AN INVESTIGATION OF THE USE OF ALTERNATIVE MATRICES IN CLINICAL AND FORENSIC TOXICOLOGY

by

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A thesis submitted to the

University of Birmingham

for the degree of

MASTER OF PHILOSOPHY

School of Biosciences

University of Birmingham

September 2012

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ACKNOWLEDGEMENTS

ABSTRACT

Blood and urine are routinely used for toxicological analysis but there could be some circumstances where the analysis of alternative matrices may prove to be more relevant or more convenient. It is not uncommon for blood and / or urine to not be available, e.g. in some post-mortem cases and it can be difficult to analyse and interpret results for matrices that are not routinely used. Oral fluid, stomach contents, vitreous humour, bile and liver were analysed alongside blood and / or urine. Techniques used included immunoassay, HPLC-DAD, LC-MS, GC-MS and GC-FID depending on the analytes to be detected. The results revealed that for drug screening the majority of drugs and metabolites that were detected in blood and urine were also detected in the alternative matrices. Where it was possible to quantify drug concentrations, little correlation was found between blood and the alternative matrices. The alternative matrices investigated have proved to be very effective for the screening of drugs and when analysed alongside traditional matrices or in conjunction with each other, the results can provide a very good insight into an individual's drug use.

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ABBREVIATIONS

6-MAM	6-monoacetylmorphine
Abs	Absorbance
Amp	Amphetamine
BSTFA-TMCS	*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane
BZE	Benzoylecgonine
CEDIA	Cloned enzyme donor immunoassay
COC	Cocaine
DHC	Dihydrocodeine
EA	Enzyme acceptor
ED	Enzyme donar
EDDP	2-ethylidene- 1,5-dimethyl-3,3-diphenylpyrrolidine
ELISA	Enzyme linked immunosorbent assay
EQC	External quality control
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionisation detection
GC-MS	Gas Chromatography-Mass Spectrometry
GC-NPD	Gas chromatograpy-nitrogen phosphurus detection
GHB	Gamma-Hydroxybutyrate or Gamma-Hydroxybutyric acid
HCl	Hydrochloric acid
HFBA	heptafluorobutyric acid
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography-diode array detector

IQC	Internal quality control
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
M3G	morphine-3-glucuronide
M6G	morphine-6-glucuronide
MA	Methamphetamine
MDA	3,4-methylenedioxyamphetamine
MDEA	Methylenedioxyethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MRM	Multiple reaction monitoring
ODT	O-desmethyltramadol
ODV	O-desmethylvenlafaxine
OF/P	oral fluid to plasma
OPI	Opiates
PK	Pharmacokinetic
Px	Prescribed
QC	Quality control
SAMHSA	Substance Abuse and Mental Health Services Administration
SIM	Selected ion monitoring
SPE	Solid phase extraction
UV	Ultra-violet
v/v	Volume to volume

| vitreous | Vitreous humour |
| vol | Volunteer |

CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION

Toxicology can be described as the science of poisons, where a poison can be any substance that causes a harmful effect, when administered to living organisms. Therefore, most drugs can act as poisons as they usually produce toxic effects at a particular dose.

Within this definition the term poison is quantitative and dose-dependent, as most substances can be harmful at a particular dose but can usually be taken without harm at some lower dose. Somewhere between these levels there exists chronic toxicity and lethal toxicity, and these levels could be effected by different circumstances, e.g. in the presence of other poisons.

Toxicity is a biological concept that is not only different from species to species but also between individuals due to differences in age, gender, size, genetics and health, (Hodgson, 2010).

As well as these complexities, some pharmacological considerations are also required when interpreting toxicological results such as method / route of exposure, how the drug or poison is absorbed, distributed, metabolised and finally excreted from the body.

1.2   AIMS AND OBJECTIVES

Blood and urine have been the traditional matrices for drug detection for many years and still provide reliable results but they are not always available. This research project aimed to determine the usefulness of a selection of alternative matrices in both Clinical and Post-mortem Toxicology.

1.   To develop and evaluate methods for detecting drugs in oral fluid.

2.   To evaluate the effectiveness of oral fluid monitoring for illicit drug use using marker compounds.

3.   To compare toxicological findings for alternative matrices with traditional matrices.

4.   Assess the interpretive usefulness of alternative matrices

1.3   CLINICAL TOXICOLOGY

This usually involves the diagnosis or treatment of patients in a hospital or clinic setting. Such uses include:-

•   Unknown drug screens - to determine or exclude drug use, for example if someone was found collapsed.

•   Therapeutic drug monitoring - if an individual is on long-term treatment for a treatable illness then it is important to measure drug levels to assess if the patient is getting the required effect from a particular dose.

•   Compliance testing for patients on replacement therapy – if an individual has become addicted to illicit drugs, they can be prescribed less harmful drugs that have similar but less harmful effects. However, it is important to test that the substitution drugs are been taken.

1.4   FORENSIC TOXICOLOGY

This assists with judicial proceedings and usually involves work for the police, HM Coroner or criminal law courts.

- Workplace drug testing – this can be a requirement set out by some employers, usually its either pre-employment screening - which is carried out on all potential employers prior to them being employed, or post-incident - after an injury, damage or near miss has occurred, or random – selecting employees at random for testing at regular intervals

- Post-mortem toxicology – this is used to determine whether drugs of poisons have caused or contributed to a death

1.5   ANALYTICAL TOXICOLOGY

The application of analytical toxicology can in effect, bring together both clinical and forensic toxicology. It can be used to describe the process and techniques, used to detect and / or measure, or exclude compounds associated with a particular investigation.

It is common practice for toxicology laboratories to perform analysis in two stages. Initially drug screening methods will be used, followed by confirmation and / or quantitative methods, where appropriate.

1.5.1 DRUG SCREENING

A drug screening technique is a qualitative assay, initially performed to test if any drugs are present in a specimen. Their main purpose is usually to provide a quick

solution for the determination of any negative samples, so that further more complicated work can be avoided,

There are many different analytical techniques that can be used for this and they can range from simple colour change or spot tests, to thin-layer chromatography (TLC), to a whole range of immunoassay procedures.

In addition, many more sophisticated analytical techniques can be used as screening methods, where this is seen as advantageous.

1.5.2 DRUG CONFIRMATION AND QUANTITATION

In forensic toxicology, courts require that the identification of a compound be beyond reasonable scientific doubt. In order to achieve this, it is important that the presence of every analyte is confirmed by a secondary method of identification. Some screening techniques already have this ability, e.g. LC-MS has both a retention parameter and a mass-spectrum parameter, to absolutely identify a particular compound and this can easily be achieved either by matching the analytical profile to a database or library on the system, or if it is not already on the system analysing a pure reference standard under the same conditions.

Some types of assay, such as immunoassay, need to be confirmed by a different technique, e.g. GC or LC. It is preferable that drugs are identified using complimentary techniques (e.g. GC and LC) or methods of detection (e.g. LC-UV and LC-MS). However, this is not always possible due to the difference in amenability of some drugs, e.g. polar or thermolabile compounds are less amenable to GC analysis.

After the presence of a drug has been absolutely confirmed, quantitation will usually be required, using the most appropriate technique. This usually involves the

extraction of a set of calibration standards, along with the test sample (in duplicate where possible) and at least one quality control (QC) standard. Results should only be accepted if the QC result is close to the expected spiked value (usually acceptable within 20%, but ideally within 10%). An appropriate internal standard should be used for all GC and HPLC techniques to help minimise matrix effects and correct for other slight variables in extraction procedures, e.g. transfer volumes. The internal standard should ideally be similar in structure to the target analyte, (Jones, 2004), (Elliott, 2009).

1.6   DRUG MATRICES

In principle, a whole range of biological specimens could be analysed to assess the presence of drugs, but in practice their suitability is limited by the ease in which the samples can be obtained and by the availability of technology to analyse them (Bennett, et al., 2003).

As blood and urine have different detection times, they are often both analysed in conjunction, and depending on the question being asked the results will usually provide a good insight to drug use and/ or exposure.

1.6.1   Blood / plasma and serum

Blood / plasma and serum are commonly used to detect and measure drugs, they can be used to determine recent or current drug use, as they have detection windows of approximately 24-48h. For this reason they are commonly used in therapeutic monitoring, as in living patients the dose of a drug is most closely correlated with its concentration in these matrices. For the same reason, blood has also been one of the primary specimens in post-mortem toxicology, as this relates to

the drug status at the time of death (Jones, 2004). However, plasma and serum are not usually an option due to the nature of post-mortem blood and extent of putrefaction. It is important to note that although the concentration of a drug or poison, found in post-mortem blood was previously assumed to be equivalent to that obtained in the blood or plasma of the deceased at the time of death, this is simply not the case. Many factors need to be taken into consideration when interpreting drug levels, as changes in drug distribution after death do occur, depending on circumstances of death, e.g. If trauma is involved and the pharmacology of the drugs, as well as the age and general health of the deceased, (Flanagan, 2011), (Elliott, 2009).

In life, blood collection can be both invasive and painful so for patients requiring long-term drug monitoring, so that blood specimens are not seen as ideal and a non-invasive alternative is sought.

1.6.2 Urine

Urine is also commonly used to detect drugs. It can be used to determine previous drug use as the detection window for most drugs is 2-3 days. It is an ideal matrix for drug screening, as it is mostly made up of water and contains relatively few endogenous compounds that interfere with analysis, (Jones, 2004).

It tends to be metabolites that are present in urine, rather than parent drugs and for some types of testing this can be seen as an advantage. For example, it is possible to distinguish illicit morphine use, from an over the counter preparation or prescribed variety, by detection of the specific metabolite

6-monoacetylmorphine (6-MAM).

In a clinic setting where drug of abuse monitoring is required, urine has become the preferred drug matrix of choice. The non-invasive technique of obtaining the sample is largely acceptable and if the individuals are supposed to be abstaining from illicit drugs then a measurement of drug is not necessarily needed as a qualitative positive or negative result will be sufficient.

However, with this type of testing, specimen adulteration, can be an issue, as some substitution drugs, such as methadone, have a high street value. For some patients the temptation to sell on their medication is too great, especially if the profit earned will be enough to buy the drug that they are addicted to. Measures will therefore need to be put into place to prevent or detect this. Addiction patients have been known to "spike" their urine with their prescribed drugs, in an attempt to get a positive result and maintain their prescription, so the testing for metabolites that urine allows can be very important.

Observed collection could eliminate these problems but due to privacy issues, the acceptability of this has raised ethical questions. Therefore, in order to guarantee the integrity of the specimens, additional tests are often performed, such as the measurement of creatinine. This is a breakdown product found in urine and a low level could suggest that the sample is not urine or has been diluted. Tests for pH will detect for any acid or alkali adulterants.

In post-mortem toxicology urine analysis can be useful, especially when used in conjunction with blood. However, although it can give a good indication of what drugs the deceased has had access to, due to the drug detection window; it is not that useful in determining the cause of death.

Also urine is not always available, in about 50% of deaths, the bladder is voided in the dying process, (Jones, 2004).

In blood the parent: metabolite ratio can help to interpret results, for example an elevated morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) compared to a lower morphine level, could indicate chronic use, but this comparison is not usually possible in urine as the parent drug is very often eliminated to the extent that it is not detected.

1.6.3 Alternative matrices

In certain circumstances the use of alternative matrices or unconventional matrices for detection of drugs can be very useful. The type of matrices will vary, but will largely depend on availability, ease of collection, analytical and testing considerations as well as interpretation of results, (Caplan, 2001).

The drug detection windows, or the length of time that a drug can be detected after ingestion, must be considered carefully when interpreting the results from different drug matrices, (Table 1.1).

As scientific techniques have become more advanced and the possibility of detecting drugs at very low concentrations has become a reality, the interest in alternative matrices has grown. Specimens of particular interest include oral fluid, sweat and hair as they benefit from non-invasive collection that can be performed relatively easily and under supervision where necessary.

Although, they are only usually available in relatively small samples, again, due to the development of more sensitive techniques, such as LC-MS, and GC-MS-MS, accurate detection and measurement of drugs is possible.

These advances have also been helped by commercial availability of collection devices, e.g. sweat patches.

A particular advent for these matrices was that they were deemed suitable to be evaluated for work place drug testing, and were included in drafts of proposed mandatory guidelines, by the regulatory board Substance Abuse and Mental Health Services Administration (SAMHSA), (SAMHSA, 2004).

Although both sweat and hair can be collected by non-invasive techniques, comparatively there are still disadvantages associated with sample collection. Sweat can be collected using a patch but this is a prolonged process where the individual is usually required to wear it for 2-3 days, which can be both inconvenient and uncomfortable.

The collection of hair is actually quite a precise science in itself. Guidelines have been proposed by Society of Hair Testing (SoHT), (Cooper, et al., 2012), for the correct methods of collection, and if these are not adhered to then any sample collected could prove useless for analysis and accurate interpretation.

Therefore, oral fluid seems to have a distinct advantage, over sweat and hair, it can be collected easily either by the old method of expectoration, (spitting), or by using a collection device which is simple, quick and easy.

In post-mortem toxicology, in some circumstances both blood and urine are not available so other specimens such as stomach contents, vitreous humour, bile, liver and other tissues, muscle or bone marrow are submitted for analysis.

In these cases it can be difficult to analyse and interpret results as these matrices are not routinely used and therefore there are often limited published data to refer to, (Fernández, et al., 2006), (Lin, et al., 1997), (Politi, et al., 2004).

Hair can be useful to determine drug history and sometimes in post-mortem toxicology this can be useful, e.g. to provide evidence of tolerance to a particular drug. However, due to the time detection window for drugs, (see Table 1.1), it is generally not very useful for determining if drugs have caused or contributed to a death, so it has limited use in this type of analysis, (Elliott, 2009).

Drug matrix	Detection times of drugs after ingestion
Stomach Contents / Saliva	Hours
Blood / Plasma	Up to 1 day
Vitreous Humour	Days
Urine / Sweat / Liver / Bile	Days – weeks
Hair	Weeks – months
Nails	Weeks – months – years

Table 1.1   Comparison of drug detection windows in different matrices

1.7   COMMON DRUGS

1.7.1 OPIATES

Opiates are any drugs or compounds derived from the opium poppy. They are a type of analgesic, which means that they are used for pain relief. The most commonly known opiates are morphine and codeine.
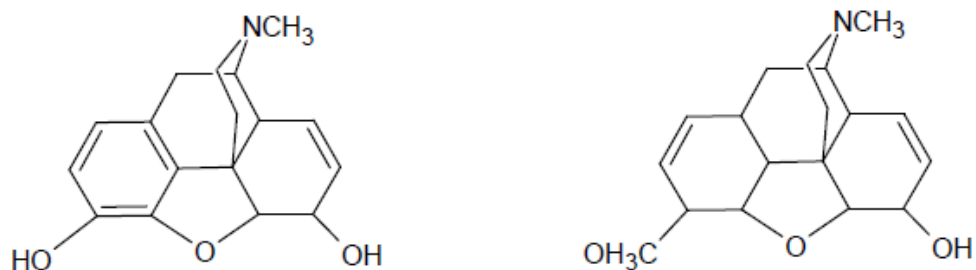
Figure 1.1   Structures of morphine (left) and codeine (right)

Morphine is available as "morphine sulphate", and it can also be prescribed in the more potent form "diamorphine", but it can also be used to produce illicit morphine or heroin which is a known drug of abuse. Heroin is usually smoked, injected or snorted if it's in its pure form.

1.7.2 COCAINE

Cocaine is a naturally occurring alkaloid found in some varieties of plant from the genus, Erythroxylum. It is a local anaesthetic, a vasoconstrictor and a powerful pyschostimulant and due to this last action, it is widely abused, (Jones, 2008). It is often "cut" with other substances, these include sugar, caffeine, lignocaine, procaine, hydroxyzine and benzocaine.
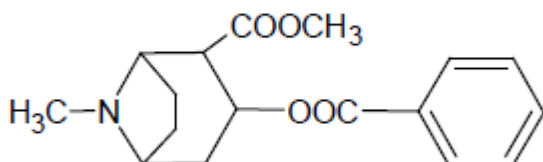
Figure 1.2   Structure of cocaine

1.7.3 AMPHETAMINES

This group of drugs, have been derived from phenylethylamine, a naturally occurring chemical. They are central nervous system (CNS) stimulants and this effect, led to these drugs being abused.

Amphetamine is still prescribed (as dexamphetamine) for narcolepsy and attention-deficit hyperactivity disorder (ADHD) in children. "Street" amphetamine is usually a powder that can be rubbed into the gums, orally ingested or snorted.
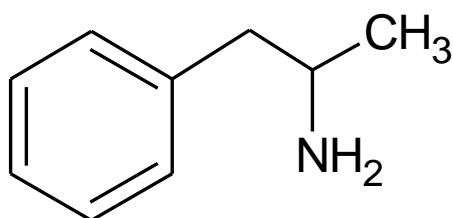
Figure 1.3   Structure of amphetamine

Methamphetamine a related drug is not prescribed. It is abused less in the UK but is very popular some areas of the world, e.g. America, Japan.

3,4-methylenedioxymethamphetamine (MDMA) sometimes known as a "designer-drug" has been abused as a stimulant since the mid to late 1980s. Other "designer-drugs" include; 4-methylthioamphetamine (4-MTA), para-methoxyamphetamine (PMA), *para*-metoxymetamphetamine (PMMA), 2,5-dimethoxy-4-methylamphetamine (DOM) and 2,5-dimethoxy-4-bromoamphetamine (DOB), (Elliott, 2009).

1.7.4 OPIOIDS

These contain synthetic compounds that provide the pharmacological properties as opiates; they are generally used as analgesics. They have a wide range of potencies, e.g. etorphine used in veterinary medicine is about 1000 times more potent than morphine. Due to their opiate-like action they are often prescribed for drug substitution programs for opiate addiction. However for the same reason, it is not unusual for them to be abused. Examples include methadone, buprenorphine, dihydrocodeine (DHC), oxycodone, tramadol and pethidine.

1.7.5 BENZODIAZEPINES

Sedative drugs prescribed for insomnia and anxiety, originally thought to be a "safer" alternative to the older sedative drugs "barbiturates". They can also be used to control seizures and treatment of alcohol or drug withdrawal symptoms. However, they are prone to be abused themselves, (Elliott, 2009). Examples include; diazepam, temazepam, lorazepam, clobazam and chlordiazepoxide.

1.7.6 ANTIDEPRESSANTS

These drugs are used to reduce the feelings of depression by altering the concentration of specific neurotransmitters in the brain. They are divided into different sub classes according to their structure and mechanism of action. Examples include citalopram, fluoxetine, venlafaxine, duloxetine, amitriptyline, dosulepin (dothiepin) and mirtazepine, (Elliott, 2009).

1.7.7 ANTICONVULSANTS / ANTIEPILEPTICS

These drugs are used to control seizures and / or fitting, e.g. they are often prescribed for epilepsy sufferers. Examples include carbamazepine, phenytoin, lamotrigine and sodium valproate.

1.7.8 ANTIPSYCHOTICS

These drugs produce tranquilising effects but without impairing consciousness. Prescribed for treating psychoses, e.g. such as schizophrenia, as well as severe anxiety. Examples include; chlorpromazine, promazine, clozapine, haloperidol and olanzapine.

1.7.9 β-BLOCKERS

These drugs are used to treat hypertension, angina, arrhythmias and anxiety. Examples include propranolol and atenolol.

1.7.10 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS)

These drugs are analgesics, used for long lasting pain relief and anti-inflammatory effects. Examples include; ibuprofen, diclofenac, salicylate and naproxen.

CHAPTER 2: EXPERIMENTAL

METHODS

2.1   ANALYTICAL TECHNIQUES

2.1.1   IMMUNOASSAY

All immunoassay techniques are based on the interaction of a target molecule (antigen) with the antibody. For drug testing, an antibody specific for the drug or drug class is used and the assay is usually based on competitive binding.

A known quantity of antibody is introduced, with a fixed quantity of labelled drug, and the test sample. Specific binding sites on the antibody bind both the drug in the test sample and the labelled drug in the assay. There is an inversely proportional relationship between labelled drug bound and unlabelled drug bound, (Hand & Baldwin, 2004).

Immunoassays for drugs can be divided into two groups:

Heterogeneous – require an additional step to separate the bound complexes and free fractions of the assay before measurement of the signal

Homogenous – do not require this step

Immunoassays have wide applications in drug testing, and there are many commercially available testing kits and analysers available. They have the advantage of fast and convenient analysis, often without any extraction methods, and are applicable to many matrices.

Some specific assays are available. They can be used to accurately quantify drugs, for example in therapeutic drug monitoring, immunoassay techniques are used routinely to quantify drugs in plasma, serum and blood.

However, for less specific assays, where the chemistry involved looks for groups of drugs, e.g. opiates, rather than specific drugs, e.g. morphine, both false negative and false positive results can occur. The manufacturers of commercially available

kits will usually carry out specific tests to calculate the cross-reactivity of common assay "interferants", and this information will be supplied with the kit. Obviously they cannot test for everything in every type of scenario so it is important for analysts using these assays to be aware of these assay limitations.

For this reason, immunoassay drug screens are usually semi-quantitative, and should be confirmed by a secondary method such as Gas Chromatography-Mass Spectrometry (GC-MS) which can provide an absolute identification of which drug within a group caused the positive screen result, and also identify any false positive screening results too. For forensic work it is essential that any immunoassay results are confirmed by a secondary method.

Enzyme linked immunosorbent assay or ELISA, is a heterogeneous immunoassay. Although, this method could appear more labour intensive than the homogeneous type this is not necessarily the case, although an additional step is often required to separate the fractions, comparatively little sample preparation is required initially, e.g. whole blood can be used without extraction which is uncommon in homogeneous methods, and also the heterogeneous assays have lower limits of detection, (Hand & Baldwin, 2004).

In ELISA, the specific antibody is coupled to a solid support. Often this is to the plastic in micro wells on a plate. An aliquot of sample to be assayed is added to micro-plate wells, followed by a solution of the same antibodies coupled to an enzyme (horseradish peroxidase). After an incubation period, the plate is washed to remove any unbound material and a colourless substrate is added to the wells. There is another incubation period during which a coloured product is produced,

(refer to figure 2.1), the intensity of the colour is measured and then used to determine the amount of antigen present in each sample, (Hames, et al., 1997).

Antigen-coated well

Wash

Specific antibody binds to antigen

Wash

Enzyme-linked antibody binds to specific antibody

Wash

Substrate is added and converted by enzyme into coloured product; the rate of colour formation is proportional to the amount of specific antibody
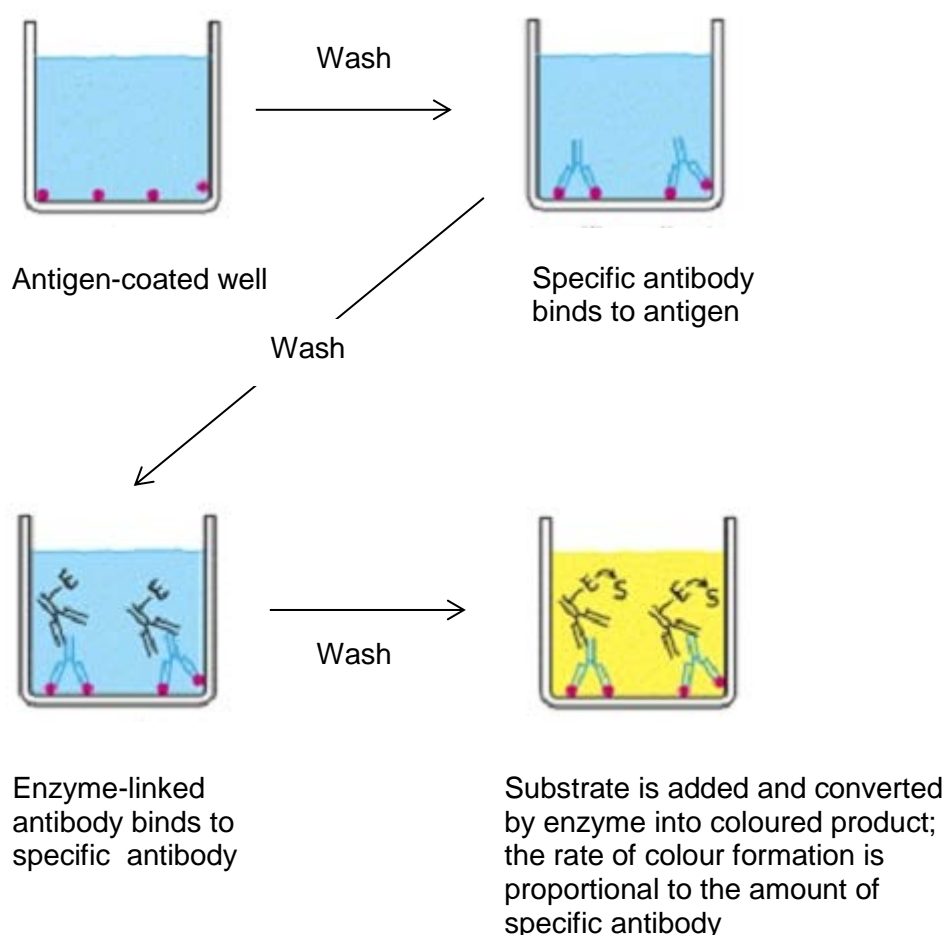
Figure 2.1   Diagram to show ELISA procedure

(Chakravarthy, 2011)

Cloned enzyme donor immunoassay (CEDIA), is a homogeneous enzyme immunoassay. This technique is based on the use of an enzyme β-galactosidase, which has been genetically engineered into two inactive fragments; one fragment is conjugated to a drug and is called the enzyme donor (ED), while the other fragment that co-exists with the antibody is known as the enzyme acceptor (EA). Drug in the

test sample competes with the ED for the binding site on the antibody. Any drug present in the sample binds to the antibody, leaving inactive enzyme fragments free to form active enzyme. The amount of active enzyme formed and resultant absorbance change are proportional to the amount of drug in the test sample. If there is no drug present in the sample, the antibody binds to the ED fragment preventing formation of active enzyme, (Henderson, et al., 1986), (Krapp, 2002).

Within the laboratory, (where I started this study), there was a fully automated immunoassay analyser that used CEDIA kits for urine drugs of abuse analysis. However, all the assays were based on urine drug cut-off levels, (as set out by the Substance Abuse and Mental Health Services Administration (SAMHSA), and as these are so much lower than levels expected / or found in oral fluid, (see Table 2.1), it was not possible to use this system for oral fluid analysis.

	Urine screening cut-off concentrations	Proposed oral fluid screening cut-off concentrations
Amphetamines	500 ng/mL	50 ng/mL
Cocaine (metabolite)	300 ng/mL	20 ng/mL
Methadone (metabolite)	100 ng/mL	20 ng/mL
Opiates	300 ng/mL	40 ng/mL
Cannabinoids	50 ng/mL	4 ng/mL

Table 2.1   Cut-off levels in urine versus oral fluid, (SAMHSA, 2004)

2.1.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE-ARRAY DETECTION (HPLC-DAD)

Chromatography can be defined as the separation of components in a mixture. Liquid chromatography (LC) produces separation based on the differential distribution of analytes between two phases. One phase is liquid, e.g. mobile phase, and the other is either a solid or a liquid that is firmly bound to a solid support, e.g. the column. When appropriate solvent conditions are reached the drug elutes off the column where it can be detected by an appropriate method.

Historically, LC was very time-consuming and it was usually only possible to analyse relatively few samples before the column would need to be re-packed. Complex separations were difficult to achieve. However, the development of high performance liquid chromatography (HPLC) changed all this. Once systems capable of quantitative analysis became commercially available, this technique became increasingly popular, recognised for its convenient automation, separation of a wide range of sample types, excellent resolution and speed.

There are a wide range of stationary phases available, they are usually described as belonging to one of four mechanistic types:-

- Adsorption chromatography – sample components are selectively adsorbed onto the surface of the solid stationary phase.

- Partition chromatography – involves a liquid stationary phase that is immiscible with the eluent and coated on an inert support. It can be either normal phase where the mobile phase is less polar than the stationary phase, or reverse phase chromatography where the opposite is true so the mobile phase is more polar than the stationary phase.

- Ion exchange chromatography - stationary phase is an ion exchange resin with anionic and cationic groups on the surface which attract solutes of opposite charge.

- Size exclusion chromatography – stationary phase is a porous gel and separation occurs on the basis of component size

Stationary phases in use today are "micro-particulate" column packings made up from uniform, porous silica particles with spherical shapes and 3 – 10 μm diameters. A typical HPLC system includes a pump, injector, column, detector and a recorder or computer, (refer to Figure 2.2). A high-pressure pump is required to move the mobile phase through the highly compacted column, this occurs at a constant flow rate e.g. 1 mL/min. Samples are injected onto the system by the auto-sampler, the mobile phase containing the analytes is pumped through the column and separation of the components occurs. Each component elutes off the column and is registered as a peak on the recorder. Detection of the eluting components can be achieved by several methods, such as ultra-violet detector (UV), photodiode array detector (UV-DAD), electrochemical (EC), fluorescence (FL), and mass-spectrometry (MS). For this study, UV-DAD was used, this consists of a large number of microdiodes, and each diode will record variations in the intensity of radiation from a particular section of the spectrum so there is a continuous monitoring of absorbance over a specified wavelength range, (e.g. 200-600 nm).

Mobile Phase Pump

Mobile Phase Reservoirs

Column Oven

Autosampler with
sample carousel

Analytical
column

Connecting (flow)
tubing

Photodiode array
detector
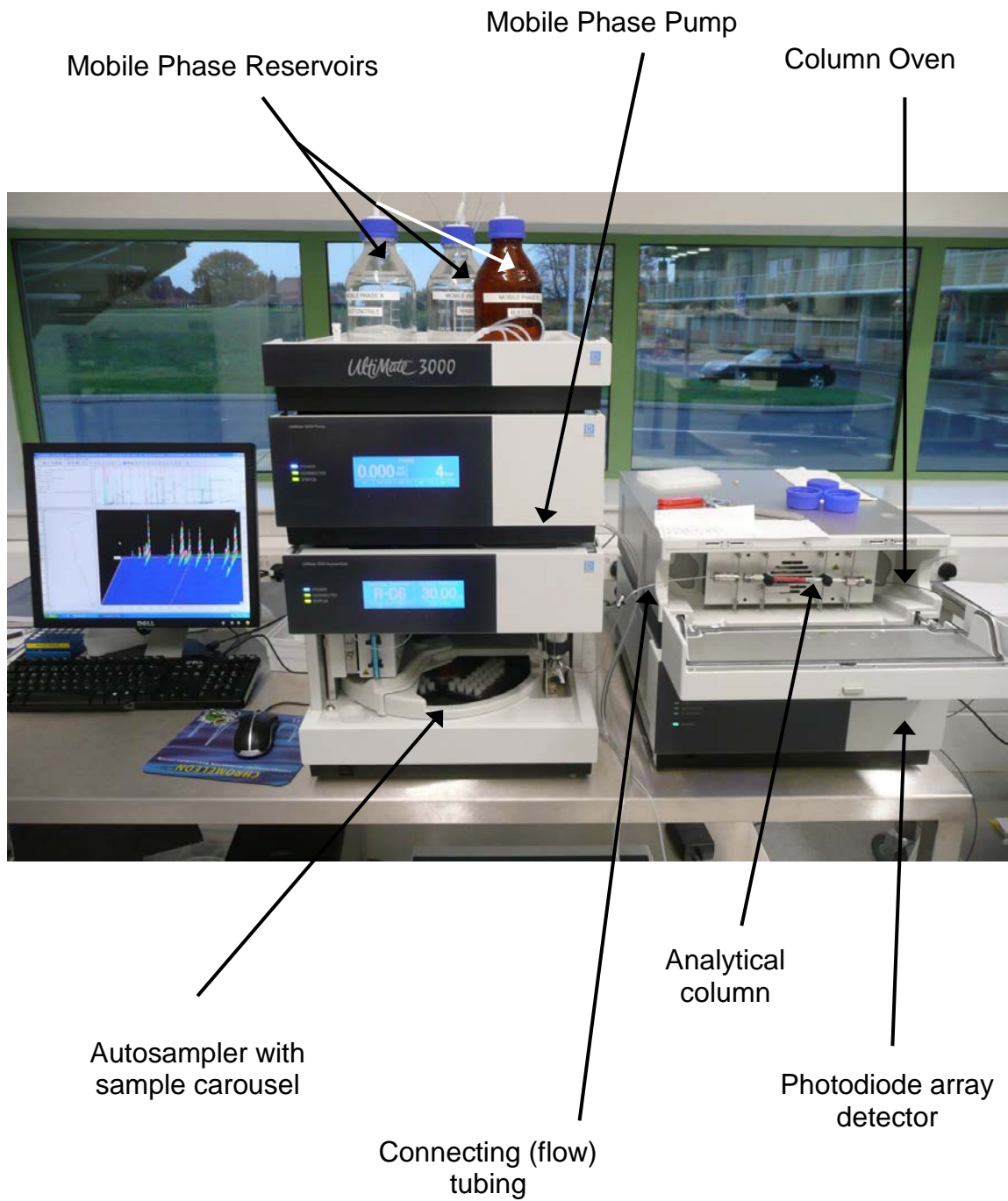
Figure 2.2   HPLC-DAD System Setup

The result is that a traditional chromatogram will be generated and for this, one particular wavelength can be chosen to observe separation. Each "peak" relates to the degree of absorbance and the concentration present, i.e. a small peak indicates a low concentration.

In addition to this, plotting of the absorbance at each wavelength produces a spectrum. This UV spectrum can be compared against UV spectra in a library of known compounds. This technique can be applied to any substance that has a suitable structure to absorb light, this usually requires a conjugated system or a chromophore, i.e. this is present in most drugs. The UV-spectra together with the retention time, provides two separate methods of identification, (Kupiec, et al., 2004), (Holme & Peck, 1998), (Elliott, 2009), (Herzler, et al., 2003).

2.1.3 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

Liquid Chromatography with Mass Spectrometry detection (LC-MS) can be used for drug screening, confirmation and quantitation. The LC system is usually an HPLC setup as previously described but it is linked to an MS, (see Figure 2.3). Mass filtration occurs in a quadrupole analyser or an ion trap. Some MS systems have triple quadrupoles, these are LC-MS-MS or tandem MS systems. Mass spectrometry (MS) is based on measurement of the mass-charge (m/z) ratio of an ionised compound. In LC-MS, energy is applied to compounds flowing into the MS from the LC system, to create an ionised compound. This is fragmented to produce a "full scan" mass spectrum, (Elliott, 2009).
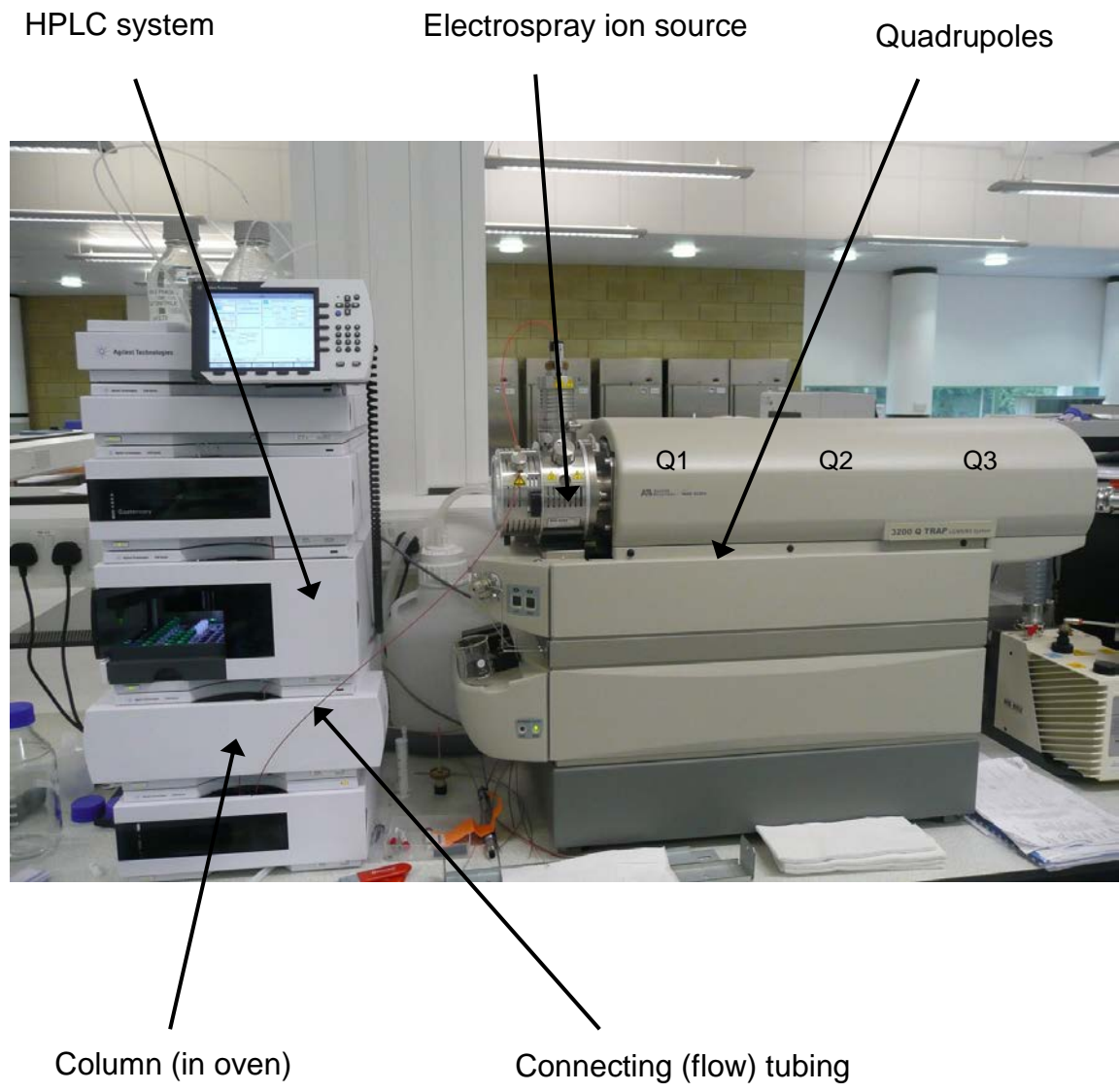
Figure 2.3   LC-MS System Setup

It is also possible to operate LC-MS using a targeted approach. This is where particular transitions can be specifically looked for, and this helps to increase the sensitivity of the system because it is scanning a smaller range. This is known as multiple reaction monitoring (MRM). LC-MS has two built in methods of identification, e.g. retention parameter and MS fragmentation pattern. Identification is primarily based on the MS, and this can be compared to a library on the system. However, it is important to note that LC-MS libraries are not very applicable between different systems and tend to be both instrument and methods specific, for this reason they are best built up in-house, (Elliott, 2009).

2.1.4 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

Gas chromatography, like LC, is a separation technique; it depends upon the partition of a solute between two phases. The mobile phase is gaseous and separation is performed in a column (containing either a solid or liquid stationary phase) that has a continuous flow of mobile phase passing through it, (usually an inert carrier gas). When a mixture of compounds is injected at the inlet, each compound partitions between the stationary phase and the gas phase as it is swept (by the carrier gas) towards the detector. Some compounds have greater affinity for the stationary phase and so take longer to reach the detector. As described with HPLC, the detector produces a signal proportional to the concentration of compound present, and each compound that elutes from the column has a characteristic retention time, this can be defined as the time interval from injection to peak detector response. The retention time of each analyte on the column, is determined by the solubility and absorption, which is largely influenced by the chemical structure (e.g. size, polarity) and temperature.
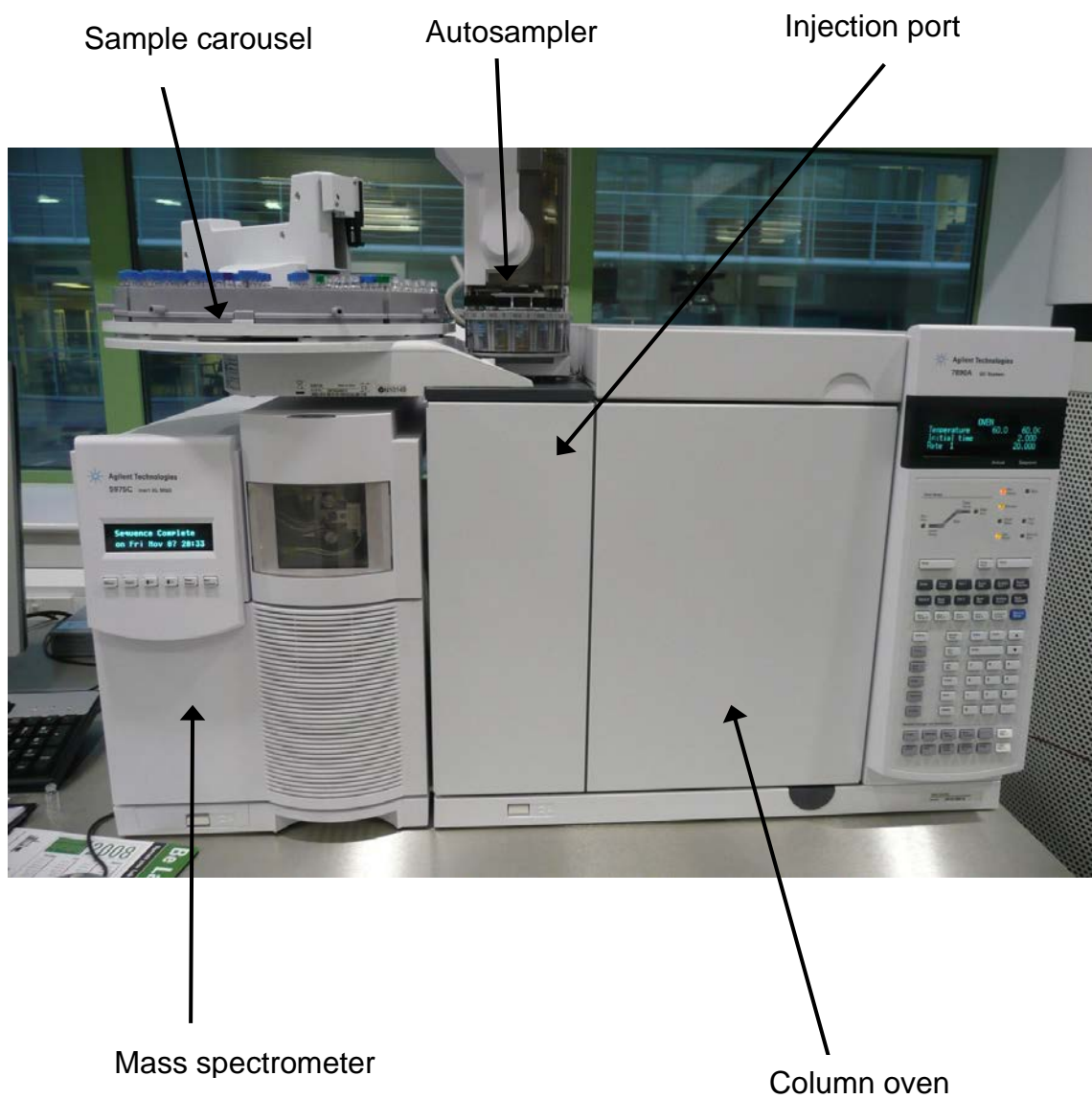
Figure 2.4   GC-MS System Setup

A typical GC system is comprised of a gas cylinder (to provide carrier gas), a sample inlet port, a column oven (to maintain temperature and keep analyte in vapour form), a column and a detector, (see Figure 2.4).

Unfortunately GC is not applicable to all compounds, non-volatile and polar compounds are not very amenable to GC. As a general rule, if a compound has sufficient volatility for its molecules to be in the gas phase at or below 400°C, without decomposing, it can probably be analysed by GC. In addition to this, small compounds (i.e. with low mass) do not fragment very well, this can be overcome by the process of derivatisation which adds more chemical groups, to produce a larger molecule that produces more distinctive fragmentation, resulting in a better mass spectrum. However, this derivatisation step complicates and lengthens sample preparation, and the reagents are often very toxic.

When the detection system is an MS, the principle of mass-spectral detection is the same as for LC-MS, as a mass-charge (m/z) ratio of an ionised compound is measured. However, with GC-MS, the compounds are in a gaseous state at high temperature. With the most common technique of electron impact (EI), the compound is bombarded with electrons, compounds absorb energy which causes them to ionise and fragment in a characteristic and reproducible manner. The molecular ion can also become fragmented so the whole drug molecule is not usually detected intact. The ions are focused and accelerated into a mass filter that allows fragments of sequentially increasing mass to enter the detector. The abundance of each mass at a given scan time produces the mass spectrum.

The MS detector can be operated in either full scan mode (collecting all the ions within a given mass range) or selected ion monitoring (SIM) mode, which collects

only pre-selected masses characteristic for the compound(s) under study, which allows for greater sensitivity. As with LC-MS, GC-MS provides two identification parameters, a retention time and a mass spectrum. Where scan mode is used, mass spectra can be compared against a library and for SIM data, the ion ratios can be compared to a reference standard. GC-MS libraries have the advantage that the data is largely applicable to all GC-MS systems, and this means that there are extensive points of reference to aid with identification, (Dawling, 2004), (Elliott, 2009), (Holme & Peck, 1998).

2.1.5   GAS CHROMATOGRAPHY WITH FLAME IONISED DETECTION (GC-FID)

A flame ionised detector (FID) depends upon the thermal energy of a flame causing some ionisation of molecules as they burn. The ions are collected by a pair of polarised electrodes and the current produced is amplified and recorded.   An FID detector responds to virtually all organic compounds, the response is dependent on the number of carbon atoms in the molecule but it is lowered if oxygen and nitrogen are also present in the molecule.

For drug analysis it is particularly useful for ethanol and other alcohols, (as these are volatile carbon chain compounds) but it can also be applied to other drugs, e.g. valproate. Chromatographic "peaks" are observed at different retention times but this technique does not have a secondary method of identification, ((Elliott, 2009), (Holme & Peck, 1998), (Dawling, 2004).

2.1.6 GAS CHROMATOGRAPHY WITH NITROGEN PHOSPHORUS DETECTION (GC-NPD)

Nitrogen-phosphorus detection (NPD) or alkali flame ionisation detection (AFID), as it is also known, involves the introduction of alkali metal vapours (usually supplied by an electrically heated bead of rubidium or caesium chloride) into the flame of an FID. This results in an enhanced response to nitrogen- or phosphorus-containing compounds. This type of detector is particularly useful for drug analysis, as most drugs contain nitrogen, while the solvents and the bulk of the co-extracted material from a biological sample do not. Like with GC-FID it only has retention time for identification with no secondary identification parameter.

It also has the disadvantage that the detecting element, often referred to as the "bead" requires a gas supply (constantly running through it), in total this means a supply of three gases, and the "life" of the "bead" is relatively short and will probably need to be replaced every few months, depending on the usage, (Dawling, 2004), (Elliott, 2009), (Holme & Peck, 1998).

2.1.7 ELISA SCREENING

2.1.7.1 SAMPLE PREPARATION

ELISA research kits for opiates and cocaine/BZE were received from "International Diagnostic Systems Corporation" (IDS) supplied by Griffols.

2.1.7.2 EXTRACTION PROCEDURE 1 (TAKEN FROM IDS KIT INSERT GUIDE)

20µL blank / calibrators / controls were pipetted into the micro plate wells and 100µL of diluted enzyme was added to each well. After 1 hour incubation at room

temperature the wells were washed and 100µL of substrate was added to each well. After 30 minutes incubation at room temperature, 100µL of stop solution was added to each well. The absorbances were measuerd with a micro plate reader at 450 nm wavelength.

2.1.8 CEDIA SCREENING

CEDIA kits for amphetamine/ecstasy, cocaine, methadone metabolite (EDDP) and opiate assays were purchased from Microgenics Corporation. These were analysed on an Olympus Chemistry Immuno AU640 Analyser which is a fully automated system. After the instrument has been calibrated, neat urine samples can simply be loaded onto the system and results will be generated sometime later.

2.1.9 LIQUID CHROMATOGRAPHY FOR SCREENING

2.1.9.1   SAMPLE PREPARATION

A range of calibrators were made from 1 mg/mL drug stocks purchased from LGC Standards (Middlesex, UK). The calibrators contained Amphetamine (AMP), Methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), the lowest calibrator was 10 ng/mL and the highest 1000 ng/mL.

Norfenfluramine was added as an internal standard (IS), (as deuterated internal standards could not be used in this experiment as the extracts were going to be ran on HPLC-DAD), this was prepared in sodium carbonate buffer and added in place of the buffer, in extraction procedure 2.

2.1.9.2 EXTRACTION PROCEDURE 2 – basic drugs

A basic extraction was used, where 500µL test sample were mixed with sodium carbonate buffer and 5 mL 1-Chlorobutane was added as the extraction solvent. The tubes were mechanically shaken and then centrifuged, after which the supernatant was removed to a clean tube and the extract was acidified with 100µL sulphuric acid (H_2SO_4 at 0.05M). The tubes were shaken and centrifuged again, then the solvent layer was aspirated and the remaining 100µL was transferred to a vial insert.

2.1.9.3 EXTRACTION PROCEDURE 3 – basic/neutral drugs

A basic/neutral extraction was used, where 500µL of test samples was mixed with 500µL of 0.2 M sodium carbonate buffer, and 5 mL hexane:ethyl acetate (7:3) was added as the extraction solvent. Following mixing and centrifugation, the supernatant was removed to a clean tube and evaporated at dryness 50°C under dry nitrogen (using a sample concentrator). The extracts were reconstituted with 100µL methanol, and vortexed, before being transferred to an appropriate vial.

2.1.9.4 EXTRACTION PROCEDURE 4 – acidic/neutral drugs

An acid extraction was used, where 500µL sample was mixed with 500µL of 0.2 M sulphuric acid , and 5 mL chloroform was added as the extraction solvent. Following mixing and centrifugation, the supernatant was removed to waste and the solvent layer was filtered into a clean tube then evaporated at dryness 50°C under dry nitrogen (using a sample concentrator). The extracts were reconstituted with 100µL methanol, and vortexed, before being transferred to an appropriate vial.

2.1.9.5 HPLC-DAD SYSTEM SETUP 1

This system consisted of a Dionex liquid chromatography system with a UV-DAD detector. Separation was performed isocratically on a 150 x 4.6mm Phenomenex Synergi Fusion-RP 4 micron column. The mobile phase was acetonitrile 70% in triethyl ammonium phosphate buffer. Data analysis was interpreted or quantified at 220nm.

2.1.9.6 LC-MS SYSTEM SETUP

The same extracts were analysed on a tandem LC-MS system, which had an Agilent 1200 series HPLC and a Q-TRAP, Applied Biosystems. Multiple reaction monitoring (MRM) was used for quantitation and two transitions were monitored for each amphetamine, (Table 2.2).

	First MRM Transition	Second MRM Transition
Amphetamine	136 / 91	136 / 65
Methamphetamine	150 / 91	150 / 119
MDA	180 / 163	180 / 135
MDMA	194 / 163	194 / 135

Table 2.2   MRM Transitions

2.1.10 GC-MS OPIATE CONFIRMATION

2.1.10.1   SAMPLE PREPARATION

Certified stock solutions of morphine, codeine, DHC and 6-MAM were purchased from LGC Standards (Middlesex, UK), along with the following deuterated standards, morphine-d3, codeine-d3, DHC-d6 and 6-MAM-d3.

The morphine and codeine stocks were used to prepare calibrators, and the deuterated stocks were used as internal standards, initial studies were carried out using both water and blank human saliva, Medidrug® Basis-line saliva (Medichem®, Steinenbronn, Germany) as a drug matrix.

2.1.10.2   EXTRACTION PROCEDURE 5

Solutions of 500µL calibrator/test/quality control, were extracted using 1mL ammonium carbonate buffer pH9 and 5 mL isopropranol:chloroform (9:1 v/v). Following mixing and centrifugation, the supernatant was removed to waste and the solvent layer evaporated at 45°C under a stream of air. Then the samples were derivatised with bis(Trimethylsilyl)Trifluoroacetamide (BSTFA-TMCS), heated at 90°C for 5 minutes and then transferred to GC-MS vials.

NB: Prior to extraction all the urine samples were hydrolysed by the addition of 100 µL *β*-glucuronidase and incubated overnight at 37°C.

2.1.10.3   GC-MS SYSTEM

An Agilent GC 6890 with a 5973 mass selective detector was used for analysis. The inlet was maintained at 250°C and the transfer line at 280°C.

The GC column was an Rtx 5ms of length 30m, internal diameter of 0.25mm and film thickness 0.25 µM, (Thames Restek UK, LTD).

2.1.10.4 GC-MS PARAMETERS 1

The column temperature was initially 130°C with a hold time of 0.5 min then it was increased 50°C/min to 300°C, with a final hold time of 1.6 min, and a total run time of 5.5 min. It was run in SIM mode detecting the ions displayed in Table 2.3.

Opiate	Target Ions	Qualifier Ions
DHC / DHC-d6	373 / 379	315, 282, 236
Codeine / Codeine-d3	178 / 181	343, 371, 234, 196
Morphine / morphine –d3	429 / 432	236, 287, 220, 324
6-MAM / 6-MAM-d3	399 / 402	266, 287, 340, 204

Table 2.3   Opiate ions used for GC-MS analysis

2.1.10.5   EXTRACTION PROCEDURE 6

Follow Procedure 5, but after evaporation, butyl acetate was added to each tube for reconstitution and the extract was then transferred to GC-MS vials.

2.1.10.6   GC-MS PARAMETERS 2

The column temperature was initially 110°C with a hold time of 1 min then it was increased to 75°C/min to 300°C, with a final hold time of 2.47 min, and a total run time of 6 min. In scan mode codeine eluted at 8.77 min, with the predominant ions 162, 229, and 299.

An investigation was carried out, and the results proved that 299 and 302 were found to be the best target ions for codeine and codeine-d3, they were used to create a SIM method.

2.1.11 GC-MS BENZOYLECGONINE CONFIRMATION

2.1.11.1   SAMPLE PREPARATION

Certified 1 mg/mL stock solutions of benzoylecgonine (BZE) were purchased from LGC Standards (Middlesex, UK) along with the deuterated internal standards, BZE-d3.

The stock solutions were used to prepare calibrators, ranging from 2 ng/mL to 8000 ng/mL, and controls were prepared from independent stock solutions at 8 ng/mL. They were extracted as described in extraction procedure 5.

2.1.11.2   GC-MS PARAMETERS 3

The same instrumentation was used as described previously, (see extraction procedure 5). The column temperature was initially 160°C with a hold time of 0.5 min then it was increased 20°C/min to 300°C, with a final hold time of 0.5 min, and a total run time of 8 min. It was run in SIM mode detecting the ions 240, 256, 361 for BZE and 243, 259, 364 for BZE-d3.

2.1.11.3   EXTRACTION PROCEDURE 7

1 mL of calibrator was extracted using 1mL acetate buffer (2M), pH 3.8 and 5 mL dichloromethane:isopropanol:ammonium hydroxide (80:20:2 v/v),
(Cone et al., 1994). Following mixing and centrifugation, the supernatant was removed to waste and the solvent layer evaporated at 45°C under a stream of air. The samples were derivatised with (BSTFA-TMCS), heated at 90°C for 5 minutes and then transferred to GC-MS vials.

2.1.12 GC-MS AMPHETAMINES CONFIRMATION

2.1.12.1 SAMPLE PREPARATION

Certified 1 mg/mL stock solutions of amphetamine (AMP), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), were purchased along with deuterated standards, MA-d5 and MDMA-d5 from LGC Standards (Middlesex, UK). These stocks were used to prepare calibrators that ranged from 2.5 ng/mL to 1000 ng/mL, and the deuterated stocks were used as internal standards.

2.1.12.2 EXTRACTION PROCEDURE 8

Calibrator/test/quality control solutions (400µL) were extracted with 50µL alkaline buffer, 200µL toluene and 25µL heptafluorobutyric acid (HFBA). Following mixing and centrifugation, the supernatant was removed and transferred to GC-MS vials.

2.1.12.3 GC-MS PARAMETERS 4

The column temperature was initially 110°C with a hold time of 1.0 min then it was increased 20°C/min to 250°C, with a total run time of 8 min.

It was run in SCAN mode and the ions displayed in Table 2.4 were extracted.

Amphetamine	Target Ions	Qualifier Ions
Amphetamine	240	118, 169
MA / d5	254 / 258	210, 218
MDA	162	135, 136, 375
MDMA / d5	254 / 258	162, 210

Table 2.4   Amphetamine ions used for GC-MS analysis

2.1.13 GHB SCREENING

2.1.13.1 SAMPLE PREPARATION

4-Hydroxybutyrate (GHB) sodium salt was purchased from Sigma-Aldrich-Fluka. A 20 mg/L high quality control (HQC), and a 4 mg/L low quality control (LQC) were spiked into plasma. GHB-d6 (100 mg/L in methanol) was purchased from LGC Standards. This was diluted into 0.05M H_2SO_4, to give a 5 mg/L working solution, this was used as the internal standard.

2.1.13.2 EXTRACTION PROCEDURE 9

100µL sample were mixed with 50µL of internal standard solution (5 mg/L GHB-D6 in dilute acid), and 500 µL acetonitrile was added as the extraction solvent. Following mixing and centrifugation, the supernatant was removed to a clean tube and evaporated to dryness 50°C under dry nitrogen (using a sample concentrator). The samples were derivatised with bis(Trimethylsilyl)Trifluoroacetamide (BSTFA-TMCS), heated at 90°C for 5 minutes and then transferred to GC-MS vials.

The column temperature was initially 60°C with a hold time of 2 mins this was increased 20°C/min to 180°C, with a final ramp of 50°C/min to 230°C with a total run time of 9 min. The target ion used for GHB was 233 m/z, this had an expected retention time of ~6.78 min.

2.1.14 BUPRENORPHINE SCREENING

2.1.14.1   SAMPLE PREPARATION

Buprenorphine and norbuprenorphine stock standards (1mg/mL) were purchased from LGC Standards (Middlesex, UK). These were used to prepare 10 ng/mL combined quality control in urine. This was extracted along with the samples, as described in extraction procedure 5.

2.1.14.2   GC-MS PARAMETERS 5

They were analysed on the same GC-MS system as previously described.

The column temperature was initially 150°C with a hold time of 0.5 min then it was increased 75°C/min to 300°C, with a total run time of 11 min. It was run in SIM mode with the target and qualifier ions, shown in Table 2.5.

Analyte	Target Ions	Qualifier Ions
Buprenorphine	450	482, 506, 539
Norbuprenorphine	468	500, 524, 557

Table 2.5   GC-MS ions for buprenorphine and metabolite norbuprenorphine

2.1.15 DRUG QUANTITATIONS

2.1.15.1   HPLC MEASUREMENT OF BASIC/NEUTRAL AND ACIDIC DRUGS

The majority of drug measurements were performed using extraction procedures 2, 3 or 4, (depending on the chemical nature of the drug, e.g. basic, neutral or acid). Suitable calibrators and QCs were prepared and extracted along with the test sample (in duplicate where possible) and in the presence of a suitable internal standard, (for full details refer to Appendix A).

2.1.15.2 HPLC MEASUREMENT OF PARACETAMOL

2.1.15.2.1 EXTRACTION PROCEDURE 10

In a tube 200 µL standard/QC/test was mixed with 200 µL internal standard solution, (2-A-P in acetonitrile, 100 mg/L). Tubes are vortex mixed and centrifuged, then 100 µL solvent layer was transferred to HPLC vials.

2.1.15.2.2 HPLC-DAD SYSTEM SETUP 2

The system setup and mobile phase used, were the same as described in setup 1. However, separation was performed isocratically on a 150 x 4.6mm Phenomenex Synergi Polar-RP 4 micron column. For specific conditions refer to Appendix A.

2.1.15.3 LC-MS MEASUREMENT OF MORPHINE AND GLUCURONIDES

Solid phase extraction is performed using Varian Bond Elut C18 (6 mL, 200 mg) SPE columns, purchased from Agilent Technologies.

2.1.15.3.1 SAMPLE PREPARATION

100 mg/L morphine, 100 mg/L M3G and 100 mg/L M6G in methanol were supplied and purchased from LGC Standards. These were used to prepare calibrator standards: 5, 10, 25, 50, 100, 250 and 500 µg/L.

These were used to prepare a 1 mg/L working internal standard solution.

2.1.15.3.2 EXTRACTION PROCEDURE 11

300 µL of blank/ standard/ QC/ sample was mixed with 1 mL of 0.5M ammonium carbonate buffer (pH 8) and 50 µL of internal standard solution (1 mg/L morphine-D3 and M3G-D3 in water).

The SPE columns are conditioned with 2 mL methanol followed by 2 mL water and 1 mL of 0.5M ammonium carbonate buffer, then 1 mL of the prepared sample is loaded onto the column. It is eluted at approximately 1 mL/min.

The SPE column is washed with 5 mL of 0.005M ammonium carbonate buffer and then flow dried under vacuum for 5 minutes. The extract is then eluted with 1 mL of 70% acetonitrile: water solution. It is evaporated to dryness under dry nitrogen at 50°C (using sample concentrator) Reconstitute by adding 100 µL of 4% Mobile Phase A: 96% Mobile Phase B solution. It is transferred to a vial insert.

2.1.15.3.3 LC-MS SYSTEM SETUP

The LC-MS setup was comprised of an Agilent 1100 series liquid chromatography system with an auto sampler coupled to an ABSciex 2000 QTRAP Mass-spectrometer.

Analysis was performed on a 150 mm x 2 mm Phenomenex Synergi Polar-RP column protected by a 4 mm x 3 mm Phenomenex Synergi Polar-RP guard column.

2.1.15.4 GC-FID MEASUREMENT

2.1.15.4.1 VALPROATE

2.1.15.4.1.1 SAMPLE PREPARATION

Valproic acid (sodium salt) was purchased from Sigma-Aldrich-Fluka, and used to make a 200 mg/L calibrator in horse plasma (purchased from TCS Biosciences), this was serially diluted to give additional calibration standards of 100, 50, 25, 12.5 mg/L. Quality control standards (QCs) were made at 30 mg/L and 150 mg/L also in horse plasma, (this was used as a blank matrix because it mimics human plasma and is commercially available). Caproic acid (hexanoic acid) was purchased from Sigma-Aldrich-Fluka, this was diluted into hydrochloric acid (1M) to give a working internal standard solution (100 mg/L).

2.1.15.4.1.2 EXTRACTION PROCEDURE 12

100 µL standard/QC/sample were mixed with 100 µL hexanoic acid solution (in HCl) and 100 µL chloroform, in a tube. The tubes were vortex mixed then centrifuged. The solvent layer from each tube, was removed and put into a GC vial insert.

2.1.15.4.1.3   GC-FID SYSTEM SETUP 1

Analysis was carried out on a ZEBRON ZB-FFAP capillary column, (15 m x 530 µm x 1 µm), with an isothermal temperature of 135°C and run time of 3.5 minutes. Valproate typically eluted at 2.2 min and hexanoic acid at 1.5 min.

2.1.15.4.2   CHLORMETHIAZOLE

2.1.15.4.2.1   SAMPLE PREPARATION AND ANALYSIS

Chlormethiazole HCl was purchased from Sigma-Aldrich-Fluka, it was used to make up calibrators of 200, 100, 50, 25 and 12.5 mg/L in horse blood, (purchased from TCS Biosciences, this was used as a blank matrix to mimic human blood). QCs were made at 30 mg/L and 150 mg/L. Extraction was carried out as described in procedure 11, (with the same internal standard), and the system was setup as described in setup 1. Chlormethiazole typically eluted at 2.1 min.

2.1.15.4.3   ETHYLENE GLYCOL

2.1.15.4.3.1   SAMPLE PREPARATION AND ANALYSIS

Ethylene Glycol was purchased from Sigma-Aldrich-Fluka, it was used to make a calibrator of 1000mg/L in horse blood. This was serially diluted to give additional calibrators of 500, 250, 125, 62.5 mg/L. A QC was made up at 400 mg/L. A solution of Propane-1.3-diol was prepared in acetonitrile (500 mg/L), this was used as the internal standard solution.

2.1.15.4.3.2   EXTRACTION PROCEDURE 13

Pipette 100 µL standard/QC/sample into a tube, add 200 µL of internal standard (propane-1.3-diol solution in acetonitrile). Then add 100 µL phenylboronic acid

solution in 2,2-dimethoxypropane. Vortex mix and centrifuge briefly. Transfer 100 µL of supernatant to a GC vial.

2.1.15.4.3.3 GC-FID SYSTEM SETUP 2

Analysis was carried out on a RESTEK RTX-5 capillary column, (15 m x 530 µm x 1.5 µm), with an isothermal temperature of 120°C and run time of 4 minutes. Ethylene glycol typically eluted at 1.2 min and propane-1,3-diol at 2.4 min.

2.1.15.5   GC-NPD SREENING

2.1.15.5.1   SAMPLE PREPARATION AND ANALYSIS

A QC standard was prepared; it contained AMP, MA, ephedrine, MDA, MDMA, 3,4-methylenedioxyethylamphetamine (MDEA), ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and methadone (all purchased from LGC Standards, Middlesex, UK) in drug free urine at 5000 ng/mL.

2.1.15.5.1.1 EXTRACTION PROCEDURE 14

700 µL of QC / test sample were pipetted into a tube, 100 µL 5M sodium hydroxide and 150 µL internal standard (prazepam 10 mg/L) were also and the tubes were vortex mixed then centrifuged. 100 µL supernatant from each tube, was transferred to a GC vial.

2.1.15.5.1.2 GC-NPD SYSTEM SETUP

A Hewlett Packard / Agilent Technologies 6890 Series gas chromatograph incorporating an NPD detector, with an Agilent HP-5 15 x 0.53 x 1.5 µm column was used for analysis. The column temperature was initially 120 °C with a hold time of 0.5 min then it was increased 50°C/min to 290°C, with a total run time of 4.9 min.

CHAPTER 3: A STUDY OF ORAL

FLUID

3.1 INTRODUCTION

3.1.1 SALIVA VERSUS ORAL FLUID

Oral fluid can be described as all of the secretions found in the mouth, these include saliva (which is secreted from the salivary glands), oral mucosal transudate (OMT which comes from the area between the teeth and gums), mucoproteins, bacteria, enzymes, food, electrolytes and cells, (Niedbala, et al., 2001), (Samyn, et al., 1999), (Höld, et al., 1996).

Oral fluid contains secretions from the submaxillary (65%), parotid (23%) and sublingual (4%) glands, (Walsh, et al., 2003).

The most important functions of human saliva, (a major component of oral fluid) are: -

- To moisten the mucous membranes of the upper aerodigestive tract in order to facilitate speech and solubilize food to ease swallowing

- To control the bacterial flora of the mouth, and establish defence and killing mechanisms

- To supply enzymes for food digestion

(Samyn, et al., 1999).

In recent papers the term saliva has been replaced by oral fluid, at least with regard to drug testing. One explanation for this change was that as a fluid mixture, the term "oral fluid" seemed more appropriate than saliva or "whole saliva" (Gallardo & Queiroz, 2008), and probably better suited than "mixed saliva" which was also used at times, (Walsh, et al., 2003).

However, a complete explanation was given by Spieler, (2004); due to the mixture of fluids in the oral cavity, (as previously described) it was agreed at the New York Academy of Sciences meeting on saliva testing in 1993, "to use the word saliva for glandular secretions collected directly from the saliva glands (most often parotid glands), and oral fluid for fluid collected by placing absorbants in the oral cavity or by expectoration" (spitting).

3.1.2 TOXICOLOGICAL APPLICATIONS OF ORAL FLUID

It has been hypothesised that oral fluid concentrations should correlate more accurately with pharmacological responses than blood or urine, (Cone, et al., 1997). The concentration of drug in oral fluid reflects the free, nonprotein-bound drug in plasma, and its lipophilic metabolites and these are the forms of drug that are able to cross the blood-brain barrier and consequently are responsible for pharmacological drug effects, (Spiehler, et al., 2002).

However, when blood is used as a matrix for drug quantitation, it is the sum of both intracellular and extracellular bound and unbound drug that is measured, whereas urine provides the measurement of accumulated analytes since the last void of the bladder, (Schramm, et al., 1992), (Cone, et al., 1997).

In a published codeine study, Kim et al., 2002, reported that detection times in oral fluid are shorter than in urine, however detection times for codeine in plasma compared to oral fluid were found to be similar but peak concentrations were found to be significantly higher in oral fluid.

Oral fluid was recognised as an appealing matrix for drug analysis as early as the seventies. Due to the relative ease of collection and non-invasive technique, it could have a wide range of applications in both clinical and forensic toxicology.

With initial experiments mostly involving therapeutic drugs, it is preferred to blood analysis for therapeutic blood monitoring of anticonvulsants in children because parents can collect oral fluid themselves and send the sample to a laboratory, and thus the collection is painless, easier and much cheaper, (Gorodischer, et al., 1997).

There is some support for its routine application with anticonvulsants and theophylline, and applications have also been described for carbamazepine, digoxin, topiramate, and methadone, (Drummer, 2006).

For drugs of abuse monitoring, an alternative such as oral fluid could be seen as non-invasive in more ways than one, as the collection method is non-invasive and it can be collected under supervision without the potential invasion of privacy, (Schramm, et al., 1992). This could eliminate the possibility of the patient adulterating or substituting the specimen before handing it to the collector, (Kim, et al., 2002), and could remove the need for validity tests.

3.1.3 MECHANISM OF DRUG TRANSFER INTO ORAL FLUID

Many of the drugs that can be detected in oral fluid are transferred from the blood by passive diffusion through the membrane lipids of the salivary glands. The drug has to pass through the capillary wall, the basal membrane and the membrane of the glandular epithelial cells, which is the rate-determining step (Haekel, 1996), (Höld, et al., 1996).

The different physiochemical properties of each drug affect their diffusion into oral fluid. These properties include: -

- pKa

- Lipid solubility

- Molecular mass

- Spatial configuration

The degree of plasma protein binding and the pH of each medium will also affect diffusion, (Samyn, et al., 1999).

However, although passive diffusion is the main route by which drugs transfer into oral fluid, it is not the only route. Active transport is thought to occur for some drugs, where their concentration is higher in oral fluid than in plasma, for example this is thought to be the case for valproic acid, (Haekel, 1996), (Höld, et al., 1996).

The characteristics of different membrane systems and the properties of different drugs, results in differences in oral fluid / plasma (OF/P) concentration ratios.

3.1.4 EFFECTS OF ORAL CONTAMINATION ON INTERPRETATION OF RESULTS

Oral cavity contamination can also be a problem with oral fluid testing, when the drug is administered orally, intra-nasally, or by smoking, or passive smoking, resulting in elevated drug concentrations at early collection times. It has already been proven that to simply rinse the oral cavity after administration does not eliminate the contaminating drug, (O'Neal, et al., 1999), (Idowu, 1982). However, research has shown that contamination can be overcome if a time delay is left between administration and collection, because this time allows for drug absorption. The appropriate absorption times for each drug will have to be determined from both literature reviews and by experimentation.

It has been reported that following administration of codeine by one of the above routes, for the first 1-2 hours elevated oral fluid/plasma ratios resulted. However, if the oral fluid was not collected until two hours after administration, to allow for drug absorption, then concentrations in oral fluid and plasma were similar and the oral fluid/plasma ratio remained constant, (O'Neal, et al., 1999).

Other studies have shown similar trends, whereby if a suitable delay time is observed prior to collection (to eliminate oral contamination), then oral fluid concentrations can be used to estimate plasma concentrations using the oral fluid/plasma ratio.

A recent study proved that the ingestion of poppy seeds could lead to a false positive morphine result in oral fluid. However, this was only true for up to one hour after ingestion. Beyond this time the morphine was not detected above the SAMHSA proposed cut-off of 40 ng/mL, (Rohrig, 2003).

This suggests that provided the two-hour absorption time is allowed to elapse after administration of seeds, then this contamination should not occur. However, this also demonstrates that the possibility of oral contamination must be taken into account when opiate data is being interpreted.

For cannabis, the main route of drug entry into the oral cavity appears to be direct deposition during use. Residues of Δ^9-tetrahydrocannabinol (THC) are sequestered in oral tissue and appear in oral fluid, contribution of THC to oral fluid from blood is known to be minimal, (Niedbala, et al., 2001). This presents a problem with interpretation of concentrations, because the measured level of THC may be elevated by oral contamination.

3.1.5 EFFECTS OF pH ON INTERPRETATION OF RESULTS

The pH of oral fluid affects the passive diffusion of drugs, and consequently the OF/P ratio of drugs. The influence of salivary pH on this transport depends upon the pK_a of the drug, the equation of *Rasmussen, 1964*, (Höld, et al., 1996).

Therefore, it is important to have a good understanding of this equation, which is based on pH partitioning, and can be used to estimate theoretical oral fluid/plasma ratios (OF/P ratios), (Hold et al., 1996).

In humans the pH of oral fluid in resting situations is 6.8, (Gallardo & Queiroz, 2008) so it is usually more acidic than plasma (pH 7.4), so basic drugs are found at higher concentrations in oral fluid than in plasma. Therefore, the OF/P ratio is equal to or less than 1 for all acidic drugs and equal to or greater than 1 for basic drugs. However, if the drug is protein bound then this will only be true for the free fraction, when it is bound the system is in equilibrium, (Haekel, 1996).

The pH is crucial to getting the correct OF/P ratio, and the pH can be easily affected, for example stimulation to cause salivation, will alter flow rate, which will alter the pH. Differences therefore in experimental methods, could account for a difference in experimentally determined OF/P ratios to theoretical OF/P ratios. This theory will need to be reviewed and could be demonstrated in volunteer studies, e.g. stimulated oral fluid versus non-stimulated.

3.1.6 COLLECTION OF ORAL FLUID

Historically collection of oral fluid involved spitting into a tube and chewing parafilm or citric acid in order to stimulate oral fluid production but both these methods had problems associated with them. Parafilm has been reported to absorb lipophilic drugs, (Paxton, 1979), and Toennes, 2005 reports that the use of citric acid or sour candy to stimulate salivary flow, has the disadvantage of increasing pH causing lower drug concentrations and affecting drug detectability. This can also complicate interpretation because although the citric acid will naturally decrease salivary pH, its purpose is to increase salivary flow which is known to increase pH so it is more basic, (Gallardo & Queiroz, 2008).

 More recently oral fluid collection devices, (originally designed for HIV research), have become commercially available. They usually consist of a cellulose pad which goes underneath the tongue, and often contain a preservative buffer for storage and / or transportation.

Analytical problems with the preservative buffer in these types of devices have been reported. Some of them contain detergent molecules that can strip the phase from LC-MS columns, reducing the life of the columns, and thus making them impractical for use, (Allen, et al., 2005).

There are several other problems associated with oral fluid collection devices. Although some have a volume indicator, the accuracy of this is questionable, and once the oral fluid has absorbed onto the pad, it needs to be extracted.

A series of in vitro experiments using different devices found that the mean collection volumes between devices ranged from 0.82 – 1.86 mL, and the percentage of the collection volume that could be recovered varied from 18% to 83%, (Crouch, 2005).

3.1.7 GUIDELINES

It is important to consider the target concentrations required for the drugs under investigation. This is directly affected by the application of the technique, whether the detection of drugs in oral fluid is intended for clinical screening or workplace drug testing.

If the service is to be used for clinical cases, such as samples from drug rehabilitation clinics, then there are no set guidelines to follow. In this case the Laboratory can define cut-off levels deemed suitable for this type of sample analysis, and confirm presence of drugs by a secondary method, where it is considered necessary.

However, if workplace testing is to be used then it is important to follow the guidelines proposed by SAMHSA. These state that all specimens must be screened using an appropriate technique, and that any positive screening results must be confirmed using a confirmatory analytical procedure such as (GC-MS) or possibly (LC-MS).

SAMHSA have drafted proposed screening cut-off concentrations and confirmatory test cut-off concentrations for common drugs of abuse in oral fluid, (see Table 3.1).

Oral Fluid	Proposed SAMHSA Cut-off concentration		Cut-off concentration when diluted 1 in 4 with buffer	
Drug	Screening	Confirmation	Screening	Confirmation
THC & metabolites	4 ng/mL	2 ng/mL	1 ng/mL	0.5 ng/mL
Cocaine metabolites	20 ng/mL	8 ng/mL	5 ng/mL	2 ng/mL
Opiates	40 ng/mL	40 ng/mL	10 ng/mL	10 ng/mL
6-acetylmorphine		4 ng/mL		1 ng/mL
Amphetamines	50 ng/mL	50 ng/mL	12.5 ng/mL	12.5 ng/mL

Table 3.1   The proposed cut-off concentrations in oral fluid, (SAMHSA, 2004)

3.2 METHOD VALIDATION

It is generally accepted that it is necessary to use validated methods in a Toxicology Laboratory, in order to produce reliable and inter-changeable data, (Bramley, et al., 2004). For forensic work, a quantitative assay should be validated for accuracy, precision, linearity, and limit of detection (LOD), (Jones, 2004).

LINEARITY

For Linear regression analysis, a calibration curve was prepared over an appropriate concentration range, (preferably with a minimum of 5 standards). This is used to calculate the regression coefficient (e.g. r^2), the calculated r^2 value must be equal to or greater than 0.98.

LIMIT OF DETECTION (LOD)

To determine the LOD, 10 blanks were extracted together with a calibration curve and suitable internal quality control standards (IQCs).

The LOD was expressed as the equivalent concentration of the mean 'blank' value (where n = 10), plus 3 SD's, which was determined relevant to the IQC response.

REPRODUCIBILITY (WITHIN BATCH)

To test the reproducibility of an assay, 10 replicates at a low concentration were extracted with 10 replicates at a high concentration. This data allowed the calculation of the accuracy (the mean concentration where n=10) and the precision (coefficient of variation, (% CV) where n=10), of the assay.

3.2.1 ELISA SCREENING

3.2.1.1 OPIATE VALIDATION

In order to test the linearity and accuracy of the ELISA opiate kit (IDS),

Morphine, codeine, 6-MAM and dihydrocodeine (DHC) curves were set upp with calibrators spiked separately at 0, 1, 2, 3, 5 and 6 ng/mL and internal quality control standards spiked at 4 ng/ml. In addition oral fluid samples with known codeine concentrations were also extracted, (these samples were from a volunteer study where the codeine concentrations had been previously measured by GC-MS, before being frozen for storage). All these were extracted using Extraction Procedure 1, (Chapter 2).

3.2.1.2   RESULTS

	[Morphine] in ng/mL	[Codeine] in ng/mL	[6-MAM] in ng/mL	[Dihydrocodeine] in ng/mL
IQC spiked at 4 ng/mL	4.26	3.74	4.39	3.68
IQC spiked at 4 ng/mL	4.34	3.63	4.52	3.96

Table 3.2   Results of IQCs calculated for the opiates

Volunteer 1	Codeine concentration in ng/mL		
Time post-dose (in h)	GC-MS	ELISA	% Difference
0	0	0	n/a
0.4	195	226.1	**15**
1.2	89	91.9	**3**
2	57	48.3	**7**
3	36	39.8	**10**
5	18	14.7	**20**
9	0	1.9	n/a
12	0	1.1	n/a

Table 3.3   Comparison of results from one volunteer in the initial pilot codeine study

3.2.1.3 ELISA COCAINE / BENZOYLECGONINE VALIDATION

In accordance with the kit insert, calibrators were spiked at 10, 20, 30 and 40 ng/mL with benzoylecgonine (BZE) (1mg/mL stock purchased from LGC Standards). These were extracted with internal quality controls (spiked at 15, 20 and 25 ng/mL). The extraction procedure was exactly the same as for the opiate assay, procedure 5.

3.2.1.4 RESULTS

IQC spiked at 15 ng/mL	16 ng/mL
IQC spiked at 20 ng/mL	20 ng/mL
IQC spiked at 20 ng/mL	22 ng/mL
IQC spiked at 25 ng/mL	26 ng/mL

Table 3.4   Results and EQCs calculated for BZE

3.2.1.5 DISCUSSION

For the opiate assay, the QC results were within an acceptable range of the spiked concentration (i.e. within 20%) for morphine, codeine, 6-MAM and dihydrocodeine, (refer to Table 3.3). As an additional validation test the codeine concentrations from some volunteer study samples were also calculated and compared to the results from a calibration curve on the GC-MS (see Table 3.4). The results showed good agreement between the two sets of data, with no more than 20% difference between the 5 values compared.

There was no codeine detected by the GC-MS method in the 2 final samples, (any codeine present must have been below the 10 ng/mL LOQ for the assay) so it was not possible to compare these.

For the BZE assay, the IQC values were also accurate, i.e. within 10% of the spiked values.

It should be noted that although the results appear to be accurate (as described above) on the Manufacture's (IDS) Certificate of Analysis their results for a Typical Standard Curve are very different to those achieved in the experiments described previously. They quote that for the zero calibrator (0 ng/mL) the absorbance should be 2.87 optical density (O.D.) but in the experiments described only a maximum absorbance of 0.84 O.D. was achieved for the 0 ng/mL calibrator with the morphine kit and even lower O.D.'s were found with the BZE kit, maximum of 0.55 O.D. It also states on the Certificate of Analysis that the minimum negative O.D. is 1.5 which could suggest that the results described previously should be rejected. However, it is acknowledged that ELISA should only be used as a semi-quantitation method, and the Manufacture's recommend that all results should be confirmed.

3.2.1.6 CONCLUSION

As a screening technique ELISA has the advantage that only a tiny amount of sample (20 μL) is required for analysis but the disadvantages are that it is both costly and time-consuming.

3.2.2 LC SCREENING VALIDATION

As the ELISA kits proved to be very expensive, other possible screening techniques were investigated for the screening of amphetamines.

3.2.2.1 METHOD

Combined calibrators for Amphetamine (AMP), Methamphetamine (MA), 3,4-methylenedioxyamphetamine   (MDA),   3,4-methylenedioxymethamphetamine (MDMA) were prepared and extracted using Extraction Procedure 2.

These extracts were analysed on two different systems, an HPLC-DAD and a tandem LC-MS system.

3.2.2.2 RESULTS
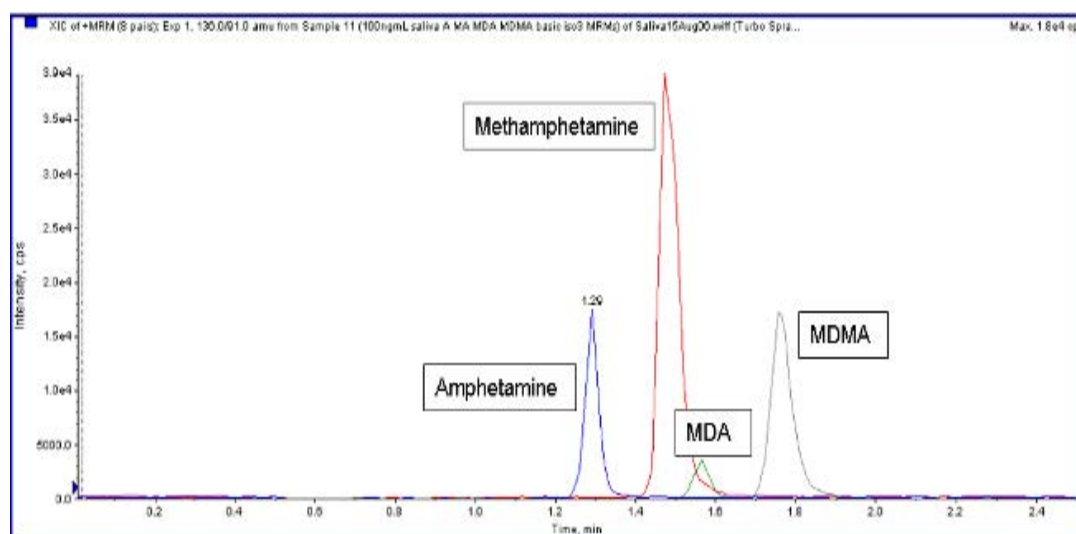
Figure 3.1   Chromatography for isocratic amphetamine LC-MS method

There was a problem with the results from the HPLC-DAD experiment as methamphetamine and MDA were found to co-elute. However this could be overcome by spiking separate standards, so that one curve would measure amphetamine and methamphetamine and a separate curve would be created to measure MDA and MDMA.

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This is not a problem with the LC-MS-MS as the extraction of MRMs allow for the quantitation of each peak when co-elution occurs. For the calibration curve to be accepted the IQCs needed to be within 20% of the spiked value (or 80% accurate). This was true for the majority of the QC values but there were 2 IQC values for MDA that were more than 20% out, so this method of analysis for MDA would require additional validation, particularly for inter-assay variability, (see Table 3.5). However, for screening of MDA it could be considered to be acceptable particularly as good separation was achieved between the amphetamines, (see Figure 3.1). It is possible that more accurate results could be achieved by using deuterated standards for each drug as an internal standard rather than using norfenfluramine as a generic IS.

3.2.2.3   FURTHER METHOD DEVELOPMENT

After the amphetamine experiments proved successful, (see Figure 3.1), it was hypothesised that a ramp could be used for LC-MS to analyse multiple basic drugs, while using the same sample extract therefore conserving valuable specimen volume. To test this hypothesis blank oral fluid was spiked at known concentrations and corresponding deuterated internal standards were added, they were extracted as before, (Extraction Procedure 2) and analysed on the LC-MS. Data was collected using multiple reaction monitoring (MRM mode) for the transitions shown in Table 3.6.

Drug	MRM	ISTD MRM	Spiked value	Calculated concentration	Accuracy (%)
Amphetamine	136/91	220/220	60	55.1	92
Amphetamine	136/65	220/220	60	57.2	95
Amphetamine	136/91	220/220	60	68.5	88
Amphetamine	136/65	220/220	60	74.7	80
Amphetamine	136/91	220/220	60	56.2	94
Amphetamine	136/65	220/220	60	55.9	93
Methamphetamine	150/91	220/220	60	60.8	99
Methamphetamine	150/119	220/220	60	61.2	98
Methamphetamine	150/91	220/220	60	73.7	81
Methamphetamine	150/119	220/220	60	73.3	82
Methamphetamine	150/91	220/220	60	51.4	86
Methamphetamine	150/119	220/220	60	53.5	89
MDA	180/163	220/220	60	67.9	88
MDA	180/135	220/220	60	74.7	80
MDA	180/163	220/220	60	85.1	**71**
MDA	180/135	220/220	60	88.6	**68**
MDA	180/163	220/220	60	65.8	91
MDA	180/135	220/220	60	70.2	85
MDMA	194/163	220/220	60	64.7	93
MDMA	194/135	220/220	60	63.5	94
MDMA	194/163	220/220	60	68.8	87
MDMA	194/135	220/220	60	72.8	82
MDMA	194/163	220/220	60	58	97
MDMA	194/135	220/220	60	57.2	95
Amphetamine	136/91	220/220	300	298	99
Amphetamine	136/65	220/220	300	289	96
Amphetamine	136/91	220/220	300	267	89

Drug	MRM	ISTD MRM	Spiked value	Calculated concentration	Accuracy (%)
Amphetamine	136/65	220/220	300	283	94
Amphetamine	136/91	220/220	300	304	99
Amphetamine	136/65	220/220	300	290	97
Methamphetamine	150/91	220/220	300	321	93
Methamphetamine	150/119	220/220	300	321	93
Methamphetamine	150/91	220/220	300	272	91
Methamphetamine	150/119	220/220	300	277	92
Methamphetamine	150/91	220/220	300	333	90
Methamphetamine	150/119	220/220	300	332	90
MDA	180/163	220/220	300	272	91
MDA	180/135	220/220	300	279	93
MDA	180/163	220/220	300	254	85
MDA	180/135	220/220	300	248	83
MDA	180/163	220/220	300	266	89
MDA	180/135	220/220	300	282	94
MDMA	194/163	220/220	300	284	95
MDMA	194/135	220/220	300	286	95
MDMA	194/163	220/220	300	263	88
MDMA	194/135	220/220	300	256	85
MDMA	194/163	220/220	300	299	100
MDMA	194/135	220/220	300	288	96

Table 3.5   LC-MS-MS IQC data for 2 different transitions for each drug

Drug / Metabolite	Transition	Collision Energy (CE) for fragmentation
Methamphetamine-d5	155 / 91	35
MDMA-d5	199 / 135	35
Cocaine	304.1 / 182 304.1 / 150	35 35
Cocaine-d3	307.1 / 185	35
Methadone	310.2 / 265 310.2 / 105	35 35
Methadone-d3	313.2 / 268.2	35
Buprenorphine	468.4 / 468.4 468.4 / 414.2	20 70
Buprenorphine-d4	472.4 / 472.4	20

Table 3.6   Additional MRM transitions

As good chromatography was achieved for these drugs in the combined standard of 10 ng/mL (see figure 3.2) the method was accepted as a possibility for the screening of oral fluid.

The possibility of including EDDP and norbuprenorphine in the screen was explored. In addition to this deuterated internal standards for amphetamine and MDA were also tested using this method. Their MRMs were determined and then added to the LC-MS method (see Table 3.7).

Validation experiments was set up to determine the lowest concentration that at which identification was possible, the limit of detection (LOD) or limit of quantitation (LOQ) for each of the drugs, refer to Table 3.8 for the results.

Drug / Metabolite	Transition	Collision Energy (CE) for fragmentation
Amphetamine-d5	141 / 91	35
MDA-d5	185.1 / 134.9	35
EDDP	278.2 / 233.9 278.2 /186.2	35 35
EDDP- d3	281.2 / 234.2	35
Norbuprenorphine	414 / 414 414 / 101.2	20

Table 3.7   Additional MRM transitions

3.2.2.4   RESULTS
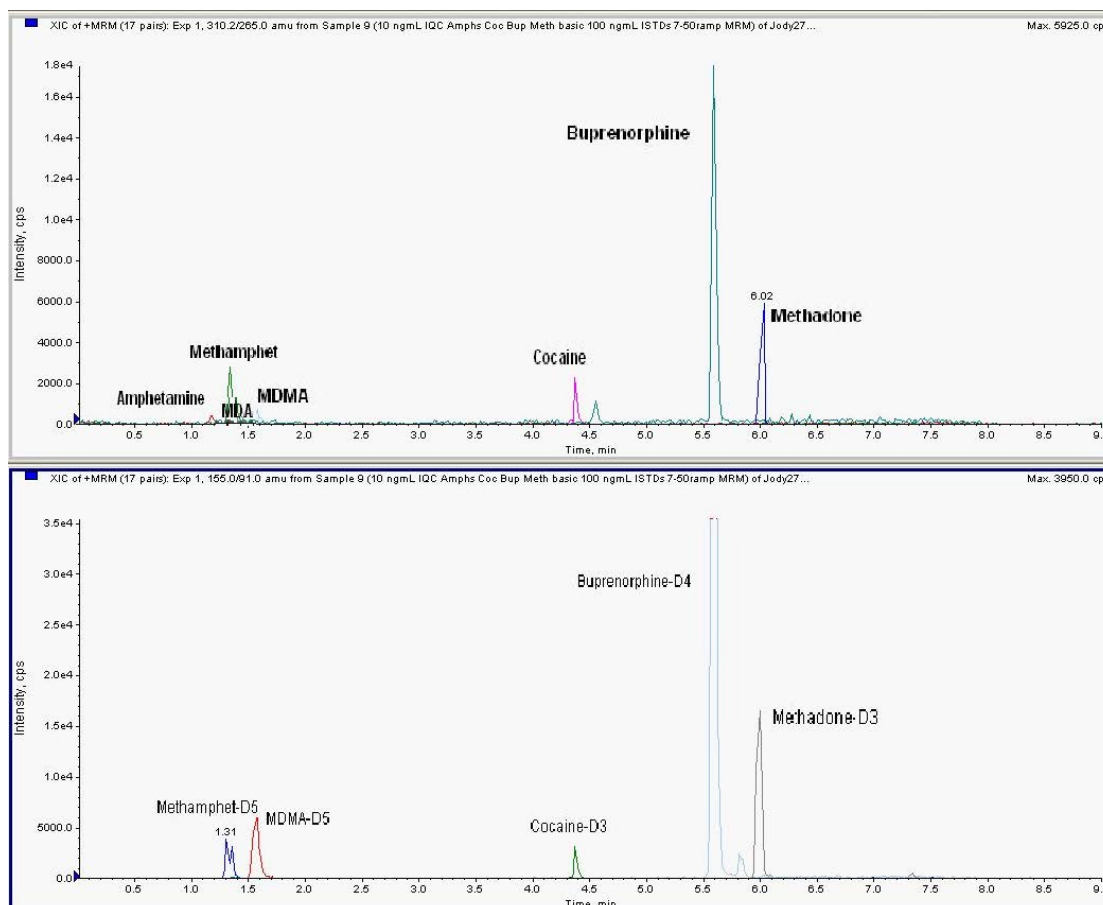
Figure 3.2   Chromatography for new ramp LC-MS Method

The results from the gradient ramp method showed good chromatography (see Figure 3.22) and demonstrated the ability to look for a wide range of drugs using a single extract, this helps to conserve sample volume which is precious when oral fluid is being analysed, due to the low volumes available. If any of the samples were found to be positive for amphetamine, methamphetamine, MDA or MDMA then it would be possible to re-inject the extract(s) and they could be specifically analysed isocratically.

Analyte	LOD / LOQ (ng/mL or µg/L)
Amphetamine	3.5
Methamphetamine	1
MDA	3.5
MDMA	5
Cocaine	1
Norbuprenorphine	2
Buprenorphine	2
EDDP	1
Methadone	1

Table 3.8   LC-MS new ramp LOD / LOQ Results

In order to further test the LC-MS method, external quality control (EQC) oral fluid samples were obtained from a proficiency testing scheme, (UKNEQAS, Cardiff Bioanalytical Services Ltd, 16 Mount Stuart Square, Cardiff CF10 5DP). These were analysed alongside in-house internal quality control samples (IQCs), refer to Table 3.9 and 3.10 for the results.

ID	Drug	Result	EQC	CV (%)	IQC 50 ng/mL	CV (%)
1	Methamphetamine	97.5	100.0	2.5	54.8	8.8
2	Methadone	90.6	110.5	18.0	60.0	16.7
3	EDDP	8.6	12.0	28.3	55.5	9.9
4	MDMA	26.2	29.9	12.4	45.5	9.9

Table 3.9   Results for external quality control oral fluid samples

ID	Drug	Result	EQC	Method mean	No of results submitted
1	Methamphetamine	97.5	100.0	128	5
2	Methadone	90.6	110.5	88.9	2
3	EDDP	8.6	12.0	No Data	0
4	MDMA	26.2	29.9	30.8	5

Table 3.10   Comparison of results for external quality control scheme

3.2.2.5   DISCUSSION

The results from the LOD / LOQ experiment are comparable with the concentrations found in similar LC–MS studies (see Table 3.10). Allen et al. (2005) reported LC-MS cut-offs of 5 μg/L for methadone and cocaine and 0.5 μg/L for EDDP and more recently ∅iestad et al. (2007) reported the following LOQs: amphetamine <6.8 μg/L, methamphetamine <3 μg/L, MDA <3.6 μg/L, MDMA <3.9 μg/L, cocaine <0.78μg/L and for methadone 4 μg/L. If these published results are compared directly with the results displayed in Table 3.8 it is evident that although there is a slight variation between the concentrations reported in each of the studies, they are all in the same region. The LOD / LOQ

concentrations could also be considered to be applicable as similar values have been used in other studies. The SAMHSA proposed cut-offs in oral fluid could also be used as an indication that the LODs / LOQs are in the correct region, although it is not clear how these proposed cut-offs were determined.

These LC-MS methods described here have been used as screening methods only but they could easily be used as quantitation methods if further validation work was carried out. However, drug screening rather than quantitation can be extremely useful in some settings, like in drug addiction clinics or prisons, where presence or absence of a drug would usually answer the question being asked.

The results from the proficiency testing scheme can provide an insight into how the assay performed quantitatively, (Tables 3.9 and 3.10) for some analytes. All IQC results were within 20% of the spiked value and for the EQCs it was only EDDP that was greater than 20% different. The scheme collated data from the 5 laboratories that participated, (Table 3.10) our results look comparable. As none of the participants reported a level for EDDP, it is difficult to comment on that performance but perhaps the lack of results could suggest that other laboratories have experienced difficulty accurately measuring this particular analyte.

3.2.2.6   CONCLUSION

The combined screen is a quick and sensitive way to analyse for amphetamine, methamphetamine, MDA, MDMA, cocaine, buprenorphine, norbuprenorphine, EDDP and Methadone. If patient samples are found to be positive for any of these drugs, it should be possible for further confirmatory tests to be carried out, as only a small amount of oral fluid sample will have been used for this test.

3.2.3 CONFIRMATION OF OPIATES IN ORAL FLUID

3.2.3.1   INTRODUCTION

It is important to be able to detect and accurately identify opiates in a variety of matrices as they are widely used in Society today. The ability to differentiate between prescribed opiates and illicit heroin is essential. This is possible by detection of the main metabolite of heroin, 6-monoacetylmorphine (6-MAM).

3.2.3.2   METHODS

Calibration curves were prepared morphine, codeine, DHC and 6-MAM and extracted as described in Chapter 2, Extraction procedure 5, they were analysed on the system described using GC-MS parameters 1.

3.2.3.3   MATRIX MATCHING

In the comparative matrix studies, water versus saliva, the morphine and codeine calibration curves were linear, and very similar, so it was considered most logical and more cost effective to use water as a drug matrix for further studies.

Although the correlation between oral fluid and water was good, these experiments revealed a problem. It became apparent that the codeine calibration curve (for both oral fluid and water) did not pass through zero.

Inspection of the GC-MS traces revealed a peak with a common ion, at the same retention time as codeine. In an attempt to estimate the size of the problem, the equation of the line was used to calculate the amount of codeine present in the blank, which was found to be 60 ng/mL. This was obviously going to be a problem when for the calculation of LOD of the assay, e.g. the

concentration of the background noise, as this would be above the proposed SAMHSA confirmatory cut-off of 40 ng/mL.

To check that the interfering peak was not contamination and that the problem was reoccurring, a series of blanks were ran on the GC-MS, blank water, blank methanol, blank buffer (from collection device) and saliva/buffer collected from 8 volunteers. The coefficient of variation (CV) between the blanks was calculated to be 9%, and the mean concentration in the blanks was calculated at 21 ng/mL. Although this is below the 40 ng/mL proposed SAMHSA cut-off, it still would not be useable with a collection device that holds a preservative buffer for transportation. This is because there is often 3 mL of buffer, which means that the detection limit required to meet the guidelines would need to be 10 ng/mL instead of 40 ng/mL.

The 234 ion was considered to be a useful alternative quantitative ion, although its abundance was much lower than the 178 ion. However, when the validation study results were re-calculated with the 234 ion, the findings were similar, with a CV calculated at 10% and the equivalent concentration of the interfering peak was 16 ng/mL.

The GC-MS was operated in scan mode in an attempt to find an alternative ion, for quantitation, no suitable alternative ion patterns were found. Attempts were made to identify the interfering peak, using the library but no matches were found.

It was hypothesised that maybe the derivatisation reagent was reacting with a component in both the oral fluid and water as well as codeine and this was causing a peak on the trace, at the same retention time.

Therefore the extraction was tried without a derivatisation step and the results were found to be much better.

3.2.3.4 CODEINE METHOD DEVELOPMENT

Initially a 10,000 ng/mL standard in butyl acetate was run on the GC-MS, on a long scan (50 – 550 m/z) to test where the peak eluted and which ions were predominant. The same instrumentation was used as described previously, (GC-MS parameters 1).

The GC-MS parameters were optimised to increase sensitivity, (GC-MS parameters 2) and the derivitisation step was removed from the extraction, (Extraction Procedure 6).

Finally, Selective ion monitoring (SIM) was used to increase sensitivity further still and allow for detection at 10 ng/mL or less. The SIM looked for the following ions: 299, 162, 188 and 214 for codeine and 229 and 302 for codeine-d3, (see Fig 3.9). The 299, 162 and 229 ions were compared to identify which would be the best for quantitation, and the 302 and 232 to check which would be best for the internal standard, using the linearity of the assay and the accuracy of the 40 ng/mL internal quality control (IQC) for assessment, (Table 3.11).

3.2.3.5 RESULTS

Ions used for quantitation	Equation of the line:	Linearity (R^2)	Concentration IQC 40 ng/mL	CV (%)
299 for codeine 232 for codeine-d3	y = 0.0234x - 0.0384	R^2 = 0.9936	45	12.5
162 for codeine 232 for codeine-d3	y = 0.0085x + 0.046	R^2 = 0.9948	46	15
229 for codeine 232 for codeine-d3	y = 0.006x + 0.0896	R^2 = 0.9917	79	97.5
299 for codeine 302 for codeine-d3	y = 0.0064x + 0.0214	R^2 = 0.9999	**42**	5
162 for codeine 302 for codeine-d3	y = 0.0023x + 0.0246	R^2 = 0.9996	44	10
229 for codeine 302 for codeine-d3	y = 0.0016x + 0.0335	R^2 = 0.9987	81	102.5

Table 3.11   Comparison of codeine ions for quantitation

In order to assess the various ion combinations, the CV was calculated, (Table 3.11), generally a CV of <20% is deemed within acceptable limits.

In this case the best target ion for codeine was found to be 299 amu and for codeine-d3 (IS) it was 302 amu, as together they gave the most accurate IQC result (CV=5%), with the best linearity, (R^2 = 0.9999).
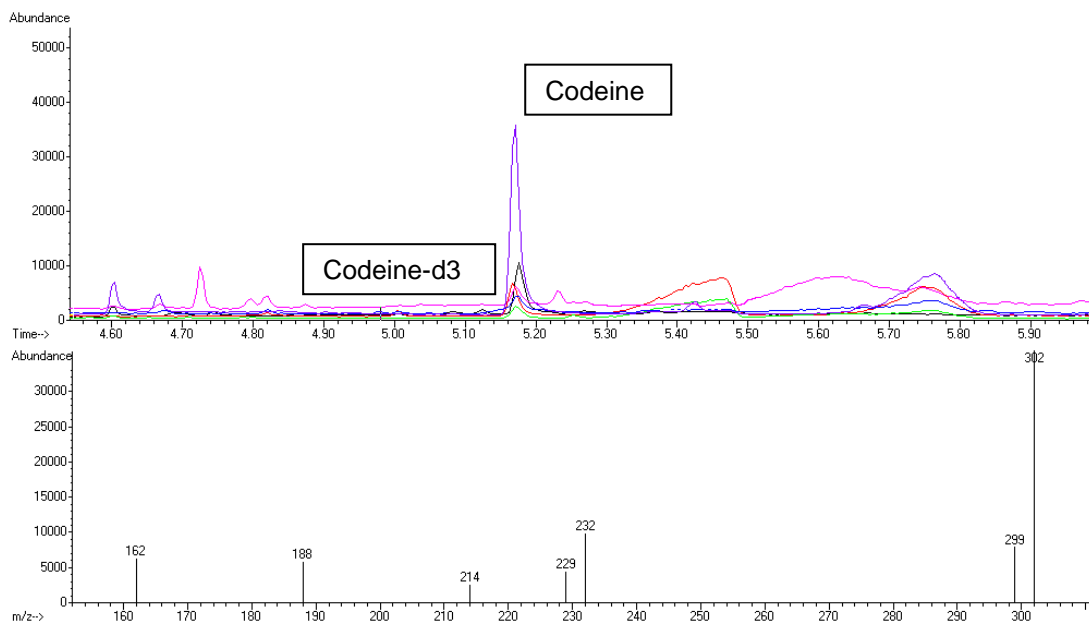
Figure 3.3   GC-MS Trace to show codeine retention time (5.17) and typical ion fragmentation pattern when ran in SIM mode
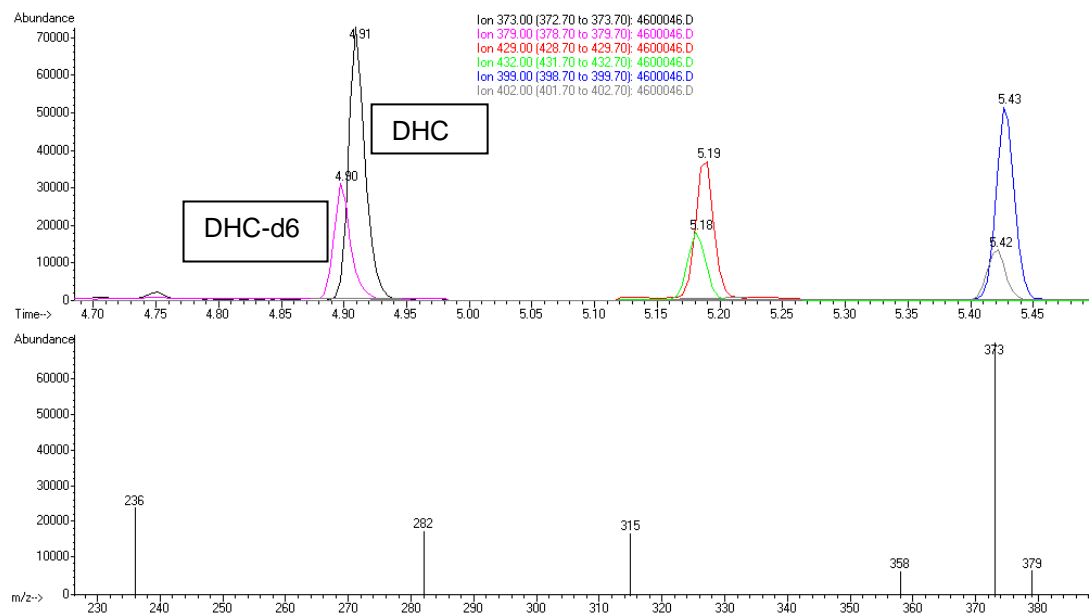
Figure 3.4   GC-MS Trace to show DHC retention time (4.91) and typical ion fragmentation pattern when ran in SIM mode
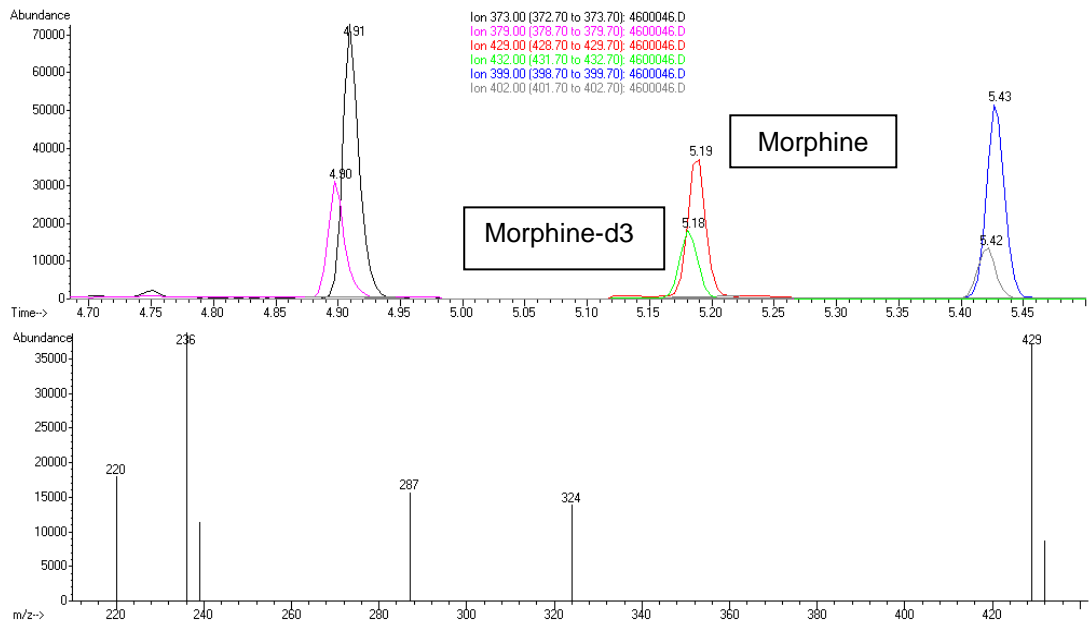
Figure 3.5   GC-MS Trace to show morphine retention time (5.18) and typical ion fragmentation pattern when ran in SIM mode
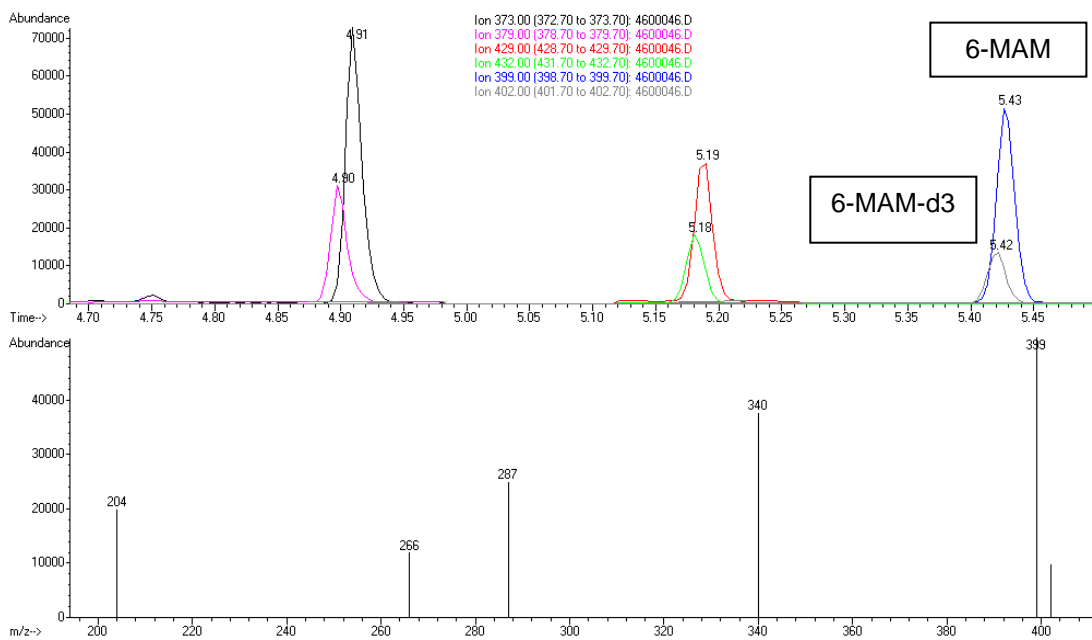
Figure 3.6   GC-MS Trace to show 6-MAM retention time (5.43) and typical ion fragmentation pattern when ran in SIM mode

Drug	Linearity ng/mL	LOD ng/mL	LOQ ng/mL	Accuracy ng/Ml n= 10	Precision % n= 10
Morphine	1000	8	10	40 = 36 400 = 398	40 = 2.5 400 = 2.6
DHC	1000	1	10	40 = 39 400 = 408	40 = 2.5 400 = 3.7
6-mam	1000	3	5	10 = 9 100 = 102	10 = 6.4 100 = 3.7
Codeine *	8000	6	10	40 = 38 400 = 402	40 = 3.2 400 = 2.8

* Results obtained from extraction procedure 2

Table 3.12   Summary of Opiate Validation Results

3.2.3.6   DISCUSSION

Despite the small volume of sample used, the results of the validation studies proved acceptable for all the opiates studied: morphine, dihydrocodeine, 6-MAM and codeine (Table 3.12). All the curves had an R^2 value of at least 0.99 and the quality control values calculated within 10% of the spiked concentration so were deemed acceptable. In addition the assays all proved to be reproducible with precision values all well below 10% and accuracy within 10% of the spiked concentration. The limit of detection values are all below 10 ng/mL, which was the target value (SAMHSA cut-off being 40, and corrected to account for 3 mL buffer, found in some brands of collection device). The limit of quantitation was taken as the first calibrator above the LOD, where a peak was detectable at the correct retention time and the ion fragmentation pattern matched sufficiently.

3.2.4 CONFIRMATION OF BENZOYLECGONINE IN ORAL FLUID

3.2.4.1   INTRODUCTION

The aim of this study was to develop and validate a method for detection and measurement of cocaine and / or its metabolites in oral fluid, by GC-MS. Studies have proved that parent cocaine, Benzoylecgonine (BZE) and ecgonine methyl ester (EME) can all be detected in oral fluid following cocaine use. However, cocaine has a pKa of 8.6 resulting in highly pH dependent concentrations being detected. In contrast the metabolites BZE and EME have pKa constants of less than 5.5 so oral fluid concentrations are less pH dependent and more consistent (Kato, et al., 1993).

It has been estimated that an oral fluid pH change from 6.5 from 7.6 could decrease the amount of cocaine detected by a factor of 12 (Jufer, et al., 2000). Therefore due to changes in oral fluid pH and the effect of different collection methods it can be concluded that that the metabolites are going to give more reliable and reproducible results when measured. BZE was found to have the longest detection time in both oral fluid and plasma when compared to other cocaine metabolites (Jufer, et al., 2000).

BZE is the cocaine metabolite that is included in the SAMSHA proposed guidelines monitoring illicit drug use by analysing with a proposed cut-off for confirmatory techniques of 8 ng/mL.

3.2.4.2   METHODS

A calibration curve was prepared in duplicate, for BZE and two different extractions were used for each, as described in Chapter 2, Extraction procedure

1 and Extraction procedure 7, (Cone, et al., 1994), they were analysed on the system previously described, using GC-MS parameters 3.

3.2.4.3   RESULTS

The two extraction procedures were compared and the BZE peak height abundance was much greater for Extraction procedure 1 compared to Extraction procedure 3.

However, there were problems with both the accuracy and sensitivity at low levels, so the decision was made to increase the extraction volume from 500 µL to 1mL.

The results showed that even when the larger sample volume of 1 mL was used for the extraction, the very low calibrators, 2 and 5 ng/mL caused problems. Although peaks were detected at these low levels the corrected results did not fit with the linear part of the line derived from the other calibrators, and the ion patterns were not consistent.
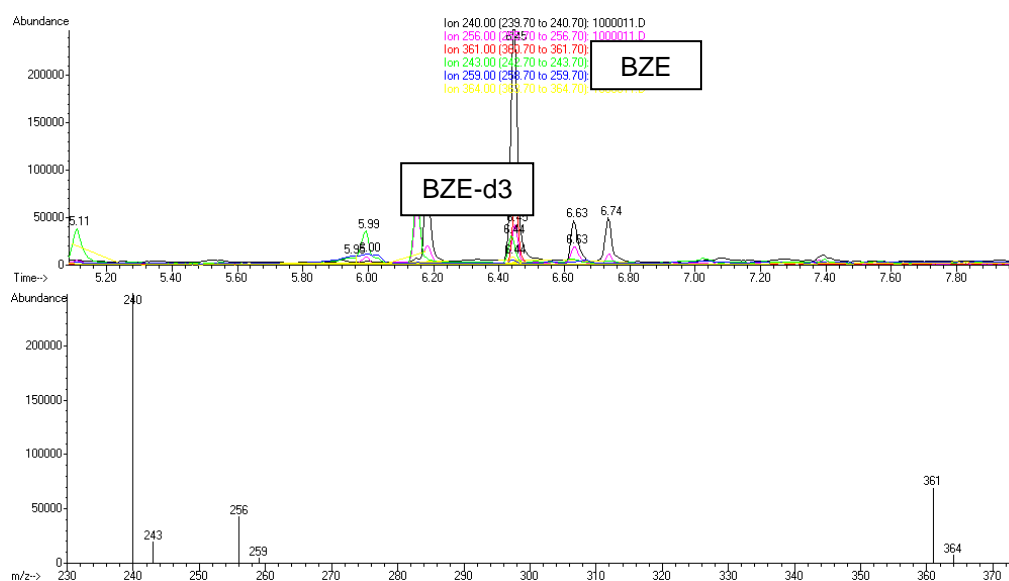
Figure 3.7   GC-MS Trace to show BZE retention time of 6.45 and ion

fragmentation pattern

Drug	Linearity ng/mL	LOD ng/mL	LOQ ng/mL	Accuracy ng/mL n = 10	Precision % n = 10
Benzoylecgonine	4000 R^2= 0.9997	13	15	32 = 33.2 320 = 315.2 15 = 14.4	32 = 7.5 320 = 1.4 15 = 8.9

Table 3.13   A Summary of the method validation results

3.2.4.4   DISCUSSION

Initially it was the aim of this project to be able to reproducibly detect BZE concentrations at 2 ng/mL and quantify at a minimum of 8 ng/mL, using only 500 µL of sample. Despite different approaches to this problem, including increasing sample volume to improve sensitivity and trying a different extraction method altogether, this has not proved to be possible with the in-house laboratory resources.

A possible solution to this problem could be to use solid-phase extraction to concentrate the drug before derivatisation (Kolbrich, et al., 2003), (Schramm, et al., 1993), (Cone, et al., 1997), (Kato, et al., 1993), (Jufer, et al., 2000), (Jenkins, et al., 1995). However, this method would prove expensive and may not turn out to be cost-effective for routine use.

Consequently, validation studies were carried out using 1 mL sample, and the limits of detection and quantitation using extraction procedure 5, were determined, (Table 3.13).

Although these cut-offs do not lie within the proposed Work-place limits, there is no reason why this validated assay could not be used for clinical analysis.

Most clinical cases involve working with samples from addiction clinics and these have much higher drug concentrations than those proposed by SAMHSA, due to routine drug use.

A few studies have been carried out which could give an indication of expected BZE levels.

In a study described by Jenkins et al. (1995) a single 44.8 mg intravenous dose of cocaine was administered, peak concentrations ranged from 428 to 1927 ng/mL. In another study with chronic cocaine users, involving large multiple doses, Jufer et al, (2000) reported that the mean Cmax for benzoylecgonine in oral fluid was 2980 ng/mL.

However these figures alone are difficult to interpret, since there seems to be a lack of data published in this area.

3.2.4.5   CONCLUSION

In conclusion, the validation parameters achieved seem acceptable for cocaine detection in clinical cases. Further studies involving patient samples from drug clinics will prove if this is true, and if this assay performance is satisfactory for that need.

3.2.5 CONFIRMATION OF AMPHETAMINES IN ORAL FLUID

3.2.5.1 INTRODUCTION

The following amphetamines have been included in the SAMHSA proposed guidelines for oral fluid testing: AMP, MA, MDA, MDMA and MDEA.

The proposed test cut-off concentration has been set at 50 ng/mL for both screening and confirmation testing for all the above amphetamines, (SAMHSA, 2004).

Within the Laboratory a urine GC-MS method was recently developed and based on the extraction method described by (Kankaanpää, et al., 2004). Problems were encountered with the measurement of MDEA. The curve was not linear and the internal quality control standards (IQCs) were not calculated to be within 10% of the spiked value. Based on these findings, the decision was made to validate the amphetamine method without MDEA, as its metabolite MDA was been measured anyway and its use in the UK is quite rare today. Therefore the measurement of MDEA in oral fluid will not be attempted in this study as if the higher concentration cut-offs in urine could not be detected in urine then the lower oral fluid cut-off concentrations would prove impossible.

3.2.5.2 METHODS

Calibration curves were prepared for AMP, MA, MDA and MDMA and extracted as described in Chapter 2, Extraction procedure 8 (Øiestad, et al., 2007), they were analysed on the system described using GC-MS parameters 4.

3.2.5.3 METHOD DEVELOPMENT

The results from this initial extraction showed that sensitivity was not good at low concentrations. The limit of quantitation was 50 ng/mL, and the lower concentrations were not detected at all. The possibility of reducing the amount of extraction solvent was investigated, in an attempt to improve sensitivity. Results proved that 150μL toluene gave the most abundant peaks so it was concluded that this would be used for future work.

In a subsequent run, problems were still encountered, and the linearity was not consistent and the quality control samples were not calculated to the correct spiked values.

The GC-MS method was converted into a selective ion monitoring (SIM) method, in order to try to increase sensitivity. The ions displayed in Table 2.3, (Chapter 2), were added to the GC-MS method, and calibrators were extracted again. However, this change did not solve any of the problems.

Although on comparison of the results from the last two experiments, a common trend was evident; the results showed that the linearity for MDMA and MA was far better (higher) and the IQCs were closer to the spiked value than for MDA and amphetamine. It was hypothesised that this could be due to the internal standards used as MDMA and MA had corresponding deuterated standards but for amphetamine MA-d5 was used and for MDA then MDMA-d5 was used.

Valtier and Cody, 1995, had observed linearity problems with amphetamine-d3, so this was not considered as an option to use. However, Valentine and Middleton, 2000, used amphetamine-d5 with HFBA derivatisation successfully

so the decision was made to try that as the IS for amphetamine, along with MDA-d5 as the IS for MDA.

When the deuterated amphetamine and MDA were received (amphetamine-d5 and MDA-d5) two standards at 100 ng/mL and two at 1000 ng/mL were extracted, each contained different amounts of internal standard to investigate the amount required to give a suitable response. A standard curve was extracted that contained standards from 10 ng/mL to 1000 ng/mL to investigate the linearity of the assay and a series of blanks to calculate the limit of detection of the assay. The target ion used to quantify amphetamine-d5 was 244 and for MDA-d5, the target ion 167 was used, these were added to the SIM program on the GC-MS prior to analysis.

3.2.5.4   RESULTS

Drug	Linearity ng/mL	LOD ng/mL	LOQ ng/mL	Accuracy ng/mL n=10	Precision % n = 10
Amp	1000	1	10	160 = 161.8 500 = 513.3	160 = 1.36 500 = 2.34
MA	1000	1	10	160 = 161.5 500 = 500.1	160 = 2.7 500 = 4.1
MDMA	1000	12	15	160 = 159.3 500 = 493.7	160 = 2.6 500 = 2.3
MDA	1000	3	10	160 = 163.7 500 = 518.9	160 = 3.4 500 = 3.2

Table 3.14   A Summary of the method validation results

3.2.5.5 DISCUSSION

The validation parameters achieved seem acceptable for detection of AMP, MA, MDA and MDMA in clinical cases. As is the case with the cocaine validation, further studies involving patient samples from drug clinics will prove if this is true, and if this assay performance is satisfactory for that required.

3.3 APPLICATION OF VALIDATED METHODS

3.3.1 AN INVESTIGATION OF THE DISTRIBUTION OF CODEINE IN ORAL FLUID FOLLOWING A SINGLE DOSE

3.3.1.1 PILOT STUDY

An initial pilot codeine study was set up with 4 volunteers, (they all completed consent forms); their demographics are given in Table 3.15. Each volunteer provided an oral fluid sample (pre-dose), before taking a single oral dose of 20 mg codeine phosphate (as Propain®) and then followed the sample collection regime below (Table 3.16)

The pre-dose sample was analysed for the presence of codeine, to ensure that no other codeine preparations were taken prior to the dose in the study.

Samples were collected at the proposed sampling times, using Quantisal™ collection devices (purchased, from Agriyork 400 Ltd, (Pocklington, UK).

 After the 1mL volume adequacy indicator had turned blue, then the Volunteers placed their saturated cellulose pad in the preservative buffer, in the storage tube. These storage tubes were labelled and kept together, in a refrigerator which was maintained between 2 and 8 °C, until they were analysed.

All of the samples were extracted to test for codeine and morphine, as a metabolite, extraction procedure 5 and extraction procedure 6 (Chapter 2).

	Gender	Age range	weight in kg	dose mg/kg	mg/70kg
Vol 1	M	20 - 29	86	0.23	16.28
Vol 2	M	20 - 29	84	0.24	16.67
Vol 3	F	20 - 29	54	0.37	25.93
Vol 4	F	20 - 29	63	0.32	22.22
Mean			**72**	**0.29**	**20.28**

Table 3.15 Demographics of volunteers involved in pilot study

Sampling time (h) following initial dose	Actual sampling time	Day of study
Pre-dose (blank)	8.30	1
0	9.00	1
0.66	9.40	1
1.33	10.20	1
2.0	11.00	1
3.0	12.00	1
5.0	14.00	1
7.0	16.00	1
9.0	18.00	1
12.0	21.00	1

Table 3.16 The collection times and times post-dose for specimen collection, following a single oral dose of codeine phosphate (20mg)

3.3.1.1.1　　RESULTS

Time post-dose in h	Codeine concentration in ng/ml			
	Vol 1	Vol 2	Vol 3	Vol 4
0	0	0	0	0
0.66	148	171	142	22
1.33	188	292	95	26
2	81	123	96	26
3	20	105	106	27
5	9	27	n/a*	8
7	0	0	0	6
9	0	0	0	2
12	0	0	0	0

*(Data missing for Volunteer 3 after 5h post-dose due to sample not being collected).

Table 3.17　　Concentration at sampling times for 4 volunteers.
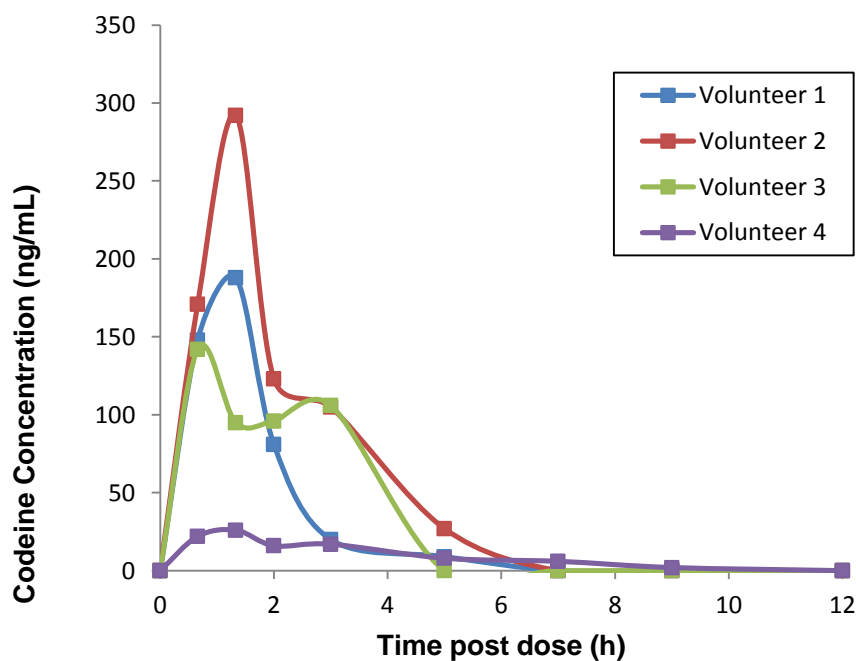
Figure 3.8　　Concentration time profile for 4 volunteers following administration of 20 mg codeine phosphate

The results of the pilot codeine study showed that beyond the 7h sampling time, codeine was not detectable in any of the volunteers. These data were used to determine that the sampling time for the second larger study should also be 12h. Morphine was not detected in any of the specimens, this was also been found to be the case in a previously reported study, (Kim, et al., 2002).

3.3.1.2   ENLARGED CODEINE STUDY

To test whether the trends found in the pilot study were representative; a larger study was subsequently set up, using the same dose and similar sample regime, (same as Table 3.16, except for the 7pm sample was omitted).

The larger study was designed to include the same 4 individuals that participated in the Pilot Study, (volunteers 1 – 4), as well as an additional 6 volunteers. For the demographics of the 10 volunteers, see Table 3.18.

The design of the study was intended to test whether the trends found in the Pilot study were typical and / or reproducible between 2 groups of individuals, but it also allowed for comparison of trends between the same individuals in 2 separate studies.

Each volunteer completed a consent form, and samples were collected, labelled, stored and extracted, as described in the Pilot Study.

As described in the Pilot study, all of the samples were extracted to test for codeine and morphine, (as a metabolite), extraction procedure 5 and extraction procedure 6 (Chapter 2).

	Gender	Age range	weight in kg	dose mg/kg	mg/70kg
Vol 1	M	20 - 29	86	0.23	16.28
Vol 2	M	20 - 29	84	0.24	16.67
Vol 3	F	20 - 29	54	0.37	25.93
Vol 4	F	20 - 29	63	0.32	22.22
Vol 5	F	30 - 39	65	0.31	21.54
Vol 6	M	20 - 29	70	0.29	20.00
Vol 7	F	20 - 29	97	0.21	14.43
Vol 8	F	20 - 29	65	0.31	21.54
Vol 9	M	40 - 49	67	0.30	20.90
Vol 10	M	40 - 49	90	0.22	15.56
Mean			**82**	**0.28**	**19.51**

Table 3.18   Demographics of the 10 volunteers that participated in the enlarged codeine study

3.3.1.2.1   RESULTS

Time post-dose in h	Codeine Concentration in ng/mL									
	Vol 1	Vol 2	Vol 3	Vol 4	Vol 5	Vol 6	Vol 7	Vol 8	Vol 9	Vol 10
0	0	0	0	0	0	0	0	0	0	0
0.66	195	319	372	0	0	265	0	90	95	85
1.33	89	172	304	64	0	194	41	97	94	294
2	57	139	187	58	41	158	126	72	77	317
3	36	107	146	53	53	104	87	53	73	212
5	18	32	48	25	45	62	57	43	40	102
9	0	15	24	12	28	42	36	40	38	45
12	0	8	11	13	16	22	21	41	30	37

Table 3.19   Concentration at sampling times for 10 volunteers

Results from the larger codeine study, (see Figure 3.99) showed that codeine was detectable from 0.66h to 12h; with codeine being detected in 10 volunteers at the final sampling time of 12h, above the 10 ng/mL LOQ. However if the proposed SAMHSA cut-off of 40 ng/mL was used to differentiate recent use, then codeine would only be detected in one volunteer at 12h, and the mean detection time would be reduced to 5.8 hours.

The samples were also analysed for morphine but it was not detected in any of the specimens, these results support those found in the pilot study where no morphine was detected either.
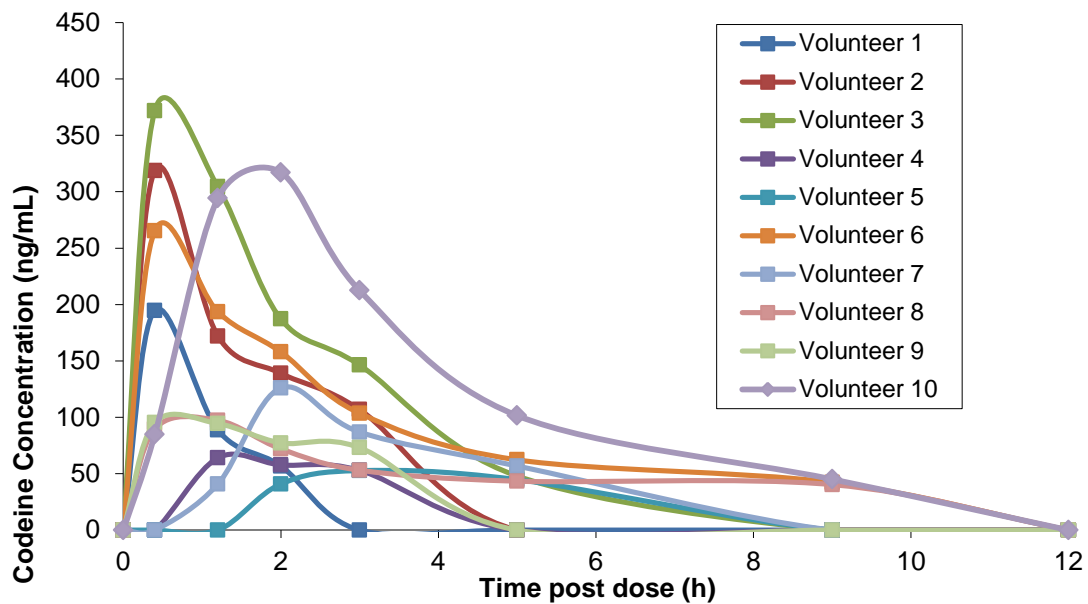
Figure 3.9   Concentration time profile for 10 volunteers following administration of 20 mg codeine phosphate

In a previously reported study involving 19 volunteers, administered a 60mg/70kg oral dose, codeine could only be detected in oral fluid for 7h when the 40 ng/mL cut-off concentration was applied (Kim, et al., 2002).

Using the Subject demographics, (see Table 3.18) it is possible to calculate the mean codeine dose per 70kg, (a weight generally considered to be average). For this study it was found to be 20 mg/70 kg, which was the actual dose given to each individual anyway. The demographic data was also used to compare male and female trends. The standard error and mean codeine concentrations were calculated at each time point for both the sexes (refer to Figure 3.10 and 3.11).
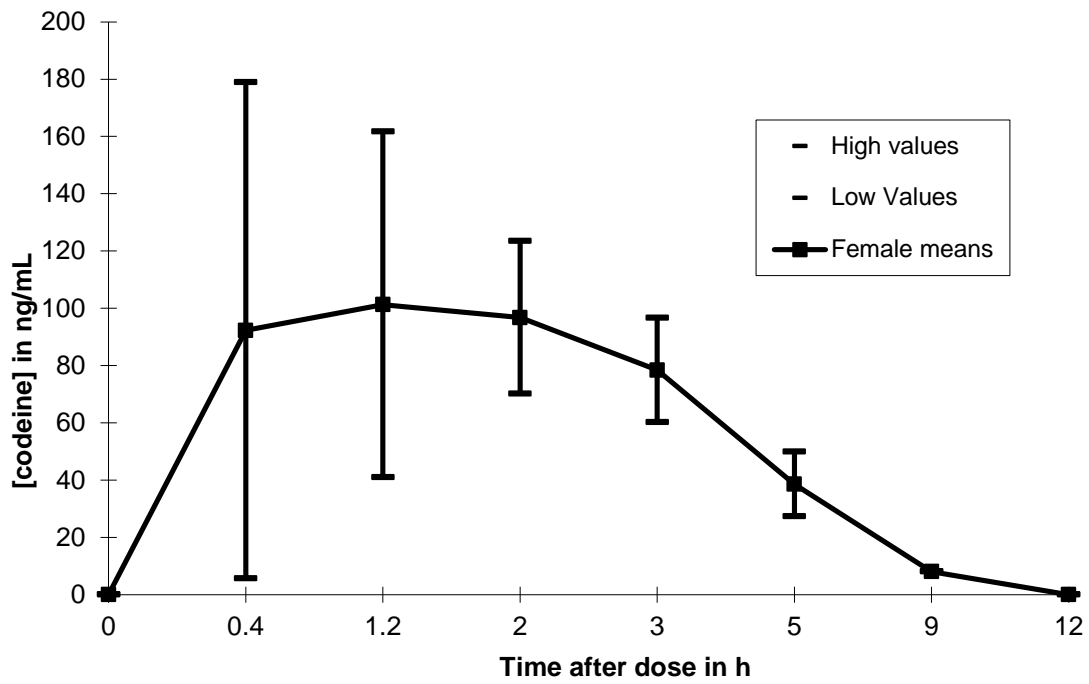
Figure 3.10   Mean +/- SE codeine concentration in oral fluid for Female

Volunteers administered 20 mg codeine orally
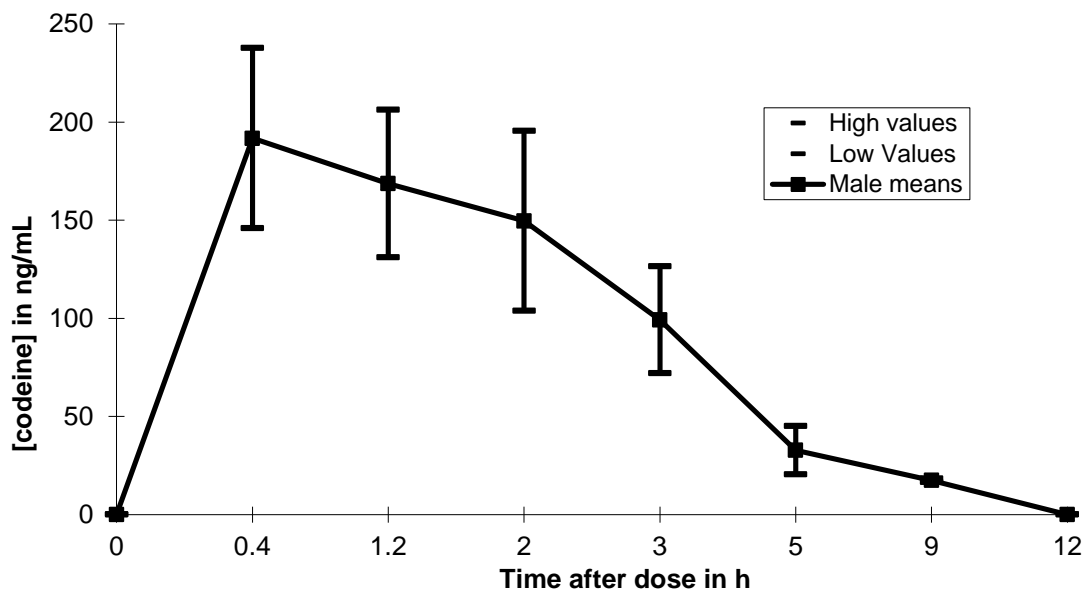
Figure 3.11   Mean +/- SE codeine concentration in oral fluid for Male

Volunteers administered 20 mg codeine orally

The results have also been calculated as dose per kg for each individual, (see Table 3.18 and Figure 3.122).

Time post-dose in h	Codeine Concentration in ng/mL per mg/kg									
	Vol 1	Vol 2	Vol 3	Vol 4	Vol 5	Vol 6	Vol 7	Vol 8	Vol 9	Vol 10
0	0	0	0	0	0	0	0	0	0	0
0.66	848	0	1005	1029	1104	0	0	290	317	386
1.33	387	221	822	555	808	0	195	313	313	1336
2	248	200	505	448	658	128	600	232	257	1441
3	157	183	395	345	433	166	414	171	243	964
5	78	86	130	103	258	141	271	139	133	464
9	0	41	65	48	175	88	171	129	127	205
12	0	45	30	26	92	50	100	132	100	168

Table 3.20   Codeine concentration for 10 volunteers in ng/mL per mg/kg
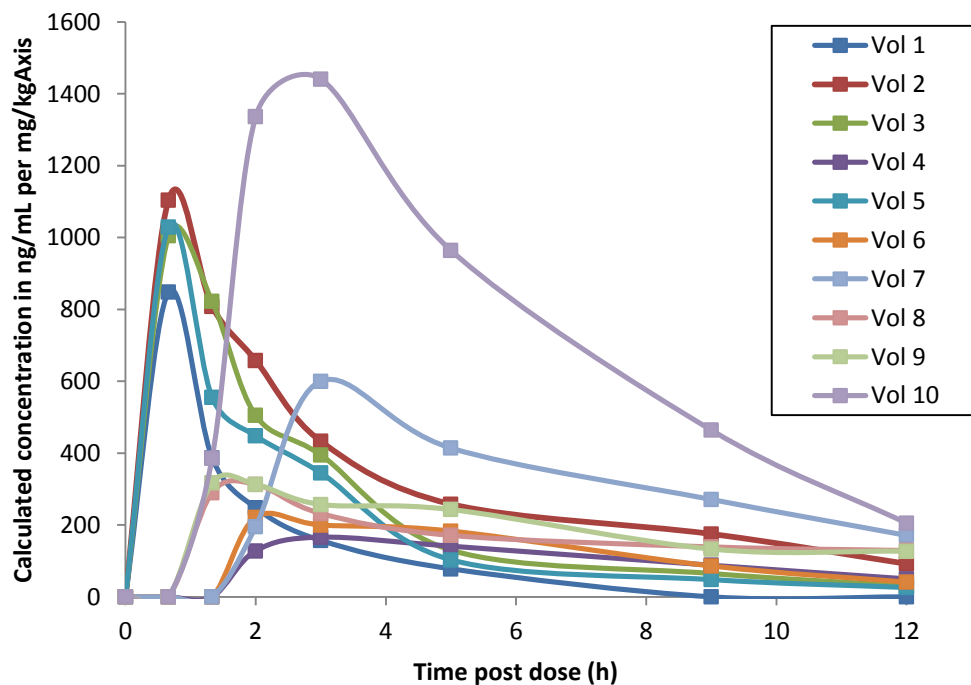
Figure 3.12   Codeine concentration for 10 volunteers in ng/mL per mg/kg

3.3.1.2.2   STATISTICAL ANALYSIS OF RESULTS

Microsoft Excel has built-in pharmacokinetic (PK) functions, (Usansky, et al., 1999), these were used to calculate the following PK parameters: Cmax which can be defined as the maximum concentration within the range, Tmax which can be defined as the time point of the maximum concentration, k which is the elimination rate constant, the half-life (t½) which can be described as the time taken for the concentration to reach half its original value and the area under the concentration-time curve (AUC), which has been calculated from time zero to the last quantifiable point (AUC_{t-0}) and also from time zero to time infinity (AUC_{t-inf}), both these functions were calculated by use of the linear trapezoidal rule, (Usansky, et al., 1999).

Pk Parameter	Vol 1	Vol 2	Vol 3	Vol 4	Vol 5	Vol 6	Vol 7	Vol 8	Vol 9	Vol 10
Cmax (µg/L)	195	265	372	53	319	64	126	97	95	317
Tmax (h)	0.66	0.66	0.66	3.00	0.66	1.33	2.00	1.33	0.66	2.00
k (h-1)	0.83	0.22	0.46	0.08	0.42	0.11	0.26	0.10	0.12	0.27
t 1/2 (h)	0.84	3.22	1.51	8.46	1.64	6.20	2.69	6.89	5.80	2.53
AUC_{t-0}	244	944	995	247	617	176	437	544	308	1323
AUC_{t-inf}	312	1140	1100	794	870	649	657	944	920	1487

Table 3.21   PK Parameters for 10 volunteers based on Table 3.20 and Figure 3.122 codeine concentration in ng/mL

	Mean	Range	SD	SEM
Cmax (µg/L)	**190**	53 – 372	119	38
Tmax (h)	**1.3**	0.66 – 3.0	0.81	0.26
k (h-1)	**0.23**	0.07 – 0.51	0.13	0.04
t 1/2 (h)	**4.16**	1.37 – 9.46	2.42	0.77
AUC_{t-0}	**584**	176 – 1323	386	122
AUC_{t-inf}	**887**	312 - 1487	320	101

Table 3.22   Table to show mean results for PK parameters

The results obtained for dose per kg for each individual were used to calculate an additional set of PK parameters, this allowed for further investigation into the handling of the drug by the individuals studied, (see Table 3.24).

Pk Parameter	Vol 1	Vol 2	Vol 3	Vol 4	Vol 5	Vol 6	Vol 7	Vol 8	Vol 9	Vol 10
Cmax (µg/L)	848	1104	1005	166	1029	221	600	313	317	1441
Tmax (h)	0.66	0.66	0.66	3.00	0.66	1.33	2.00	1.33	0.66	2.00
k (h-1)	0.51	0.20	0.31	0.11	0.31	0.17	0.12	0.07	0.10	0.16
t 1/2 (h)	1.35	3.40	2.25	6.58	2.21	4.07	5.91	9.32	6.60	4.21
AUC_{t-0}	1500	4001	2894	1159	2466	1060	2818	1919	1993	6160
AUC_{t-inf}	1611	4212	3049	1566	2811	1371	3292	2495	2724	6442

Table 3.23   PK Parameters for 10 volunteers based on Table 3.20 and

Figure 3.12, codeine concentration in ng/mL per mg/kg

	Mean	Range	SD	SEM
Cmax (µg/L)	**704**	221 - 1441	140	442
Tmax (h)	**1.3**	0.66 – 3.0	0.81	0.26
k (h-1)	**0.21**	0.07 – 0.51	0.14	0.04
t 1/2 (h)	**4.59**	1.35 – 9.32	2.49	0.79
AUC_{t-0}	**2597**	1060 – 6160	1538	487
AUC_{t-inf}	**2957**	1371 - 6442	1505	476

Table 3.24   Table to show mean results for PK parameters

3.3.1.3 RESULTS

For the second enlarged codeine study, the 10 volunteers included 4 volunteers from the pilot study. This allowed for the comparison of values between the same individuals in the two separate studies. The results were found to alter significantly between the studies even though the dose was the same and the sampling regime was very similar, (see Table 3.26).

This could be due to the impact of the many factors that affect the absorption of drugs and their subsequent passage into oral fluid.

PK values	Pilot Codeine Study				Enlarged Codeine Study			
	Cmax	Tmax	k	T ½	Cmax	Tmax	k	T ½
Vol 1	188	1.33	0.92	0.76	195	0.66	0.51	1.37
Vol 2	292	1.33	0.48	1.44	265	0.66	0.2	3.41
Vol 3	142	0.66	0.1	6.92	372	0.66	0.31	2.23
Vol 4	26	1.33	0.15	4.6	53	3	0.13	5.22

Table 3.25   Summary of the mean PK parameters for the 4 volunteers involved in both codeine studies

3.3.1.4 DISCUSSION

In both the pilot study and the larger second study, the results varied considerably between individuals, and this trend has been found in other similar studies (Kim, et al., 2002), (O'Neal, et al., 1999), (O'Neal, et al., 2000). Different people metabolise drugs at different rates and this is determined by individual phenotype. Gender, age, weight, health, environmental factors and genetic makeup all affect responses to drugs. Cytochrome P450 enzymes are involved

in drug metabolism and they exhibit genetic variability (polymorphism) that affect an individual's response to drugs. As a result, there are "fast" metabolisers that produce a short half-life or higher metabolites and "slow" metabolisers that produce a longer half-life and can accumulate parent drug or metabolites, (Lynch, 2007), (Daly, 2010), (Elliott, 2009).

In the enlarged study, there was such a huge variation between individuals that the concentration data was also calculated so that the individual differences in weight were taken into consideration, so concentrations were expressed as ng/mL per mg/kg. From the observation of the data and curves (see Tables 3.19, 3.20, 3.21 and 3.22), it is evident that there has been a shift between volunteers 2, 3 and 5, (Figure 3.99 compared to Figure 3.122) but apart from this the trends appear very similar.

Comparison of male versus female means (see Figure 3.100 and Figure 3.111) showed that although the females have a higher Cmax and shorter times, the overall elimination profiles are quite comparable. This similarity can be verified by comparing the area under the curves, which is calculated at 606 ng-h/mL for the males, compared to 571 ng-h/mL for the females.

The codeine concentrations in general were found to be much lower than in other reported studies. In a comparable study O'Neal et al. (1999), have reported that where 30 mg liquid codeine was administered to volunteers, it was found that enhanced concentrations were present for the initial samples. It was later acknowledged that this was probably due to oral contamination. However the results in this current study show no obvious evidence of oral contamination

and after 40 minutes the concentrations are only around 20% of those reported by O'Neal et al. (1999).

This could suggest that by using codeine caplets, rather than codeine linctus, the problem of oral contamination could either be overcome or at least reduced.

The results of the codeine studies reported here, show longer half life's and lower elimination rate constants than those reported in other studies indicating that clearance is reduced in the individuals included here. Looking at T1/2 values the mean for males was 3.74h and for females 3.66h compared to 2.9h for males and 2.4h for females in a similar study (Kim, et al., 2002). However this may be partly explained by the fact that the volunteers participating in these studies were naïve users e.g. had seldom or never used codeine before. Whereas in the study reported by Kim et al. (2002), the volunteers were regular opiate users and abusers, thereby introducing possible metabolic and pharmacokinetic differences.

By studying the same 4 volunteers (numbered 1 to 4) in both codeine studies, (refer to Figure 3.14 and 3.15) it can be seen that the concentrations not only vary between individuals but can also vary in one individual from day to day. For example in the pilot study the highest Cmax was from volunteer 2 and yet in the larger study it was from volunteer 3, even though the same dosing and similar sampling regimes were followed. Similar evidence for intra and inter-subject variability has been seen before (Kim, et al., 2002) (Skopp, et al., 2001).

However, the opposite scenario was also demonstrated as in both studies the results for volunteer 4 were similar and showed very little codeine absorption when compared to the other volunteers. It is possible that volunteer 4 did not

take the required tablets but as they volunteered and consented to both studies, this would be unlikely. It is possible that they were in a poor state of health for both studies but as they were well enough to attend work and as the first study was carried out in January and the second in April this also seems unlikely. The differences in absorption between volunteers 4 and 5 (with low absorption), compared to volunteers 2 and 3 (with relatively high absorption) could have been due to the presence of two different phenotypes and if this was the case, this would demonstrate a very good example of polymorphism within a small population, (n=10).

3.3.2 AN INVESTIGATION OF THE DISTRIBUTION OF DIHYDROCODEINE (DHC) IN ORAL FLUID FOLLOWING A SINGLE DOSE

3.3.2.1 INTRODUCTION

Although there seem to be quite a few published articles involving codeine, there does not seem to be as much involving DHC. In addition to this DHC has some physiochemical similarities to cocaine, such as low protein binding and a pKa value of 8.6, (same as cocaine), (Skopp, et al., 2001). Therefore as it is available over-the counter, a pilot volunteer study was designed.

The study proposed that a single oral dose of 10 mg DHC (2 tablets containing 4.98 mg dihydrocodeine per tablet, Paramol®) was to be taken at time 0, with the same sampling collection times as used for the codeine pilot study, (see Table 3.16). There were four volunteers included in this study, and they had all participated in the enlarged codeine study. As in the previous studies, each volunteer needed to provide a blank oral fluid sample before taking the DHC dose, (pre-dose blank). This was analysed for the presence of DHC, to ensure that it was indeed blank and did not contain any DHC or assay interferents.

For this proposed study, commercially available collection devices were provided by Grifols® Uk Ltd (Cambridge, UK).

Four volunteers participated in the study, (see Table 3.27 for demographics), as they also took part in the codeine studies, for continuity and for ease when comparing the studies, they have kept the same volunteer number as allocated previously.

The samples were collected, labelled, stored and extracted, as described in the Codeine Pilot Study.

	Gender	Age range	weight in kg	dose mg/kg	mg/70kg
Vol 2	M	20 - 29	84	0.24	16.67
Vol 3	F	20 - 29	54	0.37	25.93
Vol 8	F	20 - 29	65	0.31	21.54
Vol 10	M	40 - 49	90	0.22	15.56
Mean			**73**	**0.29**	**19.93**

Table 3.26   Demographics of the 4 volunteers that participated in the DHC Study

3.3.2.2   RESULTS

Time post-dose in h	DHC Concentration in ng/mL			
	Vol 2	**Vol 3**	**Vol 8**	**Vol 10**
0	0	0	0	0
0.66	241	211	110	297
1.33	235	174	153	234
2	253	95	103	250
3	112	118	36	172
5	73	67	14	151
7	26	26	0	65
9	na	18	0	78
12	na	0	0	0

na= There is no data for Volunteer 2 at the last 2 sampling times as oral fluid was not collected.

Table 3.27   DHC Concentration at sampling times for 4 volunteers
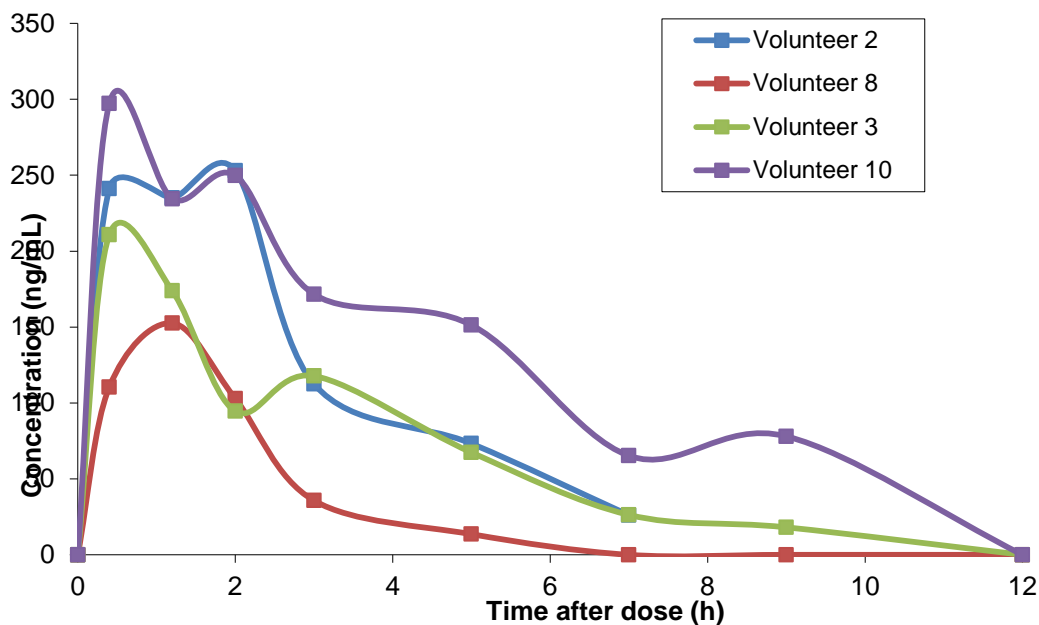
Figure 3.13 Concentration time profile for 4 volunteers following the administration of 10 mg dihydrocodeine tartrate

Microsoft Excel was used to calculate the following pharmacokinetic parameters: the maximum concentration within the range (Cmax), the time point of the maximum concentration (Tmax), the elimination rate constant (k), the half-life t ½, and the area under the curve (AUC), (see Table 3.29).

PK values	Cmax	Tmax	k	T ½	AUC	AUC_{t-inf}
Vol 2	253	2	0.36	1.9	870	890
Vol 3	211	0.66	0.29	2.38	648	710
Vol 8	153	1.33	0.56	1.23	292	317
Vol 10	297	0.66	0.18	3.89	1234	1671
Mean	229	1.16	0.35	2.35	761	897

Table 3.28 Pharmacokinetic parameters for DHC volunteer study

Due to the lack of available published studies regarding DHC in oral fluid, these PK parameters could only be compared to the PK parameters found in the codeine study for the same volunteers, following a similar sampling regime, (see Table 3.16).

PK values	Cmax	Tmax	k	T ½	AUC_{t-0}	AUC_{t-inf}
Vol 2	265	0.66	0.22	3.22	944	1140
Vol 3	372	0.66	0.47	1.51	995	1100
Vol 8	97	1.33	0.10	6.89	544	944
Vol 10	317	2	0.27	2.53	1323	1487
Mean	263	1.16	0.27	3.54	952	1168

Table 3.29   Pharmacokinetic parameters for Codeine volunteer study

The mean parameters can be seen not to vary greatly between the two types of opiates, with Cmax and Tmax being similar for both drugs. However, the results do show a marked variation between the individuals studied.

3.3.2.3   DISCUSSION

Very few DHC studies in oral fluid have been published and so there is very little data with which to compare with the findings reported here. Skopp et al. (2001) described a DHC volunteer study involving a single 60 mg dose, but this study used a much lower dose (10 mg). They found that the maximum concentrations were reached 2 – 4 hours post-dose, whereas the mean Tmax in this study was only 1.16h. They also reported a mean T1/2 of 8h compared to a mean T1/2 of 3.5h in this study but this could be explained by the difference in dose as it would generally be expected to take longer to eliminate more of the drug.

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The results in this study could show some biphasic and triphasic distribution, (see Figure 3.133) for some of the volunteers but not all of them. The same observation has not been noted by Skopp et al., (2001) after a 60 mg dose. Further studies will need to be carried out to fully evaluate this area of research. A study on a larger scale incorporating several matrices would be regarded to give a better insight into the individual handling of the drug, perhaps comparing saliva, blood and urine levels.

3.3.2.4 CONCLUSION

In conclusion, due to the variations observed both within and between the studies described, and the combined effects of flow rate, pH and collection method, further opiate studies need to be carried out before firm conclusions can be reached regarding the effectiveness of oral fluid as a means of monitoring opiate abuse.

3.3.2.5   AN INVESTIGATION OF THE DISTRIBUTION OF CODEINE IN ORAL FLUID COMPARED TO URINE FOLLOWING A SINGLE DOSE

3.3.2.6   INTRODUCTION

A codeine study was designed, to include the collection of both urine and oral fluid at designated sampling times, to allow for the comparison of the codeine time profile in these 2 different biological matrices. The design was similar to the previous codeine studies with a proposed single oral dose of 20 mg codeine phosphate (as Propain®), and proposed sampling collection times, post-dose, (see Table 3.31 and 3.32). As before each volunteer needed to provide an oral fluid sample and in addition to a urine sample before taking the codeine dose, (pre-dose blank). These were also analysed for the presence of codeine, to ensure that they have not taken any other codeine preparations prior to the dose in the study. There were 4 volunteers included in the study, and they all signed consent forms before taking part in the study. The urine was collected in plain plastic universals and the oral fluid was collected using the Quantisal™ collection devices as described previously, (purchased, from Agriyork 400 Ltd, Pocklington, UK).

All of the samples were extracted to test for codeine (extraction procedure 6) and morphine, as a metabolite, (see extraction procedure 5).

Sampling time (h) following initial dose	Actual sampling time	Day of study
Pre-dose	8.30	1
0	9.00	1
0.66	9.40	1
1.33	10.20	1
2.0	11.00	1
3.0	12.00	1
5.0	14.00	1
7.0	16.00	1
9.0	18.00	1
12.0	21.00	1

Table 3.30 The collection times and times post-dose for oral fluid collection, following a single oral dose of codeine phosphate (20mg)

Sampling time (h) following initial dose	Actual sampling time	Day of study
Pre-dose	8.30	1
0	9.00	1
0.5	9.30	1
2.0	11.00	1
4.0	13.00	1
6.0	15.00	1
9.0	18.00	1
12.0	21.00	1
24.0	9.00	2
30.0	15.00	2

Table 3.31 The collection times and times post-dose for urine collection, following a single oral dose of codeine phosphate (20mg)

3.3.2.7   RESULTS

Urine	Codeine			
Time after dose	Vol 2	Vol 8	Vol 6	Vol 10
0	0	0	0	0
0.5	70	28	13	1284
2	1325	1400	1246	2425
4	1040	1118	1653	2439
6	519	1200	949	2490
9	401	725	754	1495
12	143	49	797	908
24	0	3	244	51
30	0	0	24	7

Table 3.32   Urine concentration at sampling times for 4 volunteers
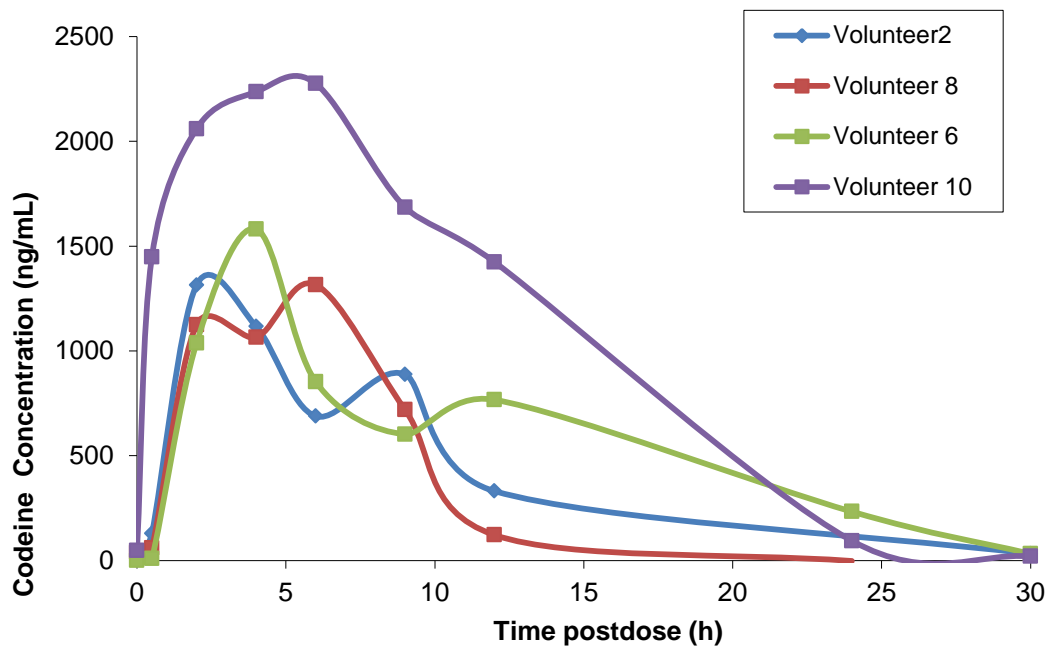
Figure 3.14   Urine codeine concentration time profile for 4 volunteers following the administration of 20 mg codeine phosphate

Urine	Morphine			
Time after dose	Vol 2	Vol 8	Vol 6	Vol 10
0	53	0	0	0
0.5	0	17	37	194
2	72	697	403	485
4	73	671	799	888
6	34	1131	340	1113
9	57	541	490	614
12	6	65	553	535
24	0	82	727	154
30	0	67	260	145

Table 3.33   Urine Morphine concentration at sampling times for 4 volunteers
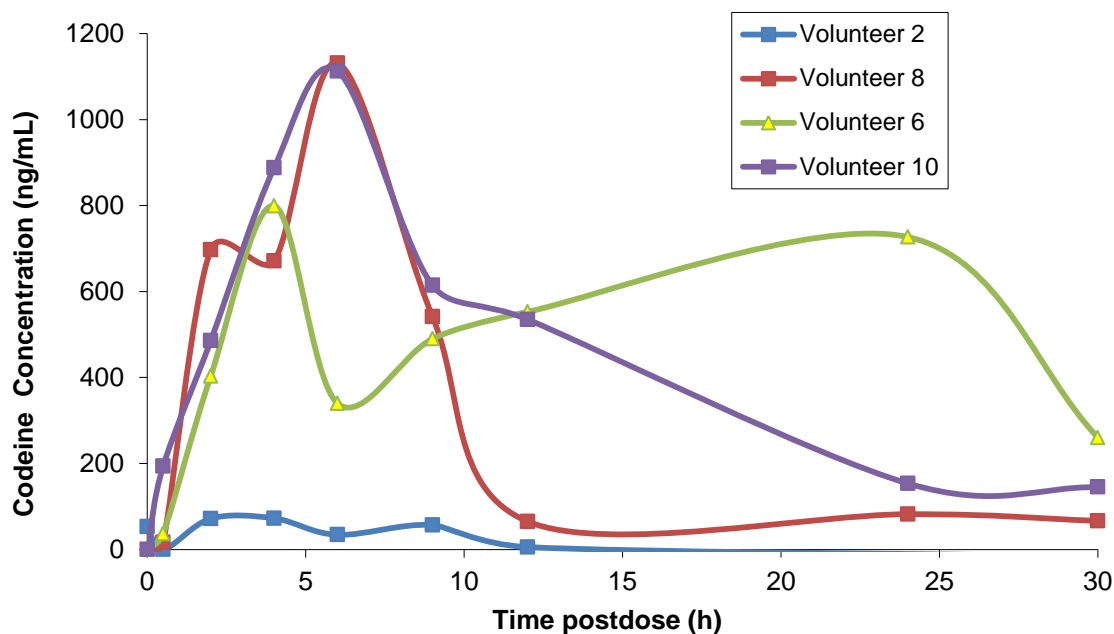
Figure 3.15   Urine morphine concentration time profile for 4 volunteers following the administration of 20 mg codeine phosphate

Oral Fluid	Codeine			
Time after dose	Vol 2	Vol 8	Vol 6	Vol 10
0	0	0	0	0
0.66	51	32	0	233
1.33	114	38	52	90
2	21	11	32	92
3	30	0	0	107
5	14	0	0	27
7	0	0	0	0
9	0	0	0	0
12	0	0	0	0

Table 3.34   Oral Fluid concentration at sampling times for 4 volunteers
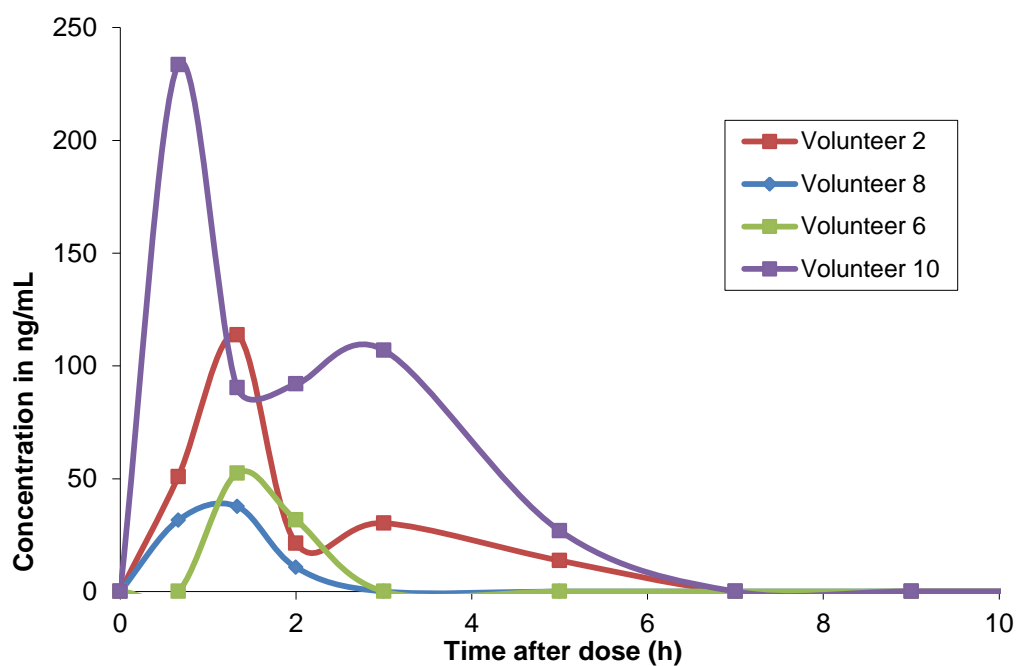
Figure 3.16   Oral fluid concentration time profile for 4 volunteers following the administration of 20 mg codeine phosphate

ORAL FLUID VERSUS URINE

The urine codeine concentrations were much higher than the oral fluid concentrations, as expected. To facilitate comparison of the 2 different matrices, the results were calculated as a percentage of the maximum concentration for each matrix, in each volunteer (see Figures 3.17 and 3.18). It should be noted that a problem with this type of urine analysis is that there will always be concentration differences between individuals due to fluid intake and bladder voiding differences.
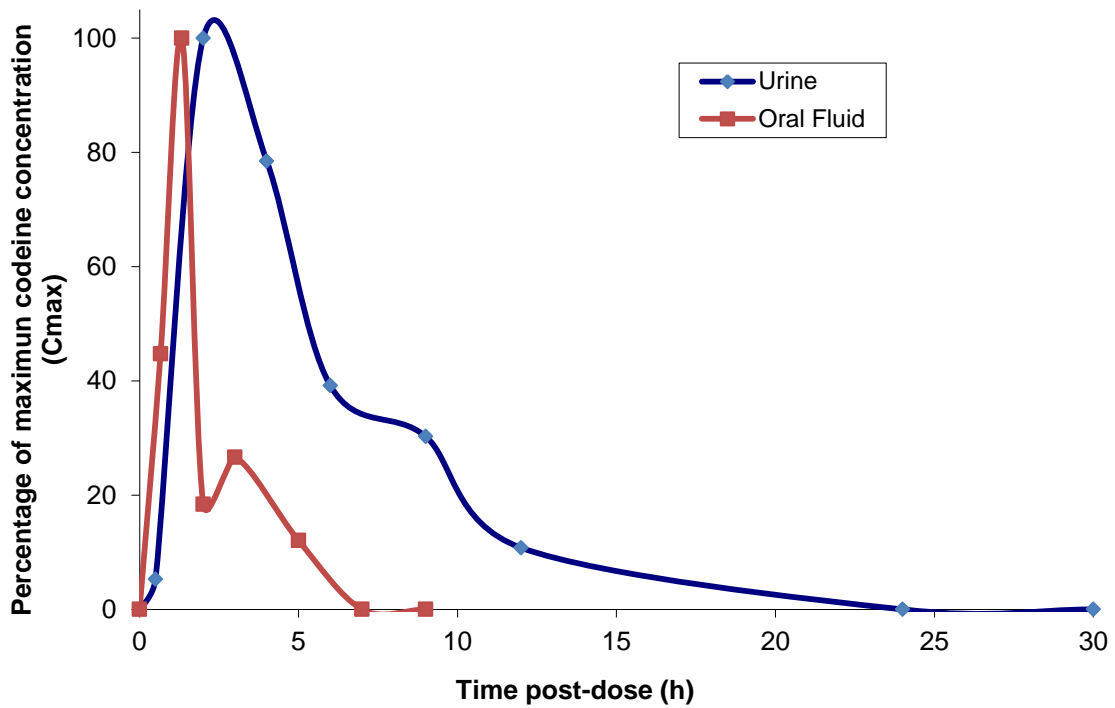
Figure 3.17   Codeine Profiles for Volunteer 2
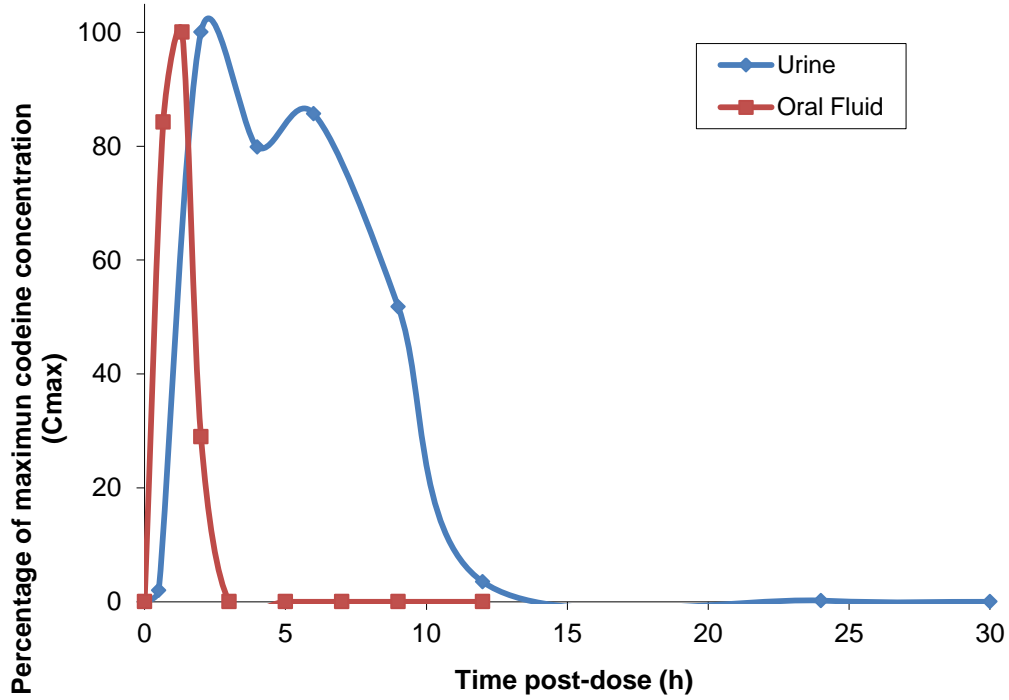
Figure 3.18   Codeine Profiles for Volunteer 8
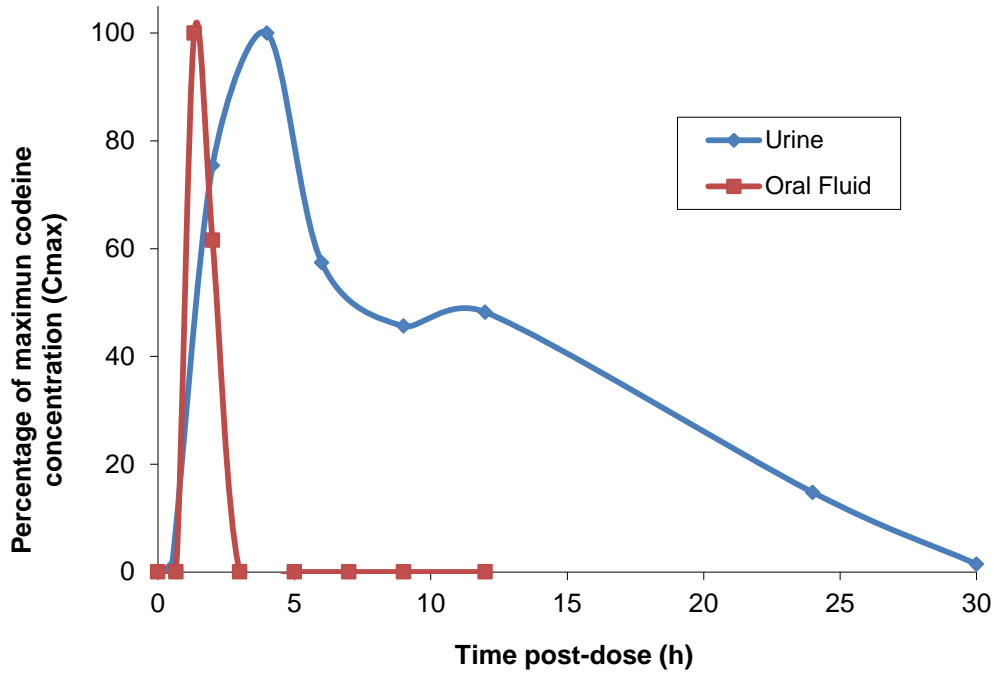
Figure 3.19   Codeine Profiles for Volunteer 6
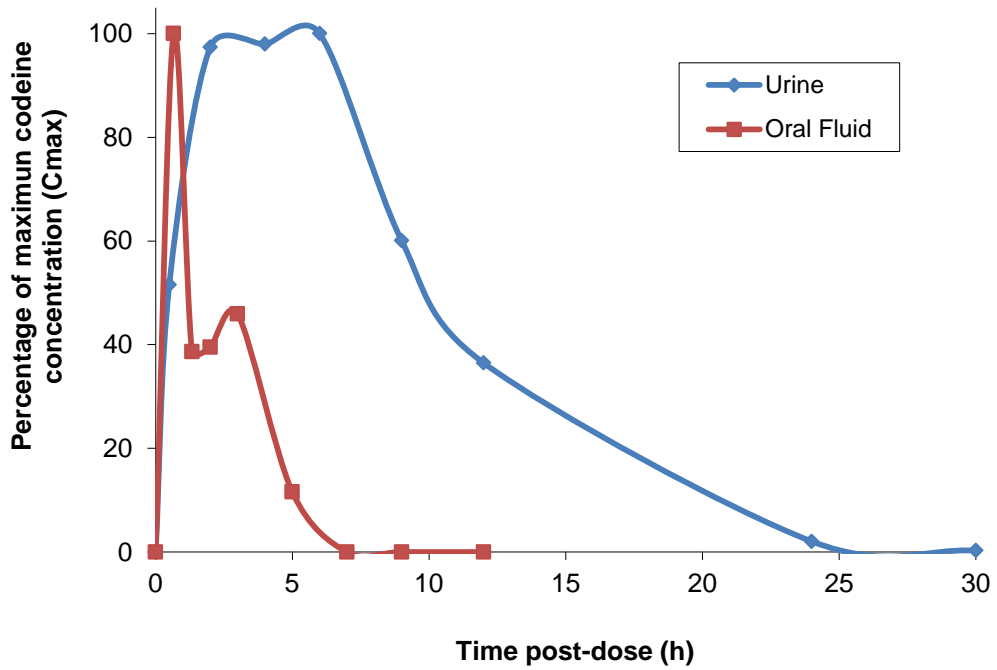
Figure 3.20   Codeine Profile for Volunteer 10

3.3.2.8 DISCUSSION

Codeine is metabolised by glucuronidation to codeine-6-glucuronide, N-demethylation to norcodeine and O-demethylation to morphine in humans, (Kim, et al., 2002). In this study only free codeine and morphine were analysed.

The urine drug concentration profiles demonstrated some biphasic distribution for both codeine and the metabolite morphine, in another study the elimination of codeine was also found to be biphasic, (Vree & Verwey-van Wissen, 1992). Whereas for oral fluid, the time concentration profiles only showed biphasic distribution in 2 out of the 4 volunteers. This is likely related to the differences in elimination profiles of the two matrices.

The results show a common trend between volunteers, they show that codeine is detected for a shorter time window in oral fluid compared to urine, and this is well documented and was to be expected. This means that the appropriate drug matrix (i.e. oral fluid or urine) can be selected in accordance to the question being asked, e.g. if its required to know whether someone has taken codeine in the past 2 – 7 hours then oral fluid could be used for analysis. However, if the query is whether any codeine has been taken in the past 24 hours then urine would need to be collected and analysed. It should be noted that the codeine dose used in this study (20 mg) was available to purchase over the counter and as such was a very low dose. If larger codeine doses (e.g. 60 mg) were used then the results would be expected to provide different time windows.

3.3.3 AN INVESTIGATION OF THE PRESENCE / ABSENCE OF SOME DRUGS OF ABUSE IN A SMALL POPULATION OF INDIVIDUALS SEEKING HELP FOR DRUG ADDICTION

3.3.3.1   INTRODUCTION

A drug-users treatment clinic agreed to participate in a small investigation to help with the completion of the oral fluid method validation and allow comparison of a relatively new drug matrix (oral fluid) to the traditional matrix for drugs of abuse (urine).

The samples were to be tested for: opiates (OPI), cocaine (COC), amphetamines (AMPS), methadone (METH) and buprenorphine (BUP).

The clinic agreed to collect approximately 20 oral fluid specimens, with corresponding urine samples, (where possible) and send them to the laboratory for analysis.

In total 17 oral specimens were sent in to the laboratory with 17 corresponding urine specimens. In addition to this 1 oral fluid sample, (3004) was received without a corresponding urine specimen. All the specimens were allocated Laboratory numbers when they were received.

3.3.3.2   ANALYTICAL METHODS

Oral fluid screening:

ELISA kits were used to screen for OPI and COC / benzoylecgonine (BZE).

LC-MS was used to screen for amphetamine (AMP), methamphetamine (MA), MDA, MDMA, COC, BUP, norbuprenorphine (NBUP), METH and

2-ethylidene- 1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP).

GC-MS was used to screen for AMP, MA, MDA and MDMA.

Oral fluid confirmation:

If the ELISA opiate screen was positive then GC-MS was used to identify whether morphine (MORPH), dihydrocodeine (DHC), codeine (COD) and / or 6-monoacetylmorphine (6-MAM) were present.

If the COC screen was positive by ELISA but negative by LC-MS then

GC-MS was still used to test specifically for BZE.

 On the urine specimens the following analytical methods were performed:

Urine screenng:

CEDIA screen for AMPS, OPI, EDDP, and BZE. In addition to these tests the urine samples were also tested for benzodiazepines, cannabinoids and creatinine, as these were already included in the service provision for urine screening, it seemed important to include them in the project.

GC-MS used to screen for BUP and NBUP.

Urine confirmation:

If the CEDIA opiate screen was positive then GC-MS was used to identify whether MORPH, DHC, COD and / or 6-MAM were present.

If the CEDIA AMPS screen was positive then GC-NPD was used to identify whether AMP, MA, MDA and / or MDMA were present.

If the CEDIA EDDP screen was positive then GC-NPD was used to test if METH (parent drug) was also present.

3.3.3.3 RESULTS

Laboratory Number	COC / BZE	OPI
3002	Negative	Negative
3004	**POSITIVE**	**POSITIVE**
3005	Negative	Negative
3007	Negative	Negative
3009	Negative	Negative
3011	Negative	Negative
3013	**POSITIVE**	Negative
3015	**POSITIVE**	**POSITIVE**
3017	**POSITIVE**	Negative
3019	**POSITIVE**	**POSITIVE**
3021	Negative	Negative
3023	Negative	Negative
3025	Negative	Negative
3027	**POSITIVE**	Negative
3029	Negative	Negative
3031	**POSITIVE**	Negative
3033	Negative	Negative
3035	Negative	Negative

Table 3.35 ELISA Screening Results on the oral fluid specimens

ELISA BZE / COC results >20 ng/mL = Positive

ELISA OPI results >40 ng/mL = Positive

Laboratory No	AMPS (Amp, MA, MDA, MDMA)	COC	BUP	METH
3002	Negative	Negative	Negative	**POSITIVE**
3004	Negative	**POSITIVE**	Negative	**POSITIVE**
3005	Negative	Negative	Negative	Negative
3007	Negative	Negative	Negative	**POSITIVE**
3009	Negative	Negative	Negative	**POSITIVE**
3011	Negative	Negative	Negative	Negative
3013	Negative	Negative	Negative	Negative
3015	Negative	**POSITIVE**	Negative	Negative
3017	Negative	**POSITIVE**	**POSITIVE**	Negative
3019	Negative	Negative	Negative	**POSITIVE**
3021	Negative	Negative	Negative	**POSITIVE**
3023	Negative	Negative	Negative	Negative
3025	Negative	Negative	Negative	**POSITIVE**
3027	Negative	Negative	Negative	Negative
3029	Negative	Negative	Negative	**POSITIVE**
3031	Negative	**POSITIVE**	Negative	**POSITIVE**
3033	Negative	Negative	Negative	**POSITIVE**
3035	Negative	Negative	**POSITIVE**	Negative

Table 3.36 LC-MS Screening Results on the oral fluid specimens

LC-MS LOD for MA, COC and METH = 1 ng/mL, LOD for Bup = 2 ng/mL

LC-MS LOD for Amp and MDA = 3.5 ng/mL, LOD for MDMA = 5 ng/mL

The LC-MS also screened for the methadone metabolite (EDDP) and the buprenorphine metabolite (norbuprenorphine) but these results are not

displayed in the table as neither of these metabolites were detected in any of the oral fluid samples.

Laboratory Number	BZE		OPI	
3004	+	**POSITIVE**	+	MORPH, COD, 6-MAM
3013	+	Negative	-	NA
3015	+	**POSITIVE**	+	MORPH, COD, 6-MAM
3017	-	**POSITIVE**	-	NA
3019	+	Negative	+	MORPH, COD, 6-MAM
3027	+	Negative	-	NA
3031	+	**POSITIVE**	-	NA

The + / - symbol refers to the ELISA screening results (see Table 3.38).

Table 3.37   GC-MS Conformation results on the oral fluid specimens

GC-MS was also used to screen for the amphetamines (Amp, MA, MDA and MDMA) in the oral fluid samples and they were all found to be negative by this method too.

Laboratory Number	AMPS	BZE	EDDP	OPI
3003	Negative	Negative	**POSITIVE**	**POSITIVE**
3006	Negative	Negative	**POSITIVE**	Negative
3008	Negative	Negative	**POSITIVE**	Negative
3010	Negative	Negative	**POSITIVE**	Negative
3012	Negative	Negative	Negative	Negative
3014	Negative	Negative	Negative	Negative
3016	Negative	**POSITIVE**	Negative	**POSITIVE**
3018	Negative	**POSITIVE**	Negative	Negative
3020	Negative	**POSITIVE**	**POSITIVE**	**POSITIVE**
3022	Negative	Negative	**POSITIVE**	Negative
3024	Negative	Negative	Negative	Negative
3026	Negative	Negative	**POSITIVE**	Negative
3028	Negative	**POSITIVE**	Negative	**POSITIVE**
3030	Negative	Negative	**POSITIVE**	Negative
3032	Negative	**POSITIVE**	**POSITIVE**	**POSITIVE**
3034	Negative	Negative	**POSITIVE**	Negative
3036	Negative	Negative	Negative	Negative

Table 3.38   CEDIA Screening Results on the urine specimens

CEDIA cut-offs are as follows: AMPS = 500 ng/mL,

BZE = 300 ng/mL, EDDP = 100ng/mL and OPI = 300 ng/mL

Every urine sample that screened positive for EDDP also tested positive for

methadone on the GC-NPD.

Laboratory Number	BUP	NBUP
3003	Negative	Negative
3006	Negative	Negative
3008	Negative	Negative
3010	Negative	Negative
3012	Negative	Negative
3014	Negative	Negative
3016	Negative	Negative
3018	**POSITIVE**	**POSITIVE**
3020	Negative	Negative
3022	Negative	Negative
3024	Negative	Negative
3026	Negative	Negative
3028	Negative	Negative
3030	Negative	Negative
3032	Negative	Negative
3034	Negative	Negative
3036	**POSITIVE**	**POSITIVE**

LOD BUP/NBUP = 2 ng/mL

Table 3.39   GC-MS Screening Results on the urine specimens

Laboratory Number	Opiate Result
3003	MORPH
3016	MORPH, COD, 6-MAM
3020	MORPH, COD, 6-MAM
3028	MORPH
3032	MORPH, COD

GC-MS LOD MORPH, COD, DHC = 50 ng/mL and 6-MAM = 5 ng/mL

Table 3.40   GC-MS Confirmation Results on the opiate positive urine specimens

	AMPS	BZE	BUP	METH	MORPH	COD	6-MAM
Oral Fluid	0	3	2	10	2	2	2
Urine	0	5	2	10	5	3	2
Difference	0	2	0	0	3	1	0

(Above data refers to n=17, as a corresponding urine was not submitted with one of the oral fluid samples).

Table 3.41 The amount of positive results in oral fluid compared to urine

Sample (Lab no)	Elisa BZE / COC Cut-off = 20 ng/ml	GC-MS BZE LOD = 13 ng/ml	LC-MS COC LOD = 1 ng/ml	Sample (Lab no)	CEDIA Cut-off = 300 ng/ml	GC-MS BZE LOD = 50 ng/ml
Oral fluid (3019)	**POSITIVE**	Negative	Negative	Urine (3020)	**POSITIVE**	**POSITIVE**
Oral fluid (3027)	**POSITIVE**	Negative	Negative	Urine (3028)	**POSITIVE**	**POSITIVE**

Table 3.42 Cocaine / BZE Discrepancies

The CEDIA screens for BZE in the above samples were positive, they were also analysed on the GC-MS to allow comparison with the oral fluid samples at a lower LOD.

Sample (Lab No)	ELISA OPI Cut-off = 40 ng/mL	GC-MS OPI LOD = 10 ng/mL	Sample (Lab No)	CEDIA Cut-off = 300 ng/mL	GC-MS OPI LOD = 50 ng/mL
Oral Fluid (3002)	Negative	Negative	Urine (3003)	**POSITIVE**	MORPH
Oral Fluid (3027)	Negative	Negative	Urine (3028)	**POSITIVE**	MORPH
Oral Fluid (3031)	Negative	Negative	Urine (3032)	**POSITIVE**	MORPH COD

Table 3.43 The Opiate Discrepancies

Although the ELISA screens for OPI were negative the above samples were also analysed on the GC-MS to allow comparison with the urine samples at a lower LOD.

3.3.3.4 DISCUSSION

When the oral fluid results are compared to the urine results (see Table 3.41), it becomes apparent that many of the results support each other. All the samples found to be positive for a drug in the oral fluid proved to be positive for at least the same drug in the corresponding urine, although sometimes analysed positive for additional drugs in the urine. This means that as the same amount of samples were found to be positive for MET, BUP or 6-MAM in both matrices then the same results would have been found whether the oral fluid or urine had been collected (or selected) for analysis.

However, the same results were not shown in both matrices for BZE, MORPH or COD, as more positive results were found in the urine samples. It is important to focus on these samples that had different results for each matrix (see Table 3.43 and 3.44).

In Table 3.43, both oral fluid samples screened positive for COC/BZE by ELISA but on confirmation they were found to be negative for both COC and BZE so the ELISA result was a false-positive result. These can be common with this type of screening technique which is why confirmation of the results is recommended. However the corresponding urine specimens were found to be positive for BZE. The reason for the different results is due to the difference in time detection windows between the matrices. BZE in urine is normally

detectable for at least 24 hours after ingestion whereas BZE / COC in oral fluid may only be detectable for a few hours after ingestion. The difference in the OPI results displayed in Table 3.44 can also be explained by the different time detection windows. The results suggest that when the oral fluid was collected OPI could not be detected as it had been more than several hours since the ingestion, however it could not have been more than 24 hours after ingestion as it was still possible to detect morphine and codeine in the urine.

The difference between the periods of time that drugs can be detected in each matrix is very important, and this must be considered when deciding which matrix is best to use. There are times when oral fluid may be the best matrix to analyse but there are also times when only urine can provide the answer to the question being asked. If both urine and oral fluid are collected and analysed, a better insight can be given to the drug-use of the individual being tested.

A significant observation is that although the clinic submitted 17 oral fluid samples with corresponding urines, for one individual only an oral fluid sample was submitted and for some reason there was noticeably less than 4 mL in total, the expected volume, (1 mL oral fluid plus 3 mL buffer). This raised several questions, firstly, would there be enough sample volume to perform all necessary confirmations, secondly had something gone wrong during collection, (to account for the short sample?), and thirdly, was it possible that the individual had deliberately sent a short sample with no urine perhaps in the hope that there would not be enough sample to confirm drug use?

However, when the screening and confirmation techniques were applied, there was sufficient sample volume to screen for amphetamines, opiates, cocaine,

methadone and buprenorphine and confirm their presence, where necessary but very little sample volume remained. The clinic had queried cannabis use on the "request form" but without a urine sample, it was not possible to test for this.

If validation work had been completed successfully for benzodiazepines and cannabinoids in oral fluid, then as the panel of drugs would have been larger, then it is unlikely that in this case at least, that there would have been sufficient sample to carry out all confirmations.

3.3.3.5   CONCLUSION

This final study has helped to show that the methods developed for oral fluid analysis are applicable to samples collected from drug-users in a treatment setting.

CHAPTER 4: A STUDY OF

POST-MORTEM TOXICOLOGY

4.1 INTRODUCTION

A post-mortem examination is carried out by a pathologist, to determine the cause of death. They are usually ordered by a coroner if the cause of death is unknown, sudden or unexpected, or at the request of a family member to provide information about illness and cause of death, (Department of Health, 2009).

The traditional matrices analysed in post-mortem (PM) toxicology are blood and urine. However, in some circumstances these are not available so other specimens such as stomach contents, vitreous humor, bile, liver and other tissues are submitted. In these cases it can be difficult to analyse and interpret results as these matrices are not routinely used and therefore there is often limited published data to refer to.

Typical cases where blood and/or urine are not available include; decomposed bodies, fire deaths, drownings, road traffic incidents and aircraft crashes. However, in cases where blood/urine is available in addition to forensic tissue and other fluids, the analysis of all the biological specimens could provide the data, to evaluate the potential interpretative usefulness of such alternative matrices.

4.1.1 ALTERNATIVE MATRICES

Historically, urine, stomach contents, bile and liver have all been routinely used for PM screening for drugs, as they are known to have the highest drug concentrations and therefore offer the greatest chance for detection.

In decomposed cases it is often only stomach contents and liver specimens that are available for analysis. However, in these cases interpretation of analytical results can be complicated, due to endogenously produced substances and destruction of drugs from the putrefactive process, (Paterson, 1993).

4.1.1.1 STOMACH CONTENTS

Stomach contents analysis actually notionally detects if any drugs have been taken close to the time of death, if a drug is present in the stomach it is there because it either has not yet been absorbed or completely absorbed, and would therefore not necessarily be detected in any other specimens collected from the body. Sometimes whole tablets are present in the stomach contents that have not been absorbed at all and it can be possible to use their shape and colour to identify them, (along with a database such as TICTAC (TicTac Communications Ltd), (Jones, 2008). The presence of whole tablets can be very useful for interpretation purposes, they could indicate "intent" in suicide situations where an alternative method of death has been used, e.g. in a hanging.

There are a few important points to consider, when analysing and interpreting stomach contents results; as metabolism does not occur in the stomach, it is only usually the parent drugs that need to be detected, and after an overdose, drug concentrations in the stomach may be quite high even after the majority of

the drug has passed into the small intestine which can make drug detection a lot easier in stomach contents compared to corresponding blood, where extensive distribution in the body could have occurred. Therefore, a low amount of drug in the stomach cannot rule out a possibility of overdose. In addition to this, the presence of a drug in the stomach contents does not necessarily mean that it has been ingested orally as passive diffusion from the blood into the stomach contents is known to occur, (Jones, 2008), (Skopp, 2004).

Unlike the other alternative matrices, with stomach contents there is not usually a low sample volume so this is not usually an issue, a portion of approximately 10 mL, is typically submitted. This creates a problem in itself though, as a portion rather than the whole stomach contents is submitted. This makes estimation of a drug dose difficult because the sampled portion could have a high concentration, for example if a 10mg tablet is taken then if the entire stomach contents was 1 L then the overall concentration would be 10 mg/L, but if only a 10mL portion was submitted and analysed this could give a high false concentration of 1 mg/mL (which is the equivalent of 1000 mg/L). Therefore, it is generally accepted that the concentration of a substance in the stomach is virtually meaningless by itself. However, if drugs are measured in stomach contents then they should only be reported as the amount of drug present in the volume or mass of stomach contents received, (Jones, 2008).

There are other problems associated with stomach contents analysis. The composition is very varied and ranges from a very thin "soup-like" consistency to a thick mass of chewed-up food, largely depending on how long before death the individual last eat. The fact that the stomach contents are rarely

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homogenous is another contributory factor into why drug concentrations cannot be accurate, unless the contents are homogenised. Soon after a dose is ingested the stomach contents concentration may be very high even if the total amount taken is not. The absence of a large amount of drug in the stomach contents does not necessarily rule out an oral overdose because it could take several hours to die from an drug overdose during which time the drug could have been absorbed and metabolised. However, consumption of an oral overdose of medicine can result in the formation of a "bezoar" (medicine mass) in the stomach, which can take several hours or more to dissipate, (Jones, 2008).

Whether or not stomach contents analysis will prove useful can be largely dependent on the type of case involved. In general it can be useful in cases where there is an alternative cause of death, for example many tablets in the stomach contents after a hanging could show suicidal intent, e.g. if an individual could take an overdose of tablets in an attempt to take their own life but then death took longer to occur then they expected, they might try an alternative suicide technique, such as hanging. In other cases however, stomach contents analysis will not be very relevant, e.g. if the deceased was a passenger in a road traffic collision, whether or not they ingested a lot of drugs before their death is probably incidental as these drugs would not have been fully absorbed and therefore are unlikely to have produced any effect or contributed to the death.

4.1.1.2   VITREOUS HUMOUR

Vitreous Humour is the clear gel that fills the posterior cavity of the eye, (Chronister, et al., 2008), (see Figure 4.1). It is usually obtained by needle puncture of the eyeball, at the sclera, (see Figure 4.1), generally this yields 2-3 mL, (Forrest, 1993).

The blood-ocular barrier restricts the entrance of drugs to the eye so that only lipophilic or very small hydrophilic substance can pass through it. The distribution equilibrium between blood and vitreous humour is determined by plasma-protein binding. During survival time, it is thought that vitreous humour levels should follow blood concentrations with a certain time delay, determined by this binding. Lipophilic drugs are eliminated quickly by diffusion through the membranes whereas hydrophilic drugs are eliminated more slowly, (Pragst, et al., 1999).

It is the preferred specimen for post-mortem ethanol measurement as post-mortem formation of ethanol does not occur in this matrix, (yet it has been shown to in blood and other tissues). This is because the interior of the eye is a sterile medium until the most advanced stages of decomposition, (Jones, 2008).

It has also been reported as very useful for the measurement of digoxin, as concentrations increased markedly in post-mortem blood (which could lead to a false diagnosis of digoxin toxicity) but conversely, were below ante mortem values in vitreous, (Vorpahl & Coe, 1978).

Vitreous humour has also been shown to be of benefit in instances where unstable drugs such as 6-monoacetylmorphine (6-MAM) and cocaine are

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involved. Conversion to morphine and benzoylecgonine, respectively, is hydrolytic and is rapid in the presence of esterase enzymes. As such, the conversion readily occurs in blood. As the vitreous humour is within the sterile and compartmentalised eye environment, the relative lack of enzymes reduces this effect and the compounds are more stable (Pragst, et al., 1999, Jones, 2008). Opiate distribution in vitreous humour specimens has also been studied, the results showed that vitreous could be used to differentiate death due to codeine overdose from heroin (morphine) abuse, (Lin, et al., 1997). Cocaine and metabolites have been compared in blood and vitreous. Results showed that although cocaine levels were higher in vitreous the metabolites were a lot lower and so although vitreous could be used to quantitate cocaine and its metabolites it was not found to be as reliable as blood, (Mackey-Bojack, et al., 2000).

There are disadvantages associated with the use of vitreous humour for drug analysis, firstly its relatively small sample volume means that analysis is limited, this can mean that choices have to be made between screening, confirmation and quantitation tests, rather than being able to cover the whole sprectrum. As very few studies have been published on drug blood concentrations compared to vitreous humour, interpretation of results can prove difficult. It is possible that this lack of published data could be due to the limited sample volume.
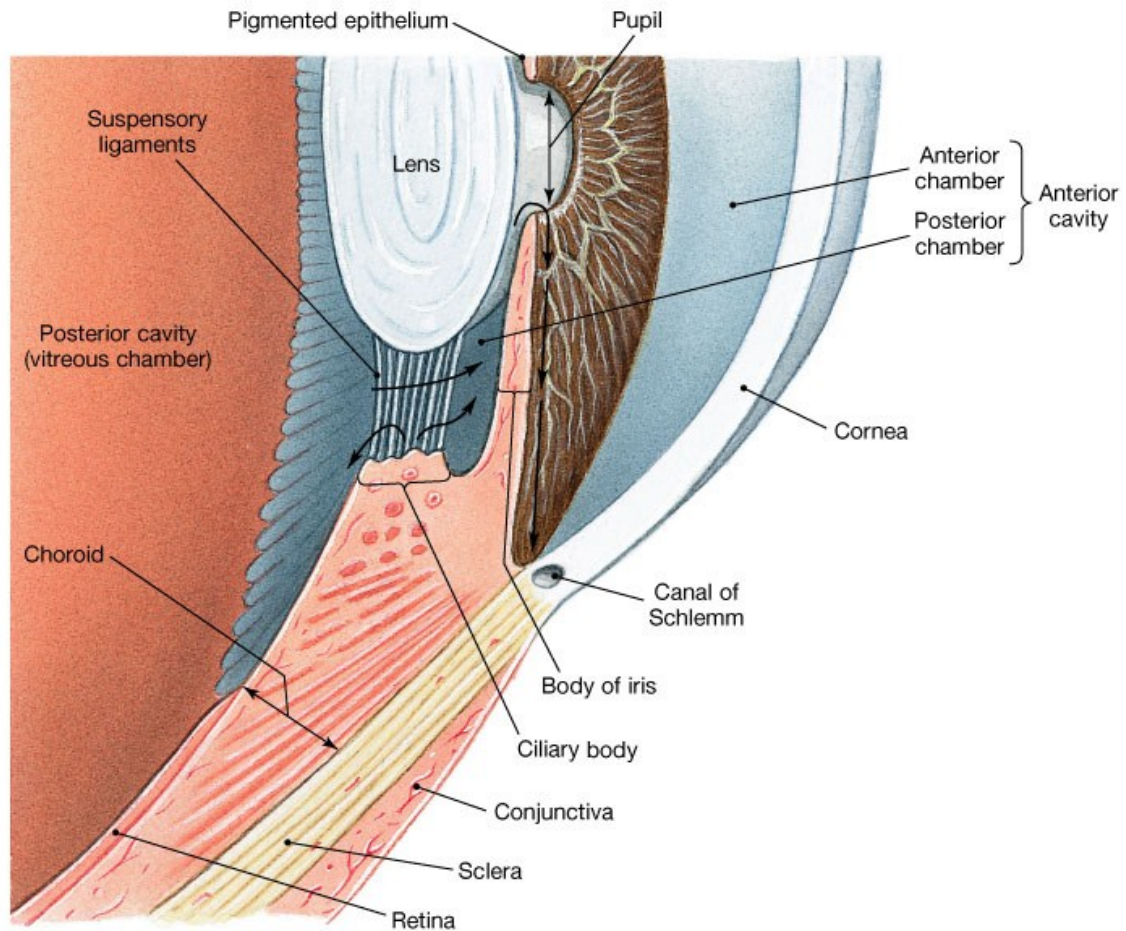
Figure 4.1   The chambers of the eye

*The ciliary body and lens divide the interior of the eye into a large posterior
cavity, also called the vireous chamber, and a smaller anyerior cavity.*

*The vitreous body helps to stabilise the shape of the eye and gives additional
physical support to the retina. It also contains specialised cells that produce
collagen fibres and proteoglycans, these are responsible for the gelatinous
consistency of the mass, (Martini, 1998).*

4.1.1.3   BILE

Historically, bile was considered valuable in post-mortem toxicology because it contains high concentrations of drug conjugates, which meant that detection was easier than in blood, with concentrations as great as 1000 times higher. However, as the instrumentation for drug detection has developed and more sensitive methods are now available, bile has been used less and less. It has been reported that the correlation between blood and bile concentrations is generally poor, (Jones, 2008).

Bile can be useful for establishing drug use, in the last few days prior to death, this can be useful in cases where drug history is unclear.

4.1.1.4   LIVER

Liver can be considered important in post-mortem toxicology because there is a large amount of tissue available, and it is relatively easy to collect and prepare compared to other tissues. Concentrations of drugs in the liver are usually higher than in the blood which makes detection easier, and they are relatively stable which is very important for the analysis of drugs that undergo post-mortem redistribution. For this reason, the liver can be more reliable than blood for measurement of drugs that are known to undergo post-mortem redistribution, e.g. tricyclic antidepressants, dextropropoxyphene and phenothiazines, (Jones, 2008). The major disadvantage with liver is that it tends to be fatty and putrefy faster than blood. It is possible to get unequal drug distribution due to diffusion of drugs from intestinal contents or from incomplete circulation and distribution

within the liver. It has also been demonstrated that post-mortem diffusion of drugs from the stomach can occur, (Jones, 2004).

4.1.2   INTERPRETATION

It used to be assumed that post-mortem blood concentrations of drugs were more or less uniform throughout the body and that concentrations measured in blood obtained at a post-mortem reflected the situation at the time of death. Therefore, interpretation of results could be based on comparison with "therapeutic" plasma concentration data. However, interpretation of post-mortem toxicology results is much more complex, and many factors need to be taken into account. These include the clinical pharmacology of the agents in question, and the circumstances under which death occurred, nature of specimens sent, changes that occur in composition after death, stability of analytes and suitability of analytical techniques, (Flanagan, 2011). It is also important to consider in cases where more than one drug has been ingested, the presence of other drugs and / or alcohol can have an impact on the overall toxic effect.

4.1.2.1   POST-MORTEM REDISTRIBUTION

One of the most important factors in sample selection and collection is the potential influence of post-mortem redistribution. The time period between death and post-mortem is very important, as this depicts how long drugs have to redistribute around the body.

It was noted back in the 1960s that different barbiturate concentrations were found in blood taken from central body cavities compared to that obtained from femoral vessels, (Curry & Sunshine, 1960). Later, a similar phenomenon was

seen in a study of digoxin where the concentration of blood collected at autopsy from various sites was higher than the concentration predicted at the time of death. This bought about the conclusion that post-mortem changes in drug concentration could occur, (Vorpahl & Coe, 1978).

In life when drugs are taken they are transported around the body in the blood to their point of action. They can also be retained or stored within larger organs, such as heart, lung or liver. However, these processes require energy and once death takes place, the supply of energy from metabolic processes is dramatically reduced. As changes in cellular biochemistry and autolysis proceed, drugs and other poisons may be released from their binding sites in the tissues and major organs. The diffusion of drugs from an area of high concentration to an area of low concentration can be expected as a natural physical process. This modification of the equilibrium between blood and tissues means that drugs can move into the surrounding blood, and this can result in an elevated concentration close to these sites, (Elliott, 2009), (Forrest, 1993), (Ferner, 2008), (Jones & Pounder, 1987).

Another consideration is that unabsorbed drug can diffuse from the bladder or the stomach. In one particular case, death was thought to occur after a long comatose period during which a large volume of urine containing drugs at high concentrations may have accumulated in the bladder. It is thought that diffusion from the bladder caused elevated drug levels of diphenhydramine and dihydrocodeine in femoral venous blood, (Moriya & Hashimoto, 2001).

Pounder et al., 1995, assessed post-mortem drug diffusion from the stomach in a human cadaver model. They found that diffusion from the stomach showed

marked case to case variability but at worst produced significant levels in both tissues and fluid samples. In order to overcome this problem they suggested that "blood be sampled from a peripheral vessel, skeletal muscle from a limb, liver from deep within the left lobe and lung from the apex rather than the base."

Typically specimens taken from "central" sites, e.g. heart, tend to give relatively "high" values for most analytes. The "peripheral" site least affected is thought to be the femoral vein for interpretation, (see Figure 4.2), but it still does not necessarily represent the concentration at the time of death, (Elliott, 2009).

This complicated interpretation of drug concentrations can be further complicated, e.g. in deaths where repeated physical CPR attempts have taken place, this could artificially redistribute the blood around the body and this could also affect the femoral vein, (Elliott, 2009).

Vitreous humour samples are thought to be least affected by post-mortem re-distribution. This is probably due to its location, it is embedded in the eye, (in the vitreous chamber), (see figure 4.1), where it is relatively isolated from blood, organs and other body fluids (Chronister, et al., 2008).
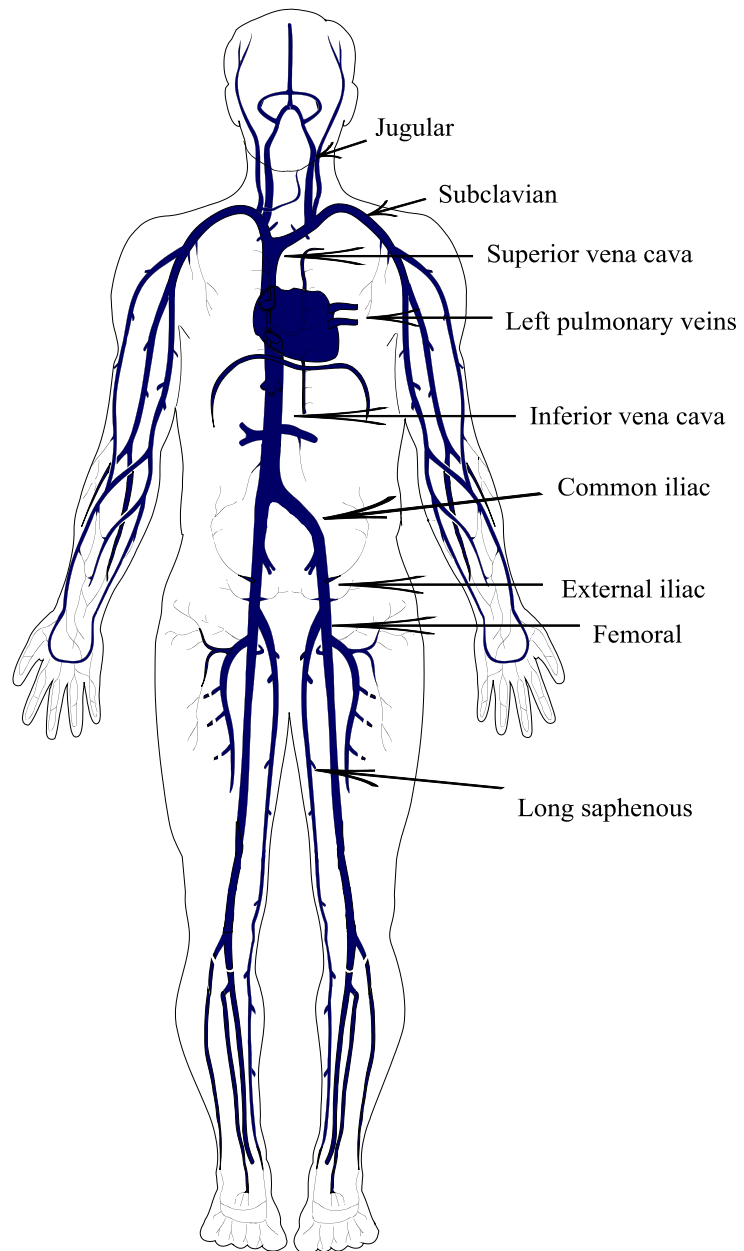
Figure 4.2   Common veins in the body.

There have been numerous studies published that prove this phenomenon, where blood samples have been collected from different veins and arteries, and drug concentrations have been measured to allow for direct comparisons.

In one such case, Pounder and Jones, 1990, found blood concentrations of doxepin that ranged from 3.6 to 12.5 mg/L and its metabolite (desmethyl-doxepin) from 1.2 to 7.5 mg/L, depending on where it was sampled from. The highest levels were found in the pulmonary vein, and the results suggest that the elevated levels could easily be from diffusion of drugs from the liver or lung, where drug concentrations were found to be very high. They also quantified the vitreous humour from the same case, and found 2.9 mg/L doxepin and 1.0 mg/L desmethyl-doxepin respectively, although generally vitreous levels are thought to be lower than in blood, these figures do not seem drastically lower than the lower blood ranges. Presumably this was due to lack of post-mortem redistribution influence of vitreous humour.

However, it has been proven that redistribution of drugs, does not affect all drugs. Jones and Pounder, 1987, compared levels of imipramine, acetaminophen (paracetamol), codeine and diphenhydramine from 10 blood sites, 24 tissue samples, cerebrospinal fluid, vitreous humour and bile. They found that imipramine and metabolite (desipramine) had the widest site-dependent concentration range and was also most highly concentrated in the organ tissues, particularly the lungs and liver. In contrast the paracetamol was relatively evenly distributed throughout the blood and the lungs with only a slightly higher concentration in the liver. Whereas the uniformity of blood levels

for diphenhydramine and codeine fell somewhere in between those of imipramine and paracetamol.

Another important aspect to take into consideration is contamination of blood during collection, e.g. stomach contents, particularly if the body has suffered internal trauma, this could also produce falsely elevated concentrations.

4.1.3   ETHICAL AND LEGAL ISSUES

There are extensive ethical and legal issues that need to be considered and / or adhered to when working with post-mortem specimens. A deceased person has rights which are exercised through the local legal framework that exists within each jurisdiction. This is usually through the coroner, who determines the type of investigation and what tests are appropriate. This does not usually include research unless prior permission is sought, (Drummer, 2007).

4.1.3.1   HUMAN TISSUE ACT 2004

The Human Tissue Act (HT Act), 2004 covers primarily England, Wales and Northern Ireland, it replaced the Human Tissue Act 1961 and came into effect on 1st September, 2006. The HT Act, 2004 established the Human Tissue Authority (HTA), to regulate activities that concern the removal, storage, use and disposal of human bodies, organs and tissue. It is a legal requirement that any premises, involved with these processes holds a valid licence, issued by the HTA, (Department of Health, 2009).

At our laboratory we hold a licence that authorises the storage of a deceased person or relevant material which has come from a human body for use for listed scheduled purposes. These include: determining the cause of death, to

establish how effective a drug or treatment has been after death, to conduct research in connection with disorders or the functioning of the human body. Apart from this, the schedule does not include any scope for research. In order to comply with the schedule, after a case has been reported, further analysis should only be carried out with consent from the family of the deceased.

4.2 MATERIALS AND METHODS

Before any analysis could take place, each case had to be examined thoroughly and an analytical plan had to be developed. This involved careful consideration of case circumstances and background, combined with assessment of the volume of each sample, to ensure that the correct testing was performed. It also confirmed that all analysis undertaken would comply with the Human Tissue Act. Qualitative screening for basic, neutral and acidic drugs was performed on alternative specimens, alongside blood where possible and appropriate.

If there was sufficient sample volume, quantitation of analytes was carried out on vitreous humour and bile, in order to compare drug levels to those found in the corresponding blood. Specialist analyses, (e.g. valproate and GHB measurement) were performed as required for the case.

4.2.1 SCREENING AND QUANTITATION

The extraction procedures for basic, neutrals and acids are detailed in Extraction Procedure 2, 3 and 4, (Chapter 2). The same procedures were used for quantitations too, except that extractions were carried out in the presence of a suitable internal standard with an appropriate range of calibrators and QCs, (for further details see Appendix A).

4.3 RESULTS

From January 2008 to March 2010, the receipt of alternative matrices as submissions for toxicological analysis was monitored, in total 476 cases were included in this study. The types of case varied dramatically with no obvious trend, except with the possible exception of "sudden infant death syndrome" (SIDS) cases because then the stomach contents is always submitted and analysed as a matter of course. Other types of cases received included: hangings, road traffic collisions, drownings or bodies found in water, fire deaths, hospital deaths, train deaths, aircraft deaths and shotgun deaths.

Results showed the following trends: 10% of cases received included the submission of alternative matrices, (Figure 4.3). Most of these cases included blood and urine but were of low volume, high viscosity and general poor state. The majority (87%) of alternative matrices submitted were stomach contents but vitreous humour was sometimes available, while bile and other matrices, e.g. liver, brain, muscle were sent occasionally, (

Figure 4.4).

Analysis of the alternative matrices revealed that stomach contents had the largest proportion of negative findings (64% of cases), with drugs detected in 71% of vitreous humour cases and 67% of bile, compared to 47% of other matrices.
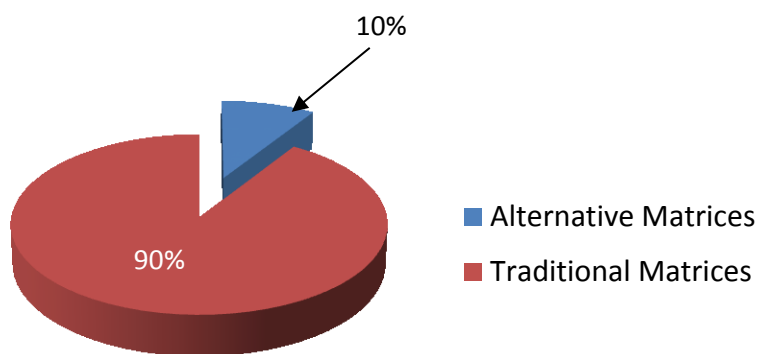
Figure 4.3   Proportion of toxicology cases where alternative matrices were submitted for analysis
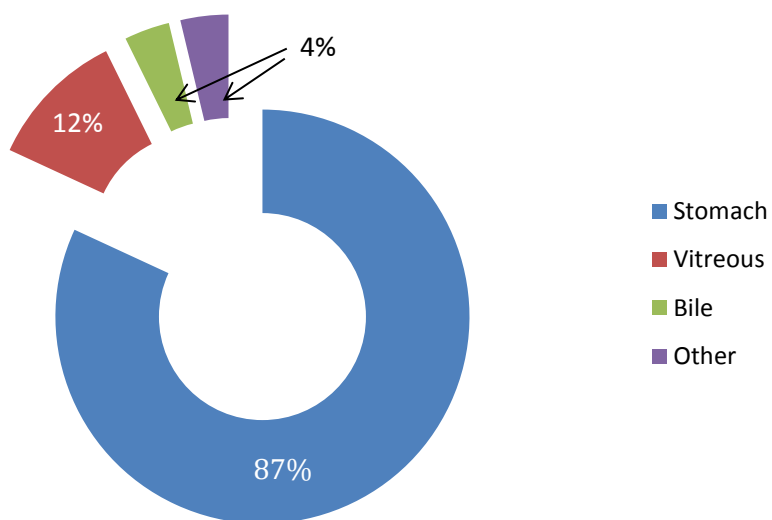
Figure 4.4   The types and proportions of alternative matrices that were submitted for analysis (some cases involve multiple specimen types)

4.3.1 DRUG SCREENING

4.3.1.1 STOMACH CONTENTS

It is not routine practice at our laboratory to request the entire stomach contents for analysis, and drugs are not quantified in this matrix. Instead, we ask for an aliquot of up to 20 mL, this can then be used to give an indication of any recent drug ingestion, prior to death. This collection procedure needed to be maintained throughout this project, (in order to comply with the Human Tissue Act), therefore all stomach contents results fall into the drug screening category. Analysis of the alternative matrices revealed that stomach contents was the most common submission, received in 87% of cases (that is 416 out of a possible 476). Out of these 36% were found to be positive for at least one drug, and in some cases for several drugs, which meant that in 64% of cases no drugs were detected. This was the largest proportion of negative findings; however, it is important to remember that a "negative" stomach contents result, does not necessarily rule out "overdose", as a cause of death, (refer to section 4.1.1.1, for explanation). With this in mind, all the cases with negative stomach contents results were reviewed to see if there were any case examples to demonstrate this, e.g. where blood levels indicate an overdose but in the stomach contents no drugs were detected. Morphine and metabolites were detected in 35 cases where no drugs were found in the stomach, see Table 4.1). In addition to these, two other cases were found where interpretation of the blood drug concentration would be consistent with an overdose but the drug responsible was not found in the stomach contents, (refer to Table 4.2).

Drug	Blood	Cause of death	Case number
morphine	y (147 ug/L)	heroin death	260
morphine	y (19 ug/L)	not morphine related	495
morphine	y (41 ug/L)	not morphine related	500
morphine	y (95 ug/L)	heroin death	660
morphine	y (21 ug/L)	not morphine related	777
morphine	y (726 ug/L)	heroin death	1215
morphine	y (213 ug/L)	heroin death	1445
morphine	y (316 ug/L)	heroin death	1970
morphine	y (142 ug/L)	heroin death	1971
morphine	y (96 ug/L	heroin death	1972
morphine	y (32 ug/L)	not morphine related	2034
morphine	y (140 ug/L)	heroin death	2101
morphine	y (945 ug/L)	possible opiate/opioid toxicity?	2167
morphine	y (126 ug/L)	heroin death	2208
morphine	y (298 ug/L)	heroin death	2261
morphine	y (1889 ug/L)	heroin death	2433
morphine	y (71 ug/L)	heroin death	2555
morphine	y (105 ug/L)	heroin death	2682
morphine	y (1346 ug/L)	heroin death	2868
morphine	y (874 ug/L)	heroin death	3162
morphine	y (316 ug/L)	heroin death	3195
morphine	y (1378 ug/L)	heroin death	3220
morphine	y (42 ug/L)	heroin death	3247
morphine	y (663 ug/L)	heroin death	3279
morphine	y (40 ug/L)	heroin death	3310
morphine	y (1167 ug/L)	heroin death	3363
morphine	y (56 ug/L)	heroin death	3474
morphine	y (227 ug/L)	heroin death	3515
morphine	y (63 ug/L)	Home - px morphine given wrong dose?	3516
morphine	y (142 ug/L)	heroin death	3662
morphine	y (126 ug/L)	heroin death	3724
morphine	y (163 ug/L)	heroin death	3837
morphine	y (162 ug/L)	heroin death	3846
morphine	y (99 ug/L)	heroin death	3903
morphine	y (1554 ug/L)	possible opiate/opioid toxicity? px morphine	4033

Table 4.1   Morphine positive results, in cases where no drugs were detected in the stomach contents

Analyte	Blood	Urine	Stomach Contents	Case Number
Amphetamine	y (4.8 mg/L)	y	nd	3810
Citalopram	y (6.47 mg/L)	y	y	4751
citalopram metabolite (Desmethylcitalapram)	y (0.37 mg/L)	y	nd	4751
Codeine	y (6.29 mg/L)	y	nd ?	4751
codeine metabolite (norcodeine)	y	y	nd	4751
Metoclopramide	nd	y	nd	4751
paracetamol	y (203 mg/L)	y	y	4751
Zopiclone	y	y	nd	4751

Key: nd=no drugs detected, o=not received or not analysed, y=detected

Table 4.2   Comparison of negative stomach contents results to blood and urine

Analytes in blood	Analyte(s) responsible for overdose	Analytes in stomach contents	Case number
ODV, Risperidone, Imipramine, Desipramine	Imipramine	Imipramine	143
Venlafaxine, Lamotrigine, Flecainide, DHC, Propranolol, Amlodipine, Paracetamol, Salicylate	Flecanide, Venlafaxine	Flecanide, Venlafaxine, Lamotrigine, Propranolol	149
Promazine And Metabolites	Promazine	Promazine	1091
Zopiclone, Valproate	Zopiclone, Valproate	Zopiclone, Valproate	1233
Amplodipine And Metab, Atenolol, Dosulepin And Metabs, Chlortalidone, Diazepam	Atenolol, Dosulepin	Atenolol, Dosulepin, Chlortalidone	1251
Citalopram And Metab, Amitriptyline And Metab Codeine, Paracetamol, Diazepam, Salicylate	Codeine, Paracetamol	Codeine, Paracetamol, Citalopram, Amitriptyline	1382
Paracetamol, Dextropropoxyphene And Metab, Diazepam + Metab	Dextropropoxyphene, Paracetamol	Dextropropoxyphene, Paracetamol	1447

Key: ODV=o-desmethylvenlafaxine, DHC=dihydrocodeine, metab=metabolite

Table 4.3   Comparison of blood and stomach contents results in drug overdose cases

There were seven other cases where blood drug concentrations indicated overdoses, and stomach contents were received. In all of these cases the drugs responsible for the overdose were detected in the stomach contents, (see Table 4.3).

There were some cases where certain drugs and metabolites were found in the stomach contents but were not detected in blood and / or urine, (see Table 4.4). These results could be important as they could help to assess how effective stomach contents would be as an alternative matrix compared to traditional matrices, e.g. if stomach contents was the only matrix received for analysis, how would this effect interpretation. It is clear that where drugs have not been detected in the blood that despite their presence in the stomach contents, an oral overdose has not occurred and this would need to be emphasised and reflected in the interpretation of any future cases where stomach contents was the only matrix analysed.

Any "obvious tablets or capsules" found in the stomach contents at post-mortem, are usually extracted and submitted for analysis in a separate tube. However, tablets submitted separately or within an aliquot of stomach contents, were not commonly found, (only seen in 1.4% of stomach contents analysed), (Table 4.5).

Drug or metabolite	Blood	Urine	Stomach contents	Case No.
Citalopram	nd	o	y	841
Diazepam	nd (AM serum)	o	y	1868
Diazepam	nd	o	y	3361
Diclofenac	nd	o	y	497
Diclofenac	nd	o	y	2260
Diclofenac	nd	nd	y	3106
Ibuprofen	nd	o	y	2545
Ibuprofen metabolites	nd	o	y	3228
Ibuprofen metabolites	nd	nd	y	3260
Lansoprazole	nd	o	y	2622
Lansoprazole	nd	nd	y	4558
Nitrazepam	nd	nd	y	3533
Omeprazole	nd	o	y	3136
Omeprazole	nd	o	y	3516
Omeprazole	nd	o	y	4009
Zolpidem	nd	o	y	841
Zolpidem	nd	o	y	3136

Key: nd=no drugs detected, o=not received or not analysed, y=detected

Table 4.4   Comparison of positive stomach contents results to blood and urine

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Case and circumstances	Tablets from stomach contents	Interpretation
Case 472 – found dead on sofa	Carbamazepine, oxycodone	Blood levels do not indicate OD
Case 2005 - Psychiatric ward - found dead in bed	oxycodone	OD - indicated from blood levels
Case 2774 – found dead on kitchen floor	codeine, fluoxetine, Paracetamol, tolterodine, zopiclone	Blood levels do not indicate OD
Case 3566 – found dead at home	Venlafaxine	OD - indicated from blood levels
Case 3685 – found dead in chair	oxycodone	OD - indicated from blood levels
Case 4732 – found dead in bed	oxycodone, warfarin	Possible excessive ingestion

Table 4.5   Results of tablet analysis, isolated from stomach contents

Although metabolites are not usually present in the stomach contents, 18 different metabolites were found and some of these were present in more than one case so this gave a total of 31 occurrences, (see Table 4.6).

Metabolite	Blood	Urine	Number of cases	Case No.
amitriptyline metabolite (nortriptyline)	y	y	3	2423, 3449, 4656
Carbamazepine metabolite	y	o	1	3823
chlordiazepoxide metabolite (demoxepam)	y	y	1	1709
chlormethiazole metabolite	y	y	1	3997
citalopram metabolite (desmethylcitalapram)	y (2/2)	y (2/2)	2	3775, 4255
clobazam metabolite (norclobazam)	y (2/2)	y (1/2)	2	1571, 2422
clozapine metabolite (norclozapine)	y (2/2)	y (2/2)	2	2807, 3143
diazepam metabolite (nordiazepam)	y (7/8)	y (3/8)	8	1155, 1215, 1709, 2101, 2188, 2367, 3195, 4423
diltiazem metabolite (deacetyldiltiazem)	y	o	1	2859
dosulepin metabolites	y	y	1	3366
nitrazepam metabolite	y	nd	1	3533
O-desmethyltramadol (ODT)	y	y	1	2862
O-desmethylvenlafaxine (ODV)	y	y	1	2040
omeprazole metabolite	y	o	1	2422
oxycodone metabolites	nd	y	1	3533
promazine metabolies	y	y	1	4423
propranolol metabolites	y (2/2)	y (2/2)	2	4255, 4448
sertraline metabolite (norsertraline)	y	y	1	2545

Key: y=detected, o=not analysed, nd=no drugs detected

Table 4.6   Metabolites detected in the stomach contents

Although, the reason for not routinely measuring drug levels in the stomach contents has been discussed at length, an exception was made in one particular case, (2473). The customer specifically requested ethylene glycol analysis on both samples submitted, these were blood and stomach contents. This is a specialist assay carried out on the GC-FID, (refer to section 2.9.3.3, extraction procedure 13). In the blood no ethylene glycol was detected above 50 mg/L (the limit of detection for this assay), in the stomach contents 205 mg/L was detected.

In another case (3194), circumstances revealed that an empty bottle of "Gamma Butyrolactone" was found in the room of the deceased. As it is known that gamma butyrolactone (GBL) is converted to gamma-hydroxybutyrate (GHB) in the body then it was necessary to do specific GC-MS analysis for GHB, (refer to section 2.7.1, extraction procedure 9). Both blood and stomach contents were analysed, the measured blood level was >1250 mg/L and it was also found to be present in the stomach contents.

VITREOUS HUMOUR

In total there were 134 analytes detected in vitreous humour but there were a further 62 analytes that were detected in other matrices that were not detected in vitreous humour.

If the screening results are compared for blood and vitreous, there were 43 analytes that were detected in blood that were not found in the corresponding vitreous humour, (see Table 4.7).

However, if the reverse scenario is looked at, positive results in vitreous that have not been detected in the blood, there are 9 examples, (see table 4.8).

If the vitreous and urine results are compared, there are 27 positive results in urine that have not been detected in the vitreous humour, (comprised of 16 parent compounds and 11 metabolites (or associates), (see table 4.10).

If vitreous positive results are compared to those not detected in urine, there are 7 examples, and 5 of these are metabolites, (see table 4.9).

If the results from blood and urine are looked at in conjunction, there are only 2 compounds that have been detected in vitreous humour that have not been found in either blood or urine, (see table 4.8 and 4.9), these results have been highlighted).

There was one case received (2584), where vitreous humour was the only matrix sent in for analysis. Case circumstances stated that the deceased had been hit by an oncoming train, and the prescription history stated that she had been prescribed antidepressants. The results showed that citalopram and metabolite were detected.

Analyte	Number of cases	Case No.
Amlodipine	3	609, 2164, 2192
Clomipramine and metabolite (norclomipramine)	1	668
Omeprazole metabolite	1	2422
Cyclizine	2	775. 2290
Cyclizine metabolite (norcyclizine)	1	2165
Diazepam	4	775, 1510, 2164, 2366
Diazepam metabolite (nordiazepam)	4	775, 1510, 2164, 2366
Duloxetine related	1	2165
Fluoxetine	1	1040
Fluoxetine metabolite (norfluoxetine)	1	939
Lansoprazole metabolite	1	1661
Levomeprazine	1	668
Lignocaine	1	1040
M3G	1	277
Methadone	1	939
Methadone metabolite (EDDP)	1	939
Mirtazepine	2	1, 668
Omeprazole	1	2290
Oxazepam	1	1650
Papaverine	1	260
Paracetamol	3	1688, 2491, 3086
Promethazine	1	2165
Risperidone	1	3086
Sertraline	2	3086, 2170
Sertraline metabolite (norsertraline)	1	2170
Temazepam	2	1, 1650
Zuclopenthixol	1	277

Key: y=detected, o=not analysed, nd=no drugs detected

Table 4.7   Analytes detected in blood but not detected in vitreous humour

Analyte	Blood	Urine	Vitreous Humour	Case No.
6-MAM	nd	o	y	260
amisulpride metabolite	nd	y	y	3
chloroquine associates	nd	nd	y	775
Codeine	nd	y	y	2291
Ibuprofen metabolites	nd	nd	y	2298
Laudanosine	nd	y	y	1
Levamisole	nd	y	y	959
Lignocaine	nd	y	y	1
omeprazole metabolite	nd	y	y	3

Key: y=detected, o=not analysed, nd=no drugs detected

Table 4.8   Analytes detected in vitreous humour that were not found in blood

Analyte	Blood	Urine	Vitreous humour	Case Number
Chloroquine associates	o	nd	y	348
Chloroquine associates	nd	nd	y	775
Ibuprofen metabolites	nd	nd	y	2298
Mirtazapine metabolites	y	nd	y	1650
Noscapine	y	nd	y	277
Paracetamol	y	nd	y	2143
Pethidine metabolite (norpethidine)	y	nd	y	3066

Key: y=detected, o=not analysed, nd=no drugs detected

Table 4.9   Analytes detected in vitreous humour that were not found in urine

Analyte	Number of cases	Case No.
6-MAM	1	1441
Amlodipine	1	609
Amlodipine metabolite	2	609, 2165
Ciprofloxacin	1	3086
Cocaine	2	939, 959
Cocaine metabolite	1	959
Cyclizine	1	775
Cyclizine metabolite (norcyclizine)	2	775, 2165
Duloxetine related	1	2165
Fluoxetine metabolite (norfluoxetine)	1	939
Lansoprazole metabolite	1	3066
Methadone	1	939
Methadone metabolite (EDDP)	1	939
Mirtazepine	1	1
Paracetamol	2	2298, 3086
Promethazine	1	2165
Quinine	1	609
Ranitidine	1	939
Risperidone	1	3086
Sertraline	2	3086, 2170
Sertraline metabolite (norsertraline)	1	2170
Temazepam	1	1650

Key: y=detected, o=not analysed, nd=no drugs detected

Table 4.10   Analytes detected in urine that were not found in vitreous humour

4.3.1.2 BILE

It was only possible to screen the bile for 12 cases; this was partly due to the low number of submissions received. However, in addition to this, problems were encountered with extraction, following the addition of solvent to the sample, mixing and centrifugation, sometimes the fluid would solidify to form a "jelly-like" consistency, once this had occurred it was not possible to continue with the extraction. As bile shows drug use in the past few days, and this is also true for urine it would make sense to compare the screening results obtained for these matrices. Unfortunately, this is only possible for 4 cases, as urine was not submitted for analysis, with the other cases.

If the bile results are compared to the both blood and urine results, 5 more analytes were found in the bile, (no urine was submitted for 4 of these cases), but in the case where urine was received (case 2753), the results show that no cyclizine was detected in the urine or blood, although it was found to be present in the bile, (see table 4.11).

There were 7 analytes that were not detected in bile that were detected in blood and / or urine, (see Table 4.11). All 7 were detected in urine and out of these an additional 2 analytes were also detected in blood, these were codeine and the venlafaxine metabolite, O-desmethylvenlafaxine.

Analyte	Blood	Urine	Bile	Case Number
Amlodipine metabolite	nd	o	y	2192
Cyclizine	nd	nd	y	2753
Doxazosin	nd	o	y	2192
Doxazosin related	nd	o	y	2192
Valproate	nd	o	y	807
6-mam	nd	y	nd	3310
Bisoprolol	nd	y	nd	2753
Codeine	y	y	nd	3310
Haloperidol	nd	y	nd	2753
Midazolam	nd	y	nd	2753
O-desmethylvenlafaxine (ODV)	y	y	nd	3310
Trazadone	nd	y	nd	563

Key: y=detected, o=not analysed, nd=no drugs detected

Table 4.11   Analytes detected in bile that were not found in blood or urine

4.3.1.3 LIVER AND OTHER MATRICES

There were 10 cases submitted where in the absence of more suitable matrices, it was deemed appropriate to analyse a different matrix to any already described, these were: liver, liver fluid, bowel, brain, lung, muscle or fluid from the pleural cavity, (see Table 4.12). For some of these cases blood and or urine were also submitted but for the majority only alternative matrices were available for analysis, so comparison of results was limited. Case circumstances have been included as it is generally due to reasons given here, that only limited specimens were available for analysis, e.g. if the body was found very decomposed (or found in water which can speed up decomposition).

4.3.1.4   ALTERNATIVE MATRICES ONLY

There was one case received (2498), where only vitreous humour and stomach contents were submitted for analysis. The case circumstances stated that the deceased was a known epileptic found unresponsive in the bath, she had been prescribed carbamazepine. The results showed that carbamazepine was detected in the vitreous at 1.53 mg/L and was also present in the stomach contents.

Case number and circumstances	Matrices received	Drugs detected
1235 suspected solvent abuse	blood	fluoxetine at 2.647 mg/L and norfluoxetine at 2.02 mg/L, butane and propane
	urine	fluoxetine and norfluoxetine
	Stomach contents	Fluoxetine (trace amount)
	brain and lung	butane and propane
2270 suspected overdose	blood	diazepam and metabolite, venlafaxine (13.8mg/L), o-desmethylvenlafaxine (5 mg/L), phenytoin (24 mg/L), propranolol, Temazepam, zopiclone and metabolite
	Urine	venlafaxine and ODV, propranolol zopiclone and metabolite
	bowel contents	venlafaxine and ODV, phenytoin, propranolol
812 recovered from river	Liver	Citalopram
1032 found hanging, decomposed	Liver	Fluoxetine
2937- found dead in bed	Liver	Codeine
2879 found hanging, decomposed	liver and muscle	venlafaxine and ODV
3504 found dead, decomposed, drug user - syringe in arm	Liver	morphine and M3G, noscapine, papaverine
	stomach contents	no drugs detected
2672 died at home	liver fluid	citalopram and metabolite, omeprazole, paracetamol, zopiclone
	vitreous humour	citalopram and metabolite, omeprazole, paracetamol
3873 recovered from stream	liver fluid	citalopram and metabolite
	stomach contents	no drugs detected
1003 found hanging, decomposed	pleural cavity	amitriptyline and metabolite, diazepam and metabolite, temazepam and metabolite, quetiapine metabolite, tramadol (33.3 mg/L) and ODT (6.83 mg/L)
	stomach contents	Tramadol
	Bile	amitriptyline and metabolite, diazepam, temazepam and metabolite, quetiapine metabolite, tramadol and ODT

Key: ODV=o-desmethylvenlafaxine, ODT= o-desmethyltramadol

Table 4.12   Results from cases where "different" matrices were analysed

4.3.2 DRUG QUANTITATION

Due to a low number of suitable submissions, limited sample of vitreous and lipid content of bile (interfering with extraction) performing drug measurements proved challenging. It was only possible to quantify drugs in vitreous (30 Cases) and/or bile (6 Cases) in relatively few cases.

4.3.2.1 VITREOUS HUMOUR

In the majority of cases analysed drug levels were found to be much lower in vitreous than in the corresponding blood, however there were a few exceptions, (Table 4.13).

In one Case, there was slightly more tramadol and metabolite, (O-desmethyltramadol) found in vitreous than in the blood, and in another case more pethidine and metabolite (norpethidine) were detected, (Table 4.13).

The results for the benzodiazepines all demonstrated lower levels in vitreous compared to blood, (Table 4.13).

During this study, 41 cases were found to be positive for tricyclic antidepressants in blood, these included amitriptyline, clomipramine, desipramine and / or dosulepin (dothiepin), but of these cases, only 2 submitted vitreous for analysis. For each of these cases, detailed analytical plans were developed and adhered to.

In Case 668, (see Table 4.14) the blood and stomach contents were screened for basic, neutral and acidic drugs. The results showed, clomipramine (+ metabolites), levomepromazine (+ metabolites), zopiclone (+ metabolites), mirtazapine (+ metabolite), diazepam (+ metabolite) and propranolol were detected in blood, with no drugs detected in the stomach, (Table 4.14). The

screening results allowed for the comparison of drug responses and clomipramine, levomepromazine, zopiclone, mirtazapine and diazepam were seen to be present at a low concentration that would be consistent with therapeutic use, so a measurement was not necessary. Propranolol was the most significant and so this was measured in both the blood and the vitreous. It was found to be 1.11 mg/L in the blood compared to 0.62 mg/L in vitreous, another example of a lower concentration in vitreous. The vitreous extract was screened for basic drugs, and levomepromazine, zopiclone (+ metabolites), and mirtazapine metabolite were all detected, but clomipramine and / or metabolite were not.

In Case 2591 the blood was screened for basic, neutral and acidic drugs and codeine, quinine, amitriptyline, bisoprolol and citalopram were detected, all the levels seen in the screen were low and so no measurements were required. The vitreous was screened for basic drugs and the results fully supported the blood, with the same drugs being detected.

Drug	Blood	Vitreous	Vitreous:Blood ratio
Benzodiazepines	**mg/L**	**mg/L**	
chlordiazepoxide	0.76	0.1	0.1316
demoxepam	2.61	0.75	0.2874
clobazam	0.41	0.1	0.2439
clobazam	0.225	0.091	0.4044
norclobazam	4.94	1.98	0.4008
diazepam	0.23	0.0715	0.3109
nordiazepam	0.6	0.0205	0.0342
temazepam	3.27	0.85	0.2599
oxazepam	0.5	0.21	0.4200
Anticonvulsants / Antiepileptics			
Carbamazepine	o	1.53	n/a
lamotrigine	1.039	0.875	0.8422
lamotrigine	1.6	o	n/a
phenytoin	9.06	< 1	n/a
phenytoin	17.32	2.67	0.1542
Valproate	nd	o	n/a
Antidepressants			
duloxetine	0.47	<0.05	n/a
Opioids			
methadone	0.46	0.1	0.2174
methadone	1.2	0.36	0.3000
methadone	0.211	0.035	0.1659
methadone	1.46	0.72	0.4932
Pethidine	9.7	10.5	1.0825
norpethidine	2.5	2.7	1.0800
tramadol	1.36	1.41	1.0368
O-desmethyltramadol	0.32	0.33	1.0313
Opiates	**ug/L**	**ug/L**	
Morphine	147	18	0.1224
Morphine	783	103	0.1315
NSAIDS	**mg/L**	**mg/L**	
ibuprofen	210.2	13.7	0.0652
salicylate	56	9	0.1607
B-blockers			
propranolol	1.11	0.62	0.5586
Antipsychotic			
olanzapine	0.0295	0.049	1.6610
Stimulant			
Amphetamine	4.8	o	n/a
Caffeine	4.28	3.93	0.9182

Table 4.13 blood vs vitreous - measured concentrations

Drug	Blood	Stomach contents	Vitreous
Clomipramine (+ metabolite)	Y (Y)	nd	Nd (nd)
Zopiclone (+metabolite)	Y (Y)	nd	Y (Y)
Levomepromazine (+ metabolite)	Y (Y)	nd	Y (nd)
Mirtazepine (+ metabolite)	Y (Y)	nd	Y (Y)
Diazepam (+ metabolite)	Y (Y)	nd	O
Propranolol	Y (1.11 mg/L)	nd	Y (0.62 mg/L)

Table 4.14   Results for Case 668

4.3.2.2   BILE

Analyte	Blood (mg/L)	Bile (mg/L)	Blood:Bile ratio
Amphetamine	4.8	29	6.04
clobazam	0.225	0.499	2.22
clobazam metabolite (norclobazam)	4.943	9.99	2.02
lamotrigine	1.6	9	5.63
Lorazepam	0.19	0.36	1.89
olanzapine	0.0295	0.2725	9.24
Valproate	nd	35	Na

Table 4.15   blood vs bile, measured concentrations

The bile levels were found to be much greater than those determined in blood, which was expected, as it is known to contain high concentrations of drugs, and many drugs have been shown to accumulate in the bile, (Skopp, 2004), (Jones, 2004).

The blood:bile ratios were calculated to see if any specific trends were evident, (Table 4.15). it is interesting that clobazam (a benzodiazepine derivative) and its metabolite norclobazam are found to both be present at the same ratio, which is twice as much in bile compared to blood, and lorazepam (a similar type of drug, benzodiazepine), comes very close to this with a ratio of 1.89. The other ratios determined were much greater, with amphetamine and lamotrigine nearly 6 times greater in bile than blood and olanzapine 10 times greater.

4.3.3 DRUG STUDIES

4.3.3.1 MORPHINE AND HEROIN RELATED COMPOUNDS

The solid phase extraction method allowed for the detection and measurement of morphine and morphine metabolites, M3G and M6G, (this allowed for the calculation of free:total morphine ratio), as well as the detection of codeine, codeine-glucuronide and the specific heroin metabolite, 6-MAM in post-mortem blood, (refer to Figure 4.5 Metabolic pathways of morphine and analogues**,**). However, as heroin is rapidly absorbed, it is not always possible to detect 6-MAM in blood and so additional heroin markers, noscapine and papaverine are also included in the analysis, (refer to Figure 4.6). These are components of the opium poppy that have come through the production process, their presence confirms heroin use rather than diamorphine or morphine, (Elliott, 2009).
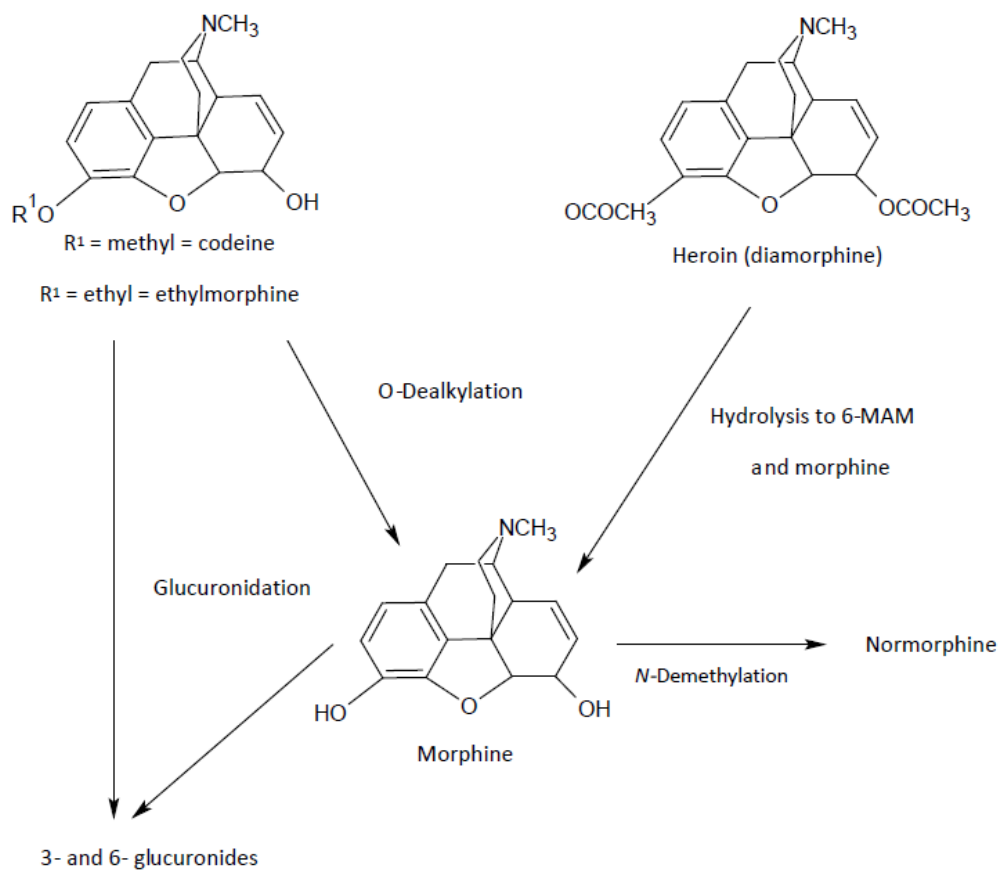
Figure 4.5 Metabolic pathways of morphine and analogues,
Redrawn from Jones, (2008)
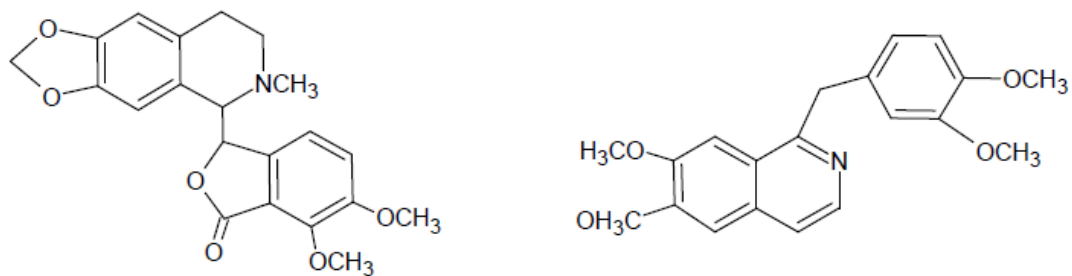
Figure 4.6 Structures of noscapine (left) and papaverine (right)

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Blood versus vitreous

It is well documented that 6-MAM is unstable and therefore not always detectable in the blood, however as vitreous humour has a very sterile environment, it has been suggested that it could be a better matrix for analysis of this metabolite. An interesting part of this study was going to test this theory.

However in reality, of all the cases where morphine analysis was required, there were only three cases that included vitreous as a submission and these were analysed specifically for morphine, (see Table 4.16).

In terms of morphine concentrations, levels were found to be a lot lower in vitreous than in blood, and the metabolites M3G and M6G were not detected in vitreous in Case 260 or 277, (above the 10 ug/L LOD for the assay). In Case 1441, the calculated concentrations for the duplicate samples were not within 20%, ordinarily this would mean the analysis would need to be repeated but this was not possible due to insufficient sample volume.

6-MAM was detected in 1/3 cases in blood and in 2/3 cases in vitreous, the opposite was found to be true for papaverine, detected in 2/3 in blood compared to 1/3 in vitreous, while noscapine was found to be present in both matrices in all 3 cases.

	Case 260	Case 277	Case 1441
Morphine	147 ug/L	783 ug/L	39 ug/L
M3G	nd (< 10 ug/L)	75 ug/L	388
M6G	nd (< 10 ug/L)	nd (< 10 ug/L)	43
6-MAM	nd	y	nd
Noscapine	y	y	y
Papaverine	y	y	nd
	Vitreous Humour		
Morphine	18 ug/L	103ug/L	y
M3G	nd (< 10 ug/L)	nd (< 10 ug/L)	y
M6G	nd (< 10 ug/L)	nd (< 10 ug/L)	y
6-MAM	y	y	nd
Noscapine	y	y	y
Papaverine	nd	y	nd
vitreous:blood ratio	0.12	0.13	n/a

Key: y = detected, nd=not detected, n/a= not applicable

Table 4.16   Morphine - blood versus vitreous

Analyte	Blood	Urine	Stomach contents	Total
Morphine	Y	Y	Y	
M3G	Y	Y	O	6 Cases
M6G	Y	Y	O	
Noscapine	Y	Y	Y	3 Cases
6-MAM	Y	Y	Y	1 Case

Key: y=detected, o=none received or not analysed

Table 4.17   Morphine - blood versus stomach contents

4.3.3.2   CODEINE

Codeine was detected in 67 of the cases included in this study, blood and urine were submitted for the majority, stomach contents was received in 63 of these cases, bile in 2, vitreous in 3 and there was one case where only liver and stomach contents were available (2937).

Of the 63 stomach contents samples analysed, codeine was only detected in 4 of them, and in each of these cases it was detected with a minimum of at least 3 other drugs, Table 4.18.

Codeine was detected in all 3 vitreous submissions but in one of the cases there was no codeine detected in the blood which was an unexpected result, Table 4.19. On investigation of the timings, it became apparent that the cases with the unexpected result had been subjected to a long time delay between date of death and collection of samples, Table 4.20.

In the case where only liver and stomach contents were submitted, the case information states that the deceased was an alcoholic, found dead in bed. In this type of case, the date found is recorded as the date of death; estimations of time of death are not routinely disclosed to the laboratory. The results showed codeine and putrefactants in the liver, and putrefactants in the stomach, (quantitation or estimation of the codeine concentration in the liver was not performed).

Case	Blood	Stomach Contents
Case 1382	Amitriptyline, Citalopram, Codeine, Diazepam, Paracetamol, Salicylate	Amitriptyline, Citalopram, Codeine, Paracetamol
Case 2034	Atenolol, Codeine, Diazepam, Ibuprofen, Morphine, M3G, M6G, Oxazepam, Salicylate, Temazepam, Trazadone, Zopiclone	Atenolol, Codeine, Diazepam, Ibuprofen, Salicylate, Temazepam, Trazadone, Zopiclone
Case 2774	Codeine, DHC, Fluoxetine, Orphenadrine, Paracetamol, Risperidone, Tolterodine, Zopiclone	Codeine, Fluoxetine, Paracetamol, Tolterodine, Zopiclone
Case 2859	Codeine, Diltiazem, Paracetamol, Temazepam, Warfarin	Codeine, Diltiazem, Paracetamol, Temazepam, Warfarin

Table 4.18   Results for codeine positive stomach contents

	Blood	**Urine**	**Vitreous**
Case 1441	Y	Y	Y
Case 2291	Nd	Y	Y
Case 2591	Y	Y	Y

Table 4.19   Codeine results – blood versus vitreous

Days elapsed	**Case 1441**	**Case 2291**	**Case 2591**
Death to collection	6 - 7	0 – 2	0
Collected to receipt	0	23	5
Receipt to analysis	0	0	1
Further Analysis	vitreous 7	vitreous 4	vitreous 6
Total days since death	13 - 14	27 – 29	12

Table 4.20   Results of timings of samples in codeine - blood versus vitreous cases

4.3.3.3   DIAZEPAM

Diazepam was one of the most commonly detected drugs, (found in 83 cases) along with its main metabolite nordiazepam (desmethyldiazepam), (refer to figure 4.9). Out of the positive cases, 72 submissions included stomach contents and 8 included vitreous. Diazepam was detected in 21 stomach contents and nordiazepam was detected in 8 cases. Diazepam was detected in 4 of the vitreous cases and nordiazepam was detected in the same 4 cases, plus an additional one. As nordiazepam is a metabolite of several different compounds, (not just diazepam) e.g chlordiazepoxide and chloazepate, this explains its presence in one of the vitreous cases and one of the stomach contents cases, as chlodiazepoxide was also detected.

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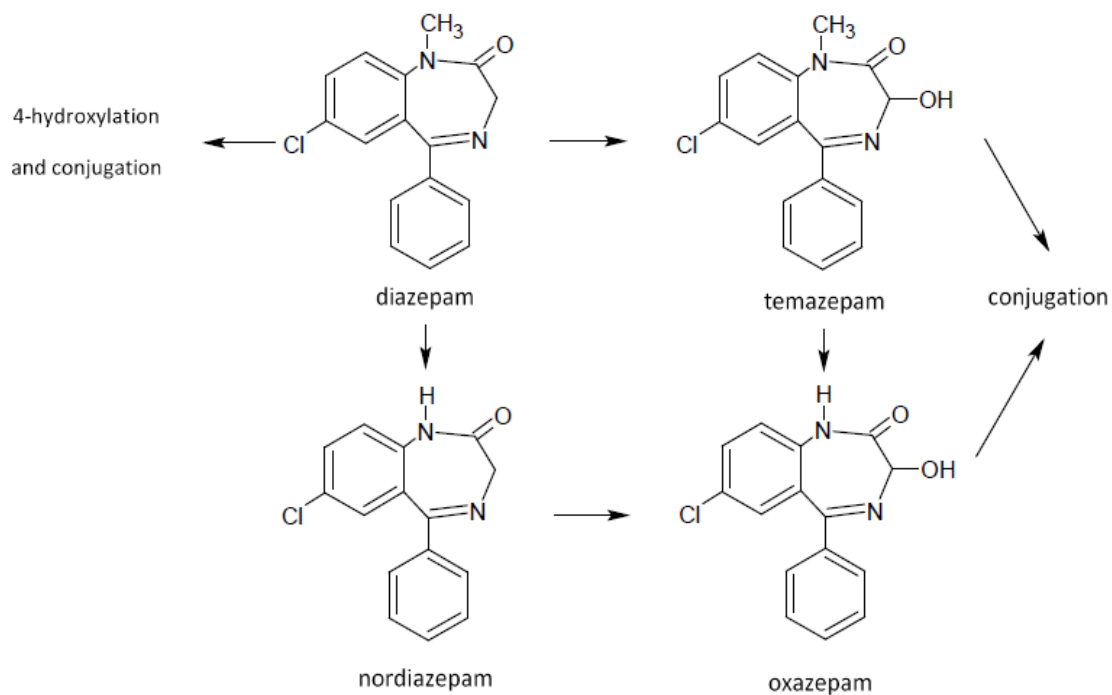
Figure 4.7   Metabolism of diazepam

Redrawn from Baselt, (2011, p. 472).

4.3.3.4   COCAINE

Cocaine was detected in 14 cases; blood and urine were submitted for every case, stomach contents was submitted for 13 of them and vitreous was sent in for 2 of them, (unfortunately bile was not submitted in any of these cases). Results showed that urine tested positive in every case, blood tested positive for 5 of them, and it was found in the stomach contents of one case and was not detected in either of the vitreous submissions.

Drug	Blood	Urine	Stomach contents	Vitreous humour	Case number
cocaine	y (6.9 mg/L)	y	y	o	491
cocaine	y	y	nd	o	698
cocaine	nd	y	nd	nd	939
cocaine	nd	y	o	nd	959
cocaine	nd	y	nd	o	1215
cocaine	y	y	nd	o	1258
cocaine	nd	y	nd	o	2261
cocaine	y	y	nd	o	2367
cocaine	nd	y	nd	o	2682
cocaine	y	y	nd	o	2767
cocaine	nd	y	nd	o	3363
cocaine	nd	y	nd	o	3422
cocaine	nd	y	nd	o	3837
cocaine	nd	y	nd	o	4255

Key: nd=no drugs detected, y=present, o=none received

Table 4.21   Results where urine tested positive for cocaine

4.3.3.5   AMPHETAMINE

A case where amphetamine overdose was the most likely cause of death, (3810) has already been considered, but it is interesting that in 3 other cases, (1971, 3290 and 4023) amphetamine was detected in the blood and / or urine but was again, not found in the stomach contents.

4.3.3.6   METHADONE

Methadone was detected in the blood of 39 cases, and the main metabolite EDDP, was detected in 12 of the same cases. 35 of these cases submitted

stomach contents for analysis and 5 submitted vitreous (4 of these were measured and have already been discussed) but only 1 submitted both. The stomach contents results showed that methadone was detected in 12 cases but the metabolite EDDP, was not detected at all.

4.4   DISCUSSION

4.4.1   DRUG SCREENING

4.4.1.1   STOMACH CONTENTS

Regarding the 35 cases where no drugs were found in the stomach but morphine was detected in the blood, four of these cases had low levels of morphine <50 ug/L, and as fatal opiate poisonings are usually associated with free morphine concentrations greater than this, (although deaths have been recorded at lower concentrations in individuals with little or no tolerance to this group of drugs), it is unlikely that in these cases, a morphine overdose was the cause of death.

In 28 of these cases either a specific heroin metabolie (6-MAM) was present or marker compound(s) were detected that confirmed heroin use, e.g. noscapine or papaverine, prior to death and in the absence of any other cause of death been found, it is most likely that the individuals in these cases died from fatal opiate toxicity. As heroin is usually smoked, injected or snorted if it's in its pure form, morphine was not necessarily expected to be detected in the stomach contents for these cases, (although it is not impossible for analytes to be detected in the stomach following routes of administration other than oral ingestion). The three remaining cases are somewhat more complicated, there is no evidence of

heroin use so it is unclear whether the morphine detected has come from heroin or morphine use, but in one case the deceased was a known heroin user prescribed methadone and the most likely cause of death was opiate or opioid toxicity so you would not necessarily expect to see morphine in the stomach contents of this case. However, in the 2 remaining cases morphine was prescribed in tablet form so the absence of morphine in the stomach contents of these cases indicates that morphine was not orally, close to the time of death.

Besides the morphine cases described, only two other cases had blood drug levels that would be regarded as consistent with an overdose, (see Table 4.2). For case 3810, amphetamine was detected at a significant level in the blood to be consistent with an overdose and it was also detected in the urine and the bile, yet it was found to be absent in the stomach contents. As previously described with heroin, this could be due to the route of administration as it can be snorted or rubbed onto the gums, ingested orally or injected, so it is possible that it could have avoided exposure to the gastric system and would therefore not necessarily be detectable in the stomach contents.

A possible scenario could be that a range of tablets were taken as an attempt at suicide and perhaps when there was no immediate effect, more tablets were taken "to speed things up", these could have been the citalopram and propranolol, then the individual died before these extra tablets had chance to be fully absorbed.

In case, 4751, blood levels suggest an overdose of both citalopram and codeine and paracetamol (available in combined preparations). In the stomach contents citalopram and paracetamol are detected but it is not clear if any codeine is

present. Paracetamol and codeine elute at very similar retention times on the HPLC-DAD system used, and as the paracetamol in the stomach was present at such a high level it was not possible to detect any codeine but that does not mean that there was none present. In addition to this the interpretation of this case is further complicated because the blood was not collected from the femoral vein (the recommended site of collection) but instead the vena cava, which is a site close to the heart. As a result of this the measured concentrations of drugs, (see Table 4.2), could be artificially elevated and therefore not reflect the concentrations that were exerting an effect.

The results that showed drugs and / or metabolites detected in the stomach contents but absent in the blood and / or urine, (see Table 4.3), could have important implications. Drugs in the stomach that are absent in the blood, and or urine, suggest very recent ingestion of these drugs prior to death, e.g. they have been ingested but not absoerbed. In the case of suicide, sometimes drugs are taken as a "back-up" plan in case the intended plan does not work or they cannot go through with it, e.g. if they planned to take their own life by hanging or shooting. Another possible scenario could be that a range of tablets were taken as an attempt at suicide and perhaps when there was no immediate effect, more tablets were taken "to speed things up", then the individual died before these extra tablets had chance to be fully absorbed and would therefore not be detected in other matrices.

The results show that ibuprofen metabolites have been detected in the stomach contents of two cases (where they have not been detected in the blood or urine).

It is unusual to see metabolites in the stomach contents, you would normally expect to see only parent drugs. In total, ibuprofen metabolites have been detected in stomach contents of 5 cases all of which also have ibuprofen present (in parent form), it is therefore possible that they are not "true" metabolites that are present in the stomach but possible break-down products from the parent compound. However, it is equally possible that the metabolites are present due to back diffusion from the bile after death. Although it is not possible to decipher exactly how these compounds came to be present in the stomach, the very acidic nature of the stomach contents would provide a favourable environment for these compounds, and the fact that they were detected in several different cases seems to support this.

A review of positive stomach contents results showed that in addition to the ibuprofen results, metabolites were detected on 31 other occasions, (see Table 4.6), this does not mean in 31 cases because some of these cases have more than one metabolite present. Perhaps the presence of more than one metabolite suggests that they have most likely got into the stomach via back diffusion rather than simultaneous breakdown of different compounds.

With reference to the results in Table 4.5, in case 2774, codeine, fluoxetine, paracetamol, tolterodine, zopiclone were detected on analysis of the tablets, but it is probable that some of the drugs were present due to contamination from the stomach contents. The results from this case also showed a discrepancy with the blood and urine as fluoxetine was only present in the stomach contents, this would indicate very recent ingestion of fluoxetine prior to death.

In Case 4732, the stomach contents were submitted for analysis in addition to the tablets that had been isolated from the stomach. These results showed up a discrepancy as the warfarin detected in the tablet was not detected in the stomach contents but it was detected in the blood. However, despite this result it is still most likely that the tablets were contaminated with warfarin from the stomach contents as these drugs are not available in combined preparations, and it is unlikely that they were contaminated before ingestion. This could highlight a potential problem with stomach contents analysis for drugs because it is possible that due to its varied composition and lack of homogeneity, the results could be different depending where the sample or aliquot was collected from i.e. proximity to where the tablet was removed from. Perhaps if more than one sample of stomach contents had been collected and analysed, warfarin would have been detected.

For case 2473, where the ethylene glycol was measured in the stomach contents, it is important to note that only an aliquot (not the entire stomach contents from the body) was received so the measurement itself is somewhat meaningless. However, the result does indicate that ethylene glycol must have been ingested sometime recently prior to death; this is information that would have been missed if the blood alone had been analysed.

For case 3194, where specific GHB analysis was carried out, although it is known that GHB exists as an endogenous compound in mammalian tissue and can be found in almost all post-mortem biological fluid, a level of >1250 mg/L is much higher than endogenous levels and consistent with recent ingestion of GBL/GHB and this is supported by the presence of GHB in the stomach.

4.4.1.2   VITREOUS HUMOUR

A total of 44 more analytes were found in blood compared to vitreous humour, this means that if only vitreous had been sent for analysis, none of these analytes would have been detected.

When the results were reviewed, no clear overdose cases would have been missed by vitreous analysis alone, but there was one case of possible excessive ingestion that would not have been picked up. This was case 939, where blood levels indicate excessive ingestion of fluoxetine (1.41 mg/L) and possibly excessive ingestion or chronic therapeutic use of methadone (0.42 mg/L), the toxicological significance of opiate/opioid drug levels are difficult to interpret, as they vary greatly from case to case, and largely depend on the deceased's dose regime and their degree of tolerance. Although fluoxetine was detected in the vitreous, methadone was not. The case circumstances state that the individual allegedly drank a bottle of methadone before jumping from a 14th floor flat, maybe the time-scale and very recent ingestion of methadone prior to death could explain its absence in the vitreous. Although if this was the explanation it would be likely that methadone would be detected in the stomach contents but it was not, so there is no real evidence to support this theory.

It is only fair to compare positive results in vitreous that are negative in blood, and there are 8 examples that demonstrate this, had the blood alone been analysed then some analytes would have been missed. However, in the cases where urine was also sent most (all but 3) of these analytes would have been detected. One exception was case 260, where urine was not submitted for analysis, in this case the presence of 6-MAM in the vitreous humour is very

significant as it indicates heroin use prior to death, (for further details refer to section 4.3.3.1 and 4.4.3.1).

There were 2 analytes: chloroquine associates and ibuprofen metabolites that were detected in vitreous that were not detected in either blood or urine, (see Table 4.8 and 4.9). As they are both metabolites, their presence in the vitreous would indicate previous use prior to death but this is unlikely to help determine the cause of death so overall for case interpretation these results are not very significant.

There were 5 other analytes that were detected in vitreous but not in urine, (see Table 4.9), but for 4 of these cases the analytes in question were present in the corresponding blood. In case 348 where no blood was submitted for analysis, chloroquine associates were detected in the vitreous but not in the urine.

More positive results were found in urine than in vitreous, this was expected as drug concentrations are known to be higher in urine than vitreous, and would therefore be easier to detect.

In terms of case 2584, the presence of citalopram in the vitreous humour indicates the ingestion of this antidepressant drug at some point prior to death but as this was the only matrix received for analysis, it is difficult to determine whether therapeutic or excessive ingestion had occurred.

4.4.1.3 BILE

Comparison of bile and urine results, show that there was only one compound that was present in the bile that was not detected in the urine (or blood) and this was cyclizine, (in case 2753).

For case 807, the circumstances revealed that an epileptic was found dead, he had been prescribed sodium valproate. Blood and bile were submitted for analysis, it was not detected in the blood above the 12.5 mg/L cut-off, and this result alone could have implied that the individual had not been taking his medication and was therefore non-compliant. However the presence of the drug in the bile suggests that he had taken his medication at some time before death, even though it is not possible to determine when or how much was taken.

The results showed that 2 analytes were detected in the bile that were absent in the blood and urine, this information could prove useful on occasion, in a similar way, as described previously for the valproate case.

4.4.1.4   LIVER AND OTHER MATRICES

For case 1235, blood, brain and lung were submitted for analysis as circumstances suggested solvent abuse and these are the best matrices for detecting solvents, as due to their volatile nature they quickly leave the blood and accumulate in the organs. Butane and propane were detected in the blood, brain and lung which confirmed the suspicions. However, fluoxetine was also detected in blood, urine and stomach contents. The fluoxetine blood levels are high and could suggest an overdose but the solvents are more likely to be cause of death. A possible scenario for these findings is that the deceased took an overdose of fluoxetine and when they didn't die very quickly - the solvents were used. This would explain the elevated fluoxetine blood levels and trace amount in the stomach.

Case 2270, was a suspected overdose case and in the absence of stomach contents the bowel was analysed to check for recent drug ingestion. Blood

results suggested that an overdose of venlafaxine had been taken prior to death, and this was was supported by the detection of this in the bowel.

The liver was the only matrix submitted for analysis in cases 812, 1032 and 2937, one drug was detected in each case, citalopram, fluoxetine and codeine, respectively. The presence of these drugs in the liver indicates that they were used at some time prior to death but with no other matrices to analyse it is not possible to determine the nature of the ingestion, e.g. whether it was recent, therapeutic or excessive. Similarly in case 2879, the detection of venlafaxine and the metabolite, o-desmethylvenlafaxine (ODV) in the liver and muscle indicates that it was taken sometime prior to death but based on these results alone, again it is not possible to determine the nature of the ingestion.

In terms of case 3504, due to the case circumstances, where the deceased was found with a syringe in their arm, specific morphine analysis was performed. This allowed for the detection of morphine, M3G, noscapine and papaverine in the liver which clearly indicated that heroin had been used prior to death and the presence of no drugs in the stomach contents seems to be consistent with other heroin deaths. However based on these results, it is not possible to determine whether such heroin use was recent or excessive prior to death.

For case 2672, the case circumstances state that the deceased died at home, and was found the same day so it is not clear why only liver fluid and vitreous humour were submitted for analysis. The results show that citalopram and metabolite, desmethylcitalopram, omperazole and paracetamol were detected in both matrices, in addition zopiclone was detected in the liver but not in the vitreous humour. The reason for this, could be due to timing and so these

results indicate previous rather than recent ingestion. However, it could be hypothesised that as zopiclone is known to act in a similar way to benzodiazepines, and be both lipophilic in nature and quite highly protein bound, that it is not often seen in vitreous. Conversely though, a review of results from this study showed that this is the only case where zopiclone has not been detected in vitreous when it has been detected in other matrices, it was found in 2 other cases in both blood and vitreous, and in another study it was detected in 8 cases in vitreous compared to 10 in urine, (Pelander, et al., 2010).

In case 3873, the presence of citalopram and metabolite in the liver fluid and detection of no drugs in the stomach contents only indicates that the drug was used at some time prior to death but probably not recently before death. However, it is impossible to determine if this drug was used excessively prior to death and therefore, overdose cannot be ruled out.

In case 1003, by the time the body was found, decomposition was quite advanced, it was not possible to collect blood from the femoral vein, so instead fluid was collected from the pleural cavity; bile and stomach contents were also collected. It is interesting that the comparison of the pleural cavity and bile screening results are very similar, only one metabolite was detected in the pleural cavity that was not present in the bile, and this was the diazepam metabolite, (nordiazepam). Tramadol and metabolite, o-desmethyltramadol (ODT) were measured in the fluid and when compared to femoral blood levels they would be consistent with an overdose prior to death, the presence of tramadol in the stomach indicated recent ingestion prior to death. However, as the blood was not collected from the recommended site (the femoral vein), the

measured concentrations of the cavity blood could have been elevated due to post-mortem redistribution resulting in the relative concentrations mimicking the more "concentrated" bile fluid content.

4.4.1.5   ALTERNATIVE MATRICES ONLY

In case 2498, the presence of carbamazepine in both vitreous humour and the stomach contents, suggests recent use prior to death. However, it is difficult to comment on the significance of the measured concentration, (1.53 mg/L) as this was the only case in this study where it was possible to measure carbamazepine in this matrix, so there are no other cases where blood was also measured, to compare it to. No published data was found to help with this interpretation.

4.4.2   DRUG QUANTITATION

4.4.2.1   VITREOUS HUMOUR

It has been reported that while the blood:vitreous ratio for some drugs is close to unity, this is not true for all drugs, (Jones, 2004) and the results from this study were rarely an exception to this, (Table 4.13).

However there were 2 cases where the measured concentrations were slightly higher in vitreous than blood, one with tramadol and ODT, and the other pethidine and norpethidine but the differences are so slight, that the significance is questionable, and they could actually be considered to be quite close to unity which would again support the literature. If the results are significant then perhaps tramadol and pethidine are exceptions to the rule, although without other similar results it is not possible to say. Another possible scenario is that

the difference in drug concentrations could be due to timing. Samples were collected 4 days after death, and received in the laboratory 12 days later. They were refrigerated on receipt and analysed 14 days later. So there were 30 days between death and analysis could this have impacted on the results. Is it possible that tramadol could have been lost from the blood but preserved in the vitreous.

For the pethidine case, the timings were reviewed: specimens collected collected 4 days after death, and received in the laboratory 9 days later. They were refrigerated on receipt and analysed 7 days later. So during the 20 days between death and analysis is it possible that pethidine could have been lost from the blood but preserved in the vitreous.

As tramadol and pethidine are both opioids notionally this may provide a structural reason to explain why there were higher concentrations in vitreous compared to blood. However, this seems unlikely as methadone (another opioid) was measured in 4 cases and found to be considerably lower in vitreous than in blood. The vitreous:blood ratios were very varied with the greatest difference reading 6 times lower in vitreous than in blood but in another case the concentration in vitreous was half that in blood. It is possible that the differences in the methadone results could be due to differences between individuals and their metabolism of methadone, as described for the codeine volunteer studies (see section 3.3.1.4). However, it might be considered that comparison of vitreous:blood ratios, rather than individual concentrations, should take these differences into account.

The observed concentrations for the benzodiazepines, lower levels in vitreous compared to blood, Table 4.13, supports the literature. A similar trend was reported in a study where diazepam, nordiazepam and temazepam blood levels were compared to vitreous in 17 cases, although the mean levels were found to be close to unity, the range of results were found to be quite varied, but in all cases the highest and mean concentrations in vitreous were lower than in blood (Scott & Oliver, 2001), this could be due to the highly lipid-soluble nature of benzodiazepines (Jones, 2004).

For highly protein bound drugs, such as tricyclic antidepressants, similar findings have also been reported, where concentrations in vitreous were again, found to be a lot lower than in blood, (Jones, 2004).

Although in case 668, (refer to Table 4.14) it was not analytically necessary to quantify the clomipramine (a tricyclic antidepressant), it seems feasible that screening result alone could support the literature as the drug was detected in the blood but not in the vireous. In terms of timing, the case information states that the deceased was found dead at home on 21/05/12, last seen on 19/05/12, with a note in a diary indicating that she planned to take her own life. All samples were collected on 27/05/08 and received on 30/05/08, the blood was screened on 02/06/08 and propranolol was quantified in both blood and vitreous on 06/06/08 and the vitreous was also screened on this date.

In another case, (case 2591), amitriptyline (another tricyclic antidepressant) was detected in the blood and the vitreous but as the levels appeared very low on the screen, no quantitations were toxicologically necessary. Without concentrations, it is difficult to comment on the significance of this result. As far

as timings go, date of death was 21/05/09, all samples were collected and received at the laboratory on 26/05/09, analysis was carried out on the blood on 27/05/09 and on the vitreous on 02/06/09. Comparison of the timings of this case with the previous, (Table 4.22), shows that analysis was carried out at least 7 days more quickly in this instance, if stability of the tricyclic antidepressants in vitreous is an issue then this time difference could account for the detection of amitriptyline in the one case, compared with the absence of clomipramine in the other.

	Case 668		Case 2591	
	Date	Days elapsed	Date	Days elapsed
Found Dead	21/05/08	(0 – 2?)	21/05/09	0
Samples collected	27/05/08	6	26/05/09	5
Samples received	30/05/08	3	27/05/09	1
Samples Analysed	02/06/08 (bl) and 06/06/08 (vit)	3 (bl) And 7 (vit)	02/06/09	6
Total days since death		12 – 14 (bl) 19 – 21 (vit)		12

Key: bl = blood, vit = vitreous humour

Table 4.22 Results of timings of samples in TCA in blood versus vitreous cases

4.4.2.2 BILE

The similarities observed between the blood:bile ratios for clobazam and lorazepam could be attributed to them both been benzodiazepines with similarities in structure, properties and mechanisms of action, (refer to figure 4.8). However, this is not a likely scenario given that similar blood:bile ratios are

also seen between amphetamine and lamotrigine which are known to be very different drugs, with very different structures, (refer to figure 4.9).
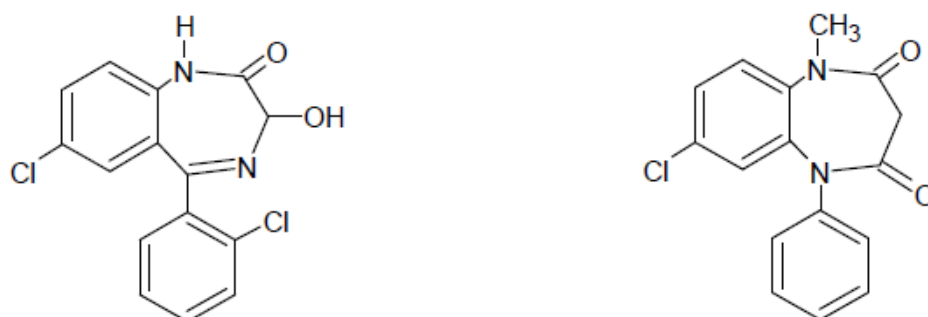
Figure 4.8   Structures of lorazepam (left) and clobazam (right)

Figure 4.9   Structures of lamotrigine (left) and amphetamine (right)

It is therefore, more likely that the similarities in blood:bile ratios are due to the timing of events rather than any structural similarities. In the valproate case, as it was not detected in the blood above the limit of detection for the assay (12.5 mg/L), it was not possible to calculate a blood:bile ratio but it has still been included in the results table (4.15), as the bile measurement proved very useful in this particular case as previously described.

4.4.3   DRUG STUDIES

4.4.3.1   MORPHINE AND HEROIN RELATED COMPOUNDS

VITREOUS HUMOUR

One possible scenario for the difference in the results between cases could be due to timing, i.e. collection time compared to time of analysis, particularly if stability is an issue. Once received in the laboratory all samples are stored in the fridge at 2-5°C, prior to analysis then stored in the freezer at -20°C, when all analysis is complete.

For Case 260, (refer to Table 4.16) the samples were received 11 days after collection, during this time the storage conditions were unknown. The blood was analysed 27 days later, and the vitreous 31 days later. Despite this delay, 6-MAM was still detectable in the vitreous and papaverine in the blood. For Case 277 the samples were received 8 days after collection, during this time the storage conditions were unknown. The blood was analysed 25 days later, and the vitreous 29 days later. After this delay, 6-MAM, noscapine and papaverine were all detectable in both matrices. This is an important result as it proves that it is possible to detect all 3 analytes in vitreous and therefore acts as a positive control which suggests that there is not a physical or structural reason why they could not be detected in this matrix. For Case 1441 all samples were submitted to the laboratory on the day of collection, both blood and vitreous were analysed 13 days after this, and noscapine alone, was detected in both matrices. The different findings in these results suggest that it is not a delay between collection and analysis that is responsible for the different results.

Although 6-MAM was detected in vitreous, in more cases than the corresponding blood, there is not enough data (n=3), to prove or disprove the theory. However, the results do demonstrate that in the cases compared, if only vitreous had been submitted for analysis, heroin use would have been detected. In another study, 25 samples of both blood and vitreous humour were analysed for 6-MAM. All the samples were from heroin deaths and 6-MAM was found in 13 of the blood samples and all of the vitreous samples, (Wyman & Bultman, 2004).

In this study, the vitreous to blood ratio is quite comparable between the 2 cases (260 and 277) but without any other data it is difficult to comment. It has been found previously that no correlation existed between 6-MAM levels in blood and vitreous humour, (Scott & Oliver, 1999).

STOMACH CONTENTS

Although specific morphine analysis was not carried out on the stomach contents, the HPLC screen used, was able to detect morphine, 6-MAM, papaverine and noscapine.

Morphine was detected in the stomach contents of 6 Cases, all of them had morphine, M3G and M6G present in the blood, with nothing to indicate heroin use, i.e. 6-MAM, papaverine or noscapine, (see Table 4.17). There was no codeine detected in the urine of any of these cases either, this can sometimes indicate heroin use as acetylcodeine is a manufacturing impurity (1-15%) of heroin, and this is readily deacetylated to produce codeine, which is subsequently metabolised to morphine, (O'Neal & Poklis, 1998), (Staub, et al., 2001).

Noscapine was detected in the stomach contents of 3 Cases, (all of which also had noscapine present in blood and urine) and 6-MAM was detected in the stomach contents in one case, (where it was also present in the blood and urine).

For the six cases where morphine was detected in the stomach contents, blood and urine it could be assumed that morphine had been ingested orally, recently prior to death.

However, for the other 4 cases, where there is evidence of diamorphine (heroin) use, the interpretation could be more complicated. As heroin is not usually orally ingested it would be easy to assume that it would not be detected in the stomach contents, however there is published data that contradicts this prediction. In one publication, Duflou et al., 2009, give details of 29 heroin overdoses, (where there was death scene evidence of intravenous use) and morphine was detected in the stomach contents of all cases. This is thought to be due to the reflux of morphine from the duodenum into the stomach, which appears to be normal after death (Duflou, et al., 2009). In another report, Kerrigan et al., 2004, found morphine in the stomach contents of a single case of a pancreatic cancer patient who was fitted with an intravenous catheter. This raises some questions about the results seen in this study: if morphine can be found in the stomach contents then is it possible that the noscapine and 6-MAM detected could have also got there via the same route? It is also necessary to question if the stomach contents collected could have been contaminated in any way i.e. during collection and is it definitely "true stomach contents" that was collected.

Although 6-MAM was only detected in the stomach contents in one case in this study, there is a report which obtained similar findings, where 23 samples of gastric contents were analysed for drugs and poisons and in one case 6-MAM was found in the blood by GC-MS and in the stomach by HPLC, (Politi et al., 2004). It is possible that the 6-MAM seen in the stomach contents is a breakdown product of diamorphine formed by hydrolysis. However, if this was the case then morphine would also be expected to be detected, (refer to fig ??), but it was not found in either of the case examples, e.g. this study or the study reported by Politi et al., 2004.

LIVER

In Case 3504, (refer to Table 4.12) a body was found in a very decomposed state, only liver and stomach contents were available for analysis. It was clear from the circumstances that heroin use was suspected so the analytical plan was to analyse the liver fluid for morphine by solid phase extraction and screen the stomach contents for the presence of any basic, neutral or acidic drugs and screen the liver for any basic drugs. .

The results showed that the screening methods detected no drugs or metabolites, but the morphine analysis detected morphine, M3G, noscapine and papaverine in the liver. This confirmed morphine and/or heroin use, at some time prior to death and this is an example where an alternative matrix (liver), proved to be useful, even without blood or urine submissions. However, it was not possible to determine from the liver specimen alone, (i.e. without a blood specimen) whether such heroin use was recent or excessive prior to death. In

addition to this, if only the stomach contents had been submitted for this case, no drugs would have been detected.

4.4.3.2 CODEINE

The presence of codeine (and other drugs) in the stomach contents, indicate recent ingestion prior to death, and all 4 cases are suspected multi-drug overdoses, Table 4.18. Although based on few findings, this scenario was found to be common in supporting literature where it states, in most fatalities involving codeine other drugs, and or alcohol is present, (Moffat, et al., 2004, p. 847).

In 3 out of the 4 cases paracetamol was also detected which could indicate that a combined preparation of codeine and paracetamol had been used.

For the blood versus vitreous results, it is necessary to consider that the time-scale could have had an impact on the unexpected result, (refer to Tables 4.19 and 4.20). It is possible that from the time of death until collection (delay of 23 days) that the codeine was lost from the blood but preserved in the more stable vitreous humour matrix.

LIVER

In case 2937, (refer to Table 4.12), the results indicated ingestion of the codeine some time prior to death but could not give any indication of whether the level ingested was significant or excessive. The presence of putrefactants suggest that the deceased may have been dead for some time before the body was found, and may have been in a decomposed state. This could explain why blood and urine were not submitted for analysis. It is likely that had blood been available, results would have proved more useful and that this is an example of

the limited use of alternative matrices (liver and stomach contents) when submitted without the traditional blood and urine matrices.

4.4.3.3 DIAZEPAM

The presence of nordiazepam in the stomach contents of 8 separate cases was an unexpected result. In 4 of these cases both diazepam and nordiazepam were detected in the stomach contents, in 2 of the cases diazepam was detected in the blood and in another nordiazepam was found in the blood. A possible scenario could be that that diazepam has broken down in the stomach contents to produce nordiazepam. However, an N-desmethylation process (removal of a methyl group, refer to Figure 4.7) is not likely to occur in the stomach because the enzymes required for the process are not present here, they are in the liver. Therefore it seems more likely that the metabolite is present in the stomach due to back diffusiuon after death.

4.4.3.4 COCAINE

Cocaine was detected in the urine of 14 cases but was only detected in 5 of the corresponding blood samples, (see Table 4.21). This is most likely because cocaine is known to be unstable in blood, and although some types of preserved container can help with stability, it can still be detected in urine for longer. Vitreous humour was received in 2 of the urine positive cases, (939 and 959) but cocaine was not detected in the vitreous of either case. However, in case 959, levamisole was detected in both the urine and the vitreous, and although this drug is used to treat roundworm infections; it is also commonly encountered

as a contaminant in some batches of cocaine. Therefore, its presence in this particular case, could help to confirm cocaine use.

The cocaine detected in the stomach contents (case 491) was an unusual result as due to the usual routes of administration i.e. Injection, inhalation or smoking, you would not necessarily expect to find it there. However, as discussed for morphine, a drug or metabolite in the stomach does not necessarily mean that it was taken orally; gastric juice (formed from extracellular fluid) is constantly being secreted into the stomach, this may contain some drugs or metabolites circulating in the blood, (Jones, 2004). In addition to this it is possible for diffusion to occur after death. Active processes stop after death and the permeability of the gut wall has been known to increase, e.g. ethanol which is absorbed from the small intestine in life, can diffuse across the stomach wall after death into adjacent tissues and blood vessels, (Ferner, 2008). For this reason, vitreous humour is commonly used for ethanol measurement as this is thought to be unaffected by post-mortem re-distribution.

In this particular case the blood cocaine level was very high (6.9 mg/L typically >2mg/L is fatal) and benzocaine was also detected in both the stomach and blood. Benzocaine is a local anaesthetic but it is also commonly used as a contaminant or "bulking agent" in some batches of cocaine.

4.4.3.5   AMPHETAMINE

The lack of amphetamine in the stomach contents could be due to the route of administration, as previously described. In all 3 cases where amphetamine was detected the individuals were known drug users, (so most likely not adverse to snorting) and case 4023, was found n possession of a white powder, although

this was not submitted for analysis, so whether it was actually amphetamine remains unconfirmed. Vitreous humour was not submitted for analysis for any of these cases, it would have been interesting to see whether amphetamine, with its lipophilic nature, would have been detected in the vitreous, as one might expect.

4.4.3.6 METHADONE

The presence of methadone in the stomach contents of 12 cases is an indication that it was taken recently prior to death and the absence of the main metabolite would be expected, as metabolism does not usually occur in the stomach. Although the presence of a drug in the stomach does not necessarily confirm or rule out an overdose, as a point of interest, with other drugs the measured blood levels have been used to indicate if a drug, (most likely) responsible for an overdose was detected in the stomach. However, it is difficult to draw any such conclusions with methadone as the toxicological significance of the blood methadone concentration depends upon the degree of tolerance possessed by the deceased, e.g. in non-addicted subjects plasma concentration of >2 mg/L could be lethal but in 13 methadone maintenance patients who died of accidental methadone overdose, the post-mortem blood concentrations ranged from 0.18-4 mg/L, (Baselt, 2011, p. 1021-1024), (Moffat, et al., 2004, p. 1231-1232).

CHAPTER 5: GENERAL DISCUSSION

The validated methods for oral fluid analysis and their application to the studies carried out, suggests that for drugs of abuse testing, oral fluid could be a suitable matrix. However, it is important to be aware of the limitations, and remember that when using oral fluid for drug detection, it will only reflect drug use in the previous 2 to 3 hours before sample collection. If a more detailed insight is required, e.g. a reflection of drug use in the past 12-24 hours then a more suitable matrix will need to be used for analysis. A good example of this was shown in the Clinic study, when results of oral fluid testing were compared to urine, results revealed that two cocaine positive and three morphine positive results would have been missed if only the oral fluid had been tested, rather than both matrices.

In addition to this, the relatively low volume of oral fluid specimens could continue to be a problem for this matrix. For example, after drug screening there might not be enough sample remaining to complete all the necessary confirmation and / or quantitation tests required. In the studies conducted, approximately 1 mL oral fluid was collected and this was added to a preservative buffer, to give a total of 4 mL sample so although this gave greater sample volume, the collection process introduced another problem, which was dilution of analytes by a factor of 4, when the levels detected in oral fluid are already considered to be relatively low. This resulted in problems with analysis as it proved difficult to validate methods with such low levels of detection and quantitation required, (refer to Table 3.1).

One of the possible outcomes from this study was to replace an existing urine drug screening provision with oral fluid. The existing service offered urine testing

for amphetamines, opiates, cocaine metabolite, methadone metabolite, benzodiazepines, cannabis as well as a validity test for urine (creatinine) and buprenorphine testing when required. Ideally, methods would have been developed and validated for all these analytes but unfortunately due to the difficulties encountered reaching the required limit of detection and the time spent on method development, this was not achieved. Unfortunately, the development of screening methods which should have been relatively straight forward, proved very difficult and time-consuming. Initially it was planned for screening to be carried out in much the same way as it was done for urine samples that was to use CEDIA immunoassay reagents, on an automated analyser that was already in the laboratory. However, the oral fluid kits were still in the "early in-house research" stages when they were required for this study and so it was not possible to obtain any kits, even for research purposes. It was for this reason that ELISA screening was investigated but as this was a new technique to our laboratory, this involved the purchase of new equipment which involved a long time-delay. The overall result was that to fill-in the time delay (required to purchase an ELISA plate-reader), the confirmation methods for opiates and benzoylecgonine were validated and in-house volunteer studies were completed, before the ELISA screening could be investigated.

The methods were validated in order of priority, (from a rehabilitation clinics point of view, at least). When validation of the most important drugs was completed a rehabilitation clinic was contacted with the idea of the study and this was set up. However, due to the length of time taken to validate the other analytes, for both screening and confirmation methods, work on

benzodiazepines and cannabis in oral fluid, never really got started. However, if these assays had been validated, the small sample volume could have been an issue and a choice would have had to be made in which confirmation tests were carried out, i.e. some kind of priority order would have needed to be established. For this reason, for the clinic study conducted, both benzodiazepines and cannabis were screened for in the corresponding urine. This means that until further work has been carried out, the oral fluid protocols put forward in this study, could not replace the existing urine service offered.

In order for oral fluid to be considered for workplace drug testing, the SAMHSA proposed cut-off levels needed to be achieved, (see Table 3.1). The results showed that although the cut-off levels were achieved for some drugs it was not possible for others, the amphetamines (except for MDMA) and the opiates, (except for 6-MAM) were successfully validated at the low levels but benzoylecgonine and 6-MAM were not, (see Table 4.23). This meant that further work would need to be undertaken, (to achieve the outstanding cut-off levels) before oral fluid could be included in a work place drug testing service. However, this would not necessarily be a difficult task as since the oral fluid work was carried out (2004 – 2007), more sensitive techniques have become available, such as tandem GC-MS which would easily detect down to the required levels. In addition to this CEDIA immunoassay kits for oral fluid analysis are now also commercially available and have been running successfully on automated analysers for over 2 years in several laboratories. This would be a lot less time-consuming than the ELISA screening techniques investigated in this study and would probably use less sample volume and be more versatile than

the LC-MS screening technique that was validated but would obviously require further confirmation of positive results, (which is not required following the LC-MS screen).

Oral Fluid (ng/mL)	Proposed SAMHSA cut-off concentration		Cut-off concentration when diluted 1 in 4 with buffer		LOQ / LOD for validated methods	
Analyte	Screen	Conf	Screen	Conf	Screen	Conf
Cocaine metabolite	20	8	5	2	1 (parent)	15
Opiates	40	40	10	10		10
6-MAM	40	4	10	1		5
Amp	50	50	12.5	12.5	3.5	10
MA	50	50	12.5	12.5	1	10
MDMA	50	50	12.5	12.5	3.5	15
MDA	50	50	12.5	12.5	5	10

Key: Screen = screening, conf = confirmation

Table 5.1   Comparison of SAMHSA cut-off levels to those achieved in the study , (SAMHSA, 2004).

There are certain situations where drug use within the past 2-3 hours could be very relevant and a good example of this is driving under the influence of drugs.

Oral fluid has the added advantage that as it reflects free, unbound parent drug, (and these are the forms that cross the blood-brain barrier and effect performance and behaviour), presence of drug(s) should correlate well with impairment, (better than with urine metabolites), (Spiehler, et al., 2002).

The idea of using oral fluid for roadside drug testing has been around for a long time but the problem of low sample volume has been a major issue. An early study was carried out in 1983, the aim was to get 3 mL per specimen (by spitting) but in reality only between 1 and 1.5 mL was actually obtained. Out of

56 drivers, cannabinoids were found in six cases and diazepam in 4, (Verstraete, 2005). In a German roadside study, carried out in 1992, 32.6% of the samples collected were essentially dry (<0.1mL), and out of the remaining samples the mean volume collected was only 0.42 mL. This meant that for the majority of samples collected they were not able to screen for the intended full panel of drugs, (Verstraete, 2005).

By the late 1990s, on-site oral fluid testing devices had been developed, and the effectiveness of some of these was tested by The Roadside Testing Assessment Project (ROSITA) which was set up by the European Commission. ROSITA 1 took place in 1999 and 2000 and involved 8 Countries, it compared 15 urine and 3 saliva on-site tests. Out of 2986 subjects, it was reported that it was possible to obtain oral fluid in nearly all the cases. The overall conclusion was that the present-generation of on-site oral fluid tests was insufficiently sensitive and / or specific to give reliable results for most classes of drugs, in addition to this the testing devices were thought to be too complex and time-consuming, (Verstraete & Puddu, 2000), (Samyn, et al., 1999a).

The ROSITA-2 project was carried out from 2003 to 2005, and it was set up to evaluate the usability and analytical reliability of 9 on-site oral fluid drug testing devices. 2046 subjects were included in this study and 2605 device evaluations were performed. Results showed that for some devices a very high percentage of failures were observed, this was apparently due to either too little or too viscous oral fluid. None of the devices met the criteria proposed during the ROSITA-1 project (sensitivity >90%, accuracy >95%) for the amphetamines, benzodiazepines and cannabis. Only one device met this criteria for cocaine

and opiates but it gave 26% failures so could not be recommended. The operational evaluations revealed further problems (apart from high failures and short samples), in general devices were complicated to operate or results were difficult to read or problems were encountered in rain or cold weather. At the end of the study none of the devices were considered reliable enough to be recommended for roadside screening of drivers, (ROSITA - Roadside Testing Assessment, 2010).

In Germany, Spain and Australia, roadside drug testing is routinely carried out yet in the UK, currently police have to demonstrate that driving has been impaired in order to prosecute. However, this is all set to change as in May, 2012, it was revealed that a new driving offence would be created, and this was confirmed in the Queen's speech. It will be an offence to drive a motor vehicle if you have certain controlled drugs in your body in excess of specified limits. Police will be equipped with hand-held devices to test oral fluid at the roadside. An expert panel have the job of deciding which drugs will be covered by the offence and the specified limits for each. The Department for Transport state that the new offence should be in place by 2015, (Department for transport, 2012), (BBC News, 2012). A decision is yet to be made about how the presence of drugs found at the roadside will be confirmed and this will need careful consideration. If more than one drug type is found to be positive, will there be sufficient sample volume to confirm all the findings and if not will a protocol be put into place to advise how such confirmatory tests could be prioritised.

In general, for the analysis of drugs in clinical cases, it is possible to choose which matrix suits your need best or even use several matrices to give a full

picture of an individual's drug usage, if this is required, desired or necessary. However, with post-mortem samples this is far from the truth. The availability of matrices is often limited by the nature of the death, e.g. in any type of vehicular crash samples can be lost as the body is dismembered, in fires samples can be burnt away, in drownings extreme water exposure can affect samples and speed up decomposition. In addition to this if a body is not found for a few days or more after death, it will start to decompose which means that bacteria that exist in the body during life, start to break it down, starting with the intestines they break out and move onto the organs, releasing digestive enzymes as they go, which help to break down organs and tissues. The greater the extent of this process the more difficult it is for the pathologist to collect samples from specific sites, in the most advanced cases it is only possible to collect blood from the central body cavity. This obviously leads to extreme difficulty for interpretation of results.

As well as these issues, following the revision of the Human Tissue Act, 2004, pathologists were suddenly unclear about what samples they were "permitted" to collect and as a result of this "confusion", far fewer samples were collected and consequently less alternative matrices were submitted for analysis.

In addition to this the HT Act also implies that if a diagnostic result can be obtained from the analysis of one sample then there is no need to analyse further samples, so this also led to the collection of less samples, e.g. the sampling of blood from more than one vein or artery was no longer an option.

This had quite a negative impact on this study, over a period of 26 months only 10% of the cases received included alternative matrices, with the majority only

sending blood and urine for toxicological analysis. This was that a lot less than expected, when the study was originally designed.

Results showed that collection of stomach contents was impacted on the least, as it made up 87% of the alternative matrix total submissions. A significant proportion of these findings were negative, e.g. no drugs were detected, and such results can prove useful to exclude recent oral ingestion of drugs prior to death. However, this does not necessarily rule out the possibility of an oral overdose because it could take several hours to die from a drug overdose and during this time most or all of the drug could have passed from the stomach to the small intestine or may even have been absorbed, (Jones, 2008).

In cases where drugs were found in the stomach contents, this largely indicates recent oral ingestion prior to death (although not necessarily as they could be present due to back diffusion after death), but it does not mean that a drug overdose has definitely been taken. There was evidence from results in this study that drugs could be detected in the stomach contents but not be present in the blood and could therefore not be responsible for an overdose. There was also evidence that often drugs found to be responsible for an overdose, (by the measurement of blood concentrations), were found in the stomach contents.

The main limitation with the analysis of stomach contents is that it is purely for drug screening purposes only (and not for quantitation).

A drug screen can be considered successful, if the results provide an answer to the question "are there any drugs present?" It is evident that the variety of stomach contents results compared to traditional matrices, and to some extent to other alternative matrices, under different case circumstances, can answer

this question, and there have been good case examples where drug screening results from vitreous humour, bile, liver and muscle could easily do the same.

However, as useful as drug screening can be, it does not really provide an answer to the all-important question: "have drugs (or poisons) either caused or contributed to the death in question?"

To answer this question it is necessary to measure the drugs detected, (through screening) and use the concentrations determined, to interpret the findings.

In order to assist with this, concentrations of drugs in bile and vitreous humour would be measured alongside those in blood to see how they compared. It was hoped that this information could then be used to interpret concentrations measured in alternative matrices, in cases where there were no traditional matrices available, as there is little published data available to help with this.

Unfortunately, mostly due to the relatively low number of suitable submissions, it was only possible to quantify drug levels, in a limited number of samples.

Comparison of blood and vitreous results, and vitreous:blood ratios (of which there are 26) shows a mixture of results, between some of the same drugs, and then between drug classes. With such a spread of results, there were no clear trends, with the exception of vitreous levels being generally lower than the corresponding blood levels. Scott and Oliver, 2001, also found inconsistencies between blood and vitreous concentrations, in their study; they found some correlation between temazepam and diazepam but no correlation for the metabolite, desmethyldiazepam, (nordiazepam).

For bile, it was only possible to measure six concentrations alongside blood and these results also showed little trend, other than that the concentrations in bile

were higher than in the blood, this was expected as many drugs have been shown to accumulate in the bile.

Overall it seems that any of the alternative matrices investigated in this study could be effectively used for drug screening. The use of oral fluid for on-site testing would mean that a sample could be collected under supervision and analysed. Any subsequent confirmatory testing could be carried out on either the remaining oral fluid sample or on a urine sample but this would only need to be collected if indicated by the screening test. In the event of a fatal road traffic collision, in order to preserve a limited blood sample, vitreous humour and stomach contents, could be used for drug screening and then any confirmatory tests and/or quantitations could be performed on the blood.

Where there is a choice of drug matrices for analysis, it is important to consider the question being asked, and in accordance with this the drug detection times for each matrix, then an informed decision can be made, on the type of analysis that would best fit the requirement.

In cases where only alternative matrices are available, it should be possible to determine if any drugs are present or absent but any specific confirmations, and or measurements could either be restricted by low sample volume, e.g. particularly for oral fluid and vitreous humour, or if they are performed could still prove difficult to interpret.

5.1 CONCLUSIONS

- Alternative matrices have proved to be very effective for the screening of drugs

- When analysed alongside traditional matrices or in conjunction with each other, the results can provide a very good insight to an individual's drug use

- Although it is possible to perform confirmatory tests and / or measure concentrations in alternative matrices, these extra tests may be prevented by limited sample volume, particularly for vitreous humour and oral fluid

- Where concentrations are determined in alternative matrices the results can prove difficult to interpret

- Alternative matrices can provide a good insight into drug use but are some way off replacing traditional matrices

- For the analysis of clinical cases urine and blood/serum or plasma will be the primary matrices, with oral fluid as a secondary choice

- For the analysis of post-mortem cases blood and urine will be the primary matrices with vitreous humour as a secondary choice but there will be circumstances where stomach contents, bile and other matrices will be used

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APPENDICES

APPENDIX A

Analyte (including metabolites)	Expected Ret.Time (mins)	Std. Range (mg/L)	ISTD + Concn (mg/L)	ISTD Expected Ret. Time (mins)	Extraction Procedure	Isocratic Conditions (% MeCN)	Total Run Time (mins)	QC concns (mg/L)
Atenolol	1.55	0.625 - 10	**Cinchonine** **0.5 mg/L**	4.42	Basic	10%	6	0.5, 5.0
Amitriptyline Nortriptyline	5.26 4.74	0.125 – 2	**Promazine** **2 mg/L**	3.98	Basic	30%	7	0.1, 10
Amphetamine	2.28	0.0625 – 1	**Cinchonine** **1 mg/L**	3.25	Basic	10%	5	0.075, 0.75
Caffeine	1.58	3.125 – 50	**Norfenfluramine** **10 mg/L**	2.48	Basic	25%	6.5	2, 20
Carbamazepine	2.34	0.625 – 10	**Clobazam** **2 mg/L**	3.97	Benzo	40%	7	1, 5

Chlordiazepoxide	1.89	0.1325 -	**Clobazam**	7.64	Benzo	30%	9	0.2, 2
Demoxepam	3.32	5	**2 mg/L**					
Citalopram	2.77	0.125 – 2	**Brompheniramine**	1.91	Basic	30%	5	0.2, 2
Clobazam	4.58	0.03125	**Nordiazepam**	3.74	Benzo	40%	8	0.1, 1
Norclobazam	3.07	– 0.5	**2 mg/L**					
Clozapine	1.99	0.25 – 4	**Desipramine**	4.6	Basic	30%	6	0.2, 2.0
			2 mg/L					
Codeine	2.9	0.3125 –	**Cinchonine**	4.43	Basic	7%	6.5	0.2, 2
		5	**2 mg/L**					
Cocaine	1.69	0.125 – 2	**Desipramine**	4.08	Basic	30%	6	0.2, 2
			2 mg/L					
Cyclizine	2.61	0.3125 –	**Promazine**	3.69	Basic	30%	6.5	0.2, 2
		5	**1 mg/L**					
Dextropropoxyphene	4.68	0.125 – 2	**Promazine**	3.8	Basic	30%	6	0.15, 5
Nordextropropoxyphene	4.16		**1 mg/L**					

Diazepam	5.75	0.3125 –	**Clobazam**	4.15	Benzo	40%	7	0.2, 2
Nordiazepam	3.84	5	**2 mg/L**					
Diltiazem	3.6	0.625 – 10	**Desipramine 2mg/L**	4.77	Basic	30%	6	0.2, 2.0
Diphenhydramine	2.71	0.625 – 10	**Desipramine 2 mg/L**	4.15	Basic	30%	6	0.5, 5
Dipipanone	10.67	0.3125 – 5	**Desipramine 2 mg/L**	4.3	Basic	30%	14	0.1, 1
Dosulepin (dothiepin)	2.6	0.125 – 2	**Clomipramine 2 mg/L**	4.41	Basic	35%	7	0.15, 1.5
Fluoxetine	3.68	0.3125 –	**Desipramine**	7.74	Basic	30%	10	0.15, 1.5
Norfluoxetine		2	**2 mg/L**					
Hydroxychloroquine	2.74	0.625 – 10	**Cinchonine 2 mg/L**	3.23	Basic	10%	6	0.5, 5
Imipramine	4.25	0.125 – 2	**Clomipramine**	7.74	Basic	30%	10	0.15, 1.5
Desipramine	3.85		**2 mg/L**					
Ibuprofen	7.75	6.25 – 100	**Naproxen 10 mg/L**	3.6	Acid	45%	9	10, 50

Lamotrigine	1.74	0.625 – 10	**Brompheniramine 5 mg/L**	3.07	Basic	25%	5	1.0, 5.0
Methadone	5.44	0.125 – 2	**Desipramine 2 mg/L**	4.14	Basic	30%	7	0.1, 1
Mirtazapine	2,25	0.625 – 10	**Brompheniramine 1 mg/L**	3.71	Basic	20%	5	0.15, 1.5
Naproxen	3.18	0.156 – 25	**Ibuprofen 100 mg/L**	6.51	Acidic	45%	13	4, 40
Nitrazepam	2.66	0.125 – 2	**Clobazam 2 mg/L**	3.79	Benzo	40%	6	0.1, 1
Olanzapine	2.73	0.3125 – 5	**Cinchonine 5 mg/L**	3.24	Basic	10%	7	0.2, 2
Oxycodone	2.93	0.125 – 2	**Cinchonine 1 mg/L**	3.52	Basic	10%	6	0.1, 1
Orphenadrine	4.11	0.625 – 10	**Desipramine 1 mg/L**	4.7	Basic	30%	6	1, 5
Paracetamol	2.56	6.35 – 100	**2-AP 50 mg/L**	4.82	10	10%	6	10, 50

Phenytoin	2.44	0.625 – 10	**Clobazam** **2 mg/L**	4.15	Benzo	40%	7	1, 5
Promazine	2.63	0.625 – 10	**Clomipramine** **2 mg/L**	4.95	Basic	35%	6.5	1, 5
Propranolol	2.26	0.625 – 10	**Desipramine** **2 mg/L**	3.36	Basic	30%	6	1, 5
Quetiapine	2.42	0.625 – 10	**Desipramine** **2 mg/L**	4.24	Basic	30%	11	0.15, 15
Quinine	1.8	1.25 – 20	**Brompheniramine** **2 mg/L**	4.48	Basic	20%	6	3, 15
Salicylate	5.09	31.25 – 500	**2-AP** **100 mg/L**	2.48	Acid	20%	6.5	75, 150
Sertraline Norsertraline	7.15 6.36	0.3125 - 5 0.03125 - 0.5	**Desipramine** **2 mg/L**	4.09	Basic	30%	11	0.15, 15
Temazepam Oxazepam	3.35 2.43	0.3125 – 5	**Clobazam** **2 mg/L**	3.72	Benzo	40%	7	0.2, 2

Trimethoprim	1.51	0.625 – 10	Norfenfluramine 2 mg/L	3.17	Basic	20%	5	1, 5
Tramadol ODT	4.45 1.98	0.125 – 2	Brompheniramine 2 mg/L	9.91	Basic	14%	12	0.75, 3
Trazadone	2.16	0.625 – 10	Desipramine 2 mg/L	4.86	Basic	30%	6	0.2, 2.0
Venlafaxine ODV	2.77 1.48	0.625 – 2.5	Brompheniramine 2 mg/L	3.22	Basic	25%	5	0.4, 4
Verapamil	6.27	0.3125 – 5	Promazine 2 mg/L	4.41	Basic	30%	8	0.4, 2.0
Zolpidem	1.65	0.125 – 2	Promazine 2 mg/L	1.89	Basic	30%	5.5	0.1, 1.0
Zopiclone	1.95	0.125 – 2	Brompheniramine 2 mg/L	3.47	Basic	25%	5	0.1, 1

Analytical conditions/methods used for drug quantitation