]

The usual procedure for hair analysis for drugs of abuse involves an initial screen by immunoassay followed by confirmation with a more sensitive and specific technique. GC-MS[71,231] and LC-MS-MS[94] methods have been reported for qualitative analysis of the common drugs of abuse in hair and simultaneous quantification of two or three targeted drug groups.[22,71,232] A recently published article does however describe the simultaneous extraction, identification and quantification of four commonly abused drug groups in hair by GC-MS.[233] LC-MS-MS methods have also been developed in some laboratories for analysing more than four commonly abused drug groups[94] but this technique is not routinely available in most toxicology laboratories.

For the analysis of hair taken from poly-drug users including drug-related deaths and patients undergoing therapy on drug treatment programmes, where several drug classes could be present in each sample, it is more efficient to simultaneously screen and quantify the different drug classes using a single extraction and analysis. The LC-MS-MS method described in this work has been validated for the simultaneous identification and quantification of: amphetamines, including amphetamine, methamphetamine, MDA, MDMA and MDEA; the most commonly abused benzodiazepine in Scotland, diazepam and its metabolites; cocaine and its metabolites namely benzoylecgonine, cocaethylene and ecgonine methyl ester; and opiates including codeine, dihydrocodeine, 6-MAM and morphine.

Several papers have evaluated different methods for extracting amphetamines[239,242], benzodiazepines[226,227], cocaine[243,244] and heroin metabolites[71,79,245] from hair. The methods used were carried out at various temperatures and involved solvent extraction, enzymatic digestion and acidic or basic digestion from cut or pulverised hair. Basic extraction conditions were found to degrade some drugs such as heroin, cocaine and benzodiazepines while the enzymatic digestion and solvent extraction methods demonstrated a very small amount, if any, degradation. Direct solvent extractions were found to give the lowest drug recoveries while complete dissolution of the hair matrix produced the highest recoveries for substances that could withstand the extreme conditions. After extraction of the drugs from the hair matrix, the analysis is carried out using very similar procedures to those used to extract drugs from blood or urine.

8.2 Drugs of Abuse

8.2.1 Amphetamines

Amphetamines are central nervous system stimulants used therapeutically in the treatment of obesity, narcolepsy and hypotension however they are also abused by athletes, addicts and mainly young people at "raves" to enhance their performance. Amphetamine abuse has been prevalent in some particular geographic areas such as Japan, Far East and the United States. However, there is an increasing consumption of amphetamine in Europe where it is reported to be the second most commonly abused drug after cannabis.[246] In this study, the classical amphetamines, amphetamine and methamphetamine, as well as the methylenedioxy-substituted amphetamines methylenedioxyethylamphetamine (MDEA), methylenedioxymethamphetamine (MDMA) and methylenedioxyamphetamine (MDA) were investigated in post-mortem hair samples. The common route of administration for these drugs is by oral means. However, amphetamine and methamphetamine have also been taken intranasally and by intravenous injection to produce euphoric effects. Methamphetamine is manufactured as the hydrochloride salt and is smoked. It resembles colourless fragments of ice (Figure 8-1) and hence is known by this streetname. The methylenedioxy-substituted amphetamines such as MDMA

(Figure 8-2) produce effects such as euphoria, enhanced energy and lowers inhibitions.

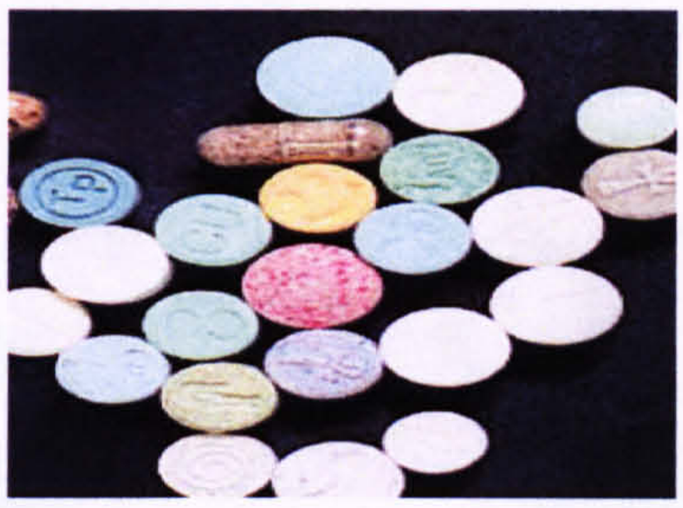


Figure 8-1 Methamphetamine HCl ("Ice") **Figure 8-2 MDMA ("ecstasy") tablets**

8.2.1.1 Pharmacokinetics and Metabolism

Pharmacokinetic data for amphetamine, methamphetamine and MDMA is given in Table 8-1. There has been little research on the pharmacokinetics of MDA and MDEA and so the data for these two compounds is not provided.

Table 8-1 Pharmacokinetic and physicochemical data for amphetamines[176]

	Half Life (hr)	Vd (L/kg)	Protein Binding (%)	pK_a
Amphetamine	7-34 (urine pH dependent)	3.2-5.6	16	9.9
Methamphetamine	6-15 (urine pH dependent)	3.0-7.0	10-20	9.9
MDMA	6-9	5.0-8.0	-	-

Amphetamines are weak bases with low molecular weights and low protein binding. Therefore, they can easily diffuse across the cell membrane and become incorporated in samples such as hair, nails, sweat and oral fluid. Hair is a very useful sample for determining an individual's past history of amphetamine abuse.

Amphetamine is metabolised by hydroxylation of the aromatic ring to *p*-hydroxyamphetamine and by deamination to phenylacetone, with subsequent oxidation to benzoic acid.[176] Glucuronide conjugates are also formed during metabolism. Methamphetamine is partially metabolised to amphetamine, which

is an active metabolite, then further metabolised in the same way as described for amphetamine. MDMA and MDEA are metabolised to form MDA. These metabolic pathways are shown in Figure 8-3-Figure 8-6.

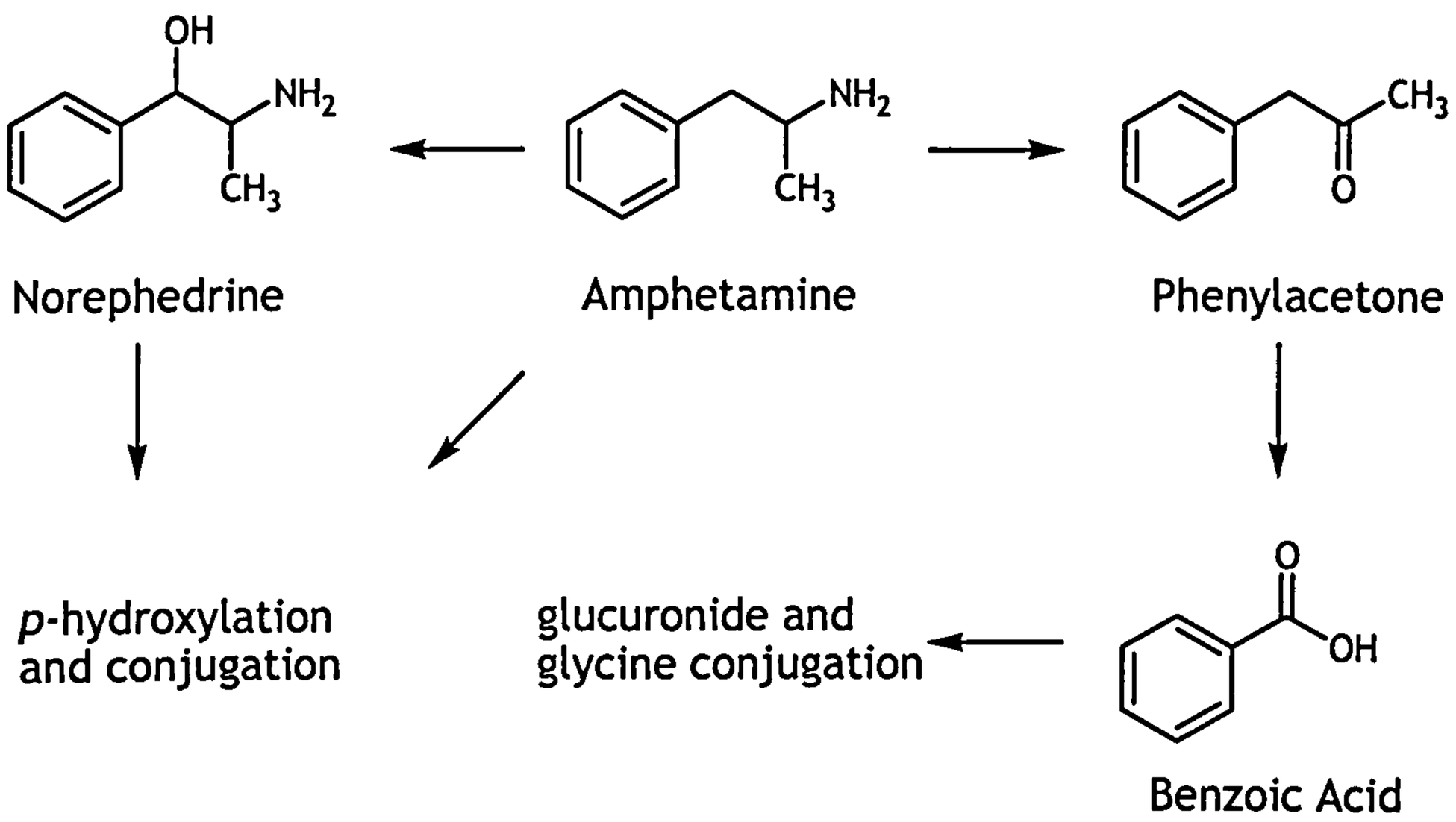


Figure 8-3 Amphetamine metabolism

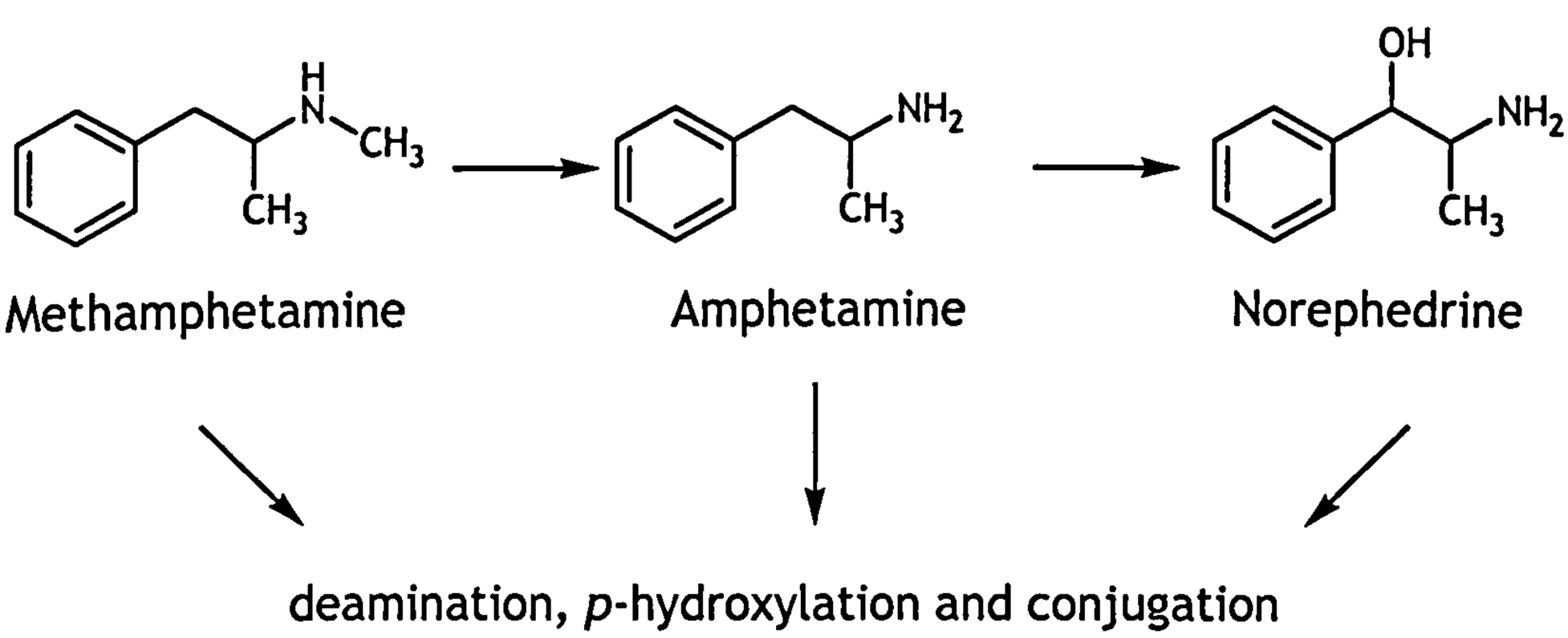


Figure 8-4 Methamphetamine metabolism

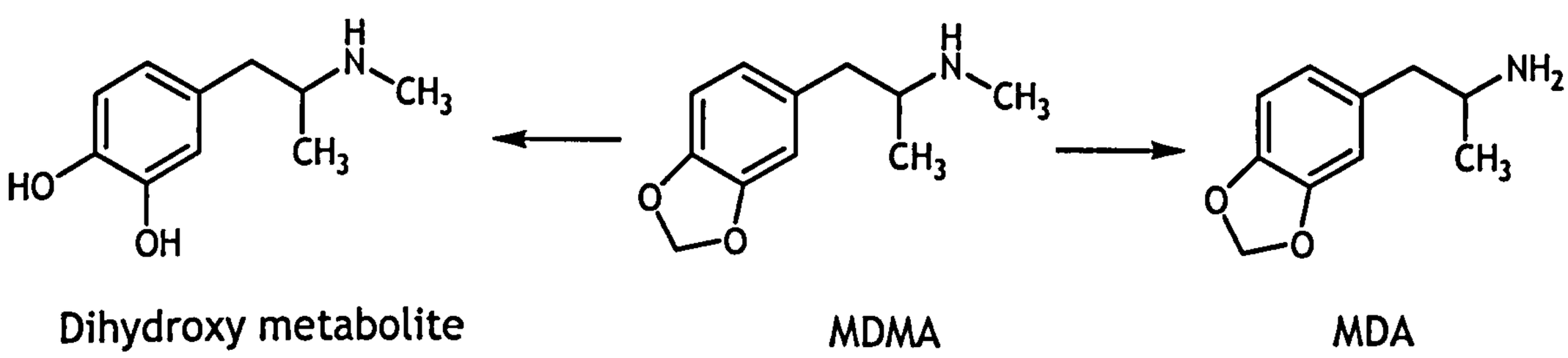
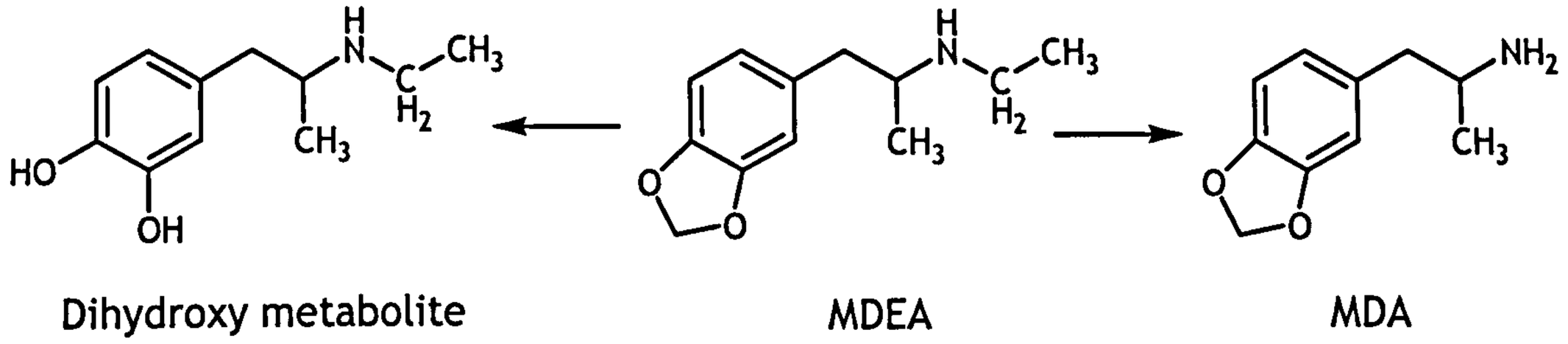


Figure 8-5 MDMA metabolism



Dihydroxy metabolite MDEA MDA

Figure 8-6 MDEA metabolism

8.2.2 Benzodiazepines

As discussed in chapter 7, benzodiazepines are commonly prescribed and abused drugs. Diazepam is prescribed under the trade name Valium® as an anti-anxiety agent, muscle relaxant or anticonvulsant. Temazepam, oxazepam and nordiazepam are metabolites of diazepam and can also be prescribed for these purposes but this is less frequent in Scotland.[179]

8.2.2.1 Pharmacokinetics and Metabolism

The pharmacokinetic properties of the drugs are summarised in the previous chapter in Table 7-1. Diazepam metabolism is described in section 7.2 and the molecular structures of diazepam metabolites are shown in Figure 7-1.

8.2.3 Cocaine

Cocaine is the most potent naturally occurring central nervous system stimulant and therefore has a long history of use and abuse.[13] Cocaine is extracted from the leaves of Erythroxylon coca (Figure 8-7) which is a South American shrub and constitutes up to 2 % of the leaf's weight.[176]

It was first produced as pure cocaine in 1855 and has been widely used by drug users as it produces feelings of euphoria. Illicit cocaine when taken by drug users is usually administered intranasally, intravenously or through smoking the free base which is known as "crack cocaine" (Figure 8-8). Only 30-70 % of a dose of "crack cocaine" is absorbed by smoking due to pyrolysis.[176] Intranasal and oral routes of administration produced lower plasma concentrations than intravenous routes due to slower absorption.[176]

Figure 8-7 Erythroxylon coca shrub

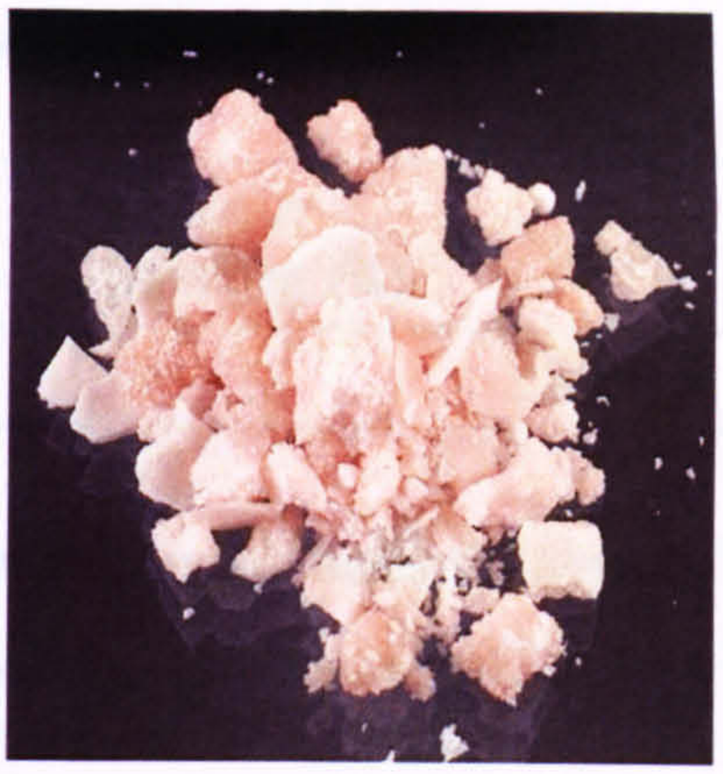


Figure 8-8 "Crack cocaine"

8.2.3.1 Pharmacokinetics and Metabolism

Cocaine is rapidly hydrolysed by one or both of the ester linkages to produce benzoylecgonine and ecgonine methyl ester which are its two main metabolites (Figure 8-9). Both of these are pharmacologically inactive. A number of minor metabolites are also produced.[176]

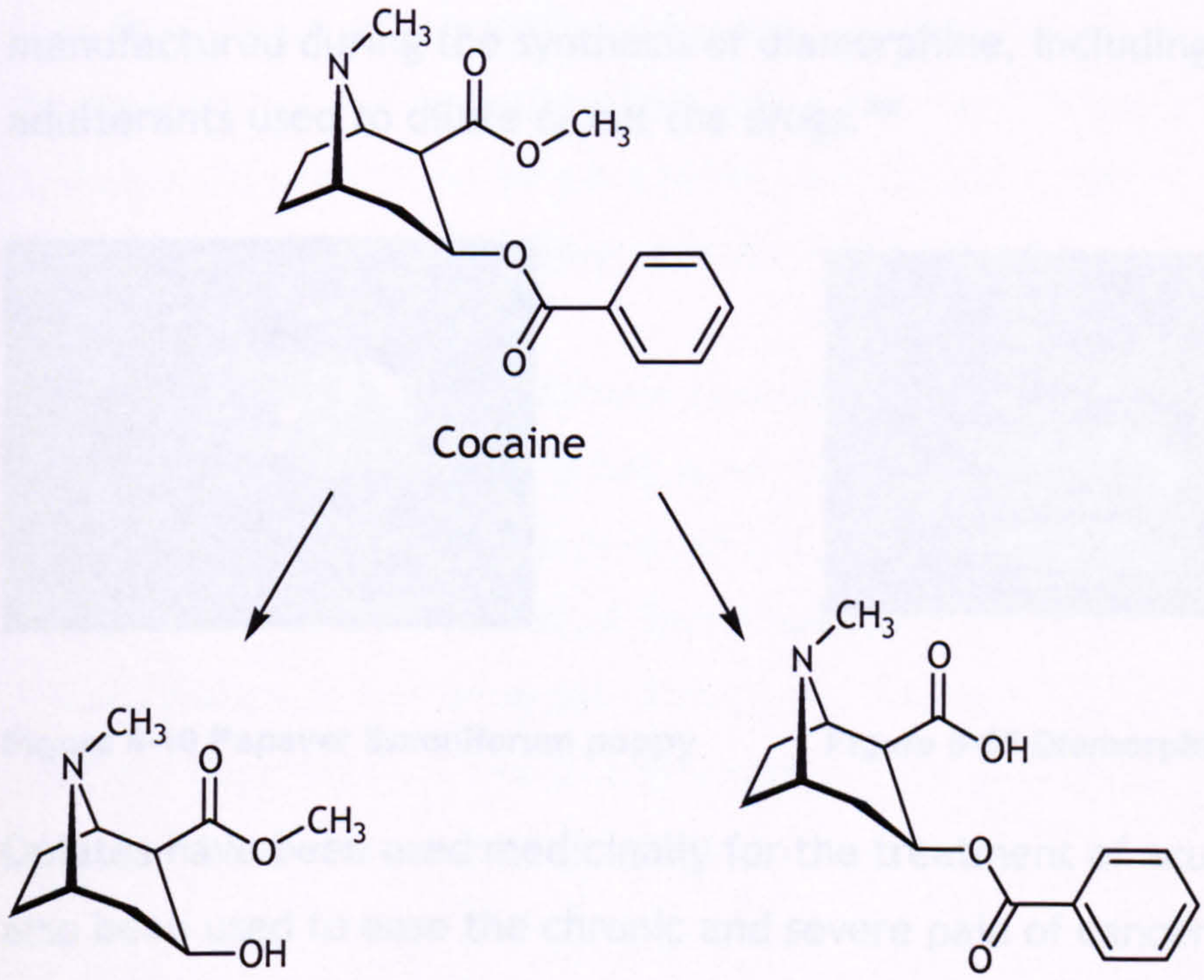


Figure 8-9 Cocaine metabolism

The pharmacokinetic and physicochemical data is presented for cocaine in Table 8-2.

Table 8-2 Pharmacokinetic and physicochemical data for cocaine[176]

	Half Life (hr)	Vd (L/kg)	Protein Binding (%)	pK_a
Cocaine	0.7-1.5	1.6-2.7	92	8.6

Cocaine is a highly lipophilic compound and consequently accumulates in fatty tissues during chronic use for a period of 2-3 weeks before excretion.[247]

8.2.4 Opiates

The term opiate describes natural opium alkaloids and the semisynthetics derived from them. Opium is a resin collected from the Papaver Somniferum poppy (Figure 8-10). It contains morphine, codeine, papaverine and noscapine. Morphine is acetylated in clandestine laboratories to produce diamorphine for the black market (Figure 8-11). Codeine is also acetylated to produce acetylcodeine during this process. In the UK, heroin is commonly defined as a mixture of impurities, reaction intermediates, and breakdown products manufactured during the synthesis of diamorphine, including the excipients and adulterants used to dilute or cut the drugs.[248]

Figure 8-10 Papaver Somniferum poppy

Figure 8-11 Diamorphine

Opiates have been used medicinally for the treatment of acute pain. They have also been used to ease the chronic and severe pain of cancer patients. Some opiates are widely abused for their euphoria-producing properties which result through oral, intravenous, intranasal or smoking routes of administration. The feeling of euphoria gained through opiate use has been associated with the psychological dependence and subsequent addiction to these drugs.[13] Opiate tolerance is known to rapidly develop to the extent that rapid dose increases are required to achieve the euphoric state.

8.2.4.1 Pharmacokinetics and Metabolism

Diamorphine is rapidly absorbed and metabolised in the liver following intravenous injection ($t_{1/2}$ in plasma is 2-6 minutes). Due to its rapid metabolism, it is not usually targeted as evidence of opiate use in body fluids. Diamorphine is rapidly metabolised via deacetylation to 6-MAM and then more slowly metabolised to morphine, which is the main pharmalogically active metabolite. 6-MAM is therefore used as a marker of diamorphine use. The metabolic pathway for diamorphine is shown in Figure 8-12. As mentioned in section 8.4.2, illicit heroin is not just composed of diamorphine but also impurities. Codeine is extracted from the Papaver Somniferum poppy as well as morphine, papaverine and noscapine. During the acetylation of morphine to diamorphine, codeine is also acetylated to produce acetylcodeine. Therefore codeine can be present as a result of acetylcodeine metabolism.[176]

Codeine is metabolised in the liver by *O*-demethylation to produce morphine and by *N*-demethylation to produce norcodeine. Both compounds are excreted as free drug and as glucuronide conjugates. Small amounts of hydrocodone, dihydrocodeine and hydromorphone have also been detected in urine.[13] Codeine is commonly prescribed for pain relief and has been used in cough remedies. The metabolic pathway for codeine is given in Figure 8-13.

Dihydrocodeine is a semisynthetic narcotic analgesis which is produced from the hydrogenation of codeine. It has been used for pain relief for example in the treatment of post-operative pain. It has also been used as an alternative to methadone in the treatment of heroin addiction.[249] It is metabolised by *O*- and *N*-dealkylation in the same set of steps as codeine. It also forms glucuronide and sulphate conjugates at the 3- and 6-hydroxy ring positions. The metabolic pathway for dihydrocodeine is given in Figure 8-14.

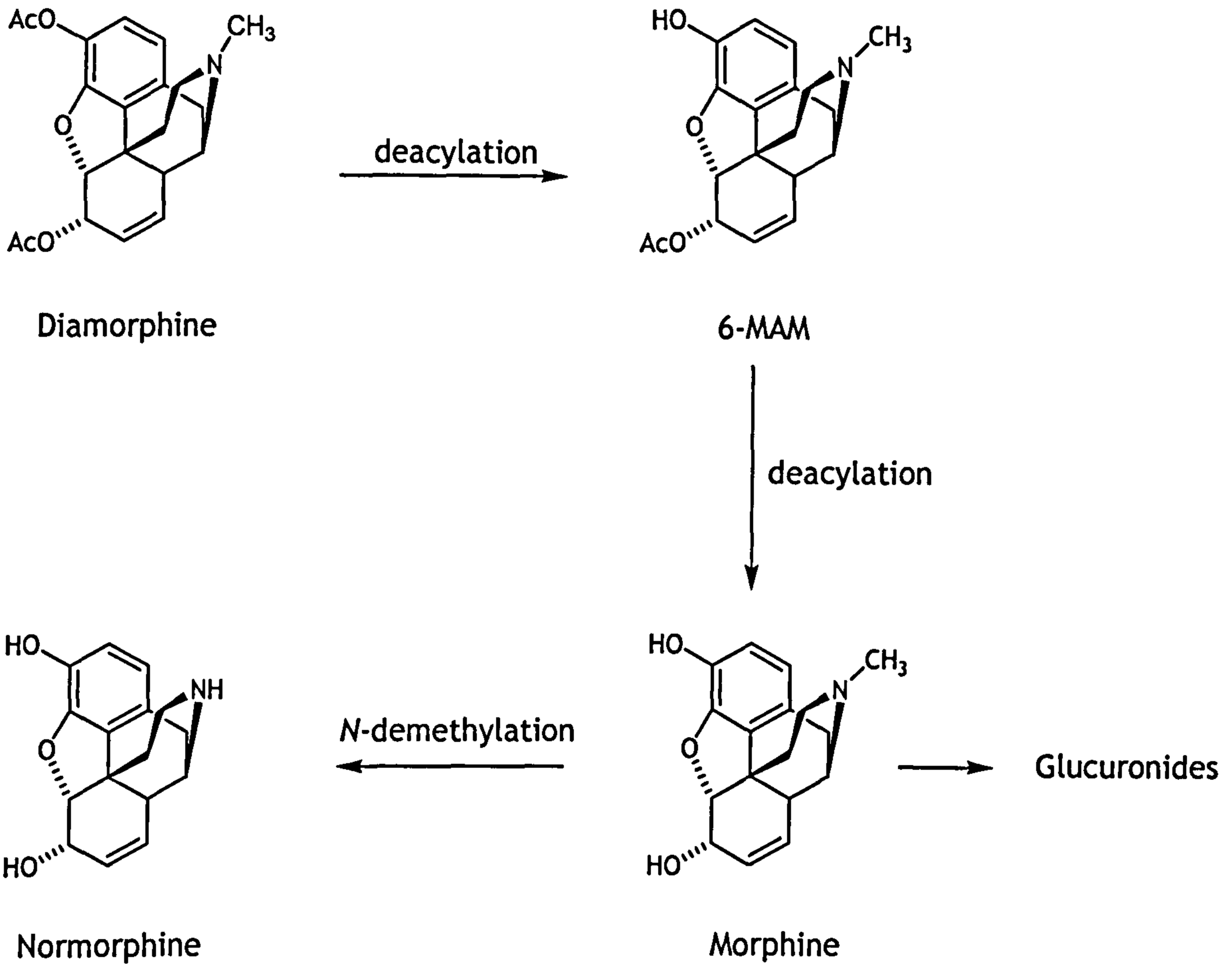
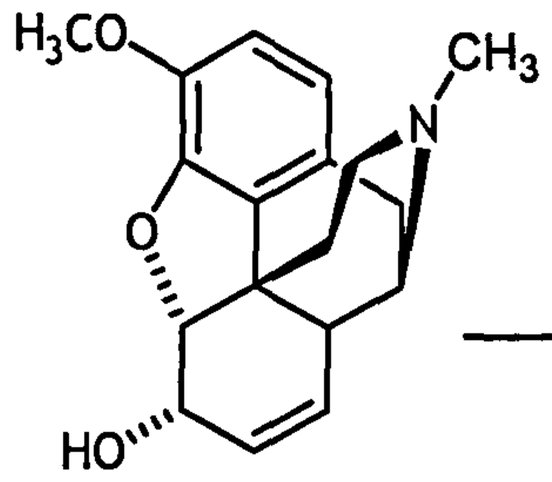
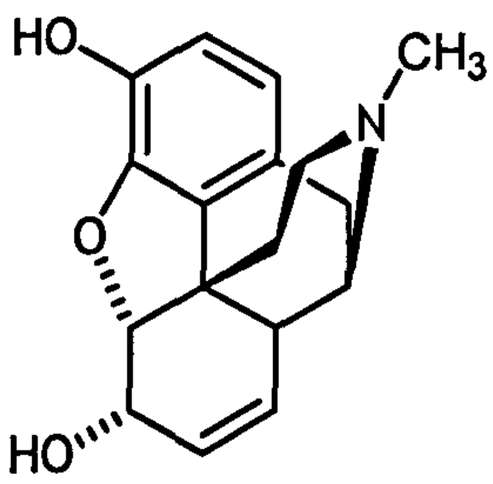


Figure 8-12 Diamorphine metabolism

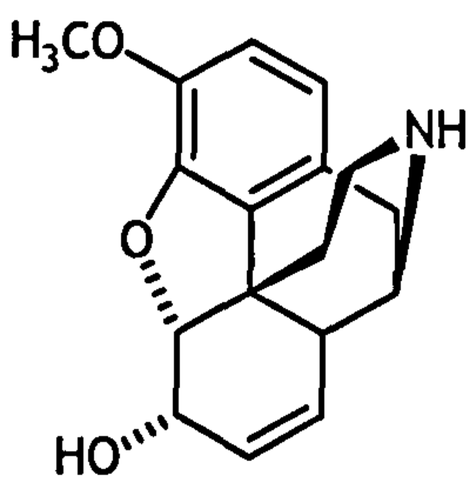


Codeine

Codeine-6-glucuronide

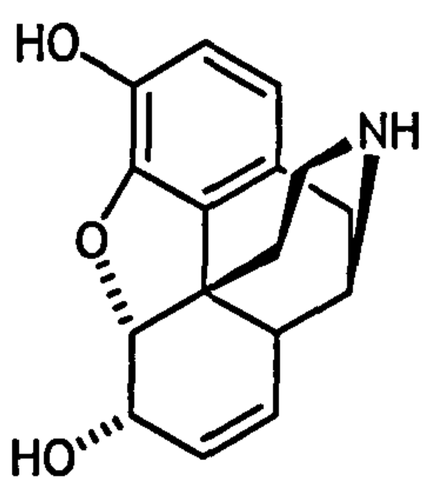


Morphine



Norcodeine

Morphine-3-glucuronide
and
Morphine-6-glucuronide



Normorphine

Figure 8-13 Codeine metabolism

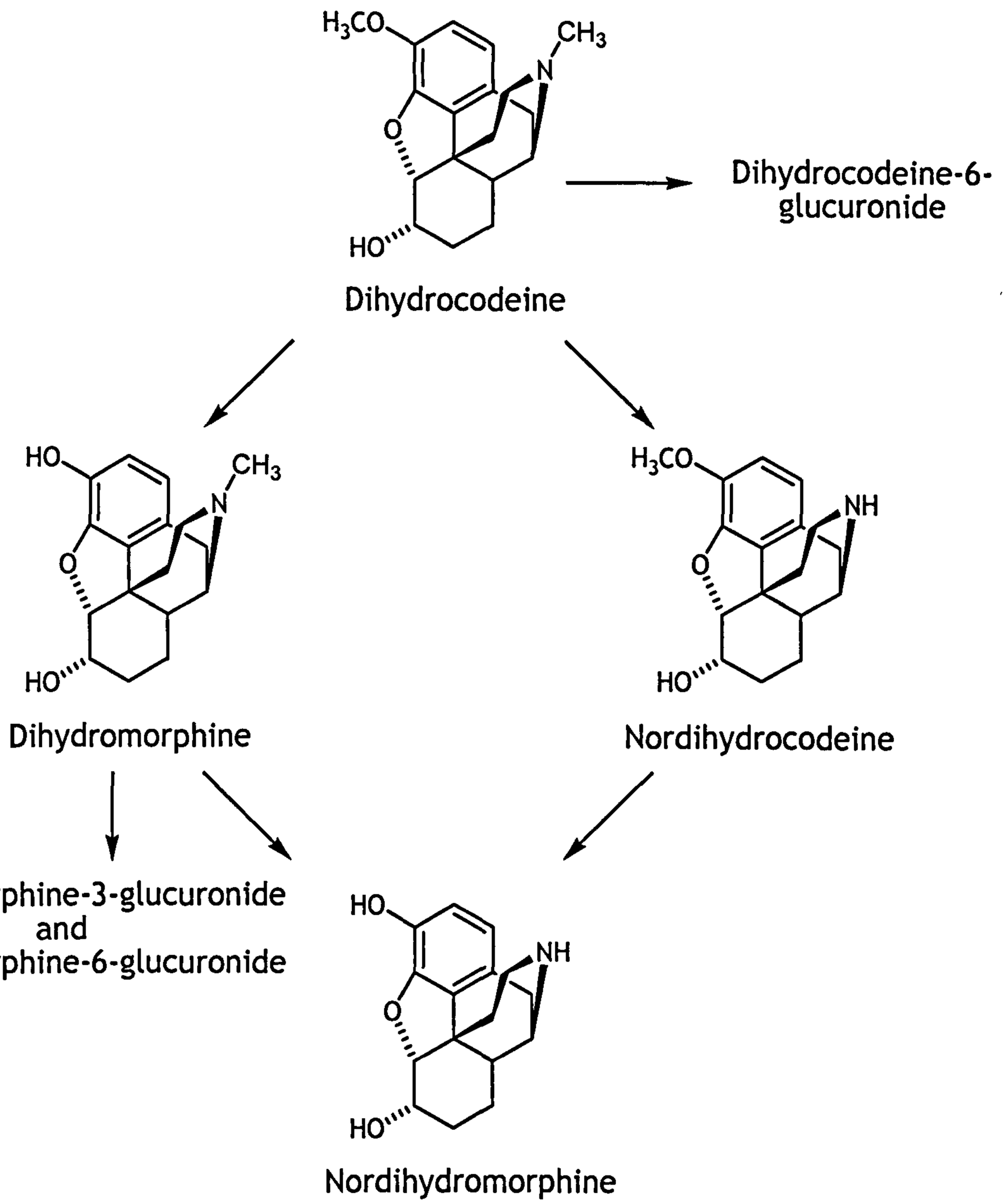


Figure 8-14 Dihydrocodeine metabolism

The pharmacokinetic and physicochemical data for these opiates is shown in Table 8-3.

Table 8-3 Pharmacokinetic and physicochemical data for opiates

	Half Life (hr)	Vd (L/kg)	Protein Binding (%)	pK_a
Diamorphine	0.03-0.1	25	40	7.6
Morphine	1.3-6.7	2-5	35	8.1, 10.0
Codeine	1.9-3.9	3.5	7-25	8.2
Dihydrocodeine	3.4-4.5	1.0-1.3	-	8.8

8.2.5 Methadone

Methadone is a morphine substitute and has been made clinically available in the United States since 1947.[176] It has many of the pharmacological properties of morphine but unlike morphine, it accumulates in the body with repeated use, causing sedative effects. Daily dosages as high as 180 mg have been used in the maintenance treatment of heroin addicts but doses of 50 mg or less can be fatal in naïve adult users.[176] The drug is produced commercially as the hydrochloride salt of the *d,l*-racemic mixture but all pharmacological activity is due to the *l*-isomer. Methadone is metabolised mainly by mono- and di-*N*-demethylation with spontaneous cyclisation of the unstable metabolites to form EDDP and EMDP (Figure 8-15). These metabolites are pharmacologically inactive and are not present to any significant extent in plasma during therapeutic methadone treatment.[176]

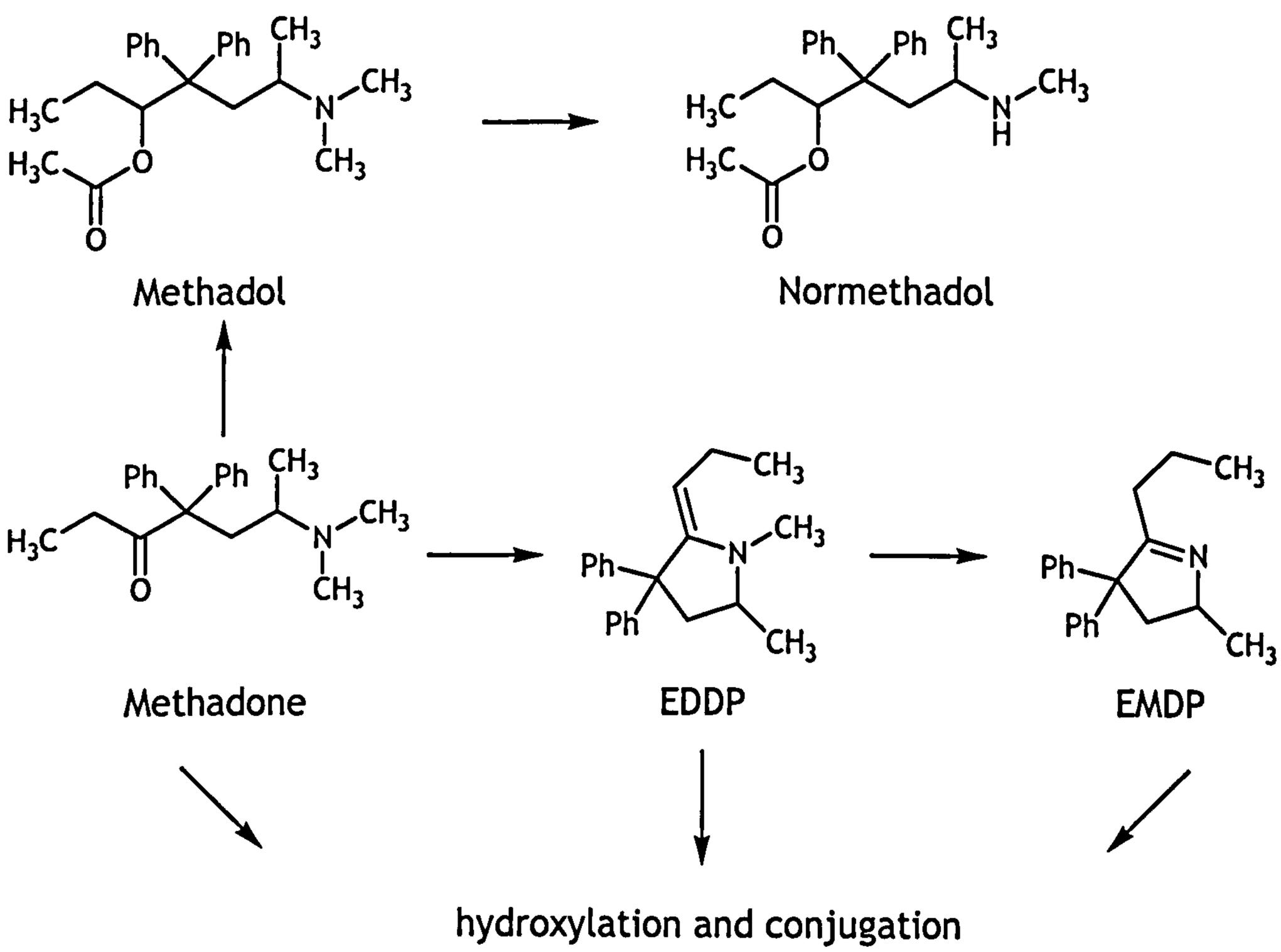


Figure 8-15 Methadone metabolism

The pharmacokinetic and physicochemical data for methadone are shown in Table 8-4.

Table 8-4 Pharmacokinetic and physicochemical data for methadone[176]

	Half Life (hr)	Vd (L/kg)	Protein Binding (%)	pK_a
Methadone	15-55	4-5	87	8.6

8.3 Aim of Study

There were two main aims of this study. The first was to develop and validate an LC-MS-MS method for the identification and quantification of common drugs of abuse in hair and apply the method to routine post-mortem hair samples. The second aim was to compare the results of the LC-MS-MS method with in-house GC-MS methods for these post-mortem samples.

8.4 Experimental

8.4.1 Samples

All samples were post-mortem scalp hair samples submitted to the toxicology laboratory of the Forensic Medicine and Science Section at the University of Glasgow for routine testing. The samples were tested for diagnostic purposes and the results were reported to the Procurator Fiscal.

8.4.2 Chemicals

Methanol, acetone, acetonitrile, acetic acid, ammonium hydroxide, ethyl acetate, formic acid, dichloromethane and propan-2-ol were purchased from BDH (Poole, UK) and were of analytical grade. Ammonium formate and sodium dodecyl sulfate were purchased from Sigma-Aldrich (Dorset, UK). *d,l*-amphetamine, *d,l*-methamphetamine, *d,l*-MDA, *d,l*-MDMA, *d,l*-MDEA, diazepam, nordiazepam, oxazepam, temazepam, cocaine, benzoylecgonine, ecgonine methyl ester, cocaethylene, morphine, 6-MAM, codeine, dihydrocodeine, methadone and EDDP were obtained from Promochem (Teddington, UK). Deuterated internal standards including *d,l*-amphetamine-d_5, *d,l*-methamphetamine-d_5, *d,l*-MDA-d_5, *d,l*-MDMA-d_5, *d,l*-MDEA-d_5, diazepam-d_5, nordiazepam-d_5, oxazepam-d_5, temazepam-d_5, cocaine-d_3, benzoylecgonine-d_3,

ecgonine methyl ester-d_3, cocaethylene-d_3, morphine-d_3, 6-MAM-d_3, codeine-d_3, dihydrocodeine-d_6, methadone-d_9 and EDDP-d_3 were obtained from Promochem (Teddington, UK). World Wide Monitoring Clean Screen® SPE columns were purchased from United Chemical technologies Incorporation (Pennsylvania, US). Bond Elut Certify™ SPE columns were purchased from Crawford Scientific (Strathaven, UK). Potassium dihydrogen phosphate was obtained from BDH (Poole, UK).

8.4.3 Standard Solutions

Individual drug stock standard solutions and deuterated drug standards were obtained as 100 µg/mL prepared in methanol. For LC-MS-MS confirmation, a combined working drug solution of *d,l*-amphetamine, *d,l*-methamphetamine, *d,l*-MDA, *d,l*-MDMA, *d,l*-MDEA, diazepam, nordiazepam, oxazepam, temazepam, cocaine, benzoylecgonine, ecgonine methyl ester, cocaethylene, morphine, 6-MAM, codeine, dihydrocodeine, methadone and EDDP was prepared at 1 µg/mL by 100-fold dilution with methanol. This was achieved by adding 25 µL of 100 µg/mL drug solution into a 25 mL volumetric flask and making up the volume to the 25 mL mark with methanol. A combined working deuterated standard solution of *d,l*-amphetamine-d_5, *d,l*-methamphetamine-d_5, *d,l*-MDA-d_5, *d,l*-MDMA-d_5, *d,l*-MDEA-d_5, diazepam-d_5, nordiazepam-d_5, oxazepam-d_5, temazepam-d_5, cocaine-d_3, benzoylecgonine-d_3, ecgonine methyl ester-d_3, cocaethylene-d_3, morphine-d_3, 6-MAM-d_3, codeine-d_3, dihydrocodeine-d_6, methadone-d_9 and EDDP-d_3 was also prepared at 1 µg/mL in the same way as the working drug solution.

8.4.4 Extraction Solutions and Mobile Phase

8.4.4.1 Preparation of Phosphate Buffer

Phosphate buffer (0.1 M, pH 6.0) was prepared as described in section 5.6.4.3. The same procedure was followed to prepare the phosphate buffer (0.1 M, pH 5.0) however the buffer pH was altered to pH 5.0 using orthophosphoric acid.

8.4.4.2 Preparation of Mobile Phase

Three mobile phases were investigated as part of the method development work. The mobile phases used in system A and B consisted of a mixture of aqueous buffer and acetonitrile. The buffers used were: 3 mM ammonium formate + 0.001 % formic acid (system A); 10 mM ammonium acetate + 0.001 % formic acid (system B). The mobile phase used in system C consisted of (1) methanol: acetonitrile: 20 mM ammonium formate (10:10:80 v/v) and (2) methanol: acetonitrile: 20 mM ammonium formate (35:35:65 v/v).

The buffer used in system A was prepared as described in section 7.5.4.2. To prepare buffer B, 0.77 g of ammonium acetate and 10 µL of concentrated formic acid were added to a 1 L volumetric flask which was then filled up to the mark with deionised water. To prepare the 20 mM ammonium formate component of mobile phase system C, 1.26 g of ammonium formate was added to a 1 L volumetric flask and the flask filled up to the mark with deionised water.

8.4.5 Instrumentation

8.4.5.1 ELISA

A MiniPrep 75 automatic pipettor from Tecan (San Jose, CA) was used. The microplate wells were washed and read using a Columbus Plus washer system and a Sunrise Remote EIA autoreader also from Tecan (Grödlg, Austria).

8.4.5.2 LC-MS-MS

LC-MS-MS analysis was carried out using a Surveyor HPLC system with an LCQ Deca XP Plus™ ion trap mass spectrometer (Thermo Finnigan, San José, CA, USA). During method development, 3 different LC columns were tested with each mobile phase described in section 8.4.4.2. The Gemini and Synergi Hydro RP columns were purchased from Phenomenex (Torrance, CA, USA). Both were C_{18} columns but the Synergi Hydro RP column also had polar end-capping. The Zorbax Stablebond (SB) Phenyl column was purchased from ChromTech (Cheshire, UK). This was a non-endcapped phenyl modified silica column. These columns were fitted with guard columns with identical packing material which

were purchased from the same companies as the LC columns. Guard column dimensions were: 4 mm x 2.0 mm, 5 μm particle size for the Gemini column; 4 mm x 2.0 mm, 4 μm particle size for the Synergi Hydro RP column; and 12.5 x 2.1 mm, 5 μm particle size for the Zorbax SB Phenyl column. A list of the mobile phases and LC columns is summarised in Table 8-5.

Table 8-5 Mobile phases and LC columns

LC Column	Mobile Phase System A	Mobile Phase System B	Mobile Phase System C
1. Gemini C18 2. Synergi Hydro RP 3. Zorbax SB Phenyl	1. 3 mM ammonium formate + 0.001 % formic acid 2. Acetonitrile	1. 10 mM ammonium acetate + 0.001 % formic acid 2. Acetonitrile	1. 10:10:80 Methanol: Acetonitrile:20 mM ammonium formate 2. 35:35:65 Methanol:Acetonitrile: 20 mM ammonium formate

8.5 LC-MS-MS Method Development

8.5.1 Optimisation of LC Column and Mobile Phase Combination

Optimum tuning parameters, precursor and product quantitation ions are shown in Table 8-6 for the drugs tested in the method. There was only 1 product ion produced during fragmentation for the amphetamines due to their low molecular weight.

Table 8-6 Optimum tuning parameters, precursor and product ions for each drug of abuse

Analyte	Sheath Gas (AU)	Auxiliary Gas (AU)	Capillary Temp (°C)	Collision Energy (%)	Precursor *m/z*	Product *m/z*
EME	10	10	210	29	200	182*, 168
Cocaine	10	10	300	30	304	182*, 150
Cocaethylene	10	10	210	30	318	196*, 150
Benzoylecgonine	10	10	300	28	290	168*, 150
Morphine	20	10	200	33	286	201*, 229
6-MAM	20	10	210	34	328	211*, 268
Codeine	10	5	240	34	300	215*, 243
Dihydrocodeine	10	5	240	33	302	245*, 201
Diazepam	20	20	300	42	285	257*, 222
Nordiazepam	20	15	300	41	271	243*, 140
Oxazepam	20	20	300	29	287	269*, 241
Temazepam	20	20	300	29	301	283*, 255
Amphetamine	15	5	210	23	136	119*
Methamphetamine	15	5	210	28	150	119*
MDA	15	15	220	21	180	163*
MDMA	15	15	280	25	194	163*
MDEA	15	15	220	20	208	163*

* = quantitation ion

The analytes shown in Table 8-6 were analysed in 3 separate injections. This was done to minimise the number of scan events per time window to achieve maximum sensitivity. It is also to maximise the number of points across the peak to obtain more accurate analyte quantifications. The first injection was for cocaine and its metabolites, opiates, methadone and EDDP. The second injection was for amphetamines and the third for benzodiazepines.

Initially, 3 mobile phase systems and 3 LC columns (Table 8-5) were investigated to determine which mobile phase system and column produced the greatest peak area response for the target analytes. Column 1 and mobile phase system A were used for the analysis of opiates, cocaine and metabolites, amphetamines and benzodiazepines in oral fluid by LC/MS/MS.[143] Column 2 and mobile phase

system B were used for the quantification of opiates and cocaine and metabolites in hair by LC-APCI-MS/MS.[241] Column 3 and mobile phase system C was used for screening of drugs of abuse in hair using LC-ESI-MS/MS.[3]

3 x 20 µL injections of 50 ng drug/200 µL mobile phase were run for each column and mobile phase system. The peak area response (PAR) data and retention times for the various columns and mobile phase systems is given in Table 8-7-8-9. The mobile phase system producing the greatest average peak area response for each drug was recorded as being 100 % and the average peak area responses for the other two mobile phase systems were calculated as a % of the optimum mobile phase system response.

Table 8-7 Relative % peak area response using Gemini C_{18} column

Analyte	System A		System B		System C	
	PAR for n=3	t_R in mins	PAR for n=3	t_R in mins	PAR for n=3	t_R in mins
EME	100.0	3.8	51.2	2.2	90.7	1.8
Cocaine	31.5	13.7	100.0	18.1	44.5	11.5
Cocaethylene	16.8	15.9	100.0	19.4	20.1	15.9
Benzoylecgonine	16.4	10.6	100.0	13.3	24.5	5.7
Morphine	100.0	5.2	42.0	8.0	45.1	2.3
6-MAM	77.9	8.6	2.7	12.9	100.0	4.8
Codeine	76.2	8.2	70.8	11.7	100.0	4.1
Dihydrocodeine	93.1	7.8	81.6	10.4	100.0	3.4
Methadone	35.4	18.7	100.0	21.4	52.6	23.8
EDDP	48.0	17.9	77.5	20.6	100.0	20.7
Oxazepam	81.2	19.2	100.0	21.4	25.5	23.6
Temazepam	12.7	20.4	100.0	22.5	59.1	25.1
Nordiazepam	100.0	20.4	17.7	22.7	2.9	25.8
Diazepam	100.0	21.5	45.1	23.8	4.9	27.2
Amphetamine	6.1	7.7	56.2	10.7	100.0	4.2
Methamphetamine	20.1	8.8	100.0	11.6	96.7	4.9
MDA	10.0	8.3	26.9	11.5	100.0	4.5
MDMA	35.8	9.3	92.4	12.2	100.0	5.1
MDEA	28.2	10.3	86.0	13.7	100.0	6.2

Using the Gemini C_{18} column, mobile phase system C produced the optimum response for the greatest number of analytes compared to the other two mobile phase systems.

Table 8-8 Relative % peak area response using Synergi Hydro RP column

Analyte	System A		System B		System C	
	PAR for n=3	t_R in mins	PAR for n=3	t_R in mins	PAR for n=3	t_R in mins
EME	70.6	4.9	13.1	5.8	100.0	1.9
Cocaine	69.1	19.4	100.0	20.7	48.2	16.8
Cocaethylene	94.3	20.8	100.0	22.1	50.8	19.9
Benzoylecgonine	100.0	10.5	99.8	13.4	49.5	6.2
Morphine	74.7	10.3	44.6	13.9	100.0	3.4
6-MAM	30.5	15.2	28.7	17.5	100.0	8.5
Codeine	41.4	14.7	33.8	17.3	100.0	7.9
Dihydrocodeine	55.4	13.8	48.4	16.6	100.0	5.7
Methadone	86.6	24.4	48.7	26.2	100.0	26.6
EDDP	46.7	23.3	20.0	25.2	100.0	23.5
Oxazepam	100.0	19.2	87.4	21.3	20.3	23.8
Temazepam	100.0	20.4	78.5	22.6	1.5	25.7
Nordiazepam	100.0	20.6	65.7	22.8	9.6	26.4
Diazepam	100.0	21.9	29.9	24.3	7.9	28.3
Amphetamine	100.0	10.8	30.0	15.3	22.3	5.2
Methamphetamine	100.0	12.1	13.9	17.2	23.9	6.5
MDA	46.6	11.7	45.3	15.8	100.0	5.6
MDMA	100.0	13.2	52.8	17.1	48.8	6.7
MDEA	100.0	13.6	44.1	18.0	47.0	8.2

Using the Synergi Hydro RP column, mobile phase system A produced the optimum response for the greatest number of analytes.

Table 8-9 Relative % peak area response using Zorbax Phenyl column

Analyte	System A		System B		System C	
	PAR for n=3	t_R in mins	PAR for n=3	t_R in mins	PAR for n=3	t_R in mins
EME	79.2	3.7	83.1	3.2	100.0	1.3
Cocaine	100.0	22.9	89.8	21.7	43.2	13.9
Cocaethylene	100.0	24.3	58.4	23.8	42.2	18.2
Benzoylecgonine	100.0	9.2	99.6	11.7	68.5	4.5
Morphine	51.8	11.5	33.7	9.5	100.0	2.1
6-MAM	89.1	18.1	40.9	16.1	100.0	6.0
Codeine	60.2	17.1	39.8	14.3	100.0	4.4
Dihydrocodeine	73.4	16.9	48.1	13.3	100.0	3.9
Methadone	100.0	28.3	21.5	29.9	91.5	25.2
EDDP	50.0	28.6	4.4	30.0	100.0	23.4
Oxazepam	44.8	17.4	100.0	19.4	9.0	18.9
Temazepam	92.1	18.6	100.0	20.5	14.7	21.2
Nordiazepam	100.0	18.6	62.8	20.5	18.1	21.6
Diazepam	100.0	19.7	63.6	21.7	22.5	23.5
Amphetamine	14.4	13.9	100.0	9.6	40.8	3.0
Methamphetamine	5.7	18.3	100.0	12.3	25.6	3.8
MDA	80.8	16.2	100.0	12.1	40.5	3.7
MDMA	61.5	19.9	100.0	14.5	53.9	5.0
MDEA	36.5	20.7	100.0	16.7	44.3	6.4

Using the Zorbax Phenyl column, mobile phase system B produced the optimum response for the greatest number of analytes.

The average peak area response was then compared for each drug using the optimum mobile phase system determined for each of the three columns. The mobile phase system and column producing the greatest average peak area response was recorded as being 100 % and the average peak area responses for the other two mobile phase systems and columns were calculated as a % of the optimum mobile phase system and column response.

Table 8-10 Relative % peak area response for the 3 optimum mobile phase and column combinations

Analyte	Relative % Peak Area Response (n=3)		
	Gemini + Mobile Phase System C	Synergi Hydro RP + Mobile Phase System A	Zorbax Phenyl + Mobile Phase System B
EME	100.0	59.5	62.9
Cocaine	46.6	67.8	100.0
Cocaethylene	48.1	100.0	71.0
Benzoylecgonine	42.4	100.0	72.3
Morphine	100.0	89.7	47.3
6-MAM	100.0	28.2	33.5
Codeine	100.0	34.2	31.2
Dihydrocodeine	100.0	43.2	43.3
Methadone	89.3	100.0	18.7
EDDP	100.0	50.5	3.2
Oxazepam	45.1	100.0	79.9
Temazepam	37.8	59.0	100.0
Nordiazepam	4.0	100.0	83.4
Diazepam	4.3	100.0	49.5
Amphetamine	85.2	68.7	100.0
Methamphetamine	31.3	71.4	100.0
MDA	89.2	33.8	100.0
MDMA	46.2	57.5	100.0
MDEA	54.0	53.6	100.0

The Zorbax Phenyl column and mobile phase system B produced the greatest average peak area response for the greatest number of analytes compared to the other two columns and mobile phase systems. This column and mobile phase combination was therefore selected for further optimisation.

8.5.2 Mobile Phase Optimisation

Since the Zorbax SB Phenyl column and mobile phase system B produced the greatest average peak area response for the greatest number of analytes, it was

decided to investigate the effect of using different concentrations of ammonium acetate and formic acid in the mobile phase on analyte response.

The ammonium acetate buffer concentrations tested were 2, 5, 10 and 25 mM. These concentrations were prepared by adding 0.15, 0.30, 0.77, 1.6 g of ammonium acetate to a 1 L volumetric flask and filling the flask up to the mark with deionised water. The formic acid concentration of 0.001 % was kept constant to determine the effects of changing buffer concentration only. The average peak area (n=3) was calculated for each analyte. The ammonium acetate concentration and the relative average % peak area response for a selection of analytes from different drug classes are shown in Figure 8-16.

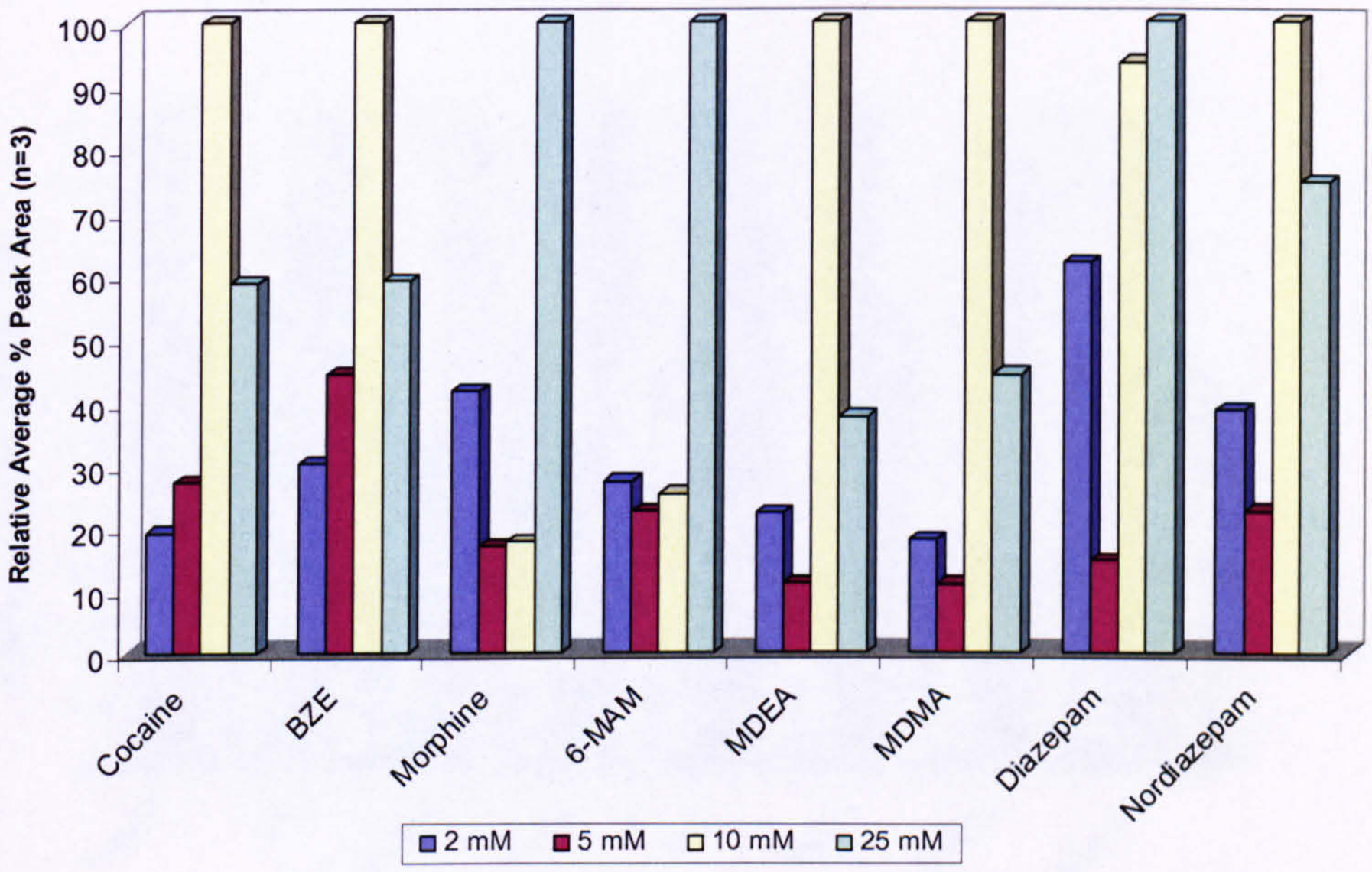


Figure 8-16 Analyte responses for increasing ammonium acetate concentration

With the exception of the opiates, analyte response decreased with the lower ammonium acetate concentrations. The highest opiate response was observed using 25 mM ammonium acetate. Higher responses were observed using 2 mM than 5 mM or 10 mM ammonium acetate.

Higher concentrations of ammonium acetate were not tested since the use of high concentrations of electrolytes has the disadvantage of forming salt clusters and cluster ions by direct solvent evaporation.[166] 10 mM ammonium acetate was selected for further work since the greatest analyte response was observed for the highest number of analytes at this concentration.

The concentration of formic acid was then investigated to determine the effect, if any, on analyte response. The 10 mM ammonium acetate was prepared using three formic acid concentrations- 0.001, 0.005 and 0.01 %. The average peak area response (n=3) was compared for each formic acid concentration. The formic acid concentration which produced the greatest average peak area response was recorded as being 100 % and the average peak area responses for the other two formic acid concentrations were calculated as a % of the optimum formic acid concentration response.

The relative average % peak area response for a selection of analytes from different drug classes is given in Figure 8-17. The results showed that the optimum formic acid concentration was 0.001 % for most analytes.

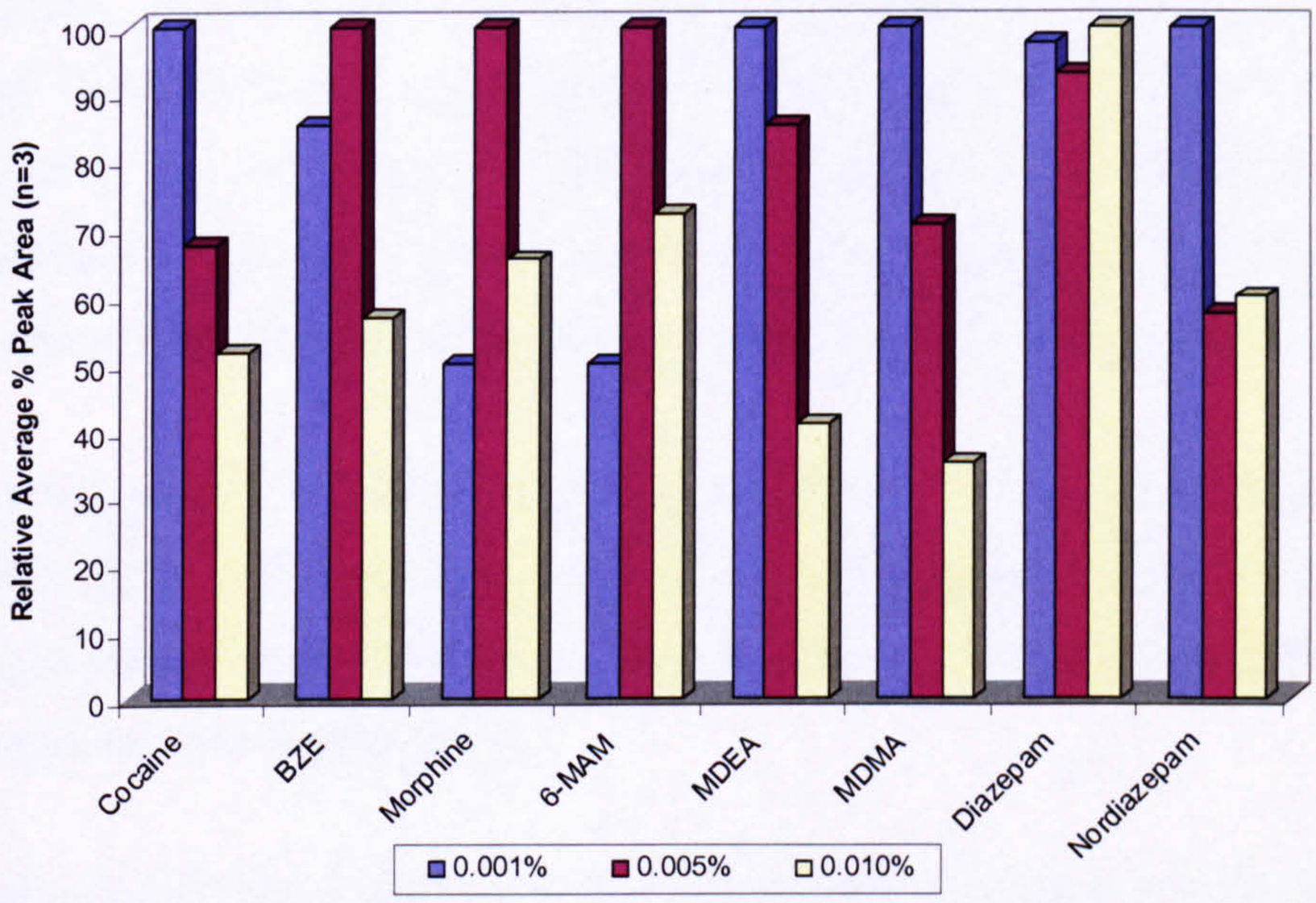


Figure 8-17 Analyte responses for increasing formic acid concentration

As the Zorbax SB phenyl column is narrow-bore, it has a smaller internal volume than a standard bore column and the flow rate for optimal column efficiency is proportionately smaller. The flow rates recommended by the column manufacturer are 0.15 to 0.25 mL/min as the column has an internal diameter of 2.1 mm. A flow rate of 0.25 mL/min was therefore selected for future work since faster flow rates result in faster sample throughput.

Although the Zorbax phenyl column was initially selected for further work, a pressure problem was encountered during method development. The pressure

would not stabilise and exceeded the instrument's maximum pressure setting of 5800 psi.

The manufacturers recommend using mobile phases such as acetonitrile/water mixtures as the column is moderately non-polar in nature so the mobile phase selection was theoretically suitable. However it was not feasible to use this column in practice. The Synergi Hydro RP column and mobile phase system A were selected for further work since this column and mobile phase combination produced a higher relative average % peak area response for more analytes compared with the Gemini column and mobile phase system C (Table 8-10).

8.5.3 Selection of Incubation Medium

A previously published SPE method which was used for the extraction of common drugs of abuse from oral fluid was used to clean up hair samples which had been incubated in various media.[143] The elution step was modified slightly from 3 mL ethyl acetate with 2 % ammonia to 1.5 mL ethyl acetate with 2 % ammonia and 1.5 mL dichloromethane/isopropanol/ammonium hydroxide (78:20:2 v/v).

In order to assess the suitability of the methanol/25 % aqueous ammonium hydroxide (20:1 v/v) incubation used for benzodiazepines in chapter 7 for extracting other drugs of abuse in hair, 2 post-mortem hair samples with positive blood results were extracted.

The hair samples were tested for the drug classes found in the corresponding blood sample. The results are shown in Table 8-11.

Table 8-11 Hair samples incubated in methanol/25 % aqueous ammonium hydroxide

Hair Sample Number	Blood result (mg/L)	Hair result (ng/mg)
1	Morphine 0.27 Codeine 0.04 Methadone 0.37 EME 0.20	Morphine 5.89 Codeine 1.55 6-MAM 70.70
2	Diazepam 0.29 Nordiazepam 0.79 Temazepam 0.05 Oxazepam 0.06 Morphine 0.14 Codeine 0.01	Codeine 0.06 Nordiazepam 1.40 Dihydrocodeine 0.26

The methanol/25 % aqueous ammonium hydroxide incubation successfully extracted opiates and benzodiazepines from post-mortem hair samples. 6-MAM, a useful marker for heroin use, was detected in hair sample 1 but was not detected in the corresponding blood sample. This is because the 6-MAM half-life is short in blood, between 6-25 minutes.[176]

Dihydrocodeine was detected in hair sample 2 and was not detected in blood. This provided additional information on the individual's prior drug use.

One concern with using the MeOH/25 % aqueous NH_4OH incubation was the potential for analyte hydrolysis, in particular cocaine and 6-MAM, as the hydrolysis of 6-MAM to morphine and also cocaine to BZE and EME has been demonstrated at alkaline pH.[208] The stability of cocaine and 6-MAM was therefore investigated using this incubation medium.

8.5.3.1 Stability of 6-MAM and cocaine using the methanol/25 % ammonium hydroxide incubation

Three samples were prepared by spiking 1.5 mL of MeOH/25 % aqueous ammonium hydroxide with 50 ng of cocaine and 6-MAM and these were subsequently left to incubate for 18 hours at room temperature. After this time, 50 ng of cocaine-d_3 and 6-MAM-d_3 were added to the vials and the vial contents

were evaporated to dryness at room temperature under nitrogen. The deuterated internal standards were added at this point in the experiment so that the results obtained were only affected by degradation during the incubation step. Three unextracted standards were prepared at the same concentration and kept in the fridge during the incubation period. 50 ng of deuterated internal standard was added to the unextracted standards at the same time as the incubated samples and the vial contents evaporated to dryness under nitrogen. The samples once dry were reconstituted in 200 µL of mobile phase initial conditions and 20 µL was injected.

The results from this experiment showed that 6-MAM was hydrolysed to a great extent (≈ 95 %) using this incubation medium. Cocaine was also significantly hydrolysed (≈ 22 %). Therefore this method does not produce true representative concentrations of 6-MAM, morphine, cocaine or benzoylecgonine in hair. Partial hydrolysis of 6-MAM will have occurred in hair sample 1 shown in Table 8-11. It was decided not to use this incubation and hence other incubations were investigated. The % recovery values are shown in Table 8-12 .

Table 8-12 % Recovery of 6-MAM and cocaine without matrix

Cocaine (% Recovery, n=3)		6-MAM (% Recovery, n=3)	
Mean	S.D.	Mean	S.D.
77.6	2.3	4.9	0.4

8.5.3.2 Stability of 6-MAM and cocaine in different incubation media

This alkaline methanol/25 % aqueous ammonium hydroxide extraction is suitable for extracting opiates and benzodiazepines from authentic hair samples as shown in section 8.5.3, although it significantly degrades 6-MAM and cocaine. Therefore, the same stability experiment as described in section 8.5.3.1 was carried out using 1, 2, 5 and 10 % aqueous ammonium hydroxide in methanol. In addition to this, the stability was assessed in aqueous acidic media (0.01, 0.1 and 1 M HCl), methanol and phosphate buffer (0.1 M, pH 5.0) by incubating for 18 hours at 45 °C.

The % recovery of 6-MAM and cocaine in the different incubation media are given in Table 8-13.

Table 8-13 % Recovery of cocaine and 6-MAM in various incubation media

Incubation Medium	Cocaine % Recovery (n=3)		6-MAM % Recovery (n=3)	
	Mean	S.D.	Mean	S.D.
Methanol	99.4	5.5	88.6	4.2
Phosphate Buffer pH 5	102.3	1.4	102.9	10.9
0.1 M HCl	101.5	5.2	47.3	5.5
0.05 M HCl	100.6	1.1	59.1	3.1
0.01 M HCl	103.7	4.3	87.4	5.3
Methanol/ 1 % NH_4OH	97.5	3.2	67.2	5.0
Methanol/ 2 % NH_4OH	91.8	3.5	37.2	3.1
Methanol/ 5 % NH_4OH	89.7	2.8	18.5	1.2
Methanol/ 10 % NH_4OH	86.1	0.5	5.5	0.5
Methanol/ 25 % NH_4OH	77.6	2.3	4.9	0.4

The extent of 6-MAM and cocaine degradation was negligible using the methanol and phosphate buffer incubations. In contrast, all concentrations of aqueous acidic media resulted in significant 6-MAM hydrolysis whereas cocaine degradation was negligible. This result was in agreement with previously published results.[245]

The results obtained for the aqueous alkaline media showed an increased 6-MAM and cocaine hydrolysis with increasing concentration of aqueous ammonium hydroxide. 6-MAM was hydrolysed to a greater extent than cocaine under alkaline conditions. Graphs of % recovery *vs* % aqueous ammonium hydroxide in methanol are shown in Figure 8-18. The 6-MAM graph demonstrates a hyperbolic decrease in % recovery with increasing ammonium hydroxide concentration, whereas the cocaine graph shows a linear decrease, with a good correlation between % recovery and % ammonium hydroxide in methanol (R^2=0.9117).

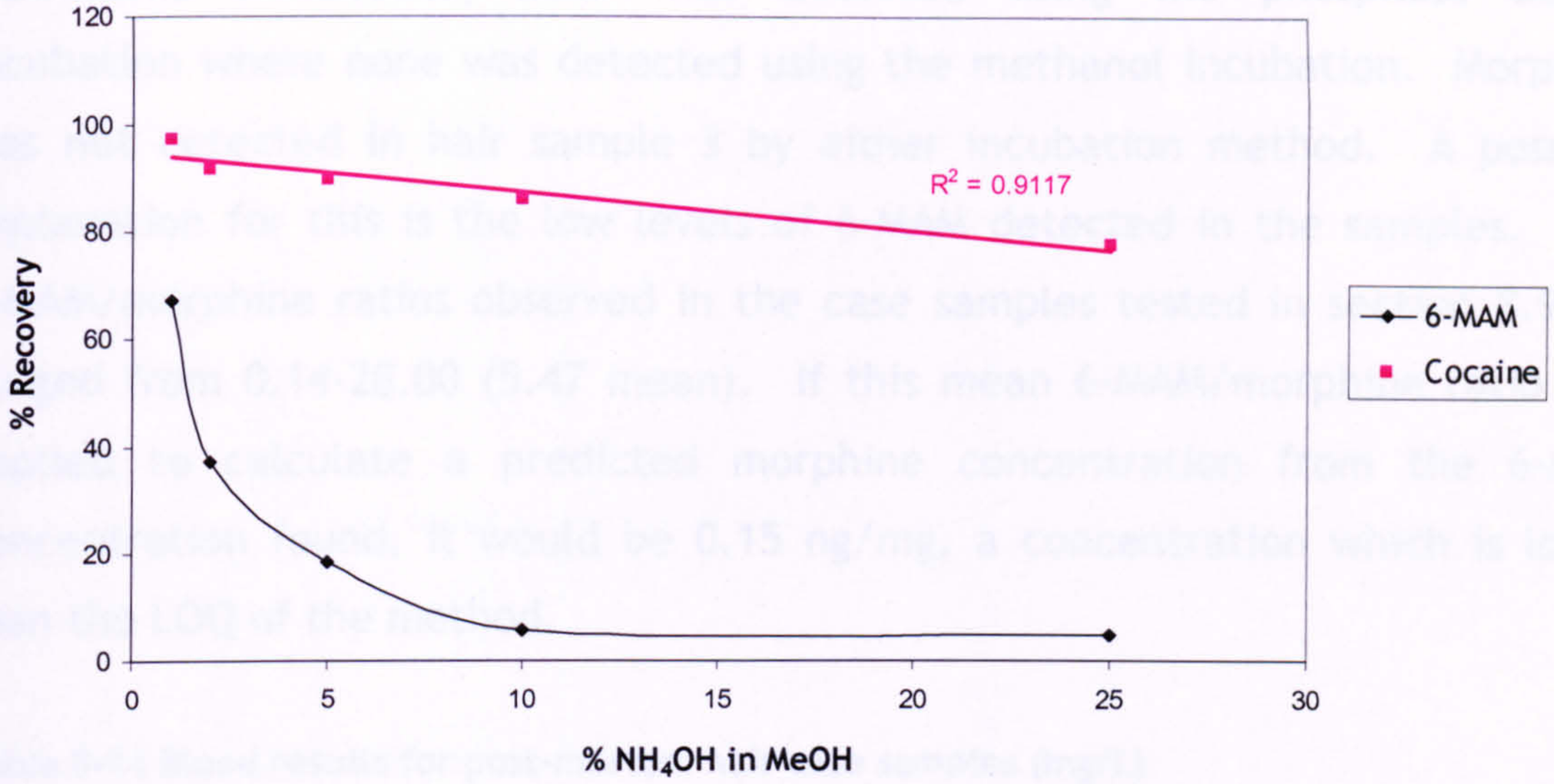


Figure 8-18 % Recovery *vs* % aqueous NH_4OH in methanol for 6-MAM and cocaine

Both the acidic and alkaline incubation media produced significant hydrolysis of 6-MAM. Cocaine showed significant hydrolysis using 25 % aqueous ammonium hydroxide in methanol. It was therefore decided to compare the methanol and phosphate buffer incubation media for future work since both cocaine and 6-MAM were relatively stable using these methods. The methanol and phosphate buffer incubation media produced 6-MAM recoveries of 88.6 % and 102.9 % respectively while cocaine recoveries were 99.4 and 102.3 % respectively.

8.5.3.3 Methanol vs Phosphate Buffer Incubation

3 post-mortem hair samples which had positive blood results were decontaminated as described before in section 6.4.1. The root-0.5 cm segment was removed and the 0.5-3.5 cm segment was cut up into smaller segments of 2-3 mm in length. This cut up segment was subsequently split to compare the methanol and phosphate buffer incubation methods. The samples were analysed for the drug classes detected in the blood. The blood and hair results are shown in Table 8-14 and Table 8-15 respectively.

With the exception of the cocaine concentration detected in hair sample 1 and the methadone concentration in hair sample 3, the phosphate buffer extraction produced higher quantitative results overall, extracting greater concentrations of EME, benzoylecgonine, 6-MAM, codeine and amphetamine. Furthermore, the

methadone metabolite, EDDP was detected using the phosphate buffer incubation where none was detected using the methanol incubation. Morphine was not detected in hair sample 3 by either incubation method. A possible explanation for this is the low levels of 6-MAM detected in the samples. The 6-MAM/morphine ratios observed in the case samples tested in section 8.9.1.2 ranged from 0.14-28.00 (5.47 mean). If this mean 6-MAM/morphine ratio was applied to calculate a predicted morphine concentration from the 6-MAM concentration found, it would be 0.15 ng/mg, a concentration which is lower than the LOQ of the method.

Table 8-14 Blood results for post-mortem hair case samples (mg/L)

Hair Sample Number	Cocaine + Metabolites	Opiates and Methadone	Amphetamines	Benzodiazepines
1	EME 5.13 COC 3.58 BZE 3.98	-	-	-
2	-	-	AMP 0.66	
3	-	MOR 0.37 COD 0.01 MD 0.17	-	DZ 0.06 NDZ 0.09 TMZ 0.02

"EME"= Ecgonine Methyl Ester, "COC"= Cocaine, "BZE"= Benzoylecgonine, "MOR"= Morphine, "COD"= Codeine, "MD"= Methadone, "AMP"= Amphetamine, "DZ"= Diazepam, "NDZ"=Nordiazepam,"TMZ"=Temazepam

Table 8-15 Methanol vs phosphate buffer hair results (ng/mg)

Hair Sample Number	Cocaine + Metabolites		Opiates		Amphetamines		Benzodiazepines	
	Methanol	Phosphate Buffer	Methanol	Phosphate Buffer	Methanol	Phosphate Buffer	Methanol	Phosphate Buffer
1	EME 0.12 COC 101.53 BZE 30.08	EME 0.51 COC 90.90 BZE 44.60	-	-	-	-	-	-
2	-	-	-	-	AMP 5.08	AMP 10.78	-	-
3	-	-	COD 0.47 6-MAM 0.84 MD 5.58	COD 0.48 6-MAM 0.94 MD 3.95 EDDP 0.02	-	-	-	-

"EME"= Ecgonine Methyl Ester, "COC"= Cocaine, "BZE"= Benzoylecgonine, "COD"= Codeine, "AMP"= Amphetamine, "MD"= Methadone

Overall, the phosphate buffer incubation was better for the simultaneous detection of cocaine and its metabolites, amphetamine and opiates. In addition, EDDP was detected in sample 3 using the phosphate buffer incubation while none was detected using the methanol incubation. Benzodiazepines were not detected in any of the samples to compare the two incubations. Furthermore, the stability data using the phosphate buffer incubation is excellent for 6-MAM and cocaine which have been shown to hydrolyse under acidic and basic conditions[245], the hair extracts are cleaner and the analysis is simpler with less sample manipulation. Analyte recoveries for the methanol incubation were generally lower for most analytes as reported in various studies.[75,208,245] It has been proposed that the generally higher recoveries obtained using a phosphate buffer incubation is a result of the water molecules penetrating the keratinised hair to a greater extent than an organic solvent.[75] Furthermore, the non-keratinous hair regions could potentially provide diffusion channels, both into and out of the hair, for small drug molecules in the presence of water.[61]

8.5.4 Comparison of SPE methods

Bond Elut Certify™ and Clean Screen® DAU SPE columns were investigated for the simultaneous extraction of the drugs of abuse from hair. Both columns are mixed-mode bonded silica, with both hydrophobic and ion-exchange properties, which are suitable for the extraction of acidic, neutral and basic analytes. The silanol groups in these columns are partially derivatised with medium length alkyl chains and cation exchange substituents which allow at least two types of analyte-sorbent interactions.[250]

8.5.4.1 Bond Elut Certify™ SPE

This method is previously published and was applied in the simultaneous screening of acidic, neutral and basic drugs from oral fluid.[143] Bond Elut Certify™ LRC cartridges were conditioned sequentially with 2 mL methanol and 2 mL phosphate buffer (0.1 M, pH 6.0). 2 mL of sample in phosphate buffer (0.1 M, pH 6.0) was applied to the cartridge and allowed to drip through without the presence of a vacuum. The cartridges were then washed with 1 mL deionised

water, 0.5 mL 0.01 M acetic acid and dried for 10 minutes under full vacuum. 50 µL of methanol was added and the cartridges were dried for a further 2 minutes under full vacuum. The acidic/neutral fraction was eluted using 4 mL acetone/dichloromethane (1:1 v/v) while the basic drugs were eluted using 1 x 1.5 mL ethyl acetate with 2 % aqueous ammonium hydroxide and 1 x 1.5 mL dichloromethane: isopropanol: aqueous ammonium hydroxide (78:20:2 v/v), with a 2 minute drying step between the two basic eluants. The acidic/neutral and the basic fractions were combined. This was particularly significant since the benzodiazepines were eluted in both fractions.

8.5.4.2 Clean Screen® SPE

This SPE was a modified version of an extraction procedure for drugs of abuse in urine provided by the SPE manufacturers. Clean Screen® (ZSDAU 020) extraction cartridges were conditioned sequentially with 3 mL methanol, 3 mL deionised water and 1 mL phosphate buffer (0.1 M, pH 5.0). The vortexed samples were loaded in 2 mL phosphate buffer (0.1 M, pH 5.0) and allowed to drip through without vacuum. The columns were then washed with 3 mL phosphate buffer (0.1 M, pH 5.0), 1 mL acetic acid (1.0 M) and dried for 5 minutes under full vacuum. The drugs were eluted using 2 mL methanol/2 % aqueous ammonium hydroxide.

8.5.4.3 Analyte recovery without hair matrix

To ensure that the SPE methods were appropriate for the drugs of abuse, the recovery was investigated in an experiment without hair matrix.

50 ng of drug was added to two test tubes along with 2 mL phosphate buffer (0.1 M, pH 5.0). Two unextracted samples were prepared at the same concentration and kept in the fridge during the extractions. 50 ng of deuterated internal standard was added to the eluant after the extraction and also to the unextracted samples. The samples were evaporated to dryness at room temperature under nitrogen and subsequently reconstituted in 200 µL of mobile phase initial conditions. 20 µL was injected for each sample. The results are displayed in Table 8-16.

Table 8-16 Bond Elut™ and Clean Screen® recovery without matrix (n=2)

Analyte	Mean % Recovery (n=2)	
	Bond Elut Certify™	Clean Screen®
EME	76.6	100.7
Cocaine	100.7	84.0
Cocaethylene	96.4	95.8
Benzoylecgonine	95.2	97.8
Morphine	77.4	87.2
6-MAM	74.1	74.6
Codeine	102.4	83.9
Dihydrocodeine	91.6	97.6
Methadone	92.9	82.6
EDDP	70.1	98.6
Oxazepam	82.9	92.7
Temazepam	94.5	99.0
Nordiazepam	93.3	97.6
Diazepam	95.9	95.2
Amphetamine	95.6	98.9
Methamphetamine	78.7	90.4
MDA	88.8	84.2
MDMA	97.6	101.2
MDEA	90.7	100.4

Without matrix, the Clean Screen® method produced higher recoveries for a greater number of analytes compared to the Bond Elut Certify™ method. The experiment was then repeated with hair matrix as discussed in section 8.5.4.4.

8.5.4.4 Analyte Recovery with Hair Matrix

10 mg ± 0.1 mg blank hair was weighed out into three vials and it was then spiked with 50 ng analyte. Two unextracted samples were prepared at the same

concentration and kept in the fridge during the extractions. The samples were then incubated in phosphate buffer (pH 5.0) as detailed in section 8.5.3.2 and extracted by the Bond Elut Certify™ and Clean Screen® methods previously described in sections 8.5.4.1 and 8.5.4.2. 50 ng of deuterated internal standard was added to the eluant after the extractions and also to the unextracted samples. The samples were evaporated to dryness at room temperature under nitrogen and subsequently reconstituted in 200 µL of mobile phase initial conditions. 20 µL was injected for each sample. The results are shown in Table 8-17.

Table 8-17 Bond Elut™ and Clean Screen® recovery with matrix

Analyte	Mean % Recovery (% RSD) (n=3) at 50 ng	
	Bond Elut Certify™	Clean Screen®
Ecgonine methyl ester	73.7 (0.5)	101.1 (5.6)
Cocaine	92.3 (5.0)	98.6 (4.8)
Cocaethylene	100.3 (6.1)	104.9 (11.1)
Benzoylecgonine	91.8 (10.0)	98.0 (6.5)
Morphine	75.9 (3.8)	94.5 (5.4)
6-MAM	94.6 (6.1)	98.3 (6.3)
Codeine	90.1 (2.2)	95.0 (6.2)
Dihydrocodeine	94.8 (5.4)	107.4 (3.6)
Methadone	124.6 (12.6)	87.8 (15.5)
EDDP	80.2 (8.3)	86.8 (3.3)
Oxazepam	90.3 (14.2)	77.0 (5.5)
Temazepam	99.3 (7.9)	107.6 (2.0)
Nordiazepam	79.8 (2.8)	99.1 (8.7)
Diazepam	81.4 (8.4)	80.5 (4.1)
Amphetamine	97.9 (8.3)	109.9 (11.9)
Methamphetamine	91.6 (3.1)	109.1 (1.5)
MDA	88.3 (2.1)	78.8 (6.0)
MDMA	78.5 (1.0)	99.5 (2.2)
MDEA	95.4 (2.8)	85.9 (2.5)

The Clean Screen® method produced higher recoveries for a greater number of analytes compared to the Bond Elut® method for spiked hair. All of these were 77 % or greater.

8.5.4.5 SPE Clean-Up

Hair was collected from five members of laboratory personnel. 10 mg ± 0.1 mg hair was washed via the procedure described previously in section 6.4.1 and subsequently extracted using the Bond Elut Certify™ and Clean Screen® methods. The chromatograms for each blank hair extract using both SPE methods were compared visually to determine which method produced the cleanest extracts overall.

The Clean Screen® method produced the cleanest extracts overall for all drug classes and for hair from all five individuals. An example LC-MS-MS chromatogram of blank hair collected from one individual using both the Bond Elut Certify™ and Clean Screen® extraction methods is shown in Figure 8-19. At the time of using this particular LC column, the diazepam retention time (t_R) was 22.7 minutes. It is apparent from the chromatograms that there was greater matrix interference at the diazepam t_R, as indicated by the larger peak area, using the Bond Elut Certify™ column compared with the Clean Screen® column.

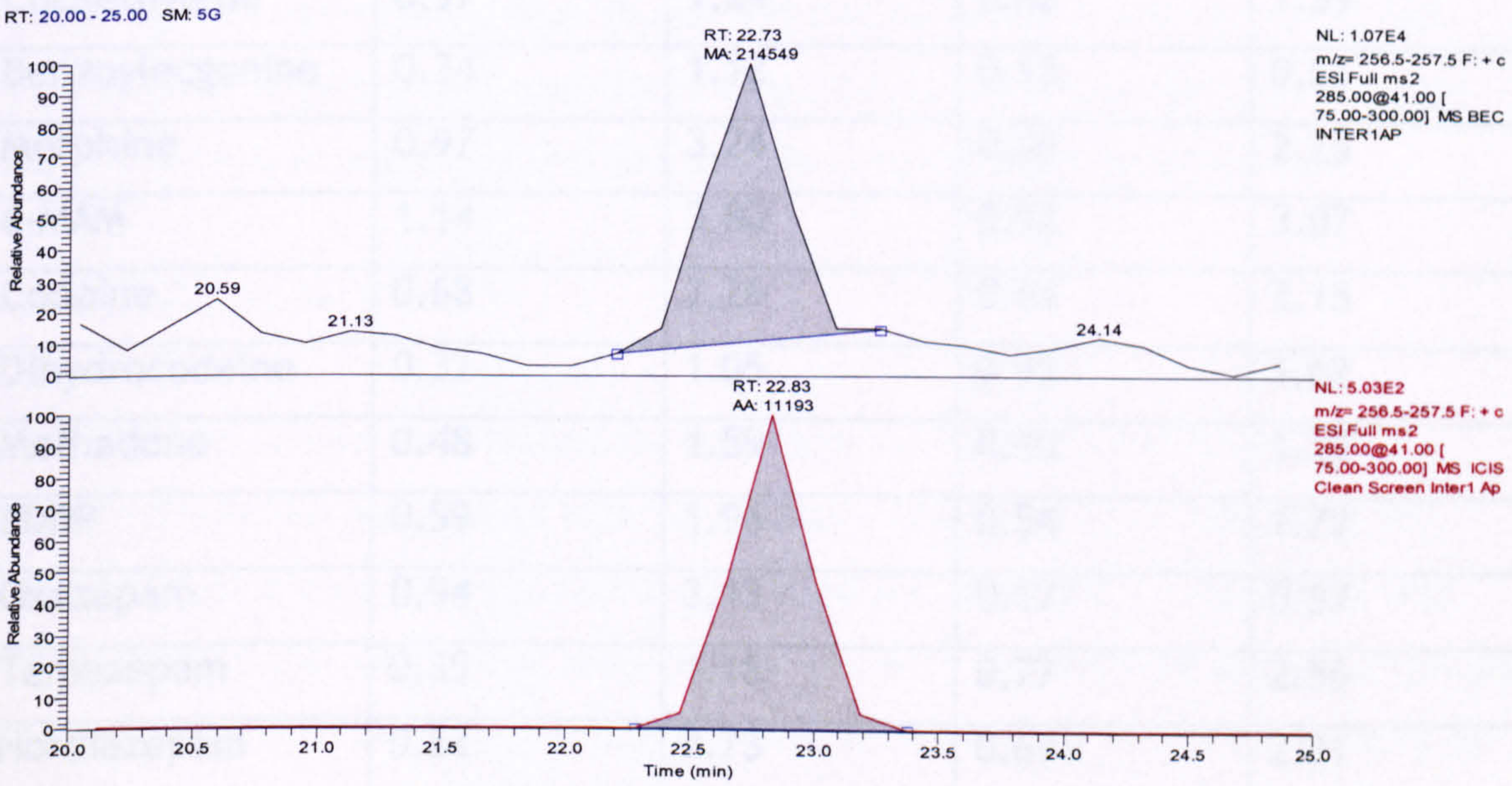


Figure 8-19 Bond Elut Certify™ vs Clean Screen® interference at diazepam t_R

8.5.4.6 LOD and LOQ

The LOD was calculated for each analyte using Equation 7-1 and Equation 7-2. The LOQ was calculated for each analyte using Equation 7-3 and Equation 7-4, however ten standard deviations ($10s_B$) of the blank was used in Equation 7-3. The LOD and LOQ values are shown in Table 8-18 for both SPE methods.

The Clean Screen® SPE method produced lower LOD and LOQ values for the majority of analytes. The LOD range was 0.15-0.92 ng/10 mg hair and the LOQ range was 0.51-3.07 ng/10 mg hair. Furthermore, there was a relatively large difference for cocaine, oxazepam and MDA. This is because of reduced matrix interference observed using the Clean Screen® method compared to the Bond Elut Certify™ method. The Clean Screen method was therefore validated in further work.

Table 8-18 LOD and LOQ values for the Bond Elut Certify™ and Clean Screen® SPE methods

Analyte	Bond Elut Certify™		Clean Screen®	
	LOD (ng/10 mg)	LOQ (ng/10 mg)	LOD (ng/10 mg)	LOQ (ng/10 mg)
EME	0.33	1.08	0.25	0.83
Cocaine	1.02	3.41	0.37	1.25
Cocaethylene	0.37	1.24	0.42	1.39
Benzoylecgonine	0.34	1.12	0.15	0.51
Morphine	0.97	3.24	0.68	2.25
6-MAM	1.14	3.80	0.92	3.07
Codeine	0.68	2.26	0.64	2.15
Dihydrocodeine	0.32	1.05	0.48	1.62
Methadone	0.48	1.59	0.40	1.32
EDDP	0.59	1.96	0.54	1.79
Oxazepam	0.94	3.13	0.17	0.57
Temazepam	0.35	1.18	0.77	2.56
Nordiazepam	0.82	2.73	0.69	2.31
Diazepam	0.33	1.10	0.56	1.56

Table 8-19 LOD and LOQ values for the Bond Elut Certify™ and Clean Screen® SPE methods (continued)

Analyte	Bond Elut Certify™		Clean Screen®	
	LOD (ng/10 mg)	LOQ (ng/10 mg)	LOD (ng/10 mg)	LOQ (ng/10 mg)
Amphetamine	0.79	2.64	0.30	1.00
Methamphetamine	0.39	1.31	0.38	1.26
MDA	0.97	3.24	0.20	0.67
MDMA	0.24	0.78	0.37	1.25
MDEA	0.26	0.88	0.26	0.86

8.6 LC-MS-MS Method Validation

In the method validation, the sample was reconstituted in 150 µL of mobile phase instead of 200 µL as used in the method development. This was to enable maximum assay sensitivity by injecting a greater quantity of drug onto the LC column.

8.6.1 Linearity

Linearity was determined over the concentration range 2-100 ng/10 mg hair. This sample weight was chosen for the validation since the hair case samples were analysed in segments and the sample weights were generally low.

8.6.2 Recovery

The % recovery was determined for each analyte at 5, 10 and 50 ng. Drug standard solution was spiked into 10 mg blank hair prior to or following SPE. 50 ng of deuterated internal standard was added after SPE. % Recovery was calculated as the mean peak area ratio of product ion/internal standard for the samples in which the standard solution was added before SPE (n=3) divided by the mean peak area ratio of product ion/internal standard for the samples in which the standard solution was added following SPE (n=3). The recovery from

authentic hair samples will however be lower than those quoted for spiked hair since the drug is incorporated.

8.6.3 Matrix Effect

Matrix effect was assessed by comparing the mean peak area ratio of product ion/internal standard of extracted blank hair samples spiked with standard solution following SPE (A) (n=6) versus the mean peak area ratio of product ion/internal standard of unextracted standards prepared in mobile phase at equivalent concentrations (B) (n=6). Matrix effect (%) was calculated for a low standard concentration of 5 ng by dividing A by B (Equation 7-5) and multiplying by 100 to express it as a percentage.

8.6.4 Intra- and Inter-Day Precision and Accuracy

Intra-and inter-day precision and accuracy were assessed over the linear range for 5 extracted hair spikes at a low, medium and high concentration (5, 10 and 50 ng). The intra-day precision was expressed as a % RSD calculated using the 5 individual values obtained on the same day. Inter-day precision was evaluated for five replicate hair extracts spiked at these concentrations on five separate days (n_{total} = 25). It was expressed as a % RSD value which was calculated using all 25 individual values.

Accuracy was calculated by dividing the mean measured concentration of 5 or 25 extracts (intra- and inter-day precision) by the theoretical spike concentration and was expressed as a percentage of the theoretical spike concentration.

8.7 Validation Problems for Methadone and EDDP

Methadone and EDDP were not included in the method validation as problems were encountered with the reproducibility of the product quantitation ion/internal standard ratio for both compounds. As demonstrated in Figure 8-20, the linearity was poor for methadone and EDDP over the range 2-100 ng. The variability of internal standard peak areas was assessed by calculating % RSD for

the peak areas of 3 consecutive injections of unextracted methadone-d_9 and EDDP-d_3 internal prepared in mobile phase at a concentration of 50 ng. The % RSD was < 20 % for both internal standards and this is acceptable precision according to published SOFT guidelines.[11] However, the % RSD for the peak areas of 3 consecutive injections of unextracted methadone and EDDP standards prepared in mobile phase at the same concentration as the internal standards (50 ng) were > 120 % for both compounds. Therefore the fragmentation of the methadone and EDDP was not consistent throughout and this resulted in poor linearity. The average peak area response for the methadone and EDDP product ions and the % RSD for these values are shown in Table 8-20.

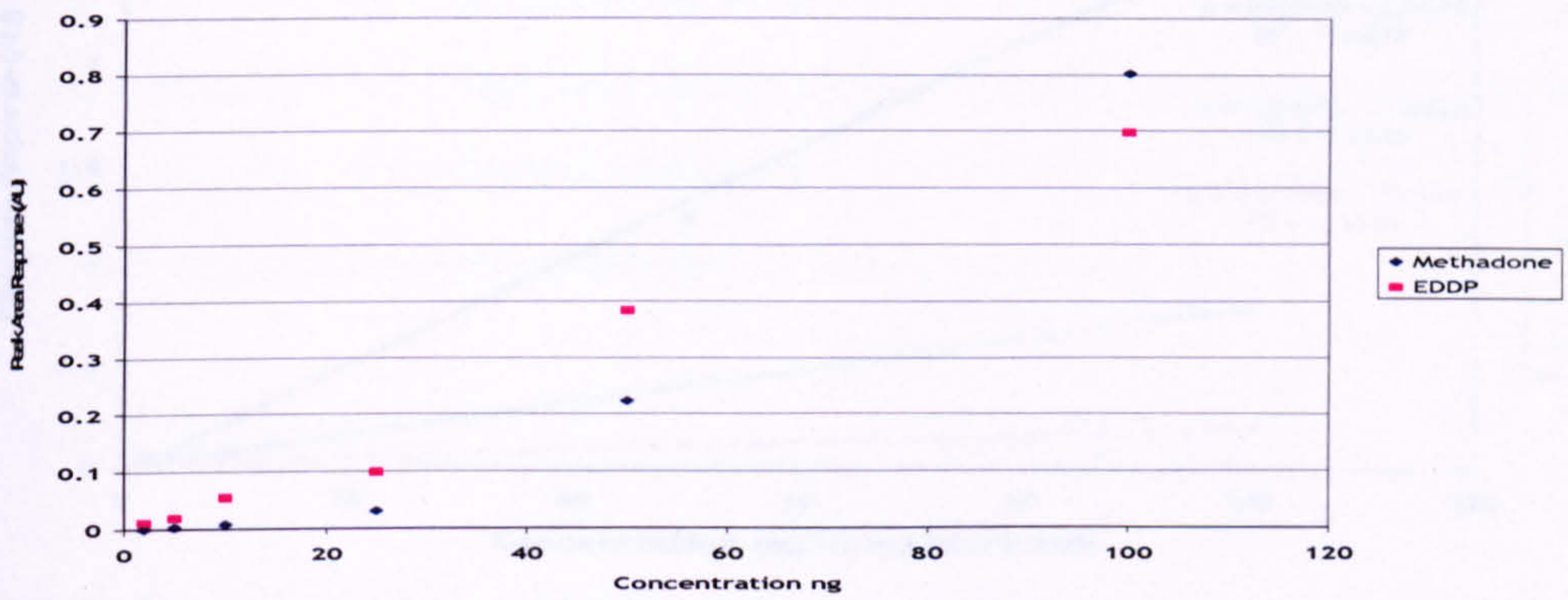


Figure 8-20 Methadone and EDDP calibration plots

Table 8-20 Methadone and EDDP product ion reproducibility

Methadone				EDDP			
Peak Area (*m/z* 265)	Average Peak Area	Standard Deviation	% RSD	Peak Area (*m/z* 249)	Average Peak Area	Standard Deviation	% RSD
55314657	21496050	29379853	136.7	446705203	187304511	226043876	120.7
6910990				82689910			
2262503				32518419			

Only one product ion was investigated for methadone and EDDP as the mass spectrums identified these as the only product ions produced after fragmentation.

8.8 LC-MS-MS Validation Results

8.8.1 Linearity

Examples of the gradient and intercept values are shown in Table 8-21, along with the correlation coefficient (R^2) values for the analyte regression lines. All regression lines had an $R^2 > 0.99$. Graphs of regression lines for selected analytes are shown in Figure 8-21.

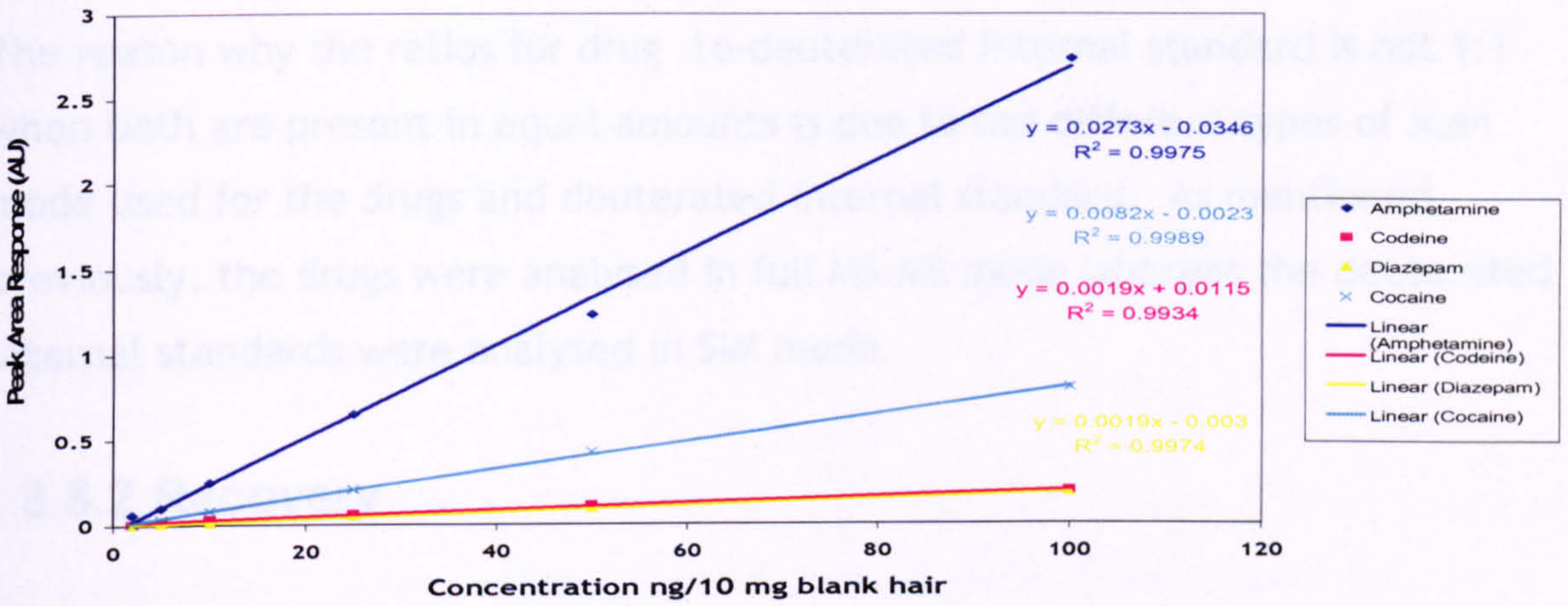


Figure 8-21 Regression lines for selected analytes

Table 8-21 Gradient, intercept and R^2 values for regression lines

Analyte	Equation of line	R^2
EME	y=0.0057x-0.0055	0.9996
Cocaine	y=0.0082x-0.0023	0.9989
Cocaethylene	y=0.0065x-0.0045	0.9998
Benzoylecgonine	y=0.0074x+0.0019	0.9981
Morphine	y=0.0033x-0.0037	0.9924
6-MAM	y=0.0018x+0.0028	0.9916
Codeine	y=0.0019x+0.0115	0.9934
Dihydrocodeine	y=0.0044x+0.011	0.9960
Oxazepam	y=0.0172x-0.0531	0.9933
Temazepam	y=0.0145x+0.0017	0.9983
Nordiazepam	y=0.0023x-0.0002	0.9987

Table 8-21 Gradient, intercept and R^2 values for regression lines (continued)

Analyte	Equation of line	R^2
Diazepam	y=0.0019x-0.003	0.9974
Amphetamine	y=0.0273x-0.0346	0.9975
Methamphetamine	y=0.0115x-0.0396	0.9902
MDA	y=0.0019x+0.002	0.9990
MDMA	y=0.0133x-0.0446	0.9972
MDEA	y=0.0018x-0.0043	0.9955

The reason why the ratios for drug -to-deuterated internal standard is not 1:1 when both are present in equal amounts is due to the different types of scan mode used for the drugs and deuterated internal standard. As mentioned previously, the drugs were analysed in full MS-MS mode whereas the deuterated internal standards were analysed in SIM mode.

8.8.2 Recovery

The recovery values for all the analytes in spiked hair are given in Table 8-22. All gave high recoveries (> 79 %) at all three concentrations and % RSD values for these were ≤ 17 %.

Table 8-22 Recovery for drugs of abuse extracted from hair (n=3)

Analyte	% Recovery (n=3)		
	5 ng/10 mg	10 ng/10 mg	50 ng/10 mg
EME	99.2 (6.6)	96.4 (3.7)	97.7 (1.0)
Cocaine	87.2 (8.9)	91.8 (4.9)	97.3 (3.1)
Cocaethylene	79.4 (13.5)	97.8 (6.6)	92.5 (2.8)
Benzoylecgonine	95.3 (8.5)	100.7 (4.5)	99.4 (3.2)
Morphine	91.2 (2.2)	80.1 (6.7)	92.3 (1.1)
6-MAM	109.6 (7.7)	104.5 (4.6)	94.3 (2.5)
Codeine	99.6 (2.3)	94.4 (5.4)	99.3 (0.2)
Dihydrocodeine	86.5 (7.5)	80.9 (3.2)	100.2 (0.7)
Oxazepam	108.0 (8.8)	96.1 (3.8)	100.6 (3.3)

Table 8-22 Recovery for drugs of abuse extracted from hair (n=3) (continued)

Analyte	% Recovery (n=3)		
	5 ng/10 mg	10 ng/10 mg	50 ng/10 mg
Temazepam	76.3 (7.2)	96.5 (9.4)	95.7 (6.4)
Nordiazepam	97.9 (5.6)	94.7 (3.7)	93.2 (2.7)
Diazepam	96.9 (6.0)	98.3 (2.1)	94.7 (0.6)
Amphetamine	108.1 (17.0)	99.6 (6.0)	91.0 (0.6)
Methamphetamine	93.6 (2.2)	106.0 (0.8)	94.2 (2.3)
MDA	82.8 (7.9)	106.2 (12.5)	97.6 (6.1)
MDMA	98.3 (12.6)	100.0 (9.7)	98.2 (0.6)
MDEA	102.5 (13.2)	104.3 (10.3)	90.8 (1.6)

8.8.3 Matrix Effect

Most analytes (12 out of 17) demonstrated ion suppression, some being affected to a greater extent than others. Oxazepam and temazepam product ions were suppressed the most. The product ions for 5 analytes demonstrated enhancement. Nordiazepam and methamphetamine product ions were enhanced the most. The results are given in Table 8-23.

The existence of the matrix effect demonstrates the importance of using deuterated internal standards in the method. Deuterated internal standards will be affected by ion suppression or enhancement to a similar extent as the corresponding standard. The use of deuterated internal standards will therefore minimise fluctuations in instrument readings and will standardise analyte responses with respect to the response provided by the deuterated internal standard.

Table 8-23 % Matrix effect at 5 ng spiked hair extracts (n=6)

Analyte	% Matrix Effect
EME	119.2
Cocaine	96.4
Cocaethylene	82.9
Benzoylecgonine	85.1
Morphine	85.4
6-MAM	110.6
Codeine	92.6
Dihydrocodeine	102.8
Oxazepam	67.6
Temazepam	63.5
Nordiazepam	130.3
Diazepam	95.5
Amphetamine	97.9
Methamphetamine	135.6
MDA	76.4
MDMA	90.4
MDEA	91.1

8.8.4 Intra-day Accuracy and Precision

The intra-day accuracy and precision values were acceptable within SOFT regulations of ± 20 %[11] for the 3 concentrations tested. The results are given in Table 8-24. Intra-day accuracy ranged from 80-116 %. Precision (% RSD) ranged from 1.4-18.7 %. As expected, lower precision was observed at the lower concentrations.

Table 8-24 Intra-day accuracy and precision (n=5)

Analyte	Spiked Quantity (ng)	Mean Measured Concentration (ng)	% RSD	% Accuracy
EME	5	5.3	5.5	106.0
	10	9.9	4.9	99.0
	50	54.8	3.8	109.6
Cocaine	5	4.1	7.2	82.0
	10	9.6	5.8	96.0
	50	46.8	3.0	93.6
Cocaethylene	5	4.8	10.0	96.0
	10	10.3	5.0	103.0
	50	48.1	3.7	96.2
BZE	5	4.6	7.1	92.0
	10	9.2	6.3	92.0
	50	49.8	3.6	99.6
Morphine	5	4.7	8.4	94.0
	10	9.1	7.5	91.0
	50	50.3	6.9	100.6
6-MAM	5	4.5	11.4	90.0
	10	8.7	5.2	87.0
	50	50.8	4.8	101.6
Codeine	5	4.7	8.9	94.0
	10	10.7	6.5	107.0
	50	54.2	2.0	108.4
Dihydrocodeine	5	4.9	7.0	98.0
	10	11.6	5.3	116.0
	50	50.2	1.4	100.4
Oxazepam	5	5.0	11.6	100.0
	10	10.6	8.9	106.0
	50	50.8	6.0	101.6

Table 8-24 Intra-day accuracy and precision (n=5) (continued)

Analyte	Spiked Quantity (ng)	Mean Measured Concentration (ng)	% RSD	% Accuracy
Temazepam	5	4.4	18.7	88.0
	10	9.4	13.9	94.0
	50	52.9	9.4	105.8
Nordiazepam	5	5.7	14.4	114.0
	10	11.0	9.6	111.0
	50	48.8	6.6	97.6
Diazepam	5	4.8	6.6	96.0
	10	9.8	5.6	98.0
	50	48.6	2.9	97.2
Amphetamine	5	5.6	9.2	112.0
	10	8.0	6.7	80.0
	50	52.1	7.0	104.2
Methamphetamine	5	5.8	6.0	116.0
	10	9.2	6.1	92.0
	50	44.3	4.4	88.6
MDA	5	4.4	9.1	88.0
	10	9.5	5.0	95.0
	50	49.6	7.1	99.2
MDMA	5	5.2	10.6	104.0
	10	8.9	6.6	89.0
	50	50.8	5.9	101.6
MDEA	5	5.7	6.7	114.0
	10	8.8	6.6	88.0
	50	55.0	3.5	110.0

8.8.5 Inter-Day Precision

The inter-day precision was generally acceptable within SOFT regulations of ± 20 %[11] for the 3 concentrations tested. Nordiazepam was slightly higher at the lower concentrations however the SOFT regulations also state that ± 25-30 % may be acceptable for some analytes. Precision (% RSD) ranged from 5.6-24.3 %. As found in the intra-day precision experiment, the % RSD was higher at lower concentrations. The % RSD range was higher for inter-day precision compared to intra-day precision due to instrumental and extraction inter-day variation and also a greater number of data values. Some analyte data in Table 8-25 was calculated for n=24. This was due to the removal of an outlier in the data sets.

Table 8-25 Inter-day precision (n=25)

Analyte	Inter-day Precision (% RSD)		
	5 ng	10 ng	50 ng
EME	5.5 (18.3)	8.8 (9.9)	49.4 (5.6)
Cocaine	4.5 (10.1)	10.1 (7.7)	50.9 (7.1)
Cocaethylene	5.1 (19.0)*	10.0 (15.8)	50.5 (10.2)
BZE	5.3 (22.5)	10.1 (12.0)	48.6 (8.8)
Morphine	5.1 (16.4)	9.8 (15.5)*	55.2 (11.0)
6-MAM	4.6 (20.5)	10.5 (13.6)	51.9 (8.2)
Codeine	5.0 (18.7)	10.9 (17.5)	54.3 (9.3)
DHC	4.8 (18.2)	10.6 (12.9)	51.9 (8.5)
Oxazepam	5.2 (16.8)	9.9 (14.2)*	49.7 (10.8)*
Temazepam	5.2 (20.2)*	10.1 (18.9)	51.9 (10.6)
Nordiazepam	5.6 (24.3)*	9.2 (22.9)*	47.4 (10.6)
Diazepam	4.7 (13.3)	9.8 (12.2)	47.3 (9.2)
AMP	5.0 (16.9)	9.3 (15.7)	48.9 (12.2)
MAMP	4.9 (20.7)	8.9 (8.9)	51.5 (8.6)
MDA	5.6 (20.8)	10.6 (19.4)	52.0 (19.6)
MDMA	5.1 (15.9)	8.9 (10.8)	52.6 (10.0)
MDEA	4.8 (15.1)	9.4 (11.0)	52.4 (9.8)

*n=24

8.8.6 LOD and LOQ

The LOD and LOQ values for the Clean Screen® method are provided in Table 8-19. The LOD values ranged from 0.15-0.92 ng/10 mg hair and the LOQ ranged from 0.51-3.07 ng/10 mg hair.

8.9 Case Samples

The validated LC-MS-MS method was used to analyse 17 post-mortem scalp hair samples. All samples were submitted to the toxicology laboratory of the Forensic Medicine and Science Section at the University of Glasgow for routine testing. The samples were tested for diagnostic purposes and the results were reported to the Procurator Fiscal.

The hair samples were decontaminated as described in section 6.4.1, segmented and split between 3 vials where sufficient sample was available. This was carried out for the purposes of comparing the LC-MS-MS results with GC-MS results obtained using routine in-house methods and also with ELISA screening results obtained using the validated methods described in chapters 6 and 7.

8.9.1 Comparison of LC-MS-MS vs Routine GC-MS Methods

8.9.1.1 Methods

The validated LC-MS-MS method analysed each case sample for amphetamines, benzodiazepines, cocaine and its metabolites and opiates while the GC-Ms methods analysed individual drug groups.

The GC-MS methods for opiates and cocaine were detailed in section 6.6.4. In brief, the GC-MS method for amphetamine extraction from hair involved ultrasonicating the hair for 1 hour in phosphate buffer (pH 6.0) then incubating at 40 °C for 2 hours. The extracts were cleaned up using Bond Elut Certify™ SPE cartridges. The amphetamines were derivatised at 60 °C for 20 minutes using PFPA/ethyl acetate (2:1 v/v) prior to GC-MS analysis.

The LOD and LOQ were approximately 1 ng/30 mg and 2 ng/30 mg respectively for all compounds in the in-house GC-MS methods.

8.9.1.2 Results

The LC-MS-MS and GC-MS results are given in Table 8-26. The drug groups analysed by GC-MS are provided in the footnote for Table 8-26. The abbreviations in Table 8-26 are as follows: "MOR"=Morphine; "6-MAM"=6-Monoacetylmorphine; "COD"=Codeine; "DHC"=Dihydrocodeine; "COC"=Cocaine; "BZE"=Benzoylecgonine; "COCAETH"=Cocaethylene; "EME"=Ecgonine Methyl Ester; "AMP"=Amphetamine; "DZ"=Diazepam; "NDZ"=Nordiazepam; "OXAZ"=Oxazepam; and "TMZ"=Temazepam.

Table 8-26 LC-MS-MS vs GC-MS results

Hair Sample Number	LC-MS-MS Weight (mg)	LC-MS-MS Results (ng/mg)	GC-MS Weight (mg)	GC-MS Results (ng/mg)
(1)† Root- 0.5 cm	2.85	0.91 MOR 0.97 6-MAM 1.38 COD 2.93 DHC	2.56	NEG for MOR NEG for 6-MAM 0.08 COD 4.98 DHC
(1)† 0.5-3.5 cm	23.80	2.02 MOR 1.31 6-MAM 0.37 COD 1.22 DHC 0.10 DZ 0.37 NDZ	23.36	6.14 MOR 2.87 6-MAM 1.08 COD 3.32 DHC
(2)† Root-0.5 cm	2.05	1.67 MOR 1.96 6-MAM NEG for COD 0.34 DZ 2.26 NDZ	2.21	0.18 MOR NEG for 6-MAM 0.41 COD

†GC-MS analysis for opiates and cocaine only, NT=Not Tested, NEG=Negative

Table 8-26 LC-MS-MS vs GC-MS results (continued)

Hair Sample Number	LC-MS-MS Weight (mg)	LC-MS-MS Results (ng/mg)	GC-MS Weight (mg)	GC-MS Results (ng/mg)
(2) † 0.5-3.5 cm	25.45	1.23 MOR 0.12 6-MAM 0.45 COD 0.13 DHC 0.06 COC 0.42 BZE 1.03 MDMA 0.22 OXAZ 0.10 TMZ 0.52 DZ 0.67 NDZ	25.63	3.11 MOR NEG for 6-MAM 0.75 COD 0.16 DHC 0.09 COC 0.63 BZE
(3) † Root-0.5 cm	2.74	NEG for all drug groups	2.76	NEG for opiates and cocaine
(3) † 0.5-3.5 cm	19.09	0.09 MOR 0.52 6-MAM 0.21 COD NEG for DHC 0.29 COC 0.16 BZE 1.26 MDMA 0.12 DZ 0.36 NDZ	19.48	0.01 MOR 0.72 6-MAM 0.13 COD 0.05 DHC 1.28 COC 0.29 BZE
(3) † 3.5-6.5 cm	18.60	NEG for MOR 0.53 6-MAM NEG for COD NEG for DHC 0.96 COC 0.45 BZE 1.60 MDMA	18.97	0.08 MOR 1.06 6-MAM 0.05 COD 0.05 DHC 3.85 COC 0.64 BZE NT

†GC-MS analysis for opiates and cocaine only, NT=Not Tested, NEG=Negative

Table 8-26 LC-MS-MS vs GC-MS results (continued)

Hair Sample Number	LC-MS-MS Weight (mg)	LC-MS-MS Results (ng/mg)	GC-MS Weight (mg)	GC-MS Results (ng/mg)
(4) † Root-0.5 cm	3.21	0.58 MOR 0.77 6-MAM 0.32 DHC 1.40 NDZ	3.39	NEG for MOR NEG for 6-MAM NEG for DHC
(4) † 0.5-3.5 cm	21.12	0.16 MOR 0.58 6-MAM 0.20 COD 0.09 DHC 0.86 COC 0.11 BZE 0.19 AMP 1.42 MDMA 0.04 DZ 0.29 NDZ	21.11	0.24 MOR 0.71 6-MAM 0.09 COD 0.19 DHC 0.61 COC 0.21 BZE
(5)‡ Root-0.5 cm	1.71	1.39 6-MAM	2.11	NEG for 6-MAM
(5) ‡ 0.5-3.5 cm	12.62	0.22 MOR 0.49 6-MAM 0.32 COD 10.18 COC 3.11 BZE 0.43 COCAETH 0.24 EME 0.11 MDMA 0.17 DZ 0.52 NDZ	12.47	0.11 MOR NEG for 6-MAM 0.10 COD
(6) † Root-0.5 cm	1.03	NEG for all drug groups	1.33	NEG for opiates

†GC-MS analysis for opiates and cocaine only, ‡ GC-MS analysis for opiates only, NT=Not tested, NEG=Negative

Table 8-26 LC-MS-MS vs GC-MS results (continued)

Hair Sample Number	LC-MS-MS Weight (mg)	LC-MS-MS Results (ng/mg)	GC-MS Weight (mg)	GC-MS Results (ng/mg)
(6) † 0.5-3.5 cm	18.49	2.32 COD	18.31	5.52 COD
(7)‡ Root-0.5 cm	1.49	NEG for MOR NEG for 6-MAM 0.13 COD	1.68	5.23 MOR 3.03 6-MAM 1.75 COD
(7)‡ 0.5-3.5 cm	8.38	1.10 MOR 4.55 6-MAM 0.11 COD	8.62	2.77 MOR 3.36 6-MAM 0.32 COD
(8)‡ Root-0.5 cm	1.05	NEG for DHC	1.52	1.91 DHC
(8)‡ 0.5-3.5 cm	9.24	NEG for MOR 0.58 6-MAM 0.08 COD 0.21 DHC	9.34	0.24 MOR 0.85 6-MAM NEG for COD 1.07 DHC
(9)† Root-0.5 cm	3.16	0.71 6-MAM 2.05 COC 0.31 BZE 0.25 COCAETH	3.24	1.49 6-MAM 4.78 COC 1.56 BZE NEG for COCAETH
(9)† 0.5-3.5 cm	28.55	0.04 MOR 1.12 6-MAM NEG for COD 13.99 COC 4.73 BZE 0.21 EME 0.54 COCAETH 0.21 AMP 0.18 DZ 0.17 NDZ	28.72	0.45 MOR 1.55 6-MAM 0.13 COD 19.34 COC 4.92 BZE Not reported 1.07 COCAETH

†GC-MS analysis for opiates and cocaine only ‡ GC-MS analysis for opiates only, NT=Not Tested, NEG=Negative

Table 8-26 LC-MS-MS vs GC-MS results (continued)

Hair Sample Number	LC-MS-MS Weight (mg)	LC-MS-MS Results (ng/mg)	GC-MS Weight (mg)	GC-MS Results (ng/mg)
(10)† Root-0.5 cm	3.27	0.60 COC 0.18 BZE 0.24 COCAETH 0.31 DZ 0.34 NDZ	3.34	2.40 COC 1.25 BZE NEG for COCAETH
(10)† 0.5-3.5 cm	29.47	0.08 6-MAM 2.50 COC 1.88 BZE 0.05 COCAETH 0.15 EME 0.26 OXAZ 0.14 TMZ 0.24 DZ 0.64 NDZ	29.71	0.20 6-MAM 5.09 COC 2.35 BZE 0.10 COCAETH Not reported
(11)‡ 0.5-3.5 cm	8.55	NEG for COD 0.49 MDMA	8.86	0.27 COD
(11)‡ 3.5-6.5 cm	8.69	0.02 COD	9.00	0.48 COD
(12)‡ Root-0.5 cm	0.35	14.48 DHC	0.84	11.26 DHC
(12) ‡ 0.5-3.5 cm	10.40	0.87 MOR 0.12 6-MAM 16.18 DHC 0.46 MDMA 3.51 OXAZ 0.68 DZ 0.92 NDZ	10.36	0.64 MOR NEG for 6-MAM 14.60 DHC

†GC-MS analysis for opiates and cocaine only, ‡ GC-MS analysis for opiates only, NT=Not Tested, NEG=Negative

Table 8-26 LC-MS-MS vs GC-MS results (continued)

Hair Sample Number	LC-MS-MS Weight (mg)	LC-MS-MS Results (ng/mg)	GC-MS Weight (mg)	GC-MS Results (ng/mg)
(13)‡ Root-0.5 cm	0.21	NEG for all drug groups	0.54	NEG for opiates
(13)‡ 0.5-3.5 cm	3.79	NEG for MOR 0.71 6-MAM	3.58	0.05 MOR 0.19 6-MAM
(14)‡ 0.5-1.5 cm	1.25	NEG for COD	1.47	2.57 COD
(15)† Root-0.5 cm	1.75	3.54 MOR 1.31 6-MAM NEG for COD NEG for DHC	2.45	5.71 MOR 1.05 6-MAM 2.59 COD 0.83 DHC
(15)† 0.5-3.5 cm	18.66	1.03 MOR 1.32 6-MAM 0.31 COD 10.72 DHC	18.94	3.01 MOR 2.28 6-MAM 1.27 COD 12.45 DHC
(16) † Root-0.5 cm	4.90	NEG for all drug groups	5.36	NEG for opiates
(16) † 0.5-3.5 cm	20.62	NEG for all drug groups	21.10	NEG for opiates
(16) † 3.5-6.5 cm	17.30	NEG for all drug groups	25.63	NEG for opiates
(17) * Root-0.5 cm	2.49	5.59 AMP	3.02	NEG for AMP
(17)* 0.5-3.5 cm	18.21	7.29 AMP	18.01	1.75 AMP

†GC-MS analysis for opiates and cocaine only, ‡ GC-MS analysis for opiates only, NT=Not Tested, NEG=Negative

In general, the LC-MS-MS method was better for detecting a wider range of analytes compared to the GC-MS methods which targeted individual drug groups using similar sample weights between the two methods.

8.9.1.3 Qualitative Root Results

Some analytes were detected in root samples of low weight by LC-MS-MS (≤ 3.27 mg) due to the low LOD/LOQ of the method where none were detected using GC-MS: morphine in root samples 1 and 4; 6-MAM in root samples 2, 4 and 5; dihydrocodeine in root sample 4; and cocaethylene in root samples 9 and 10. Morphine was detected in the blood samples corresponding to root samples 1 and 4. 6-MAM was detected in the blood samples corresponding to root samples 2 and 5. No 6-MAM was detected in the blood sample corresponding to root sample 4, probably due to its short half life of 2-4 minutes in blood.[176] There was no dihydrocodeine detected in the blood sample corresponding to root sample 4. The routine blood method does not analyse for cocaethylene so it is not possible to compare cocaethylene results for blood with the root results for samples 9 and 10.

The GC-MS method detected opiates in root samples of low weight (≤ 2.05 mg) where LC-MS-MS detected none: morphine and 6-MAM in root sample 7; codeine in root samples 2 and 15; and dihydrocodeine in root samples 8 and 15. The blood results for morphine, 6-MAM and codeine showed excellent correlation with the root samples however no dihydrocodeine was detected in the blood samples corresponding to root samples 8 and 10. The GC-MS method has a higher opiate recovery than the LC-MS-MS method as it is targeting the opiate drug group so this could explain the detection of these opiates by GC-MS where LC-MS-MS failed to detect them. However the opiate concentrations detected by GC-MS in these root samples were relatively high and so another explanation for the LC-MS-MS results being negative for these samples may be due to uneven splitting of root segments between the two methods. The root-0.5 cm segment of a growing hair would contain incorporated drug whereas a hair at the end of its growth cycle would not have incorporated drug into the root segment.

8.9.1.4 Qualitative results for segments excluding roots

A higher GC-MS recovery for opiates and cocaine and its metabolites was observed in the 0.5-3.5 cm (and in 1 case 3.5-6.5 cm segment) which had a

sufficiently high sample weight compared with root segments. Although the LC-MS-MS method produced lower recoveries for opiates and cocaine and its metabolites compared to the GC-MS method, it generally detected the analytes in the 0.5-3.5 cm segments which were detected by GC-MS. However, the analytes that were detected by GC-MS but not LC-MS-MS were morphine (sample 3, 8 and 13), codeine (sample 3, 9 and 11) and dihydrocodeine (sample 3). The analyte concentrations in these samples as determined by GC-MS were low. The blood results corresponding to hair samples 3, 8 and 13 were positive for morphine. Codeine was detected in the blood samples corresponding to hair samples 9 and 11. The blood and hair results for hair sample 3 were both negative for codeine and dihydrocodeine where the hair results for GC-MS were positive. The better opiate recovery obtained using the GC-MS method explains the positive GC-MS results where the LC-MS-MS results were negative.

The LC-MS-MS method did however detect some analytes in the 0.5-3.5 cm segments where GC-MS failed to detect them. This was 6-MAM in samples 2, 5 and 12 and codeine in sample 8. The concentrations detected in these samples as determined by LC-MS-MS were also low. The results for the blood samples corresponding to hair samples 2 and 5 were positive for 6-MAM. However no 6-MAM was found in the blood sample corresponding to hair sample 12 and no codeine was detected in the blood sample corresponding to hair sample 8.

The LC-MS-MS and GC-MS results showed excellent qualitative correlation for cocaine, benzoylecgonine and cocaethylene in the 5 positive samples tested for these analytes by both methods. The GC-MS method recovery was higher than that for LC-MS-MS as higher quantities of cocaine, benzoylecgonine and cocathylene were detected in the 0.5-3.5 cm segments. EME was identified and quantified in samples 9 and 10 by LC-MS-MS however the GC-MS method was incapable of identifying and quantifying EME due to a co-eluting peak in the method and therefore EME concentrations were not reported for GC-MS. The corresponding blood results for hair sample 9 did not detect EME but did detect benzoylecgonine. The corresponding blood results for hair sample 10 detected EME as well as cocaine and benzoylecgonine.

A higher quantity of amphetamine was recovered in sample 17 using the LC-MS-MS method. This was the only sample that was tested for amphetamines using GC-MS. The LC-MS-MS method also detected amphetamine in the root where none was detected by GC-MS. The LC-MS-MS method involved incubating the hair overnight in phosphate buffer (pH 5.0) while the GC-MS method involved incubating the hair for 2 hours in phosphate buffer (pH 6.0). The higher amphetamine recovery for the LC-MS-MS method could be a result of the longer incubation time or the pH of the extraction. The blood result for this hair sample was also positive for amphetamine at a concentration of 0.66 mg/L. The LC-MS-MS method also detected amphetamine in samples 4 and 9 but as the GC-MS method did not test for amphetamine and there was a lack of sample to analyse using the routine GC-MS method for amphetamine, it was not possible to compare these. The blood results corresponding to hair samples 4 and 9 were negative for amphetamine. MDMA was detected by LC-MS-MS in 6 of the 17 samples (samples 2, 3, 4, 5, 10 and 12) which were not tested for amphetamines by GC-MS. The corresponding blood results for these 6 samples were negative for MDMA.

The GC-MS method did not test for benzodiazepines. However the LC-MS-MS method detected diazepam and nordiazepam in the 0.5-3.5 cm (and in 1 case 3.5-6.5 cm) segments of 8 samples. Oxazepam and temazepam were also detected in 2 of these 8 samples.

8.9.1.5 Discussion

8.9.1.5.1 Points to consider for interpretation

It is not possible to directly correlate the amount of drug taken with the amount of drug detected in hair because the dose is unknown in all cases. Also studies have demonstrated that different quantities of drug incorporate into hair of individuals who have received the same dose.[25,251]

Using the LC-MS-MS method, each of the 12 opiate positive samples tested positive for 6-MAM, which is a marker of heroin use. The detection of 6-MAM is

however not definitive of heroin use as it is possible that 6-MAM could be present as a reaction side-product of the incomplete conversion of morphine to diamorphine during the acetylation process. In hair samples collected from 20 known heroin users, 6-MAM was predominantly found over morphine and codeine and usually in higher concentrations.[13,252] In one study it was proposed that to confirm heroin use, a morphine/codeine ratio of 5:1 should be in place for low morphine levels (< 1 ng/mg) and 2:1 for morphine concentrations > 1 ng/mg. The same authors also found a 6-MAM/morphine ratio range of 1.3-10.0.[1] Another group of authors proposed that 6-MAM/morphine ratios and 6-MAM/codeine ratios should be greater than 7.14 and 50 respectively to interpret the results as consistent with heroin use. In another study, the same authors defined three levels of use (low, medium and high) in relation to the 6-MAM marker found in hair after heroin use.[253] Low use was defined as containing < 2 ng/mg, medium 2-10 ng/mg and high > 10 ng/mg 6-MAM. The cut-off proposed in this study was 0.5 ng/mg 6-MAM. An important point of consideration when applying these recommended ratios is the type of extraction used. In both of these papers proposing 6-MAM/morphine and 6-MAM/codeine ratios, the opiates were extracted by acidic hydrolysis. 6-MAM can hydrolyse to some extent under acidic conditions so the ratios proposed using acidic extraction will be different to the ratios observed using a phosphate buffer extraction in which 6-MAM is stable. It is more meaningful to compare the ratios obtained using the same type of extraction medium.

7 out of 8 cocaine positive samples tested positive for benzoylecgonine which suggests cocaine use. The presence of benzoylecgonine is not however definitive of cocaine use as it was shown in one study that small quantities of benzoylecgonine were formed in hair as a result of environmental contamination with cocaine.[254] In addition, this study also found that benzoylecgonine could be absorbed from illicit cocaine contamined with benzoylecgonine. Current Society of Hair guidelines propose a benzoylecgonine/cocaine ratio of 5 %.[100] It was proposed that this value was not appropriate for some cocaine users' hair which can produce levels < 5 %.[255]

Amphetamine and MDMA were detected in 8 out of 17 samples. No MDA was detected in the MDMA positive hair samples; however, these would be reported as positive according to current SoHT guidelines which recommend a cut-off of 0.2 ng/mg for individual amphetamines.[100] There are no recommendations in these guidelines for a cut-off value for the MDMA metabolite, MDA.

Diazepam and nordiazepam were detected in 8 out of 17 samples which also contained heroin metabolites. There are currently no recommended cut-off values for benzodiazepines in hair.

MDMA, EME, oxazepam and temazepam were not detected in the root-0.5 cm segments by LC-MS-MS where the 0.5-3.5 cm segments were positive. The drug group specific GC-MS methods did not detect cocaethylene or amphetamine in the root-0.5 cm segments where the 0.5-3.5 cm segments were positive. LC-MS-MS found these root segments positive for cocaethylene and amphetamine.

As there are no definitive markers of heroin or cocaine use in hair per se, it is very useful to examine an individual's past drug use history if provided in the pathologist's background report. Other additional information from the police reports may include drug findings at the location of death which could aid the toxicology interpretation. An example is the recovery of a syringe filled with heroin in situ. This information would be very useful in a suspected heroin death where no 6-MAM was found in the hair. Table 8-27 provides information on the individual case histories.

Table 8-27 Case Histories

Case Number	Age	Prescription and Illicit Drugs	Useful findings *in situ*/at post-mortem	Cause of Death
1	32	History of heroin abuse. History of overdoses and depression. Not on any prescribed medication at the time of death.	Needles in a small bag recovered from his bedroom. 3 recent needle puncture marks on arms.	Bronchopneumonia due to heroin intoxication.
2	-	-	-	-
3	30	History of multi-substance abuse including heroin and cocaine. History of extensive alcohol abuse. Not on any prescribed medication at the time of death.		Heroin and alcohol intoxication.
4	27	Not known. History of mental illness. Being treated for schizophrenia.	Needle puncture mark in right groin.	Bronchopneumonia due to aspiration of gastric contents due to morphine intoxication.
5	30	Known to abuse anabolic steroids and possibly heroin. He was taking a large number of herbal tablets and other substances, including Tamoxifen. Not known.	Various tablets found in his house plus a box of needles.	Heroin intoxication.
6	33	History of chronic alcohol abuse. Using street methadone (about 150 mL per week). Liver problems.	Not applicable.	Gastrointestinal haemorrhage due to oesophageal ulcers due to chronic alcohol abuse.

"-"= No information (external post-mortem)

Table 8-27 Case Histories (continued)

Case Number	Age	Prescription and Illicit Drugs	Useful findings *in situ*/at post-mortem to aid toxicology interpretation	Primary cause of death
7	39	History of heroin abuse.	A syringe was found on the floor next to the fireplace. A sharps container with syringes was also found. Potential needle marks noted on the hands, feet and bend of right elbow.	Heroin intoxication.
8	35	History of drug misuse.	2 puncture wounds in the bend of the right elbow.	Heroin intoxication.
9	20	History of drug abuse. Prescribed olanzapine. Regular cannabis user. Never known to have taken heroin. Taking olanzapine for schizophrenia.	A number of drug productions were recovered from the scene.	Morphine (heroin) intoxication.
10	16	No previous history of heroin use. On day of death, took a bag of Valium tablets and 3 g cocaine to step-brother's house.	Drug paraphernalia at scene including tinfoil and citric acid found in bedroom however step-brother was known to be an intravenous drug user. Multiple needle puncture marks in body.	Cocaine, heroin and diazepam intoxication.

Table 8-27 Case Histories (continued)

Case Number	Age	Prescription and Illicit Drugs	Useful findings *in situ*/at post-mortem to aid toxicology interpretation	Primary cause of death
11	32	History of alcohol and heroin dependency. Prescribed campral, chlordiazepoxide, propanolol, dothiepin and vitamins. History of self-harm, depression. A number of previous overdose attempts. Placed on methadone programme in 2002, taking 50 mg daily. Liver tests abnormal in 2005.	Drug paraphernalia found in the room. Tourniquet around the right arm with a syringe lying on the floor between the legs and arms. Several needle puncture marks in the bend of the right elbow and a tourniquet round the lower arm.	Heroin and alcohol intoxication.
12	32	Previously addicted to heroin. Prescribed methadone 20 mL daily, oxazepam, epilim, dihydrocodeine, diazepam, sertraline and olanzapine. Severe brain injury following a road traffic accident from which he subsequently had epilepsy and a compound fracture in his left leg. Long history of self-harm. Been on methadone programmes had a number of episodes where he cut himself and overdoses.	Large quantity of medication next to bed including sertraline, olanzapine and a methadone bottle.	Sertraline, methadone and morphine overdose.

Table 8-27 Case Histories (continued)

Case Number	Age	Prescription and Illicit Drugs	Useful findings *in situ*/at post-mortem to aid toxicology interpretation	Primary cause of death
13	36	Heroin addiction. Not on any prescribed medication. Known to have smoked hash.	Needle puncture mark in the bend of the left elbow.	Heroin intoxication.
14	34	Chronic alcohol abuse. Prescribed several medications for hypertension and depression. Chronic alcohol abuse, depression and hypertyension	-	Cerebro-vascular accident ('stroke')
15	35	Longstanding drug abuse since 1988. Initially Temgesic and temazepam but subsequently diamorphine and benzodiazepines. Currently prescribed beclometasone and salbutamol inhalers, dihydrocodeine and diazepam. History of asthma. Attended numerous rehabilitation centres.	Drug paraphernalia associated with the body, an empty syringe pack and a used syringe below it. An empty diazepam box and a nitrazepam bottle were also found. Possible needle puncture mark detected and there was a blood trail on the right arm.	Heroin intoxication.
16	37	Alcoholic for 15 years. Used cannabis in past.	Cannabis resin found on bedside cabinet nearby along with 2 empty Buckfast bottles and several empty beer cans.	Fatty degeneration of liver due to chronic alcohol abuse.

Table 8-27 Case Histories (continued)

Case Number	Age	Prescription and Illicit Drugs	Useful findings *in situ*/at post-mortem to aid toxicology interpretation	Primary cause of death
17	52	Known to use cannabis. Past history of depression and arthritis.	Several syringes found lying near to the deceased. Also some foil, plastic cup with a residue and a burner. Drugs found were citalopram, Celecoxib and Arthrotec.	Amphetamine intoxication.

8.9.1.5.2 Quantitative Root Results

The opiate ranges and number of positive root-0.5 cm segments found by LC-MS-MS and GC-MS are shown in Table 8-28.

Table 8-28 Opiate concentration ranges and number of opiate positive root segments for LC-MS-MS and GC-MS

Analyte	LC-MS-MS		GC-MS	
	Concentration Range (ng/mg)	Number of Positive Samples	Concentration Range (ng/mg)	Number of Positive Samples
Morphine	0.58-3.54	4	0.18-5.71	3
6-MAM	0.71-1.96	6	1.05-3.03	3
Codeine	0.13-1.38	2	0.08-2.59	4
Dihydrocodeine	0.32-14.48	3	0.83-11.26	4

The results in Table 8-28 show that the LC-MS-MS method detected morphine and 6-MAM in a greater number of root-0.5 cm segments compared to the GC-MS method. Conversely, the GC-MS method detected codeine and dihydrocodeine in a greater number of root-0.5 cm segments. The lowest concentration from the

LC-MS-MS and GC-MS ranges for 6-MAM and dihydrocodeine was found using LC-MS-MS. In contrast, the lowest concentration from the LC-MS-MS and GC-MS ranges for morphine and codeine was found using GC-MS. The method sensitivity appears to be better for 6-MAM and dihydrocodeine using LC-MS-MS while the method sensitivity appears to be better for morphine and codeine using GC-MS. The difference in method sensitivity may however be due to the uneven splitting of root-0.5 segments between the two methods. As mentioned previously, drugs ingested when a hair is in its growth phase would be incorporated into the hair whereas no drugs would be incorporated into the hair at the end of its growth cycle. The highest concentration from the LC-MS-MS and GC-MS ranges for morphine, 6-MAM and codeine was found using the GC-MS method. The higher opiate recovery of the GC-MS method would explain this.

In the 4 root samples (samples 1, 2, 4 and 15) containing both 6-MAM and morphine, the 6-MAM/morphine ratios ranged from 0.37-1.33 (mean 0.99) using the LC-MS-MS method. A 6-MAM/morphine ratio range of 0.37-6.45 was observed for hair samples in another study which used a phosphate buffer (pH 5.0) extraction.[84] 6-MAM and morphine were detected together in 2 root samples (samples 7 and 15) using GC-MS. The 6-MAM/morphine ratios were 0.58 and 0.18 (mean 0.38) in these samples. These ratios are lower than the ratios proposed in other studies which used acidic hydrolysis to extract opiates from hair, as discussed in section 8.9.1.5.1. As expected, the mean 6-MAM/morphine ratio for the GC-MS method was lower than that for the LC-MS-MS method. This is because of the different extraction methods used in the two methods. The GC-MS method used hydrochloric acid for part of the extraction and this may have resulted in some hydrolysis of 6-MAM to morphine, leading to lower ratios. On the other hand, the phosphate buffer extraction used in the LC-MS-MS method does not cause hydrolysis of 6-MAM so the ratios should be higher. Acetylated opiates (6-MAM and acetylcodeine) were found to significantly decompose (70-86 %) using 0.1 M hydrochloric acid at 55 °C for 16 hours and the reproducibility of the hydrolysis was poor.[82]

Codeine was only detected in 1 root sample (sample 1) using LC-MS-MS. 6-MAM and morphine were also present in this root sample by LC-MS-MS. The

morphine/codeine ratio was 0.66 in this sample which is in the reported range of 0.38-6.45 which was found for hair samples extracted using a phosphate buffer extraction.[84] Codeine was detected in 4 root samples (samples 1, 2, 7 and 15) using GC-MS. In root sample 1, codeine was found by GC-MS without any other heroin metabolites so the results could have been interpreted as codeine use. However the LC-MS-MS results for this root sample detected 6-MAM, morphine and codeine which could indicate heroin use. Heroin metabolites were detected in the other 3 root samples. 2 of these root samples (samples 7 and 15) would have been reported as positive using the criteria proposed in section 8.9.1.5.1. As the GC-MS method results in the partial hydrolysis of 6-MAM to morphine, the morphine/codeine ratios should be higher using this method compared to the LC-MS-MS method which uses a phosphate buffer extraction which does not cause 6-MAM hydrolysis.

Dihydrocodeine was detected in root segment 8 (1.9 ng/mg) where LC-MS-MS failed to detect it. The LC-MS-MS sample weight and GC-MS sample weight were very low in this case therefore the better recovery of the GC-MS method could explain this result. Alternatively, the root-0.5 cm segments may not have been split evenly between the two methods. Hair growing at the time of ingestion would have incorporated dihydrocodeine whereas hair at the end of its growth cycle would not. There are a limited number of scientific papers concerning dihydrocodeine levels in hair. One study found 13 hair samples collected from suspected heroin users positive for dihydrocodeine in the range 1.2-31.2 ng/mg.[256] Both the LC-MS-MS and GC-MS methods detected lower dihydrocodeine concentrations in root segments 4 and 15 respectively than the lowest concentration reported in this study. The rest of the positive samples did however have concentrations within this reported range.

Cocaine and benzoylecgonine were detected in 2 root segments (samples 9 and 10) using LC-MS-MS and GC-MS. The root sample weights were > 3 mg for both methods. Cocaethylene was also detected in these samples by LC-MS-MS but not by GC-MS. Root segment 9 contained 2.05 ng/mg cocaine, 0.31 ng/mg benzoylecgonine and 0.25 ng/mg cocaethylene as determined by LC-MS-MS. The GC-MS method detected higher cocaine and benzoylecgonine levels (4.78 ng/mg

cocaine and 1.56 ng/mg benzoylecgonine) in root sample 9. The higher recovery of the extraction method could explain these results. As the root segment weights were similar for LC-MS-MS and GC-MS analysis, it appears that the LC-MS-MS method sensitivity is better for cocaethylene however the result could also be due to uneven splitting of the root segments. The ratio of benzoylecgonine/cocaine was ≥ 0.05 in the positive root samples analysed by both methods. This ratio is recommended cut-off proposed by the SoHT.[100]

As mentioned previously, amphetamine was detected in root sample 17 where none was detected by GC-MS. GC-MS did not test for benzodiazepines however the LC-MS-MS method detected diazepam (samples 2 and 10) and nordiazepam (samples 2, 4 and 10) were detected in the ranges of 0.31-0.34 ng/mg and 0.34-2.26 ng/mg respectively. Nordiazepam/diazepam ratios were 1.10 and 6.65 for these samples. These ratios are within the ranges reported by other studies.[221]

8.9.1.5.3 Quantitative results for segments excluding roots

The drug ranges and number of segments excluding roots found positive by LC-MS-MS and GC-MS are summarised in Table 8-29. The opiate ranges were much broader than the ranges obtained for the root segments shown in Table 8-28.

Table 8-29 Drug ranges and number of positive segments excluding roots

Analyte	LC-MS-MS		GC-MS	
	Concentration Range (ng/mg)	Number of Positive Segments	Concentration Range (ng/mg)	Number of Positive Segments
Morphine	0.04-2.02	10	0.01-6.14	12
6-MAM	0.08-4.55	12	0.19-3.36	10
Codeine	0.02-2.32	10	0.05-5.52	13
Dihydrocodeine	0.09-16.18	6	0.05-14.60	8
Cocaine	0.06-13.99	7	0.09-19.34	6
Benzoylecgonine	0.11-4.73	7	0.21-4.92	6
Cocaethylene	0.04-0.53	3	0.10-1.07	2
EME	0.21-0.24	2	Not reported	N/A
Amphetamine	0.19-7.29	3	1.75	1
MDMA	0.46-1.60	6	Not tested	N/A
Diazepam	0.04-0.52	7	Not tested	N/A

Table 8-29 Drug ranges and number of positive segments excluding roots (continued)

Nordiazepam	0.17-0.67	7	Not tested	N/A
Oxazepam	0.22-0.26	2	Not tested	N/A
Temazepam	0.10-0.14	2	Not tested	N/A

Using LC-MS-MS, 6-MAM was detected in 12 segments without roots in this current study which suggests heroin use. 7 of these samples contained morphine and codeine which can support heroin consumption when 6-MAM is also present. The morphine and codeine ranges as determined by LC-MS-MS were 0.04-2.02 ng/mg and 0.02-2.32 ng/mg. For GC-MS, the morphine and codeine ranges were 0.01-6.14 ng/mg and 0.05-5.52 ng/mg respectively. The higher range for GC-MS for morphine and codeine highlights the better extraction capability of the GC-MS method compared to the LC-MS-MS method. The morphine/codeine ratios in these samples ranged from 0.43-10.00 (mean 3.35). Morphine was detected at a higher concentration than codeine in 4 of these 7 samples. As well as being a metabolite of 6-MAM, morphine is also a metabolite of codeine. In samples 3 and 4, the codeine concentration is higher than the morphine concentration. However due to the possible consumption of codeine, these ratios can be higher and as found in samples 3 and 4, can be found at a greater concentration than morphine. In these cases, it is extremely important to detect 6-MAM to distinguish heroin use from codeine use. The morphine/codeine ratios reported in another study ranged from 0.38-6.45 as reported for 23 morphine and codeine positive hair samples which used a method involving a phosphate buffer (pH 5.0) extraction.[84] According to the criteria discussed in section 8.9.1.5.1, a sample should be reported positive for heroin use when there is a morphine/codeine ratio of 5: 1 in cases where the morphine level is < 1 ng/mg. Samples 3 and 4 would be reported as negative for heroin use according to these reported ratios. However 6-MAM was found in these samples and the 6-MAM/morphine ratios were 5.8 and 3.6 respectively which would fit the criteria proposed by one of the studies[1] for reporting heroin use but not the other study which proposes a 6-MAM/morphine ratio > 7.14.[253] The information provided in the pathology reports for sample 3 was that the individual had a past history of multi-substance abuse, including heroin abuse. Morphine, diazepam, nordiazepam and alcohol were found in the blood sample. The information provided in the

pathology report for sample 4 provided no information on past drug abuse history. Morphine, diazepam, nordiazepam, oxazepam and procyclidine were detected in the blood sample. It would appear that the proposed morphine/codeine ratios of 5:1 are not appropriate in these cases where the background information indicates past heroin use and where no information to support codeine use was found.

Using LC-MS-MS, 9 out of 12 opiate positive segments without roots contained both morphine and 6-MAM. In contrast using GC-MS, 5 out of 12 opiate positive segments without roots contained both 6-MAM and morphine. In general there was good correlation between the qualitative opiate results for LC-MS-MS and GC-MS. However some analytes were detected by LC-MS-MS where GC-MS failed to detect them and vice versa.

The 6-MAM concentrations detected by LC-MS-MS and GC-MS were in the range 0.08-4.55 ng/mg and 0.19-3.36 ng/mg respectively, with most being categorised as low (91.6 %) users according to the proposals in the previously mentioned study.[253] The concentrations detected in sample 7 by LC-MS-MS and GC-MS were indicative of medium level use. According to this proposed categorisation, 3 of the segments would be reported as negative using the LC-MS-MS method and 1 of these segments would be reported as positive since the 6-MAM concentrations were < 0.5 ng/mg. This finding highlights the importance of establishing cut-off values appropriate to a particular method. However, recent recommendations by the SoHT state that a cut-off of 0.2 ng/mg 6-MAM is acceptable for interpreting heroin use.[100] Using LC-MS-MS, 6-MAM levels detected in 3 segments (samples 2, 10 and 12) were below this cut-off level. The GC-MS results for samples 2 and 12 did not detect any 6-MAM so these results would have been reported as negative by both methods. The GC-MS method detected 6-MAM at the SoHT cut-off concentration of 0.2 ng/mg in sample 10 where the LC-MS-MS found a concentration < 0.2 ng/mg.

There was no background information available for sample 2. There was drug paraphernalia and multiple needle marks on the individual from which sample 10 was collected, which is consistent with heroin use. Morphine, codeine, EME,

cocaine, benzoylecgonine, diazepam, nordiazepam, oxazepam and temazepam were found in the blood sample. Information from the pathology reports revealed that hair sample 12 was collected from an individual with a previous addiction to heroin and had been attending methadone programmes. Morphine was detected in the blood sample, as well as diazepam, nordiazepam, oxazepam, temazepam, methadone, sodium valproate and sertraline.

The 6-MAM/morphine ratios are shown in Table 8-30. With the exception of sample 3, the ratios for the GC-MS method are lower than those for the LC-MS-MS method. This is a result of the different extraction methods used. The LC-MS-MS ratios for 6-MAM/morphine are mostly higher than the ratios determined in another study which used a phosphate buffer (pH 5.0) extraction.

Table 8-30 6-MAM/morphine ratios in hair by LC-MS-MS and GC-MS

Hair Sample Number	6-MAM/Morphine by LC-MS-MS	6-MAM/Morphine by GC-MS
(1) 0.5-3.5 cm	0.65	0.47
(2) 0.5-3.5 cm	0.10	N/A
(3) 0.5-3.5 cm	5.78	72.00
(3) 3.5-6.5 cm	N/A	13.30
(4) 0.5-3.5 cm	3.63	2.96
(7) 0.5-3.5 cm	4.14	0.58
(8) 0.5-3.5 cm	N/A	3.54
(9) 0.5-3.5 cm	28.00	3.44
(12) 0.5-3.5 cm	0.14	N/A
(13) 0.5-3.5 cm	N/A	3.80
(15) 0.5-3.5 cm	1.28	0.76

As mentioned previously, dihydrocodeine levels have not been commonly reported in hair. The LC-MS-MS and GC-MS concentrations found in the segments excluding roots ranged from 0.09-16.18 ng/mg and 0.05-14.60 ng/mg. The LC-MS-MS method appears to extract more dihydrocodeine than the GC-MS method. The concentrations found by both methods were lower than the range reported in another study.[247] Dihydrocodeine was found in hair samples 12 and 15 at relatively high levels. The background reports stated that the individuals from which samples 12 and 15 were collected were being currently prescribed dihydrocodeine.

Cocaine and benzoylecgonine were detected in 7 segments excluding roots by LC-MS-MS. The benzoylecgonine/cocaine metabolite to parent drug ratios were ≥ 0.05 in all samples which is the cut-off value proposed by the SoHT.[97] Further suggestion of cocaine use was the presence of cocaethylene in 3 of these segments (samples 5, 9 and 10) and the presence of EME in 2 segments (samples 9 and 10). Cocaine concentrations by LC-MS-MS ranged from 0.06-13.99 ng/mg, benzoylecgonine from 0.11-4.73 ng/mg, cocaethylene from 0.04-0.53 ng/mg and 0.21-0.24 ng/mg EME. These ranges correspond with other studies.[81,233] One study which used phosphate buffer (pH 5.0) incubation for the extraction of cocaine and its metabolites from 103 hair samples from known drug users detected cocaine levels in 74 of these samples ranging between 0.01-21.37 ng/mg, benzoylecgonine levels between 0.03-10.51 ng/mg and cocaethylene levels between 0.05-1.26 ng/mg.[81] The method did not analyse for EME. Benzoylecgonine/cocaine ratios in this study ranged form 0.43-1.85. There was excellent qualitative agreement between the LC-MS-MS and GC-MS results for cocaine and benzoylecgonine. The GC-MS method was applied for the analysis of cocaine and its metabolites in 6 segments excluding roots. Cocaine and benzoylecgonine were detected in these segments also found to be positive by LC-MS-MS. Cocaethylene was also detected in samples 9 and 10. As mentioned previously, EME results were not reported for the GC-MS method due to an interferent at the EME retention time. Quantitatively, the GC-MS method generally extracted greater quantities of cocaine and its metabolites due to a higher extraction recovery. The benzoylecgonine/cocaine metabolite to parent drug ratios were ≥ 0.05 in all samples. Cocaine concentrations by GC-MS ranged from 0.09-19.34 ng/mg, benzoylecgonine from 0.21-4.92 ng/mg and cocaethylene from 0.10-1.07 ng/mg. The cocaine range found is lower than reported in another study which used acidic hydrolysis with hydrochloric acid at 50 °C overnight to extract cocaine and its metabolites from hair.[248] Benzoylecgonine concentrations were however within the range reported in this study.

For the segments excluding roots, the LC-MS-MS concentrations of amphetamine in hair ranged from 0.19-7.29 ng/mg which is in agreement with other studies. The GC-MS method was only applied to sample 17 and found a concentration of

1.75 ng/mg where the LC-MS-MS detected a level of 7.29 ng/mg. Amphetamine was also found in the blood sample at a concentration of 0.66 mg/L and the cause of death was amphetamine intoxication. One study reported amphetamine concentrations of 0.01-4.46 ng/mg in hair of 40 volunteers who were on drug rehabilitation programmes and were known drug users.[2]

The GC-MS method for amphetamines was only applied to sample 17 however the LC-MS-MS method analysed for amphetamines in all hair samples. MDMA was detected in 4 samples (samples 2, 3, 4 and 5) by LC-MS-MS. Concentrations ranged from 0.46-1.60 ng/mg which corresponds with other scientific literature. According to the current SoHT guidelines these would be reported as positive.[97] 1 of the samples (sample 4) was positive for both MDMA and amphetamine. The presence of amphetamine in this sample could be due to ingesting amphetamine tablets or alternatively the amphetamine could be present as an impurity in the MDMA tablets. There was no MDMA metabolite, MDA detected in the samples. A possible explanation for this is the low levels of MDMA detected in the samples. One study examined MDA/MDMA metabolite to parent drug ratios in hair samples collected from 40 suspected drug users.[249] The MDA/MDMA ratios ranged from 0.03-1.10 (mean 0.15). If this mean MDA/MDMA ratio was applied to calculate a predicted MDA concentration range from the MDMA concentration range found, it would be 0.70-2.4 ng/mg. The lower value in this range is the LOQ of the method and this may explain why MDA was not reported as positive in this sample. In one study, MDMA concentrations ranging from 0.12-6.4 ng/mg were detected in the hair of 20 known users.[250] The range of MDMA concentrations in another study analysing hair collected from suspected abusers was 0.56-34.06 ng/mg.[249] MDA was also detected in this study in the range 0.13-6.44 ng/mg.

The GC-MS method did not analyse for benzodiazepines. The LC-MS-MS method however detected nordiazepam most frequently in the hair samples tested in this study and was generally present at a higher level than diazepam, as found in other studies.[214,218] These results were consistent with the findings of the benzodiazepine study carried out in chapter 7. Diazepam concentrations ranged from 0.04-0.52 ng/mg, nordiazepam from 0.17-0.67 ng/mg, 0.22-0.26 ng/mg oxazepam and 0.10-0.14 ng/mg temazepam. Oxazepam and temazepam were

generally detected at lower levels than found in the other study in chapter 7 as a result of the different extraction method used. They were also detected in fewer samples compared to diazepam and nordiazepam. This would suggest diazepam use in these cases. However, oxazepam was detected at a higher concentration in sample 12 compared to diazepam and nordiazepam which may indicate combined diazepam and oxazepam use.

The LC-MS-MS chromatograms for hair samples that were positive for each drug class are given in Figures 8-22-8-25.

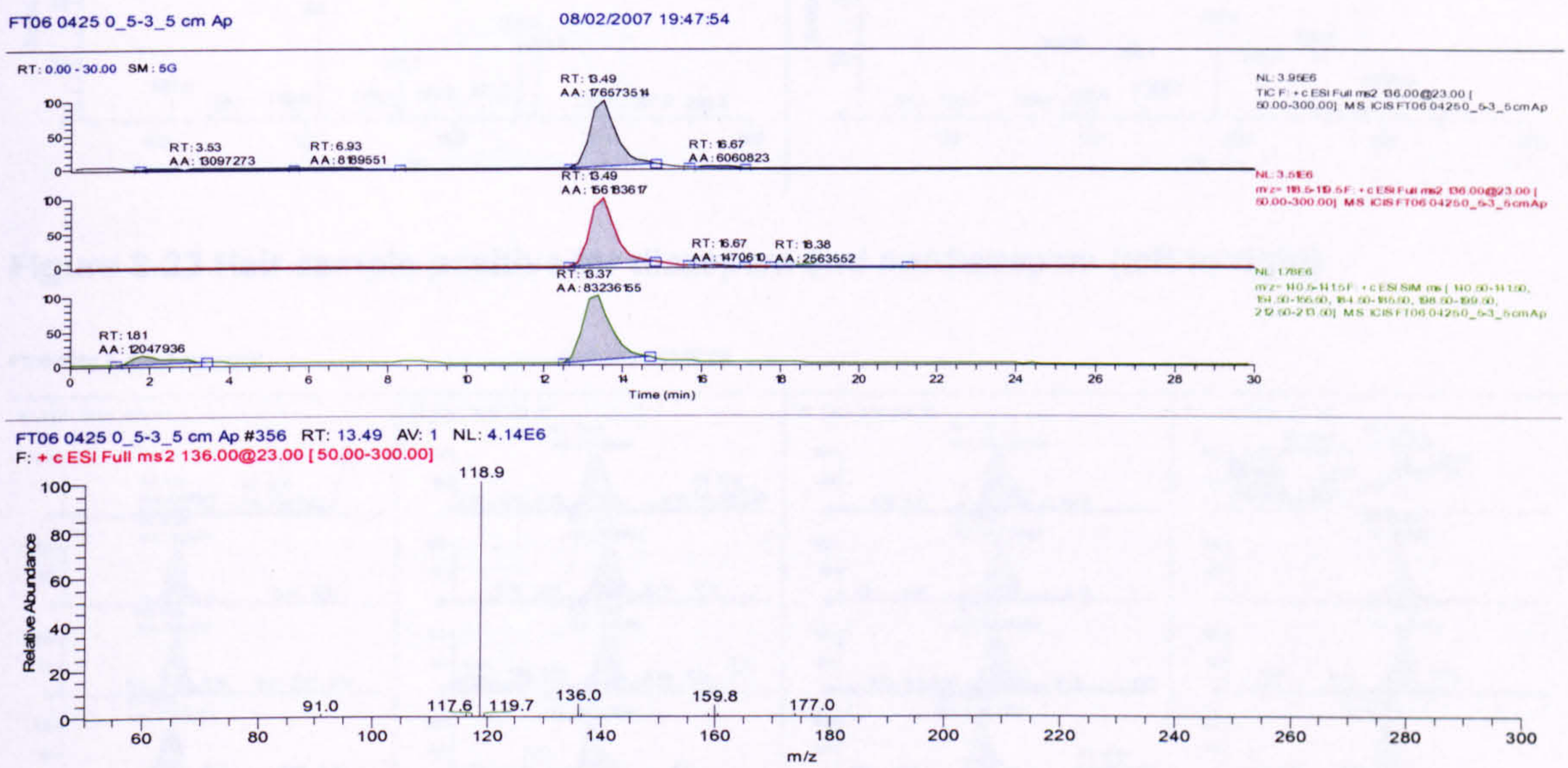


Figure 8-22 Hair sample positive for amphetamine

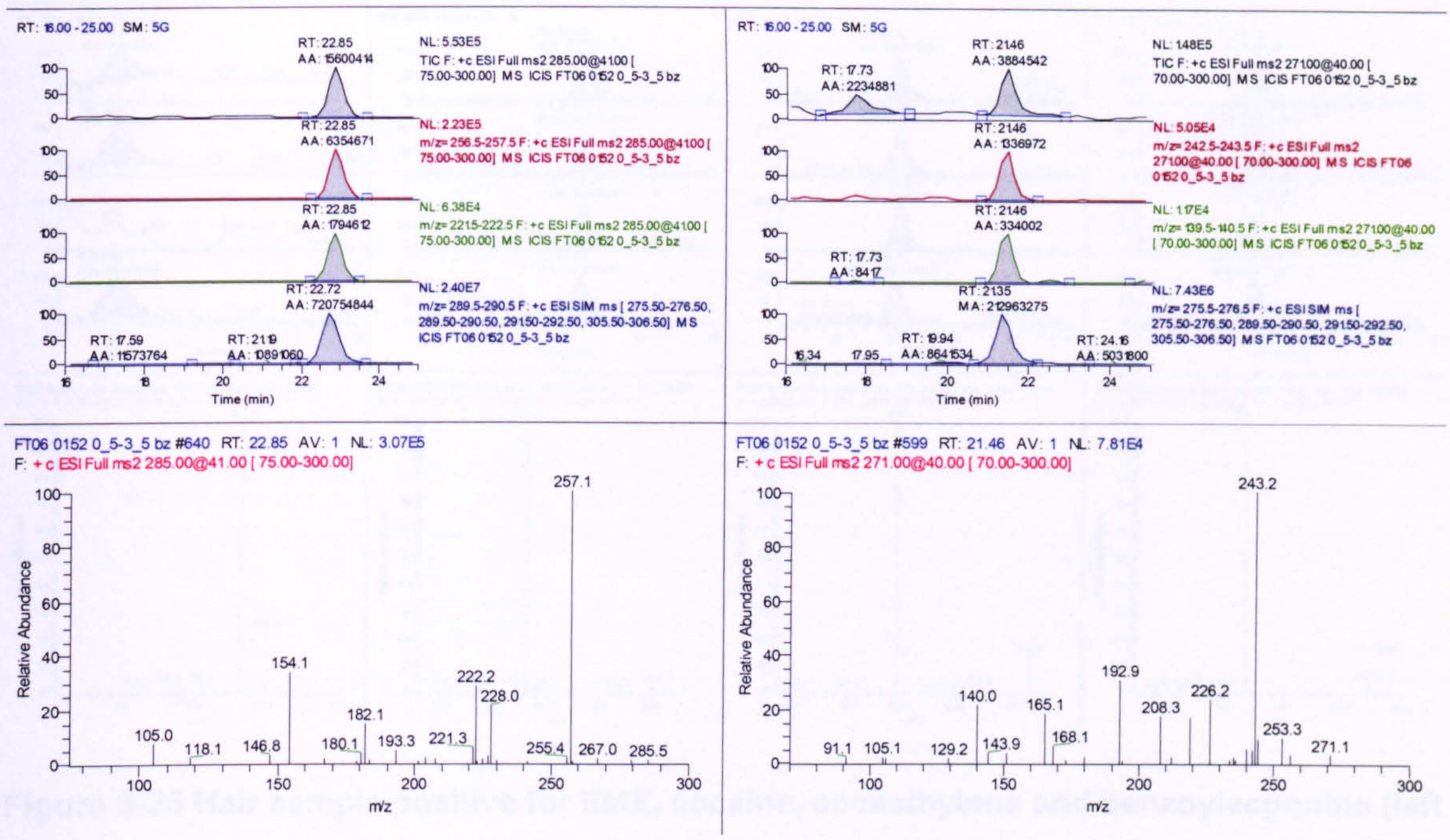


Figure 8-23 Hair sample positive for diazepam and nordiazepam (left to right)

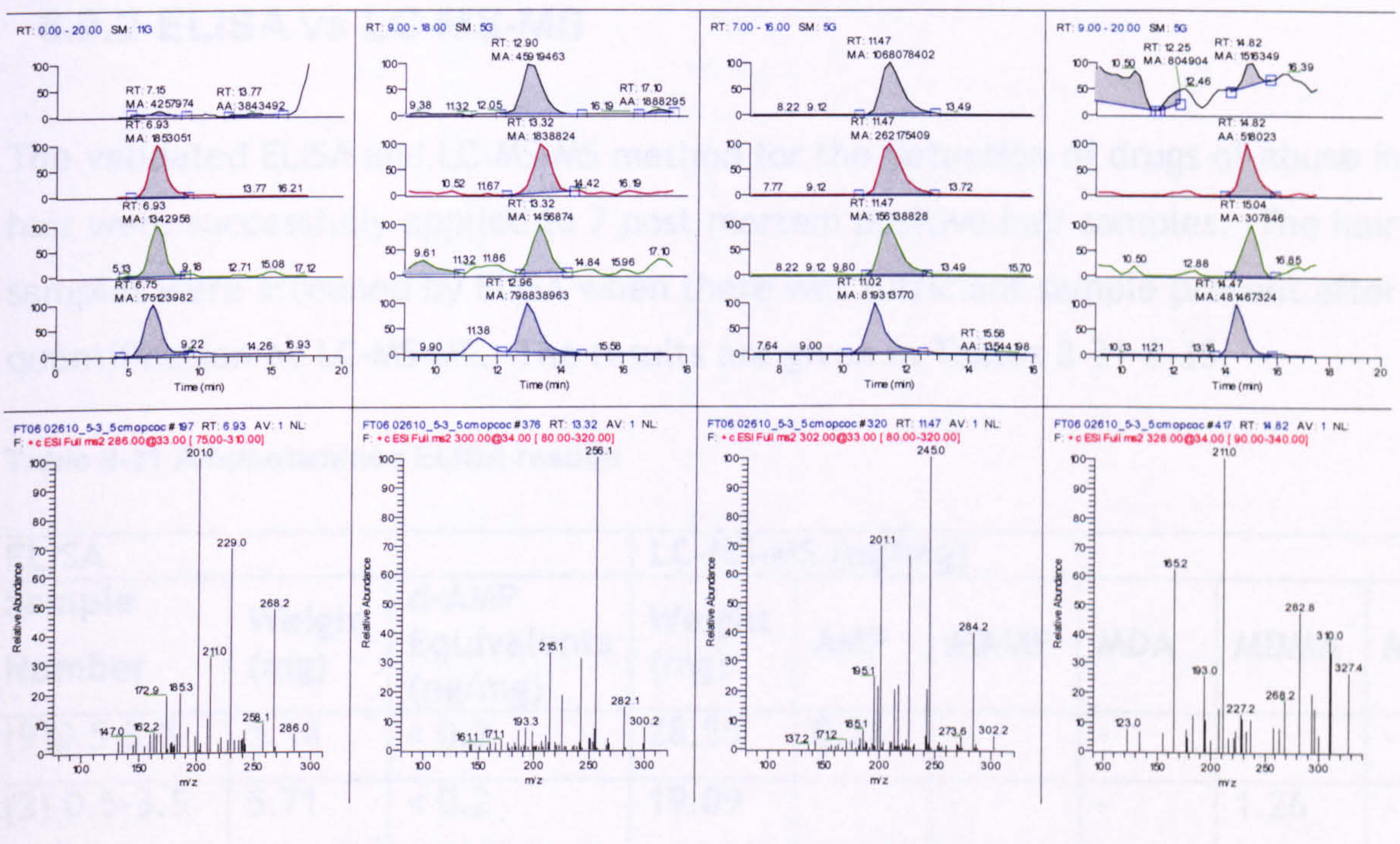


Figure 8-24 Hair sample positive for morphine, codeine, dihydrocodeine and 6-MAM (left to right)

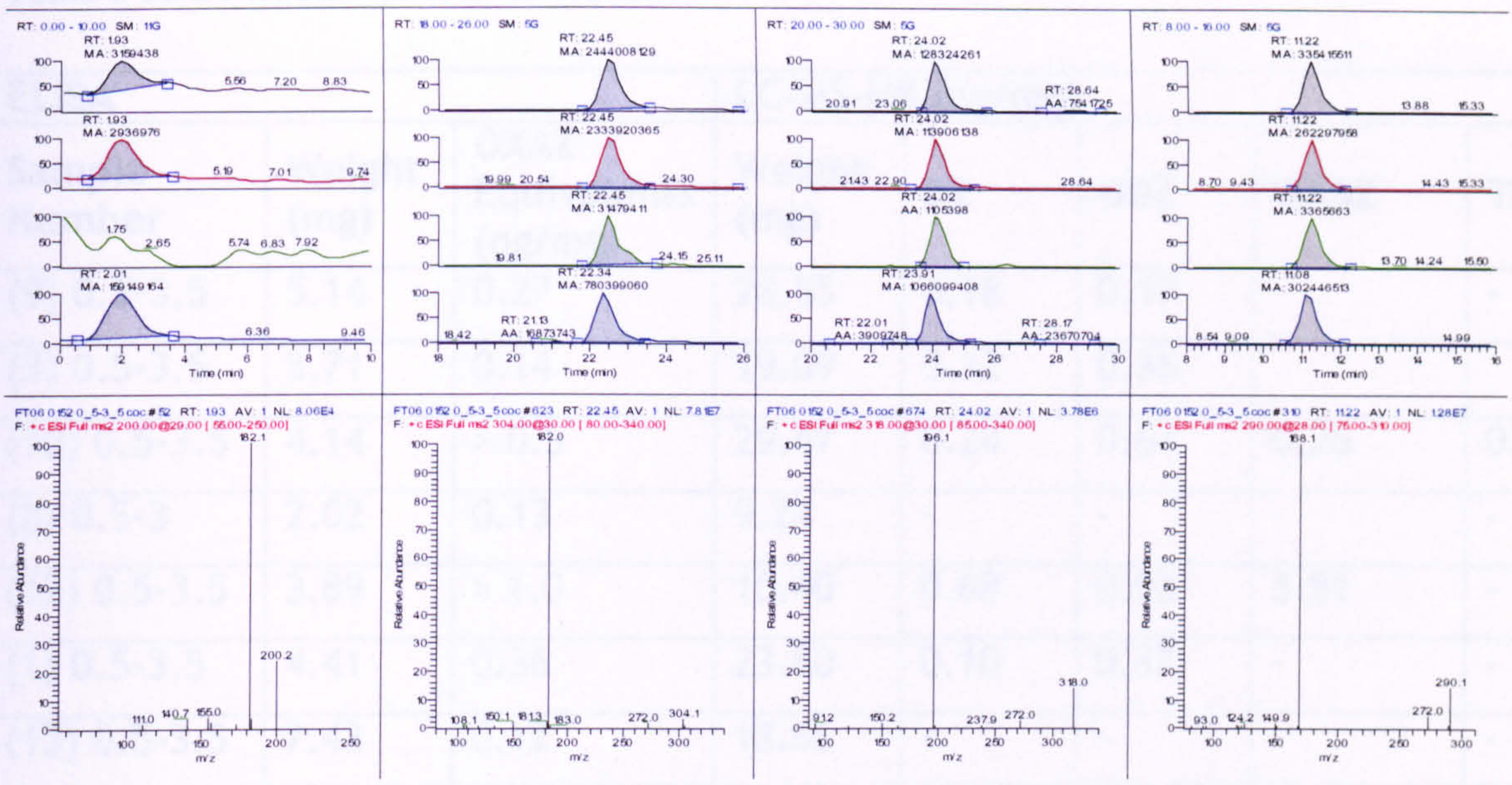


Figure 8-25 Hair sample positive for EME, cocaine, cocaethylene and benzoylecgonine (left to right)

8.9.2 ELISA vs LC-MS-MS

The validated ELISA and LC-MS-MS method for the detection of drugs of abuse in hair were successfully applied to 7 post-mortem positive hair samples. The hair samples were screened by ELISA when there was sufficient sample present after quantification by LC-MS-MS. The results are given in Tables 8-31-8-35.

Table 8-31 Amphetamines ELISA results

ELISA			LC-MS-MS (ng/mg)					
Sample Number	Weight (mg)	d-AMP Equivalents (ng/mg)	Weight (mg)	AMP	MAMP	MDA	MDMA	MDEA
(9)0.5-3.5	5.14	< 0.2	28.55	0.21	-	-	-	-
(3) 0.5-3.5	5.71	< 0.2	19.09	-	-	-	1.26	-
(10) 0.5-3.5	4.14	< 0.2	29.47	-	-	-	-	-
(8) 0.5-3	2.02	< 0.2	9.24	-	-	-	-	-
(12) 0.5-3.5	3.89	< 0.2	10.40	-	-	-	0.46	-
(1) 0.5-3.5	4.41	< 0.2	23.80	-	-	-	-	-
(15) 0.5-3.5	7.43	< 0.2	18.66	-	-	-	-	-

"AMP"=Amphetamine, "MAMP"=Methamphetamine

Table 8-32 Benzodiazepine ELISA results

ELISA			LC-MS-MS (ng/mg)				
Sample Number	Weight (mg)	OXAZ Equivalents (ng/mg)	Weight (mg)	DZ	NDZ	OXAZ	TMZ
(9) 0.5-3.5	5.14	0.27	28.55	0.18	0.17	-	-
(3) 0.5-3.5	5.71	0.14	19.09	0.12	0.36		
(10) 0.5-3.5	4.14	> 0.5	29.47	0.24	0.64	0.26	0.14
(8) 0.5-3	2.02	0.13	9.24	-	-	-	-
(12) 0.5-3.5	3.89	> 1.0	10.40	0.68	0.92	3.51	-
(1) 0.5-3.5	4.41	0.36	23.80	0.10	0.37	-	-
(15) 0.5-3.5	7.43	0.12	18.66	-	-	-	-

"DZ"= Diazepam, "NDZ"= Nordiazepam, "OXAZ"=Oxazepam, "TMZ"=Temazepam

Table 8-33 Cocaine ELISA Results

ELISA			LC-MS-MS (ng/mg)				
Sample Number	Weight (mg)	COC Equivalents (ng/mg)	Weight (mg)	COC	BZE	COCAETH	EME
(9) 0.5-3.5	5.14	> 2.5	28.55	13.99	4.73	0.53	-
(3) 0.5-3.5	5.71	0.64	19.09	0.29	0.16	-	-
(10) 0.5-3.5	4.14	> 2.5	29.47	0.60	0.18	0.04	-
(8) 0.5-3	2.02	< 0.5	9.24	0.08	-	-	-
(12) 0.5-3.5	3.89	< 0.5	10.40	-	-	-	-
(1) 0.5-3.5	4.41	< 0.5	23.80	-	-	-	-
(15) 0.5-3.5	7.43	< 0.5	18.66	-	-	-	-

"COC"= Cocaine, "BZE"= Benzoylecgonine, "COCAETH"= Cocaethylene, "EME"=Ecgonine Methyl Ester

Table 8-34 Methamphetamine ELISA results

ELISA			LC-MS-MS (ng/mg)					
Sample Number	Weight (mg)	*d*-MAMP Equivalents (ng/mg)	Weight (mg)	AMP	MAMP	MDA	MDMA	MDEA
(9) 0.5-3.5	5.14	0.24	28.55	0.21	-	-	-	-
(3) 0.5-3.5	5.71	0.60	19.09	-	-	-	1.26	-
(10) 0.5-3.5	4.14	< 0.2	29.47	-	-	-	-	-
(8) 0.5-3	2.02	< 0.2	9.24	-	-	-	-	-
(12) 0.5-3.5	3.89	0.31	10.40	-	-	-	0.46	-
(1) 0.5-3.5	4.41	< 0.2	23.80	-	-	-	-	-
(15) 0.5-3.5	7.43	0.42	18.66	-	-	-	-	-

"AMP"=Amphetamine, "MAMP"=Methamphetamine

Table 8-35 Opiate ELISA Results

ELISA			LC-MS-MS (ng/mg)				
Sample Number	Weight (mg)	MOR Equivalents (ng/mg)	Weight (mg)	MOR	6-MAM	COD	DHC
(9) 0.5-3.5	5.14	0.43	28.55	0.04	1.12	-	-
(3) 0.5-3.5	5.71	< 0.2	19.09	0.09	0.52	-	-
(10) 0.5-3.5	4.14	< 0.2	29.47	-	0.08		
(8) 0.5-3	2.02	< 0.2	9.24	-	0.58	-	-
(12) 0.5-3.5	3.89	> 1.0	10.40	0.87	0.12	-	16.18
(1) 0.5-3.5	4.41	0.20	23.80	2.02	-	-	-
(15) 0.5-3.5	7.43	> 1.0	18.66	1.03	1.32	0.31	10.72

"MOR"=Morphine, "6-MAM"= 6-monoacetylmorphine, "COD"= Codeine, "DHC"= Dihydrocodeine

The sample weights used for ELISA were low (< 10 mg and in most cases < 5 mg). The drug levels detected in some samples using LC-MS-MS were low even using a higher sample weight of 10-30 mg therefore the sample weight limited the ELISA method.

The methamphetamine kit produced a greater number of true results compared with the amphetamine kit which produced 3 FN results. This is due to the

different cross reactivity of the 2 kits. The methamphetamine kit has a very high cross reactivity with MDMA and 2 of the samples were confirmed as positive using LC-MS-MS for MDMA.

The benzodiazepine kit produced 5 TP and 2 FP results using a cut-off of 0.1 ng/mg oxazepam. The 2 FP concentrations were calculated as the actual cut-off value. There may have been benzodiazepines present in these samples other than diazepam and its metabolites which could have cross reacted with the ELISA kit to produce these FP results.

Using the recommended SoHT cut-off value of 0.5 ng/mg cocaine, the kit detected 3 TP, 3 TN and 1 FN result. The FN result produced a low positive result for cocaine and so using this cut-off, the sample would have been reported as negative.

The opiates kit detected 4 TP and 3 FN results using a cut-off of 0.2 ng/mg morphine. The FN results were samples which contained low levels of opiates as determined by LC-MS-MS.

On the whole, ELISA is a useful method for screening provided there is sufficient sample (> 10 mg). The validated LC-MS-MS method was capable of detecting drug levels in very low weight root samples as shown in Table 8-26. This was not the case for ELISA screening of low sample weights as there were FN results for all drug classes tested except benzodiazepines. Furthermore the superior LC-MS-MS specificity means that screening and quantification could be carried out using the LC-MS-MS method described.

8.10 Conclusion

The developed and validated LC-MS-MS method is capable of simultaneously identifying and quantifying amphetamines, diazepam and its metabolites, cocaine and its metabolites and opiates from one hair sample of 8-30 mg. It is also capable of detecting these drug groups in root segments of low weight (in some cases < 1 mg). Although the opiate and cocaine recoveries are generally lower from real hair samples using the LC-MS-MS compared to the in-house GC-

MS methods targeting specific drug groups, the sensitivity and therefore the qualitative data was better, especially for morphine, 6-MAM and cocaethylene in low weight root samples.

As a result of the lower opiate and cocaine recoveries using this LC-MS-MS method compared to GC-MS, it should be borne in mind that there may be cases where the GC-MS method would detect the analyte where the LC-MS-MS method would not. This has been shown in a few samples for morphine and codeine. Dihydrocodeine was however detected in 2 samples where LC-MS-MS failed to detect it. The LC-MS-MS and GC-MS data showed excellent qualitative correlation for cocaine, benzoylecgonine and cocaethylene but the GC-MS method extracted greater quantities of cocaine and benzoylecgonine. Cocaethylene and EME were detected in some samples by LC-MS-MS were GC-MS detected none. A disadvantage of the GC-MS method is its inability to report EME levels due to a co-eluting interferent peak at the EME retention time. Amphetamine recovery was higher using the LC-MS-MS in 1 sample compared to GC-MS.

The method was useful for the analysis of 17 drugs of abuse from post-mortem hair in forensic toxicology cases. Maximum information is obtained from 1 hair sample which is extremely useful when the sample weight is limited. As it is currently not possible to relate the quantity of drug detected in hair with amount of drug ingested or the frequency of drug use, a qualitative screening method may provide all the required information for as accurate an interpretation as possible at this time. The ability of the LC-MS-MS method to extract and analyse a greater number of drug groups from one hair sample highlights the advantages of using this method over GC-MS which targets individual drug groups and requires splitting the sample.

9 Conclusion

An investigation was carried out into the potential use of ELISA and LC-MS-MS for the analysis of biological samples in forensic toxicology. Both analytical techniques have been found to be extremely useful however as with any technique, each has its own set of benefits and limitations.

A highly sensitive and specific ELISA method was developed for the successful detection of buprenorphine in 21 urine samples. This level of sensitivity is essential as therapeutic concentrations of buprenorphine can be low. The limit of detection was 0.5 ng/mL. Norbuprenorphine cross reactivity was relatively high which is a good feature of the kit since norbuprenorphine levels in urine are generally higher than buprenorphine levels. Using 0.5 ng/mL as the ELISA cut off, the sensitivity and specificity were both 100 % compared with an in-house LC-MS method.

A single ELISA method was successfully validated for amphetamines, benzodiazepines, cocaine, methamphetamine, methadone and opiates in hair and subsequently applied to the screening of case samples. The kits were found to be very sensitive with LOD values ≤ 0.1 ng/mg using a 10 mg hair sample. Intra-day precision and inter-day precision levels were acceptable (< 9 % and < 18 % respectively) and appropriate cross reactivity was demonstrated with drugs and drug metabolites relevant to hair.

The ELISA cut-off values used for the post-mortem hair samples in chapters 6 and 8 were recommended by the Society of Hair Testing (SoHT) for amphetamines, cocaine and opiates. The ELISA sensitivity and specificity were 89 % and 79 % for 27 opiate positive samples. The 3 FN results could be explained by low sample weights ≤ 5 mg and in 1 case, the presence of a low level of dihydrocodeine which has been shown to cross react with the kit by 43 %. The ELISA sensitivity for 20 benzodiazepine positive hair samples was 100 % however specificity was 67 % using a cut-off value of 0.1 ng/mg. It was shown that FN results were produced at higher ELISA cut-off values. In the collaboration with the Korean laboratory, 29 methamphetamine positive hair

samples were screened at a cut-off of 0.5 ng/mg. 28 out of 29 samples were TP results and there was 1 FN which was very close to the ELISA cut-off value.

There were a relatively small number of cocaine, methadone and amphetamine positive hair samples available for ELISA screening as the majority of the sample required to be analysed by a more specific confirmatory method first. Therefore no substantiated conclusions can be drawn regarding the recommended SoHT cut-off values for cocaine and amphetamine. Interestingly, 2 of the 3 FN cocaine results contained only cocaine at concentrations < 0.5 ng/mg as determined by GC-MS. This could be a result of contamination or low use. According to current SoHT guidelines, these 2 samples would be reported as negative as the guidelines stipulate that a benzoylecgonine/cocaine ratio ≥ 0.05 should be used as a cut-off. There were only 3 samples confirmed as positive for amphetamines by LC-MS-MS. 2 of these samples were positive for MDMA and tested negative using the amphetamines ELISA due to very low MDMA cross reactivity but tested positive using the methamphetamine kit which has a high MDMA cross reactivity. 1 sample was positive for amphetamine and only tested positive using the methamphetamine kit. The LC-MS-MS concentration calculated for amphetamine was the actual cut-off so this explains the negative result using the amphetamines kit. 5 out of 6 methadone positive samples screened as positive at a cut-off of 0.2 ng/mg. The 1 FN result was probably a result of a very low sample weight (1 mg) as the GC-MS concentration detected was 3.7 ng/mg.

The ELISA method was applied successfully for the detection of methamphetamine in hair of methamphetamine abusers. The kit demonstrated a sensitivity and specificity of 97 % and 100 % respectively using a cut-off value of 0.5 ng/mg *d*-methamphetamine. The amphetamine to methamphetamine ratios ranged from 0.02-0.159 in 28 samples excluding roots.

An LC-MS-MS method was successfully validated for the detection of 9 benzodiazepines in hair as described in chapter 7. The method was sufficiently sensitive for their detection in post-mortem hair samples. 13 post-mortem samples were found to be positive for benzodiazepines using this method. A

mild alkaline incubation lasting 16 hours was shown to be most suitable for benzodiazepine extraction and there was no significant hydrolysis observed. Recoveries in spiked hair ranged from 53-98 % and the LOD range was 0.03-0.62 ng/30 mg hair. The matrix effect ranged from 77-105 % with most analytes displaying ion suppression. The evaporation step in the SPE method was identified as means of 7-aminoflunitrazepam and chlordiazepoxide loss, which is partly responsible for the lower recoveries of these analytes.

Also in chapter 7, a preliminary study was carried out into the use of a diazepam molecularly imprinted polymer for the detection of benzodiazepines in post-mortem hair case samples. It was shown that it can be applied successfully for this purpose and detected a higher number of diazepam positive cases compared to the classical SPE method because of higher extraction recovery and the excellent molecular recognition of the template molecule (diazepam) imparted by the imprinting process.

The method developed in chapter 8 was successfully validated for 17 drugs of abuse in hair. It enabled the simultaneous identification and quantification of amphetamines, diazepam and its metabolites, cocaine and its metabolites and opiates from 1 hair extract using 3 LC-MS-MS injections. 3 injections were used to enhance assay sensitivity by minimising the number of scan events per time window. This also maximised the number of points across the peak to obtain more accurate analyte quantifications.

17 post-mortem hair samples were analysed using this method and the results were compared to the GC-MS results obtained by in-house methods. At least one drug class was detected in 16 out of 17 samples. 1 sample was negative by both the LC-MS-MS and GC-MS methods. In general, there was a good qualitative correlation between LC-MS-MS and GC-MS results for opiates, cocaine and amphetamine, particularly in the 0.5-3.5 cm segments which were of sufficiently high sample weight. Morphine and codeine were detected in 2 segments excluding roots using GC-MS where LC-MS detected none. However this did not affect the reported result in accordance with current SoHT guidelines. Dihydrocodeine was also detected in 2 segments excluding roots by GC-MS where

LC-MS-MS failed to detect it. On the other hand, the LC-MS-MS method detected cocaethylene and EME in 2 samples where GC-MS did not detect any and in the case of EME could not report the GC-MS result due to co-eluting interferent at the EME retention time. Overall, the LC-MS-MS method was capable of testing for more drug groups using one hair sample and extraction method compared to the drug class specific GC-MS methods which involved splitting the hair sample and the use of different extraction methods. On the other hand, the GC-MS methods generally extracted greater quantities of opiates and cocaine and its metabolites since they were targeting these particular drug groups. The methods had been developed and validated with a view to detecting one particular drug group therefore the extraction and analysis conditions were optimum for that drug group. The LC-MS-MS method however was developed and validated with a view to detecting 4 different drug groups. Therefore, a compromise on extraction and analysis conditions had to be reached for the LC-MS-MS method. The LC-MS-MS method detected amphetamine, MDMA, diazepam and its metabolites in some samples however the GC-MS method did not test for these drug groups as there was insufficient sample after testing for opiates and in some cases cocaine and its metabolites. The ability of the LC-MS-MS method to extract and analyse a greater number of drug groups from one hair sample highlights the advantages of using this method over GC-MS which targets individual drug groups and requires splitting the sample.

In conclusion, ELISA screening is a highly sensitive method which can be applied for the detection of drugs of abuse in hair and urine. LC-MS-MS has demonstrated excellent reliability, sensitivity and specificity and it is an extremely useful instrument in a forensic toxicology laboratory. Using LC-MS-MS, the sensitivity and therefore the qualitative data was better for post-mortem samples discussed in chapter 8, especially for 6-MAM and morphine in low weight root samples. The LC-MS-MS method was useful for the analysis of targeted unknown drugs from hair in forensic toxicology cases and could even be used in place of ELISA for a more specific screening procedure or in cases where minimum sample is available. However GC-MS is still better for some compounds and should ideally be used in tandem with LC-MS-MS to enable the analysis of a large number of compounds.

10 Further Work

The application of ELISA for the detection of drugs of abuse in hair has been demonstrated. The ELISA study in this thesis was limited by the number of samples available and the weight of the sample. It was therefore not possible to draw any definitive conclusions about the suitability of proposed cut-off values by the Society of Hair Testing (SoHT). Screening a greater number of samples of sufficient weight (> 10 mg) in order to recommend appropriate in-house values would therefore be an area of interest for future work. In addition, ELISA kits which have recently become commercially available e.g. zolpidem and fluoxetine should be tested for potential use in hair screening.

Although the LC-MS-MS method in chapter 7 was successfully validated and applied in the analysis of 13 post-mortem hair samples from chronic users, only 6 of the 9 benzodiazepines included in the method were detected in the samples. The method should be applied to hair samples containing chlordiazepoxide, flunitrazepam and 7-aminoflunitrazepam to ensure that the method is suitable for extracting these drugs when they are actually incorporated. As some 7-aminoflunitrazepam and chlordiazepoxide were lost during the evaporation step in the method, an experiment should be carried out to test their recovery when tartaric acid is added to the extract prior to evaporating the extracts to dryness using nitrogen. Further work is also required to investigate the detection of single dose benzodiazepine in hair which can be used to sedate a victim in a "date-rape" situation. With respect to the molecularly imprinted polymer work, the possibility of mixing two or more sorbents in one cartridge to detect a particular drug group should be investigated. Further work is also required to investigate the ability of the MISPE to detect benzodiazepines in single dose hair samples.

The newly validated LC-MS-MS method described in chapter 8 should be applied to a greater number of hair samples and the results compared with current GC-MS methods to obtain more statistically valid data. This data could be used to establish whether the guidelines and cut-offs proposed by the Society of Hair Testing are appropriate for the LC-MS-MS method. There is scope for the

inclusion of other analytes in the method which would improve the method's screening capability. An in-house LC-MS-MS library could be built up for this method although this is more difficult than for GC-MS as the LC-MS spectra vary depending on instrument, chromatographic conditions and fragmentation voltages. However if the instrument used in chapter 8 was to be used for all routine hair analysis then this would be possible. The matrix effect observed for 17 drugs of abuse in the method ranged between 64-136 %. An experiment using an atmospheric pressure chemical ionisation (APCI) source could be carried out to compare validation results with the electrospray (ESI) results as APCI is thought to produce less matrix effects than ESI. This is because of the presence of excess reagent ions which produce charged species in APCI, making it less susceptible to matrix effects.

References

[1] Moeller MR, Fey P, Sachs H. *Hair analysis as evidence in forensic cases.* Forensic Science International (1993); 63: 43-53.

[2] Cairns T, Hill V, Schaffer M, Thistle W. *Amphetamines in washed hair of demonstrated users and workplace subjects.* Forensic Science International (2004); 145: 137-142.

[3] Tagliaro F, De Battisti Z, Lubli G, Neri C, Manetto G, Marigo M. *Integrated use of hair analysis to investigate the physical fitness to obtain the driving license: a casework study.* Forensic Science International (1997); 84: 129-135.

[4] Brewer C. *Hair analysis as a tool for monitoring and managing patients on methadone maintenance. A discussion.* Forensic Science International (1993); 63: 277-283.

[5] Lewis D, Moore C, Morrissey P, Leikin J. *Determination of drug exposure using hair: application to child protective cases.* Forensic Science International (1997); 84: 121-128.

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

[7] Kintz P (Ed.). *Drug testing in hair*, Florida, United States: CRC Press Inc., 1996

[8] Gaillard Y, Vayssette F, Pepin G. *Compared interest between hair analysis and urinalysis for doping controls. Results for amphetamines, corticosteroids and anabolic steroids in racing cyclists.* Foensic Science International (2000); 107: 361-379.

[9] Vinner E, Vignau J, Thibault T, Codaccioni X, Brassart C, Humbert L, Lhermitte M. *Hair analysis of opiates in mothers and newborns for evaluating opiate exposure during pregnancy.* Forensic Science International (2003); 133: 57-62.

[10] Gaillard Y, Pepin G. *Testing hair for pharmaceuticals.* Journal of Chromatography B (1999); 1-2: 231-246.

[11] *SOFT/AAFS Forensic Laboratory Guidelines* 2006. Available on-line at http://www.soft-tox.org/docs/Guidelines%202006%20Final.pdf, accessed 20th April 2007.

[12] Harkey MR. *Anatomy and physiology of hair.* Forensic Science International (1993); 63: 9-18.

[13] Kintz P (Ed.). *Analytical and practical aspects of drug testing in hair*, CRC Press, Florida, United States (2007).

[14] Dawber R. *Hair: Its structure and response to cosmetic preparations.* Clinics in Dermatology (1996); 14: 105-112.

[15] Pragst F. *Illegal and therapeutic drug concentrations in hair segments-aimetable of drug exposure?* Forensic Science Review (1998); 10: 81-111.

[16] Prota G. *Melanins, melanogenesis and melanocytes: looking at their functional significance from the chemist's viewpoint*. Pigment Cell Research (2000); 13: 283-293.

[17] Montagna W, Ellis RA (Eds.). *The biology of hair growth*, New York, Academic Press, 1958.

[18] Potsch L, *A discourse on human hair fibers and reflections on the conservation of drug molecules*. International Journal of Legal Medicine (1996); 108: 285-293.

[19] Borges CR, Wilkins DG, Rollins DE. *Amphetamine and N-acetylamphetamine incorporation into hair: an investigation of the potential role of drug basicity in hair color bias*. Journal of Analytical Toxicology (2001); 25: 221-227.

[20] Gygi SP, Wilkins DG, Rollins DE. *Distribution of codeine and morphine into rat hair after long-term daily dosing with codeine*. Journal of Analytical Toxicology (1995); 19: 387-391.

[21] Nakahara Y, Kikura R. *Hair analysis for drugs of abuse XIII: effect of structural factors on incorporation of drugs into hair:the incorporation rates of amphetamine analogs*. Archives of Toxicology (1996); 70: 841-849.

[22] Scheidweiler KB, Huestis MA. *Simultaneous quantification of opiates, cocaine and its metabolites in hair by LC-APCI-MS-MS*. Analytical Chemistry (2004); 76: 4358-4363.

[23] Nakahara Y, Shimamine M, Takahashi K. *Hair analysis for drugs of abuse III. Movement and stability of methoxyphenamine (as a model compound of*

methamphetamine) along hair shaft with hair growth. Journal of Analytical Toxicology (1992); 16: 253-257.

[24] Puschel K, Thomasch P, Arnold W. *Opiate levels in hair.* Forensic Science International (1983); 21: 181-186.

[25] Henderson GL. *Mechanisms of drug incorporation into hair.* Forensic Science International (1993); 63: 19-29.

[26] Rollins DE, Wilkins DG, Gygi SP, Slawson MH, Nagasawa NH. *Testing for drugs of abuse in hair-experimental observations and indications for future research.* Forensic Science Review (1997); 9: 23-25.

[27] Cone EJ, Hillsgrove MJ, Jenkins AJ, Keenan RM, Darwin WD. *Sweat testing for heroin, cocaine, and metabolites.* Journal of Analytical Toxicology (1994); 18: 298-305.

[28] Kintz P, Tracqui A, Mangin P. *Sweat testing for benzodiazepines.* Journal of Forensic Science (1996); 41: 851-854.

[29] Kintz P. *Value of hair analysis in post-mortem toxicology.* Forensic Science International (2004); 142: 127-134.

[30] Henderson GL, Harkey MR, Zhou C, Jones RT, Jacob P. *Incorporation of isotopically labelled cocaine and metabolites into human hair, part 3: 1, dose-response relationships.* Journal of Analytical Toxicology (1996); 20: 1-12.

[31] Stout PR, Ruth JA. *Deposition of [3H]cocaine, [3H]nicotine and [3h]flunitrazepam in mouse hair melanosomes after systemic administration.* Drug Metabolism and Disposition (2000); 28: 286-291.

[32] Potsch L, Moeller MR. *On pathways for small molecules into and out of human hair fibers.* Journal of Forensic Sciences (1996); 41: 121-125.

[33] Stout PR, Ruth JA. *Comparison of in vivo and in vitro deposition of rhodamine and fluorescein in hair.* Drug Metabolism and disposition (1998); 26: 943-948.

[34] Potsch L, Skopp G, Moeller MR. *Influence of pigmentation of the codeine content of hair fibers in guinea pigs.* Journal of Forensic Sciences (1997); 42(6): 1095-1098.

[35] Borges CR, Roberts JC, Wilkins DG, Rollins DE. *Cocaine, benzoylecgonine, amphetamine and N-acetylamphetamine binding in melanin subtypes.* Journal of Analytical Toxicology (2003); 27: 1-10.

[36] Testorf MF, Kronstrand R, Svensson SP, Lundstrom I, Ahlner J. *Characterization of [3H]flunitrazepam binding to melanin*, Analytical Biochemistry (2001); 298: 259-264.

[37] Nakahara Y, Takahashi K, Kikura R. *Hair analysis for drugs of abuse, X: effect of physicochemical properties of drugs on the incorporation rates into hair.* Biological Pharmaceutical Bulletin (1995): 41; 121-125.

[38] Kronstrand R, Ahlner J, Dizdar N, Larson G. *Quantitative analysis of desmethylselegiline, methamphetamine and amphetamine in hair and plasma*

from Parkinson patients on long-term selegiline medication. Journal of Analytical Toxicology 2003: 27; 135-141.

[39] Rothe M, Pragst F, Thor S, Hunger J. *Effect of pigmentation on the drug deposition in hair of grey-haired subjects.* Forensic Science International (1997); 84: 53-60.

[40] Scott KS, Nakahara Y. *A study into the rate of incorporation of eight benzodiazepines into rat hair.* Forensic Science International (2003); 133: 47-56.

[41] Rollins DE, Wilkins DG, Krueger GG, Augsburger MP, Mizuno A, O'Neal C, Borges CR, Slawson MH. *The effect of hair color on the incorporation of codeine into human hair.* Journal of Analytical Toxicology (2003); 27: 545-551.

[42] Nakahara Y, Kikura R, Takahashi K. *Hair analysis for drugs of abuse XX: Incorporation and behaviours of seven methamphetamine homologs in the rat hair root.* Life Sciences (1998); 63 (10): 883-893.

[43] Nakahara Y, Hanajiri R. *Hair analysis for drugs of abuse XXI. Effect of para-substituents on benzene ring of methamphetamine on drug incorporation into rat hair.* Life Sciences (2000); 66 (7): 563-574.

[44] Jurado C, Kintz P, Menedez M, Repetto M. *Influence of the cosmetic treatment of hair on drug testing.* International Journal of Legal Medicine (1997); 110: 159-163.

[45] Wickett RR. *Permanent waving and straightening of hair.* Cutis: Cutaneous Medical Practitioner (1987); 39: 496.

[46] Delauder SF, Kidwell DA. *The incorporation of dyes into hair as a model for drug binding.* Forensic Science International (2000); 93-104.

[47] Skopp G, Potsch L, Moeller MR. *On cosmetically treated hair- aspects and pitfalls of interpretation.* Forensic Science International (1997); 84: 43-52.

[48] Cirimele V, Kintz P, Mangin P. *Drug concentrations in human hair after bleaching.* Journal of Analytical Toxicology (1995); 19: 331.

[49] Yegles M, Marson Y, Wennig R. *Influence of bleaching on stability of benzodiazepines in hair.* Forensic Science International (2000); 107: 87-92.

[50] Joseph RJ, Su T-P, Cone EJ. *In vitro binding studies of drugs to hair: Influence of melanin and lipids on cocaine binding to Caucasoid and Africoid hair.* Journal of Analytical Toxicology (1996); 20: 338-344.

[51] Green SJ, Wilson JF. *The effect of hair color on the incorporation of methadone into hair in the rat.* Journal of Analytical Toxicology (1996); 20: 121-123.

[52] Khumalo NP, Doe PT, Dawber RP, Ferguson DJ. *What is normal black African hair? A light scanning electron-microscopic study.* Journal of the American Academy of Dermatology (2000); 45: 814.

[53] Kidwell DA, Lee EH, Delauder SF, *Evidence for bias in hair testing and procedures to correct bias.* Forensic Science International (2000); 107:39-61.

[54] Mieczkowski T, Newel R. *An evaluation of patterns of racial bias in hair assays for cocaine: black and white arrestees compared.* Forensic Science International (1993); 63: 85-98.

[55] Kelly RC, Mieczkowski T, Sweeney SA, Bourland JA. *Hair analysis for drugs of abuse. Hair color and race differentials or systematic differences in drug preferences.* Forensic Science International (2000); 107: 63-86.

[56] Mieczkowski T, Newel R. *Statistical examination of hair color as a potential biasing factor in haor analysis.* Forensic Science International (2000); 107: 13-38.

[57] Han E, Yang W, Lee J, Park Y, Kim E, Lim M, Chung H. *Correlation of methamphetamine results and concentrations between head, axillary and pubic hair.* Forensic Science International (2005); 147: 21-24.

[58] Mangin P, Kintz P. *Variability of opiates concentrations in human hair according to their anatomical origin: head, axillary and pubic regions.* Forensic Science International (1993); 63: 77-83.

[59] Skopp G, Potsch L, Aderjan R. *Experimental investigations on hair fibres as diffusion bridges and opiates as solutes in solution.* Journal of Forensic Sciences (1996); 41: 117-120.

[60] Potsch L, Skopp G. *Stability of opiates in hair fibers after exposure to cosmetic treatment.* Forensic Science International (1996); 81: 95-102.

[61] Baumgartner WA, Hill VA, Blahd WH. *Hair analysis for drugs of abuse.* Journal of Forensic Sciences (1989); 34(6): 1433-1453.

[62] Potsch L, Skopp G, Becker J. *Ultrastructural alterations and environmental exposure influence the opiate concentrations in hair of drug addicts.* Journal of Legal Medicine (1995); 107: 301-305.

[63] Schaffer M, Wang W-L, Irving J. *An evaluation of two wash procedures for differentiation of external contamination versus ingestion in the analysis of human hair samples for cocaine.* Journal of Analytical Toxicology (2002); 26: 485.

[64] Cairns T, Hill V, Schaffer M, Thistle W. *Removing and identifying drug contamination in the analysis of human hair.* Forensic Science International (2004); 145: 97.

[65] Cone EJ, Yousefnejad D, Darwin WD, Maguire T. *Testing human hair for drugs of abuse II. Identification of unique cocaine metabolites in hair of drug abusers and evaluation of decontamination procedures.* Journal of Analytical Toxicology (1991); 15: 250-255.

[66] Wilkins DG, Valdez AS, Kreuger GG, Rollins DE. *Quantitative analysis of 1-a-acetyl-N-normethadol and 1-a-acetyl-N,N-dinormethadol in human hair by positive ion chemical ionisation mass spectrometry.* Journal of Analytical Toxicology (1997); 21; 420-426.

[67] Blank DL, Kidwell DA. *Decontamination procedures for drugs of abuse in hair: are they sufficient?* Forensic Science International (1995); 70: 13-38.

[68] Wang W-L, Cone EJ. *Testing human hair for drugs of abuse, IV:environmental cocaine contamination and washing effects.* Forensic Science International (1995); 70: 39.

[69] Nakahara Y, Kikura R, Takahashi K. *Hair analysis for drugs of abuse , VIII: effective extraction and determination of 6-acetylmorphine and morphine in hair with trifluoroacetic acid-methanol for the confirmation of retrospective heroin use by gas chromatography-mass spectrometry.* Journal of Chromatography B Biomedical Applications (1994); 657: 93-101.

[70] Kintz P, Tracqui A, Mangin P. *Detection of drugs in human hair for clinical and forensic applications.* International Journal of Legal Medicine (1992); 105: 1-4.

[71] Moeller MR, Fey P, Wennig R. *Simultaneous determination of drugs of abuse (opiates cocaine and amphetamine) in human hair by GC/MS and its application to a methadone treatment program.* Forensic Science International (1993); 63: 185-206.

[72] Romano G, Barbera N, Lombardo I. *Hair testing for drugs of abuse: evaluation of external cocaine contamination and risk of false positives.* Forensic Science International (2001); 123: 119.

[73] Romolo FR, Rotolo MC, Palmi I, Pacifici R, Lopez A. *Optimized conditions for simultaneous determination of opiates, cocaine and benzoylecgonine in hair.* Forensic Science International (2003); 138: 17-26.

[74] Kintz P, Mangin P. *What constitutes a positive result in hair analysis: Proposal for the establishment of cut-off values.* Forensic Science International (1995); 70: 3.

[75] Romano G, Barbera N, Spadaro G, Valenti V. *Determination of drugs of abuse in hair: evaluation of external heroin contamination and risk of false positives*. Forensic Science International (2003); 131: 98-102.

[76] Eser HP, Potsch L, Skopp G, Moeller MR. *Influence of sample preparation on analytical results: drug analysis (GC/MS) on hair powder using various extraction methods*. Forensic Science International (1997); 84: 271-279.

[77] Personal Communication from Mrs Yvonne Cruickshank, Forensic Science Unit, University of Strathclyde.

[78] Jurado C, Gimenez MP, Menedez M, Repetto M. *Simultaneous quantification of opiates, cocaine and cannabinoids in hair*. Forensic Science International (1995); 70: 165-174.

[79] Balikova MA, Habrdova V. *Hair analysis for opiates: evaluation of washing and incubation procedures*. Journal of Chromatography B Analytical Technology Biomedical Life Sciences (2003); 789: 93-100.

[80] Uhl M, *Determination of drugs in hair using GC/MS/MS*. Forensic Science International (1997); 84: 281-294.

[81] Lachenmeier K, Musshoff F, Madea B. *Determination of opiates and cocaine in hair using automated enzyme immunoassay screening methodologies followed by gas chromatographic-mass spectrometric (GC-MS) confirmation*. Forensic Science International (2006); 159: 189-199.

[82] Allen DL, Scott KS, Oliver JS.*Comparison of solid-phase extraction and supercritical fluid extraction for the analysis of morphine in blood.* Journal of Analytical Toxicology (1999); 23: 216-218.

[83] Edder P, Staub C, Veuthey JL, Pierroz I, Haerdi W. *Subcritical fluid extraction of opiates in hair of drug addicts.* Journal of Chromatography B (1994); 658: 75-86.

[84] Sachs H. *History of hair analysis.* Forensic Science International (1997); 84: 7-16.

[85] Cone EJ, Darwin WD, Wang W.L. *The occurrence of cocaine, heroin and metabolites in hair of drug abusers.* Forensic Science International (1993); 63: 55-68.

[86] Jones J, Tomlinson K, Moore C. *The simultaneous determination of codeine, morphine, hydrocodone, hydromorphone, 6-acetylmorphine, and oxycodone in hair and oral fluid.* Journal of Analytical Toxicology (2002); 26: 171-175.

[87] Kintz P, Mangin P. *Simultaneous determination of opiates, cocaine and major metabolites of cocaine in human hair by gas chromatography/mass spectrometry (GC/MS).* Forensic Science International (1995); 73: 93-100.

[88] Goldberger BA, Caplan YH, Maguire T, Cone EJ. *Testing human hair for drugs of abuse III: identification of heroin and 6-acetylmorphine as indicators of heroin use.* Journal of Analytical Toxicology(1991); 15: 226-231.

[89] Spokert F, Pragst F. *Use of headspace solid-phase micro-extraction (HS-SPME) in hair analysis for organic compounds.* Forensic Science International (2000); 107; 129-148.

[90] Spokert F, Pragst F. *Determination of methadone and its metabolites EDDP and EMDP in human hair by headspace solid-phase microextraction and gas chromatography-mass spectrometry.* Journal of Chromatography B (2000); 746: 255-264.

[91] Ariffin, M M. Miller EI, Cormack PAG, Anderson RA. *Molecularly imprinted solid-phase extraction of diazepam and its metabolites from hair samples.* Analytical Chemistry (2007); 79(1): 256-262.

[92] Anderson RA, Ariffin MM, Cormack PAG, Miller EI. *Comparison of molecularly imprinted solid-phase extraction (MISPE) with classical solid-phase extraction (SPE) for the detection of benzodiazepines in post-mortem hair samples.* Forensic Science International (2007). Article In Press.

[93] Sachs H, Raff I. *Comparison of quantitative results of drugs in human hair by GC/MS.* Forensic Science International (1993); 63: 207-216.

[94] Kronstrand R, Nystrom I, Strandberg J, Druid H. *Screening for drugs of abuse in hair with ion spray LC-MS-MS.* Forensic Science International (2004);145: 183-190.

[95] Tagliaro F, Valentini R, Manetto G, Crivellente F, Carli G, Marigo M. *Hair analysis using radioimmunoassay, high performance liquid chromatography and capillary electrophoresis to investigate chronic exposure to heroin, cocaine and/or ecstasy in applicants for driving licenses.* Forensic Science International (2000); 107: 121-128.

[96] Tagliaro F, Poiesi C, Aiello R, Dorizzi R, Ghielmi S, Marigo M. *Capillary electrophoresis for the investigation of illicit drugs in hair: determination of cocaine and morphine.* Journal of Chromatography (1993); 638: 303-309.

[97] Society of Hair Testing. *Recommendations for hair testing in forensic cases.* Forensic Science International (2004); 145: 83-84.

[98] Department of Health and Human Services. Substance Abuse and Mental Health Services Administration. Proposed revisions to mandatory guidelines for federal workplace drug testing programs. Federal Register April 13th 2004; 69 (71).

[99] Gosling JP (Ed.). *Immunoassays*, Oxford University Press, New York, United States (2000).

[100] Smith FP (Ed), Siegel, JA (Series Ed.). *Handbook of Forensic Drug Analysis*, London: Elsevier Academic Press, 2005.

[101] Spiehler VR, Collison IB, Sedgwick PR, Perez SL, Le SD, Farnin DA. *Validation of an automated microplate enzyme immunoassay for screening of postmortem blood for dtugs of abuse.* Journal of Analytical Toxicology (1998); 22: 573-579.

[102] Kemp P, Sneed G, Kupiec T, Spiehler V. *Validation of a micro-titer plate ELISA for screening of postmortem blood for opiates and benzodiazepines.* Journal of Analytical Toxicology (2002); 26: 504-512.

[103] Cirimele V, Kintz P, Lohner S, Ludes B, *Enzyme immunoassay validation for the detection of buprenorphine in urine*. Journal of Analytical Toxicology (2003); 27: 103-105.

[104] Miller EI, Torrance HJ, Oliver JS. *Validation of the Immunalysis® Microplate ELISA for the detection of buprenorphine and its metabolite norbuprenorphine in urine*. Journal of Analytical Toxicology (2006); 30 (2): 115-119.

[105] Moore C, Feldman M, Harrison E, Irvan D, Kuntz D, Ross W, Giorgi N, Agrawal A, Rana S, Vincent M, Soares J. *Analysis of cocaine and metabolites in hair, oral fluid and urine*. Annales de Toxicologique Analytique (SFTA) (2005); XVII No.4: 221-228.

[106] Moore C, Feldman M, Giorgi N, Ross W, Harrison E, Irvan D, Kuntz D, Agrawal A, Rana S, Vincent M, Soares J. *Analysis of amphetamines in hair, oral fluid and urine*. Annales de Toxicologique Analytique (SFTA) (2005); XVII No. 4: 229-236.

[107] Cooper G, Wilson L, Reid C, Baldwin D, Hand C and Spiehler V. *Validation of the Cozart microplate ELISA for detection of opiates in hair*. Journal of Analytical Toxicology 2003; 27: 591-586.

[108] Miller EI, Wylie FM and Oliver JS, *Detection of benzodiazepines in hair using ELISA and LC-MS-MS*. Journal of Analytical Toxicology 2006; 30 (7): 441-448.

[109] Han E, Miller E, Lee J, Park Y, Lim M, Chung H, Wylie FM and Oliver JS, *Validation of the Immunalysis® microplate ELISA for the detection of methamphetamine in hair*. Journal of Analytical Toxicology (2006); 30 (6): 380-385.

[110] Negrusz A, Moore C, Deitermann D, Lewis D, Kaleciak K, Kronstrand R, Feeley B, Niedbala RS. *Highly Sensitive micro-Plate enzyme immunoassay screening and NCI-GC-MS confirmation of flunitrazepam and its major metabolite 7-aminoflunitrazepam in hair*. Journal of Analytical Toxicology (1999); 23: 429-435.

[111] Barnes AJ, Kim I, Schepers R, Moolchan ET, Wilson L, Cooper G, Reid C, Hand C, Huestis MA. *Sensitivity, specificity, and efficiency in detecting opiates in oral fluid with the cozart opiate microplate EIA and GC-MS following controlled codeine administration*. Journal of Analytical Toxicology (2003); 27: 402-407.

[112] Kacinko SL, Barnes AJ, Kim I, Moolchan ET, Wilson L, Cooper GA, Reid C, Baldwin D, Hand CW, Huestis MA. *Performance characteristics of the cozart rapiscan oral fluid drug testing system for opiates in comparison to ELISA and GC/MS following controlled codeine administration*. Forensic Science International (2004); 20 (141) 41-48.

[113] http://www.biosystemdevelopment.com/site_graphics/elisa.jpg, accessed 19th March 2007.

[114] http://chemistry.umeche.maine.edu/CHY431/Antibody.jpg, accessed 19th March 2007.

[115] Campbell NA, Reece JB, Mitchell LG, *Biology 5th edition*, United States: Jim Green, 1999.

[116] Bona CA, Bonilla FA. *Textbook of Immunology 2nd revised edition*. The Netherlands: Harwood Academic Publishers GmbH, (1996).

[117] Cody JT, Valtier S. *Effects of Stealth™ adulterant on immunoassay testing for drugs of abuse*. Journal of Analytical Toxicology (2001); 25: 158-165.

[118] ElSohly MA, Jones AB, *Drug testing in the workplace: could a positive test for one of the mandated drugs be for reasons other than illicit use of the drug?* Journal of Analytical Toxicology (1995); 19: 450-458.

[119] Jaffee WB, Trucco E, Levy S, Weiss RD. Is this urine really negative? A systematic review of tampering methods in urine drug screening and testing. Journal of Substance Abuse Treatment (2007). In press. Available on-line from 16th January 2007.

[120] Goldberger BA, Caplan YH. *Effect of glutaraldehyde (UrinAid) on detection of abused drugs in urine by immunoassay*. Clinical Chemistry (1994); 40: 1605-1606.

[121] Wu AH, Bristol B, Sexton K, Cassella-McLane G, Holtman V, Hill DW. *Adulteration of urine by "Urine Luck"*. Clinical Chemistry (1999); 45: 1051-1057.

[122] Dasgupta A, Chughtai O, Hannah C, Davis B, Wells A. *Comparison of spot tests with AdultaCheck 6 and Intect 7urine test strips for detecting the presence of adulterants in urine specimens.* Analytica Chimica Acta (2004); 348: 19-25.

[123] Kupiec T, DeCicco L, Spiehler V, Sneed G, Kemp P. *Choice of an ELISA assay for screening postmortem blood for amphetamine and/or metamphetamine.* Journal of Analytical Toxicology (2002); 26 (7): 513-518.

[124] Maurer, HH, Fritz CF. *Toxicological determination of pholcodine and its metabolites in urine and hair using radio immunoassay, fluorescence polarisation immunoassay, enzyme immunoassay and gas chromatography-mass spectrometry.* International Journal of Legal Medicine (1990);104: 43-46.

[125] Fraser AD, Howell P. *Oxaprozin cross-reactivity in three commercial immunoassays for benzodiazepines in urine.* Journal of Analytical Toxicology (1998); 22: 50-54.

[126] Dietzen DJ, Ecos K, Friedman D, Beason, S. *Positive predictive values of abused drug immunoassays on the Beckman Synchron in a veteran population.* Journal of Analytical Toxicology (2001); 25: 174-178.

[127] Beck O, Lin Z, Brodin K, Borg S, Hjemdahl P. *The online screening technique for urinary benzodiazepines: comparison with EMIT, FPIA and GC-MS.* Journal of Analytical Toxicology (1997); 21: 554-557.

[128] Spector S, Parker C. *Morphine radioimmunoassay.* Science (1970); 168: 1347-1348.

[129] Wainer BH, Fitch FW, Rothberg RM, Fried J. *Morphine-3-succinyl-bovine serum albumin: an immunogenic hapten-protein conjugate*. Science (1972); 176: 1143-1144.

[130] Moore KA, Werner C, Zannelli RM, Levine B, Smith ML. *Screening postmortem blood and tissues for nine cases of drugs of abuse using automated microplate immunoassay*. Forensic Science International (1999); 106: 93-102.

[131] Armbruster DA, Schwarzhoff RH, Pierce BL, Hubster EC. *Method comparison of EMIT II and online with RIA for drug screening*. Journal of Forensic Sciences (1993); 38: 1326-1341.

[132] http://www.immunalysis.com/Tech%20Briefs.htm. Accessed 19th March 2007.

[133] Van Bocxlaer JF, Clauwaert KM, Lambert WE, Deforce DL, Van den Eeckhout, EG and De Leenheer AP. *Liquid chromatography-mass spectrometry in forensic toxicology*, Mass Spectrometry Reviews (2000);19: 165-214.

[134] Rook EJ, Hillebrand MJX, Rosing H, van Ree JM, Beijnen JH. *The quantitative analysis of heroin, methadone and their metabolites and the simultaneous detection of cocaine, acetylcodeine and their metabolites in human plasma by high-performance liquid chromatography coupled with tandem mass spectrometry*. Journal of Chromatography B. (2005); 824: 213-221.

[135] Maralikova B, Weinmann W. *Confirmatory analysis for drugs of abuse in plasma and urine by high-performance liquid chromatography-tandem mass spectrometry with respect to criteria by compound identification*. Journal of Chromatography B. (2004); 811: 21-30.

[136] Naidong W, Lee JW, Jiang X, Wehling M, Hulse JD, Lin PP. *Simultaneous assay of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human plasma using normal-phase liquid chromatography-tandem mass spectrometry with a silica and an aqueous organic mobile phase*. Journal of Chromatography B. (1999); 735: 255-269.

[137] Wood M, Laloup M, del Mar Ramirez Fernandez M, Jenkins KM, Young MS, Ramaekers JG, De Boeck G, Samyn N. *Quantitative analysis of multiple illicit drugs in preserved oral fluid by solid-phase extraction and liquid chromatography-tandem mass spectrometry*. Forensic Science International (2005); 150: 227-238.

[138] Maurer HH. *Multi-analyte procedures for screening for and quantification of drugs in blood, plasma, or serum by liquid chromatography-single stage or tandem mass spectrometry (LC-MS or LC-MS/MS) relevant to clinical and forensic toxicology*. Clinical Biochemistry (2005); 38 (4): 310-318.

[139] Wylie FM, Torrance H, Anderson RA, Oliver JS. *Drugs in oral fluid Part I. Validation of an analytical procedure for licit and illicit drugs in oral fluid.* Forensic Science International (2005); 150: 191-198.

[140] Liang HR, Foltz RL, Meng M, Bennett P. *Method development and validation for quantitative determination of methadone enantiomers in human plasma by liquid chromatography/tandem mass spectrometry*. Journal of Chromatography B (2004); 806: 191-198.

[141] Kronstrand R, Nystrom I, Josefsson M, Hodgins S. *Segmental ion spray LC-MS-MS analysis of benzodiazepines in hair of psychiatric patients*. Journal of Analytical Toxicology (2002); 26: 479-484.

[142] McClean S, O'Kane E, Hillis J, SmythWF. *Determination of 1,4-benzodiazepines and their metabolites by capillary electrophoresis and high-performance liquid chromatography using ultraviolet and electrospray ionisation mass spectrometry*. Journal of Chromatography A. (1999); 838: 273-291.

[143] LeBeau M. *Analysis of biofluids for flunitrazepam and metabolites by electrospray liquid chromatography/mass spectrometry*. Journal of Forensic Sciences (2000); 45 (5): 1133-1141.

[144] Kintz P, Villain M, Cirimele V, Pepin G, Ludes B. *Windows of detection of lorazepam in urine, oral fluid and hair, with a special focus on drug-facilitated crimes*. Forensic Science International (2004); 145: 131-135.

[145] Chèze M, Villain M, Pepin,G. *Determination of bromazepam, clonazepam and metabolites after a single intake in urine and hair by LC-MS/MS Application to forensic cases of drug facilitated crimes*. Forensic Science International (2004); 145: 123-130.

[146] Kintz P, Villain M, Concheiro M, Cirimele V. *Screening and confirmatory method for benzodiazepines and hypnotics in oral fluid by LC-MS/MS*. Forensic Science International (2005);150: 213-220.

[147] Villain M, Concheiro M, Cirimele V, Kintz P. *Screening method for benzodiazepines and hypnotics in hair at pg/mg level by liquid chromatography-mass spectrometry/mass spectrometry*. Journal of Chromatography B. (2005); 825: 72-78.

[148] Samyn N, De Boeck G, Wood M, Lamers CTJ, De Waard D, Brookhuis KA, Verstraete AG, Riedel WJ, *Plasma, oral fluid and sweat wipe ecstasy concentrations in controlled and real life conditions*. Forensic Science International (2002); 128: 90-97.

[149] Laloup M, Tilman G, Maes V, De Boeck G, Wallemacq P, Ramaekers J, Samyn N. *Validation of an ELISA-based screening assay for the detection of amphetamine, MDMA and MDA in blood and oral fluid*. Forensic Science International (2005); 153: 29-37.

[150] Jeanville PM, Estape ES, Needham SR, Cole MJ. *Rapid confirmation/quantitation of cocaine and benzoylecgonine in urine utilizing high performance liquid chromatography and tandem mass spectrometry*. Journal of the American Society for Mass Spectrometry (2000); 11: 257-263.

[151] Srinivasan K, Wang P, Eley AT, White CA, Bartlett MG. *Liquid chromatography-tandem mass spectrometry analysis of cocaine and its metabolites from blood, amniotic fluid, placental and fetal tissues:study of the metabolism and distribution of cocaine in pregnant rats*. Journal of Chromatography B (2000); 745: 287-303.

[152] Weinmann W, Vogt S, Goerke R, Muller C, Bromberger A. *Simultaneous determination of THC-COOH and THC-COOH-glucuronide in urine samples by LC/MS/MS*. Forensic Science International (2000);113: 381-387.

[153] Breindahl T, Andreasen K. *Determination of 11-nor-D9-tetrahydrocannabinol-9-carboxylic acid in urine using high-performance liquid chromatography and electrospray ionization mass spectrometry*. Journal of Chromatography B (1999); 732: 155-164.

[154] Johansen SS, Jensen JL. *Liquid chromatography-tandem mass spectrometry determination of LSD, ISO-LSD, and the main metabolite 2-oxo-3-hydroxy-LSD in forensic samples and application in a forensic case*. Journal of Chromatography B (2005); 825: 21-28.

[155] Hoja H, Marquet P, Verneuil B, Lotfi H, Dupuy J-L, Lachatre G. *Determination of LSD and N-demethyl-LSD in urine by liquid chromatography coupled to electrospray ionization mass spectrometry*. Journal of Chromatography B (1997); 692: 329-335.

[156] Mueller CA, Weinmann W, Dresen S, Schreiber A, Gergov M. *Development of a multi-target screening analysis for 301 drugs using a QTrap liquid chromatography/tandem mass spectrometry system and automated library searching*. Rapid Communications in Mass Spectrometry 19: 1332-1338.

[157] Mortier KA, Maudens KE, Lambert WE, Clauwaert KM, Van Bocxlaer JF, Deforce DL, Van Peteghem CH, De Leenheer AP. *Simultaneous quantitative determination of opiates, amphetamines, cocaine and benzoylecgonine in*

oral fluid by liquid chromatography quadrupole-time-of-flight mass spectrometry. Journal of Chromatography B (2002); 779: 321-330.

[158] Polettini A (Ed.). *Applications of LC-MS in toxicology*, London, Pharmaceutical Press (2006).

[159] Dams R, Benijts T, Gunther W et al. *Influent of the eluent composition on the ionization efficiency for morphine of pneumatically assisted electrospray, atmospheric-pressure chemical ionization and sonic spray.* Rapid Communications in mass spectrometry (2002); 16: 1072-1077.

[160] Tyrefors N, Hyllbrant B, Ekman, L et al. *Determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human serum by solid-phase extraction and liquid chromatography-mass spectrometry with electrospray ionisation*. Journal of Chromatography A (1996); 729: 279-285.

[161] Ardrey R. *Liquid Chromatography-Mass Spectrometry: An Introduction.* Chicester, England, (2003).

[162] http://www.waters.com/WatersDivision/ContentD.asp?watersit=EGOO-66YNU9. Accessed 25th April 2007.

[163] Thermo Finnigan LCQ Deca Hardware Manual. Re-printed July 2005.

[164] Ariffin MM, Anderson RA. *LC/MS/MS analysis of quaternary ammonium drugs and herbicides in whole blood*. Journal of Chromatography B. (2006); 842: 91-97.

[165] Maurer, HH. *Liquid chromatography-mass spectrometry in forensic and clinical toxicology*. Journal of Chromatography B (1998); 713: 3-25.

[166] Oliveira EJ, Watson, DG. *Liquid chromatography-mass spectrometry in the study of the metabolism of drugs and other xeniobiotics*. Biomedical Chromatography (2000); 14: 351-372.

[167] Gelpi E. Biomedical and biochemical applications of liquid chromatography-mass spectrometry. Journal of Chromatography A (1995); 703: 59-80.

[168] Souverain S, Rudaz S, Veuthey J-L. *Matrix effect in LC-ESI-MS-MS and LC-APCI-MS-MS with off-line and on-line extraction procedures*. Journal of Chromatography A. (2004); 1058: 61-66.

[169] Liang HR, Foltz RL, Meng M et al. *Ionization enhancement in atmospheric chemical ionization and suppression in electrospray ionization between target drugs and stable-isotope-labeled internal standards in quantitative liquid chromatography/tandem mass spectrometry*. Rapid Communications in Mass Spectrometry (2003); 17: 2815-2821.

[170] Matuszewski BK, Constanzer ML, Chavez-Eng CM. *Strategies for the assessment of matrix-effect in quantitative bioanalytical methods based on HPLC-MS/MS*. Analytical Chemistry (2003); 75 3019-3030.

[171] Baselt RC, Cravey RH, *Disposition of toxic drugs and chemicals in man*, 4th edition, Chemical Technology Institute Foster City, California, United States (1995).

[172] Mycek MJ, Harvey RA, Champe PC. *Lippincott's Illustrated Reviews: Pharmacology*, 2nd edition. Lippincott Williams & Wilkins, Philadelphia, PA, (2000).

[173] *Buprenorphine- an alternative treatment for opioid dependence*. National Institute on Drug Abuse, Research Monograph Number 121 (1992).

[174] http://www.isdscotland.org/isd/files/final_F06.xls. Accessed 30th April 2007.

[175] Seymour A, Black M, Oliver JS. *Drug related deaths in the Strathclyde region of Scotland, 1995-1998*. Forensic Science International (2001); 122: 52-59.

[176] Seymour A, Oliver JS. *Role of drugs and alcohol in impaired drivers and fatally injured drivers in the Strathclyde region of Scotland, 1995-1998.* Forensic Science International (1999); 103: 89-100.

[177] Personal Communication from Dr Fiona M Wylie, Forensic Medicine and Science Section, University of Glasgow.

[178] Hammersley R, Cassidy MT, Oliver JS. *Drugs associated with drug-related deaths in Edinburgh and Glasgow, November 1990 to October 1992*. Addiction (1995); 90: 959-965.

[179] Cirimele V, Etienne S, Villain M, Ludes B, Kintz P. *Evaluation of the One Step™ ELISA kit for the detection of buprenorphine in urine, blood and hair specimens*. Forensic Science International (2004); 143: 153-156.

[180] Tracqui A, Kintz P, Ludes B. *Buprenorphine-related deaths among drug addicts in France: a report on 20 fatalities.* Journal of Analytical Toxicology (1998); 22: 430-434.

[181] Labat L, Fontaine B, Delzenne C, Doublet A, Marek MC, Tellier D, Tonneau M, Lhermitte M, Frimat P. *Prevalence of psychoactive substances in truck drivers in the Nord-Pas-de Calais region (France).* Forensic Science International (2007). Article In Press. Available on-line

[182] de los Cobos, Martin A, Etcheberrigaray A. *A controlled trial of daily versus thrice-weekly buprenorphine administration for the treatment of opioid dependence.* Drug Alcohol Dependency (2000); 59: 223-233.

[183] Vincent F, Bessard J, Vacheron J, Mallaret M, Bessard G. *Determination of buprenorphine and norbuprenorphine in urine and hair by gas chromatography-mass spectrometry.* Journal of Analytical Toxicology (1999); 23: 270-279.

[184] Hoja H, Marquet P, Verneuil B. *Determination of buprenorphine and norbuprenorphine in whole blood by liquid chromatography-mass spectrometry.* Journal of Analytical Toxicology (1997); 21: 160-165.

[185] Kintz P. *Deaths involving buprenorphine: a compendium of French cases.* Forensic Science International (2001); 121: 65-69.

[186] Lai SH, Yao YJ, Lo DST. *A survey of buprenorphine related deaths in Singapore.* Forensic Science International (2006); 162: 80-86.

[187] Nielsen S, Dietze P, Lee N, Dunlop A, Taylor D. *Concurrent buprenorphine and benzodiazepines use and self-reported opioid toxicity in opioid substitution treatment.* Addiction (2007); 102 (4): 616.

[188] Debrabandere L, Van Boven M, Daenens P. *Analysis of buprenorphine in urine specimens.* Journal of Forensic Sciences (1992); 37: 82-89.

[189] Böttcher M, Beck O. *Evaluation of buprenorphine CEDIA assay versus GC-MS and ELISA using urine samples from patients in substitution treatment.* Journal of Analytical Toxicology (2005); 29(8): 769-776.

[190] Alemany G, Gamundi A, Rossello C, Nicolau MC. *A simple thin-layer chromatographic method for the analysis of buprenorphine in urine.* Biomedical Chromatography (1996); 10: 146-147.

[191] Mercolini L, Mandrioli R, Conti M, Leonardi C, Gerra G, Raggi MA. *Simultaneous determination of methadone, buprenorphine and norbuprenorphine in biological fluids for therapeutic drug monitoring purposes.* Journal of Chromatography B (2007); 847: 95-102.

[192] George S, George C, Chauhan M. *The development and application of a rapid gas chromatography-mass spectrometry method to monitor buprenorphine withdrawal protocols.* Forensic Science International (2004); 143: 121-125.

[193] Lisi AM, Kazlauskas R, Trout GJ. *Gas chromatographic-mass spectrometric quantitation of urinary buprenorphine and norbuprenorphine after derivatization by direct extractive alkylation.* Journal of Chromatography B (1997); 11: 67-77.

[194] Kronstrand R, Seldén TG, Josefsson M. *Analysis of buprenorphine, norbuprenorphine and their glucuronides in urine by liquid chromatography-mass spectrometry.* Journal of Analytical Toxicology (2003); 27(7): 464-470.

[195] Spiehler V. *Hair analysis by immunological methods from the beginning to 2000.* Forensic Science International (2000); 107: 249-259.

[196] Quinn CP, Semenova VA, Elie CM, Romero-Steiner S, Greene C et al. *Specific, sensitive and quantitative enzyme-linked immunosorbent assay for human immunoglobulin G antibodies to anthrax toxin protective antigen.* Emerging Infectious Diseases (2002). Article available on-line at Medscape. Accessed 7th May 2007.

[197] Baumgartner AM, Jones PF, Black CT. *Detection of phencyclidine in hair.* Journal of Forensic Sciences (1981); 26: 576-581.

[198] Baumgartner WA, Jones PF, Black CT, Blahd WH. *Radioimmunoassay of cocaine in hair.* Journal of Nuclear Nedicine (1982); 26: 790-792.

[199] Sachs H, Arnold W. *Results of comparative determination of morphine in human head hair using RIA and GC/MS.* Journal of Clinical Chemistry and Clinical Biochemistry (1989); 27: 873-877.

[200] Strano-Rossi S, Bermejo-Barrera, Chiarotti M. *Segmental hair analysis for cocaine and heroin abuse determination.* Forensic Science International (1995); 70: 211-216.

[201] Tagliaro F, Valentini R, Manetto G, Crivellente F, Carli G, Marigo M. *Hair analysis by using radioimmunoassay, high performance liquid chromatography*

and capillary electrophoresis to investigate chronic exposure to heroin, cocaine and/or ecstasy in applicants for driving licenses. Forensic Science International (2000); 107: 121-128.

[202] Sramek JJ, Baumgartner WA, Ahrens TN, Hill VA, Cutler NR. *Detection of benzodiazepines in human hair by radioimmunoassay*. Annals of Pharmacotherapy (1009); 26: 469-471.

[203] Segura J, Stramesi C, Redón A, Ventura M, Sanchez CJ, González G, San L, Montagna M. *Immunological screening of drugs of abuse and gas chromatographic-mass spectrometric confirmation opiates and cocaine in hair*. Journal of Chromatography B (1999); 724: 9-21.

[204] Miki A, Katagi M, Tsuchihashi H. *Recent improvements in forensic hair analysis for illicit drugs*. Journal of Health Science (2003); 49(5): 325-332.

[205] Kintz P, Mangin P. *Analysis of opiates in human hair with FPIA, EMIT and GC-MS*. Journal of Forensic Medicine (1991); 7: 129-132.

[206] Kintz P, Ludes B, Mangin P. *Detection of drugs in human hair using Abbot ADx with confirmation by gas chromatography/mass spectrometry (GC/MS)*. Journal of Forensic Sciences (1992); 37(1): 328-331.

[207] Moore C, Deitermann D, Lewis D, Feeley B, Niedbala RS. *The detection of cocaine in hair specimens using micro-plate enzyme immunoassay*. Journal of Forensic Sciences (1999); 44: 609.

[208] Musshoff F, Driever F, Lachenmeier K, Lachenmeier DW, Banger M, Madea B. *Results for hair analyses for drugs of abuse and comparison with self-reports and urine tests*. Forensic Science International (2006); 156: 118-123.

[209] Han, E, Park Y, Yang W, Lee J, Lee S, Kim E, Lim M, Chung H. The study of metabolite-to-parent drug ratios of methamphetamine and methylenedioxymethamphetamine in hair. Forensic Science International (2006); 161: 124-129.

[210] Halapy E, Kreiger N, Cotterchio M, Sloan M. *Benzodiazepines and risk for breast cancer*. Annals of Epidemiology (2006); 16: 632-636.

[211] Salamone SJ (Ed.). *Benzodiazepines and GHB, Detection and Pharmacology*. Humana Press Incorporation, New Jersey, United States (2001).

[212] Personal Communication from Dr. Anthony Martin, Forensic Medicine and Science Section, University of Glasgow.

[213] Steentoft A, Teige B, Holmgren P et al. *Fatal poisoning in Nordic drug addicts in 2002*. Forensic Science International (2006); 160:148-156.

[214] Yegles M, Mersch F, Wennig R. *Detection of benzodiazepines and other psychotropic drugs in human hair by GC/MS*. Forensic Science International (1997); 84: 211-218.

[215] Muller RK, Thieme D (Eds.). *Progress in hair analysis for illegal drugs, Workshop Proceedings of the International Society of Hair Testing, June 18 to 20, 2000, Kreischa*, Sport and Buch Strauss, Cologne, Germany (2000) p22-34.

[216] Negrusz A, Bowen AM, Moore CM, Dowd SM, Strong MJ. *Disposition of clonazepam and 7-aminoclonazepam in hair following a single dose of Klonopin™.* Journal of Analytical Toxicology (2002); 26: 471-478.

[217] Cirimele V, Kintz P, Mangin P. *Detection and quantification of lorazepam in human hair by GC-MS/NCI in a case of traffic accident.* International Journal of Legal Medicine (1996); 108: 265-267.

[218] Cirimele V, Kintz P, Ludes B. *Screening for forensically relevant benzodiazepines in human hair by gas chromatography-negative ion chemical ionization-mass spectrometry.* Journal of Chromatography B (1997); 700: 119-129.

[219] El Mahjoub A, Staub C. *Determination of benzodiazepines in human hair by on-line high performance liquid chromatography using a restricted access extraction column.* Forensic Science International (2001); 123: 17-25.

[220] Couper FJ, McIntyre IM, Drummer OH. *Extraction of psychotropic drugs from human scalp hair.* Journal of Forensic Sciences (1995); 40: 83-86.

[221] Chèze M, Duffort G, Deveaux M, Pepin G. *Hair analysis by liquid chromatography-mass spectrometry in toxicological investigation of drug-facilitated crimes: report of 128 cases over the period June 2003-May 2004 in metropolitan Paris.* Forensic Science International (2005); 153: 3-10.

[222] Hold KM, Crouch DJ, Wilkins DG, Rollins DE, Maes RA. *Detection of alprazolam in hair by negative ion chemical ionization mass spectrometry.* Forensic Science International (1997); 84: 201-209.

[223] Sachs H, Kintz P. *Testing for drugs in hair: critical review of chromatographic procedures since 1992.* Journal of Chromatography B (1998); 713: 147-161.

[224] Kintz P, Cirimele V, Vayssette F, Mangin P. *Hair analysis for oxazepam and nordiazepam by gas chromatography-negative-ion chemical ionization mass spectrometry.* Journal of Chromatography B (1996); 677: 241-244.

[225] Cirimele V, Kintz P, Mangin P. *Determination of chronic flunitrazepam abuse by hair analysis using GC-MS-NCI.* Journal of Analytical Toxicology (1996); 20: 596-598.

[226] Cirimele V, Kintz P, Staub C, Mangin P. *Testing human hair for flunitrazepam and 7-aminoflunitrazepam by GC/MS-NCI.* Forensic Science International (1997); 84: 189-200.

[227] Irving RC, Dickson SJ. *The detection of sedatives in hair and nail samples using tandem LC-MS-MS.* Forensic Science International (2007); 166: 58-67.

[228] Miller JN, Miller JC. *Statistics and Chemometrics for Analytical Chemistry*, 4th edition, Pearson Education Limited, Harlow, England (2000).

[229] Kintz P, Villain M, Cirimele V, Pépin G, Ludes B. Windows of detection of lorazepam in urine, oral fluid and hair, with a special focus on drug-facilitated crimes. Forensic Science International (2004); 45: 131-135.

[230] Paterson S, McLachlan-Troup, Cordero R, Dohnal M, Carman S. *Qualitative screening for drugs of abuse in hair using GC-MS.* Journal of Analytical Toxicology (2001); 25(3): 203-208.

[231] Kintz P, Cirimele V. *Interlaboratory comparison of quantitative determination of amphetamine and related compounds in hair samples.* Forensic Science International (1997); 84: 151-156.

[232] Brewer WE, Galipo RC, Sellers KW, Morgan SL. *Analysis of cocaine, benzoylecgonine, codeine, and morphine in hair by supercritical fluid extraction with carbon dioxide modified with methanol.* Analytical Chemistry (2001); 73 (11): 2371-2376.

[233] Cordero R, Paterson S. *Simultaneous quantification of opiates, amphetamines, cocaine and metabolites and diazepam and metabolite in a single hair sample using GC-MS.* Journal of Chromatography B (2007); 850: 423-431.

[234] Offidani C, Strano-Rossi S, Chiarotti M. *Improved enzymatic hydrolysis of hair.* Forensic Science International (1993); 63: 171-174.

[235] Offidani C, Carnevale A, Chiarotti M. *Drugs in hair: a new extraction procedure.* Forensic Science International (1989); 41: 35-39.

[236] Polettini A, Stramesi C, Vignali C, Montagna M. *Determination of opiates in hair: effects of extraction methods on recovery and on stability of analytes.* Forensic Science International (1997); 84: 271-279.

[237] Pellegrini M, Rosati F, Pacifici R, Zuccaro P, Romolo FS, Lopez A. *Rapid screening method for the determination of ecstasy and amphetamines in urine samples using gas chromatography-mass spectrometry.* Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences (2002); 769(2): 243-251.

[238] Cone EJ, Weddington WW Jr. *Prolonged occurrence of cocaine in human saliva and urine after chronic use.* Journal of Analytical Toxicology (1989); 13: 65-68.

[239] White P. *Crime Scene to Court, The Essentials of Forensic Science*, 1st edition, The Royal Society of Chemistry, Thomas Graham House, Science Park, Milton Road, Cambridge (1999).

[240] Seymour A, Black M, Oliver JS, Jay J. *Dihydrocodeine-drug of use or misuse?* British Journal of General Practice (2001): 404-405.

[241] Franke JP, de Zeeuw RA. *Solid-phase extraction procedures in systematic toxicological analysis.* Journal of Chromatography B (1998); 713: 51-59.

[242] Rollins DE, Wilkins DG, Krueger GG, Augsburger MP, Mizuno A, O'Neal C, Borges CR, Slawson MH. *The effect of hair color on the incorporation of codeine into human hair.* Journal of Analytical Toxicology (2003); 27(8): 545-551.

[243] Gaillard Y, Pepin G. *Simultaneous solid-phase extraction on C_{18} cartridges of opiates and cocainins for an improved quantitation in human hair by Gc-MS: one year of forensic applications.* Forensic Science International (1997); 86 (1-2): 49-59

[244] Pepin G, Gaillard Y. *Concordance between self-reported drug use and findings in hair about cocaine and heroin.* Forensic Science International (1997); 84: 37-41.

[245] Wang WL, Cone EJ. Testing human hair for drugs of abuse IV. Environmental cocaine contamination and washing effects. Forensic Science Internaltional (1995); 70 (1-3): 39-51.

[246] Cairns T, Hill V, Schaffer M, Thistle W. *Levels of cocaine and its metabolites in washed hair of demonstrated cocaine users and workplace subjects.* Forensic Science International (2004); 145: 175-181.

[247] Sachs H, Denk R, Raff I. *Determination of dihydrocodeine in hair of opiate addicts by GC/MS.* International Journal of Legal Medicine (1993); 105: 247-250.

[248] Jurado C, Giménez MP, Menéndez M, Repetto M. *Simultaneous quantification of opiates, cocaine and cannabinoids in hair.* Forensic Science International (1995); 70: 165-174.

[249] Han E, Yang W, Lee J, Park Y, Kim E, Lim, M, Chung H. *The prevalence of MDMA/MDA in both hair and urine in drug users.* Forensic Science International (2005); 152: 172-177.

[250] Allen DL, Oliver JS. *The use of supercritical fluid extraction for the determination of amphetamines in hair.* Forensic Science International (2000); 107 (1-3): 191-199.

Appendix 1 Publications in Support of this Thesis

1. Miller EI, Torrance HJ, Oliver JS. Validation of the Immunalysis® microplate ELISA for the detection of buprenorphine and its metabolite norbuprenorphine in urine. Proceedings of the Society of Forensic Toxicologists meeting, Tennessee, United States, 2005.

2. Miller EI, Torrance HJ, Oliver JS. Validation of the Immunalysis® microplate ELISA for the detection of buprenorphine and its metabolite norbuprenorphine in urine. Journal of Analytical Toxicology (2006); 30 (2): 115-119.

3. Han E, Miller E, Lee J, Park Y, Lim M, Chung H, Wylie FM, Oliver JS. Validation of the Immunalysis® Microplate ELISA for the detection of methamphetamine in hair. Proceedings of the Society of Hair Testing meeting, Vadstena, Sweden, 2006.

4. Han E, Miller E, Lee J, Park Y, Lim M, Chung H, Wylie FM, Oliver JS. Validation of the Immunalysis® Microplate ELISA for the detection of methamphetamine in hair. Journal of Analytical Toxicology (2006); 30 (6): 380-385.

5. Miller EI, Wylie FM, Oliver JS. Detection of benzodiazepines in hair using ELISA and LC-ESI-MS-MS. Proceedings of The International Association of Forensic Toxicologists meeting, Ljubljana, Slovenia, 2006.

6. Ariffin MM, Miller EI, Cormack PAG, Anderson RA. Benzodiazepine detection in hair by MISPE v. SPE. Proceedings of The International Association of Forensic Toxicologists meeting, Ljubljana, Slovenia, 2006.

6. Miller EI, Wylie FM, Oliver JS. Detection of benzodiazepines in hair using ELISA and LC-ESI-MS-MS. Journal of Analytical Toxicology (2006); 30 (7): 441-448.

7. Ariffin MM, Miller EI, Cormack PAG, Anderson RA. Molecularly imprinted solid-phase extraction of diazepam and its metabolites from hair samples. Analytical Chemistry (2007); 79: 256-262.

8. Anderson RA, Ariffin MM, Cormack PAG, Miller EI. Comparison of molecularly imprinted solid-phase extraction (MISPE) with classical solid-phase extraction (SPE) for the detection of benzodiazepines in post-mortem samples. Forensic Science International. Published on web 27 April 2007.

9. Miller EI, Wylie FM, Oliver JS. Simultaneous identification and quantification of cocaine and its metabolites, opiates, amphetamines and diazepam and its metabolites from a single hair extract. Proceedings of the Society of Hair Testing meeting, Cardiff, Wales, 2007.

TECHNICAL NOTE: Validation of the Immunalysis® Microplate ELISA for the Detection of Buprenorphine and Its Metabolite Norbuprenorphine in Urine

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The purpose of this study was to validate the Immunalysis Buprenorphine Microplate enzyme-linked immunosorbent assay (ELISA) for the detection of buprenorphine in urine samples. Sixty-nine urine samples were obtained from volunteers on the Subutex® treatment program and from routine samples submitted to the laboratory for buprenorphine testing. For ELISA analysis, samples were diluted 1:10 with K_2HPO_4 (0.1M, pH 7.0). The limit of detection was calculated as 0.5 ng/mL buprenorphine. The intra-assay and interday precision was 3.8% (n = 10) and 8.6% (n = 50) respectively at 1 ng/mL buprenorphine. At a low concentration of norbuprenorphine (1 ng/mL), the immunoassay demonstrated a cross-reactivity of 78%. A higher cross-reactivity of 116% was observed at a higher concentration of norbuprenorphine (10 ng/mL). Dihydrocodeine, codeine, tramadol, morphine, propoxyphene, methadone, and EDDP were tested at concentrations of 10 ng/mL and 10,000 ng/mL and demonstrated no cross-reactivity with the assay. For liquid chromatography-tandem mass spectrometry (LC-MS-MS), deuterated internal standard mixture, 1M acetate buffer (pH 5.0), and b-glucuronidase were added to the standards and samples, which were then incubated for 3 h at 60°C. After incubation, 3 mL K_2HPO_4 (0.1M, pH 6.0) was added and the pH altered to pH 6.0 using 1M KOH. Buprenorphine and norbuprenorphine were subsequently extracted by solid-phase. Twenty-one samples were confirmed positive and 48 samples were confirmed negative by LC-MS-MS. Using a cut-off value of 0.5 ng/mL buprenorphine, the immunoassay demonstrated a sensitivity and specificity of 100%.

Validation of the Immunalysis® Microplate ELISA for the Detection of Methamphetamine in Hair

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The object of this study was to validate the Immunalysis Methamphetamine Microplate ELISA for detecting methamphetamine in hair. Twenty-nine scalp hair samples were obtained as routine cases submitted to the National Institute of Scientific Investigation in Seoul by the police. The hair samples were washed with 0.1% sodium dodecyl sulfate, distilled water, and dichloromethane. The samples were screened using the Immunalysis Methamphetamine Microplate ELISA and confirmed using gas chromatography-mass spectrometry (GC-MS). Twenty-eight hair samples were screened and confirmed as positive for methamphetamine. For ELISA analysis, the samples were extracted by incubation in monobasic phosphate buffer for 1 h at 60°C. For GC-MS, the samples were extracted for 20 h in methanol containing 1% hydrochloric acid. The methanol/acid solution was evaporated to dryness and the resulting residue was derivatized with trifluoroacetic anhydride. Methamphetamine and amphetamine were detected using selective ion monitoring (SIM) mode. The Immunalysis Methamphetamine Microplate ELISA demonstrated a sensitivity and specificity of 97% and 100%, respectively, using a cut-off concentration of 0.5 ng/mg d-methamphetamine. The ELISA kit showed 63% cross-reactivity with d,l-methamphetamine and did not cross-react to any significant extent with the licit l-methamphetamine isomer. The intra- and interassay precisions were 2.5% and 3.7%, respectively.

Detection of Benzodiazepines in Hair Using ELISA and LC-ESI-MS-MS

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This study was designed to validate an enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for the detection of nine benzodiazepines in hair. Sixteen hair case samples were tested from drug-related deaths where a positive benzodiazepine blood result was obtained. The case samples were decontaminated with 0.1% sodium dodecyl sulfate, distilled water, and dichloromethane. For ELISA analysis, the samples were extracted by incubation in monobasic phosphate buffer for 1 h and then neutralized with dibasic phosphate buffer. They were diluted 1:5 with phosphate buffer saline (PBS) prior to analysis. For LC-MS-MS, the samples were incubated overnight in methanol/25% ammonium hydroxide (20:1). The benzodiazepines were extracted by solid phase. Thirteen samples were confirmed positive by LC-MS-MS. The benzodiazepines detected included diazepam, nordiazepam, temazepam, oxazepam, nitrazepam, and lorazepam. Using a cut-off concentration of 0.1 ng/mg oxazepam, the Immunalysis® Benzodiazepine Microplate ELISA demonstrated a sensitivity and specificity of 100% and 81%, respectively, compared with LC-MS-MS results.

Benzodiazepine detection in hair by MISPE v. SPE.

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Abstract

Background: This preliminary study compares the results of benzodiazepine analysis in scalp hair samples in ten post-mortem cases using a validated conventional solid-phase extraction (SPE) system and an anti-diazepam molecularly imprinted polymer solid-phase extraction (MISPE) system.

Methods: The ten hair samples tested were from drug-related deaths in which a positive benzodiazepine blood result was obtained. The case samples were decontaminated with 0.1 % sodium dodecyl sulfate, distilled water and dichloromethane, incubated overnight in methanol/25 % ammonium hydroxide (20:1) and subsequently extracted by SPE or MISPE followed by LC-MS-MS analysis.

Results: Diazepam, nordiazepam, oxazepam, temazepam and nitrazepam were detected in the samples using both extraction methods. The recovery values for diazepam, nordiazepam, oxazepam, temazepam and nitrazepam in MISPE and SPE methods were between 73-103 % and 69-91 % respectively. LOD levels between 0.06-0.39 ng/30 mg hair and 0.03-0.24 ng/30 mg hair were obtained with the MISPE and SPE methods, respectively. Diazepam was detected in more case samples using MISPE due to the lower LOD values and higher extraction recovery, as a result of excellent recognition of the template. The LOD and recovery values for diazepam by MISPE are 0.09 ng/30 mg hair and 93 %, respectively compared to 0.13 ng/30 mg and 69 % by SPE. Nitrazepam levels detected by both methods were very similar. The MISPE method however was

less sensitive for temazepam, nordiazepam and oxazepam determination compared to the conventional SPE method.

Conclusion: The molecularly imprinted polymer used for this study demonstrated good selectivity for the 5 benzodiazepines tested, especially the template substance, diazepam. The MISPE method coulod be used as an alternative to conventional SPE extraction for the analysis of benzodiazepine positive hair samples collected from chronic users.

Keywords: Benzodiazepines; Hair; SPE; MISPE; LC-MS-MS

Simultaneous identification and quantification of cocaine and its metabolites, opiates, amphetamines and diazepam and its metabolites from a single hair extract

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A liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and validated for the simultaneous identification and quantification of opiates, cocainics, amphetamines and diazepam and its metabolites from a single hair extract. Three different LC columns (Gemini C_{18}, Synergi Hydro RP and Zorbax Phenyl) and three different mobile phase combinations were investigated. Two mixed mode SPE extraction cartridges (Bond Elut Certify® and Clean Screen® ZSDAU 020) were compared, with the Clean Screen® cartridge producing higher recoveries, lower LODs and cleaner extracts. Methanol and phosphate buffer (pH 5) incubation methods were compared for three authentic hair samples, with the phosphate buffer incubation producing better qualitative and quantitative results. The optimised validated method used the Synergi Hydro RP column with 3 mM ammonium formate + 0.001 % formic acid/Acetonitrile mobile phase, Clean Screen extraction cartridge and phosphate buffer incubation.

The authentic hair samples used in the validation were from cases where the blood result was positive for at least one drug class. After segmentation, the samples were decontaminated by washing with 0.1 % sodium dodecyl sulfate, deionised water and dichloromethane. Drugs were extracted for 18 h at 45 °C using phosphate buffer (pH 5), followed by SPE clean-up using a mixed mode Clean Screen® extraction cartridge. Analytes were identified by their full MS/MS spectra and quantification was based on data from the major product ion. Deuterated internal standards were used for all analytes and the data was collected using selected ion monitoring (SIM) mode. The method was applied to

15 authentic post-mortem scalp samples, detecting at least one drug class in each sample.