# An Investigation into the Potential Use of Liquid Chromatography – Mass Spectrometry in Forensic Toxicology

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

by

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*For Grandma Lamb*

♓

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# Summary

Liquid chromatography – mass spectrometry (LC-MS) is the ultimate combination of gentle separation and the unrivalled specificity of a mass spectrometer. Finally compounds not suited to gas chromatography (GC) can be analysed without compromising the extensive qualitative data the mass spectrometer provides. The aim of this project was to evaluate the use of LC-MS in the forensic toxicology setting. To investigate if its use could solve problems encountered while using other instrumentation including downtime, limits of detection, chromatography and specificity.

Benzodiazepines as a class, are known for their undesirable chromatographic behaviour when using GC. One way to improve their chromatographic behaviour is to derivatise. This is not only time consuming but uses harmful chemicals. Even with derivatisation, these drugs can decompose in the injection port, before they are even applied to the column. Routinely, diazepam and its metabolites are analysed by high pressure liquid chromatography (HPLC) with ultraviolet (UV) detection. The chromatography is improved, but specificity is compromised. The use of LC-MS was investigated as an alternative to classical HPLC.

Using LC-MS two methods were developed for the analysis of these drugs in whole blood.

Increasing awareness of drug facilitated sexual assault, has led to an increase in the number of cases to be analysed for drugs such as Rohypnol®. This prompted the development and validation of a sensitive and specific method for its detection in blood using solid phase extraction and liquid chromatography – mass spectrometry.

To enhance the qualitative data and cope with degraded samples, the use LC-MS was used in the analysis of diazepam and its three metabolites. A method was developed and validated and is now used in the routine analysis of blood samples in the laboratory.

The LC-MS proved invaluable in the analysis of sildenafil, Viagra®, in a post-mortem blood sample received in the laboratory. A single quadrupole mass spectrometer was used to develop and validate a method to determine if the dose was therapeutic or toxic.

The use of oral fluid as an alternative specimen to blood or urine was investigated. This was through the British Roadside Impairment Test Evaluation (BRITE) project, evaluating the field impairment test (FIT), and the IMMORTAL project, analysing oral fluid from a random selection of drivers around the city of Glasgow. LC-MS in combination with GC-MS was used to screen for over 50 licit and illicit drugs in 1mL of oral fluid. LC-MS-MS was then used to identify and quantitate 21 drugs of abuse and their metabolites using a similar sample size. The use of one extraction and the reliability of LC-MS proved invaluable in these projects.

Currently urine is used to assess recent drug use in rehabilitation centres. The use of oral fluid as an alternative was evaluated. Blood, urine and oral fluid, collected using two different devices, were volunteered from patients on admission to a residential rehabilitation centre for 5 consecutive days. Opiates and benzodiazepines were monitored, to assess the detection window of past heroin use. Oral fluid concentrations were compared with respective blood samples to give and an indication of levels likely to be found.

# List of Abbreviations

| | |
|---|---|
| 7-AF | 7-Aminoflunitrazepam |
| 7-AF-$d_7$ | 7-Aminoflunitrazepam-$d_7$ |
| APCI | Atmospheric Pressure Chemical Ionisation |
| API | Atmospheric Pressure Ionisation |
| 6-MAM | 6-monoacetylmorphine |
| BRITE | British Roadside Impairment Test Evaluation |
| CGC | Capillary Gas Chromatograpy |
| CI | Chemical Ionisation |
| $\Delta^9$-THC | delta-9-tetrahydrocannabinol |
| $d_3$ | tri-deuterated |
| $d_4$ | tetra-deuterated |
| $d_6$ | hexa-deuterated |
| $d_9$ | nona-deuterated |
| DHC | Dihydrocodeine |
| ECD | Electron Capture Detector |
| EDDP | 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine |
| EI | Electron Impact |
| ESI | Electrospray Ionisation |
| FIT | Field Impairment Test |
| FLU | Flunitrazepam |
| FLU-$d_7$ | Flunitrazepam-$d_7$ |
| GC | Gas Chromatography |
| GC-EI-MS | Gas Chromatography-Electron Impact-Mass Spectrometry |
| GC-MS | Gas Chromatography – Mass Spectrometry |
| HPLC | High Pressure Liquid Chromatography |
| IMMORTAL | Impaired Motorists, Method of Roadside Testing and Assessment for Licensing |
| LC-MS | Liquid Chromatography – Mass Spectrometry |
| LC-MS-MS | Liquid Chromatography – Tandem Mass Spectrometry |
| LLE | Liquid Liquid Extraction |
| LOD | Limit of Detection |
| LOQ | Limit of Quantitation |
| MDA | Methylenedioxyamphetamine |

| | |
|---|---|
| MDEA | Methylenedioxyethamphetamine |
| MDMA | Methylenedioxymethamphetamine |
| MS | Mass Spectrometry |
| NPD | Nitrogen Phosphorous Detector |
| $R^2$ | Linear Correlation Coefficient |
| ROSITA | Roadside Testing Assessment |
| RSD | Relative Standard Deviation |
| SIM | Single Ion Monitoring |
| SPE | Solid Phase Extraction |
| SPME | Solid Phase Micro Extraction |
| UV | Ultraviolet |

# 1 Introduction

The use of LC-MS in all sectors of analytical chemistry has been increasing at an exponential rate in recent years[1]. After mastering how to couple liquid chromatography to mass spectrometry with the development of atmospheric pressure ionisation (API), LC-MS has revolutionised the way routine forensic toxicology is carried out. Before the advent of LC-MS the compromise would be the use of UV spectra for drugs not amenable to GC-MS. Now these drugs can have both the qualitative data of MS with the chromatography of HPLC. LC-MS is also ideal for developing and validating a method quickly, downtime is much less compared to GC-MS, and analytes are easily identified initially by their pseudo-molecular ion.

One class of drugs found to chromatograph well by HPLC are the benzodiazepines. In forensic toxicology, post-mortem blood is often used for analysis, and since this can be substantially degraded, the products can cause increased interference in the detector signal. Diazepam and its metabolites N-desmethyldiazepam, temazepam and oxazepam are routinely analysed in whole blood. If a mass spectrometer could be used for detection, specificity would be greater leading to a decrease in the background noise and hence better detection limits and less interferents to be concerned about.

Flunitrazepam is becoming renowned as a "date rape" drug, and its identification in bodily fluids increasingly requested. By using LC-MS, flunitrazepam and its metabolite 7-aminoflunitrazepam can be chromatographed easily and positively identified, to very low concentrations. This is essential when the memory of the victim may be affected, the report of a crime may be delayed, and the amount administered may be only 1 or 2 mg.

Oral fluid has gained much interest in recent years. Being an ultrafiltrate of blood, oral fluid drug concentrations are related to the unbound, pharmacologically active fraction in blood. This has resulted in its use for therapeutic drug monitoring[2,3]. However, recently its use as a roadside specimen, to identify impaired drivers, has been of utmost interest. Projects have been carried out to evaluate the impairment effects of drugs on driving and their detection and subsequent presentation to the judicial system, such as ROSITA[4]. The use of field impairment tests (FIT) have also been of interest to objectively assess the ability of the driver at the roadside. As part of the evaluation of FIT, oral fluid was taken when the driver was found not to be impaired by FIT. Only 1 mL of oral fluid was

obtained and had to be screened for a wide range of drugs of abuse, prescription and over the counter drugs. The use of GC-MS for its screening ability of unknowns, as well as the reliability of LC-MS would complement each other, and cover an extensive range of analytes.

Using an LC-MS-MS gives even greater specificity, and can produce mass spectra that look similar to GC-MS spectra with many qualitative ions.

# 2 Liquid Chromatography – Mass Spectrometry

## 2.1 Introduction

The technique of separating compounds using chromatography has been in practice since the beginning of the 20th century. Tswett described the separation of plant pigments by percolating an extract through a glass column packed with calcium carbonate in 1903[5]. Chromatography relies on two phases, a stationary and mobile phase. The stationary phase can be solid spheres packed into a column or a liquid coated onto a capillary wall. The mobile phase can be liquid or gas and flows through and around the stationary phase. When compounds are introduced to this environment, they will behave differently depending on the types of stationary and mobile phases present. Different analytes are retained on the stationary phases for varying amounts of time. The two most common methods used to encourage the elution of all analytes are raising the temperature, as in gas chromatography, or increasing the percentage of organic solvent as in reverse phase liquid chromatography.

### *2.1.1 Gas Chromatography*

In Capillary Gas Chromatography (CGC), the stationary phase consists of a liquid coated onto the inside wall of a silica capillary. The capillary can be up to 30 m in length and as little as 0.1 mm in internal diameter. The mobile phase is an inert gas such as helium, which carries the compounds through the column and on to the detector. To separate a mixture, the temperature of the column is increased over time to an upper limit e.g. 300 °C. Volatile and inert compounds with few polar functional groups elute first. Compounds with many functionalities are preferentially attracted to the stationary phase and therefore require higher temperatures to elute them. They are retained for different lengths of time, dictated by their structure. Analytes which elute quickly have short retention times and more complex molecules are retained for longer.

To observe analytes eluting from the column a detector is needed. Typically a flame ionisation detector (FID) is used for organic compounds. This is a non-specific detector as it gives quantitative but not qualitative information about the analyte. Other detectors include nitrogen phosphorous detector (NPD) or electron capture detector (ECD). Neither of these gives detailed structural information about the analyte. All rely on unique

retention times as qualitative data. Using mass spectrometry as a detector can give an enormous amount of structural information making it very specific and almost negating the need for resolution in the CGC. The gaseous effluent from the gas chromatograph makes it a relatively simple process to attach the end of the column to a mass spectrometer ion source. As the gas enters the mass spectrometer, it is bombarded with electrons as with electron impact ionisation, EI. This ionisation is referred to as "hard ionisation" as it fragments the analyte into many unique daughter ions. This technique gives immense structural information and very specific qualitative data. Thus Gas Chromatography – Mass Spectrometry (GC-MS) has become the standard analytical technique for forensic toxicology.

Nonetheless, there are some compounds for which GC-MS is not completely satisfactory. If a compound is thermally labile, it may decompose in the GC injection port e.g. temazepam. This problem can be overcome by changing the functional groups, making them more stable and less reactive with the stationary phase. However, derivatisation adds a step onto the extraction method and the chemicals used can be toxic. It introduces another source of possible error and consumes more time and reagents.

### *2.1.2 Liquid Chromatography*

In benzodiazepine analysis, reversed phase high pressure liquid chromatography, RP-HPLC, is commonly used. Liquid chromatography uses columns packed with small spheres, 3 μm in diameter, as the stationary phase, and a composition of aqueous and organic solvents as the mobile phase.

When using reversed phase liquid chromatography, increasing the organic content of the mobile phase has the same effect as increasing the temperature in gas chromatography. In this way even extremely polar drugs are eluted from the column. HPLC is typically coupled with ultraviolet (UV) detectors, measuring the changes in absorbance at different wavelengths, as different compounds elute off the column and flow through the detector cell. This technique relies on compounds being UV absorbent. The UV detector works well for the benzodiazepines, as they have specific absorbency wavelengths.

In order to use mass spectrometry with liquid chromatography, the mobile phase must be removed in such a way as to allow the analytes to enter the mass spectrometer which is

kept under vacuum. This has only become possible in the last two decades therefore allowing the successful interface of liquid chromatography with mass spectrometry.

## 2.2 Liquid Chromatography – Mass Spectrometry

### *2.2.1 The Mobile Phase*

The mobile phase in LC-MS is very important for both the separation and ionisation of analytes. It usually comprises of two parts, the organic phase and the aqueous phase. Routinely in HPLC the aqueous part is made up of phosphate buffers at varying molarities and pH's. Phosphate buffers cannot be used in LC-MS because they are not volatile and will block the entrance to the source, by crystallising out. The main alternatives are acetate or formate buffers. Salts present in the mobile phase can affect the spectra by forming adducts with the analyte. In toxicology, buffers are commonly used to control the pH of a sample for efficient extraction. It is important to have not only good recovery in the extraction used, but also a clean extract with no buffer present, to minimise adduct formation. If adducts are formed then the number of analyte molecules remaining to produce the pseudo molecular ion, $[M+H]^+$, is reduced therefore decreasing sensitivity.

Buffers or additives can be used but there are conflicting views on how changing the mobile phase in this way affects the fragmentation patterns. One group showed that changes in acetonitrile concentration did not cause dramatic variation in spectra, except that the acetonitrile adduct was more often visible at high concentrations of acetonitrile[6]. In an earlier paper, the authors commented that the extent of fragmentation of morphine-3-glucuronide and morphine-6-glucuronide was greatly dependent on the acetonitrile concentration, i.e. fragmentation increased with increasing acetonitrile concentration[7]. A second group opposed this theory of fragmentation increasing with acetonitrile concentration. They carried out controlled experiments that showed fragmentation was stable and independent of the composition of the mobile phase, ranging from 0-90% acetonitrile[8]. A third group argued that the different batches of mobile phase used had varied their fragmentation of LSD[9].

Another reason for the use of buffers in the mobile phase is to increase the response of the analyte. Formic acid is used routinely, with varying concentrations, especially when analysing basic drugs. One group used both ammonium acetate (5mM) and formic acid

(0.1%) in the mobile phase for optimal sensitivity and separation of their analytes[10]. They also noted that the addition of formic acid was essential in preventing the hydrolysis of cocaine. Another group claims that the lowest possible concentration of formic acid was best for ion production, but too low a concentration would result in insufficient control of separation pH and thus a less robust method[11]. With respect to analysing 1,4-benzodiazepines, one group found the addition of traces of acid e.g. 0.05% trifluoroacetic acid did not enhance the ion yield[12]. Using trifluoroacetic acid as a buffer is uncommon in LC-MS and the authors do not comment on any other acids tested.

The addition of a buffer has also been seen to be preferential for more reproducible retention times[13]. Another reason for using a buffer in the mobile phase is to ionise the analytes before they are injected onto the system by reconstituting samples in the mobile phase. It has been demonstrated that electrospray ionisation is most efficient with compounds already ionised in solution[14]. For example using a moderately acidic mobile phase for basic compounds of interest. The acid mobile phase had an excess of $H^+$ ions encouraging the addition of a proton on to a basic drug.

The many differing opinions on the cause of spectral variation suggest that the reasons for these variations exist in each system to some extent, and are dependent on the analyte concerned. System history has its part to play in spectra variation, especially if the instrument is being used for a variety of analytes and mobile phase systems.

### 2.2.2 The Column

Columns used in HPLC are between 200 and 300 mm in length with an internal diameter of 4.6 mm. The flow rate is usually ~1 mL/min. When using LC-MS the interface dictates that the flow rate must be lower so that there is less mobile phase to evaporate in the source, but the high pressure must still be present. Smaller columns are therefore used with a maximum length of 150 mm and an internal diameter of 2.1 mm.

### 2.2.3 The Interface

In general, LC-MS uses atmospheric pressure ionisation, API, to interface the liquid chromatograph with the mass spectrometer. As the LC flow emerges from the column it is directed through a capillary to the ion source of the mass spectrometer. The interface is

positioned orthogonally to the source entrance in order to avoid excess contamination of the source. The source is under vacuum, however the interface is at atmospheric pressure. As the effluent sprays out of the interface a high percentage of it is lost while the rest is drawn into the source through the entrance cone.

The probe is set at a high temperature e.g. 300 °C, which is dependent on the flow-rate. This high temperature and the nitrogen flow helps to desolvate the liquid emerging from the capillary. Source conditions can be optimised for varying mobile phases, flow-rates and analytes used.

In LC-MS there are two common interfaces used, atmospheric pressure chemical ionisation, APCI and electrospray ionisation, ESI. In most instruments the interfaces can be interchanged easily, allowing the use of either.

Both ESI and APCI use soft ionisation to ionise the analytes as opposed to the hard ionisation used in GC-EI-MS. By using soft ionisation, the analyte is not exposed to such forces and often only the pseudo molecular ion is formed. If it is a basic compound then a proton is added $[M+H]^+$, or if an acidic compound a proton is removed $[M-H]^-$. When using buffers in the mobile phase there is an increased chance of adduct formation with the analyte molecules. Ammonium, sodium and potassium are common adducts resulting in the analyte molecular weight plus 18, 23, and 39 respectively, these are denoted as $[M+18]^+NH_4^+$, $[M+23]^+Na^+$ and $[M+39]^+K^+$.

One study showed that in APCI mode, using a single quadrupole mass spectrometer, spectra are dependent on the system history. A 6-MAM standard was analysed and four adducts were found, when analysed eight months later there were no adducts. The authors suggest that the heated vaporiser used in the APCI interface is a possible source of variability in fragmentation and adduct formation. They found that ESI showed better long-term reproducibility[6].

### 2.2.3.1 Atmospheric Pressure Chemical Ionisation

When using APCI, the probe is inserted into a heated nebulizer. The capillary, which carries the liquid from the HPLC column and into the probe, is situated in a second cylinder. Nitrogen gas is passed between the cylinders to aid the conversion of liquid out of the capillary end into a stream of droplets. The mobile phase is evaporated quickly and

the analytes are charged while in the gaseous state. This process is shown in Figure 2-1. A corona discharge pin is located just at the opening of the probe. A potential is applied to this, aiding ionisation of the buffer molecules present in the mobile phase. These ionised buffer molecules collide with the analyte molecules and protons are transferred. As high temperatures are involved, APCI is not suitable for compounds that are thermally labile as there may be thermal degradation.

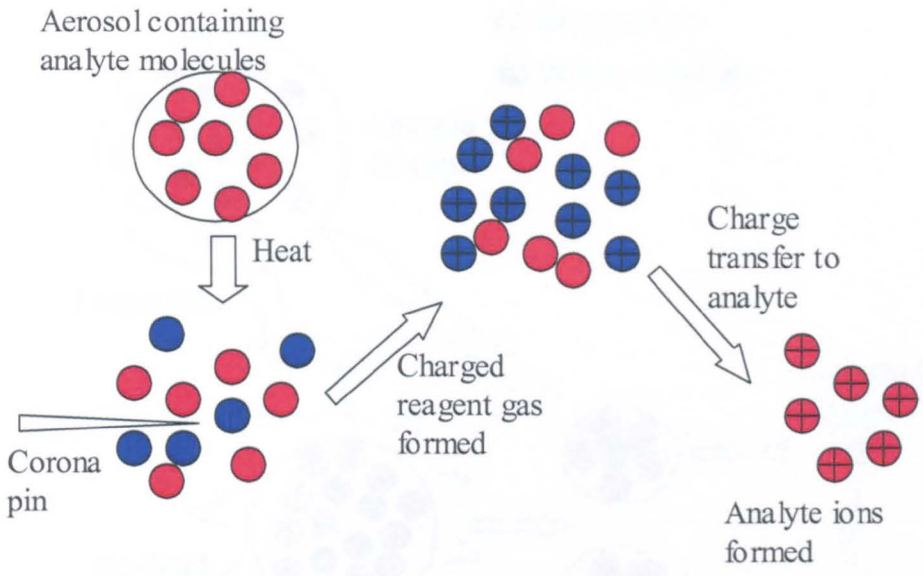



**Figure 2-1 : Atmospheric pressure chemical ionisation process**

### 2.2.3.2 Electrospray Ionisation

Electrospray ionisation has been used more frequently than APCI, probably because of its ability to ionise larger molecules, such as proteins and more polar molecules. The ESI interface has been used to analyse benzodiazepines[15,16,12,13], cannabinoids[17,18], cocaine[19,20,21], lysergic acid diethylamide[9,14], and opiates[7,11,22]. One group, using ESI, has developed a broad drug screening method which can be used to identify seventeen basic drugs from a variety of classes[23].

In contrast to the APCI interface, ESI ionises the analytes in the effluent as they spray out of the interface followed by the evaporation of the solvent. Since the analytes are ionised in solution and then desolvated, thermally labile molecules can be ionised without degradation[24]. The ESI probe has a voltage applied, which charges the molecules as they exit the probe. This probe voltage can be varied from 0 to 5 kV.

As droplets flow out of the ESI probe they are full of charged ions. As the solvent evaporates the droplets become smaller and the charges are brought closer together. When the repulsive forces of the charged species on the surface of the droplet are sufficient to overcome the cohesive forces of surface tension, columbic explosions occur which split the droplets into smaller droplets. This cycle continues until the analyte ion is sufficiently desolvated to enter the source, refer to Figure 2-2.

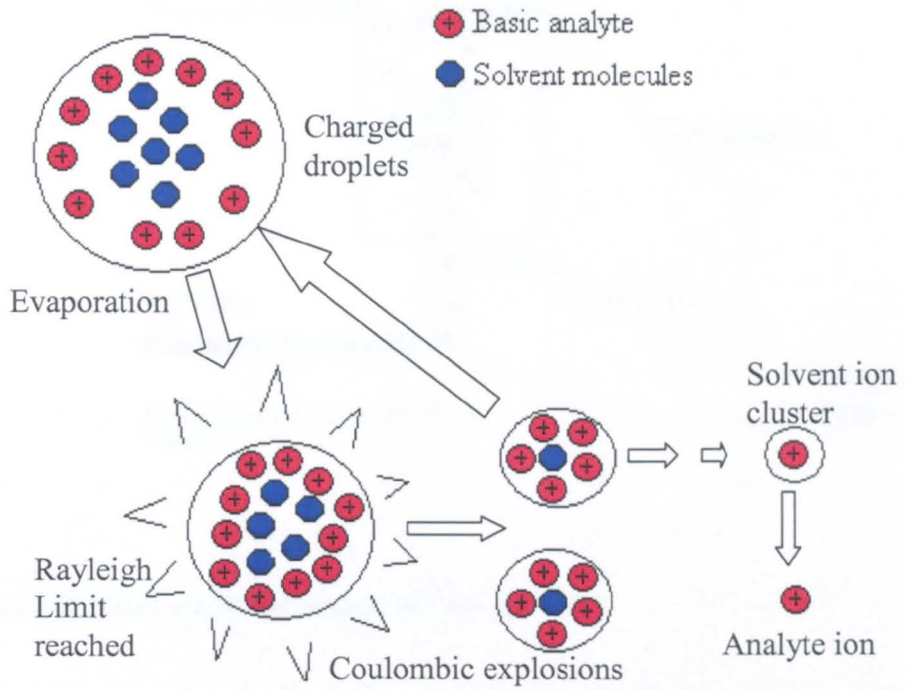



**Figure 2-2 : Electrospray ionisation process**

## 2.2.4 The Ion Source

On leaving the probe the analytes will be charged but will not necessarily have formed the pseudo molecular ion. The charged molecules enter the source via the skimmer or entrance cone, shown in Figure 2-3. In-source fragmentation can be achieved by applying a voltage to the skimmer cone. It can be varied from 0 to 100 V. The aim of this voltage is to fragment the analyte further, to obtain more qualitative information or to knock off any adducts from the molecular ion. Increasing the voltage applied to the skimmer cone will increase fragmentation. In the ThermoFinnigan AQA instrument, this voltage can be changed rapidly between scans, providing the ability to monitor a drug eluting from the column, at different voltages showing the pseudo molecular ion and a qualitative ion. Applying a voltage to the skimmer cone to induce fragmentation is also known as "cone

voltage" fragmentation. From the skimmer cone the ions are directed into the mass analyser. In the ThermoFinnigan AQA instrument this is a single quadrupole.

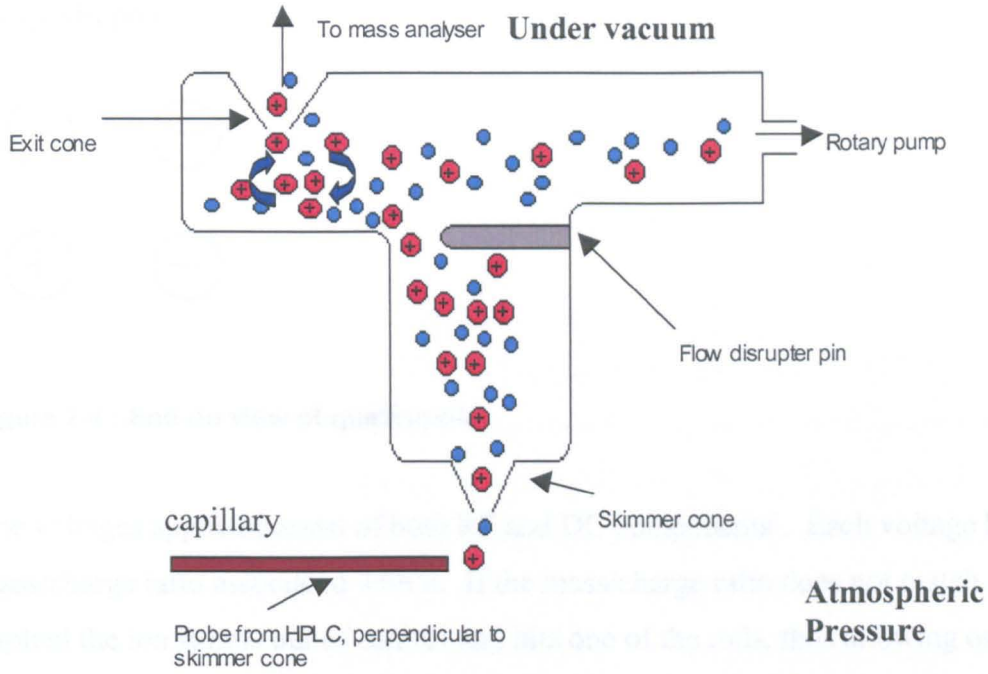



**Figure 2-3 : Diagram of ion source in Finnigan AQA**

The ion source in the LCQ instrument is very similar to the AQA. "In-source" fragmentation can be performed, but with the use of the ion trap to fragment ions further, it is not essential. As the charged analyte molecules leave the probe they enter the source through the skimmer cone and down a long transfer line to the ion trap.

## *2.2.5 The Analyser*

There are many analysers available for LC-MS, the quadrupole, ion trap, time of flight and magnetic sector, not to mention hybrids of these. For this project two different instruments were used, the ThermoFinnigan AQA, a single quadrupole mass analyser, and the ThermoFinnigan LCQ Advantage, an ion trap analyser.

### 2.2.5.1 Single Quadrupole, ThermoFinnigan AQA

The quadrupole is the most common of analysers and can be used singularly as in the ThermoFinnigan AQA or in tandem (triple quadrupole) to give greater sensitivity and more

spectral information. Its function is to filter the ions produced in the source into their mass/charge ratios. The quadrupole is made up of four rods parallel to each other making up four vertices of a cuboid. Figure 2-4 shows the view an ion would have travelling into the quadrupole.

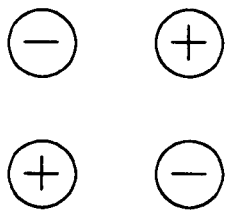


**Figure 2-4 : End-on view of quadrupole**

The voltages applied consist of both RF and DC components[1]. Each voltage has a mass/charge ratio associated with it. If the mass/charge ratio does not match the voltage applied the ion spirals out of control and hits one of the rods, thus allowing only ions of a certain mass/charge ratio to pass through onto the detector. When using full scan analysis the quadrupoles scan through a range of mass/charge ratios. If Single Ion Monitoring (SIM) is used the quadrupoles identify specific ions determined by the user.

### 2.2.5.2 Ion Trap, ThermoFinnigan LCQ Advantage

The ion trap analyser is formed out of three stainless steel electrodes. The ring electrode, which looks like a doughnut, and two endcaps which sit on either side forming a cavity within. The endcaps have small holes in the centre of them to allow ions to enter and exit the trap. It is sometimes described as a three-dimensional quadrupole[24]. Instead of an ion having a stable trajectory following through the analyser and onto the detector, it is trapped. Figure 2-5 shows a cross section of the trap.

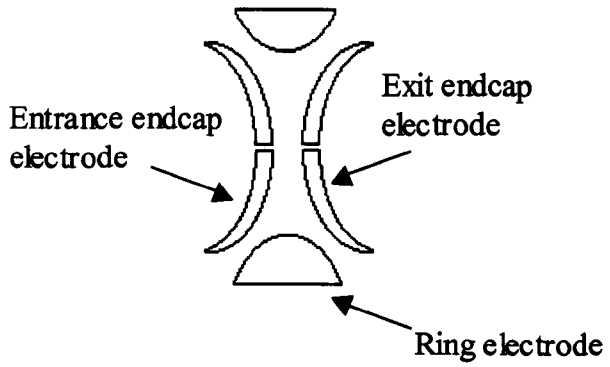



**Figure 2-5 : Cross section of ion trap**

Helium is present within the ion trap cavity at a partial pressure of ~0.1 Pa. Helium is an important part of the trap as it greatly enhances sensitivity. When ions enter the cavity they collide with the helium atoms, thus reducing their kinetic energy and the amplitude of their oscillations. This results in the ions being focused into the centre of the cavity instead of being allowed to spread out. The helium atoms are also used in tandem mass spectrometry (MS-MS) to collide with the parent ion with sufficient energy to dissociate it into one or more daughter ions.

When all the ions above a specified mass/charge ratio are trapped, a voltage is applied to the endcap electrodes and the voltage on the ring electrode is increased to eject all the ions sequentially as in Full MS mode. For MS-MS analyses an RF voltage is applied to the endcap electrodes to cause collision induced dissociation. The trap now contains the product ions of the pseudo-molecular ion. As before, these ions are scanned out by increasing the ring electrode voltage while simultaneously applying a voltage to the endcap electrode to facilitate ejection of the ions. Ions of increasing mass/charge ratio become unstable and are ejected from the trap to go on to the detector[25].

## *2.2.6 The Detector*

Once an ion has successfully made it through the analyser it reaches the detector. The main component in the detector is the photomultiplier tube. The ion strikes a conversion dynode that emits electrons. These electrons hit a phosphor screen that emits photons. The photons go on to the photocathode in front of the photomultiplier where an electron cascade is created. The cascade magnifies the original signal by ~1,000,000 times and it is this signal that is then converted into a voltage that can be analysed and processed by the computer on board the instrument[26].

An example of a mass spectra produced using an ion trap to obtain MS-MS data is shown in Figure 2-6. This shows the number of specific daughter ions produced which can all be used are further qualitative data. The chromatogram is produced by plotting each ion in the spectra over time. The area under the peak is related to the relative abundance of each ion in the spectra.

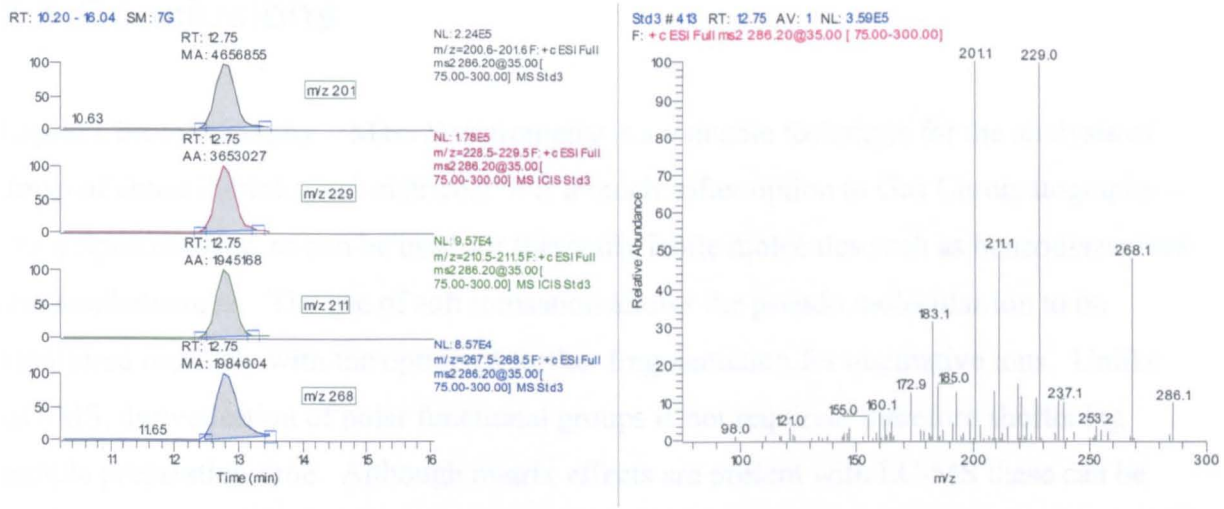



**Figure 2-6 : Typical extracted chromatogram and MS-MS mass spectra obtained using an ion trap analyser.**

## *2.2.7 Matrix Effect*

Matrix effects from biological samples, e.g. blood, urine and oral fluid, play an important role in LC-MS. They can be described as a change in the ionisation process of an analyte, due to a co-eluting compound from the matrix. Ionisation of the analyte molecule occurs at the interface of the LC-MS. A complex series of charge-transfer and ion-transfer reactions occur to ionise the analyte molecule[27]. It is clear that the matrix constituents present in the eluent will affect the efficiency of ionisation resulting in ion suppression or enhancement. ESI has been found to be more susceptible to matrix effects than APCI due to the different processes used to ionise analyte molecules[28].

As ionisation of analytes is dependent on the matrix constituents present, problems arise when the matrix is not consistent between individuals, affecting reproducibility, precision and accuracy. Ways to reduce the impact of the matrix effects include extraction techniques, such as solid phase extraction to minimise the amount of matrix injected into

the system, and the use of deuterated internal standards, as these elute at the same time as the analyte and are therefore affected in the same way by any matrix components present.

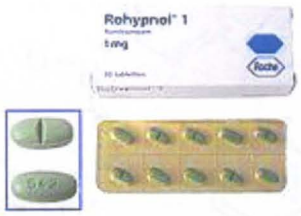
Matrix effects highlight the importance of robust and suitably validated methods with the use of deuterated internal standards where possible.

## 2.3 Conclusions

Liquid Chromatography – Mass Spectrometry is a valuable technique for the analysis of drugs of abuse in biological matrices. It is a much softer option to Gas Chromatography – Mass Spectrometry, so can be used for thermally labile molecules such as benzodiazepines and amphetamines. The use of soft ionisation allows the pseudo molecular ion to be monitored routinely with the option of further fragmentation for qualitative ions. Unlike GC-MS, derivatisation of polar functional groups is not required, therefore shortening sample preparation time. Although matrix effects are present with LC-MS these can be minimised with suitable extraction techniques and the use of deuterated internal standards. With the use of LC-MS-MS, more complex spectra can be produced, akin to GC-MS.

# 3 Flunitrazepam in Whole Blood

## 3.1 Introduction




Flunitrazepam (Rohypnol®) is used for its sedative and anaesthetic properties. Its alleged use in connection with drug facilitated sexual assault has gained wide publicity. Consequently the manufacturers, Roche Products Limited, altered the drug formulation making it harder to dissolve the drug in drinks and added a blue dye, making it more easily detectable[29]. With the increased public awareness of the drug, there has been an increase in the number of requests for flunitrazepam analysis, however there are several problems to overcome. The drug belongs to a potent group of hypnotic benzodiazepines that are therapeutically effective at low doses. A 2 mg tablet can cause sedation after 20-30 minutes which can last several hours[30]. A further delay in sample collection can occur as victims of drug facilitated sexual assault can be reluctant to report a crime and are sometimes even unaware that a crime has taken place due to drug induced amnesia. This delay in sample collection results in a significant clearance in the amount of drug present in the blood or urine. One group found that 24 hours after the administration of a 2 mg dose of Rohypnol®, free 7-aminoflunitrazepam levels in serum varied from 3.7-5.3 ng/mL[30]. To be able to detect such low levels of the drug, the method of analysis must be specific (to minimise the signal/noise ratio) and have good recovery from the matrix to allow very low detection limits.

## 3.2 Chemistry

Flunitrazepam or 5-(2-Fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one is a hypnotic benzodiazepine. When using liquid chromatography – mass spectrometry the pseudo-molecular ion is typically formed. Flunitrazepam forms a positive ion in the mass spectrometer. This is achieved by the addition of a proton to the structure thereby giving the analyte a positive charge and an increase in molecular weight of 1 amu. The most likely place for protonation to occur is at the cyclic ketone (highlighted in green in Figure 3-1). Flunitrazepam's metabolite 7-aminoflunitrazepam can also be protonated at this site. 7-Aminoflunitrazepam is similar in structure to

flunitrazepam except that it has a primary amine instead of a nitro group; this is shown in Figure 3-1 (highlighted in yellow).

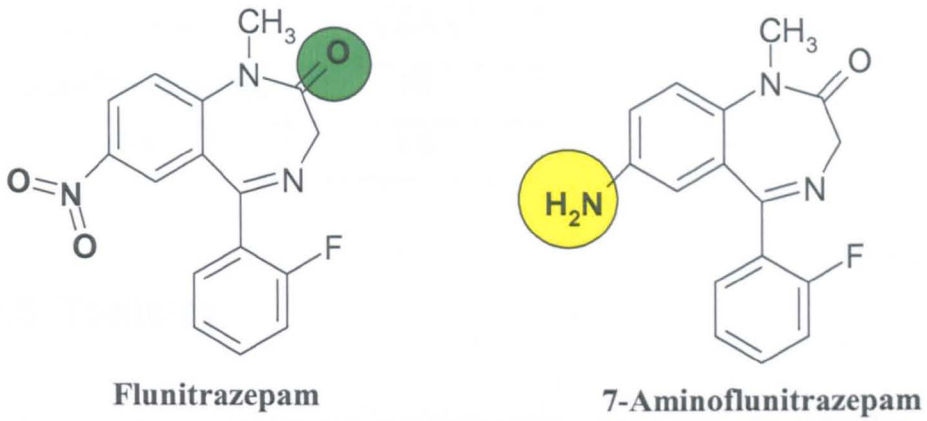



**Figure 3-1 : Structure of flunitrazepam and 7-aminoflunitrazepam**

This amine group is the likely primary site of protonation, but as the ketone group is still available, there are two potential sites to produce the pseudo-molecular ion. In both the single quadrupole instrument and the ion trap, 7-aminoflunitrazepam gives a higher response than flunitrazepam at the same concentration. Steric hindrance of the cyclic ketone may account for this.

## 3.3 Metabolism and Excretion

Flunitrazepam is metabolised to its major metabolite 7-aminoflunitrazepam by reduction of the nitro group to an amine. 7-aminoflunitrazepam is present in the plasma at concentrations of 2-4 ng/mL up to 24 hours after a single dose (2 mg) of flunitrazepam[31]

## 3.4 Pharmacokinetics

After a 2 mg dose of flunitrazepam, administered orally, peak blood concentrations of 6 ng/mL occur after 2 hours[32]. Pharmacokinetic data for flunitrazepam is summarized in Table 3-1.[31]

**Table 3-1 : Pharmacokinetic and physiochemical data for flunitrazepam**

| | Flunitrazepam |
|---|---|
| **Half Life (hours)** | 9-25 |
| **Vd (L/Kg)** | 3.4-5.5 |
| **Protein Binding (%)** | 78 |
| **pKa** | 1.8 |

## 3.5 Toxicity

A flunitrazepam overdose can result in ataxia, drowsiness, hypotension, respiratory depression and coma.[31] 28 mg of flunitrazepam has resulted in death, with kidney levels of 0.5 mg/kg for both the parent drug and its metabolite. Flunitrazepam's potency at low concentrations and its rapid half-life make it a likely drug for drug facilitated sexual assault.

## 3.6 Previous Work

Analysis of Flunitrazepam has been largely done by GC-MS[30,33,34,35,36,37,38,39] and HPLC[40,41,42,43,44,45,46,47,48,49]. Owing to its rapid metabolism and the low doses of flunitrazepam administered, it is essential to have as low detection limits as possible. Using GC-MS, detection limits of between 10 ng/mL[30] to 100 ng/mL in blood[34] for flunitrazepam have been achieved. Using HPLC, detection limits for flunitrazepam range from 3.5 ng/mL[47] to 20 ng/mL[48] in blood. As LC-MS is a relatively new technique there are fewer reports on the analysis of flunitrazepam in blood[15,50,51]. Using the technique, detection limits varying from 0.025 ng/mL[50] to 0.5 ng/mL[15] have been achieved.

Most LC-MS instruments have the ability to change between an APCI and ESI probe with ease. Different groups have reported the use of both APCI and ESI probes with similar results. A variety of mobile phase buffers have also been employed from 0.03 % ammonium hydroxide to 50 mM ammonium formate producing comparable data. These reports would suggest that there are no set rules for the choice of probe and mobile phase in relation to the analyte.

LC-MS-MS has also been used for flunitrazepam analysis; one group[52] used a ThermoFinnigan LCQ ion trap and achieved a detection limit of, 0.25ng/mL, similar to that using a single quadrupole.

## 3.7 Aim

The aim of the following project was to develop a reliable and sensitive method for the detection of flunitrazepam and its major metabolite 7-aminoflunitrazepam in blood, using Liquid Chromatography – Mass Spectrometry (LC-MS).

## 3.8 Method Development

The ThermoFinnigan AQA single quadrupole LC-MS was used for this project equipped with a P4000 quaternary pump, and AS3000 autosampler. For the development of this LC-MS method a Phenomenex Luna $C_{18}$ column, 150 x 2.0 mm, with 3 μm packing material was used. A C18 column was chosen as this is the most commonly used stationary phase for flunitrazepam analysis[15,40,50,52]. Initially the mobile phase comprised of 60 % methanol and 40 % deionised water, pumped isocratically, with a flow rate of 0.15 mL/min. These conditions were found to resolve the two analytes from each other and the solvent front. During the method development, the use of buffers in the mobile phase and the effect of changing the probe would be investigated.

### *3.8.1 Chemicals*

Methanol, acetonitrile and hexane were HPLC grade obtained from VWR International (UK). Analytical grade potassium dihydrogen phosphate and ammonium acetate were supplied by BDH laboratory supplies (Poole, England). Analytical grade concentrated ammonia, glacial acetic acid and concentrated formic acid were purchased from Sigma® Chemicals Co. (UK).

### *3.8.2 Standards*

Stock standards of flunitrazepam, 7-aminoflunitrazepam, flunitrazepam-$d_7$ and 7-aminoflunitrazepam-$d_7$ were purchased from Promochem as 100 μg/mL methanolic solutions. A working drug solution of 1 μg/mL, for spiking blank blood, was prepared by

taking 50 µL of each standard (flunitrazepam and 7-aminoflunitrazepam) and adding 4900 µL of methanol. A working internal standard solution was similarly prepared using the deuterated standards.

## *3.8.3 Blank Blood*

Time expired packed red blood cells were provided from the blood bank for blank blood. These were suspended in a ratio of 1:1 with isotonic saline solution which was made by dissolving 9.5 g of sodium chloride in 1 L of deionised water.

## *3.8.4 Solutions*

### 3.8.4.1 Preparation of 5, 7.5 and 10mM Ammonium Acetate Solutions

A 1 M solution of ammonium acetate was prepared first by adding 38.54 g of ammonium acetate to a 500 mL volumetric flask and making up to volume with deionised water. 5, 7.5 and 10 mL of this solution was added to 3 x 1 L volumetric flasks and made up to volume with deionised water resulting in 5, 7.5 and 10 mM solutions.

### 3.8.4.2 Preparation of 0.002, 0.004, 0.01, 0.05 and 0.1 % Formic Acid Solutions

20, 40, 100, 500, and 1000 µL of concentrated formic acid was added to 5 x 1 L volumetric flasks, which were made up to volume with deionised water to produce 0.002, 0.004, 0.01, 0.05 and 0.1 % solutions.

### 3.8.4.3 Preparation of 0.1 M, pH 6.0 Phosphate Buffer

6.81 g of potassium dihydrogen phosphate was weighed out into a 500 mL volumetric flask and 450 mL of deionised water was added. The pH was adjusted to 6.0 with 1 M potassium hydroxide solution, then made to 500 mL with deionised water.

## *3.8.5 Optimisation of Skimmer Cone Voltage*

An unextracted standard was prepared by adding 100 µL of working drug solution and 100 µL of working internal standard solution to a vial and blowing down under nitrogen at

35°C. This was reconstituted in 100 µL of mobile phase (60:40 methanol:deionised water). 20 µL of the standard was injected twice varying the skimmer cone voltage from 20-45 V for one injection and from 45-60 V for a second injection. The pseudo molecular ions were monitored in SIM mode. Table 3-2 shows the pseudo molecular ions monitored.

**Table 3-2 : Molecular weight and pseudo molecular ions for flunitrazepam and 7-aminoflunitrazepam**

| Analyte | Molecular weight (amu) | $[M+H]^+$ (m/z) |
|---|---|---|
| Flunitrazepam | 313 | 314 |
| Flunitrazepam-$d_7$ | 320 | 321 |
| 7-Aminoflunitrazepam | 283 | 284 |
| 7-Aminoflunitrazepam-$d_7$ | 290 | 291 |

The areas produced for the pseudo molecular ion at each skimmer cone value were plotted and an optimum found. After 45 V the pseudo-molecular had fragmented and was no longer detectable. Figure 3-2 shows graphically how the peak areas changed for each ion as the skimmer cone voltage was varied.

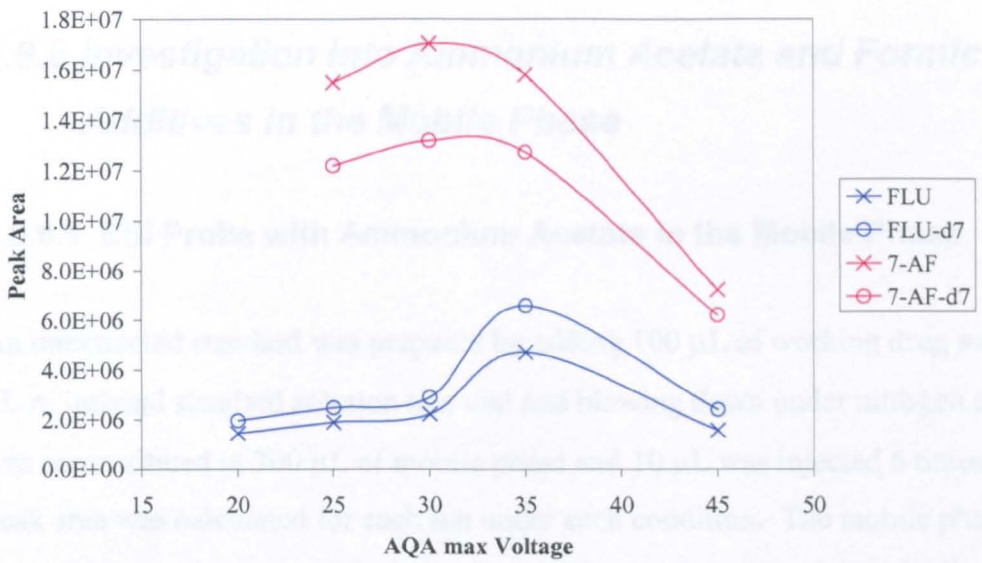



**Figure 3-2 : Optimisation of skimmer cone voltage for flunitrazepam and 7-aminoflunitrazepam and their deuterated standards.**

It is expected that the analyte and its deuterated standard produce the same curve on the graph as they have a common structure, and will therefore fragment similarly.

A qualifier ion for both analytes was found by increasing the skimmer cone voltage and fragmenting the pseudo molecular ion. In a SIM experiment both the pseudo molecular ion and qualifier ion can be monitored by alternating between each voltage. Table 3-3 shows the Skimmer cone values used for the rest of the method development. It was decided unnecessary to fragment the deuterated standards, as only the pseudo-molecular ion was needed for quantitation.

**Table 3-3 : Pseudo molecular ions, qualifier ions and skimmer cone voltages used for analysis**

| Analyte | Pseudo molecular ion (Skimmer cone voltage) | Qualifier ion (Skimmer cone voltage) |
|---|---|---|
| Flunitrazepam | 314 (35 V) | 268 (45 V) |
| Flunitrazepam-$d_7$ | 321 (35 V) | N/A |
| 7-Aminoflunitrazepam | 284 (30 V) | 226 (55 V) |
| 7-Aminoflunitrazepam-$d_7$ | 291 (30 V) | N/A |

## *3.8.6 Investigation into Ammonium Acetate and Formic Acid as Additives in the Mobile Phase*

### 3.8.6.1 ESI Probe with Ammonium Acetate in the Mobile Phase

An unextracted standard was prepared by adding 100 µL of working drug solution and 100 µL of internal standard solution to a vial and blowing down under nitrogen at 35°C. This was reconstituted in 200 µL of mobile phase and 10 µL was injected 6 times. An average peak area was calculated for each ion under each condition. The mobile phase composition was kept at 40 % aqueous and 60 % methanol run isocratically at 0.15 mL/min. The first condition used deionised water as the aqueous part of the mobile phase. Ammonium acetate was then used instead of deionised water at concentrations of 5 mM, 7.5 mM and 10 mM. The results of the experiment are shown in Figure 3-3.

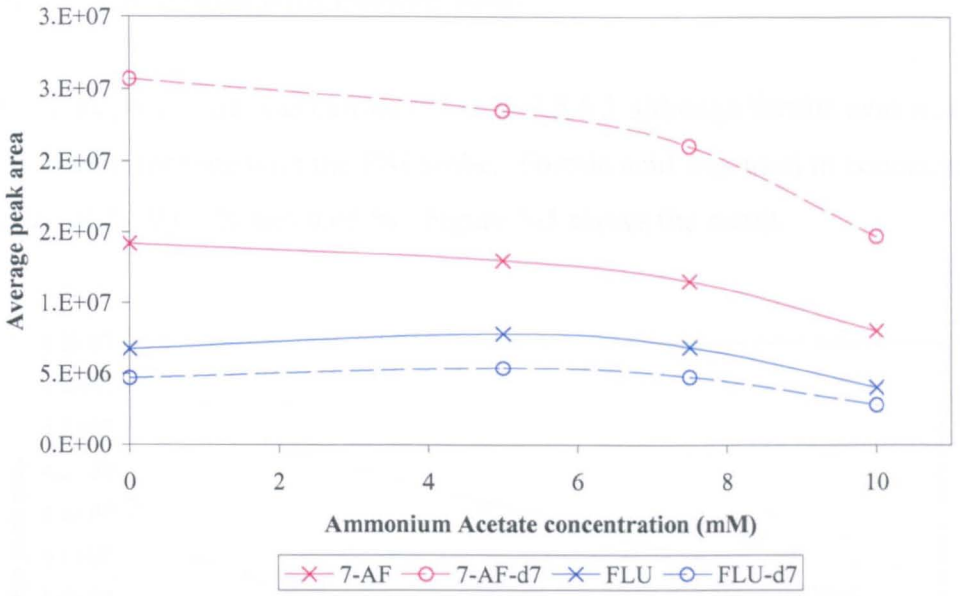



**Figure 3-3 : Effect of ammonium acetate concentration on response using an ESI probe**

### 3.8.6.2 APCI Probe with Ammonium Acetate

An unextracted standard was prepared as before in 3.8.6.1 and injected 6 times for each condition. An average peak area was calculated for each ion under each condition. The same ammonium acetate concentrations were used as before, changing only the probe to APCI. The graph in Figure 3-4 shows the result.

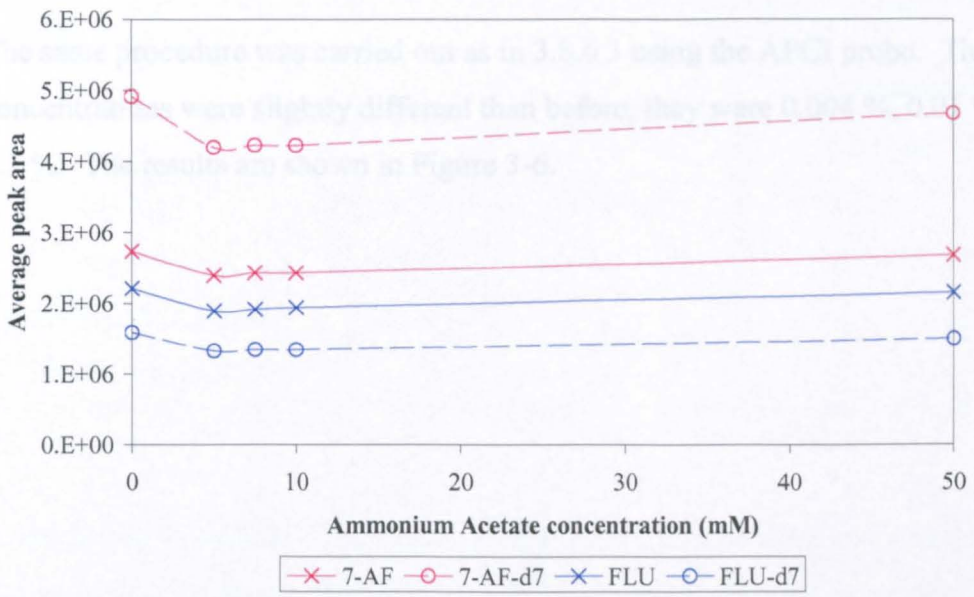



**Figure 3-4 : Effect of ammonium acetate concentration on response using an APCI probe**

### 3.8.6.3 ESI Probe with Formic Acid

The same procedure was carried out as in 3.8.6.1 although formic acid was used instead of ammonium formate with the ESI probe. Formic acid was used in concentrations of 0.002 %, 0.004 %, 0.01 % and 0.05 %. Figure 3-5 shows the result.

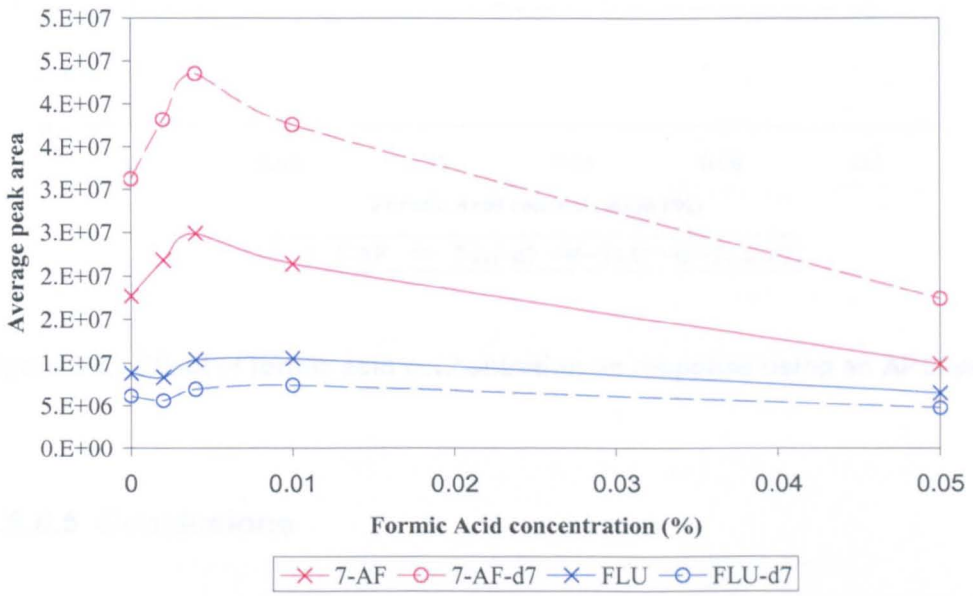



**Figure 3-5 : Effect of formic acid concentrations on response using an ESI probe**

### 3.8.6.4 APCI Probe with Formic Acid

The same procedure was carried out as in 3.8.6.3 using the APCI probe. The formic acid concentrations were slightly different than before, they were 0.004 %, 0.01 %, 0.05 %, and 0.1 %. The results are shown in Figure 3-6.

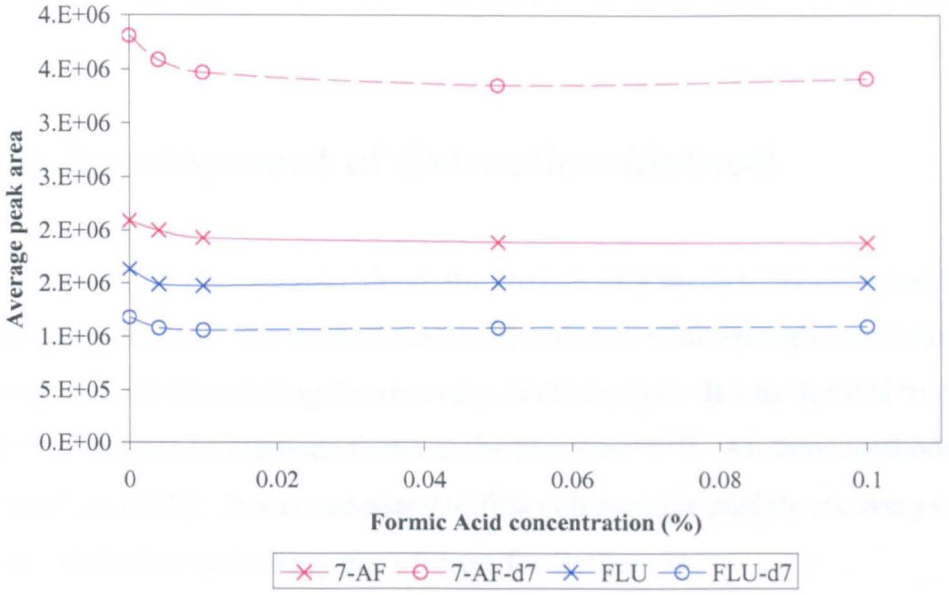



**Figure 3-6 : Effect of formic acid concentration on response using an APCI probe**

### 3.8.6.5 Conclusions

The graphs show that at the higher concentrations of both additives the response of both analytes is either unchanged or decreased. By comparing the maximum responses achieved with each combination of probe and additive an optimum can be found. In Table 3-4 the maximum responses are displayed.

**Table 3-4 : Maximum responses (7-aminoflunitrazepam-$d_7$) found for each combination of additive and probe**

| | **Electrospray Ionisation** | | **Atmospheric Pressure Chemical Ionisation** | |
|---|---|---|---|---|
| *Additive (amount)* | *Formic Acid (0.004 %)* | *Ammonium Acetate (0)* | *Formic Acid (0)* | *Ammonium Acetate (0)* |
| **Maximum Response (Arb. Units)** | 45,000,000 | 25,000,000 | 4,000,000 | 5,000,000 |

From the above table it is clear that electrospray ionisation gives a higher response than atmospheric pressure chemical ionisation for flunitrazepam and its metabolite without any additives present. Also when any ammonium acetate was added to the mobile phase the response decreased with both probes. The optimum mobile phase and probe was found to

be 60 : 40, methanol : 0.004 % formic acid in deionised water, using electrospray ionisation.

## 3.9 Development of Extraction Method

To analyse flunitrazepam in blood, the analyte first needs to be extracted from the matrix and concentrated. The method needs to be able to wash enough interfering compounds away without diminishing the recovery of the analyte. It was decided to evaluate the use of three extraction methods found in the literature[15,51,12]. All three methods use solid phase extraction (SPE). It was necessary to first compare the analyte recovery of the three methods before optimising one of them for further use.

### *3.9.1 Comparison of Three Extraction Methods*

The details of the three methods compared are found in Table 3-5 below.

**Table 3-5 : Details of three extraction methods used for comparison**

| Method | SPE Column | Wash Sequence | Elution Solvent |
|---|---|---|---|
| A[15] | CleanScreen DAU (Octyl+benzyl sulfonic acid) | 1 mL elution solvent<br>3 mL Methanol<br>3 mL DI water<br>2 mL phosphate buffer (0.1 M, pH 6)<br>*Add Sample*<br>2 mL DI water<br>2 mL 20:80 Acetonitrile:phosphate buffer<br>Dry<br>2 mL Hexane<br>Dry<br>2 X 2 mL DI Water<br>Dry | 2.5 mL 98 % ethyl acetate 2 % $NH_3$ |
| B[51] | Bond Elut $C_{18}$ | 1 mL Methanol<br>1 mL DI Water<br>2 mL Ammonium Carbonate Buffer (0.01 M, pH 9.3)<br>*Add Sample*<br>2 mL Ammonium Carbonate Buffer<br>Dry | 2 X 0.5 mL Methanol:0.5 M acetic acid, into tube with 5 µL 0.001 M HCl |
| C[12] | Bond Elut $C_{18}$ | 2 mL Methanol<br>2 mL DI Water<br>*Add Sample*<br>2 mL DI Water<br>Dry | 1 mL Methanol |

Initially the recoveries of flunitrazepam and 7-aminoflunitrazepam were calculated for the three extraction methods, without changing any factors. This was carried out by extracting in duplicate 50 ng of each analyte from 1 mL of blank blood with each extraction method. An unextracted standard of the same concentration, 50 ng, was also prepared at the same time in duplicate. 50 μL of a 1 μg/mL solution of flunitrazepam-$d_7$ and 7-aminoflunitrazepam-$d_7$ was added after the extraction to all extracted and unextracted samples. The ratios between the analyte and its respective deuterated standard were calculated. By dividing the extracted ratio by the unextracted ratio and multiplying by 100, a percentage recovery was calculated for each extraction. Table 3-6 shows the recoveries obtained experimentally and the recoveries reported in the literature.

**Table 3-6 : Recoveries of flunitrazepam and 7-aminoflunitrazepam using three different extraction methods**

| | Recovery (%) | | | |
|---|---|---|---|---|
| | **Flunitrazepam** | | **7-Aminoflunitrazepam** | |
| | **Experimental @ 50 ng (n=2)** | **Literature** | **Experimental @ 50 ng (n=2)** | **Literature** |
| A[15] | 71 | 74 @ 30 ng | 24 | 26 @ 30 ng |
| B[51] | None Detected | 93 | 31 | 92 |
| C[12] | 40 | 102 (serum) | 55 | Not Analysed |

To establish which extraction gave the cleanest extract, the chromatograms were analysed visually. A comparison of the chromatograms is shown in Figure 3-7 below.

## *3.9.2 Conclusions*

Using method B flunitrazepam was not detected and 7-aminoflunitrazepam had a much lower recovery than that reported in the literature. This extraction method was used with an APCI probe in the report, the ESI probe was used in this investigation which is thought to suffer from the matrix effect more, this could be the cause of the dramatic loss in analytes in the extraction.

By comparing an injection from each extract it was clear that using method A gave the cleanest extract with little interference. Method C was worse with an interefering peak co-

eluting with 7-aminoflunitrazepam, followed by method B which gave the dirtiest chromatogram.

## *3.9.3 Optimisation of Extraction Method*

It was decided to continue using method A and try and improve the recovery of 7-aminoflunitrazepam. The first step was to investigate if any analyte was being eluted from the solid phase with the washes. The hexane wash was explored first.

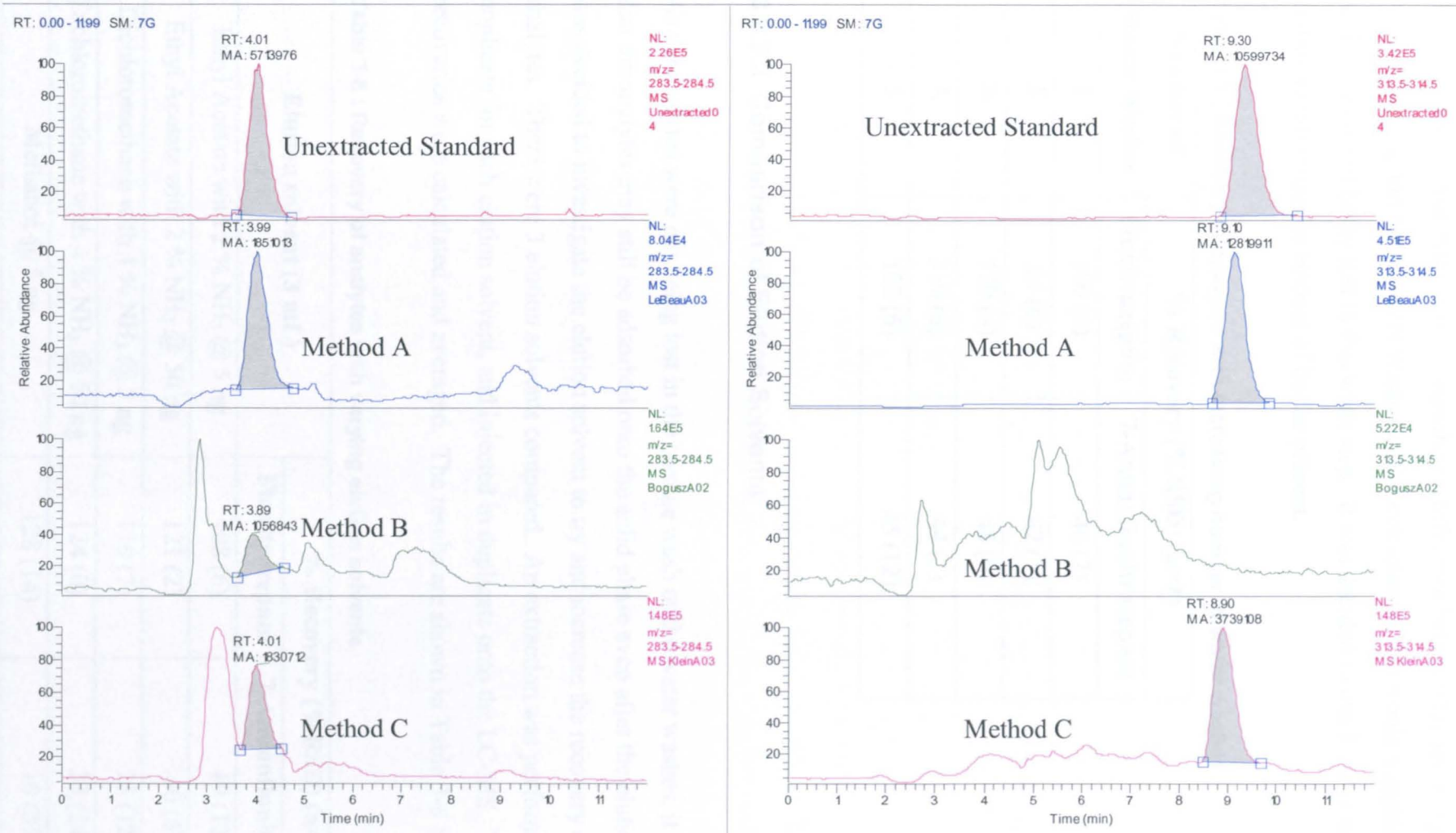
### 3.9.3.1 Monitoring Hexane Wash

An extraction of 50 ng of analyte in whole blood was carried out in duplicate. An unextracted 50 ng standard was also prepared in duplicate. After the first drying stage 2 mL of hexane was added and collected. 50 ng of deuterated internal standards were added to the hexane wash and unextracted samples. These were then blown down under nitrogen with no heat and reconstituted in 100 μL of mobile phase, they were injected in duplicate. It was clear from the hexane wash chromatogram that there was no flunitrazepam or 7-aminoflunitrazepam present. This showed that the analytes were not being lost in the hexane wash, so this step was retained in the extraction method.

### 3.9.3.2 Variation of the Number of Water Washes

In the paper an unspecified number of water washes are performed after the hexane wash to ensure that all phosphate buffer has been washed off. If potassium ions from the buffer are left with the analytes, adducts can form and reduce the response of the molecular ion in the MS. It was postulated that some of the analytes were being washed away with these water washes. This was investigated by comparing the recoveries of the analytes with an increasing number of water washes used.

In duplicate, the recovery of analytes was calculated using 1, 2, 3, 4 and 5 water washes before elution. The samples were injected in duplicate and the average recovery for each number of water washes found. The results are shown in Table 3-7.




**7-aminoflunitrazepam** **flunitrazepam**

**Figure 3-7 : Chromatograms comparing extractions procedures**

The results show that there was no significant difference between the recovery of analytes with increasing number of water washes in the extraction. This would suggest that analytes were not being lost in this wash step. It was decided to use 3 water washes before elution to minimise the amount of buffer present.

**Table 3-7 : Recovery of analytes with increasing number of water washes**

| Number of Water Washes | % Recovery (%RSD) (n=2) | |
|---|---|---|
| | Flunitrazepam | 7-Aminoflunitrazepam |
| 1 | 100 (6) | 46 (2) |
| 2 | 95 (6) | 42 (1) |
| 3 | 120 (4) | 48 (9) |
| 4 | 110 (8) | 44 (3) |
| 5 | 102 (6) | 45 (12) |

### 3.9.3.3 Comparison of Elution Solvents

As the analytes were not being lost in the hexane wash or the water washes, it was thought that the analytes may still be adsorbed onto the solid phase even after the elution step. It was decided to investigate the elution solvent to try and increase the recovery of the analytes. There were 3 elution solvents compared. An extraction was performed in duplicate for each elution solvent, and injected in duplicate onto the LC-MS. The recoveries were calculated and averaged. The results are shown in Table 3-8 below.

**Table 3-8 : Recovery of analytes with varying elution solvents**

| Elution solvent (3 mL) | % Recovery (%RSD) (n=2) | |
|---|---|---|
| | Flunitrazepam | 7-Aminoflunitrazepam |
| Ethyl Acetate with 2 % $NH_3$ @ 5 ng | 108 (6) | 49 (12) |
| Ethyl Acetate with 2 % $NH_3$ @ 50 ng | 121 (2) | 40 (5) |
| Dichloromethane with 4 % $NH_3$ @ 5 ng | 110 (7) | 25 (12) |
| Dichloromethane with 4 % $NH_3$ @ 50 ng | 124 (0) | 33 (24) |
| Methanol @ 5 ng | 128 (14) | 10 (37) |
| Methanol @ 50 ng | 109 (5) | 47 (11) |

Using dichloromethane with 4 % $NH_3$ as the elution solvent decreased the recovery of 7-aminoflunitrazepam. Ethyl acetate with 2 % $NH_3$ and methanol gave similar recoveries for both analytes. By looking at the chromatograms produce from each extract, Figure 3-8 it was clear that the methanol extract contained more interferents than the ethyl acetate extract. Therefore it was decided to continue using ethyl acetate with 2 % $NH_3$ as the elution solvent.

## 3.10 Method Validation

The complete method used for validation and subsequent case samples was as follows.

### *3.10.1 Sample Preparation*

Spiked blood samples were prepared by adding the appropriate amount of working drug solution (1, 2, 5, 10, 25, 50, 100 µL) and internal standard solution (100 µL) to test tubes, followed by 3.5 mL of 0.1 M, pH 6 phosphate buffer and 1 mL of blank blood. A blank was prepared without any standards present and another with internal standard only to assess any contamination of the deuterated standards.

### *3.10.2 Extraction Method*

3.5 mL of 0.1 M, pH 6.0 phosphate buffer was added to 1 mL of whole blood. 100 µL of 1 µg/mL solution of flunitrazepam-$d_7$ and 7-aminoflunitrazepam-$d_7$ was added as an internal standard. This was vortexed and centrifuged for 10 minutes at 3000 rpm. CleanScreen DAU® cartridges were conditioned with 1 mL ethyl acetate with 2 % $NH_3$, 3 mL methanol, 3 mL deionised water and 2 mL 0.1 M, pH 6.0 phosphate buffer prior to sample application. The cartridges were washed with 2 mL deionised water and 2 mL phosphate buffer (01 M, pH 6) : acetonitrile (80 : 20), then dried under full vacuum for 1 minute. 2 mL of hexane was applied and dried under full vacuum for a further minute. 3 x 2 mL aliquots of deionised water were applied to the cartridges drying under full vacuum for 1 minute between each addition of water. After the third aliquot of water the cartridges were left at full vacuum for 10 minutes to dry thoroughly. The analytes were then eluted with 2.5 mL ethyl acetate with 2 % $NH_3$. This was blown down under nitrogen at 30 °C until dry. Samples were reconstituted in 100 µL of mobile phase (60 : 40 methanol : 0.004 % formic acid), with 20 µL injected in duplicate.

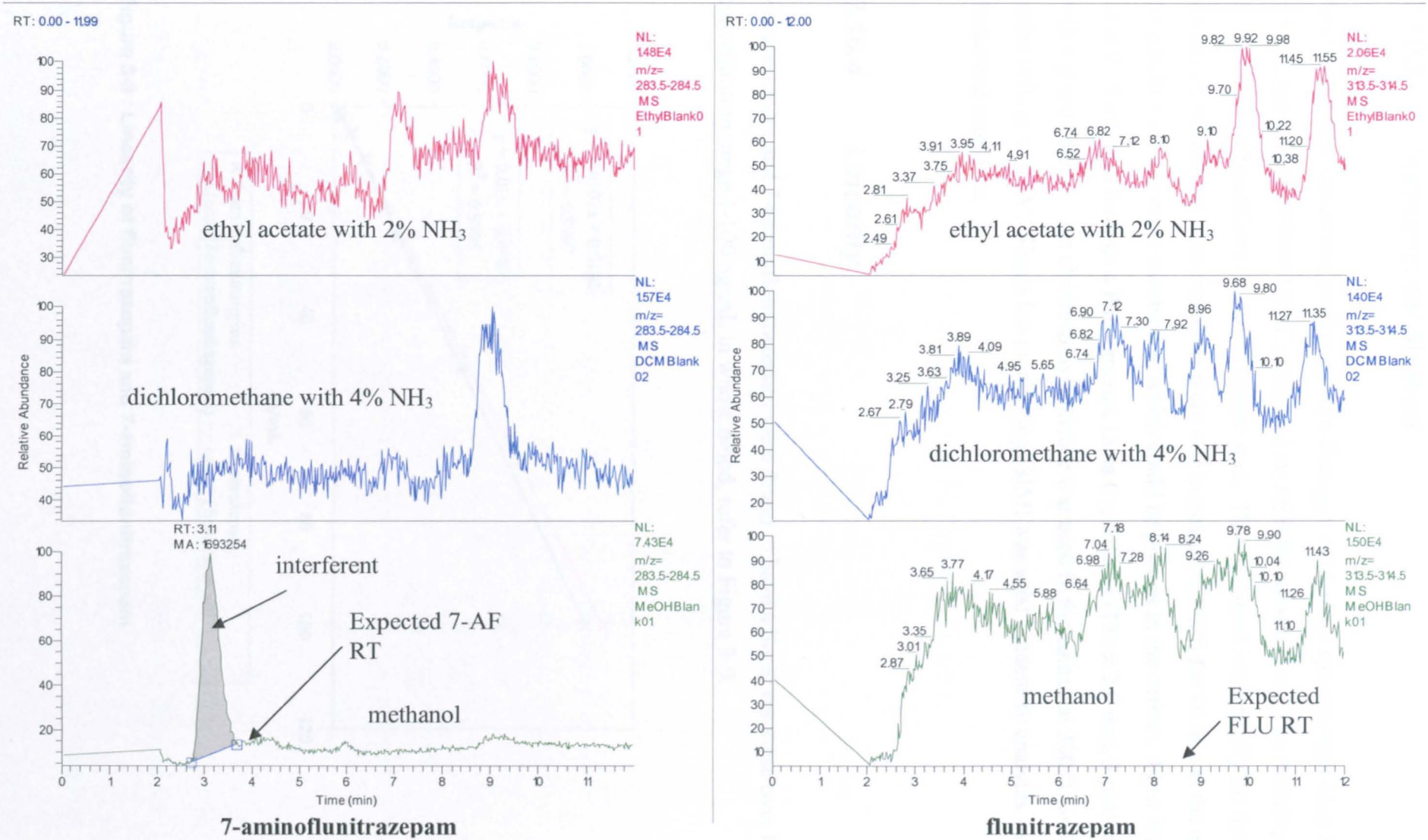



**Figure 3-8 : Comparison of blank chromatograms using different elution solvents**

### 3.10.3 Instrumental Method

Analysis of the extracts was performed on a Finnigan AQA mass spectrometer (San Jose, CA) equipped with a P4000 HPLC pump and an AS3000 autosampler. The mobile phase used was 60 : 40 methanol : 0.004 % formic acid. This was used isocratically for the separation of analytes. The organic content was increased to 90 % for 10 min at the end of the run, to elute any other substances, which could be present in the extract. Flow rate was set at 0.15 mL/min through a Phenomenex Luna $C_{18}$ column (150 x 2.0 mm, 3 μm), fitted with a guard column. An electrospray interface was used in the source at 300 °C and a probe voltage of 4 kV. Single ion monitoring (SIM) was used to identify analytes and their deuterated standards.

### 3.10.4 Linearity

Flunitrazepam and 7-aminoflunitrazepam were found to have a linear response over the concentration range 1-100 ng/mL in whole blood, refer to Figure 3-9.

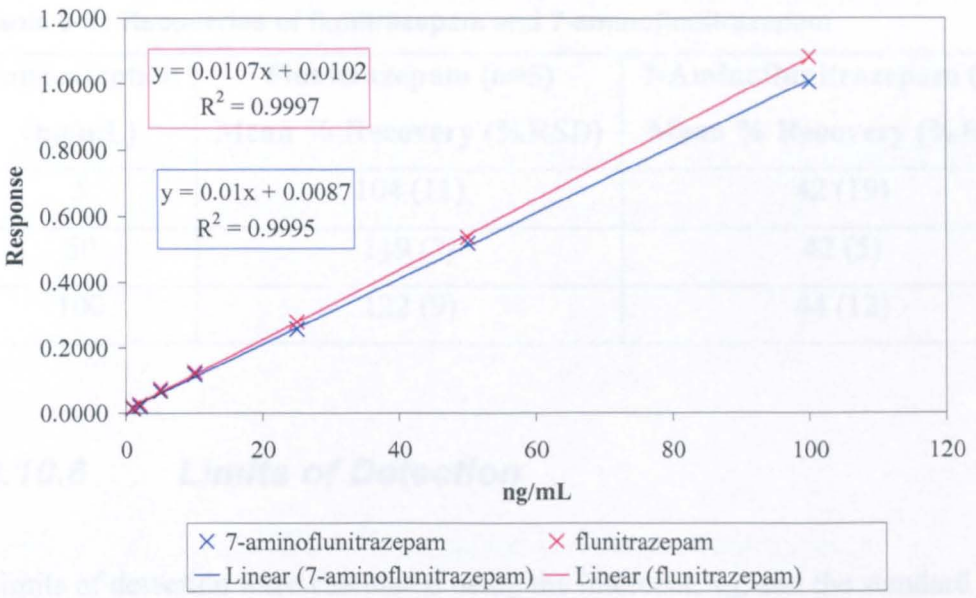



**Figure 3-9 : Linearity of flunitrazepam and 7-aminoflunitrazepam**

### 3.10.5 Recovery

Relative recoveries of flunitrazepam and 7-aminoflunitrazepam were calculated at three concentrations (5, 50 and 100 ng/mL). Each concentration was extracted five times without internal standard present. Two unextracted standards were also prepared at each concentration without internal standard and were kept in the fridge throughout the extraction. Before blowing down the samples under nitrogen to dryness, 100 ng of internal standard was added to each vial including the unextracted standards. Once the samples were dry, they were reconstituted in 100 µL of mobile phase and 20 µL was injected in duplicate.

The recovery of each drug was calculated using the following equation.

$$\text{Recovery}(\%) = \frac{\text{Peak Area Ratio of Extracted Standard}}{\text{Peak Area Ratio of Unextracted Standard}} \times 100 \qquad \textbf{Equation 1}$$

Recoveries for flunitrazepam and 7-aminoflunitrazepam at each concentration are shown in Table 3-9 with an average recovery of 115 % and 43 % respectively.

**Table 3-9 : Recoveries of flunitrazepam and 7-aminoflunitrazepam**

| Concentration (ng/mL) | Flunitrazepam (n=5) Mean % Recovery (%RSD) | 7-Aminoflunitrazepam (n=5) Mean % Recovery (%RSD) |
|---|---|---|
| 5 | 104 (11) | 42 (19) |
| 50 | 119 (7) | 42 (5) |
| 100 | 122 (9) | 44 (12) |

### 3.10.6 Limits of Detection

Limits of detection were calculated using the intercept, $y_B$, and the standard error of the regression line, $s_B$.[53] The equations used are shown below, where m = the gradient.

$$y_{LOD} = y_B + 3s_B \qquad \textbf{Equation 2}$$

$$\text{L.O.D.} = \frac{(y_{LOD} - y_B)}{m} \qquad \textbf{Equation 3}$$

The limits of detection for flunitrazepam and 7-aminoflunitrazepam were found to be 0.2 and 0.5 ng/mL respectively.

### 3.10.7 Limits of Quantitation

Limits of quantitation were calculated using the same method as in 3.10.6 except that 10 times the standard error of the line was used. The equations were changed to equations 4 and 5.

$$y_{LOQ} = y_B + 10s_B \qquad \textbf{Equation 4}$$

$$\text{L.O.Q.} = \frac{(y_{LOQ} - y_B)}{m} \qquad \textbf{Equation 5}$$

Limits of quantitation for flunitrazepam and 7-aminoflunitrazepam were calculated as 0.8 and 1.6 ng/mL respectively.

### 3.10.8 Inter-Day Precision

Inter-day precision was calculated using five sets of linearity data acquired on five different days. The %RSD of the five values for two concentrations, 5 and 50 ng/mL were calculated. The results are shown in Table 3-10.

**Table 3-10 : Inter-day precision data for flunitrazepam and 7-aminoflunitrazepam**

| Concentration (ng/mL) | Flunitrazepam (%RSD) (n=5) | 7-Aminoflunitrazepam (%RSD) (n=5) |
|---|---|---|
| 5 | 5.2 (9.0) | 5.0 (8.5) |
| 50 | 48.9 (1.1) | 50.4 (2.7) |

### 3.10.9 Intra-Day Precision between Injections

Extracted standards were prepared at two concentrations, 5 and 50 ng/mL. They were reconstituted in 150 µL of mobile phase and 20 µL was injected 5 times consecutively. The %RSD of the peak area ratios was calculated at each concentration for each drug. The results are shown in Table 3-11.

**Table 3-11 : Intra-day precision data for flunitrazepam and 7-aminoflunitrazepam**

| Concentration (ng/mL) | Flunitrazepam (%RSD) (n=5) | 7-Aminoflunitrazepam (%RSD) (n=5) |
|---|---|---|
| 5 | 4.5 (2.8) | 5.0 (3.3) |
| 50 | 49.3 (2.8) | 50.8 (0.8) |

## 3.11 Discussion

In the process of developing the final LC-MS method, the additives in the mobile phase were varied, and LC-MS conditions optimised for the target analytes. It was found that using very small amounts of additives, 0.004% formic acid, in the mobile phase gave the best response for the two analytes. This is in contrast to many published methods which use up to 0.1% formic acid. The amount of additive will affect the chromatography of analytes. In the case of flunitrazepam analysis the most important factor was sensitivity and as only the parent drug and its major metabolite were analysed, resolution was found not to be a problem when varying buffers. The limits of quantitation achieved were very low, 0.8 ng/mL and 1.6 ng/mL for flunitrazepam and 7-aminoflunitrazepam respectively.

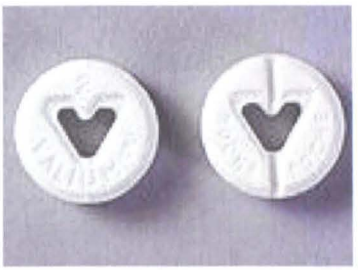
Precision between five different days was found to be less than 10 % for both analytes at a concentration of 5 ng/mL. Precision between injections was found to be less than 4 % for both analytes at a concentration of 5 ng/mL. This precision at low concentrations and the low limits of detection enable this method to be applied to drug facilitated sexual assault case samples.

## 3.12 Conclusions

A liquid chromatography – mass spectrometry method was developed and validated for the extraction and quantitation of flunitrazepam and its major metabolite 7-aminoflunitrazepam from whole blood. The method has been applied to case samples in the Forensic Medicine and Science Dept at the University of Glasgow, all have been found negative.

# 4 Diazepam and Metabolites in Whole Blood

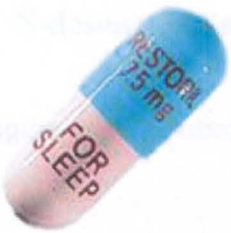
## 4.1 Introduction



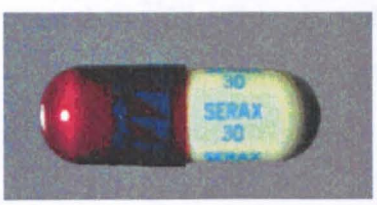
Diazepam is one of the most frequently prescribed drugs in the United States and is more commonly known by its pharmaceutical name, Valium®. It is prescribed as an anti-anxiety agent, muscle relaxant or anticonvulsant[31].

In the UK, diazepam has been associated with an increased risk of road traffic accidents[54], and linked to fatalities from drugs of abuse[55]. In a French study of 900 injured drivers, benzodiazepines were the most frequently observed psychoactive therapeutic drug[56].

One of the concerns of diazepam is that the metabolites are all pharmacologically active to some degree. N-desmethyldiazepam is at least as active as diazepam, and temazepam and oxazepam are also active but are not accumulated in the blood significantly.




Temazepam is a hypnotic benzodiazepine and causes sedation and drowsiness[31]. It was rescheduled in 1996 from schedule 4 to schedule 3 of the Misuse of Drugs Regulations 1985[57]. This resulted in a stricter control of prescribing and thus a decrease in availability, drug users looked to alternatives such as diazepam. In the Strathclyde region of Scotland there was a decrease in the number of post-mortem cases testing positive for temazepam in 1996 and 1997 and an increase in diazepam positive cases[58].




Oxazepam is an anti anxiety agent similar to diazepam but less potent, used for mild anxiety. It has been used in the United States since 1965, and is also available in the UK.

Owing to their continued use as anti anxiety and hypnotic agents, and their contribution to road traffic accidents and drug related deaths, it is essential to have a reliable and sensitive technique for the detection and quantitation of diazepam and its metabolites in forensic case samples.

## 4.2 Chemistry

The metabolic pathway of diazepam and the structures of itself and its metabolites are shown in Figure 4-1 below.

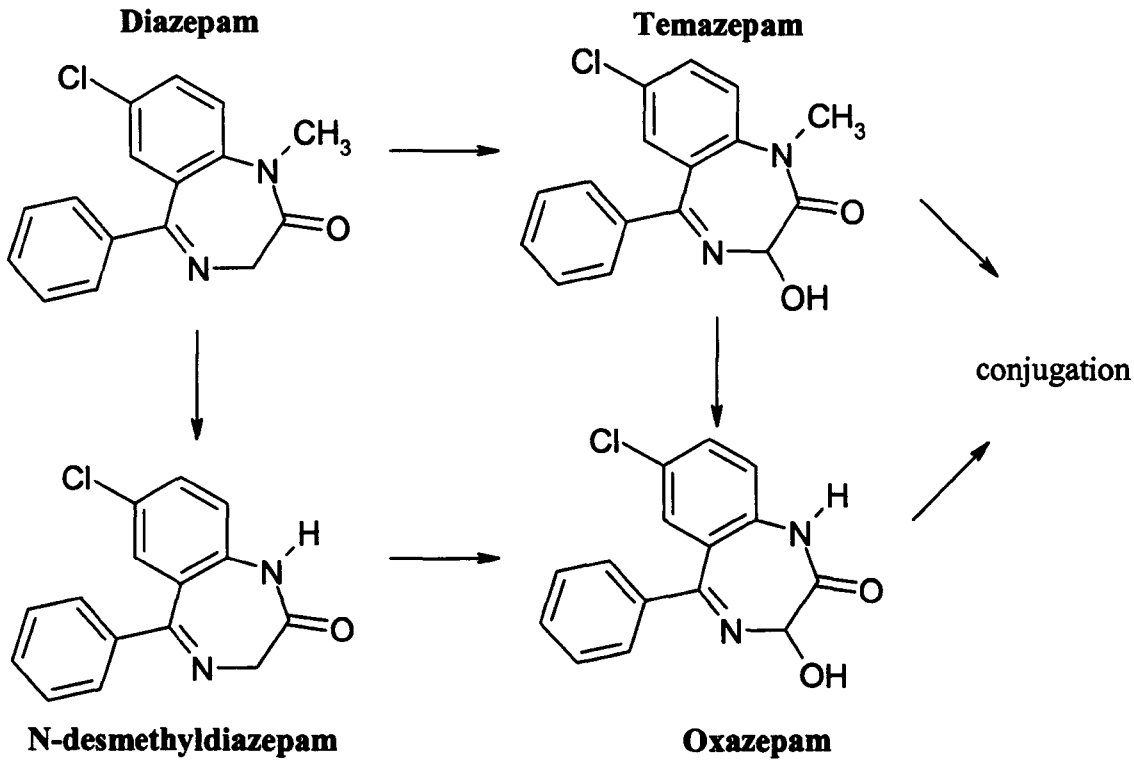



**Figure 4-1 : Metabolic pathway of diazepam**

Diazepam and its metabolites all have a ketone group, which could be the site of ionisation for these molecules. The hydroxyl group next to the ketone in temazepam and oxazepams structure is also a potential ionisation site. All four molecules also have a chlorine atom in their structure. Chlorine atoms exist as stable isotopes, $^{37}Cl$ has 2 more neutrons in the atom than $^{35}Cl$. This gives the potential for two pseudo-molecular ions, one with $^{35}Cl$ and one with $^{37}Cl$ at a ratio of 3:1. Monitoring both pseudo-molecular ions offers another piece of qualitative information.

## 4.3 Metabolism and Excretion

Diazepam metabolises by N-demethylation to form the active metabolite N-desmethyldiazepam[31]. These compounds are then converted to their 3-hydroxy derivatives

temazepam and oxazepam. Temazepam and oxazepam are also active drugs but not to the same extent, as they do not accumulate in blood.

## 4.4 Pharmacokinetics

Peak blood concentrations of diazepam averaged 148 ng/mL at 1 hour after a single 10 mg oral dose; this had fallen to 37 ng/mL in 24 hours. Its metabolite N-desmethyldiazepam had reached its peak concentration of 29 ng/mL after 24 hours. A single 30 mg oral dose of temazepam administered to healthy volunteers resulted in an average peak plasma concentration of 870 ng/mL, after 1.4 hours. Following a single 15 mg dose of oxazepam peak serum levels were reached after 1.5 hours with a concentration of 310 ng/mL[31]. The pharmacokinetic properties of diazepam and its metabolites are summarized in the Table 4-1.

**Table 4-1 : Pharmacokinetic and physiochemical properties of diazepam and its metabolites**

| | Diazepam | Temazepam | N-desmethyldiazepam | Oxazepam |
|---|---|---|---|---|
| **Half life (hr)** | 21-37 | 3-13 | 31-97 | 4-11 |
| **Vd (L/kg)** | 0.7-2.6 | 0.8-1.0 | - | 0.7-1.6 |
| **Protein Binding (%)** | 96 | 97 | 97 | 87-94 |
| **$pK_a$** | 3.4 | 1.3 | 3.5, 12.0 | 1.7, 11.6 |

## 4.5 Toxicity

Diazepam over dosage results in drowsiness, ataxia and muscular weakness. Serum concentrations of up to 20 mg/L have occurred in hospitalised patients exhibiting light coma[31]. Temazepam can cause sedation and drowsiness in normal doses, and a single oral dose of 20 mg at night has been shown to impair driving the next morning[31]. Oxazepam has been found to impair driving with blood concentrations of between 0.2 and 8 mg/L.[31] Diazepam is rarely found alone in fatal cases, and it more likely to be in combination with other drugs e.g. alcohol and heroin.

## 4.6 Previous Work

Like flunitrazepam, diazepam and its metabolites have been primarily analysed by HPLC[42,47,48]. Benzodiazepines, in general, chromatograph well using liquid

chromatography. They also have a tendency to decompose in the injection ports of gas chromatographs, temazepam especially. Despite this gas chromatography has been used successfully in the analysis of benzodiazepines[59,60,61,37,34]. Using liquid chromatography for its gentle separation and mass spectrometry for its specificity would be an ideal way to obtain reproducible, sensitive and qualitative information. Liquid chromatography – mass spectrometry has been used in benzodiazepine analysis in recent years[12,16,62,63,64,65]. Of these papers, four analyse specifically for diazepam and its three metabolites temazepam, N-desmethyldiazepam and oxazepam. Of these four, only two are extracted from whole blood the other two use hair in one and *calliphora vicina* larvae in the other. Using restricted-access solid-phase microextraction, one group has quoted limits of quantitation as 20, 20, 30 and 35 ng/mL for diazepam, N-desmethyldiazepam, temazepam and oxazepam respectively[64]. Another group using liquid-liquid extraction obtained limits of quantitation of 13, 14, 5 and 7 ng/mL[65]. This was achieved using an ion trap mass spectrometer yielding MS-MS data.

## 4.7 Aim

The aim was to develop a method for the analysis of diazepam and its metabolites temazepam, N-desmethyldiazepam and oxazepam using liquid chromatography – single quadrupole mass spectrometry. Benzodiazepines are routinely analysed by HPLC with UV or diode array detection. If the extract is particularly dirty then there can be problems with interference, and the drug cannot be quantified. If the extract is very concentrated then the response can go off the scale of the detector and hence cannot be quantified without further dilution steps. At the other extreme, detection limits for HPLC-UV are higher than for LC-MS. A more specific technique will generally give better detection limits because the noise will be less. If LC-MS were to be used, these problems could be overcome.

## 4.8 Method Development

### *4.8.1 Chemicals*

Methanol, acetonitrile and hexane were HPLC grade and obtained from VWR International (UK). Analytical grade potassium dihydrogen phosphate, ammonium acetate were supplied by BDH laboratory supplies (Poole, England). Analytical grade concentrated

ammonia, glacial acetic acid and concentrated formic acid were purchased from Sigma® Chemicals Co. (UK).

## 4.8.2 Standards

Stock standards of diazepam, N-desmethyldiazepam, temazepam and oxazepam were purchased from Promochem as 100 μg/mL methanolic solutions. The $d_5$ deuterated standards of each drug were also purchased as 100 μg/mL methanolic solutions. A working drug solution of 1 μg/mL, for spiking blank blood, was prepared by taking 50 μL of each standard and adding 4800 μL of methanol. A working internal standard solution was prepared in the same way using the deuterated standards.

## 4.8.3 Blank Blood

Time expired packed red blood cells were provided from the blood bank for blank blood. These were suspended in a ratio of 1:1 with isotonic saline solution which was made by dissolving 9.5 g of sodium chloride in 1 L of deionised water.

## 4.8.4 Solutions

### 4.8.4.1 Preparation of 5 and 10 mM Ammonium Acetate Solutions

A 1 M solution of ammonium acetate was prepared first by adding 38.54 g of ammonium acetate to a 500 mL volumetric flask and making up to volume with deionised water. 5 and 10 mL of this solution was added to 2 x 1 L volumetric flasks and made up to volume with deionised water resulting in 5 and 10 mM solutions.

### 4.8.4.2 Preparation of 0.002 %, 0.004 %, 0.008 %, 0.01 % and 0.05% Formic Acid Solutions

20, 40, 80, 100, and 500 μL of concentrated formic acid was added to 5 x 1 L volumetric flasks, which were made up to volume with deionised water to produce 0.002, 0.004, 0.008, 0.01 and 0.05 % solutions.

#### 4.8.4.3 Preparation of 0.1 M, pH 6.0 Phosphate Buffer

6.81 g of potassium dihydrogen phosphate was weighed out into a 500 mL volumetric flask and 450 mL of deionised water was added. The pH was adjusted to 6.0 with 1 M potassium hydroxide solution, then made up to 500 mL with deionised water.

### *4.8.5 Liquid Chromatography – Mass Spectrometry*

The instrument used for this work was a ThermoFinnigan AQA LC-MS. It consisted of a TSP P4000 quaternary pump, a TSP AS3000 autosampler, a UV4000 Diode Array Detector, and a single quadrupole mass spectrometer. The interface between the liquid chromatograph and the mass spectrometer could be interchanged between ESI, electrospray ionisation, and APCI, atmospheric pressure chemical ionisation. The probe temperature was set at 300 °C. The mobile phase consisted of 50:50 acetonitrile:deionised water mixture, run isocratically at 0.15 mL/min.

### *4.8.6 Optimisation of Skimmer Cone Voltage*

100μL of a 1 μg/mL solution was blown down under nitrogen at 35°C and reconstituted in 100μL of mobile phase, this was done for each drug individually. Two methods were set up using full scan analysis and scanning at 4 different skimmer cone voltages, duplicate injections were carried out on both. The first used 25, 30, 35, and 40 V and the second used 45, 50, 55 and 60 V. The UV detector was also used at 242nm. The UV response could be used as an internal standard, and account for inconsistent injection volumes. The ratio between the pseudo-molecular ion and the UV response was calculated at each skimmer cone voltage. The optimum was found for each analyte and this was the value used in SIM experiments.

The following skimmer cone values shown in Table 4-2 were found to be optimum for diazepam and its metabolites.

**Table 4-2 : Optimum skimmer cone values for diazepam and metabolites**

| Analyte | Optimum Skimmer Cone Value (V) |
|---|---|
| Diazepam | 40 |
| N-Desmethyldiazepam | 35 |
| Temazepam | 25 |
| Oxazepam | 30 |

These values were used to get the best sensitivity for the analytes; it is a compromise between separating the analyte from any adducts and not fragmenting the pseudo molecular ion too much. It was found that temazepam fragments readily to a daughter ion of m/z = 255, this could be seen starting to form at 35 V. Figure 4-2 shows the decrease in m/z = 301 ion and the increase in m/z = 255 ion with increasing skimmer cone voltages.

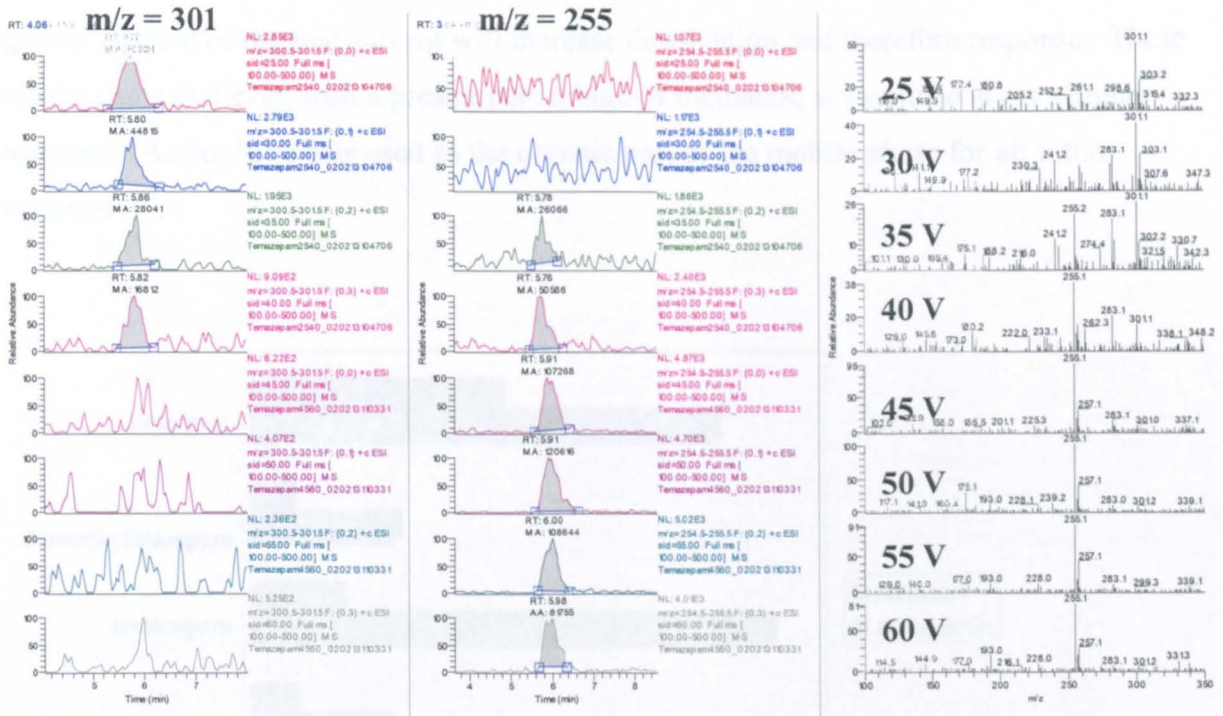



**Figure 4-2 : Effect of increasing skimmer cone voltage from 25 V to 60 V on temazepam mass spectra**

### 4.8.7 Methanol vs. Acetonitrile in the Mobile Phase

To assess the effect of methanol compared to acetonitrile in the mobile phase, two chromatographic conditions were used. Two 100 ng unextracted standards were prepared for each analyte, with no deuterated standards present. The UV detector was used in tandem with the mass spectrometer at 242 nm. The peak for the analyte observed with the UV detector was used as an internal standard. The two chromatographic conditions were (A) methanol : deionised water (70:30) and (B) acetonitrile : water (50:50), both isocratically for 10 minutes at a flow rate of 0.2 mL/min. Each analyte was injected individually on each system, and the ratios between the molecular ion and UV response calculated. The results are shown graphically in Figure 4-3.

It s clear from these results that using acetonitrile : water (50:50) as the mobile phase gives a higher response for all four analytes than using methanol : water (70:30). Although the total organic solvent content in each condition is different, it is generally thought that a greater amount of organic solvent will increase desolvation and therefore response. These results show that even with a greater percentage of methanol, acetonitrile gives a higher response. Acetonitrile was used as the organic part of the mobile phase for all future analyses.

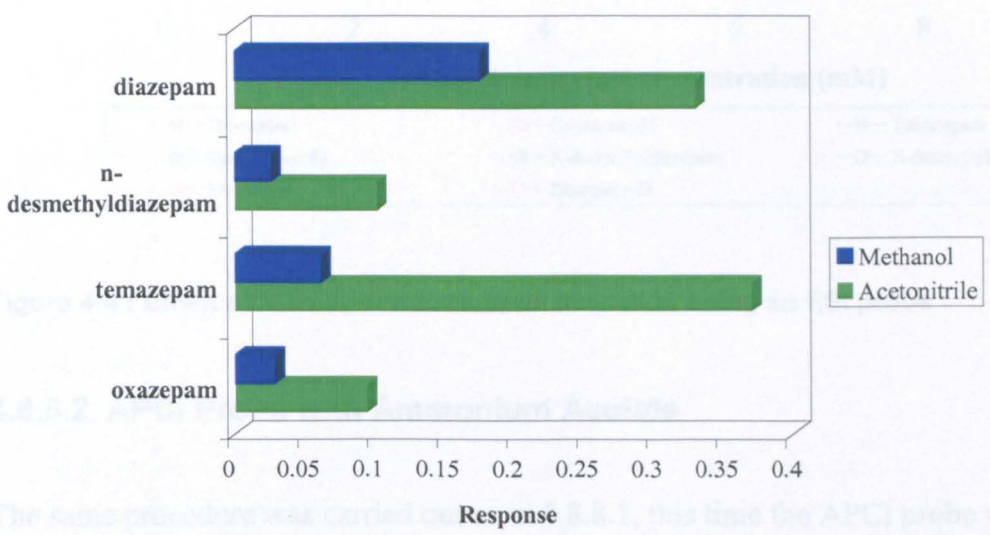



**Figure 4-3 : Difference in response of analytes using methanol and acetonitrile in the mobile phase**

## 4.8.8 Investigation into Ammonium Acetate and Formic Acid as Additives in the Mobile Phase

### 4.8.8.1 ESI Probe with Ammonium Acetate

A standard was prepared by adding 100 µL of 1 µg/mL solution to a vial and blowing down under nitrogen to dryness. This was reconstituted in 200 µL of mobile phase and 10 µL was injected 6 times onto the LC-MS. An average peak area was calculated for each ion under each mobile phase composition. The mobile phase composition was 50:50 acetonitrile : aqueous buffer. Initially a mobile phase composition of 50:50 acetonitrile : deionised water was used. The deionised water was substituted for increasing amounts of ammonium acetate, specifically 5 and 10 mM. The results are shown in the Figure 4-4.

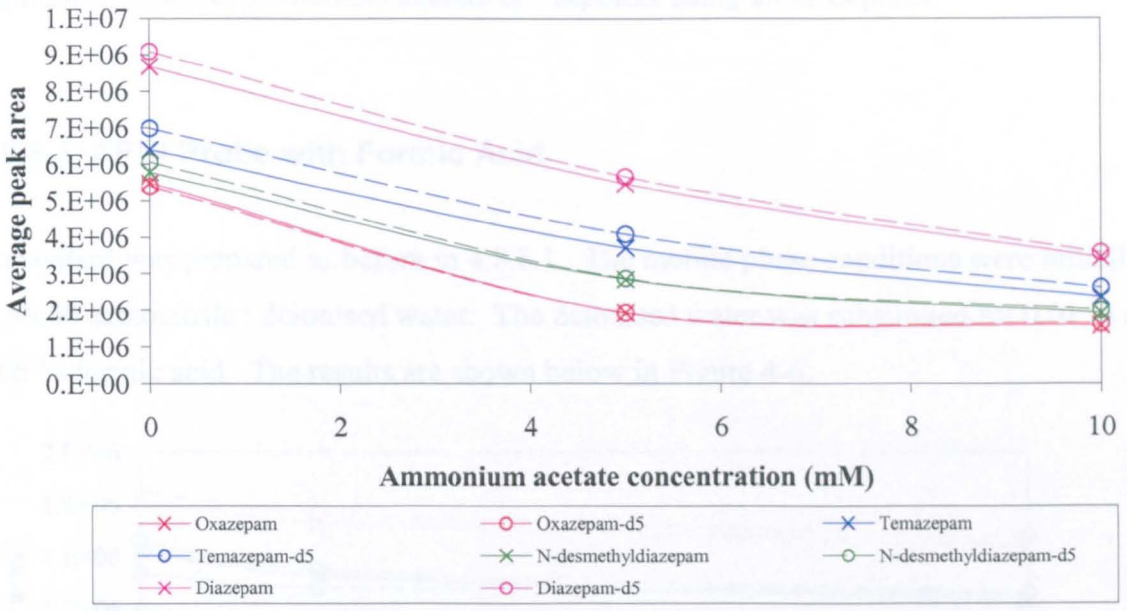



**Figure 4-4 : Effect of ammonium acetate on response using an ESI probe**

### 4.8.8.2 APCI Probe with Ammonium Acetate

The same procedure was carried out as in 4.8.8.1, this time the APCI probe was used instead of ESI. Additives are routinely used in APCI to transfer charge between the probe and the analyte. The results are shown in Figure 4-5.

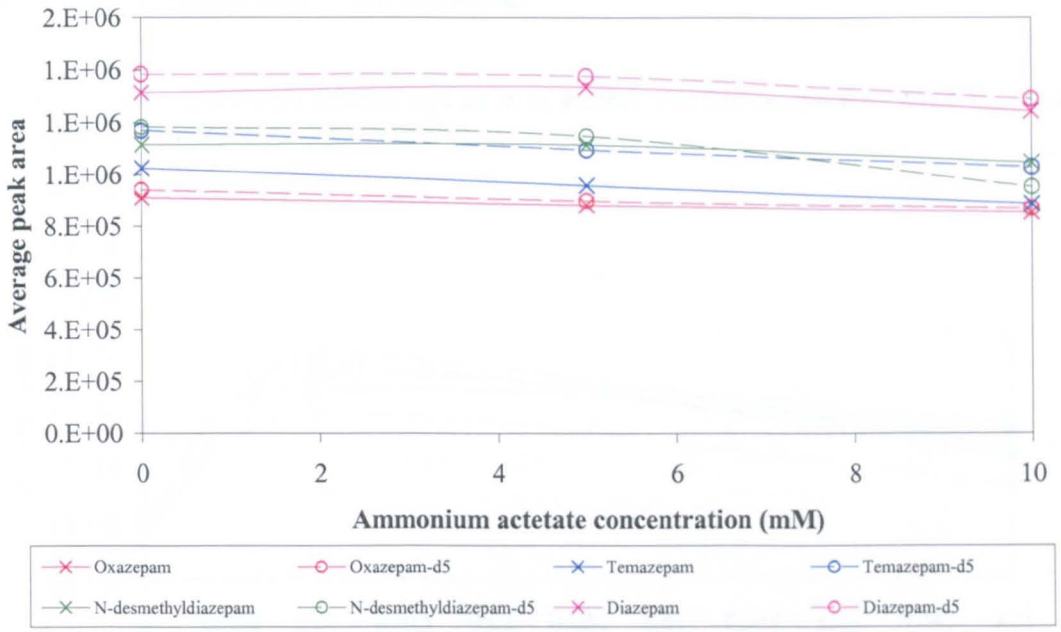



**Figure 4-5 : Effect of ammonium acetate on response using an APCI probe**

### 4.8.8.3 APCI Probe with Formic Acid

A standard was prepared as before in 4.8.8.1. The mobile phase conditions were initially at 50:50 acetonitrile : deionised water. The deionised water was substituted for 0.01 % and 0.05 % formic acid. The results are shown below in Figure 4-6.

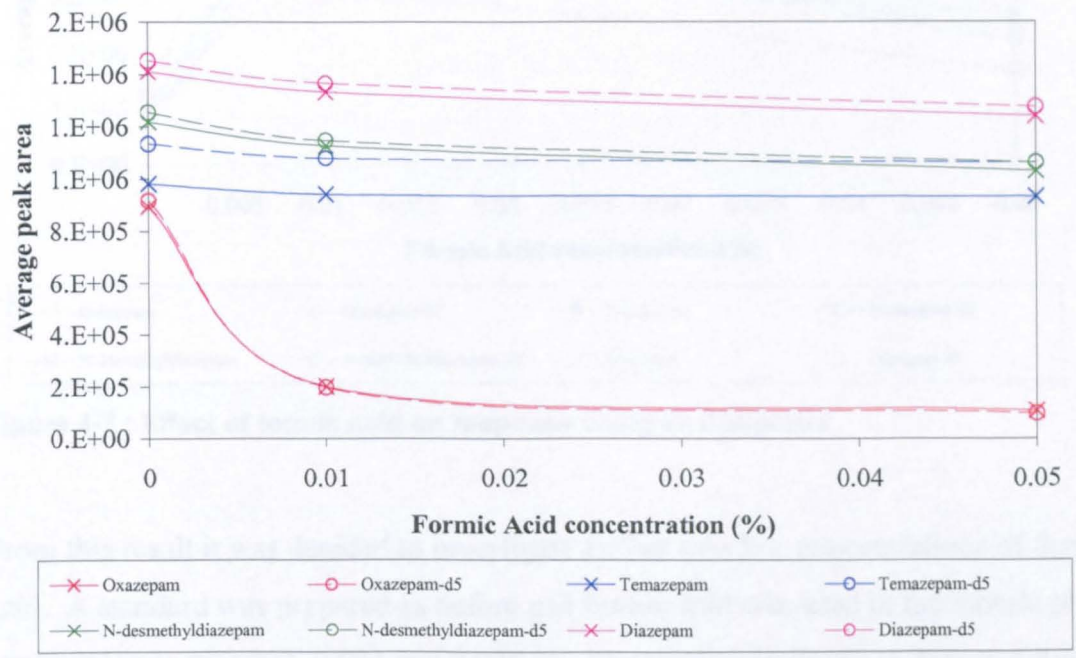



**Figure 4-6 : Effect of formic acid on response using an APCI probe**

### 4.8.8.4 ESI Probe with Formic Acid

**The same procedure was carried out as in 0, except the ESI probe was used instead of the APCI probe. The results are shown in**

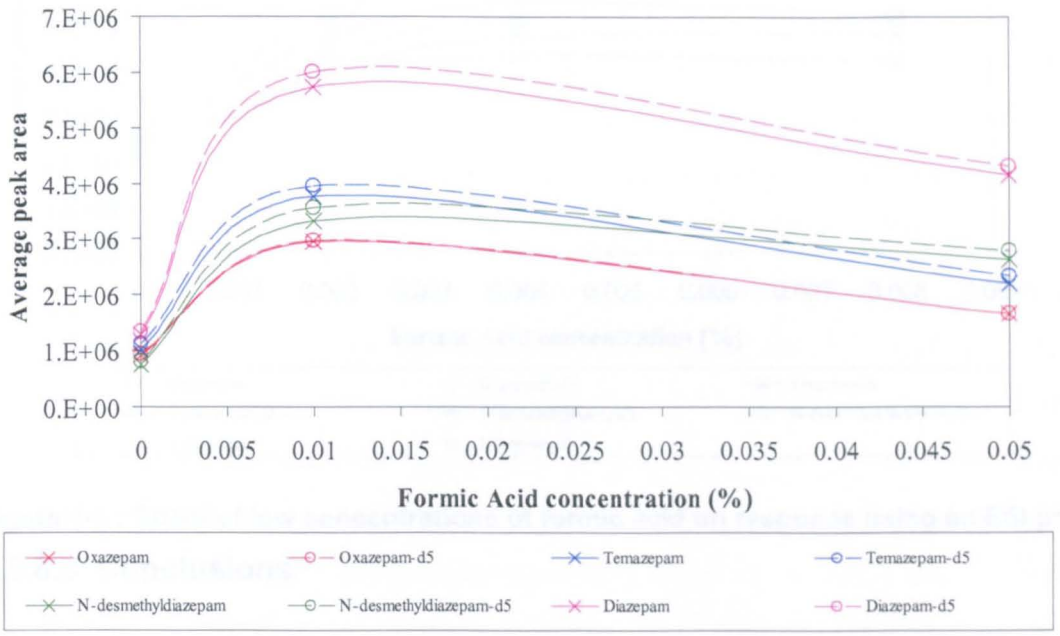



Figure 4-7 below.

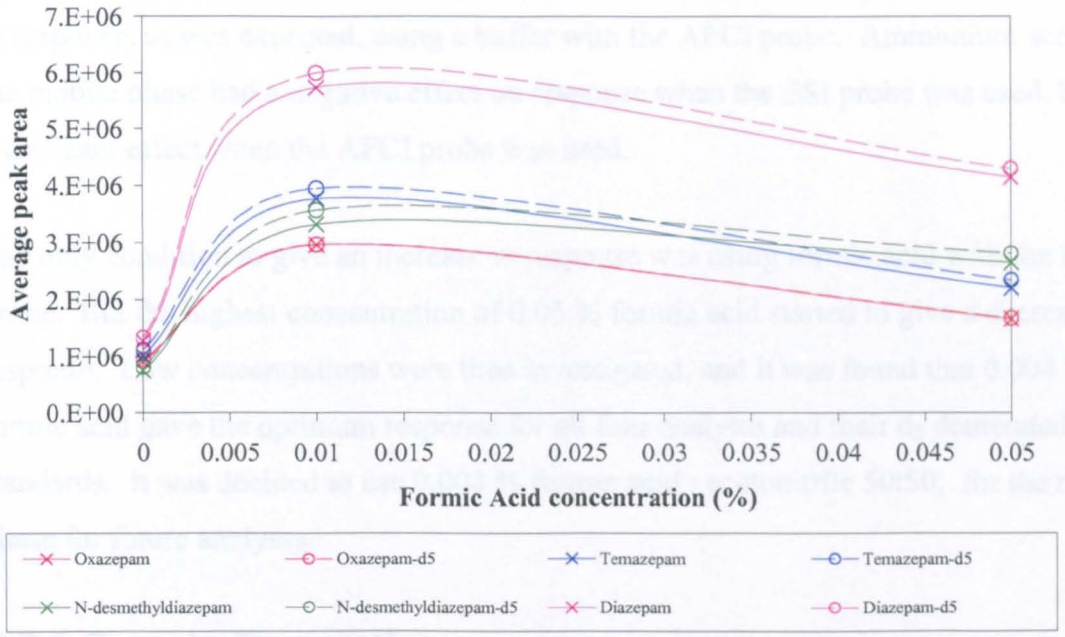



**Figure 4-7 : Effect of formic acid on response using an ESI probe**

From this result it was decided to investigate further into low concentrations of formic acid. A standard was prepared as before and formic acid was used in the mobile phase at concentrations of 0.002, 0.004 and 0.008 %. The results are shown in Figure 4-8 below.

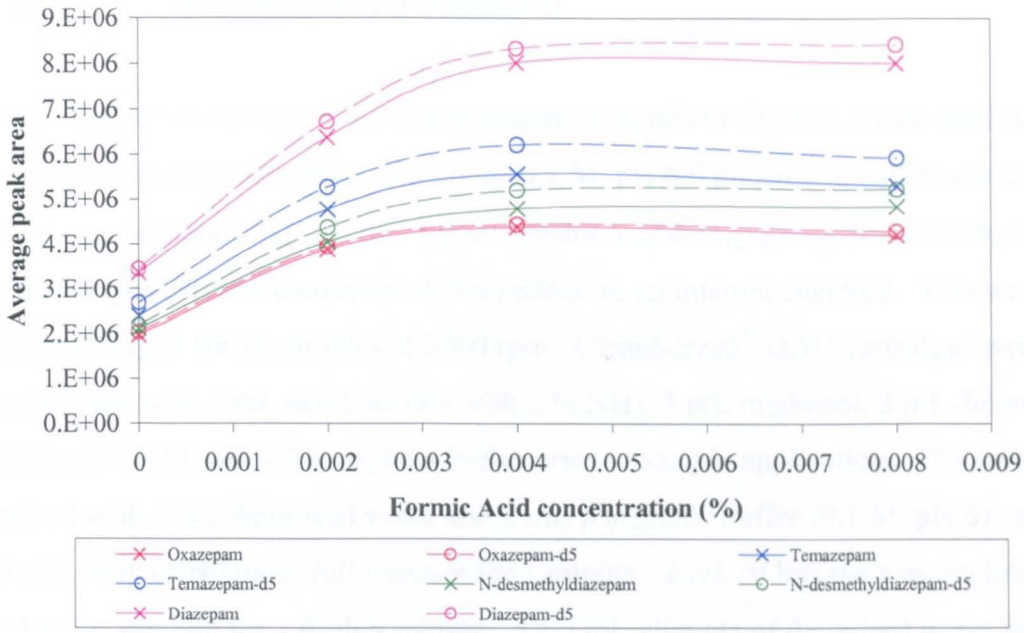



**Figure 4-8 : Effect of low concentrations of formic acid on response using an ESI probe**

### 4.8.8.5 Conclusions

From the results it is clear that using both additives with the APCI probe either produced a detrimental effect on the response of all analytes or no effect at all. There was no increase in response, as was expected, using a buffer with the APCI probe. Ammonium acetate in the mobile phase had a negative effect on response when the ESI probe was used, but no significant effect when the APCI probe was used.

The only condition to give an increase in response was using formic acid with the ESI probe. But the highest concentration of 0.05 % formic acid started to give a decrease in response. Low concentrations were then investigated, and it was found that 0.004 % formic acid gave the optimum response for all four analytes and their $d_5$ deuterated standards. It was decided to use 0.004 % formic acid : acetonitrile 50:50, for the mobile phase for future analyses.

## *4.8.9 Sample Preparation*

Spiked blood samples were prepared by adding the appropriate amount of working drug solution (1, 2, 5, 10, 25, 50, 100 µL) and internal standard solution (100 µL) to test tubes, followed by 3.5 mL of 0.1 M, pH 6 phosphate buffer and 1 mL of blank blood. A blank was prepared without any standards present and another with internal standard only to assess any contamination of the deuterated standards.

### 4.8.10 Extraction Procedure

The extraction method used for flunitrazepam analysis in Chapter 3 was employed for diazepam and its metabolites. 3.5 mL of 0.1 M, pH 6.0 phosphate buffer was added to 1 mL of whole blood. 100 μL of 1 μg/mL solution of diazepam-$d_5$, N-desmethyldiazepam-$d_5$, temazepam-$d_5$ and oxazepam-$d_5$ was added as an internal standard. This was vortexed and centrifuged for 10 minutes at 3000 rpm. CleanScreen® DAU cartridges were conditioned with 1 mL ethyl acetate with 2 % $NH_3$, 3 mL methanol, 3 mL deionised water and 2 mL 0.1 M, pH 6.0 phosphate buffer prior to sample application. The cartridges were washed with 2 mL deionised water and 2 mL phosphate buffer (0.1 M, pH 6) : acetonitrile 80 : 20, then dried under full vacuum for 1 minute. 2 mL of hexane was applied and dried under full vacuum for a further minute. 3 x 2 mL aliquots of deionised water were applied to the cartridges drying under full vacuum for 1 minute between each addition of water. After the third aliquot of water the cartridges were left at full vacuum for 10 minutes to dry thoroughly. The analytes were then eluted using 3 mL ethyl acetate with 2 % $NH_3$. This was blown down under nitrogen at 30 °C until dry. Samples were reconstituted in 100 μL of mobile phase (50 : 50 acetonitrile : 0.004 % formic acid), with 20 μL injected in duplicate.

## 4.9 Method Validation

For qualitative information about diazepam and its metabolites, the two pseudo-molecular ions were monitored $^{35}Cl$ and $^{37}Cl$. This combined with the relative retention time, with respect to the relevant deuterated standard, was used for positive identification of each analyte.

### 4.9.1 Linearity

Diazepam, N-desmethyldiazepam, temazepam and oxazepam were found to have a linear response over the concentration range 1-100 ng/mL in whole blood. A 100 ng/mL of whole blood, extracted standard is shown in Figure 4-9.

## *4.9.2 Recovery*

The recovery was assessed at three concentrations, 5, 50 and 100 ng/mL and calculated as in Chapter 3.10.5. The results are displayed in Table 4-3.

**Table 4-3 : Recovery of diazepam and metabolites**

| | **Mean % Recovery (%RSD) (n=5)** | | |
|---|---|---|---|
| **Analyte** | **5 ng/mL** | **50 ng/mL** | **100 ng/mL** |
| Diazepam | 87 (3) | 87 (2) | 87 (3) |
| N-desmethyldiazepam | 81 (4) | 86 (4) | 79 (2) |
| Temazepam | 87 (6) | 85 (5) | 88 (2) |
| Oxazepam | 78 (7) | 84 (3) | 81 (4) |

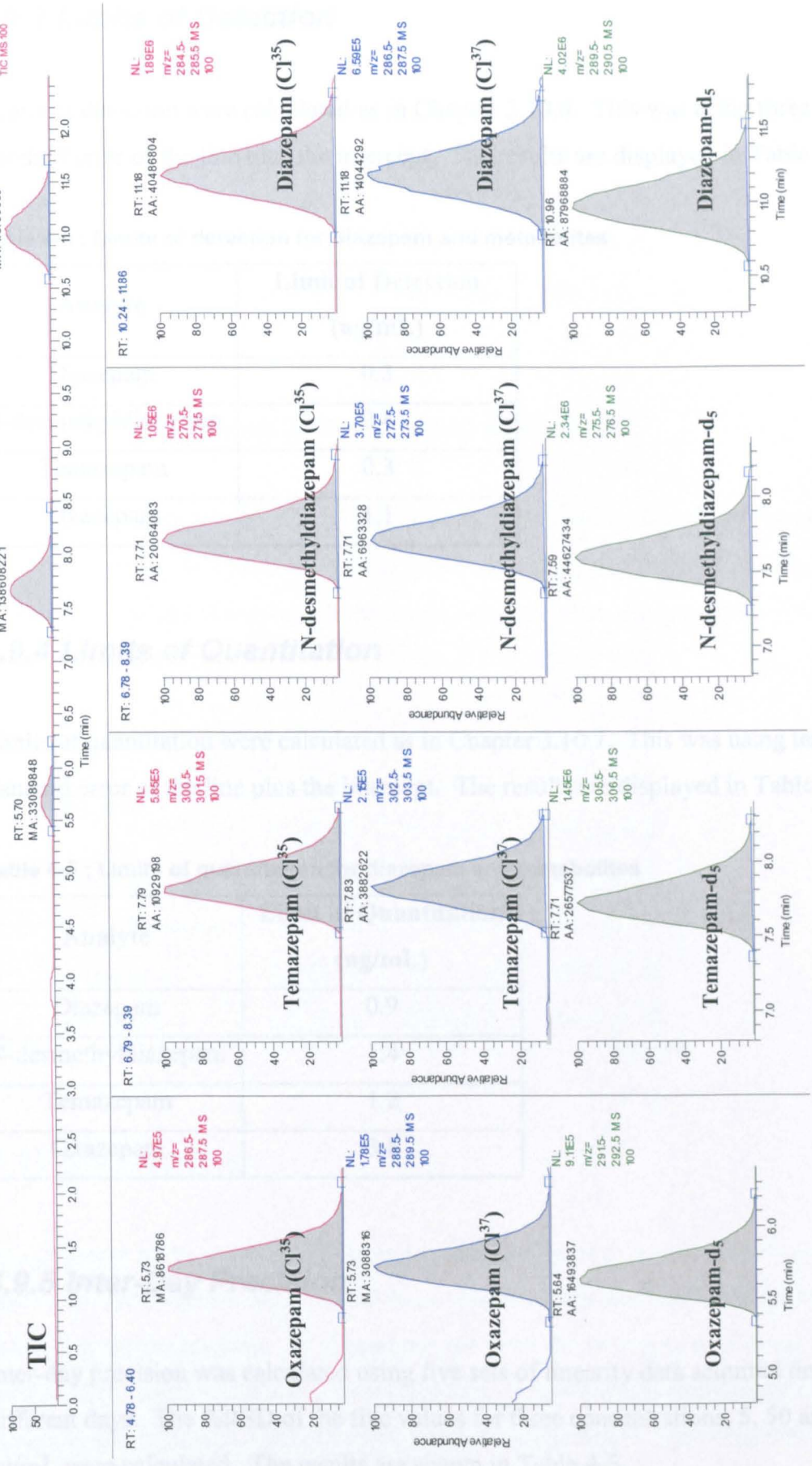



**Figure 4-9 : Example chromatogram of 100 ng of diazepam and its metabolites extracted from 1 mL of whole blood**

### 4.9.3 Limits of Detection

Limits of detection were calculated as in Chapter 3.10.6. This was using three times the standard error of the line plus the intercept. The results are displayed in Table 4-4.

**Table 4-4 : Limits of detection for diazepam and metabolites**

| Analyte | Limit of Detection (ng/mL) |
|---|---|
| Diazepam | 0.3 |
| N-desmethyldiazepam | 0.4 |
| Temazepam | 0.3 |
| Oxazepam | 1.1 |

### 4.9.4 Limits of Quantitation

Limits of quantitation were calculated as in Chapter 3.10.7. This was using ten times the standard error of the line plus the intercept. The results are displayed in Table 4-5.

**Table 4-5 : Limits of quantitation for diazepam and metabolites**

| Analyte | Limit of Quantitation (ng/mL) |
|---|---|
| Diazepam | 0.9 |
| N-desmethyldiazepam | 1.4 |
| Temazepam | 1.2 |
| Oxazepam | 3.8 |

### 4.9.5 Inter-Day Precision

Inter-day precision was calculated using five sets of linearity data acquired on five different days. The %RSD of the five values for three concentrations, 5, 50 and 100 ng/mL were calculated. The results are shown in Table 4-6.

**Table 4-6 : Inter-day precision of diazepam and its metabolites**

| Analyte | Inter-Day Precision (%RSD) (n=5) | | |
|---|---|---|---|
| | 5 ng/mL | 50 ng/mL | 100 ng/mL |
| Diazepam | 5.1 (8.6) | 49.1 (1.7) | 100.2 (1.0) |
| N-desmethyldiazepam | 4.9 (7.6) | 50.2 (2.2) | 99.3 (0.9) |
| Temazepam | 4.9 (10.1) | 49.4 (2.9) | 99.9 (0.8) |
| Oxazepam | 4.8 (9.2) | 49.3 (2.0) | 100.3 (0.7) |

### *4.9.6 Intra-Day Precision between Injections*

Extracted standards were prepared at three concentrations, 5, 50 and 100 ng/mL. They were reconstituted in 150 µL of mobile phase and 20 µL was injected 5 times. The %RSD of the peak area ratios was calculated at each concentration for each drug. The results are shown in Table 4-7.

**Table 4-7 : Intra-day precision of diazepam and its metabolites**

| Analyte | Intra-Day Precision (%RSD) (n=5) | | |
|---|---|---|---|
| | 5 ng/mL | 50 ng/mL | 100 ng/mL |
| Diazepam | 4.9 (4.0) | 49.5 (1.7) | 99.5 (1.9) |
| N-desmethyldiazepam | 4.7 (11.6) | 51.3 (2.6) | 99.4 (1.9) |
| Temazepam | 4.6 (7.0) | 49.7 (1.5) | 100.5 (1.4) |
| Oxazepam | 4.5 (5.8) | 48.0 (5.7) | 101.0 (1.0) |

## 4.10 Discussion

The optimum response of diazepam and its metabolites was found using 0.004% formic acid in the mobile phase which is the same concentration that was found be the optimum for flunitrazepam analysis. As all analytes are from the same group of drugs, benzodiazepines, this is not wholly unexpected. The difference in response between methanol and acetonitrile in the mobile phase was significant, indicating that both the organic solvent and the aqueous buffer are capable of affecting the ionisation of an analyte.

The validated method shows good linearity throughout the range (1-100 ng/mL) and detection limits are all less than 1.5 ng/mL. Recoveries greater than 78 % were observed for all analytes at low, medium and high concentrations and intra and inter day precision was less than 2 % at 100 ng/mL. As diazepam and its metabolites have chlorine in their molecules, two pseudo-molecular ions are produced in the mass spectrometer one with chlorine$^{35}$ and one with chlorine$^{37}$ at a ratio of 3:1. The second molecular ion produced can be used as a qualifier ion, and the ratio between the two ions should be constant. This is further qualitative information ensuring reliable results.

## 4.11 Conclusions

A method has been developed and validated for the extraction of diazepam, N-desmethyldiazepam, temazepam and oxazepam from whole blood, using solid phase extraction and liquid chromatography-mass spectrometry for analysis.

# 5 Sildenafil in Whole Blood

## 5.1 Introduction


Although originally marketed as an anti hypertensive agent, sildenafil citrate has been used for the treatment of erectile dysfunction, since its approval by the U.S. Food and Drug Administration (FDA) in March 1998. Since then the drug has been widely available and used recreationally. A study showed that 2% of dance music enthusiasts in the UK, with an average age of 28 years, had used sildenafil recreationally[66].

## 5.2 Chemistry

The molecular structure of sildenafil is shown in Figure 5-1. Sildenafil is chemically known as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine citrate. It has a molecular weight of 474 amu, producing a pseudo-molecular ion of m/z = 475 in the LC-MS source.

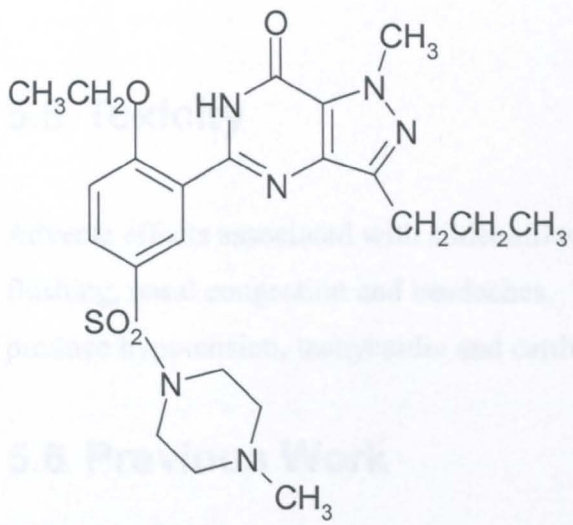



**Figure 5-1 : Molecular structure of sildenafil**

## 5.3 Metabolism and Excretion

Metabolism of sildenafil is mainly through biotransformation; with 80 % of these products excreted in the faeces. N-desmethylsildenafil is a major plasma metabolite and has half the pharmacological activity of its parent drug[31].

## 5.4 Pharmacokinetics

One study found peak plasma concentrations of sildenafil occur between 0.5 and 2.5 hours after oral administration. Peak plasma concentrations of 159 ng/mL were found with sildenafil barely detected at 24 hours (0.8 ng/mL).[67] Pharmacokinetic data for sildenafil is summarised in Table 5-1[31].

**Table 5-1 : Pharmacokinetic and physiochemical data for sildenafil**

| | Sildenafil |
|---|---|
| **Half Life (hours)** | 1.3-3.4 |
| **Vd (L/Kg)** | 1.0-1.5 |
| **Protein Binding (%)** | 96 |
| **pKa** | 8.7 |

## 5.5 Toxicity

Adverse effects associated with sildenafil tend to be transient and moderate; they include flushing, nasal congestion and headaches. When taken in overdose amounts sildenafil may produce hypotension, tachycardia and cardiac arrest[31].

## 5.6 Previous Work

Several LC-MS methods have been published, most using tandem mass spectrometry, which is not always available[68,69,70]. One group[71] reported the use of a single quadrupole LC-MS instrument using liquid-liquid extraction. Presented here is a simple, fast, specific method using solid phase extraction and a single quadrupole mass spectrometer for the determination of sildenafil in whole blood.

## 5.7 Aim

A post-mortem blood sample was submitted to the laboratory for sildenafil analysis. The aim was to develop a method for the detection and quantitation of sildenafil citrate, the active drug in Viagra®, in whole blood.

## 5.8 Method Development

After some initial experiments with mobile phase composition and flow rates the following conditions were found sufficient for analysis. Separation was performed on a Phenomenex Luna C18(2) 150 x 2.0 mm i.d. with 3 μm packing, kept at 35 °C. A guard column with identical packing material was used to prolong the column life. Mobile phase comprised (A) 10 mM ammonium bicarbonate and (B) acetonitrile, with the gradient shown in Table 5-2

**Table 5-2 : Mobile phase gradient for sildenafil analysis**

| Time (min) | Flow (mL/min) | A (%) | B (%) |
|---|---|---|---|
| 0.0 | 0.3 | 50 | 50 |
| 5.0 | 0.3 | 90 | 10 |
| 5.1 | 0.5 | 90 | 10 |
| 7.0 | 0.5 | 90 | 10 |
| 7.1 | 0.3 | 50 | 50 |
| 12.0 | 0.3 | 50 | 50 |

### *5.8.1 Chemicals*

Methanol, acetonitrile and hexane were HPLC grade and obtained from VWR International (UK). Analytical grade potassium dihydrogen phosphate, ammonium acetate and ammonium bicarbonate were supplied by BDH laboratory supplies (Poole, England). Analytical grade concentrated ammonia, glacial acetic acid and concentrated formic acid were purchased from Sigma® Chemicals Co. (UK).

### *5.8.2 Standards*

A stock standard of sildenafil was prepared by weighing 2.43 mg of sildenafil citrate and dissolving this in 1.729 mL of methanol, this gave a solution of 1 mg/mL free sildenafil. A

second solution of 100 μg/mL sildenafil was prepared by taking 500 μL of 1 mg/mL stock solution and adding 4500 μL of methanol. This was then diluted down further taking 50 μL of 100 μg/mL solution and adding 4950 μL of methanol producing a 1 μg/mL working solution. Cocaine-$d_3$ was purchased from Radian International as a 100 μg/mL methanolic solution. A working internal standard solution was prepared by taking 50 μL of 100 μg/mL solution and adding 4950 μL of methanol.

## *5.8.3 Blank Blood*

Time expired packed red blood cells were provided from the blood bank for blank blood. These were suspended in a ratio of 1:1 with isotonic saline solution which was made by dissolving 9.5 g of sodium chloride in 1 L of deionised water.

## *5.8.4 Solutions*

### 5.8.4.1 Preparation of 10 mM Ammonium Bicarbonate Buffer

0.7906 g of ammonium bicarbonate was weighed into a 1 L volumetric flask and made up to volume with deionised water, resulting in a 10 mM solution.

### 5.8.4.2 Preparation of 0.1 M, pH 6.0 Phosphate buffer

6.81 g of potassium dihydrogen phosphate was weighed out into a 500 mL volumetric flask and 450 mL of deionised water was added. The pH was adjusted to 6.0 with 1 M potassium hydroxide solution, then made up to 500 mL with deionised water.

### 5.8.4.3 Preparation of 0.01M Acetic Acid

57 μL of glacial acetic acid was placed in a 100 mL volumetric flask this was made up to volume with deionised water, resulting in a 0.01 M solution.

## *5.8.5 Optimisation of Skimmer Cone Voltage*

In-source collision or cone voltage fragmentation can be used in the LC-MS source to provide more structural information without the aid of tandem MS. The major difference with in-source fragmentation is that it is indiscriminate and any substance present in the

source will have the voltage applied. This can give complicated spectra, but it can also give a daughter ion for qualitative purposes. The optimum skimmer cone voltage was found for the parent and daughter ions. A standard was prepared for injection by adding 100 µL of 1 µg/mL sildenafil solution to a vial, this was blown down under nitrogen at 35°C and reconstituted in 200 µL of mobile phase. 20 µL was injected onto the LC-MS in duplicate for each condition. In an injection the skimmer cone voltage can be set at 4 different levels for each ion monitored. Table 5-3 displays the voltages set for each injection.

**Table 5-3 : Skimmer cone voltages used to optimise sildenafil response**

| Injection | Skimmer cone voltages (V) |
|---|---|
| 1,2 | 5, 10, 15, 20 |
| 3,4 | 25, 30, 35, 40 |
| 5,6 | 45, 50, 55, 60 |
| 7,8 | 65, 70, 75, 80 |

As the skimmer cone voltage is increased the molecular ion response increases until it starts to fragments. At this point the response of the molecular ion is at its optimum. By increasing the voltage further the daughter ion increases in response until it starts to fragment further. This second voltage is used for optimum response of the daughter ion. Figure 5-3 shows the difference in peak area and spectra produced with increasing the skimmer cone voltage.

The final results showed that 30 V was optimum for the pseudo molecular ion m/z = 475 and 60 V was optimum for the daughter ion m/z = 283. The proposed fragmentation is shown in Figure 5-2.

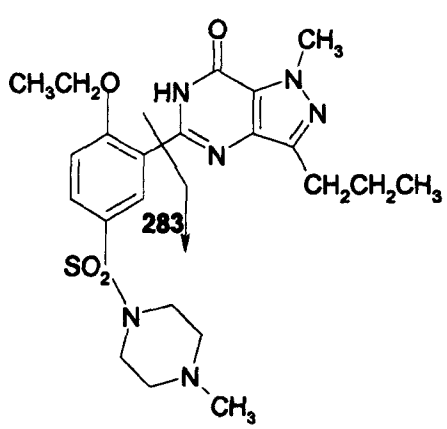



**Figure 5-2 : Proposed fragmentation of sildenafil in the source**

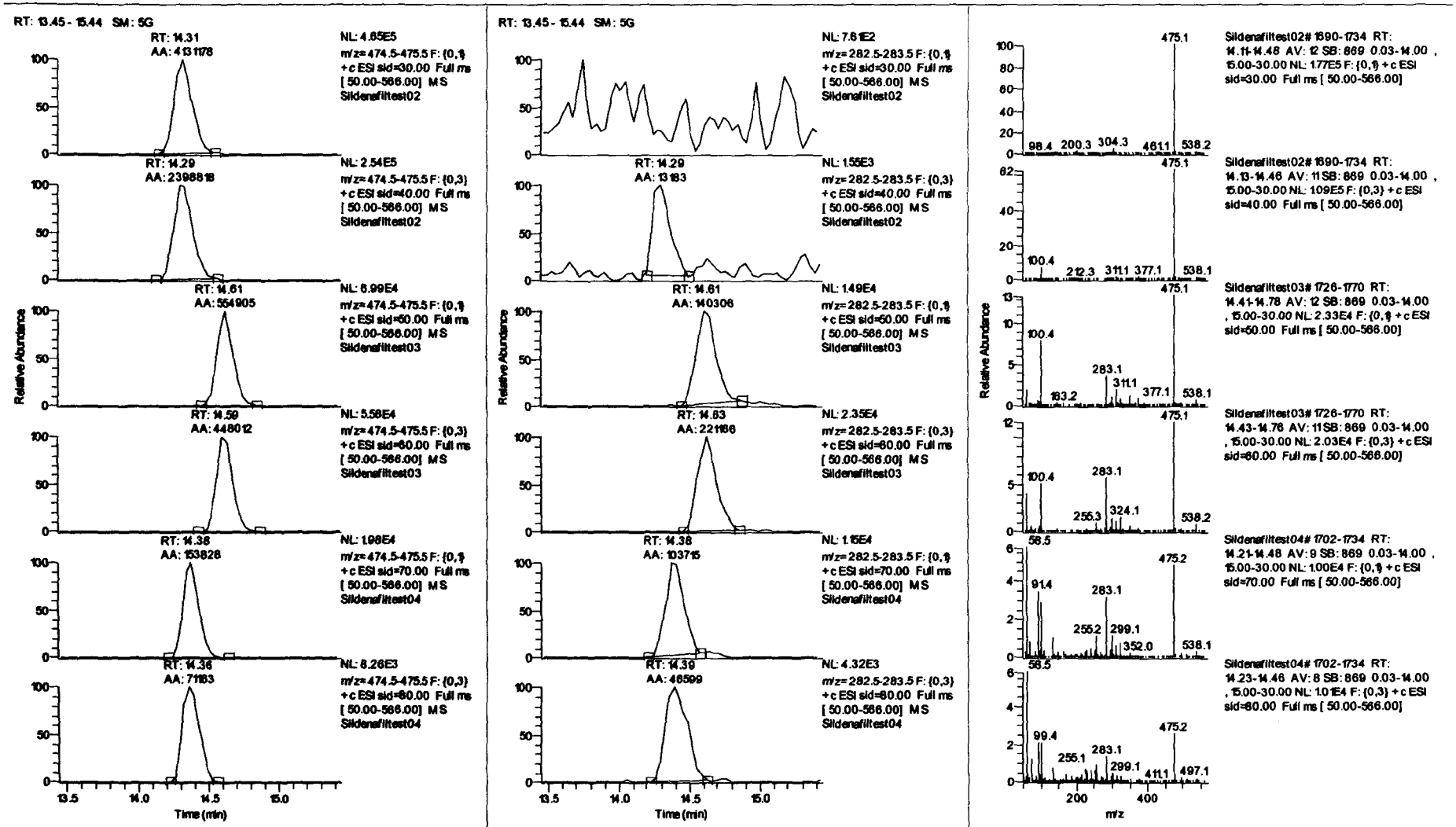



**Figure 5-3 : Variation in sildenafils spectra with increasing skimmer cone voltage**

# 5.9 Method Validation

## 5.9.1 Sample Preparation

Spiked blood samples were prepared by adding the appropriate amount of working drug solution (1, 2, 5, 10, 25, 50, 100, 200, 500 μL) and internal standard solution (200 μL) to test tubes, followed by 3.5 mL of 0.1 M, pH 6 phosphate buffer and 1 mL of blank blood. A blank was prepared without any standards present and another with internal standard only to assess any contamination of the deuterated standards.

## 5.9.2 Extraction Procedure

The extraction method employed was based on a solid phase extraction method used for extracting acidic and basic drugs simultaneously[72]. To 1 mL of whole blood, 3.5 mL of 0.1 M, pH 6.0 phosphate buffer and 200 μL of internal standard was added, vortexed and centrifuged at 3000 rpm for 10 minutes. Bond Elut Certify® LRC cartridges were condition with 2 mL of methanol followed by 2 mL of phosphate buffer (0.1 M, pH 6). The sample was applied and allowed to drip through with no vacuum applied. To wash the cartridges 1 mL of deionised water followed by 0.5 mL of 0.01 M acetic acid was used. The cartridges were dried for 10 minutes with full vacuum applied, before adding 50 μL of methanol and drying for a further 2 minutes. 4 mL of acetone : chloroform, 1:1, was applied; this was used as a clean-up step and was not collected. Elution was achieved using 2 mL of ethyl acetate with 2 % ammonia. The sample was blown down to dryness under nitrogen at 30 °C. 60 μL of mobile phase, acetonitrile and 10 mM ammonium bicarbonate (50:50), was used to reconstitute the sample, before injecting 20 μL onto the LC-MS system. Duplicate injections of each extract were made.

## 5.9.3 Linearity

Linearity of response for sildenafil was demonstrated over a wide dynamic range 1-500 ng/mL. This is shown graphically in Figure 5-4. Although plotting the daughter ions response with concentration only gives an $R^2$ value of 0.9864, this ion would not be used for quantitation but rather qualitative purposes. Its linearity shows that the fragmentation is consistent and that the ratio between the pseudo molecular ion and the daughter ion should be stable.

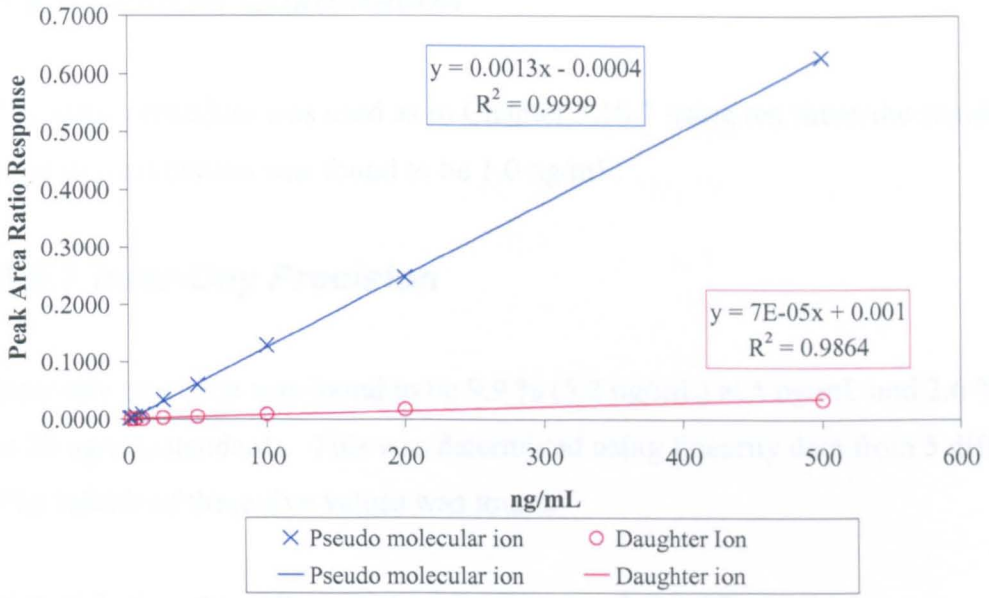



**Figure 5-4 : Linearity of sildenafil**

## 5.9.4 Recovery

The Bond Elut Certify® extraction method gave a mean recovery of 60 % for sildenafil. Recovery was calculated at 3 different concentrations 5, 50 and 500 ng/mL extracted 5 times refer to Chapter 3.10.5 for calculation. The mean recovery found at each concentration is shown in Table 5-4.

**Table 5-4 : Recovery of sildenafil**

| Concentration (ng/mL) | Sildenafil (n=5) Mean % Recovery (%RSD) |
|---|---|
| 5 | 61 (13) |
| 50 | 57 (17) |
| 500 | 63 (11) |

## 5.9.5 Limit of Detection

Limits of detection were calculated as in Chapter 3.10.6. This was using three times the standard error of the line plus the intercept. The limit of detection for sildenafil was found to be 0.3 ng/mL.

### 5.9.6 Limit of Quantitation

The same procedure was used as in Chapter 3.10.7 using ten times the standard error. The limit of quantitation was found to be 1.0 ng/mL.

### 5.9.7 Inter-Day Precision

Inter-day precision was found to be 9.9 % (5.2 ng/mL) at 5 ng/mL and 2.6 % (49.2 ng/mL) at 50 ng/mL standards. This was determined using linearity data from 5 different days. The %RSD of these five values was found.

### 5.9.8 Intra-Day Precision between Injections

Intra-day precision was found by injecting spiked standards 5 times into the system and calculating the %RSD, which was found to be 7.2 % (6.9 ng/mL) at 5 ng/mL and 4.0 % (47.5 ng/mL) at 50 ng/mL standards.

## 5.10 Discussion

Most LC-MS methods using single quadrupole instruments suffer from limited mass spectral data. This is due to the type of ionisation used in LC-MS. Atmospheric pressure ionisation techniques are termed soft ionisation, as they gently ionise the molecule with little fragmentation. This is very different to the ionisation used in GC-MS, where electron impact is used, termed hard ionisation. More energy is applied which breaks the molecule down into several fragments, and a complex spectrum is produced, with various ions to choose from to identify and quantify with. A specific daughter ion for sildenafil was found by alternating between a low and high skimmer cone voltage to produce the parent and daughter ion respectively. The fragmentation was also found to be consistent between concentrations resulting in a ratio between the parent and daughter ion that could be used as another qualitative piece of data.

## 5.11 Conclusions

This method demonstrates that the use of a single quadrupole LC-MS is sufficient to identify and quantify sildenafil in whole blood. The method was applied to a post-mortem whole blood sample, refer to Chapter 10.1.

# 6 Oral Fluid

## 6.1 Introduction

Oral fluid was first used almost 30 years ago for therapeutic drug monitoring[2]. It has since been evaluated for use in the forensic field, recognising its advantages over other matrices[73]. It is a non-invasive sampling technique that does not require a medical practitioner to perform. Thus, a police officer or researcher is able to obtain a sample for analysis. Unlike urine sampling there is little chance of adulteration, and it is thought to be more acceptable to donate oral fluid than blood or urine. In recent years there has been great interest in the use of saliva testing at the roadside to identify potentially impaired drivers.

Oral fluid is the name given to the mixture of fluids collected in the oral cavity and is made up of several components, including saliva, oral mucosal transudate, epithelial cells, food debris and oral micro-organisms. Saliva is defined as the fluid produced by the salivary glands in the mouth. Oral mucosal transudate can be defined as a serum derived fluid that enters saliva from the gingival crevice and across oral mucosal surfaces[74] or fluid that comes from the area between the teeth and gums[75].

In the course of this study two types of oral fluid collection devices were used, the modified Omni-Sal® device and the Intercept® device. Each device has a different approach to the collection of oral fluid. The modified Omni-Sal® device collects ~1 mL of oral fluid using a pad without stimulation, shown in Figure 6-1. This pad is placed in the mouth until an indicator turns blue at the end of the stick. This informs the user that 1 mL of oral fluid has been collected. The pad is then placed in a collection tube, which has 2 mL of buffer. Once the sample has been transported to the lab, the pad is separated from the stick, and the resultant mixture filtered. A mixture of buffer:oral fluid 2:1 is then available for analysis. A correction factor (multiply by 3) is applied when quoting a result in ng/mL of oral fluid.

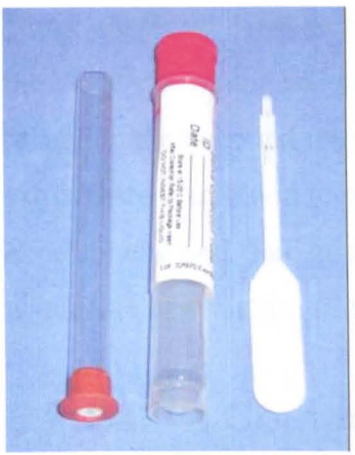


**Figure 6-1 : The modified Omni-Sal® oral fluid collection device**

The Intercept® device collects oral mucosal transudate using a pad with stimulation, shown in Figure 6-2. The pad is impregnated with common salts and gelatin, which stimulates saliva flow. The pad is placed between the lower gum and cheek and left for 2-5 minutes. Once the pad is removed from the mouth it is placed in a collection tube with 0.8 mL of buffer. A group of authors showed that the average volume obtained with the Intercept® device is 0.38 $\pm$ 0.19 (SD) with a range of 0.05 to 0.8 mL[76]. Taking this into account the same correction factor as before is required. The stick is snapped off as the pad is inserted into the tube, and a lid secures the sample for transport. In the laboratory the bottom bit of plastic is snapped off to reveal a hole. The collection tube is placed in a second tube and then placed in the centrifuge at 3000 rpm for 10 minutes. The oral fluid and buffer are separated from the pad into the second tube ready for analysis. All of these matrices include saliva in their composition and therefore the knowledge of the chemistry of saliva is vitally important.

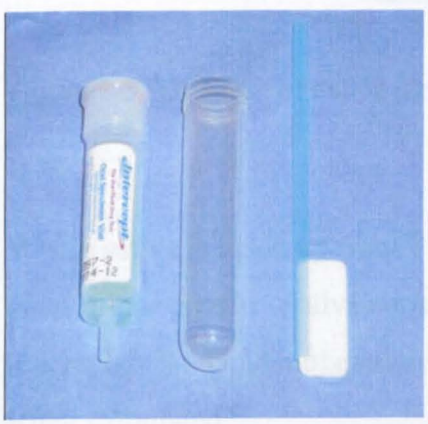


**Figure 6-2 : The Intercept® oral fluid collection device**

Drugs are found in saliva by transportation over a thin layer of epithelial cells separating the blood circulation and saliva duct. This means that the drug concentration in saliva is related to the unbound fraction of drug circulating in the blood. This allows the use of saliva instead of blood to predict current drug use. A study assessed the use of expectorated oral fluid as a means of determining impairment in drivers and found it to be a valuable new matrix for the detection of drugs[77].

## 6.2 Saliva Composition

In the mouth three major paired glands produce saliva. The parotid gland, at the roof of the mouth, produces a watery serous fluid, the sublingual glands, at the sides of the mouth produce a mixture of serous fluid and mucin that makes the saliva sticky, and the submandibular gland produces a mixture like the sublingual gland and is positioned at the base of the tongue.

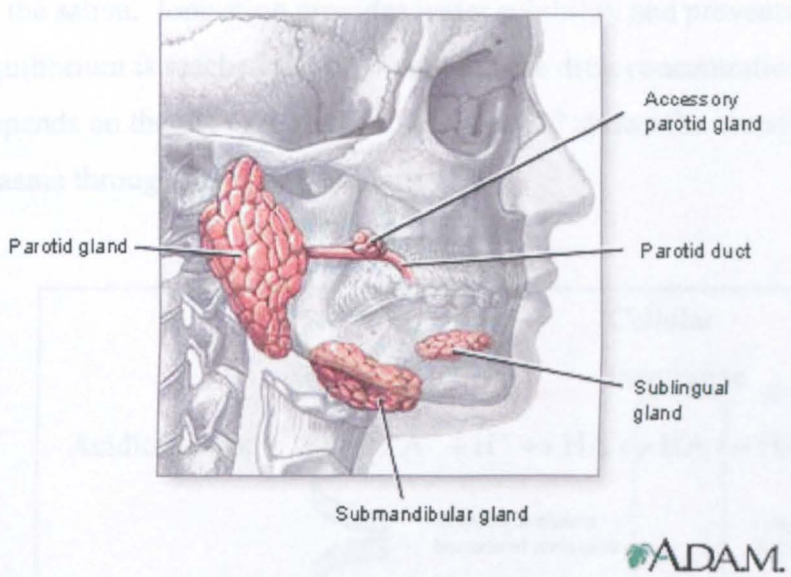



**Figure 6-3 : Diagram of saliva glands in the mouth[78]**

Whole saliva is made up from 71 % submandibular, 25 % parotid and 4 % sublingular and other minor glands. Saliva consists mostly of water, 99 %, some proteins, 0.3 %, and mucin, 0.3 %. Unstimulated saliva has a pH range of 5.6-7.0, but this can increase to 8.0 when stimulated[79].

## 6.3 Drug Transport

Drugs are transported into saliva across a thin layer of epithelial cells that separate the saliva duct from the systemic circulation. The external carotid artery provides the arterial blood, which flows in the opposite direction to the salivary flow within the duct[80]. Drugs can be transported by three ways active transport, diffusion and passive diffusion. Active transport (secretion) involves the movement of very small molecules such as $Li^+$, this can cause a higher concentration in saliva than in plasma. Diffusion occurs through water-filled pores and involves molecules with a molecular weight less than 100 amu e.g. ethanol. Most drugs of interest have molecular weights above 100 amu and are transported via passive diffusion across a concentration gradient. Passive diffusion of drugs occurs in both directions until equilibrium is reached. As the concentration of drugs in plasma is changing with metabolism, passive diffusion is occurring constantly in both directions. Lipoidal biological membranes are not permeable to ionised molecules. Therefore transfer rates of drugs into saliva are determined by their lipid solubility[81]. Drugs need to have some lipophilicity to be able to cross the membrane, but also water solubility to be retained in the saliva. Ionisation provides water solubility and prevents back diffusion. Once equilibrium is reached the ratio between the drug concentration in saliva and in plasma depends on the pH of the saliva. Figure 6-4[79] shows the transfer of drugs from saliva into plasma through the cell membrane.

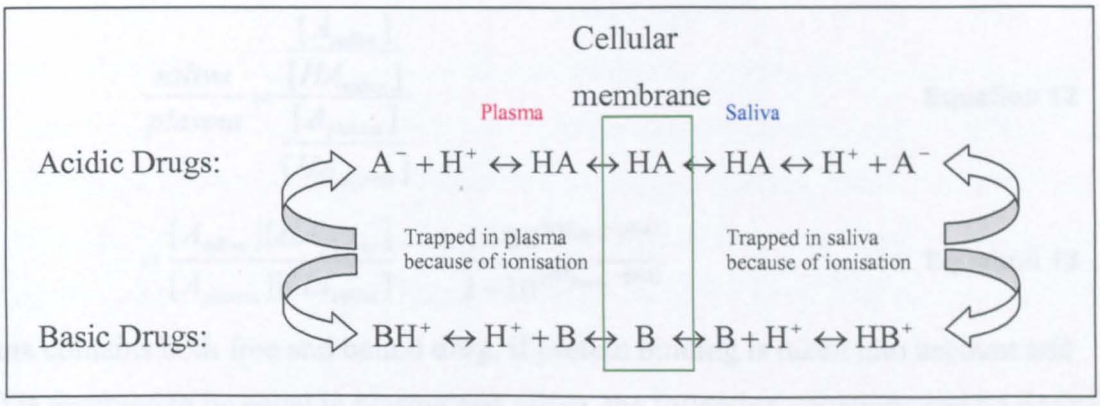



**Figure 6-4 : Drug transport between plasma and saliva**

Saliva/plasma ratios are derived from two equations, the Henderson-Hasselbalch equation, Equation 6, and the equation for mass balance, Equation 7[79].

$$pH = pKa + \log \frac{[A^-]}{[HA]} \qquad \textbf{Equation 6}$$

$$[A] = [A^-] + [HA] \qquad \textbf{Equation 7}$$

where pKa is the pH at which the drug is 50% ionised

$[A^-]$ is the concentration of the ionised form of the drug

[HA] is the concentration of the non-ionised form of the drug

[A] is the total concentration of drug in both forms

By solving both equations:

$$[A^-] = [A] - [HA] \quad \text{therefore} \quad pH = pKa + \log \frac{[A]\text{-}[HA]}{[HA]} \qquad \textbf{Equation 8}$$

$$\log \frac{[A]\text{-}[HA]}{[HA]} = pH - pKa \qquad \textbf{Equation 9}$$

$$\frac{[A]\text{-}[HA]}{[HA]} = 10^{(pH-pKa)} \qquad \textbf{Equation 10}$$

$$\frac{[A]}{[HA]} = 1 + 10^{(pH-pKa)} \qquad \textbf{Equation 11}$$

As the above equation can be applied to both saliva and plasma, the saliva/plasma ratio can be calculated by:

$$\frac{saliva}{plasma} = \frac{\frac{[A_{saliva}]}{[HA_{saliva}]}}{\frac{[A_{plasma}]}{[HA_{plasma}]}} \qquad \textbf{Equation 12}$$

$$= \frac{[A_{saliva}][HA_{plasma}]}{[A_{plasma}][HA_{saliva}]} = \frac{1 + 10^{(pH_{saliva}-pKa)}}{1 + 10^{(pH_{plasma}-pKa)}} \qquad \textbf{Equation 13}$$

Plasma contains both free and bound drug, if protein binding is taken into account and [HA] is assumed to be equal in plasma and saliva, the following equations can be derived:

**Acidic Drugs** →

$$\frac{\text{saliva}}{\text{plasma}} = \frac{1 + 10^{(pH_{saliva}-pKa)}}{1 + 10^{(pH_{plasma}-pKa)}} \times \frac{\text{free}_{plasma}}{\text{free}_{saliva}} \qquad \textbf{Equation 14}$$

**Basic Drugs** →

$$\frac{\text{saliva}}{\text{plasma}} = \frac{1 + 10^{(pKa-pH_{saliva})}}{1 + 10^{(pKa-pH_{plasma})}} \times \frac{\text{free}_{plasma}}{\text{free}_{saliva}} \qquad \textbf{Equation 15}$$

These equations predict that it is the amount of unbound drug in plasma that will affect the amount of drug in saliva. As it is only the unbound drug that is pharmacologically active, and this is reflected in the saliva concentration, saliva analysis has great potential for determining recent drug use.

## 6.4 Drugs of Abuse in Saliva

Drugs that are not ionisable or are un-ionised within the salivary pH range are most suitable for analysis in saliva. This means a change in saliva pH or flow rate does not effect the saliva drug concentration. If a drug is ionised in the saliva, it will not be able to diffuse back into the plasma resulting in the drug concentrations in the two matrices is not being in equilibrium and therefore the saliva drug concentration can not predict the plasma concentration. For drugs that have a pKa less than 5.5 and greater than 8.5, saliva pH has little effect on drug concentrations[82]. Unfortunately some drugs of abuse have pKa values close to 8.5 and can be affected by saliva pH. As saliva flow increases, as does the bicarbonate content which makes the saliva more alkaline[80]. High saliva flow rates can result in a pH of 7.4 - 7.6, much higher than the normal range of 5.6-7.0. Stress, anxiety and medication can all depress the flow of saliva and lower its pH. Saliva flow and pH are at their lowest in the early morning and peak at about 4 pm. Unstimulated saliva flow rates range from 0.3 to 0.5 mL/min, with 2% citric acid stimulated saliva, the flow rate ranges from 1.0 to 3.0 mL/min[83]. Stimulated saliva can be collected by citric acid under the tongue, chewing parafilm wax or Teflon, or sucking on pebbles. Not only do these methods increase flow rate therefore increasing alkalinity and decreasing saliva/plasma ratios, there is the possibility of adsorption of drugs onto the parafilm wax or Teflon[73]. As well as drugs of abuse having pKa values close to the pH of saliva, there is also the problem of contamination. Sources of contamination include orally ingested, smoked or internasally administered drugs. Contamination produces elevated drug concentrations in the saliva, thus making prediction of the plasma drug concentration difficult. Drugs of abuse found in saliva can infer recent drug use, but concentrations showed be looked at with caution, taking saliva collection and contamination issues into account.

## 6.4.1 Amphetamines

Amphetamines as a class include the classical amphetamines, amphetamine and methamphetamine and the methylenedioxy derivatives, methylenedioxymethamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA), and methylenedioxyamphetamine (MDA) as shown in Figure 6-5. Amphetamine and methamphetamine are commonly abused by oral administration. They have been used both intranasally and by intravenous injection. Methamphetamine hydrochloride also known as "ice" is usually smoked. Users experience a "rush" which is not attained when taken orally or intranasally[84]. The methylenedioxy derivatives seem to produce different effects to the classical amphetamines such as feelings of euphoria, happiness, increased energy and feeling close to others. Some authors described these effects as "entactogenic"[85]. Young people at "raves" increasingly use these drugs because of the unique feeling they produce[86]. MDMA, MDEA and MDA are usually taken orally as pills.

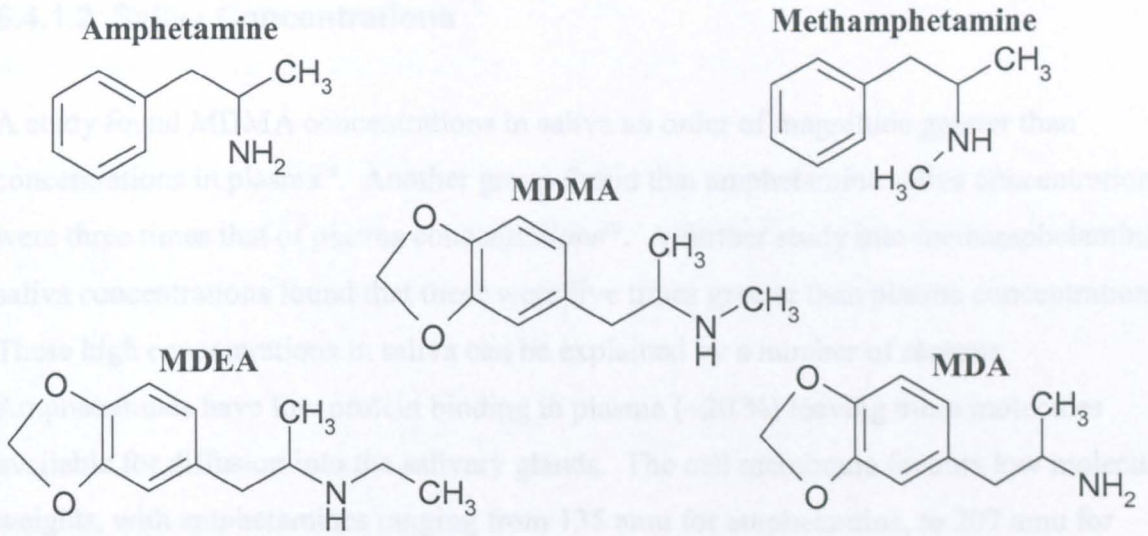



**Figure 6-5 : Molecular structures of amphetamine, methamphetamine, MDMA, MDEA and MDA**

### 6.4.1.1 Metabolism and Pharmacokinetics

Amphetamine is metabolised by hydroxylation of the aromatic ring to *p*-hydroxy-amphetamine and by deamination to benzylmethylketone, followed by oxidation to benzoic acid[87]. Methamphetamine is partially metabolised to amphetamine, which is then metabolised further[88]. MDMA and MDEA both metabolise to MDA. Although not

extensively researched it is thought that a substantial portion of MDA is excreted unchanged[31].

Pharmacokinetic data for amphetamine, methamphetamine and MDMA is shown in Table 6-1[31]. There has been little research done on the pharmacokinetics of MDEA and MDA. We can assume they will have similar protein binding and pKa values to amphetamine and methamphetamine.

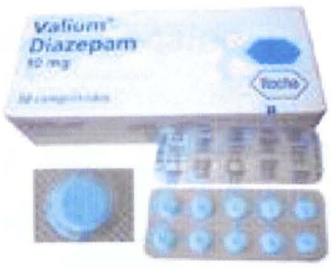
**Table 6-1 : Pharmacokinetic and physiochemical data for the amphetamine, methamphetamine and MDMA**

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

### 6.4.1.2 Saliva Concentrations

A study found MDMA concentrations in saliva an order of magnitude greater than concentrations in plasma[89]. Another group found that amphetamine saliva concentrations were three times that of plasma concentrations[90]. A further study into methamphetamine saliva concentrations found that these were five times greater than plasma concentrations[91]. These high concentrations in saliva can be explained by a number of reasons. Amphetamines have low protein binding in plasma (~20 %) leaving more molecules available for diffusion into the salivary glands. The cell membrane favours low molecular weights, with amphetamines ranging from 135 amu for amphetamine, to 207 amu for MDEA. Saliva is slightly more acidic than plasma therefore amphetamines are ionised in saliva and cannot diffuse back into plasma. Thus they accumulate in the saliva elevating saliva concentrations; this is sometimes referred to as "ion trapping"[91]. MDMA is known to act on serotonergic neurotransmission leading to vasoconstriction and a reduction in saliva production concentrating drugs in this matrix[92]. Individuals often experience dry mouth[93]. Peak oral fluid MDMA concentrations have been reported as 1215 ng/mL[94] (75 mg dose) and 3375 ng/mL (100 mg dose). The high concentrations of amphetamines in saliva make them particularly suited for oral fluid analysis, to show recent drug use. One still has to be aware of the potential of contamination through oral and intranasal ingestion.

## 6.4.2 Benzodiazepines




Benzodiazepines as a class include the most frequently prescribed and abused drugs diazepam and temazepam. Diazepam is prescribed as an antianxiety agent, muscle relaxant or anticonvulsant. Temazepam is a metabolite of diazepam and a prescribed drug in its own right. It is used for insomnia and is short acting. Both of these drugs have been associated with abuse in the west of Scotland. Between 1995 and 1998 a study into drug related deaths in the Strathclyde region of Scotland showed that 45 % of cases were positive for diazepam and 33 % positive for temazepam[58]. A further study into the prevalence of drugs in impaired drivers during 1995 and 1998 found benzodiazepines in 82 % of cases. With temazepam the most frequently encountered then diazepam[95].

### 6.4.2.1 Metabolism and Pharmacokinetics

The metabolism of diazepam is shown in Figure 4-1. The pharmacokinetic properties of the diazepam and its metabolites are shown in Table 6-2 below.

**Table 6-2 : Pharmacokinetic and physiochemical data for 1,4-benzodiazepines**

| | Diazepam | Temazepam | N-desmethyldiazepam | Oxazepam |
|---|---|---|---|---|
| **Half life (hr)** | 21-37 | 3-13 | 31-97 | 4-11 |
| **Vd (L/kg)** | 0.7-2.6 | 0.8-1.0 | | 0.7-1.6 |
| **Protein Binding (%)** | 96 | 97 | 97 | 87-94 |
| **$pK_a$** | 3.4 | 1.3 | 3.5, 12.0 | 1.7, 11.6 |

### 6.4.2.2 Saliva Concentrations

Diazepam (Valium®) is a long-acting lipophilic benzodiazepine, which exhibits extensive protein binding (96-99 %) allowing only a small percentage to diffuse through into saliva. Concentrations in saliva are found in the 0 – 20 ng/mL range and are observed up to 8 hours after dosing[88]. A metabolite of diazepam, N-desmethyldiazepam, has also been found in saliva. After chronic dosing saliva concentrations were approximately equivalent to diazepam, 1.2 – 23 ng/mL[88]. There seems to be little research on the analysis of benzodiazepines in oral fluid. This may be due to the high protein binding of the 1,4-

benzodiazepines in blood. One group attempted to analyse diazepam in saliva after a single oral dose but found their chromatographic method lacked enough sensitivity[96].

## 6.4.3 Cocaine

Cocaine is extracted from the leaves of *Erythroxylum coca*, which contain up to 2 % in weight[31]. Illicit cocaine is sold as cocaine hydrochloride usually administered intranasally or intravenously. Cocaine base "crack cocaine" is used for smoking. Cocaine is absorbed into the blood stream from all sites of application including mucous membranes, the gastrointestinal tract and lung aveoli[88]. Lung alveoli are especially efficient at cocaine absorption, and some users prefer a low cocaine dose smoked than a high dose injected. Intranasal and oral routes of administration produce lower plasma concentrations over a prolonged time, due to slower absorption[97]. Cocaine is a CNS stimulant and produces feelings of well-being and euphoria[88].

### 6.4.3.1 Metabolism and Pharmacokinetics

Once in the blood stream, cocaine is hydrolysed to its two major metabolites, benzoylecgonine (BZE) and ecgonine methyl ester (EME) and a number of minor metabolites[31]. Cocaine's pharmacokinetic data is presented in Table 6-3 and the structures of cocaine and its metabolites are shown in Figure 6-6.

**Table 6-3 : Pharmacokinetic and physiochemical data for cocaine**

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

Peak concentrations of cocaine occur in plasma within 2 hours after oral administration[98]. The half life of cocaine in plasma was determined as 1.5 hours after acute administration[98] and 3.8 hours after frequent use[99]. As a result of cocaine's lipophilicity it is thought that cocaine is accumulated in the fatty tissues and CNS during chronic use and excreted over a

period of 2-3 weeks[100]. Plasma half lives for BZE and EME have been reported as 6.4 hours and 3.7 hours respectively[98].

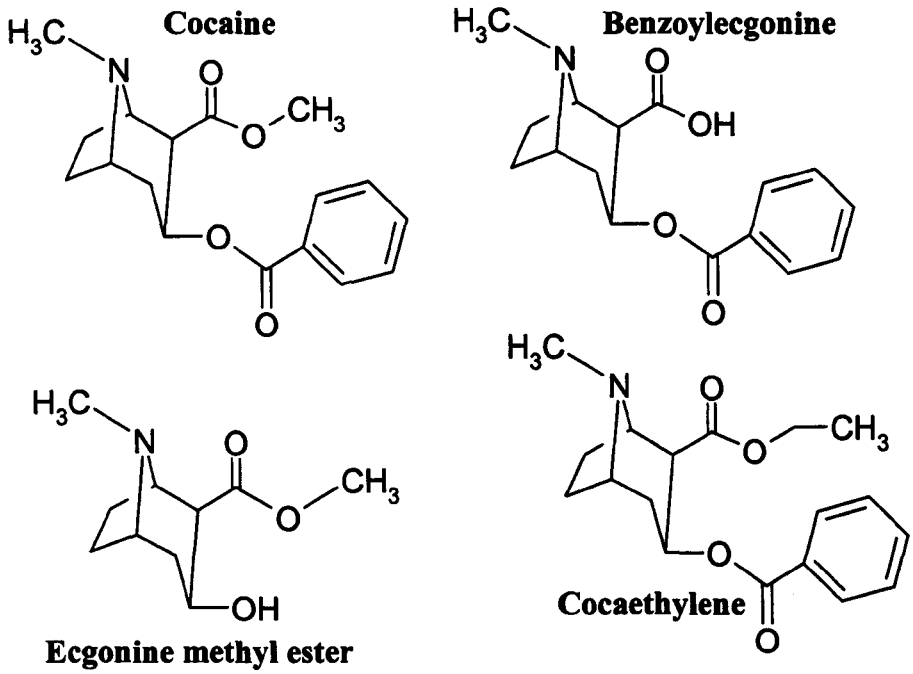



**Figure 6-6 : Molecular structures of cocaine and its metabolites**

### 6.4.3.2 Saliva Concentrations

Cocaine concentrations in plasma correlate significantly with saliva concentrations and subjective effects[101]. The individual saliva cocaine concentrations were frequently found to be higher than respective plasma concentrations. Another study found that detection times for cocaine in saliva were longer than in plasma[102]. These findings are consistent with cocaine having a saliva/plasma ratio of >1. BZE and EME are less lipid-soluble than cocaine and are found in lower concentrations in saliva than plasma samples collected at the same time[103]. In one study however, EME was found in higher concentrations in saliva compared to plasma[98]. In the same study a mean peak EME saliva concentration of 11,566 ng/mL was found whereas the mean peak BZE saliva concentration was 2,980 ng/mL and the mean peak cocaine saliva concentration was 1,978 ng/mL. These atypical results may be due to the chronic administration of cocaine, subsequent accumulation and prolonged elimination. Cocaine could be found in the saliva of these chronic users for 5-10 days after abstinence. Another group found that cocaine saliva concentrations were ~2.4 fold higher than plasma concentrations, BZE saliva concentrations were a 1/5$^{th}$ of plasma concentrations and EME saliva concentrations were equivalent to plasma concentrations[99].

This group studied the levels of cocaine and its metabolites during abstinence, which may account for the differences in results.

If cocaine is administered intranasally or smoked, contamination of the oral cavity can occur. This will artificially elevate the saliva concentrations. The extent of contamination by the intranasal route of administration was found to be highly variable and lasted longer (2 hours) than contamination by the smoked route (0.25 hours)[102]. The difference in extent of contamination by the different routes is thought to be due to ion trapping of the drug in saliva and successive swallowing resulting in rapid clearance of the drug while smoking. All routes of administration reach the limit of detection (1 ng/mL) within 12 hours[102].

Another factor effecting cocaine levels in saliva is the method of collection, whether saliva flow is stimulated or unstimulated. A study found that there was a 5 fold reduction in cocaine, BZE and EME saliva concentrations after stimulation[104].

Cocaine was first detected in the saliva of an impaired driver in 1984[77]. Since then various studies have been performed on the deposition of cocaine and its metabolites in saliva. It is thought that plasma cocaine concentrations are significantly correlated with saliva concentrations[101], if cocaine is administered intravenously or there has been time for contamination to clear from the oral cavity, ~2 hours[102]. There is a longer window of detection in saliva than plasma, due to the high saliva/plasma ratio and possible accumulation of cocaine in the fatty tissues of chronic users[100]. Saliva analysis of cocaine can be indicative of recent use and if contamination is known not be a factor then plasma concentrations can be predicted.

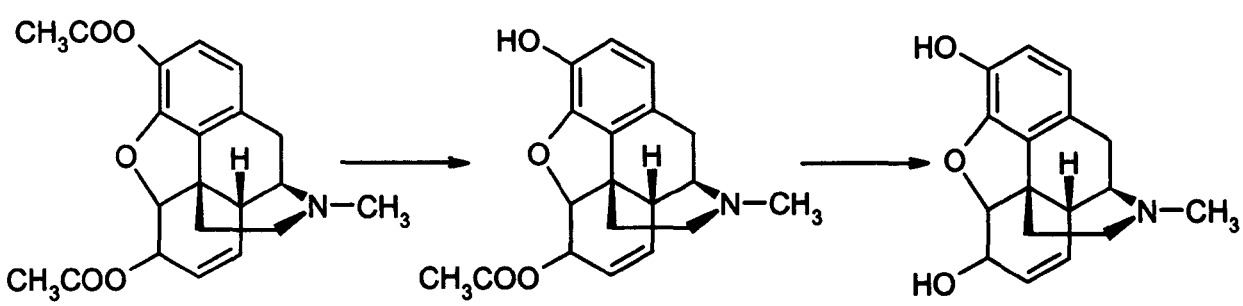
### 6.4.4 Opiates: Morphine, Codeine and 6-monoacetylmorphine

Opium is the dried exudate of the poppy *Papaver Somniferum*, it contains morphine and codeine amongst other natural alkaloids[88]. Clandestine laboratories acetylate morphine to diamorphine to be sold on the black market. This procedure results in the conversion of codeine to acetylcodeine. In the USA heroin and diamorphine are interchangeable names. In the UK heroin is commonly defined as the mixture of the impurities, reaction intermediates, breakdown products manufactured during the synthesis of diamorphine, and excipients and adulterants used to dilute or cut the drug[105].

### 6.4.4.1 Metabolism and Pharmacokinetics

Diamorphine is metabolised rapidly in plasma with a half-life of 2-6 minutes[31]. For this reason it is rarely the primary target for opiate detection in body fluids. Diamorphine metabolises to morphine via 6-monoacetylmorphine (6-MAM), this allows the detection of 6-MAM to be conclusive of diamorphine use. As codeine is extracted from the poppy at the same time as morphine, it is also present in heroin, and hence can be detected in body fluids after heroin administration. The metabolism of diamorphine is shown in Figure 6-7. Codeine found in body fluids from heroin overdose victims is due to the impure heroin, and not metabolism[31].

Codeine metabolises to morphine, by O-demethylation, and norcodeine via N-demethylation. Both are excreted in their free form or their glucuronide conjugates in urine. Norcodeine is found in trace amount in serum, and therefore it is unlikely to find any appreciable amounts in saliva. Morphine can be found in serum up to 10% of the codeine concentration within 6-8 hours[31].




**Diamorphine** **6-acetylmorphine** **Morphine**

**Figure 6-7 : Metabolism of diamorphine**

Codeine is a commonly prescribed opiate used for pain relief and cough suppression, its structure is shown in Figure 6-8. It can also be abused for both its euphoric and depressant effects, and to prevent opiate withdrawal. Owing to its availability there have been several studies on codeine in oral fluid[96,106,107,108,109].

**Table 6-4 : Pharmacokinetic and physiochemical data for diamorphine, morphine and codeine**

| | Diamorphine | Morphine | Codeine |
|---|---|---|---|
| **Half life (hr)** | 2-6 minutes | 1.3-6.7 | 1.9-3.9 |
| **Vd (L/kg)** | 25 | 2-5 | 3.5 |
| **Protein Binding (%)** | 40 | 35 | 7-25 |
| **$pK_a$** | 7.6 | 8.1, 10 | 8.2 |

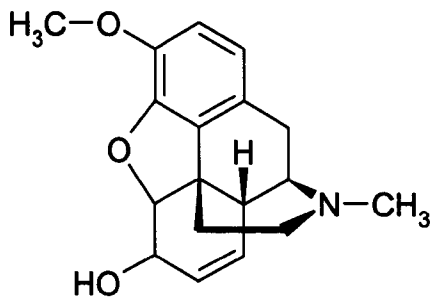



**Figure 6-8 : Chemical structure of codeine**

### 6.4.4.2 Saliva Concentrations

Morphine plasma concentrations have been shown to peak at 0.25 – 0.5 hours, while saliva concentrations tend to be slightly delayed reaching maximum at 0.5 hours. Morphine concentrations found in saliva tend to be lower than the equivalent plasma samples. One study found a peak morphine plasma concentration of 150 ng/mL following a 20mg dose. The equivalent peak morphine saliva concentration was 38 ng/mL. Both matrices gave positive morphine results up to 24 hours[106].

The same study found that in contrast to morphine, codeine saliva concentrations were higher than plasma concentrations. After a 120 mg dose of codeine, peak plasma concentrations of 272 ng/mL were observed compared to 308 ng/mL in saliva. A different study found that codeine saliva concentrations remained 3 – 4 times greater than plasma concentrations throughout 24 hours[107]. Codeine was even found to be present for a longer period of time in saliva (36 hr) than in plasma (24 hr)[106]. This is supported by another group of authors who found that codeine was detected 5 – 9 hours longer in oral fluid than in plasma[109] and another group who found codeine was detected in saliva 12 hours longer than in plasma[107].

One group studied the effect of collection method on saliva concentrations of codeine and found that "spitting" gave consistently higher results than any of the collection devices, even a cotton roll without any stimulating salts impregnated. This could be due to adsorption of drugs onto the device or the stimulation effect of the device. They found spitting to give 3.5 times higher results than acidic stimulation and 1.3 – 2.0 times higher results than any other device[108]. They also concluded that there was considerable

interindividual variability in peak codeine concentrations between collection methods and within each collection group.

One point to note is that even after brushing teeth, oral contamination is still present, supporting a theory of small depots of drug in the oral cavity releasing over a period of time[107].

### 6.4.5 Morphine Substitutes: Methadone, Buprenorphine and Dihydrocodeine

Methadone, buprenorphine and dihydrocodeine are all used in the treatment of heroin addiction.

Methadone is a morphine substitute, and possesses many of the pharmacologic properties of morphine. However it can accumulate in the body with repeated administration and cause sedative effects. Since 1965 it has been used for opioid maintence patients, with daily doses as high as 180 mg. It is known that doses of 50 mg or less can be fatal in nontolerant adults[31].

Buprenorphine is a synthetic thebaine derivative with both analgesic and opioid antagonist properties[31]. It is used as a morphine substitute like methadone. Buprenorphine has not been found to be a contributor in drug related deaths or impaired driving[58,95]. A study into drug related deaths in Edinburgh and Glasgow found 1 death positive for buprenorphine and concluded that the lack of positive samples implied that the drug is very unlikely to lead to death[110].

Dihydrocodeine is a semisynthetic narcotic analgesic, prepared by the reduction of codeine and can be prescribed as an alternative to methadone[111]. Its structure is shown in Figure 6-9.

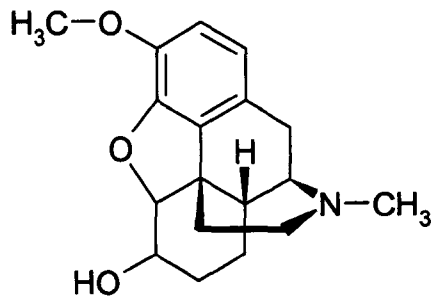


**Figure 6-9 : Chemical structure of dihydrocodeine**

### 6.4.5.1 Metabolism and Pharmacokinetics

**Table 6-5 : Pharmacokinetic and physiochemical data for methadone, buprenorphine and dihydrocodeine**

| | Methadone | Buprenorphine | Dihydrocodeine |
|---|---|---|---|
| Half life (hr) | 15-55 | 2-4 | 3.4-4.5 |
| Vd (L/kg) | 4-5 | 2.5 | 1.0-1.3 |
| Protein Binding (%) | 87 | 96 | - |
| $pK_a$ | 8.6 | 8.5, 10.0 | 8.8 |

Methadone it metabolised to 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) by mono-N-demethylation with spontaneous cyclization as shown in Figure 6-10. EDDP is not found to any significant extent in plasma during therapeutic usage of methadone. Methadone plasma concentrations peak at 4 hours with an average value of 0.83 mg/L with chronic administration of 100-200 mg daily oral dose[31].

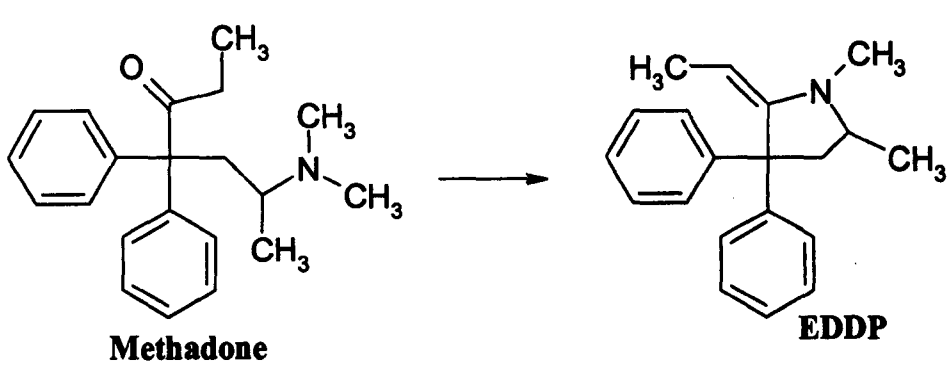



**Figure 6-10 : Metabolism of methadone to EDDP**

Blood concentrations of buprenorphine tend to be very low. 2 mg of sublingual administered buprenorphine in 6 patients gave an average peak plasma concentration of 1.6 ng/mL at 1.3 hours. Buprenorphine is metabolised to norbuprenorphine which is also pharmacologically active their structures are shown in Figure 6-11. In the urine free buprenorphine and norbuprenorphine levels may be up to 20 ng/mL in abuse cases.[31]

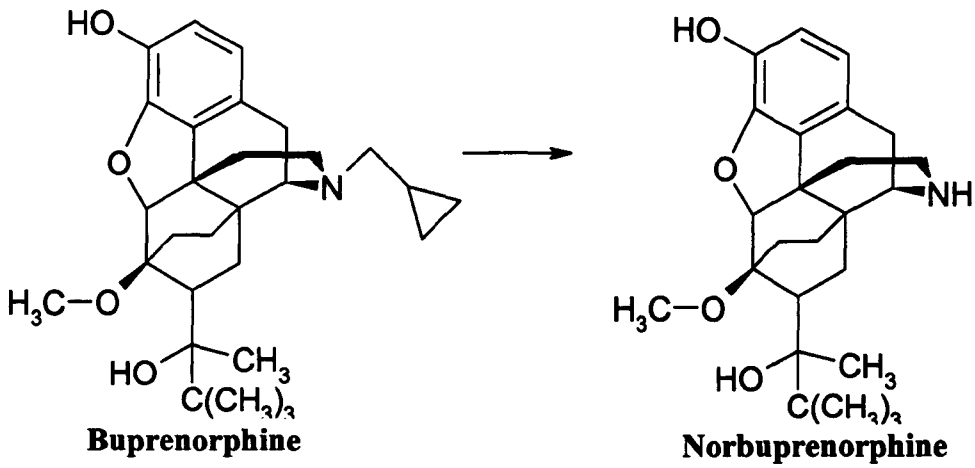



**Figure 6-11 : Metabolism of buprenorphine**

Dihydrocodeine is thought to undergo the same biotransformation steps as codeine[31]. Peak plasma concentrations of dihydrocodeine of 70 ng/mL have been found at 1.6 hours after a 30mg oral dose.

### 6.4.5.2 Saliva Concentrations

There are conflicting views on the use of oral fluid for methadone analysis. The theoretical saliva/plasma ratio is calculated by using the pH of oral fluid and plasma. As the pH of oral fluid is not constant the theoretical saliva/plasma ratio can vary also. A value of 1.3 was calculated by one group, who concluded that methadone was an ideal candidate for oral fluid analysis, as there would be a good linear relationship between the two matrices[112]. However a more recent study found no correlation between methadone saliva and serum concentrations[113].

Saliva concentrations of buprenorphine are substantially less than plasma with a saliva/plasma ratio of 0.05-0.41, following intramuscular administration[88]. It would be unlikely to find buprenorphine or its metabolite norbuprenorphine in oral fluid due to the

very low levels found in plasma. Oral contamination could indicate its recent ingestion but no information on plasma levels could be deduced.

After oral administration of 60 mg dihydrocodeine to six subjects dihydrocodeine was found in saliva for up to 12-24 hours and the half-life in saliva was twice that of blood. After chronic administration of dihydrocodeine, the parent drug was the main analyte in saliva with only a few samples positive for N-nordihydrocodeine.[114]

## 6.5 Conclusions

It is known that some drugs can collect in oral fluid through passive diffusion over lipid membranes in the salivary glands. It is also understood that blood and urine are not preferred body fluids to give or obtain. A medical practitioner needs to take blood, and urine gives us information on what drugs have been taken in the past and not what is in the circulatory system at present. Oral fluid analysis provides information on drugs which are in the blood stream at the time of collection, or in the case of contamination, drugs which have been ingested within the past few hours. Owing to the advantages of oral fluid analysis, it has the potential to find a place in roadside testing and heroin rehabilitation centres.

# 7 Drugs of Abuse in Oral Fluid Using LC-MS

## 7.1 Introduction

As part of a departmental project, 60 oral fluid samples were to be analysed for a wide spectrum of licit and illicit drugs. Samples were collected using the modified Omni-Sal® device, supplied by Cozart Bioscience Ltd. This device collects 1 mL of oral fluid through a pad placed at the side of the mouth. This was then diluted with 2 mL of buffer to preserve the sample until analysis. As sample volume was limited it was decided to use both the GC-MS and LC-MS to screen the samples for as many analytes as possible. A single extraction procedure was needed and the extract would be split for injection onto both instruments.

## 7.2 Aim

Most of the common drugs of abuse have been analysed by LC-MS in the past. These include morphine[115,116,117,118], methadone[119,120,121], buprenorphine[122], amphetamines[123,124] and combinations of these, opioids and cocaine[125], and opiates, amphetamine and cocaine[126]. The aim of this project was to develop and validate a method for the extraction of twenty drugs of abuse and their metabolites from 1 mL of oral fluid and identify them using liquid chromatography – mass spectrometry.

## 7.3 Method Development

Initially the pseudo molecular ions for the analytes must be identified and the voltage applied to the skimmer cone (AQA max voltage) optimised. Once the ions are identified the chromatography can be improved by changing the mobile phase to try and resolve the analytes. The initial mobile phase conditions were 50:50 acetonitrile :0.004 % formic acid at 0.15 mL/min using a Phenomenex Luna C18(2) 150 x 2.0 mm, 3 µm packing. These conditions were chosen as they had worked well in benzodiazepine analysis, refer to Chapter 4.

### 7.3.1 Chemicals

Methanol, acetonitrile and hexane were HPLC grade and obtained from VWR International (UK). Analytical grade potassium dihydrogen phosphate, ammonium acetate and ammonium bicarbonate were supplied by BDH laboratory supplies (Poole, England). Analytical grade concentrated ammonia, glacial acetic acid and concentrated formic acid were purchased from Sigma® Chemicals Co. (UK).

### 7.3.2 Standards

Stock standards of analytes were purchased from Promochem as 100 μg/mL methanolic solutions. The deuterated standards of each drug were also purchased as 100 μg/mL methanolic solutions. The analyte and deuterated standards used are shown in Table 7-1. In the first instance 1 μg/mL solutions of each analyte were prepared so they could be injected singularly. This was done by taking 10 μL of 100 μg/mL solution and adding 990 μL of methanol. A working drug solution of 1 μg/mL for each drug, was prepared by taking 250 μL of each standard into a 25 mL volumetric flask and making up to volume with methanol. A working internal standard solution was prepared in the same way using the deuterated standards.

**Table 7-1 : Purchased standards and deuterated standards**

| Analyte | Deuterated Standard |
|---|---|
| Amphetamine | Amphetamine-$d_5$ |
| Methamphetamine | Methamphetamine-$d_5$ |
| MDA | MDA-$d_5$ |
| MDMA | MDMA-$d_5$ |
| MDEA | MDEA-$d_5$ |
| Diazepam | Diazepam-$d_5$ |
| N-desmethyldiazepam | N-desmethyldiazepam-$d_5$ |
| Temazepam | Temazepam-$d_5$ |
| Oxazepam | Oxazepam-$d_5$ |
| Cocaine | Cocaine-$d_3$ |
| Benzoylecgonine | Benzoylecgonine-$d_3$ |
| Cocaethylene | Cocaethylene-$d_3$ |

| Analyte | Deuterated Standard |
|---|---|
| Morphine | Morphine-$d_3$ |
| Codeine | Codeine-$d_3$ |
| 6-MAM | 6-MAM-$d_3$ |
| Dihydrocodeine | Dihydrocodeine-$d_6$ |
| Methadone | Methadone-$d_9$ |
| EDDP | EDDP-$d_3$ |
| Buprenorphine | Buprenorphine-$d_4$ |
| Norbuprenorphine | Norbuprenorphine-$d_3$ |

## *7.3.3 Spiked Oral Fluid Samples*

Drug free oral fluid was collected from healthy volunteers using Omni-Sal® devices with proprietary modifications as supplied by Cozart Biosciences Ltd., Abingdon, UK. Oral fluid, 1mL, was collected with each device as indicated by a colour change at the end of the stick. This was then placed into 2 mL of buffer and the stick was twisted to detach the pad. This mixture of oral fluid and buffer (1:2 v/v) was then expressed through a filter to separate it from the pad and was used as the matrix for preparation of oral fluid standards.

## *7.3.4 Solutions*

### 7.3.4.1 Preparation of 0.1 M, pH 6.0 Phosphate Buffer

6.81 g of potassium dihydrogen phosphate was weighed out into a 500 mL volumetric flask and 450 mL of deionised water was added. The pH was adjusted to 6.0 with 1 M potassium hydroxide solution, then made to 500 mL with deionised water.

### 7.3.4.2 Preparation of 0.004% Formic Acid Solution

40 μL of concentrated formic acid was placed in a 1 L volumetric flask and made up to volume with deionised water.

### 7.3.4.3 Preparation of Ammonium Formate and Formic Acid Buffers

Table 7-2 show the amounts of ammonium formate and concentrated formic acid that were added to a 1 L volumetric flask and made up to volume with deionised water.

**Table 7-2 : Amounts of ammonium formate and concentrated formic acid used to make mobile phase buffers**

| Final Concentration in 1 litre | Ammonium Formate (g) | Formic Acid (μL) |
|---|---|---|
| 20 mM Ammonium Formate + 0.001 % Formic Acid | 1.261 | 10 |
| 10 mM Ammonium Formate + 0.001 % Formic Acid | 0.631 | 10 |
| 5 mM Ammonium Formate + 0.001 % Formic Acid | 0.315 | 10 |
| 3 mM Ammonium Formate + 0.001 % Formic Acid | 0.189 | 10 |
| 2 mM Ammonium Formate + 0.001 % Formic Acid | 0.126 | 10 |
| 3 mM Ammonium Formate + 0.002 % Formic Acid | 0.189 | 20 |
| 3 mM Ammonium Formate + 0.004 % Formic Acid | 0.189 | 40 |

## *7.3.5 Optimisation of Skimmer Cone Voltage*

Single standards were prepared by taking 100 μL of a 1 μg/mL solution into a vial and blowing down under nitrogen. This was reconstituted in 200 μL of mobile phase and 10 μL injections were made. Full scan mode was used, setting the skimmer cone voltage to 5, 10, 20 and 30 V for the first injection and 40, 50, 60 and 70 V for the second injection. The skimmer cone values for diazepam and its metabolites were taken from previous work, refer to Chapter 4. The results are shown in Table 7-3 below.

**Table 7-3 : Pseudo molecular ions and skimmer cone values for 20 analytes**

| Analyte | Molecular weight (amu) | Pseudo molecular ion $[M+H]^+$ (m/z) | Skimmer cone voltage (V) |
|---|---|---|---|
| Amphetamine | 135 | 136 | 5 |
| Methamphetamine | 149 | 150 | 10 |
| MDA | 179 | 180 | 10 |
| MDMA | 193 | 194 | 10 |
| MDEA | 207 | 208 | 10 |
| Diazepam | 284 | 285 | 40 |
| N-desmethyldiazepam | 270 | 271 | 35 |
| Temazepam | 300 | 301 | 25 |
| Oxazepam | 286 | 287 | 30 |
| Cocaine | 303 | 304 | 10 |

| Analyte | Molecular weight (amu) | Pseudo molecular ion $[M+H]^+$ (m/z) | Skimmer cone voltage (V) |
|---|---|---|---|
| Benzoylecgonine | 289 | 290 | 20 |
| Cocaethylene | 317 | 318 | 20 |
| Morphine | 285 | 286 | 20 |
| Codeine | 299 | 300 | 30 |
| 6-MAM | 327 | 328 | 30 |
| Dihydrocodeine | 301 | 302 | 5 |
| Methadone | 309 | 310 | 10 |
| EDDP | 277 | 278 | 20 |
| Buprenorphine | 467 | 468 | 30 |
| Norbuprenorphine | 413 | 414 | 40 |

All of the analytes eluted within a couple of minutes of the solvent front, so it was clear that a gradient would have to be used to resolve the analytes and a higher buffer content in the initial conditions to separate the analytes from the solvent front.

### *7.3.6 Optimisation of Mobile Phase Conditions*

Although it is widely believed that basic drugs ionise more efficiently with an acidic buffer in the mobile phase, it has been shown that a high pH buffer such as ammonium bicarbonate can give satisfactory results[127]. A mixture of ammonium formate and formic acid has also been used in the mobile phase for the analysis of a complex mixture of opioids and cocaine[125]. It was decided to investigate the effect of using both ammonium formate and formic acid at different concentrations in the mobile phase. A formic acid concentration of 0.001% was used and the amount of ammonium formate added was changed to 20, 10, 5, 3, and 2 mM. Each mobile phase conditions were equilibrated on the LC system before 5 injections of drug mixture were run. The average peak area was calculated for each drug, a selection of analytes is shown in Figure 7-1.

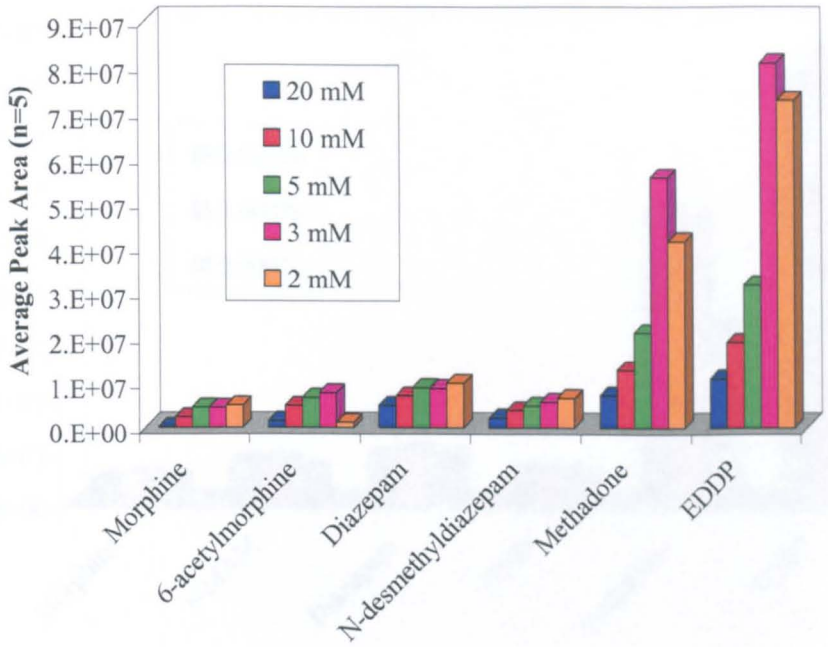



**Figure 7-1 : Variation of response with decreasing ammonium formate concentration**

As before in Chapter 4, response of analytes increased with lower amounts of buffer present. It was decided to use 3mM ammonium formate and change the concentration of formic acid to see what effect it had on analyte response. Three concentrations of formic acid were investigated, 0.001, 0.002 and 0.004 %, with 3 mM ammonium formate. The results are shown in Figure 7-2. From the results it was shown that 3 mM ammonium formate with 0.001 % formic acid gave the optimum response for the analytes.

A group of authors who used 10 mM ammonium formate and 0.001 % formic acid in the mobile phase used a Synergi Polar RP column for separation[125]. During this project a C18 column was used. It was decided to investigate the use of a basic buffer compared to an acidic buffer and the use of a Synergi Polar RP column compared to a C18 column. The systems used for comparison are detailed in Table 7-4.

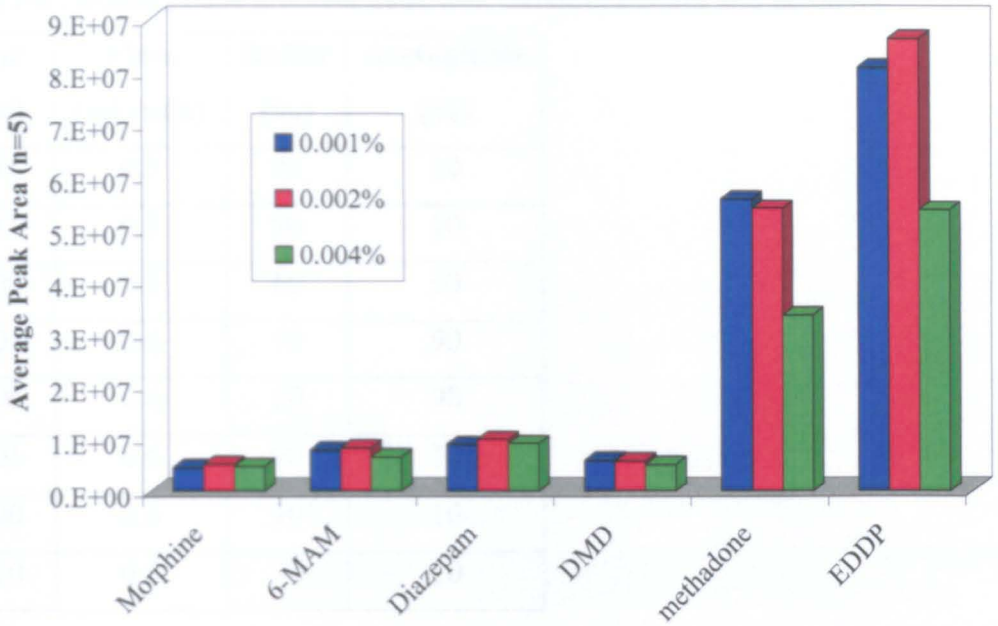



**Figure 7-2 : Variation of response with increasing formic acid concentration**

**Table 7-4 : Different columns and buffers investigated**

| System | Column | Buffer |
|---|---|---|
| 1 | Phenomenex C18 Luna 150 x 2 mm, 3 μm packing | 3 mM ammonium formate + 0.001 % formic acid |
| 2 | Phenomenex C18 Luna 150 x 2 mm, 3 μm packing | 10 mM ammonium bicarbonate |
| 3 | Phenomenex Synergi Polar RP 150 x 2 mm, 3 μm packing | 3 mM ammonium formate + 0.001 % formic acid |

Unfortunately the basic buffer was too high in pH to run through the Polar RP column without damaging it. The gradient used is shown in Table 7-5. Analysis was done in SIM mode in two injections. The first injection included the opioids, benzodiazepines and cocaine and its metabolites. The second injection included the five amphetamines. Two injections were needed because the instrument could not monitor all of the ions at the same time. Five injections for each condition were performed consecutively, and the mean area calculated for each analyte.

**Table 7-5 : Mobile phase gradient used with different buffers and columns**

| Time (min) | Flow (mL/min) | Buffer (%) | Acetonitrile (%) |
|---|---|---|---|
| 0.00 | 0.3 | 90 | 10 |
| 2.00 | 0.3 | 90 | 10 |
| 15.00 | 0.3 | 10 | 90 |
| 15.01 | 0.6 | 10 | 90 |
| 30.00 | 0.6 | 10 | 90 |
| 30.01 | 0.6 | 90 | 10 |
| 40.00 | 0.6 | 90 | 10 |
| 40.10 | 0.3 | 90 | 10 |

#### 7.3.6.1 Results

Figure 7-3 shows the difference in response of analytes between each column and mobile phase. It was clear that using the C18 column (systems 1 & 2) gave a greater response for every analyte except methadone and EDDP. As methadone and EDDPs response was so much greater than the other analytes it was thought a compromise would be to pick the best conditions for the smaller response analytes. The difference in response between an acidic and basic buffer on a C18 column was not consistent for every analyte. It was decided to use the basic buffer with the C18 column (system 2), as this gave the greatest response for most of the analytes of interest. This is in contrast to the hypothesis that when analysing basic drugs, acidic buffers should be used in the mobile phase to promote ionisation.

### *7.3.7 Sample Preparation*

Spiked oral fluid samples were prepared by adding the appropriate amount of working drug solution (5, 10, 25, 50, 100, 200 µL) and internal standard solution (200 µL) to test tubes, followed by 3.5 mL of 0.1 M, pH 6 phosphate buffer and 1 mL of blank oral fluid. A blank was prepared without any standards present and another with internal standard only to assess any contamination of the deuterated standards.

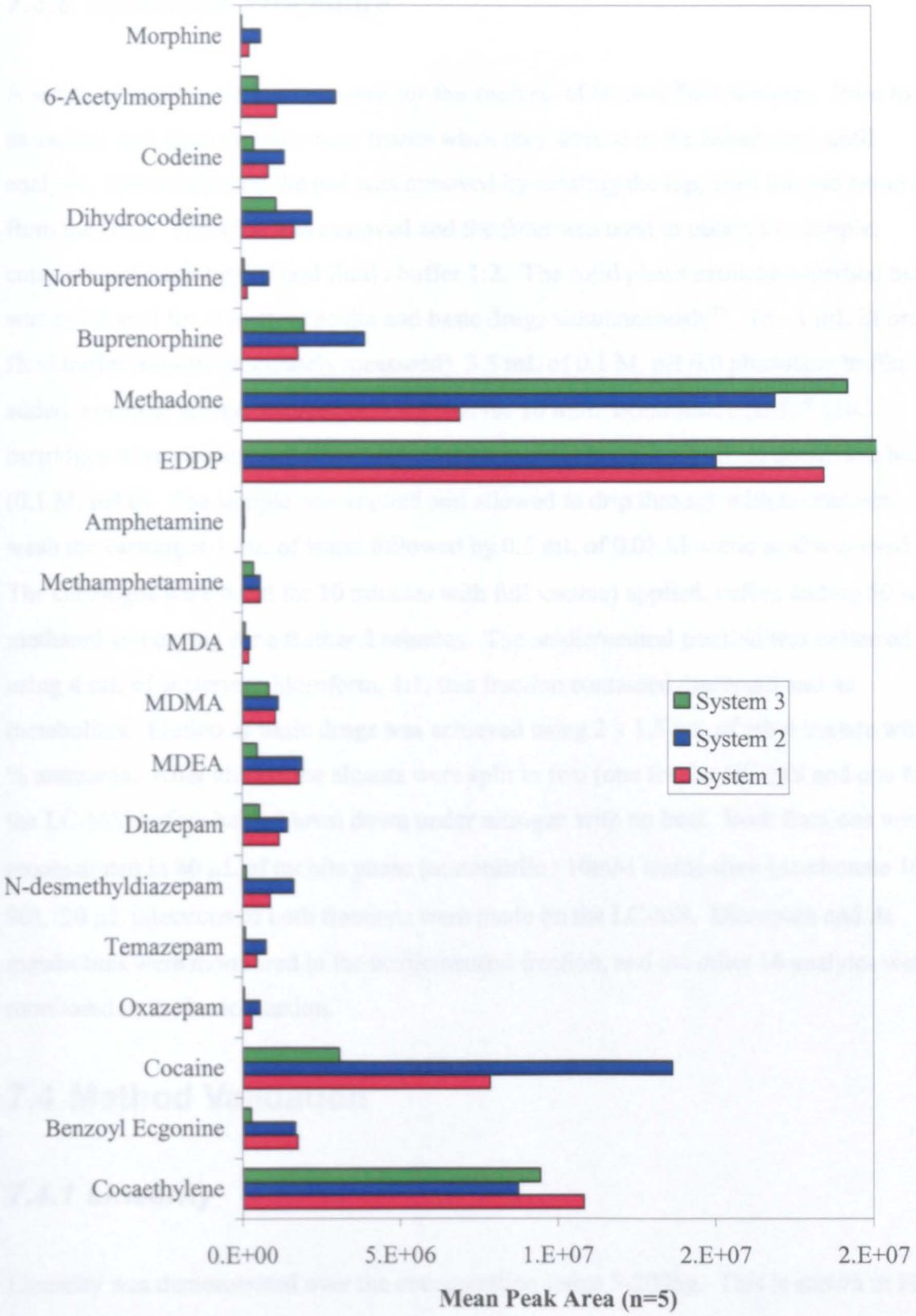



**Figure 7-3 : Difference in mean peak area for 20 analytes using different mobile and stationary phases**

### 7.3.8 Extraction Procedure

A single extraction method was used for the analysis of 60 oral fluid samples. Prior to extraction oral fluid samples were frozen when they arrived in the department until analysis. Once defrosted the pad was removed by twisting the top, until the pad came free from the stick. The stick was removed and the filter was used to obtain the sample, consisting of a mixture of oral fluid : buffer 1:2. The solid phase extraction method used, was developed for extracting acidic and basic drugs simultaneously[72]. To ~1 mL of oral fluid:buffer mixture (accurately measured), 3.5 mL of 0.1 M, pH 6.0 phosphate buffer was added, vortexed and centrifuged at 3000 rpm for 10 min. Bond Elut Certify® LRC cartridges were conditioned with 2 mL of methanol followed by 2 mL of phosphate buffer (0.1 M, pH 6). The sample was applied and allowed to drip through with no vacuum. To wash the cartridges 1 mL of water followed by 0.5 mL of 0.01 M acetic acid was used. The cartridges were dried for 10 minutes with full vacuum applied, before adding 50 μL of methanol and drying for a further 2 minutes. The acidic/neutral fraction was collected using 4 mL of acetone : chloroform, 1:1, this fraction contained diazepam and its metabolites. Elution of basic drugs was achieved using 2 x 1.5 mL of ethyl acetate with 2 % ammonia. After elution the eluents were split in two (one for the GC-MS and one for the LC-MS) before being blown down under nitrogen with no heat. Both fractions were reconstituted in 80 μL of mobile phase (acetonitrile : 10mM ammonium bicarbonate 10 : 90). 20 μL injections of both fractions were made on the LC-MS. Diazepam and its metabolites were monitored in the acidic/neutral fraction, and the other 16 analytes were monitored in the basic fraction.

## 7.4 Method Validation

### 7.4.1 Linearity

Linearity was demonstrated over the concentration range 5-200ng. This is shown in Figure 7-4 to Figure 7-8.

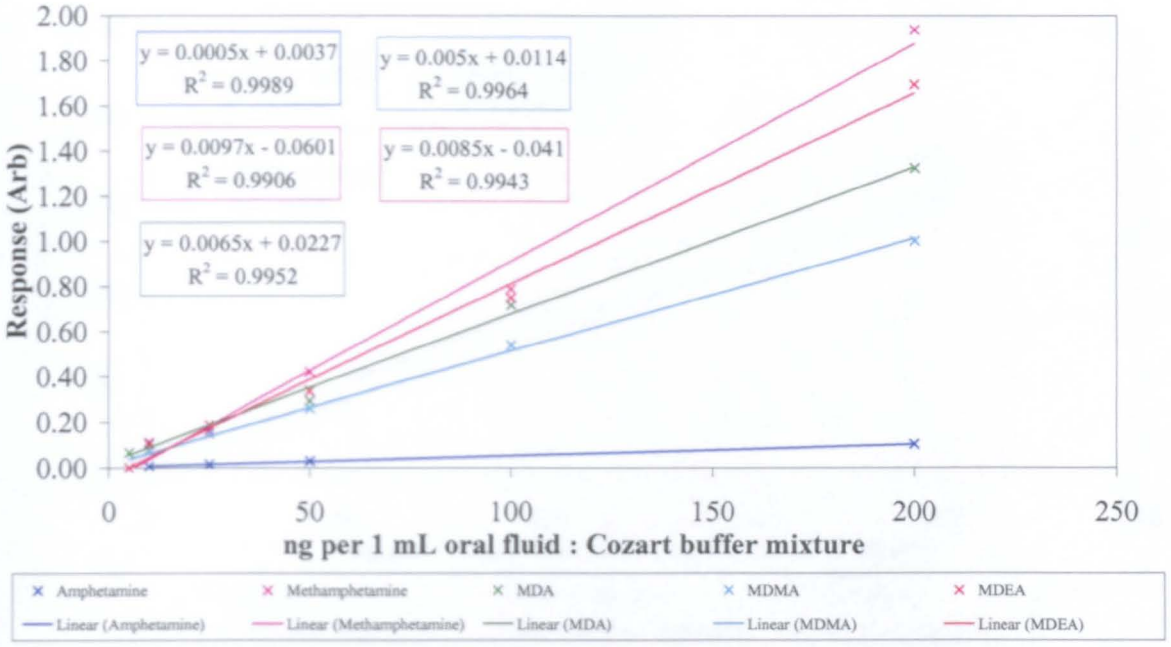



**Figure 7-4 : Linearity data for amphetamine, methamphetamine, MDA, MDMA and MDEA**

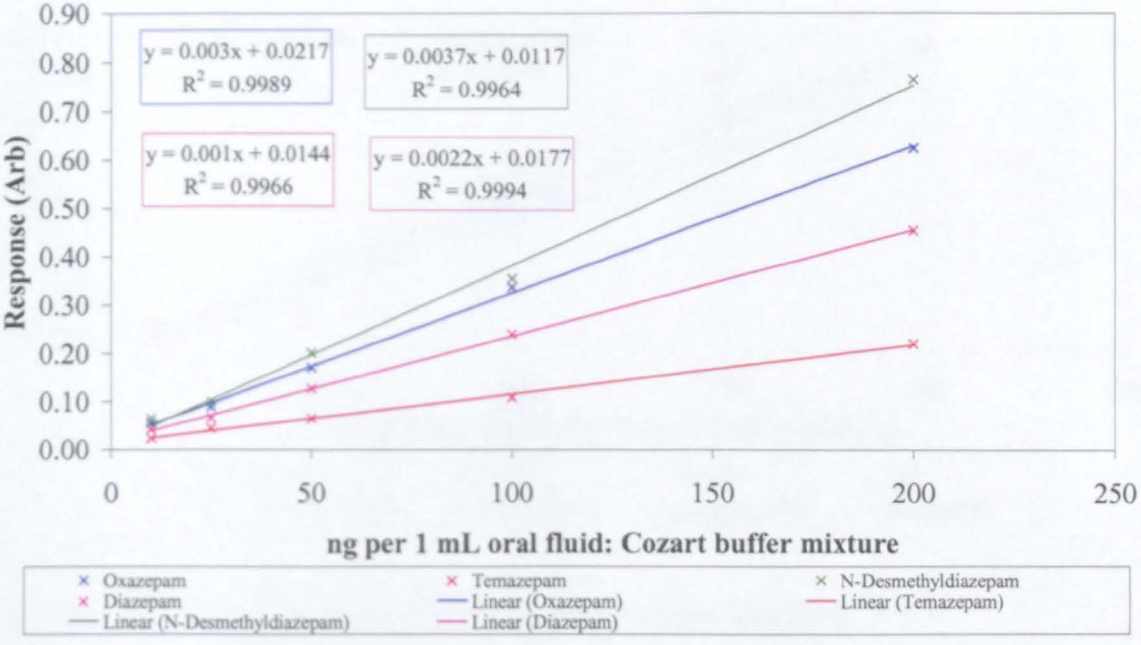



**Figure 7-5 : Linearity data for oxazepam, temazepam, N-desmethyldiazepam and diazepam**

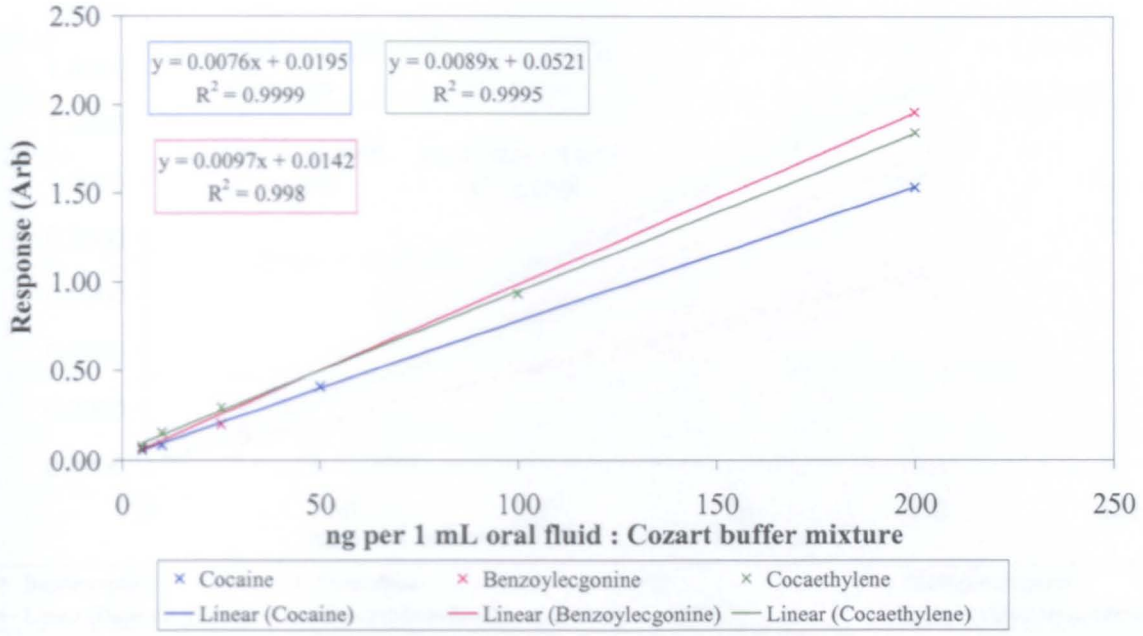



**Figure 7-6 : Linearity data for cocaine, benzoylecgonine and cocaethylene**

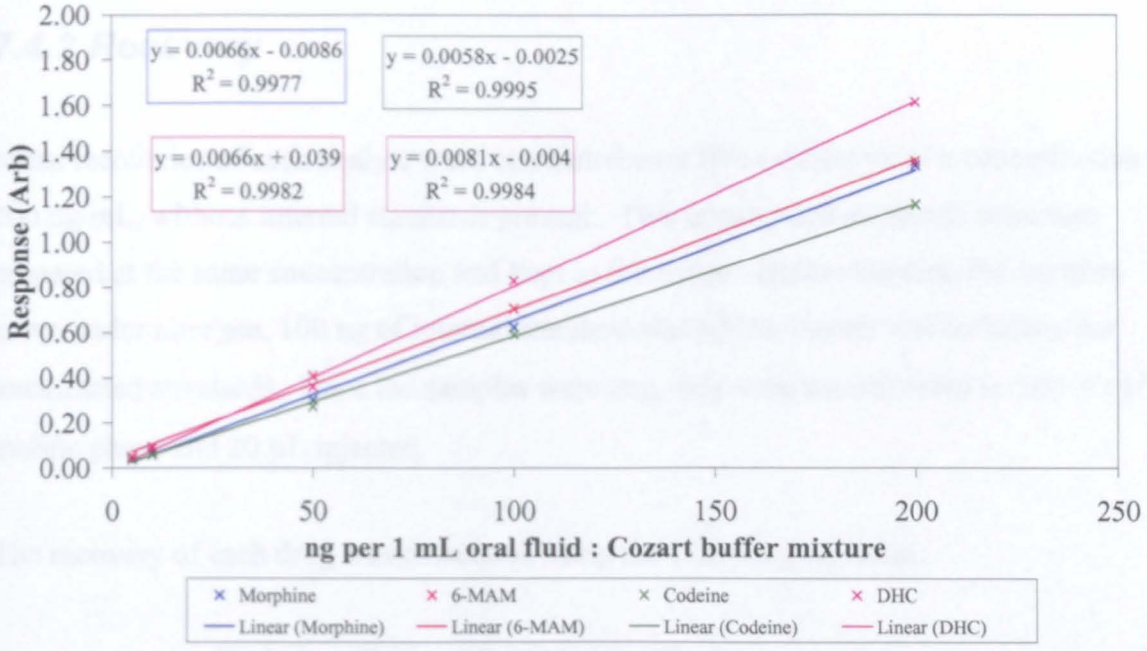



**Figure 7-7 : Linearity data for morphine, 6-MAM, codeine and DHC**

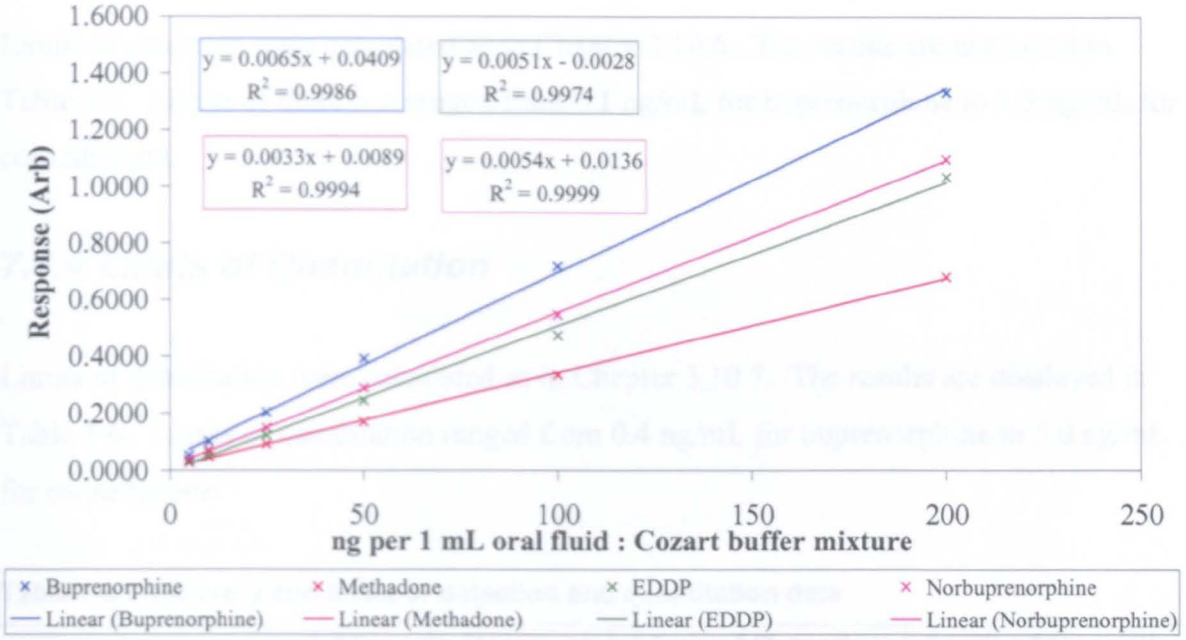



**Figure 7-8 : Linearity data for buprenorphine, methadone, EDDP and norbuprenorphine**

## *7.4.2 Recovery*

Mean recoveries of each analyte were calculated over five extractions at a concentration of 200 ng/mL, without internal standards present. Two unextracted standards were also prepared at the same concentration and kept in the fridge. Before blowing the samples down under nitrogen, 100 ng of internal standard was added to each vial including the unextracted standards. Once the samples were dry, they were reconstituted in 100 µL of mobile phase and 20 µL injected.

The recovery of each drug was calculated using the following equation.

$$\text{Recovery}(\%) = \frac{\text{Peak Area Ratio of Extracted Standard}}{\text{Peak Area Ratio of Unextracted Standard}} \times 100 \qquad \textbf{Equation 16}$$

The recoveries calculated for each analyte is shown in Table 7-6. Recovery for each analyte varied from 58 % for benzoylecgonine to 105 % for cocaethylene.

### 7.4.3 Limits of Detection

Limits of detection were calculated as in Chapter 3.10.6. The results are displayed in Table 7-6. Limits of detection ranged from 0.1 ng/mL for buprenorphine to 1.5 ng/mL for cocaethylene.

### 7.4.4 Limits of Quantitation

Limits of quantitation were calculated as in Chapter 3.10.7. The results are displayed in Table 7-6. Limits of quantitation ranged from 0.4 ng/mL for buprenorphine to 5.0 ng/mL for cocaethylene.

**Table 7-6 : Recovery and limits of detection and quantitation data**

| Analyte | Mean % Recovery (%RSD) (n=5) | Limit of Detection (ng/mL) | Limit of Quantitation (ng/mL) |
|---|---|---|---|
| Amphetamine | 59 (11.1) | 1.1 | 3.8 |
| Methamphetamine | 85 (10.7) | 1.3 | 4.5 |
| MDA | 88 (9.0) | 1.1 | 3.6 |
| MDMA | 90 (3.9) | 1.3 | 4.4 |
| MDEA | 94 (5.0) | 1.2 | 4.1 |
| Diazepam | 80 (1.6) | 0.5 | 1.5 |
| N-desmethyldiazepam | 64 (1.2) | 0.7 | 2.5 |
| Temazepam | 93 (2.0) | 1.3 | 4.3 |
| Oxazepam | 89 (0.9) | 0.7 | 2.3 |
| Cocaine | 60 (1.7) | 0.8 | 2.5 |
| Benzoylecgonine | 58 (13.6) | 1.4 | 4.7 |
| Cocaethylene | 105 (11.0) | 1.5 | 5.0 |
| Morphine | 97 (9.4) | 1.5 | 4.9 |
| Codeine | 90 (8.8) | 0.8 | 2.5 |
| 6-MAM | 79 (7.5) | 1.5 | 4.8 |
| Dihydrocodeine | 85 (5.3) | 1.3 | 4.4 |
| Methadone | 96 (4.3) | 1.4 | 4.6 |
| EDDP | 92 (3.7) | 1.2 | 3.9 |
| Buprenorphine | 98 (0.8) | 0.1 | 0.4 |
| Norbuprenorphine | 89 (7.7) | 0.4 | 1.2 |

## *7.4.5 Inter-Day Precision*

Inter-day precision was calculated using five sets of linearity data acquired on five different days. The %RSD of the five values for two concentrations, 50 and 200 ng/mL were calculated. The results are shown in Table 7-7.

**Table 7-7 : Inter-day precision for 20 analytes**

| Analyte | Inter-day precision (%RSD) (n=5) | |
|---|---|---|
| | **50ng** | **200ng** |
| Amphetamine | 53 (4.2) | 199 (5.6) |
| Methamphetamine | 47 (6.2) | 199 (2.2) |
| MDA | 49 (13.4) | 200 (1.3) |
| MDMA | 48 (7.4) | 199 (1.5) |
| MDEA | 49 (2.5) | 200 (1.5) |
| Diazepam | 52 (4.5) | 197 (1.1) |
| N-desmethyldiazepam | 52 (4.0) | 198 (1.4) |
| Temazepam | 51 (2.9) | 200 (1.8) |
| Oxazepam | 52 (3.9) | 200 (0.8) |
| Cocaine | 48 (10.6) | 198 (1.7) |
| Benzoylecgonine | 53 (7.2) | 201 (0.8) |
| Cocaethylene | 47 (4.0) | 195 (8.0) |
| Morphine | 45 (9.6) | 200 (2.2) |
| Codeine | 51 (11.7) | 201 (1.5) |
| 6-MAM | 52 (10.9) | 199 (1.8) |
| Dihydrocodeine | 51 (10.1) | 199 (0.9) |
| Methadone | 51 (3.3) | 201 (1.3) |
| EDDP | 49 (4.7) | 201 (0.8) |
| Buprenorphine | 51 (4.4) | 199 (1.3) |
| Norbuprenorphine | 50 (5.2) | 200 (0.8) |

### 7.4.6 Intra-Day Precision between Injections

Extracted standards were prepared at two concentrations 50 and 200 ng/mL. They were injected 5 times consecutively. The %RSD of the peak area ratios was calculated at each concentration for each drug. The results are shown in Table 7-8.

**Table 7-8 : Intra-day precision of 5 consecutive injections**

| Analyte | Intra-day precision 5 injections (%RSD) (n=5) | |
|---|---|---|
| | 50ng | 200ng |
| Amphetamine | 45 (4.3) | 212 (9.5) |
| Methamphetamine | 50 (4.6) | 203 (6.8) |
| MDA | 51 (8.1) | 205 (9.8) |
| MDMA | 50 (1.7) | 201 (3.0) |
| MDEA | 51 (3.9) | 200 (1.4) |
| Diazepam | 49 (1.4) | 199 (5.2) |
| N-desmethyldiazepam | 52 (1.4) | 198 (2.1) |
| Temazepam | 50 (3.8) | 197 (2.1) |
| Oxazepam | 51 (0.5) | 202 (2.2) |
| Cocaine | 51 (3.6) | 202 (2.8) |
| Benzoylecgonine | 49 (8.7) | 203(7.4) |
| Cocaethylene | 49 (7.6) | 202 (4.1) |
| Morphine | 44 (2.5) | 201 (7.7) |
| Codeine | 56 (5.1) | 200 (4.4) |
| 6-MAM | 56 (8.0) | 200 (8.2) |
| Dihydrocodeine | 46 (7.1) | 200 (8.6) |
| Methadone | 45 (2.4) | 201 (1.3) |
| EDDP | 49 (1.7) | 200 (1.0) |
| Buprenorphine | 51 (2.6) | 203 (3.0) |
| Norbuprenorphine | 54 (6.5) | 194 (5.9) |

### 7.4.7 Intra-Day Precision between Extracts

Five extracted standards at two concentrations, 50 and 200 ng/mL, were prepared simultaneously. The %RSD of the peak area ratios were calculated for each extraction, at each concentration, for each drug. The results are shown in Table 7-9.

**Table 7-9 : Intra-day precision of 5 extractions**

| Analyte | Intra-day precision 5 extractions (%RSD) (n=5) | |
|---|---|---|
| | 50ng | 200ng |
| Amphetamine | 43 (9.2) | 194 (10.8) |
| Methamphetamine | 48 (9.7) | 197 (5.6) |
| MDA | 49 (9.3) | 195 (7.2) |
| MDMA | 51 (7.5) | 200 (3.8) |
| MDEA | 48 (4.2) | 200 (3.3) |
| Diazepam | 51 (3.2) | 197 (2.2) |
| N-desmethyldiazepam | 52 (3.4) | 197 (1.2) |
| Temazepam | 51 (2.8) | 203 (1.6) |
| Oxazepam | 51 (2.7) | 200 (2.0) |
| Cocaine | 46 (7.3) | 197 (2.9) |
| Benzoylecgonine | 51 (8.0) | 200 (8.0) |
| Cocaethylene | 52 (3.7) | 199 (2.9) |
| Morphine | 49 (7.0) | 202 (8.9) |
| Codeine | 49 (8.1) | 201 (5.2) |
| 6-MAM | 59 (8.1) | 200 (5.9) |
| Dihydrocodeine | 51 (6.4) | 196 (5.2) |
| Methadone | 50 (2.4) | 198 (1.8) |
| EDDP | 50 (1.9) | 201 (1.6) |
| Buprenorphine | 51 (2.7) | 200 (3.0) |
| Norbuprenorphine | 51 (5.4) | 198 (4.1) |

## 7.5 Discussion

While investigating the buffers used in the mobile phase, it was noted that low concentrations of acidic buffers, 3 mM ammonium formate, enhance response greater than higher concentrations, 10 mM ammonium formate. This is concurrent with earlier findings for diazepam and its metabolites in Chapter 4. The use of a basic buffer in the mobile phase, ammonium bicarbonate, was evaluated and found to improve sensitivity compared to using ammonium formate and formic acid. These findings suggest that it is not always necessary to use an acidic buffer when analysing basic drugs, and the use of a high pH buffer could be an alternative.

All linear correlation coefficients were greater than 0.99, ranging from 0.9906 for methamphetamine to 0.9999 for cocaine and norbuprenorphine. For most analytes recoveries were between 80 and 100 %. Benzoylecgonine had the lowest recovery of 58 %, and cocaethylene had the highest recovery of 105 %. Limits of detection for all analytes were less than or equal to 1.5 ng/mL, and limits of quantitation were all less than 5 ng/mL. Intra and Inter – day precision was less than 11 % at 200 ng/mL for all analytes.

## 7.6 Conclusions

A liquid chromatography – mass spectrometry method was developed and validated for the extraction and quantitation of 20 drugs of abuse and their metabolites from oral fluid. Analytes were identified by their retention time with respect to the deuterated standard, and using the pseudo-molecular ion. Further qualitative data was acquired using the GC-MS in full scan mode.

This method was applied to 60 oral fluid samples collected by the modified Omni-Sal® device, refer to Chapter 10.2.

# 8 The Detection of Drugs of Abuse in Oral Fluid Using LC-MS-MS

## 8.1 Introduction

As part of the European project, Impaired Motorists, Methods of Roadside Testing and Assessment for Licensing, otherwise known as IMMORTAL (Deliverable R4.2), the University of Glasgow was required to analyse 1396 oral fluid samples, collected from drivers, for a wide range of drugs. Samples were collected in and around the city of Glasgow from drivers stopped at random. Drivers were asked to give an oral fluid specimen by use of the modified Omni-Sal® device, supplied by Cozart Biosciences.

## 8.2 Aim

The aim of this project was to develop and validate an LC-MS-MS method for the identification and quantitation of 21 drugs of abuse and their metabolites extracted from oral fluid specimens.

### *8.2.1 Chemicals*

Methanol and acetonitrile were HPLC grade obtained from VWR International (UK). Analytical grade potassium dihydrogen phosphate, ammonium formate were supplied by BDH laboratory supplies (Poole, England). Analytical grade concentrated ammonia, glacial acetic acid and concentrated formic acid were purchased from Sigma® Chemicals Co. (UK).

### *8.2.2 Standards*

Stock standards of analytes were purchased from Promochem as 100 μg/mL methanolic solutions. The deuterated standards of each drug were also purchased as 100 μg/mL methanolic solutions. The analyte and deuterated standards used are shown in Table 8-1. In the first instance 1 μg/mL solutions of each analyte were prepared so they could be infused singularly. This was done by taking 10 μL of 100 μg/mL solution and adding 990 μL of methanol. A working drug mixture with a concentration of 1 μg/mL for each drug,

was prepared by taking 250 μL of each standard into a 25 mL volumetric flask and making up to volume with methanol. A working internal standard solution was prepared in the same way using the deuterated standards.

**Table 8-1 : Purchased standards and deuterated standards**

| Analyte | Deuterated Standard |
|---|---|
| Amphetamine | Amphetamine-$d_5$ |
| Methamphetamine | Methamphetamine-$d_5$ |
| MDA | MDA-$d_5$ |
| MDMA | MDMA-$d_5$ |
| MDEA | MDEA-$d_5$ |
| Diazepam | Diazepam-$d_5$ |
| N-desmethyldiazepam | N-desmethyldiazepam-$d_5$ |
| Temazepam | Temazepam-$d_5$ |
| Oxazepam | Oxazepam-$d_5$ |
| Cocaine | Cocaine-$d_3$ |
| Benzoylecgonine | Benzoylecgonine-$d_3$ |
| Ecgonine Methyl Ester | Ecgonine Methyl Ester-$d_3$ |
| Cocaethylene | Cocaethylene-$d_3$ |
| Morphine | Morphine-$d_3$ |
| Codeine | Codeine-$d_3$ |
| 6-MAM | 6-MAM-$d_3$ |
| Dihydrocodeine | Dihydrocodeine-$d_6$ |
| Methadone | Methadone-$d_9$ |
| EDDP | EDDP-$d_3$ |
| Buprenorphine | Buprenorphine-$d_4$ |
| Norbuprenorphine | Norbuprenorphine-$d_3$ |

### *8.2.3 Solutions*

#### 8.2.3.1 Preparation of 0.1 M, pH 6.0 Phosphate Buffer

6.81 g of potassium dihydrogen phosphate was weighed out into a 500 mL volumetric flask and 450 mL of deionised water was added. The pH was adjusted to 6.0 with 1 M potassium hydroxide solution, then made to 500 mL with deionised water.

#### 8.2.3.2 Preparation of 3 mM ammonium formate with 0.001 % formic acid

0.189 g of ammonium formate was placed into a 1 L volumetric flask with 10 µL concentrated formic acid. This was made up to volume with deionised water.

## 8.3 Method Development

This project involved the use of an LC-MS with an ion trap analyser which allowed MS-MS data to be collected for each analyte. Previously only SIM mode could be carried out on the single quadrupole instrument. As a result of the soft ionisation process in LC-MS, full scan MS data regularly consists of the pseudo-molecular ion only. Using tandem MS allows the pseudo-molecular ion to be fragmented to obtain specific daughter ions. This gives another element of specificity, and therefore greater accuracy in quantitation.

Each analyte was infused into the source, by-passing the chromatographic column. This allows a constant signal of analyte ions. Mobile phase was also allowed to flow into the source at the same time, trying to reconstruct a real chromatographic run environment. The pseudo-molecular ions were known from previous work (Chapter 7). Each analytes pseudo-molecular ion was "tuned" automatically by the instrument. This optimises settings within the optics of the mass spectrometer for each analyte.

Whilst infusing the analyte, the daughter ions were determined, by increasing the collision energy applied and recording what ions were produced at different collision energies. In this way the collision energy and daughter ions specific to that analyte are determined. The collision energy and daughter ions determined are found in Table 8-2.

**Table 8-2 : Analyte precursor and daughter ions and collision energies**

| Analyte | Precursor ion (m/z) | Collision energy (%) | Product ion(s) (m/z) |
|---|---|---|---|
| Amphetamine | 136 | 22 | 119 |
| Methamphetamine | 150 | 27 | 119, 91 |
| MDA | 180 | 20 | 163 |
| MDMA | 194 | 25 | 163 |
| MDEA | 208 | 25 | 163 |
| Diazepam | 285 | 42 | 257, 222 |
| N-desmethyldiazepam | 271 | 40 | 243, 208 |
| Temazepam | 301 | 25 | 283, 255 |
| Oxazepam | 287 | 40 | 269, 241 |
| Cocaine | 304 | 35 | 182 |
| Benzoylecgonine | 290 | 28 | 168 |
| Ecgonine methyl ester | 200 | 28 | 182 |
| Cocaethylene | 318 | 28 | 196 |
| Morphine | 286 | 35 | 201, 229, 268 |
| 6-MAM | 328 | 35 | 211, 268 |
| Codeine | 300 | 35 | 215, 243, 282 |
| Dihydrocodeine | 302 | 35 | 245, 201, 227 |
| Methadone | 310 | 25 | 265 |
| EDDP | 278 | 38 | 249 |
| Buprenorphine | 468 | 36 | 414, 396 |
| Norbuprenorphine | 414 | 32 | 396, 340 |

## *8.3.1 Fragmentation Patterns in the Ion Trap*

Amphetamine and MDA both lose a fragment equal to 17 amu. This can be attributed to a $-NH_3^+$ group being knocked off the main structure. Amphetamines fragmentation is shown in Figure 8-1. Methamphetamine and MDMA both lose a fragment equal to 31 amu, suggesting the loss of a $-NH_2^+ - CH_3$ group. Methamphetamine seems to fragment further to an ion m/z = 91. This can be explained by the loss of a part of the carbon branch as shown in Figure 8-2. MDEA is similar in structure to MDMA, except its alkyl chain is

one carbon longer, giving it its higher molecular weight. The daughter ion is the same as shown in Figure 8-3.

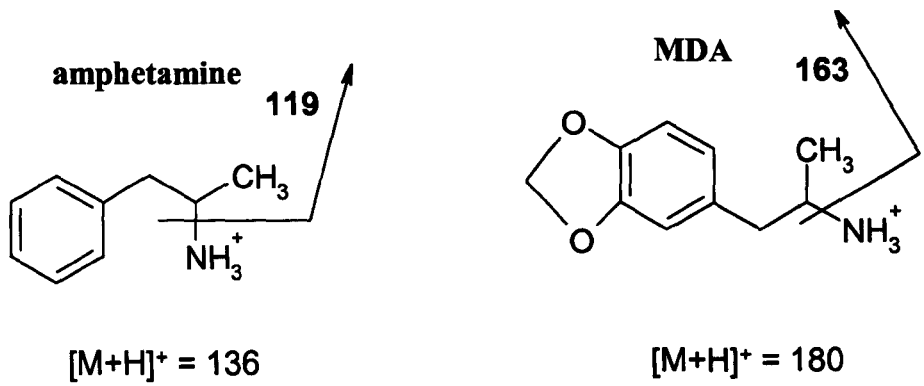



**Figure 8-1 : Proposed fragmentation of amphetamine and MDA in the ion trap**

By looking at the fragmentation patterns of these amphetamines the common m/z 119 ion for amphetamine and methamphetamine is apparent as is the common m/z 163 ion for MDA, MDMA and MDEA.

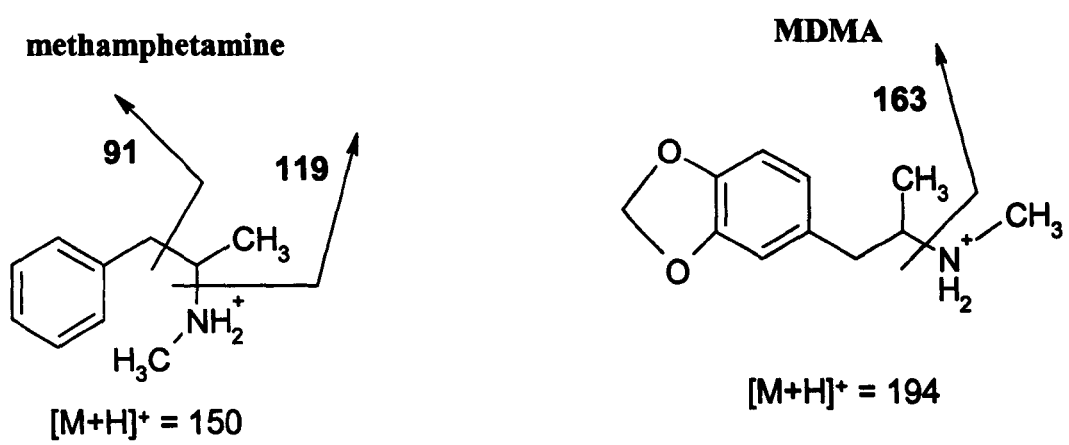



**Figure 8-2 : Proposed fragmentation of methamphetamine and MDMA in the ion trap**

As a result of the amphetamines being so similar in structure, it is difficult to resolve them all on the liquid chromatography column. Therefore there is some overlapping of chromatographic peaks. When using the single quadrupole analyser in Chapter 7, it was impossible to tell which daughter ion came from which drug. By using MS-MS data, although the amphetamines have common daughter ions, the analyser can differentiate between them by knowing which parent ion they came from. This is one of the major advantages of using MS-MS data, and what gives it its specificity.

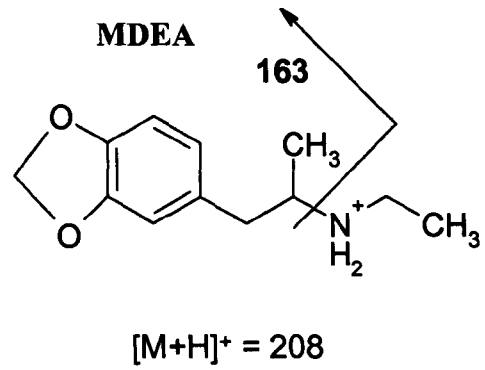



**Figure 8-3 : Proposed fragmentation of MDEA in the ion trap**

Diazepam metabolises to N-desmethyldiazepam and are therefore very similar in structure, differing only in a methyl group. In the ion trap, they fragment in a similar manner by first losing a –CO group, and then a chlorine atom. A proposed scheme for diazepam is shown in Figure 8-4. Diazepam and N-desmethyldiazepam metabolise to temazepam and oxazepam respectively, with the addition of a hydroxyl group to the 7-membered ring. It is thought that temazepam and oxazepam undergo the loss of a $-H_2O^+$ fragment before the loss of a –CO group as with diazepam and N-desmethyldiazepam. One would expect the loss of the chlorine atom next, but this is not observed in the MS-MS spectra. This may be observed in $MS^3$ data. Unfortunately the instrument did not have the relevant computer software to do this.

Cocaine, benzoylecgonine and cocaethylene all have a common loss of 122 amu from their pseudo-molecular ion. This can be attributed to the loss of a benzene ring, carbonyl and oxygen group from the structure. Their proposed fragmentations are shown in Figure 8-5. The same fragmentation of cocaine has been shown by GC-MS with chemical ionisation which is a soft ionisation like ESI[128]. Ecgonine methyl ester fragments to the same daughter ion as cocaine m/z 182, but this is with the loss of a $-H_2O^+$.

The opiates including dihydrocodeine have very complex ring systems in their structures, making it very difficult to propose a fragmentation pattern. The ring system must be broken somewhere to give the m/z 201 ion for morphine. The loss of a $-H_2O^+$ fragment from morphine would explain the m/z 268 ion, but this ion is very small compared to m/z 201.

Methadone and EDDP tend to fragment to just one daughter ion, by the loss of a branch from the structure. This is shown graphically in Figure 8-6.

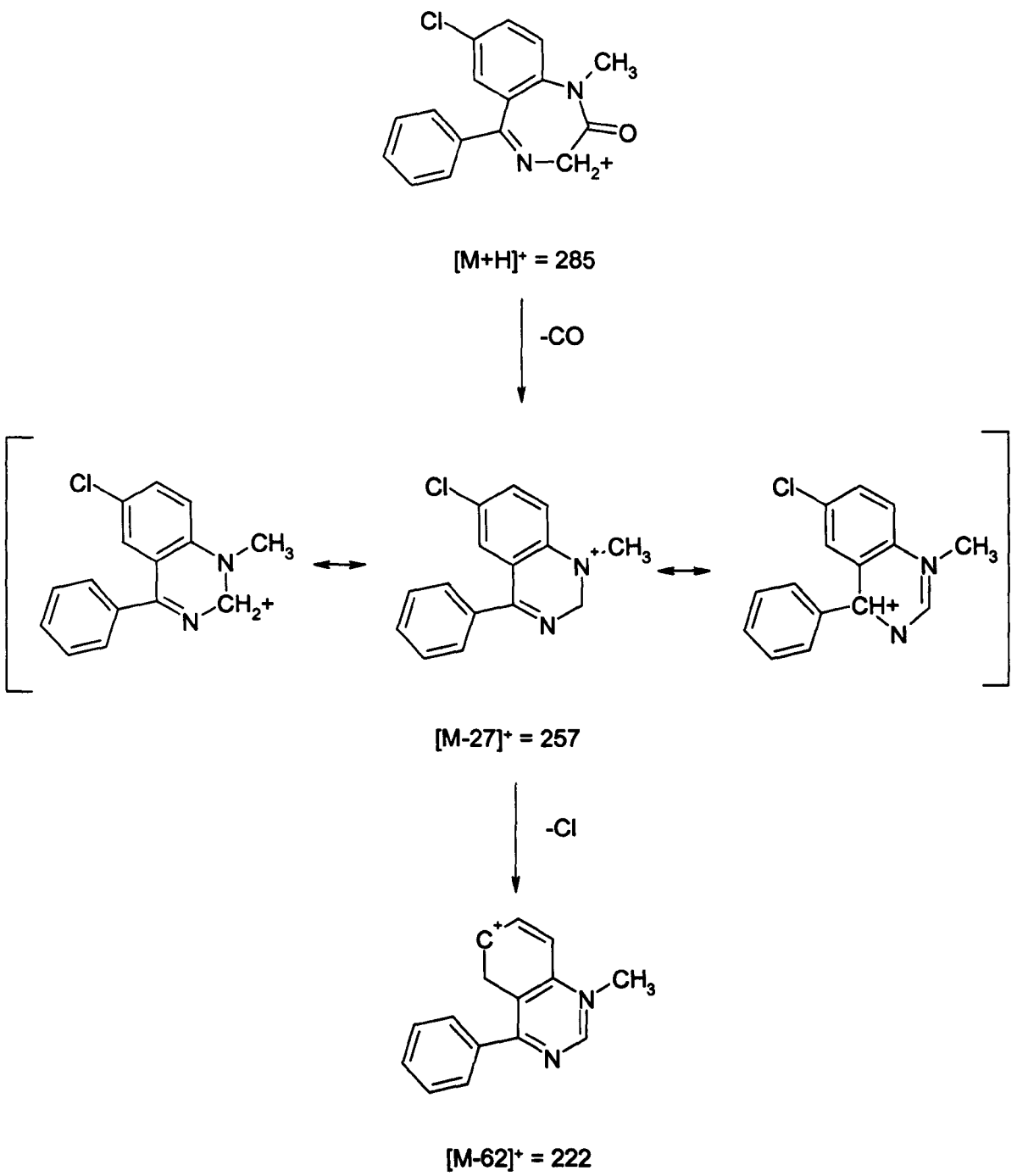



**Figure 8-4 : Proposed fragmentation of diazepam in the ion trap**

Buprenorphine fragments with the loss of a methylcyclopropane group to norbuprenorphines pseudo-molecular ion. This in turn fragments with the loss of a $-H_2O^+$ group, producing a m/z 396 daughter ion observed in both buprenorphine and norbuprenorphines $MS^2$ spectra. This is shown in Figure 8-7.

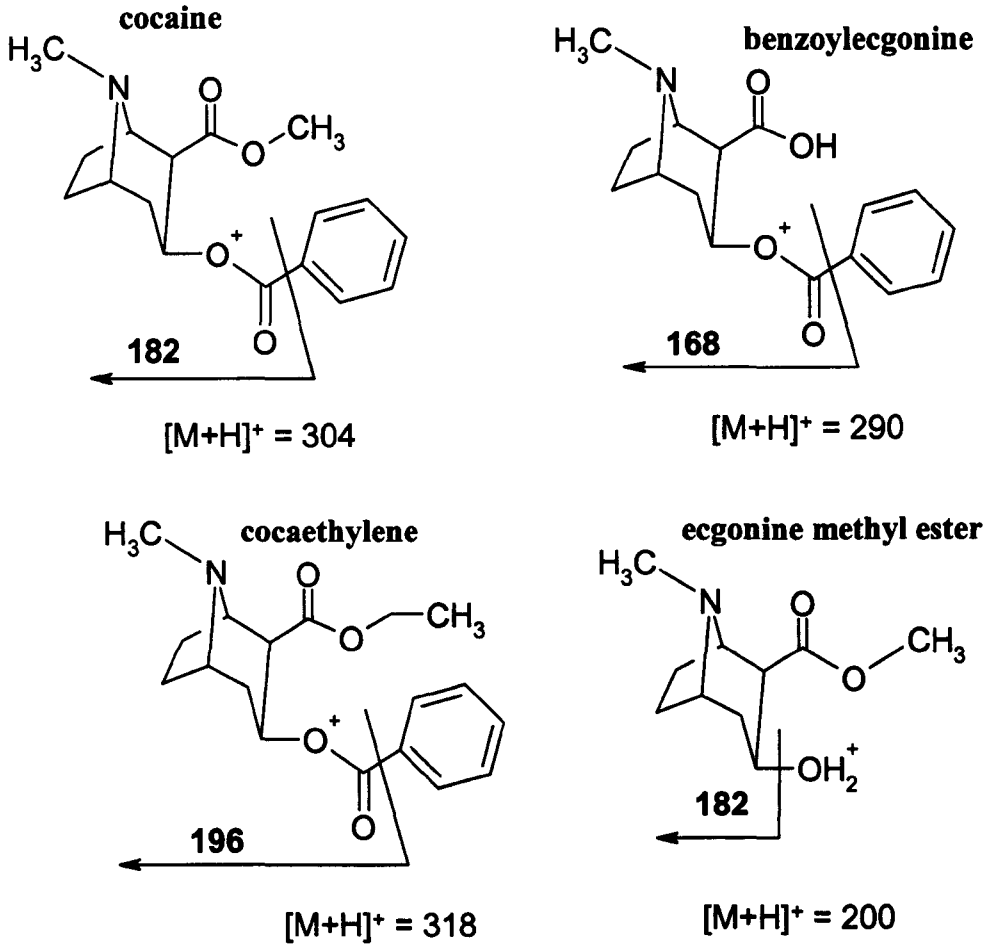



**Figure 8-5 : Proposed fragmentation of cocaine, benzoylecgonine, ecgonine methyl ester and cocaethylene**

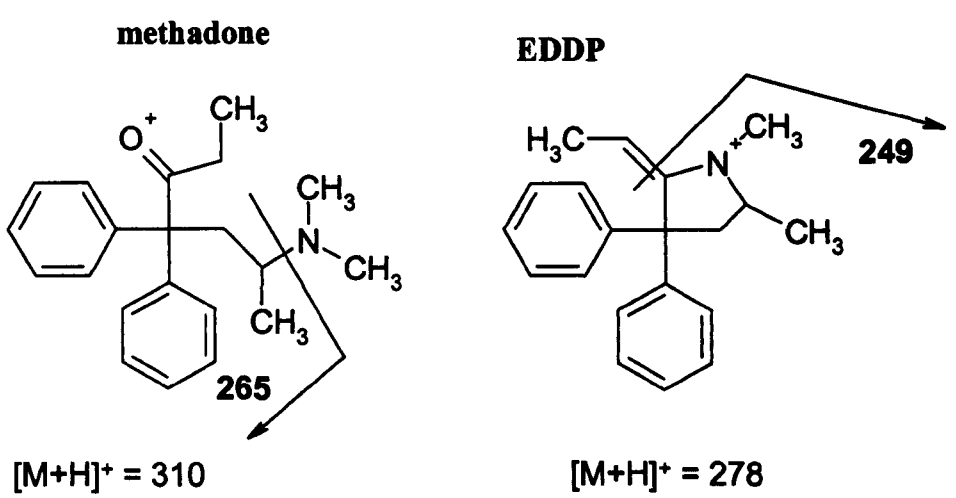



**Figure 8-6 : Proposed fragmentation of methadone and EDDP**

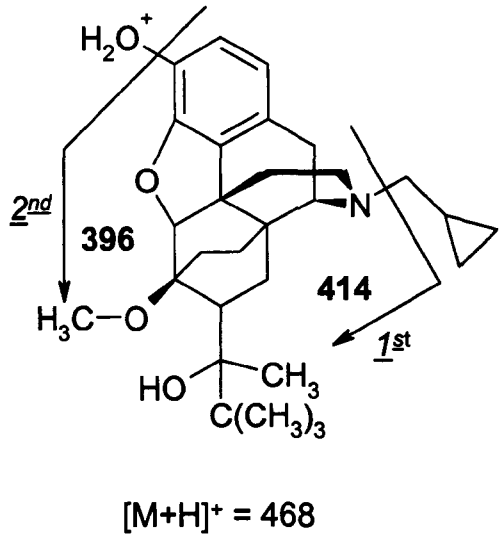



**Figure 8-7 : Proposed fragmentation of buprenorphine**

## *8.3.2 Chromatography Conditions*

It was decided to use mobile phase conditions previously investigated (Chapter 7). This was 3 mM ammonium formate + 0.001 % formic acid, and acetonitrile on a Phenomenex C18 Luna column. Although 10 mM ammonium bicarbonate gave greater response on the signal quadrupole instrument, the analytes peak shape was found to deteriorate quicker than the acidic buffer.

Using the gradient shown in Table 8-3 and a Phenomenex Luna C18 150 x 2.0 mm, 3 μm packing, the retention times for the analytes were determined.

**Table 8-3 : Mobile phase gradient conditions**

| Time (min) | Flow (mL/min) | 3 mM ammonium formate + 0.001 % formic acid (%) | Acetonitrile (%) |
|---|---|---|---|
| 0 | 0.3 | 95 | 5 |
| 13 | 0.3 | 74 | 26 |
| 22 | 0.3 | 20 | 80 |
| 26 | 0.3 | 5 | 95 |
| 29 | 0.3 | 5 | 95 |
| 30 | 0.3 | 95 | 5 |
| 36 | 0.3 | 95 | 5 |

Once retention times were found, analytes could be split up into segments or retention windows. Each segment has different scan events thereby looking for different analytes and their deuterated standards in each segment. It is important to try and minimise the number of scan events in each segment, to maximise the number of data points across the chromatographic peak. It was noted that looking for many ions at the same time, reduced the number of data points across the peak and thereby reducing reproducibility of peak areas. Unfortunately many analytes eluted very close to one another, which resulted in poor reproducibility between injections. To overcome this problem it was decided to inject the sample twice and monitor different analytes each time. Although this increased the total run time on the instrument, reproducibility between injections was much better.

The extraction used was the same as the one used previously in Chapter 7. This produced two fractions. The first fraction was analysed for diazepam and its metabolites in one injection. The second fraction was analysed for the amphetamines, cocaine and its metabolites in one injection and the opioids and metabolites in a second injection. The mobile phase conditions were kept the same for every injection, with only the scan events changed for relevant analytes. The different segments and scan events are detailed in Table 8-4. Full MS-MS data was acquired for analytes and SIM data was acquired for the deuterated standards as further qualitative data was not required for them.

**Table 8-4 : Segments and scan events for each analyte**

| Injection | Analyte | Internal Standard | Segment | Scan Events |
|---|---|---|---|---|
| 1 | Oxazepam | $d_5$ | 1 | Full MS-MS m/z 287<br>SIM m/z 292 |
| 1 | Temazepam | $d_5$ | 2 | Full MS-MS m/z 301 & 271<br>SIM m/z 306 & 276 |
| 1 | Nordiazepam | $d_5$ | | |
| 1 | Diazepam | $d_5$ | 3 | Full MS-MS m/z 285<br>SIM m/z 290 |
| 2 | Ecgonine methyl ester | $d_3$ | 1 | Full MS-MS m/z 200<br>SIM m/z 203 |
| 2 | Amphetamine | $d_5$ | 2 | Full MS-MS m/z 136<br>SIM m/z 141 |

| Injection | Analyte | Internal Standard | Segment | Scan Events |
|---|---|---|---|---|
| 2 | MDA | $d_5$ | 3 | Full MS-MS m/z 180, 150 & 194 SIM m/z 185, 155 & 199 |
| 2 | Methamphetamine | $d_5$ | | |
| 2 | MDMA | $d_5$ | | |
| 2 | MDEA | $d_5$ | 4 | Full MS-MS m/z 208 & 290 SIM m/z 213 & 293 |
| 2 | Benzoylecgonine | $d_3$ | | |
| 2 | Cocaine | $d_3$ | 5 | Full MS-MS m/z 304 SIM m/z 307 |
| 2 | Cocaethylene | $d_3$ | 6 | Full MS-MS m/z 318 SIM m/z 321 |
| 3 | Morphine | $d_3$ | 1 | Full MS-MS m/z 286 SIM m/z 289 |
| 3 | Codeine | $d_3$ | 2 | Full MS-MS m/z 300 & 302 SIM m/z 303 & 308 |
| 3 | Dihydrocodeine | $d_6$ | | |
| 3 | 6-MAM | $d_3$ | 3 | Full MS-MS m/z 328 SIM m/z 321 |
| 3 | Norbuprenorphine | $d_3$ | 4 | Full MS-MS m/z 414 SIM m/z 417 |
| 3 | EDDP | $d_3$ | 5 | Full MS-MS m/z 278 SIM m/z 281 |
| 3 | Methadone | $d_9$ | 6 | Full MS-MS m/z 310 & 468 SIM m/z 319 & 472 |
| 3 | Buprenorphine | $d_4$ | | |

Using the same mobile phase gradient for each injection allowed the queuing up of samples, and therefore the instrument could be run almost continuously.

## 8.4 Method Validation

The sample preparation and extraction method used in conjunction with the LC-MS-MS was the same as in the previous chapter, 7.3.7 and 7.3.8. This extraction method had been used successfully for the analysis of 20 drugs of abuse and their metabolites, and was used

to validate the LC-MS-MS method for 21 drugs of abuse and metabolites extracted from 1 mL oral fluid.

### 8.4.1 Linearity

Linearity was demonstrated over the range 1 – 200 ng/mL. All analytes gave a correlation coefficient > 0.99. Figure 8-8 shows examples of linearity data obtained for 5 drugs and Table 8-5 shows linearity equations and correlation coefficient data for all analytes.

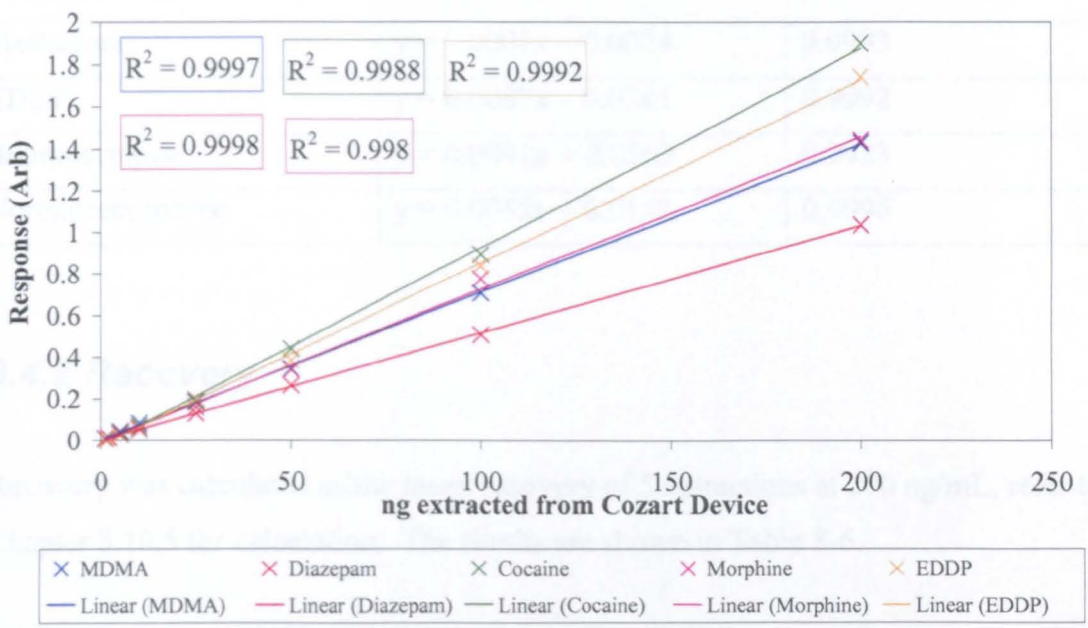



**Figure 8-8 : Linearity graphs for MDMA, diazepam, cocaine, morphine and EDDP**

**Table 8-5 : Linearity data for all 21 drugs of abuse and their metabolites**

| Analyte | Linearity Equation | $R^2$ value |
|---|---|---|
| Amphetamine | y = 0.0068x – 0.014 | 0.9956 |
| Methamphetamine | y = 0.0066x + 0.0136 | 0.9947 |
| MDMA | y = 0.0071x + 0.0032 | 0.9997 |
| MDEA | y = 0.0063x + 0.0039 | 0.9934 |
| MDA | y = 0.0057x – 0.02 | 0.9978 |
| Diazepam | y = 0.0052x + 0.0007 | 0.9998 |
| N-desmethyldiazepam | y = 0.0051x + 0.006 | 0.9994 |
| Temazepam | y = 0.0044x + 0.0029 | 0.9986 |

| Analyte | Linearity Equation | $R^2$ value |
|---|---|---|
| Oxazepam | y = 0.0056x – 0.0138 | 0.9982 |
| Cocaine | y = 0.0095x – 0.0268 | 0.9988 |
| Benzoylecgonine | y = 0.0085x – 0.0094 | 0.9989 |
| Cocaethylene | y = 0.0067x – 0.0342 | 0.9974 |
| Ecgonine methyl ester | y = 0.0054x – 0.0119 | 0.9953 |
| Morphine | y = 0.0073x – 0.0057 | 0.998 |
| Codeine | y = 0.0079x – 0.0151 | 0.9986 |
| 6-monoacetylmorphine | y = 0.0062x + 0.0009 | 0.9969 |
| Dihydrocodeine | y = 0.0078x + 0.0524 | 0.9906 |
| Methadone | y = 0.0007x – 0.0024 | 0.9993 |
| EDDP | y = 0.0087x – 0.0241 | 0.9992 |
| Buprenorphine | y = 0.0091x + 0.0569 | 0.9923 |
| Norbuprenorphine | y = 0.0082x – 0.0142 | 0.9996 |

### 8.4.2 Recovery

Recovery was calculated as the mean recovery of 5 extractions at 200 ng/mL, refer to Chapter 3.10.5 for calculation. The results are shown in Table 8-6.

### 8.4.3 Limits of Detection

Limits of detection were calculated as before in Chapter 3.10.6. This was using 3 times the standard error of the line plus the intercept. The results are shown in Table 8-6.

### 8.4.4 Limits of Quantitation

Limits of quantitation were calculated as before in Chapter 3.10.7. This was using 10 times the standard error of the line plus the intercept. The results are shown in Table 8-6.

**Table 8-6 : Recovery, LOD and LOQ data**

| Analyte | Mean % Recovery (% RSD) (n=5) | LOD (ng/mL) | LOQ (ng/mL) |
|---|---|---|---|
| Amphetamine | 75 (8.8) | 0.4 | 1.2 |
| Methamphetamine | 100 (6.4) | 0.4 | 1.3 |
| MDA | 74 (8.3) | 1.0 | 3.3 |
| MDMA | 98 (14.8) | 0.8 | 2.5 |
| MDEA | 80 (12.4) | 1.2 | 4.0 |
| Diazepam | 65 (5.8) | 0.3 | 0.9 |
| N-desmethyldiazepam | 57 (4.0) | 0.4 | 1.2 |
| Temazepam | 109 (11.0) | 0.5 | 1.6 |
| Oxazepam | 77 (3.3) | 0.8 | 2.5 |
| Cocaine | 89 (2.1) | 0.9 | 3.1 |
| Benzoylecgonine | 63 (12.4) | 3.4 | 11.4 |
| Ecgonine methyl ester | 30 (6.3) | 0.3 | 0.9 |
| Cocaethylene | 95 (7.0) | 1.0 | 3.4 |
| Morphine | 52 (7.3) | 0.5 | 1.7 |
| 6-MAM | 93 (2.6) | 0.3 | 1.0 |
| Codeine | 98 (7.4) | 0.5 | 1.8 |
| Dihydrocodeine | 92 (5.3) | 0.5 | 1.7 |
| Methadone | 96 (9.0) | 0.7 | 2.2 |
| EDDP | 95 (2.7) | 0.2 | 0.8 |
| Buprenorphine | 109 (10.7) | 0.5 | 1.8 |
| Norbuprenorphine | 91 (1.8) | 0.5 | 1.6 |

### *8.4.5 Inter-Day Precision*

Inter-day precision was calculated using five sets of linearity data acquired on five different days. The %RSD of the five values for two concentrations 25 and 200 ng/mL were calculated. These results are shown in Table 8-7.

**Table 8-7 : Inter-day precision**

| Analyte | Inter-day precision (% RSD) (n=5) | |
|---|---|---|
| | 25 ng | 200 ng |
| Amphetamine | 25 (5.8) | 201 (0.9) |
| Methamphetamine | 26 (12.0) | 200 (0.8) |
| MDA | 26 (6.9) | 202 (1.2) |
| MDMA | 26 (9.0) | 201 (1.9) |
| MDEA | 24 (3.0) | 199 (2.8) |
| Diazepam | 25 (2.7) | 198 (0.7) |
| N-desmethyldiazepam | 26 (10.0) | 200 (1.2) |
| Temazepam | 23 (8.2) | 196 (2.4) |
| Oxazepam | 24 (6.3) | 202 (1.6) |
| Cocaine | 22 (2.9) | 202 (0.4) |
| Benzoylecgonine | 24 (13.8) | 198 (1.7) |
| Ecgonine methyl ester | 24 (11.3) | 200 (1.4) |
| Cocaethylene | 23 (2.6) | 204 (1.2) |
| Morphine | 25 (5.0) | 202 (1.5) |
| 6-MAM | 23 (3.5) | 200 (0.6) |
| Codeine | 25 (2.6) | 202 (0.7) |
| Dihydrocodeine | 27 (5.7) | 199 (1.9) |
| Methadone | 23 (12.2) | 208 (2.2) |
| EDDP | 25 (3.5) | 201 (1.5) |
| Buprenorphine | 25 (8.3) | 201 (1.5) |
| Norbuprenorphine | 24 (3.3) | 201 (0.6) |

### *8.4.6 Intra-Day Precision between Injections*

Extracted standards were prepared at two concentrations 25 and 200 ng/mL. They were injected 5 times consecutively. The %RSD of the peak area ratios were calculated at each concentration for each drug. These results are shown in Table 8-8.

**Table 8-8 : Intra-day precision between 5 consecutive injections**

| Analyte | Intra-day precision between 5 injections (% RSD) (n=5) | |
|---|---|---|
| | 25 ng | 200 ng |
| Amphetamine | 27 (6.0) | 208 (3.4) |
| Methamphetamine | 24 (12.4) | 199 (4.7) |
| MDA | 25 (8.9) | 198 (8.6) |
| MDMA | 25 (8.0) | 202 (1.8) |
| MDEA | 27 (2.6) | 202 (1.2) |
| Diazepam | 26 (4.0) | 203 (2.1) |
| N-desmethyldiazepam | 29 (6.6) | 197 (9.8) |
| Temazepam | 23 (7.6) | 196 (6.9) |
| Oxazepam | 28 (3.8) | 204 (3.1) |
| Cocaine | 25 (3.0) | 207 (3.0) |
| Benzoylecgonine | 23 (5.4) | 200 (6.0) |
| Ecgonine methyl ester | 25 (2.8) | 200 (0.9) |
| Cocaethylene | 25 (1.0) | 201 (2.1) |
| Morphine | 22 (6.0) | 199 (5.7) |
| 6-MAM | 24 (6.4) | 209 (3.8) |
| Codeine | 22 (10.5) | 209 (2.0) |
| Dihydrocodeine | 24 (5.8) | 199 (2.8) |
| Methadone | 22 (12.7) | 193 (4.3) |
| EDDP | 24 (6.0) | 198 (1.4) |
| Buprenorphine | 28 (7.9) | 202 (5.0) |
| Norbuprenorphine | 21 (5.1) | 206 (2.2) |

## *8.4.7 Intra-Day Precision between Extracts*

Five extracted standards at two concentrations 25 and 200 ng/mL were prepared simultaneously. The %RSD of the peak area ratios were calculated for each extraction, at each concentration, for each drug. These results are shown in Table 8-9.

**Table 8-9 : Intra-day precision between 5 extracts**

| Analyte | Intra-day precision between 5 extracts (% RSD) (n=5) | |
|---|---|---|
| | 25 ng | 200 ng |
| Amphetamine | 24 (8.9) | 201 (9.5) |
| Methamphetamine | 19 (10.4) | 199 (3.6) |
| MDA | 18 (10.2) | 206 (7.7) |
| MDMA | 19 (12.4) | 200 (8.4) |
| MDEA | 28 (5.7) | 203 (5.2) |
| Diazepam | 29 (4.3) | 203 (2.7) |
| N-desmethyldiazepam | 24 (5.9) | 201 (10.3) |
| Temazepam | 23 (6.4) | 201 (1.8) |
| Oxazepam | 26 (6.4) | 201 (4.2) |
| Cocaine | 24 (2.8) | 201 (4.2) |
| Benzoylecgonine | 19 (19.7) | 209 (9.1) |
| Ecgonine methyl ester | 24 (7.2) | 200 (3.7) |
| Cocaethylene | 24 (6.2) | 201 (5.6) |
| Morphine | 27 (5.1) | 200 (2.0) |
| 6-MAM | 26 (3.4) | 203 (3.1) |
| Codeine | 27 (9.4) | 202 (5.8) |
| Dihydrocodeine | 23 (5.5) | 199 (3.7) |
| Methadone | 33 (11.4) | 203 (8.0) |
| EDDP | 28 (2.9) | 202 (1.9) |
| Buprenorphine | 25 (10.4) | 199 (8.2) |
| Norbuprenorphine | 27 (8.5) | 203 (6.3) |

## 8.5 Discussion

Linearity was demonstrated over 1 – 200 ng/mL for all analytes. Although some recoveries were not as high as expected, all limits of detection were below 3.4 ng/mL. Limits of detection were similar to those found using a single quadrupole, showing there is little difference in the sensitivity of the two analysers.

To obtain a %RSD of less than 10% at 200ng/mL for repeat injections, three injections of each sample were needed. Although this increased the total run time, 21 drugs of abuse and their metabolites were analysed from one extract of 1mL of sample. A variation of 20% is generally considered acceptable[129]. Effectively it is instrument time, not analysts time that is the rate determining step.

## 8.6 Conclusions

The developed method successfully extracted and analysed 21 drugs of abuse and their metabolites. The ion trap analyser proved invaluable in producing greater specificity than a single quadrupole. This greater specificity allowed the use of this technique alone, without confirmation from a GC-MS.

This method was successfully applied to 1396 oral fluid samples donated from a random selection of drivers in and around the city of Glasgow. Refer to Chapter 10.3.

# 9 Comparison of Oral Fluid Collection Devices for the Detection of Drugs of Abuse and Their Metabolites

## 9.1 Introduction

The advantages of oral fluid analysis have been discussed in Chapter 6. This project involved the collection of various biological matrices from opiate dependent subjects while attending a residential crisis centre. Presently, urine analysis is used to determine opiate use when the patients are first admitted. Urine concentrations are accumulated over a period of time and are not a true reflection of what is circulating in the body at the time of collection. Another problem of urine analysis is the potential for adulteration if not passed under supervision. Blood would give circulating concentrations of drugs, but poses the problem of a medical practitioner taking the sample, risk of infection and with respect to opiate addicts, difficulty in obtaining a sample.

Oral fluid analysis could solve some of these problems. It can be taken by a non-medically trained care worker, it would give information on the drugs circulating in the body at the time of collection and it is a much more acceptable body fluid to donate.

## 9.2 Aim

The aim of this project was to analyse a variety of sample matrices, namely urine, blood and oral fluid. Two oral fluid samples were collected, one using the modified Omni-Sal® device and another using the Intercept® collection device. The modified Omni-Sal® device was used first followed by the Intercept device to prevent the salts present in the Intercept® device affecting the modified Omni-Sal® device. The samples to be collected were taken back to the laboratory for opiate and benzodiazepine analysis using LC-MS-MS, there was no initial screening test carried out. An extraction method used previously was employed.

## 9.3 Chemicals

Methanol and acetonitrile were HPLC grade obtained from VWR International (UK). Analytical grade potassium dihydrogen phosphate, ammonium formate were supplied by BDH laboratory supplies (Poole, England). Analytical grade concentrated ammonia, glacial acetic acid and concentrated formic acid were purchased from Sigma® Chemicals Co. (UK).

## 9.4 Standards

Stock standards of analytes were purchased from Promochem as 100 μg/mL methanolic solutions. The deuterated standards of each drug were also purchased as 100 μg/mL methanolic solutions. The analyte and deuterated standards used are shown in Table 9-1. A working drug mixture with a concentration of 1 μg/mL for each drug, was prepared by taking 250 μL of each standard into a 25 mL volumetric flask and making up to volume with methanol. A working internal standard solution was prepared in the same way using the deuterated standards.

**Table 9-1 : Purchased standards and deuterated standards**

| Analyte | Deuterated Standard |
|---|---|
| Diazepam | Diazepam-$d_5$ |
| N-desmethyldiazepam | N-desmethyldiazepam-$d_5$ |
| Temazepam | Temazepam-$d_5$ |
| Oxazepam | Oxazepam-$d_5$ |
| Morphine | Morphine-$d_3$ |
| Codeine | Codeine-$d_3$ |
| 6-MAM | 6-MAM-$d_3$ |
| Dihydrocodeine | Dihydrocodeine-$d_6$ |

## 9.5 Spiked urine, blood and oral fluid samples

Time expired packed red blood cells were provided from the blood bank for blank blood. These were suspended in a ratio of 1:1 with isotonic saline solution which was made by dissolving 9.5 g of sodium chloride in 1 L of deionised water.

Drug free urine and oral fluid, collected using the two different devices, was obtained from healthy volunteers.

## 9.6 Solutions

### *9.6.1 Preparation of 0.1 M, pH 6.0 Phosphate Buffer*

6.81 g of potassium dihydrogen phosphate was weighed out into a 500 mL volumetric flask and 450 mL of deionised water was added. The pH was adjusted to 6.0 with 1 M potassium hydroxide solution, then made to 500 mL with deionised water.

### *9.6.2 Preparation of 3 mM Ammonium Formate with 0.001 % Formic Acid*

0.189 g of ammonium formate was placed into a 1 L volumetric flask with 10 µL concentrated formic acid. This was made up to volume with deionised water.

## 9.7 LC-MS-MS Conditions

### *9.7.1 LC Conditions*

The column used for analysis was a Phenomenex Luna C18, 150 x 2.0 mm, 3 µm packing. A guard column packed with identical stationary phase was used prior to the column to extend column lifetime. Mobile phase consisted of 3 mM ammonium formate + 0.001 % formic acid and acetonitrile. The gradient conditions are shown in Table 9-2. The column oven was maintained at 35 °C throughout the analysis.

**Table 9-2 : Mobile phase conditions**

| Time (min) | Flow (mL/min) | 3 mM ammonium formate + 0.001 % formic acid (%) | Acetonitrile (%) |
|---|---|---|---|
| 0 | 0.3 | 95 | 5 |
| 13 | 0.3 | 74 | 26 |
| 22 | 0.3 | 20 | 80 |
| 26 | 0.3 | 5 | 95 |
| 29 | 0.3 | 5 | 95 |
| 30 | 0.3 | 95 | 5 |
| 36 | 0.3 | 95 | 5 |

### *9.7.2 MS Conditions*

Full MS-MS data was collected for each analyte, while deuterated internal standards were monitored using SIM analysis. All mass spectral data was collected in electrospray positive ion mode. The following settings were applied throughout the analysis: transfer capillary temperature, 280 °C; probe voltage, 4.5 kV; sheath and auxiliary gases were set to 20 and 15 units on the data system arbitrary scale.

## 9.8 Method Validation for Urine Specimens

Spiked urine samples were prepared by adding the appropriate amount of working drug solution (5, 10, 25, 50, 100, 200 µL) and internal standard solution (100 µL) to test tubes, followed by 3.5 mL of 0.1 M, pH 6 phosphate buffer and 1 mL of blank urine. A blank was prepared without any standards present and another with internal standard only to assess any contamination of the deuterated standards.

The extraction method was the same as that used in Chapter 7.3.6. 1 mL of urine was added to 3.5 mL of phosphate buffer (0.1 M, pH 6.0) and 100 ng of internal standard mixture, before vortex mixing and centrifuging. The solid phase extraction procedure involved conditioning Bond Elut Certify® columns with 2 mL methanol and 2 mL phosphate buffer (0.1 M, pH 6) before the sample was applied. 2 mL of deionised water, followed by 0.5 mL of 0.01 M acetic acid was applied before drying under full vacuum for 10 minutes. 50 µL of methanol was then applied before drying once more for 2 minutes.

Diazepam and its metabolites were eluted using 4 mL of acetone : chloroform 1:1. Opiates were eluted using 2 x 1.5 mL ethyl acetate with 2 % ammonia. Each fraction was blown down under nitrogen at 35 °C until dryness. Samples were reconstituted in 80 µL of mobile phase, before 20 µL was injected onto the LC-MS-MS.

There were two fractions for each sample, and these were injected separately onto the LC-MS-MS. The first fraction contained diazepam, N-desmethyldiazepam, temazepam and oxazepam. The second fraction contained morphine, 6-MAM, codeine and dihydrocodeine.

## 9.8.1 Linearity

Linearity was demonstrated over 5 – 200 ng/mL for each analyte.

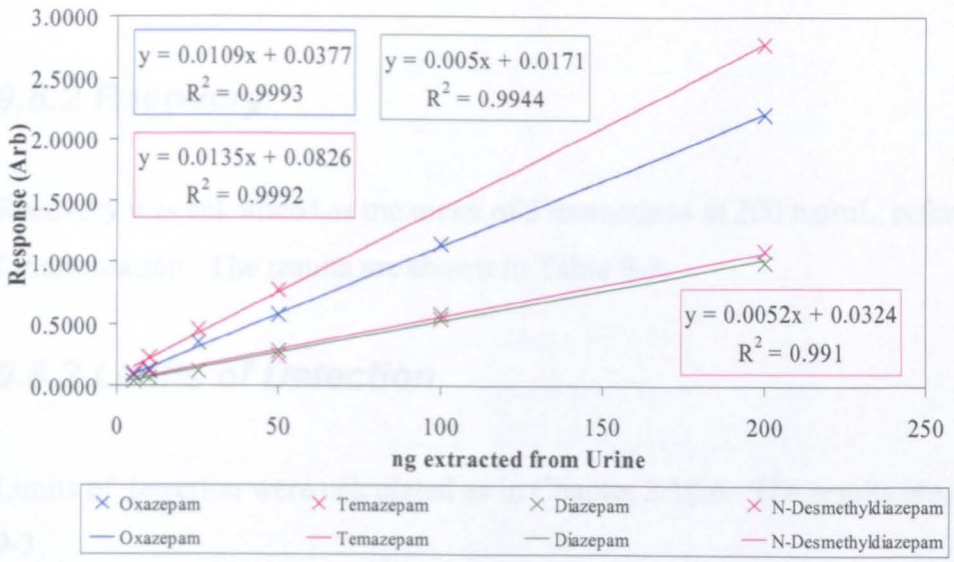



**Figure 9-1 : Linearity of diazepam and metabolites in urine**

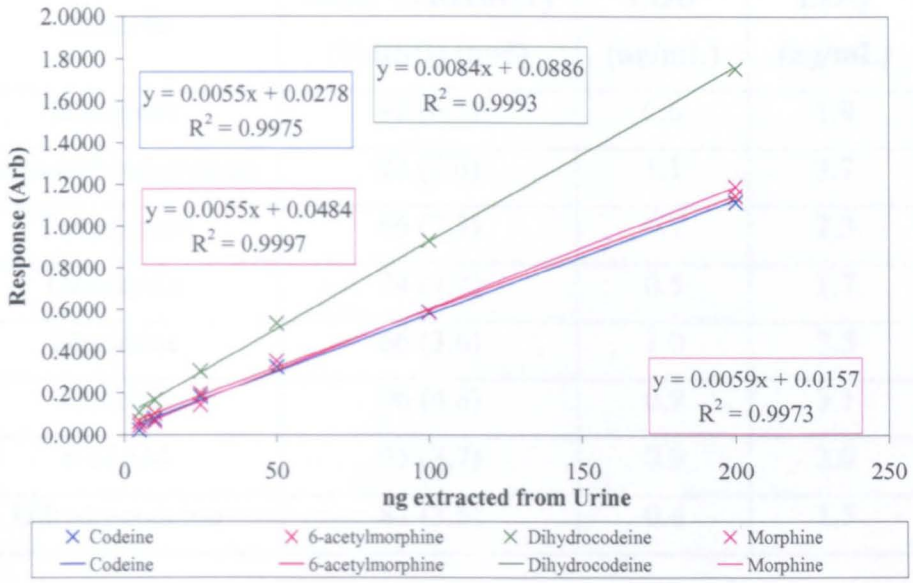



**Figure 9-2 : Linearity of morphine, codeine, 6-MAM and dihydrocodeine in urine**

## 9.8.2 Recovery

Recovery was calculated as the mean of 5 extractions at 200 ng/mL, refer to Chapter 3.10.5 for calculation. The results are shown in Table 9-3.

## 9.8.3 Limits of Detection

Limits of detection were calculated as in Chapter 3.10.6. The results are shown in Table 9-3

## 9.8.4 Limits of Quantitation

Limits of quantitation were calculated as in Chapter 3.10.7. The results are shown in Table 9-3.

**Table 9-3 : Recovery, LOD and LOQ for urine specimens**

| Analyte | Mean % Recovery (%RSD) (n=5) | LOD (ng/mL) | LOQ (ng/mL) |
|---|---|---|---|
| Diazepam | 92 (6.5) | 0.6 | 1.9 |
| N-desmethyldiazepam | 92 (5.6) | 1.1 | 3.7 |
| Temazepam | 66 (7.3) | 0.7 | 2.3 |
| Oxazepam | 94 (4.7) | 0.5 | 1.7 |
| Morphine | 66 (3.6) | 1.0 | 3.5 |
| Codeine | 96 (4.8) | 0.9 | 3.1 |
| 6-MAM | 95 (3.7) | 0.9 | 3.0 |
| Dihydrocodeine | 81 (3.5) | 0.4 | 1.5 |

## *9.8.5 Inter-Day Precision*

Inter-day precision was calculated using five sets of linearity data acquired on five different days. The %RSD of the five values for two concentrations, 50 and 200 ng/mL were calculated. The results are shown in Table 9-4.

**Table 9-4 : Inter-day precision for urine specimens**

| Analyte | Inter-day precision (%RSD) (n=5) | |
|---|---|---|
| | 50 ng | 200 ng |
| Diazepam | 50 (5.8) | 198 (1.2) |
| N-desmethyldiazepam | 46 (12.8) | 201 (1.4) |
| Temazepam | 50 (11.5) | 200 (0.1) |
| Oxazepam | 51 (3.4) | 200 (0.8) |
| Morphine | 55 (3.9) | 199 (0.3) |
| Codeine | 56 (2.3) | 197 (0.9) |
| 6-MAM | 57 (7.1) | 199 (0.4) |
| Dihydrocodeine | 57 (1.4) | 199 (1.4) |

### 9.8.6 Intra-Day Precision between Injections

Extracted standards were prepared at two concentrations, 50 and 200 ng/mL. They were injected 5 times consecutively. The %RSD of the peak area ratios were calculated at each concentration for each drug. The results are shown in Table 9-5.

**Table 9-5 : Intra-day precision between injections for urine specimens**

| Analyte | Intra-day precision 5 injections (%RSD) (n=5) | |
|---|---|---|
| | 50 ng | 200 ng |
| Diazepam | 49 (2.9) | 199 (1.5) |
| N-desmethyldiazepam | 56 (6.4) | 203 (1.4) |
| Temazepam | 55 (2.3) | 202 (3.4) |
| Oxazepam | 51 (4.0) | 200 (3.2) |
| Morphine | 54 (3.0) | 199 (3.2) |
| Codeine | 45 (3.3) | 200 (3.7) |
| 6-MAM | 56 (3.2) | 200 (1.6) |
| Dihydrocodeine | 49 (1.9) | 199 (2.5) |

### 9.8.7 Intra-Day Precision between Extracts

Five extracted standards at two concentrations, 50 and 200 ng/mL, were prepared simultaneously. The %RSD of the peak area ratios were calculated for each extraction, at each concentration, for each drug. The results are shown in Table 9-6.

**Table 9-6 : Intra-day precision between extracts for urine specimens**

| Analyte | Intra-day precision 5 extracts (%RSD) (n=5) | |
|---|---|---|
| | 50 ng | 200 ng |
| Diazepam | 51 (4.1) | 199 (4.9) |
| N-desmethyldiazepam | 50 (7.1) | 198 (8.5) |
| Temazepam | 47 (6.0) | 199 (9.8) |
| Oxazepam | 50 (2.7) | 201 (3.9) |
| Morphine | 51 (4.5) | 194 (2.6) |
| Codeine | 49 (6.3) | 199 (7.7) |
| 6-MAM | 49 (4.1) | 196 (2.9) |
| Dihydrocodeine | 50 (2.3) | 199 (1.6) |

## 9.9 Method Validation for Whole Blood Specimens

The same solid phase extraction method described in 9.8 was used with 1 mL of whole blood.

### *9.9.1 Linearity*

Linearity was demonstrated over 5 – 200 ng/mL for each analyte.

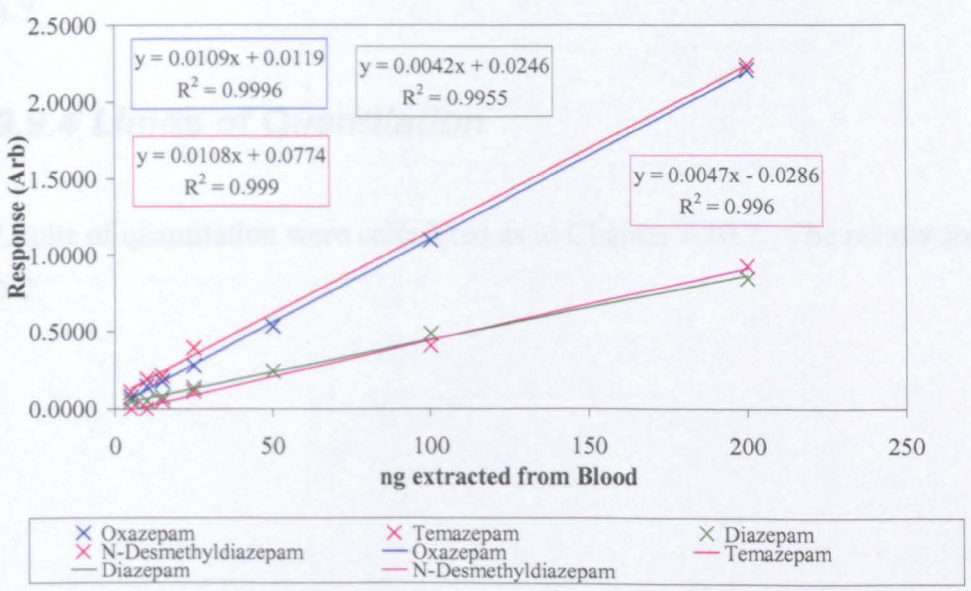



**Figure 9-3 : Linearity of diazepam and metabolites in whole blood**

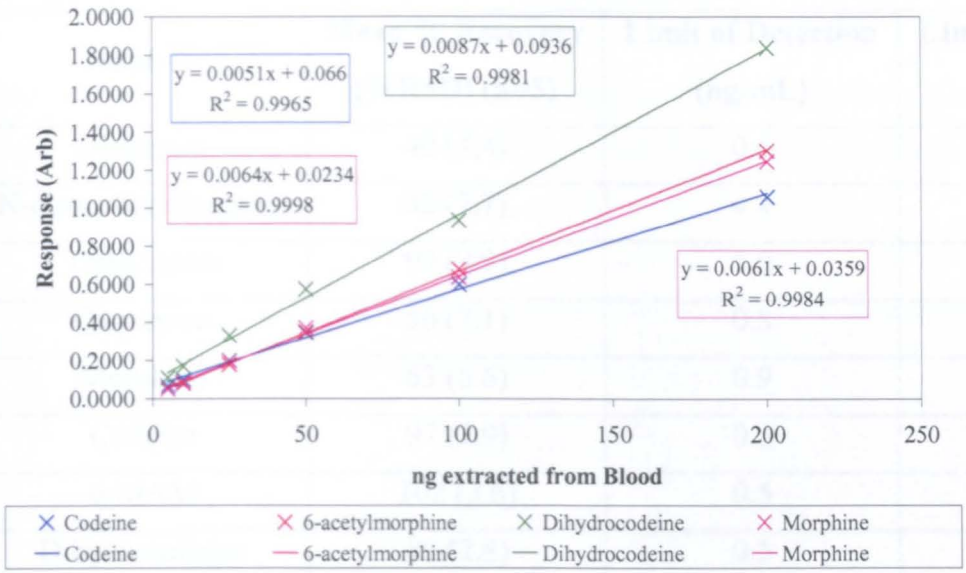



**Figure 9-4 : Linearity of morphine, codeine, 6-MAM and dihydrocodeine in whole blood**

## 9.9.2 Recovery

Recovery was calculated as the mean of 5 extractions at 200 ng/mL, refer to Chapter 3.10.5 for calculation. The results are shown in Table 9-7.

## 9.9.3 Limits of Detection

Limits of detection were calculated as in Chapter 3.10.6. The results are shown in Table 9-7.

## 9.9.4 Limits of Quantitation

Limits of quantitation were calculated as in Chapter 3.10.7. The results are shown in Table 9-7.

**Table 9-7 : Recovery, LOD and LOQ for whole blood specimens**

| Analyte | Mean % Recovery (%RSD) (n=5) | Limit of Detection (ng/mL) | Limit of Quantitation (ng/mL) |
|---|---|---|---|
| Diazepam | 40 (4.4) | 0.6 | 2.0 |
| N-desmethyldiazepam | 32 (7.7) | 4.4 | 14.7 |
| Temazepam | 99 (9.9) | 1.0 | 3.4 |
| Oxazepam | 56 (7.1) | 0.8 | 2.7 |
| Morphine | 63 (6.6) | 0.9 | 3.1 |
| Codeine | 97 (3.9) | 0.8 | 2.6 |
| 6-MAM | 102 (3.6) | 0.5 | 1.8 |
| Dihydrocodeine | 83 (2.8) | 0.5 | 1.5 |

## *9.9.5 Inter-Day Precision*

Inter-day precision was calculated using five sets of linearity data acquired on five different days. The %RSD of the five values for two concentrations, 50 and 200 ng/mL were calculated. The results are shown in Table 9-8.

**Table 9-8 : Inter-day precision for whole blood specimens**

| Analyte | Inter-day precision (%RSD) (n=5) | |
|---|---|---|
| | 50 ng | 200 ng |
| Diazepam | 53 (1.9) | 199 (1.1) |
| N-desmethyldiazepam | 55 (28.1) | 203 (2.8) |
| Temazepam | 46 (7.2) | 201 (1.3) |
| Oxazepam | 50 (5.1) | 201 (0.7) |
| Morphine | 52 (8.1) | 201 (1.5) |
| Codeine | 52 (7.7) | 197 (1.4) |
| 6-MAM | 54 (9.0) | 199 (0.6) |
| Dihydrocodeine | 57 (6.7) | 195 (0.6) |

### 9.9.6 Intra-Day Precision between Injections

Extracted standards were prepared at two concentrations, 50 and 200 ng/mL. They were injected 5 times consecutively. The %RSD of the peak area ratios was calculated at each concentration for each drug. The results are shown in Table 9-9.

**Table 9-9 : Intra-day precision between injections for whole blood specimens**

| Analyte | Intra-day precision 5 injections (%RSD) (n=5) | |
|---|---|---|
| | **50 ng** | **200 ng** |
| Diazepam | 51 (6.7) | 201 (4.0) |
| N-desmethyldiazepam | 50 (12.2) | 199 (8.8) |
| Temazepam | 49 (5.8) | 197 (3.7) |
| Oxazepam | 47 (4.6) | 201 (3.7) |
| Morphine | 55 (2.6) | 200 (1.3) |
| Codeine | 53 (2.0) | 199 (2.9) |
| 6-MAM | 53 (3.5) | 198 (1.9) |
| Dihydrocodeine | 54 (1.0) | 201 (2.4) |

### 9.9.7 Intra-Day Precision between Extracts

Five extracted standards at two concentrations, 50 and 200 ng/mL, were prepared simultaneously. The %RSD of the peak area ratios were calculated for each extraction, at each concentration, for each drug. The results are shown in Table 9-10.

**Table 9-10 : Intra-day precision between extracts for whole blood specimens**

| Analyte | Intra-day precision 5 extracts (%RSD) (n=5) | |
|---|---|---|
| | 50 ng | 200 ng |
| Diazepam | 53 (3.3) | 201 (1.0) |
| N-desmethyldiazepam | 50 (8.4) | 201 (6.8) |
| Temazepam | 50 (7.8) | 199 (5.1) |
| Oxazepam | 49 (3.5) | 201 (1.5) |
| Morphine | 52 (2.9) | 198 (3.7) |
| Codeine | 50 (3.5) | 201 (2.7) |
| 6-MAM | 50 (2.8) | 199 (2.5) |
| Dihydrocodeine | 50 (2.5) | 200 (2.9) |

## 9.10 Method Validation for the Intercept® Device

The same solid phase extraction method described in 9.8 was used with 0.5 mL of the oral fluid:buffer mixture obtained from the device.

### *9.10.1 Linearity*

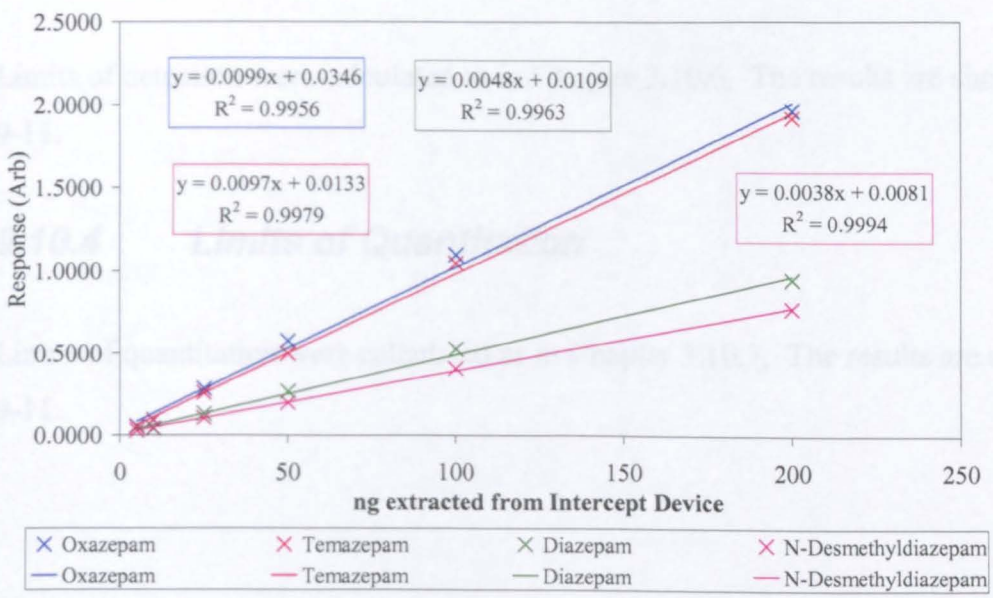



**Figure 9-5 : Linearity for diazepam and metabolites for the Intercept® collection device**

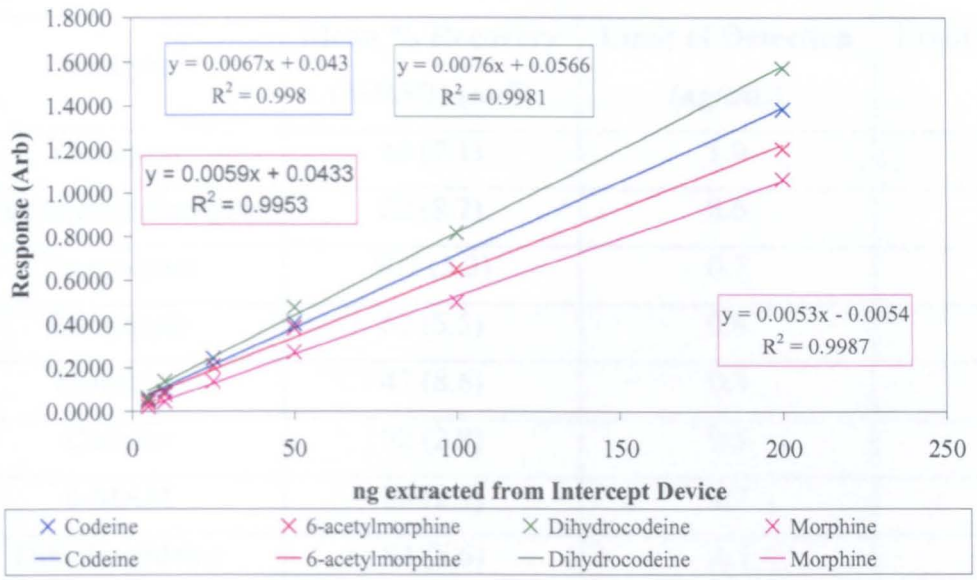



**Figure 9-6 : Linearity for morphine, codeine, 6-MAM and dihydrocodeine for the Intercept® collection device**

## 9.10.2 Recovery

Recovery was calculated as the mean of 5 extractions at 200 ng/mL. The results are shown in Table 9-11.

## 9.10.3 Limits of Detection

Limits of detection were calculated as in Chapter 3.10.6. The results are shown in Table 9-11.

## 9.10.4 Limits of Quantitation

Limits of quantitation were calculated as in Chapter 3.10.7. The results are shown in Table 9-11.

**Table 9-11 : Recovery, LOD and LOQ for the Intercept® collection device**

| Analyte | Mean % Recovery (%RSD) (n=5) | Limit of Detection (ng/mL) | Limit of Quantitation (ng/mL) |
|---|---|---|---|
| Diazepam | 46 (7.1) | 1.9 | 6.2 |
| N-desmethyldiazepam | 22 (8.7) | 8.6 | 28.6 |
| Temazepam | 103 (5.3) | 0.7 | 2.5 |
| Oxazepam | 67 (5.5) | 0.6 | 2.0 |
| Morphine | 47 (8.6) | 0.4 | 1.4 |
| Codeine | 92 (2.9) | 0.5 | 1.7 |
| 6-MAM | 86 (2.9) | 0.7 | 2.5 |
| Dihydrocodeine | 84 (5.6) | 0.3 | 1.1 |

## *9.10.5 Inter-Day Precision*

Inter-day precision was calculated using five sets of linearity data acquired on five different days. The %RSD of the five values for two concentrations, 50 and 200 ng/mL were calculated. The results are shown in Table 9-12.

**Table 9-12 : Inter-day precision for the Intercept® collection device**

| Analyte | Inter-day precision (%RSD) (n=5) | |
|---|---|---|
| | 50 ng | 200 ng |
| Diazepam | 51 (0.5) | 200 (1.2) |
| N-desmethyldiazepam | 47 (5.5) | 202 (0.8) |
| Temazepam | 50 (9.3) | 202 (1.2) |
| Oxazepam | 50 (0.2) | 201 (2.4) |
| Morphine | 53 (3.2) | 199 (0.3) |
| Codeine | 49 (12.4) | 198 (0.2) |
| 6-MAM | 52 (6.1) | 201 (0.3) |
| Dihydrocodeine | 53 (9.6) | 198 (0.3) |

### 9.10.6 Intra-Day Precision between Injections

Extracted standards were prepared at two concentrations, 50 and 200 ng/mL. They were injected 5 times consecutively. The %RSD of the peak area ratios was calculated at each concentration for each drug. The results are shown in Table 9-13.

**Table 9-13 : Intra-day precision between injections for Intercept® collection device**

| Analyte | Intra-day precision 5 injections (%RSD) (n=5) | |
|---|---|---|
| | 50 ng | 200 ng |
| Diazepam | 50 (4.6) | 199 (1.2) |
| N-desmethyldiazepam | 54 (10.7) | 203 (3.7) |
| Temazepam | 55 (12.9) | 204 (3.3) |
| Oxazepam | 51 (3.6) | 200 (2.8) |
| Morphine | 52 (1.4) | 200 (5.3) |
| Codeine | 52 (3.7) | 200 (4.6) |
| 6-MAM | 50 (9.2) | 201 (9.8) |
| Dihydrocodeine | 50 (9.5) | 199 (5.2) |

### 9.10.7 Intra-Day Precision between Extracts

Five extracted standards at two concentrations, 50 and 200 ng/mL, were prepared simultaneously. The %RSD of the peak area ratios were calculated for each extraction, at each concentration, for each drug. The results are shown in Table 9-14.

**Table 9-14 : Intra-day precision between extracts for Intercept® collection device**

| Analyte | Intra-day precision 5 extracts (%RSD) (n=5) | |
|---|---|---|
| | 50 ng | 200 ng |
| Diazepam | 49 (7.0) | 200 (3.6) |
| N-desmethyldiazepam | 48 (7.0) | 201 (7.1) |
| Temazepam | 53 (9.7) | 198 (5.0) |
| Oxazepam | 49 (3.3) | 200 (2.9) |
| Morphine | 45 (9.7) | 199 (5.6) |
| Codeine | 54 (3.8) | 200 (3.5) |
| 6-MAM | 50 (2.7) | 198 (3.7) |
| Dihydrocodeine | 53 (8.7) | 199 (1.6) |

## 9.11 Discussion

During the course of this project the same extraction and LC-MS method was validated for each matrix analysed. When using LC-MS the matrix has an important role to play in the ionisation of analytes, it can enhance or suppress ionisation, recognized as the "matrix effect". By using an extensive extraction technique such as solid phase extraction, the analyte is retained while much of the matrix is lost in the clean up steps. However, some constituents of the matrix will always be retained and go on to effect the recovery of analytes and hence limits of detection. It has been shown by one group that solid phase extraction offers cleaner extracts and was preferred for plasma analysis of morphine, while protein precipitation was sufficient for oral fluid analysis, and sample dilution was suitable for the high concentrations found in urine[130]. Another group have reported the presence of endogenous phospholipids found in plasma which are not washed away during solid phase extraction using mixed-mode columns, and elute late on in the chromatographic run[131]. Diazepam and its metabolites elute late on in the chromatographic run and the difference in their recoveries over the different matrices could be accounted for by interference from matrix constituents. All analytes have recoveries greater than 66 % for urine analysis. Diazepam has a recovery of 92 % from urine but only 46 % from the Intercept® device and 40 % from blood. The same drop in recovery is true for N-desmethyldiazepam and oxazepam. In general recoveries are best for urine then blood and lastly the Intercept® collection device. In line with the low recovery of N-desmethyldiazepam from blood and

the Intercept® device, limits of detection change from 1.1 ng/mL in urine, to 4.4 ng/mL in blood and 8.6 ng/mL in the Intercept® device. Intra and inter- day precision was similar for each matrix, less than 10 % at 200 ng/mL.

## 9.12 Conclusions

The method used for analysis was taken from previous work (Chapter 8) and validated successfully for the additional matrices blood, urine and the Intercept® device.

The method was applied to 48 whole blood samples, 87 urine samples, 91 oral fluid samples collected by the Intercept® device and 95 oral fluid samples collected by the modified Omni-Sal® device. These samples were collected from 20 opiate addicts in a rehabilitation centre, refer to Chapter 10.

# 10 Case Samples

## 10.1 Sildenafil in Whole Blood

### *10.1.1 Post-Mortem Case Sample*

A male, 29 years old, was found dead in his bed with sildenafil tablets missing from a packet beside his bed. It was suspected that the deceased could have taken an overdose of sildenafil. At the post-mortem, femoral whole blood was collected and this was subsequently analysed for sildenafil using the method in Chapter 5. The concentration in the blood was found to be within the expected therapeutic range of concentrations for this drug, and not the cause of death. A single 50 mg dose of sildenafil citrate has produced peak plasma concentrations of 0.260 mg/L[31]. Using the pseudo-molecular ion as the quantitation ion gave a level of 0.093 mg/L of blood. If the daughter ion was used instead, the level was found to be 0.088 mg/L. These results demonstrate the consistency of fragmentation that takes place within the source. Figure 10-1 shows the chromatogram produced for the blood sample.

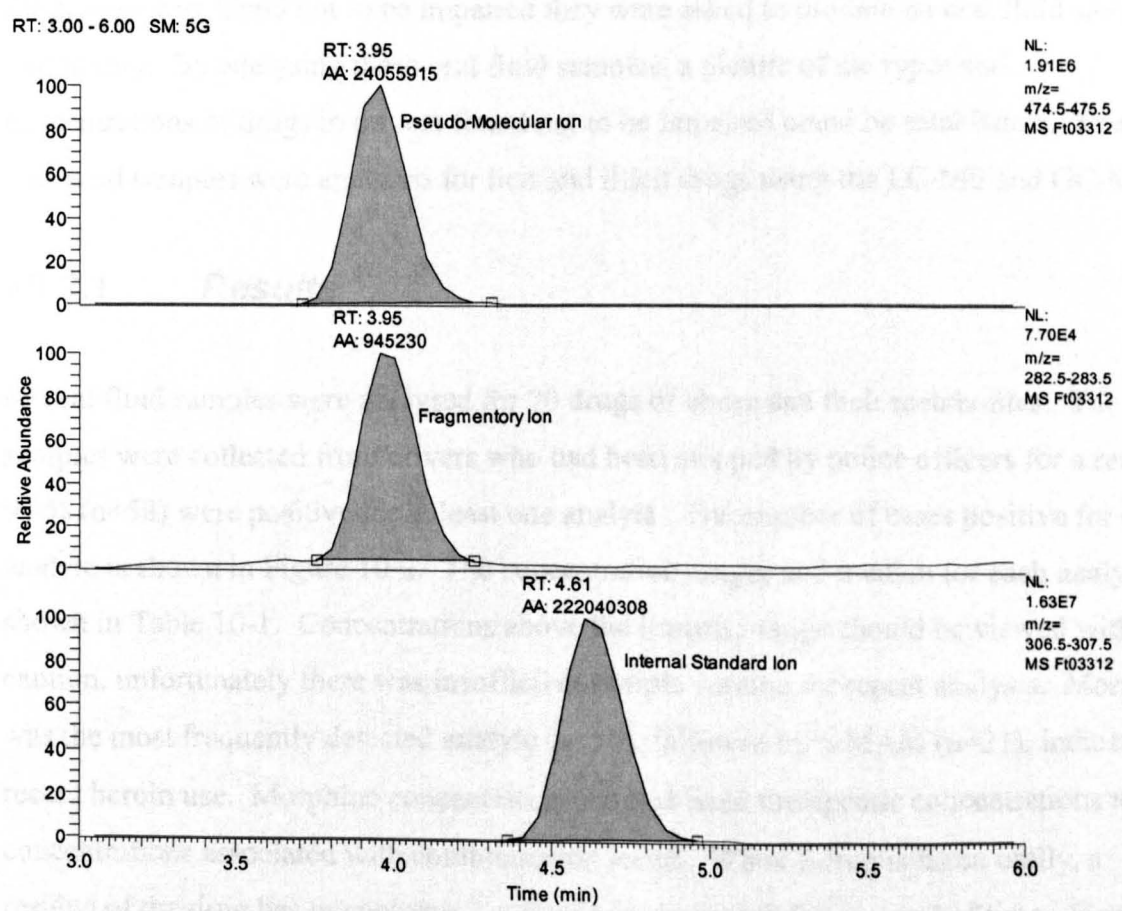



**Figure 10-1 : Sample chromatogram of sildenafil case sample**

### 10.1.2 Conclusions

The method developed and validated in Chapter 5 was successfully applied to a post-mortem case sample. The result was within the therapeutic range and therefore not the cause of death.

## 10.2 Detection of Drugs of Abuse in Oral Fluid by LC-MS

The method developed in Chapter 7 was used to analyse 60 oral fluid samples collected by the modified Omni-Sal® device, supplied by Cozart Biosciences Ltd. This was part of a UK project, British Roadside Impairment Test Evaluation (BRITE), which involved the evaluation of the Field Impairment Test (FIT) for use at the roadside in the UK.

FIT is a battery of tests to help the police officer decide if the driver is under the influence of drugs. FIT includes four attention tests, "walk and turn", "one legged stand", "finger to nose" and the Romberg test. Pupillary examination is also included giving a total of five tests to evaluate the driver. The police officer makes a judgement on whether the subject is impaired or not on the totality of the results of the tests. For the purposes of this project, if the subject was found not to be impaired they were asked to provide an oral fluid sample voluntarily. By analysing these oral fluid samples, a picture of the types and concentrations of drugs in drivers found not to be impaired could be established. These oral fluid samples were analysed for licit and illicit drugs using the LC-MS and GC-MS.

### 10.2.1 Results

60 oral fluid samples were analysed for 20 drugs of abuse and their metabolites. The samples were collected from drivers who had been stopped by police officers for a reason. 97 % (n=58) were positive for at least one analyte. The number of cases positive for each analyte is shown in Figure 10-2. The concentration ranges and median for each analyte are shown in Table 10-1. Concentrations above the linearity range should be viewed with caution, unfortunately there was insufficient sample volume for repeat analysis. Morphine was the most frequently detected analyte (n=50), followed by 6-MAM (n=21), indicating recent heroin use. Morphine concentrations ranged from therapeutic concentrations up to concentrations associated with contamination levels. When a drug is taken orally, a residue of the drug has the potential to remain in the mouth for a period of time. If oral

fluid is collected immediately after oral administration the resulting concentration will be a combination of that in the saliva and drug residue in the mouth. A past study has shown that with controlled intravenous administration of 5, 10 and 20 mg heroin hydrochloride, a peak saliva concentration of 20 ng/mL of morphine was reached. In the same study, 2.6 mg smoked heroin base was administered in a controlled manner, and peak saliva levels reached 3531 ng/mL of morphine[132]. A median morphine concentration of 25 ng/mL would suggest intravenous use, or heroin smoked several hours before sample collection. However the very high concentrations are indicative of recently smoked heroin.

Morphine was found without 6-MAM or codeine in 27 cases. These concentrations ranged from 2–175 ng/mL with a median of 11 ng/mL. Morphine, 6-MAM and codeine were found together in 19 cases, the morphine result ranging from 8–5361 ng/mL with a median of 156 ng/mL. Morphine and codeine were found together in 2 cases, and morphine and 6-MAM in another two cases. There were no instances when codeine was found alone, indicating that codeine was not present from prescribed or over the counter medications but from impure "street heroin" use.

6-MAM was found with morphine in 42 % (n=21) of morphine positive cases. A workplace study found 6-MAM to be present in 67 % of the morphine positive case samples[133]. This study employed a different collection device, which could account for the increased presence of 6-MAM compared to this study or it may be due to the variations in the time between administration and collection and/or the purity of the heroin.

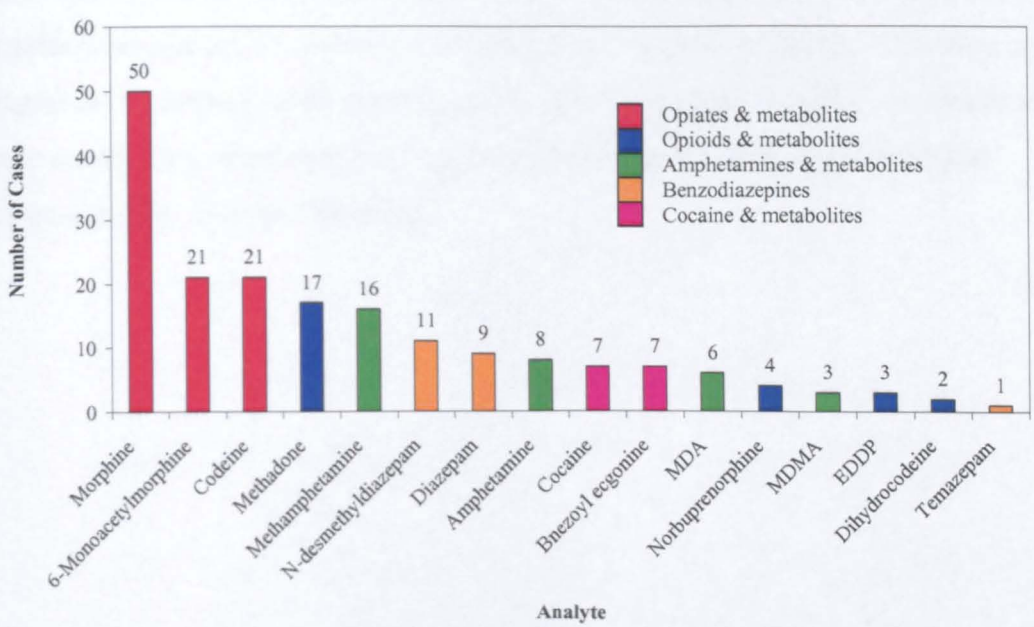



**Figure 10-2 : Number of samples positive**

Methadone was present in 28% (n=17) of all cases, with its metabolite EDDP present in only 5% (n=3). EDDP was not found without methadone. EDDP is not found at any significant level in plasma, during therapeutic usage of methadone, therefore it is not unexpected to see very few cases positive for EDDP. Methadone concentrations ranged from 4 – 597 ng/mL with a mean of 82 ng/mL and a median of 11 ng/mL. Methadone oral fluid concentrations have been found to show great inter-individual variability[134].

Methamphetamine was found in 27% (n=16) of all case samples. The levels found were very low, apart from one sample at 50ng/mL. This sample was also positive for MDMA, MDA and amphetamine. A previous study analysing oral fluid collected by the modified Omni-Sal® device from heroin addicts, found 11 % positive for amphetamines, mainly methamphetamine, and at very low concentrations, around LOD of 1 – 10 ng/mL[135].

N-desmethyldiazepam, diazepam and temazepam were found in 18 % (n=11), 15 % (n=9) and 2 % (n=1) of all cases respectively. All analytes were found alone, except in one case where N-desmethyldiazepam and temazepam were found together. The concentrations of the benzodiazepines were quite low ranging from 5 – 51 ng/mL. Benzodiazepines have a high protein binding capacity, allowing only a small percentage to diffuse into saliva. A past study has shown similar concentrations to those presented here[87].

Amphetamine was found positive in 13 % (n=8) of all case samples. Amphetamine was only found present with MDMA in one case, which was also positive for MDA and methamphetamine. In this case 6525 ng/mL of amphetamine was found with 50 ng/mL of methamphetamine, 245 ng/mL of MDMA and 27 ng/mL of MDA. This case could be explained by impure amphetamine, and/or the combination of drugs administered. Three case samples had amphetamine levels over 3000 ng/mL indicating intranasal contamination, through "snorting".

**Table 10-1 : Drug concentrations detected in oral fluid case samples**

| Analyte | n | Mean (ng/mL) | Median (ng/mL) | Concentration Range (ng/mL) |
|---|---|---|---|---|
| Morphine | 50 | 224 | 25 | 2 – 5361 |
| 6-MAM | 21 | 637 | 29 | 2 – 5400 |
| Codeine | 21 | 74 | 15 | 2 – 468 |
| Methadone | 17 | 82 | 11 | 4 – 597 |
| Methamphetamine | 16 | 6 | 2 | 2 – 50 |
| N-desmethyldiazepam | 11 | 13 | 5 | 1 – 51 |
| Diazepam | 9 | 5 | 5 | 2 – 15 |
| Amphetamine | 8 | 1662 | 178 | 43 – 6525 |
| Cocaine | 7 | 275 | 172 | 36 – 863 |
| Benzoylecgonine | 7 | 201 | 124 | 18 -746 |
| MDA | 6 | 11 | 7 | 1 – 27 |
| Norbuprenorphine | 4 | 19 | 17 | 1 -43 |
| MDMA | 3 | 313 | 245 | 225 – 468 |
| EDDP | 3 | 5 | 4 | 4 – 8 |
| Dihydrocodeine | 2 | 2 | 2 | 2, 2 |
| Temazepam | 1 | - | - | 32 |

Cocaine and its metabolite benzoylecgonine were found in 12 % (n=7) of all case samples. In every sample both cocaine and benzoylecgonine were present. Cocaine values ranged from 36 – 863 ng/mL, while benzoylecgonine had similar values ranging from 18 – 746 ng/mL. Previous studies, using stimulated saliva collection, have shown that smoking and intranasally administered cocaine, results in saliva concentrations in the thousands initially, decreasing to limits of detection within 12 hours. Intravenous administration of cocaine hydrochloride results in peak concentrations of cocaine in the hundreds, decreasing to trace amount after 12 hours[104,132]. The difference in magnitude can be accounted for by oral contamination from the route of administration. The results presented here suggest that the oral contamination has cleared, and cocaine has been taken within the last few hours.

The results for cocaine and benzoylecgonine were plotted against each other to assess if there was any correlation. The graph in Figure 10-3 shows the results.

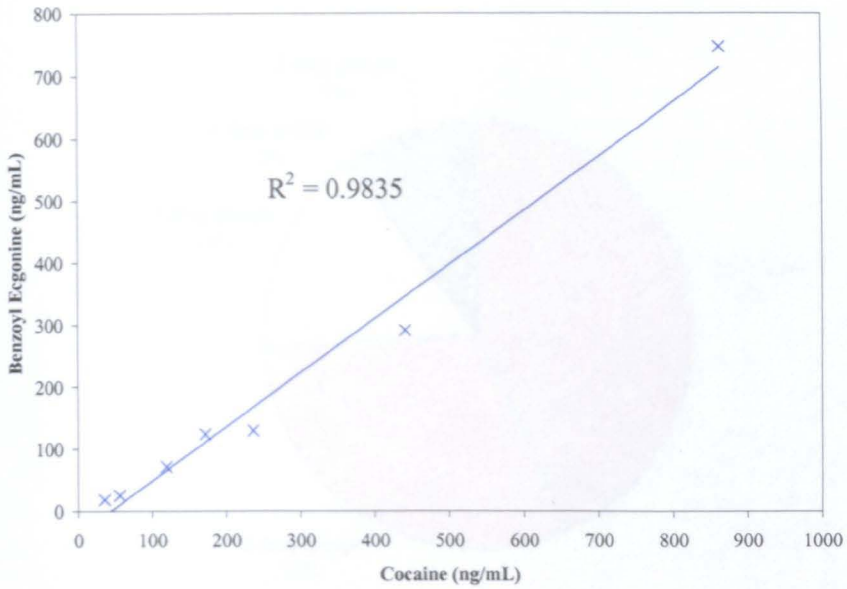



**Figure 10-3 : Correlation between cocaine and benzoylecgonine oral fluid results**

This showed significant correlation between cocaine and benzoylecgonine oral fluid concentrations. The concentrations of cocaine and benzoylecgonine in the same sample were in the same range unlike the studies previously mentioned, where benzoylecgonine levels were a magnitude lower than cocaine concentrations. This could be due to the stimulated conditions used to collect the saliva in these previous studies. Stimulation of saliva production results in an increase in saliva pH which would drive benzoylecgonine to its unionised form leaving it free to cross the lipid membrane back into the blood. At a lower pH benzoylecgonine would exist in its ionised form which would be trapped in the saliva.

MDMA was found in 5 % (n=3) of all case samples. Its metabolite MDA was also found in the same three case samples and in an additional two at very low levels. MDMA was found at concentrations ranging from 225 – 468 ng/mL, with a median of 245 ng/mL. These levels are below what would be expected for oral contamination and suggest recent use.

Of the 58 samples found positive, 42 % (n=25) had one drug group, 31 % (n=18) 2 drug groups, 16 % (n=9) 3 drug groups, 2 % (n=1) 4 drug groups and 9 % (n= 5) 5 drug groups.

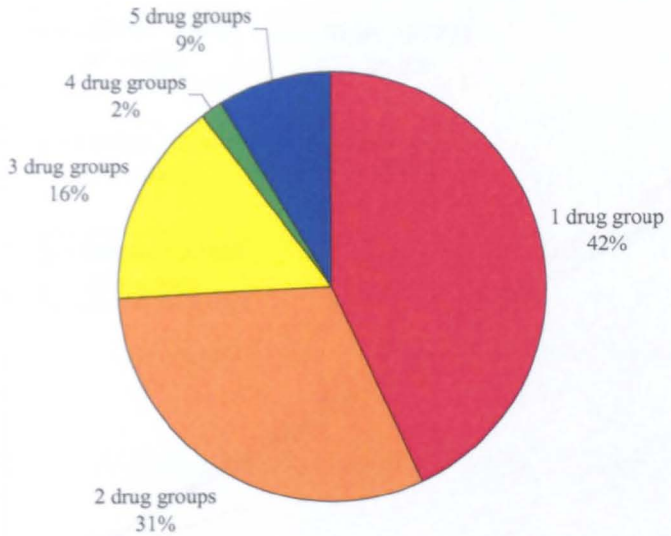



**Figure 10-4 : Number of drug groups present in positive samples**

Figure 10-4 shows the extent of polydrug use in this population of samples. Less than half, 42%, of the samples had one drug group present. Polydrug use has been reported previously amongst drivers in blood and urine samples[95,136,137]. The most common drug group taken in combination with other drug group was the opiates, namely morphine attributed to heroin use in combination with benzodiazepines and methadone.

### *10.2.2 Comparison of LC-MS and GC-MS Results*

Some analytes were quantified using both the GC-MS and the LC-MS. Correlation between the instruments for these analytes are shown below in Figure 10-5. The higher sensitivity of the LC-MS resulted in some discrepancies between the two instruments results.

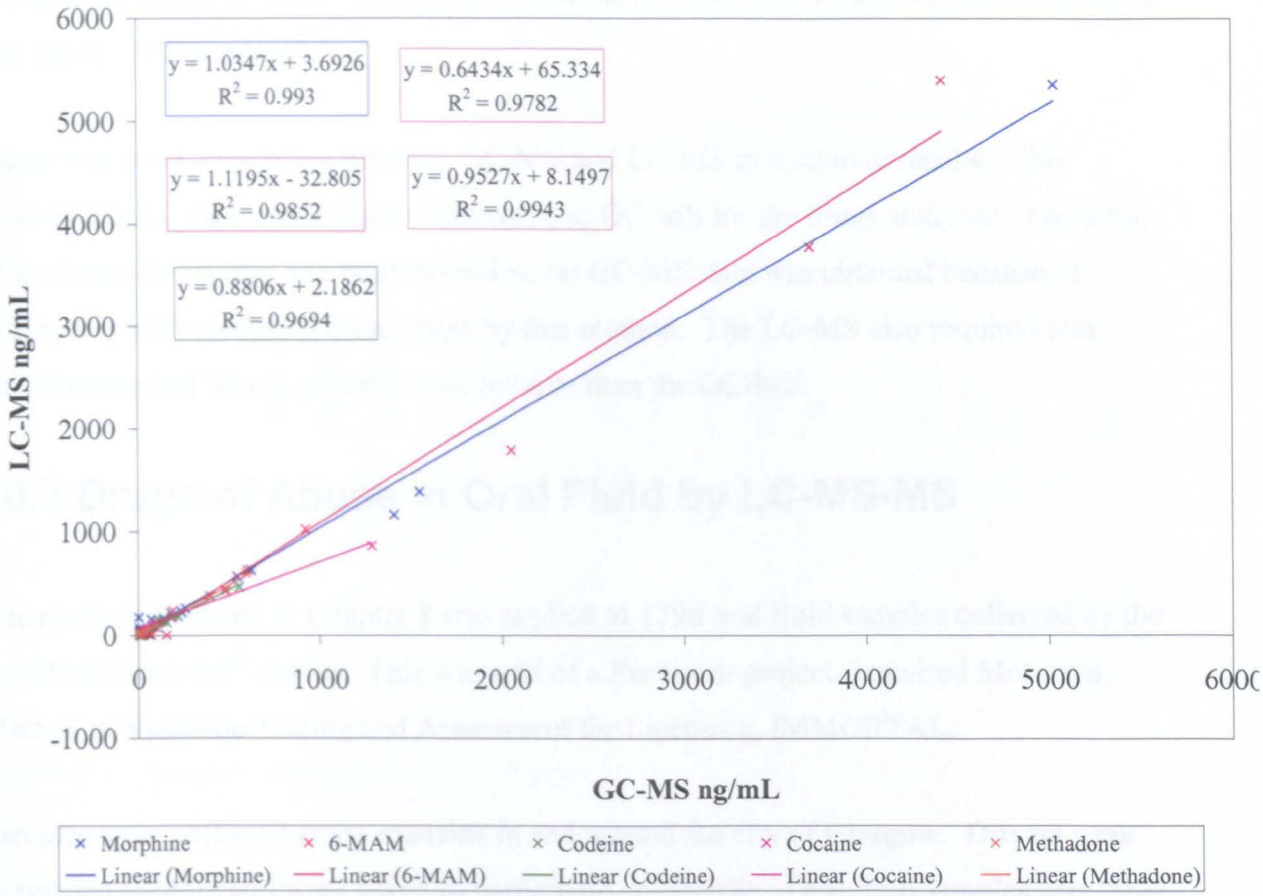



**Figure 10-5 : Correlation between GC-MS and LC-MS results**

## 10.2.3 Discussion

The samples analysed were obtained from drivers who were stopped for a reason by the police officer, but were later found to be unimpaired using FIT. The results show that 97% of these samples were positive for at least one drug group, indicating that although drugs were found in the drivers' system they may not have exerted an impairing effect at the time FIT was carried out. It could also indicate tolerance to drugs in the individual or that more training is required for the police officers. Morphine, indicating heroin use, was the most commonly detected drug, which is in line with previous studies on drivers and drug deaths in the West of Scotland area[58,95,138].

Cannabinoids were analysed in the acidic/neutral fraction of the extraction on the GC-MS. There have been papers published which describe the analysis of cannabinoids using LC-MS, but using the present chromatographic conditions $\Delta^9$-THC did not elute from the

column. This meant that $\Delta^9$-THC analysis was not possible on the LC-MS for this project and the GC-MS was used.

There was good correlation between GC-MS and LC-MS quantitative results. This showed that LC-MS was a viable alternative to GC-MS for the drugs analysed. For some of the benzodiazepines and buprenorphine, no GC-MS data was obtained because of difficulties with analysing these drugs by this method. The LC-MS also required less maintenance and was in general more reliable than the GC-MS.

## 10.3 Drugs of Abuse in Oral Fluid by LC-MS-MS

The method validated in Chapter 8 was applied to 1396 oral fluid samples collected by the modified Omni-Sal® device. This was part of a European project, Impaired Motorists, Method of Roadside Testing and Assessment for Licensing, IMMORTAL.

Samples were collected at the roadside in and around the city of Glasgow. Drivers were stopped at random and were asked to participate voluntarily. Oral fluid samples was taken and transported to the Forensic Medicine and Science Department, where they were frozen on arrival until the analysis was carried out.

The samples were analysed for 21 drugs of abuse and their metabolites by LC-MS-MS. Prescription drugs and cannabinoids were analysed by GC-MS, refer to Appendix 1 for GC-MS methodology[139].

### *10.3.1 Results*

1396 oral fluid samples were analysed for 21 drugs of abuse and their metabolites. 12.8 % (n=178) were positive for at least one drug. The number of cases positive for each analyte is shown in Figure 10-6. The concentration ranges and median for each analyte are shown in Table 10-2.

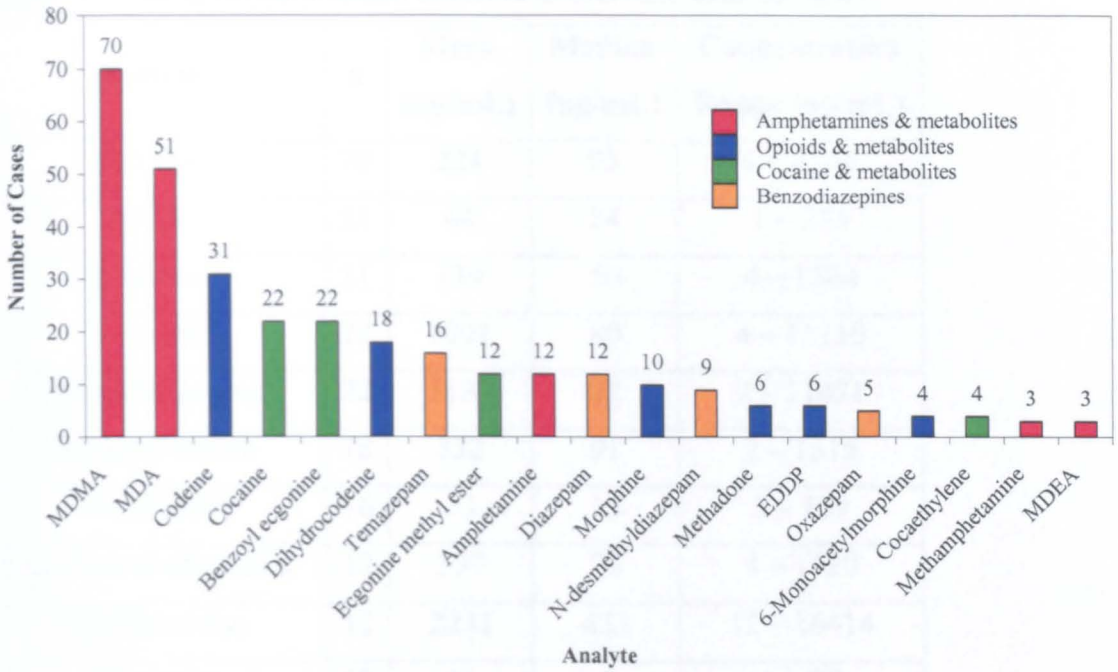



**Figure 10-6 : Number of positive case samples**

MDMA, was the most frequently detected drug (n=70) followed by its metabolite MDA, (n=51). The concentrations detected for MDMA, range from trace amounts to contamination levels, 1 – 3144 ng/mL. Only 4 cases out of 70 indicated contamination with MDMA levels greater than 1000 ng/mL. The median was 93 ng/mL indicating drug use within 24 hours[89].

MDA was found without MDMA in 3 cases, at very low levels, 1, 9 and 9 ng/mL. This would suggest that the MDA positive cases are from the metabolism of MDMA and not from the ingestion of MDA alone.

There are 48 cases where both MDMA and MDA are found in the sample. By plotting the MDMA and MDA concentrations, it is shown that there is little correlation between the two, Figure 10-7. This is in contrast to cocaine and benzoylecgonine in the previous set of case samples (Figure 10-3).

**Table 10-2 : Drug concentrations detected in oral fluid case samples**

| Analyte | n | Mean (ng/mL) | Median (ng/mL) | Concentration Range (ng/mL) |
|---|---|---|---|---|
| MDMA | 70 | 224 | 93 | 1 – 3144 |
| MDA | 51 | 44 | 24 | 1 – 275 |
| Codeine | 31 | 139 | 50 | 4 – 1504 |
| Cocaine | 22 | 1001 | 80 | 4 – 11110 |
| Benzoylecgonine | 22 | 1134 | 82 | 2 – 11471 |
| Dihydrocodeine | 18 | 332 | 91 | 2 – 1315 |
| Temazepam | 16 | 33 | 13 | 3 – 189 |
| Ecgonine methyl ester | 12 | 337 | 76 | 4 – 1520 |
| Amphetamine | 12 | 2231 | 433 | 12 – 16414 |
| Diazepam | 12 | 11 | 8 | 1 – 28 |
| Morphine | 10 | 784 | 12 | 2 – 7442 |
| N-desmethyldiazepam | 9 | 46 | 16 | 4 – 221 |
| Methadone | 6 | 1578 | 667 | 8 – 6949 |
| EDDP | 6 | 16 | 12 | 8 – 38 |
| Oxazepam | 5 | 14 | 10 | 4 – 33 |
| 6-MAM | 4 | 1906 | 11 | 3 – 7600 |
| Cocaethylene | 4 | 46 | 32 | 2 – 119 |
| Methamphetamine | 3 | 34 | 2 | 1 – 98 |
| MDEA | 3 | 10 | 13 | 4 – 14 |

After MDMA and MDA the next most frequently detected drug was codeine with 17.4 % (n= 31) of case samples found positive. Codeine was found without any other drug in 23 case samples. A sample positive for codeine only suggests codeine use through over the counter medication. Of the other eight case samples positive for codeine, two were positive for dihydrocodeine, also indicating over the counter medication. Four were positive for morphine, and one of those also positive for 6-MAM. Finding codeine and morphine in oral fluid could be the result of codeine use alone (as codeine metabolises to morphine) or heroin use. 6-MAM present in a sample is conclusive of heroin use, as 6-MAM is the metabolite of diamorphine, found in heroin. The other two cases had a small amount of EDDP in one and MDMA in the other.

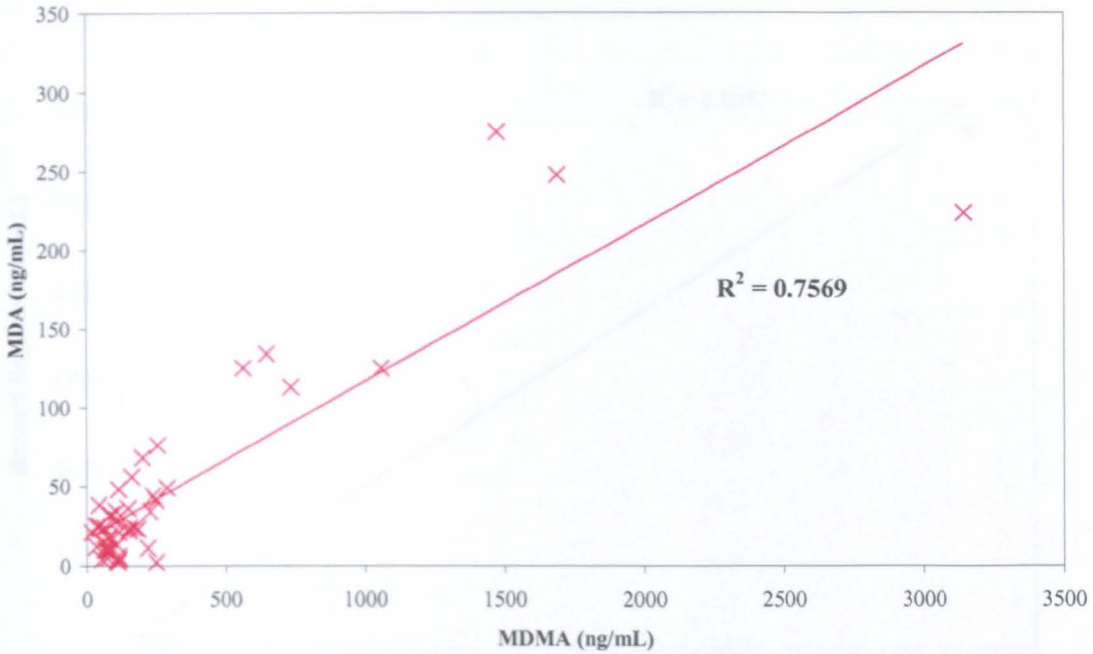



**Figure 10-7 : Correlation between MDMA and MDA concentration in oral fluid**

Cocaine and its metabolite benzoylecgonine were the next most frequently detected drugs, both in 12.4 % (n = 22) of positive case samples. Ecgonine methyl ester, another cocaine metabolite, was found in 6.7 % (n = 12) of positive case samples. Benzoylecgonine and ecgonine methyl ester were found without cocaine in only four cases. Cocaine and benzoylecgonine were found together in nineteen cases, and cocaine and both its metabolites were found together in ten cases. In the previous set of oral fluid data, there was significant correlation between cocaine and benzoylecgonine. When the current data was plotted the correlation is not as significant as before, with an $R^2$ value of 0.8957, Figure 10-8. This may be because of the widespread range in levels found in this project compared with the previous study.

Temazepam was found in 19 % (n = 16) of positive samples. It was found without diazepam, N-desmethyldiazepam and oxazepam in seven samples. This finding indicates either the use of temazepam or diazepam. An additional five samples had only one benzodiazepine present. Diazepam was next most frequently detected (6.7 %) benzodiazepine after temazepam, followed by N-desmethyldiazepam, 5.1 %, and oxazepam, 2.8 %.

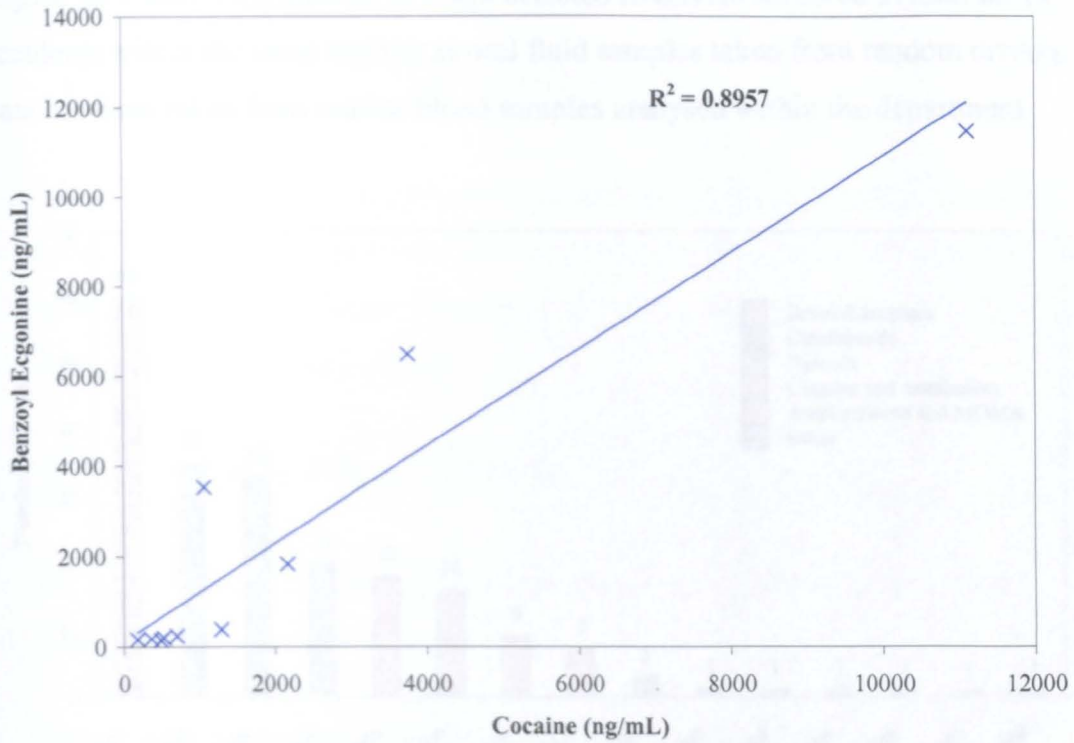



**Figure 10-8 : Correlation between cocaine and benzoylecgonine oral fluid concentrations**

## *10.3.2 Discussion*

12.8 % of samples collected from drivers at random were found positive for at least one drug. The most frequently found drug was MDMA and its metabolite MDA, with median concentrations representing use within the last 24 hours. In one study it was shown that although vehicle control is not greatly affected after MDMA ingestion, the decreased sense of risk taking has a major part to play in accident involvement[140]. This is confirmed by a further study showing that MDMA use is not consistent with safe driving and that impairment can last for a considerable time after last use[141]. Codeine was the next most frequently detected drug with 25 out of 31 cases representing over the counter medications. Cocaine use was observed in 22 cases, with a median concentration of 80 ng/mL, this would indicate use within the last 2-8 hours depending on the route of administration[102]. Only five out of the 22 cases showed concentrations of 1000 ng/mL indicating oral contamination and use within the last couple of hours prior to sampling.

The majority of sample positives can be explained by over the counter medications or concentrations indicating use within the last 24 hours, and not necessarily exerting an impairing effect on the driver.

Figure 10-9 shows the number of drugs detected in drivers involved in road traffic accidents within the same vicinity as oral fluid samples taken from random drivers. This data has been taken from routine blood samples analysed within the department.

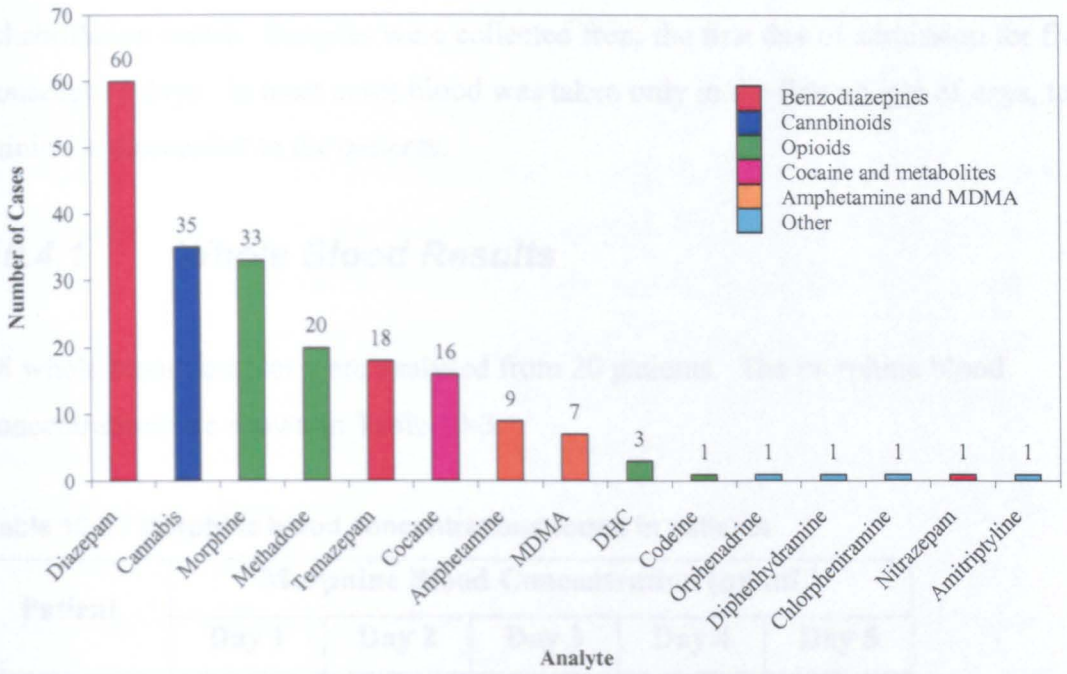



**Figure 10-9 : Number of positive samples from drivers involved in road traffic accidents in the same vicinity as random oral fluid samples collected**

In drivers involved in road traffic accidents, diazepam is the most frequently detected drug. Cannabinoids, morphine, methadone and temazepam are the next most frequently detected drugs. The five most frequently detected drugs all have sedative properties[142,143]. This is in contrast to oral fluid samples collected from non-impaired drivers where MDMA was the most frequently detected drug, followed by codeine and cocaine. MDMA and cocaine both produce stimulating effects[142,143]. As discussed earlier there have been studies on the effect of MDMA on driving, which suggest that it does have some impairment effects[140,141]. MDMA found in drivers who are non-impaired can be explained by either contamination of the oral cavity from oral administration or tolerance from the effect of the drug through repeated use. If an individual frequently takes a drug, tolerance to its effects builds up and the user needs to take a higher dose to experience the same effect. This would result in high concentrations of the drug in oral fluid, without the impairing effect.

$\Delta^9$-THC was analysed on the GC-MS, results for this are shown in Appendix 1[144].

# 10.4 Comparison of Oral Fluid Collection Devices for the Detection of Drugs of Abuse

Blood, urine and oral fluid samples were taken from patients attending a residential rehabilitation centre. Samples were collected from the first day of admission for five consecutive days. In most cases blood was taken only in the first couple of days, to minimise discomfort to the patients.

## *10.4.1 Whole Blood Results*

48 whole blood samples were analysed from 20 patients. The morphine blood concentrations are shown in Table 10-3.

**Table 10-3 : Morphine blood concentrations found in patients**

| Patient | Morphine Blood Concentration (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| GY31290 | 0 | ns | 0 | 0 | 6 |
| MMG4167 | 12 | 11 | 8 | 3 | 0 |
| SL16771 | 7 | ns | 0 | ns | ns |
| MT171060 | 0 | ns | ns | 0 | 0 |
| BA3181 | 19 | ns | ns | 0 | ns |
| HD180669 | 0 | 0 | ns | ns | 0 |
| CL180981 | 7 | ns | 0 | ns | ns |
| JC271273 | 0 | 4 | 0 | ns | ns |
| CD221180 | 28 | 0 | 3 | ns | 0 |
| MM22676 | 13 | 0 | 0 | ns | ns |
| DT4676 | 0 | 0 | 0 | ns | ns |
| SC191082 | ns | 7 | ns | 1 | ns |
| JS18981 | ns | ns | 0 | 0 | ns |
| SM051273 | 3 | ns | 0 | ns | ns |
| JM011178 | 0 | ns | ns | ns | ns |
| JL300578 | 4 | ns | 0 | ns | ns |
| SD220384 | 3 | ns | ns | ns | ns |
| AC290980 | 16 | ns | ns | ns | ns |

| Patient | Morphine Blood Concentration (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| PT020681 | 0 | ns | ns | ns | ns |
| DMcC51174 | 3 | ns | ns | 0 | ns |

ns : no sample provided

From the above results it can be seen that there are many samples missing from the data set. In many cases, collection of blood was found to be either too distressing for the patient, or too difficult for the medical practitioner. Many of the results were negative and for those which were positive, levels were low. Levels ranged from 3-28 ng/mL with a mean and median of 10 and 7 ng/mL, respectively. Morphine metabolises rapidly and has a half-life of 1.3-6.7 hours[31]. One study has shown morphine concentrations in plasma to decline to the sensitivity of the analytical method (0.6 ng/mL) by 24 hours after 20 mg and 10 mg doses of intramuscular morphine[106]. The self reported time between the last heroin use to blood collection ranged from 7 hours (AC290980) to 4-5days (BA3181). This could explain the low positive results found. The low positive results for morphine found in later days could be explained by patients re-using within the rehabilitation centre. 6-MAM was not detected in any blood samples. This is not surprising considering the low concentrations of morphine found and the rapid metabolism of 6-MAM to morphine. Codeine was also absent in the blood samples, codeine is a by-product of the production of heroin. It can be found in blood after heroin use but levels are a magnitude less than morphine. Considering the low morphine levels, we would not expect codeine to be present.

Dihydrocodeine was found in most patients. When a patient is first admitted into the centre, dihydrocodeine is given to the patient by the care workers until they are seen by the doctor who can then prescribe them methadone. The levels of dihydrocodeine found are shown in Table 10-4.

**Table 10-4 : Dihydrocodeine blood concentrations found in patients**

| Patient | Dihydrocodeine Blood Concentration (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| GY31290 | 570 | ns | 1 | 0 | 0 |
| MMG4167 | 375 | 53 | 0 | 0 | 0 |
| SL16771 | 0 | ns | 0 | ns | ns |
| MT171060 | 269 | ns | ns | 0 | 0 |
| BA3181 | 221 | ns | ns | 0 | ns |
| HD180669 | 0 | 0 | ns | ns | 0 |
| CL180981 | 216 | ns | 0 | ns | ns |
| JC271273 | 358 | 15 | 0 | ns | ns |
| CD221180 | 0 | 0 | 0 | ns | 0 |
| MM22676 | 0 | 245 | 339 | ns | ns |
| DT4676 | 0 | 0 | 0 | ns | ns |
| SC191082 | ns | 403 | ns | 221 | ns |
| JS18981 | ns | ns | 0 | 0 | ns |
| SM051273 | 0 | ns | 0 | ns | ns |
| JM011178 | 235 | ns | ns | ns | ns |
| JL300578 | 310 | ns | 1 | ns | ns |
| SD220384 | 294 | ns | ns | ns | ns |
| AC290980 | 0 | ns | ns | ns | ns |
| PT020681 | 69 | ns | ns | ns | ns |
| DMcC51174 | 12 | ns | ns | 0 | ns |

ns : no sample provided

Diazepam and its three metabolites were all detected in blood. Diazepam was found in 92 % (n=44) of samples and in all but one patient DMcC51174. Results are shown in Table 10-5. Patients are prescribed diazepam if they are suffering from withdrawal symptoms from recent use.

**Table 10-5 : Diazepam blood concentrations found in patients**

| Patient | Diazepam Blood Concentration (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| GY31290 | 241 | ns | 672 | 434 | 362 |
| MMG4167 | 375 | 420 | 594 | 474 | 342 |
| SL16771 | 8 | ns | 449 | ns | ns |
| MT171060 | 0 | ns | ns | 26 | 0 |
| BA3181 | 596 | ns | ns | 344 | ns |
| HD180669 | 47 | 21 | ns | ns | 3 |
| CL180981 | 247 | ns | 399 | ns | ns |
| JC271273 | 312 | 226 | 261 | ns | ns |
| CD221180 | 120 | 126 | 146 | ns | 44 |
| MM22676 | 903 | 854 | 677 | ns | ns |
| DT4676 | 309 | 342 | 370 | ns | ns |
| SC191082 | ns | 1787 | ns | 1266 | ns |
| JS18981 | ns | ns | 327 | 472 | ns |
| SM051273 | 1538 | ns | 310 | ns | ns |
| JM011178 | 385 | ns | ns | ns | ns |
| JL300578 | 684 | ns | 774 | ns | ns |
| SD220384 | 687 | ns | ns | ns | ns |
| AC290980 | 301 | ns | ns | ns | ns |
| PT020681 | 97 | ns | ns | ns | ns |
| DMcC51174 | 0 | ns | ns | 0 | ns |

ns : no sample provided

### 10.4.2 Discussion

Blood concentrations of morphine were very low, if it was present. 6-MAM and codeine were not found at all, highlighting the rapid metabolism of heroin. Dihydrocodeine and the benzodiazepines were found in many samples at high concentrations, resulting from administration within the rehabilitation centre.

### 10.4.3 Oral Fluid Collected by the Modified Omni-Sal® Device Results

95 oral fluid samples collected by the modified Omni-Sal® device were analysed for opiates and benzodiazepines. This device was accepted well by the patients, the main disadvantage was the time taken to collect a sample. The device has a window at the end of the stick which turns blue when 1 mL of oral fluid is collected. Opiate addicts often suffer from "dry mouth" prolonging the time taken to collect 1mL; it took one patient 30 minutes. Only 5 samples were not collected, either because the patient left the centre early, or the centre had run out of the collection devices.

Assuming that morphine levels in oral fluid are from the diffusion of morphine from blood into saliva, it would be expected that the levels found in oral fluid would be comparable to those found in blood. The morphine concentrations found in the oral fluid samples are shown in Table 10-6.

In day 1, 10 samples were found positive for morphine. In whole blood on day 1, 11 samples were found positive with 4 samples absent from analysis. Although there seems to be less positive results in oral fluid, the mean concentration was 83 ng/mL with a median of 48 ng/mL. The concentrations ranged from 4 – 437 ng/mL. A study showed that following intramuscular injection of 20 mg morphine sulphate, equilibrium between saliva and plasma concentrations was reached after 45 minutes. They found that plasma levels were similar or greater than saliva levels[88]. Intramuscular injections of morphine ensure no contamination of the oral cavity. The highest level of morphine found in these oral fluid samples, was 437 ng/mL (JL300578), this could be as a result of oral contamination from smoking heroin. The median in oral fluid is almost 7 times higher than that in blood.

**Table 10-6 : Morphine oral fluid concentrations collected using the modified Omni-Sal® device**

| Patient | Morphine Oral Fluid Concentrations (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| GY31290 | 11 | 0 | 0 | 23 | 306 |
| MMG4167 | 0 | 0 | 66 | 0 | 0 |
| SL16771 | 0 | 0 | 0 | 0 | 0 |
| MT171060 | 21 | 0 | 0 | 0 | 0 |
| BA3181 | 0 | 0 | 0 | 0 | ns |
| HD180669 | 0 | 0 | 0 | 0 | 0 |
| CL180981 | 0 | 0 | 0 | 0 | 0 |
| JC271273 | 0 | 0 | 0 | 0 | ns |
| CD221180 | 98 | 4 | 0 | 0 | 0 |
| MM22676 | 54 | 0 | 0 | 9 | 0 |
| DT4676 | 0 | 0 | 0 | 0 | 0 |
| SC191082 | 52 | 3 | 0 | 9 | 0 |
| JS18981 | 43 | 0 | 0 | 0 | 0 |
| SM051273 | 4 | 0 | 0 | 0 | 0 |
| JM011178 | 0 | 0 | 0 | ns | ns |
| JL300578 | 437 | 42 | 0 | 0 | 0 |
| SD220384 | 0 | 0 | 0 | ns | 0 |
| AC290980 | 99 | 0 | 0 | 0 | 0 |
| PT020681 | 0 | 0 | 0 | 0 | 0 |
| DMcC51174 | 6 | 0 | 0 | 0 | 0 |

ns : no sample provided

6-MAM was not present in any blood samples, this was not the case with oral fluid samples. Using the modified Omni-Sal® device, 6-MAM was found in 9 out of the 20 patients analysed. The full set of results is shown in Table 10-7. JL300578 shows a high level of 6-MAM, 282 ng/mL which would support the hypothesis of oral contamination through smoking. A study evaluating the use of oral fluid testing for the detection of opiate use in drug users found high concentrations of 6-MAM which was attributed to the breakdown of heroin during smoking rather than the diffusion from the blood. The authors also found in a few cases opiates could be detected in oral fluid up to five days after last reported use[145].

**Table 10-7 : 6-MAM oral fluid concentrations collected using the modified Omni-Sal® device**

| Patient | 6-MAM Oral Fluid Concentrations (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| GY31290 | 0 | 0 | 0 | 0 | 0 |
| MMG4167 | 0 | 0 | 92 | 0 | 0 |
| SL16771 | 0 | 11 | 0 | 0 | 0 |
| MT171060 | 40 | 15 | 0 | 0 | 0 |
| BA3181 | 0 | 0 | 0 | 0 | ns |
| HD180669 | 0 | 0 | 0 | 0 | 0 |
| CL180981 | 0 | 0 | 0 | 0 | 0 |
| JC271273 | 0 | 0 | 18 | 5 | ns |
| CD221180 | 0 | 0 | 0 | 0 | 0 |
| MM22676 | 30 | 0 | 0 | 0 | 0 |
| DT4676 | 0 | 0 | 0 | 0 | 0 |
| SC191082 | 36 | 0 | 0 | 0 | 0 |
| JS18981 | 0 | 0 | 0 | 0 | 0 |
| SM051273 | 0 | 0 | 0 | 0 | 0 |
| JM011178 | 0 | 0 | 0 | ns | ns |
| JL300578 | 282 | 25 | 3 | 0 | 7 |
| SD220384 | 0 | 0 | 8 | ns | 0 |
| AC290980 | 0 | 0 | 0 | 0 | 0 |
| PT020681 | 0 | 0 | 65 | 10 | 2 |
| DMcC51174 | 0 | 0 | 0 | 0 | 0 |

ns : no sample provided

Codeine was also found in these oral fluid samples, where none was found in the respective blood samples. These could be explained by the oral contamination through smoking, or the oral self-administration of an over the counter preparation. The full set of codeine results are shown in Table 10-8. Codeine positive samples are consistent with morphine positive samples. The median of codeine concentrations was 11 ng/mL compared with the median morphine concentration of 48 ng/mL. Although the detection of morphine and codeine is not conclusive of heroin use, the patients were known heroin abusers.

**Table 10-8 : Codeine oral fluid concentrations collected using the modified Omni-Sal® device**

| Patient | Codeine Oral Fluid Concentrations (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| GY31290 | 24 | 0 | 0 | 0 | 0 |
| MMG4167 | 0 | 0 | 0 | 0 | 0 |
| SL16771 | 0 | 0 | 0 | 0 | 0 |
| MT171060 | 0 | 0 | 0 | 0 | 0 |
| BA3181 | 14 | 0 | 0 | 0 | ns |
| HD180669 | 3 | 1 | 0 | 0 | 0 |
| CL180981 | 0 | 0 | 0 | 0 | 0 |
| JC271273 | 0 | 0 | 0 | 0 | ns |
| CD221180 | 45 | 0 | 0 | 0 | 0 |
| MM22676 | 35 | 0 | 0 | 0 | 0 |
| DT4676 | 0 | 0 | 0 | 0 | 0 |
| SC191082 | 17 | 10 | 0 | 0 | 0 |
| JS18981 | 11 | 4 | 0 | 0 | 0 |
| SM051273 | 4 | 0 | 0 | 0 | 0 |
| JM011178 | 0 | 0 | 0 | ns | ns |
| JL300578 | 20 | 6 | 0 | 0 | 6 |
| SD220384 | 0 | 0 | 0 | ns | 0 |
| AC290980 | 21 | 0 | 0 | 0 | 0 |
| PT020681 | 0 | 0 | 0 | 0 | 0 |
| DMcC51174 | 0 | 0 | 0 | 0 | 0 |

ns : no sample provided

Dihydrocodeine was found in 43 % (n=41) of samples, with a median of 379 ng/mL and results ranging from 1 – 15058 ng/mL. In blood, dihydrocodeine was found positive in 44 % (n=21) of samples, with a median of 235 ng/mL and a range of 1 – 570 ng/mL. The highest blood concentration of 570 ng/mL was from GY31290 on day 1, the corresponding oral fluid concentration was the highest found. Dihydrocodeine levels are elevated in oral fluid samples compared to blood. The full set of dihydrocodeine results are shown in Table 10-9.

**Table 10-9 : Dihydrocodeine oral fluid concentrations collected using the modified Omni-Sal® device**

| Patient | Dihydrocodeine Oral Fluid Concentrations (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| GY31290 | 15058 | 767 | 17 | 13 | 0 |
| MMG4167 | 1736 | 66 | 0 | 0 | 0 |
| SL16771 | 0 | 0 | 0 | 0 | 0 |
| MT171060 | 3134 | 198 | 0 | 0 | 0 |
| BA3181 | 836 | 232 | 3 | 0 | ns |
| HD180669 | 1598 | 181 | 37 | 1 | 0 |
| CL180981 | 379 | 57 | 3 | 0 | 0 |
| JC271273 | 869 | 27 | 5 | 0 | ns |
| CD221180 | 0 | 0 | 0 | 0 | 0 |
| MM22676 | 0 | 671 | 715 | 2541 | 846 |
| DT4676 | 0 | 0 | 0 | 0 | 0 |
| SC191082 | 1069 | 4610 | 610 | 2702 | 1143 |
| JS18981 | 0 | 0 | 0 | 0 | 0 |
| SM051273 | 0 | 0 | 0 | 0 | 0 |
| JM011178 | 707 | 90 | 19 | ns | ns |
| JL300578 | 629 | 304 | 0 | 0 | 0 |
| SD220384 | 1541 | 121 | 55 | ns | 10 |
| AC290980 | 0 | 0 | 0 | 0 | 0 |
| PT020681 | 892 | 24 | 0 | 0 | 0 |
| DMcC51174 | 0 | 0 | 0 | 0 | 0 |

In contrast with the opiates, diazepam was found in only 59 % (n=56) of oral fluid samples, compared with 92 % (n=44) of blood samples. The number of oral fluid samples with a corresponding blood sample was 48. Of these, 92 % (n=44) were positive for diazepam in blood, and 52 % (n=25) were positive for diazepam in oral fluid. The blood concentrations had a median of 353 ng/mL with a range of 3 – 1787 ng/mL and the oral fluid concentrations had a median of 17 ng/mL with a range of 2 – 329 ng/mL. Oral fluid concentrations of diazepam are a magnitude less than blood concentrations. Diazepam is highly protein bound in plasma. Therefore it is only the low amount of free drug which is able to diffuse into saliva.

Similarly, N-desmethyldiazepam, temazepam and oxazepam had considerably lower concentrations in oral fluid compared to blood. N-desmethyldiazepam was found in 42 % (n=40) of oral fluid samples compared with 92 % (n=44) of blood samples. Temazepam was found in 18 % (n=17) of oral fluid samples compared with 94 % (n=45) of blood samples. Similarly oxazepam was found in 20 % (n=19) of oral fluid samples compared with 83 % (n=40) of blood samples. The median concentrations of diazepam and its metabolites in blood and oral fluid collected by the modified Omni-Sal® device are shown in Table 10-10.

**Table 10-10 : Median concentrations of diazepam and its metabolites in blood and oral fluid collected by the modified Omni-Sal® device.**

| Median (ng/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Diazepam** | | **N-desmethyldiazepam** | | **Temazepam** | | **Oxazepam** | |
| *Oral Fluid* | *Blood* | *Oral Fluid* | *Blood* | *Oral Fluid* | *Blood* | *Oral Fluid* | *Blood* |
| 22 | 353 | 26 | 380 | 3 | 55 | 8 | 51 |

## *10.4.4 Discussion*

The modified Omni-Sal® device was easy to use and generally accepted by the patients. However, for some patients it took up to 30 minutes to provide a sample as a result of "dry mouth". Morphine concentrations were elevated in the oral fluid specimen compared with blood levels, although the number of positive samples was less in oral fluid than blood.

6-MAM was found in 15 oral fluid samples, but not in any of the corresponding blood samples. This could be useful for confirmation of heroin use, as 6-MAM is a unique marker, unlike morphine.

Diazepam and its metabolites were found more frequently in blood compared to oral fluid, and concentrations were much higher in blood. This could be as a result of the high protein binding capacity of the benzodiazepines in blood resulting in low concentrations in saliva.

### 10.4.5 Oral Fluid Collected by the Intercept® Device Results

91 oral fluid samples were collected by the Intercept® device. Instead of waiting until a specific volume was absorbed like the modified Omni-Sal® device, this collection device was placed in the mouth for between 2 – 5 minutes. The pad is impregnated with a salt to stimulate saliva production, and placed between the lower gum and cheek. A mixture of oral mucosal transudate, saliva and other debris in the mouth is absorbed by the pad. After collection, the pad is removed and placed in a tube with buffer, the stick snapped off and the tube sealed. Some patients preferred this device to the modified Omni-Sal® device because it was quick, but they didn't like the after taste from the salt.

In day 1 samples, only 6 were found positive for morphine, compared with 10 using the modified Omni-Sal® device and 11 in whole blood with 4 samples missing results. The full set of morphine results are shown in Table 10-11. In total 9 samples were found positive for morphine with a mean concentration of 60 ng/mL and a median of 22 ng/mL. The results ranged from 2 – 312 ng/mL. As before, oral fluid concentrations were greater than in whole blood, although the actual number of positives was less. The concentrations found in oral fluid samples collected using the Intercept® device were less than those obtained from the modified Omni-Sal® device. Morphine has a pKa of 8.1 and 10, and therefore its concentration in saliva is greatly affected by a change in saliva pH. By stimulating saliva flow, the bicarbonate concentration in the saliva increases making it more alkaline. This rise in pH can lower the saliva/plasma ratio and this could account for the lower concentrations of morphine found using this device compared to the other oral fluid device. Also because the saliva flow is stimulated, more saliva is secreted but drugs don't have time to diffuse in, giving a dilution effect. Another possibility is that the Intercept® collection device only collects ~0.4 mL of oral fluid compared to 1 mL by the modified Omni-Sal® collection device.

6-MAM was found in 5 % (n=5) of samples, compared with 16 % (n=15) of samples collected with the modified Omni-Sal® device. The mean concentration found was 49 ng/mL with a median of 34 ng/mL and a range of 7 – 133 ng/mL. The highest concentration of 133 ng/mL was found in JL300578 on day 1, this was also the highest 6-mam result, 282 ng/mL, using the modified Omni-Sal® device.

Codeine was found in only 2 samples at 8 and 9 ng/mL, compared with 15 samples using the modified Omni-Sal® device with a mean of 15 ng/mL and a median of 11 ng/mL. In the sample with 8 ng/mL of codeine morphine was also present at 61 ng/mL.

Dihydrocodeine was found in 35 % (n=32) of samples, with a mean and median of 1002 and 773 ng/mL respectively. Concentrations ranged from 20 – 4598 ng/mL. Although the number of samples found positive was less than that found using the modified Omni-Sal® device (43 %, median 379 ng/mL), the median concentration was greater. Dihydrocodeine has a pka of 8.8; it therefore, may not be as affected by stimulation than the other opiates.

**Table 10-11 : Morphine oral fluid concentrations collected using the Intercept® Device**

| Patient | Morphine Oral Fluid Concentrations (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| GY31290 | 22 | 0 | 0 | 0 | 0 |
| MMG4167 | ns | 0 | 9 | 0 | 0 |
| SL16771 | 0 | 0 | 0 | 0 | 0 |
| MT171060 | 0 | 0 | 0 | 0 | 0 |
| BA3181 | 0 | 2 | 0 | 0 | ns |
| HD180669 | 0 | 0 | 0 | 0 | 0 |
| CL180981 | 0 | 0 | 0 | 0 | 0 |
| JC271273 | 10 | 14 | 0 | 0 | ns |
| CD221180 | 0 | 0 | 0 | 0 | 0 |
| MM22676 | 61 | 0 | 0 | 0 | 0 |
| DT4676 | 0 | 0 | 0 | 0 | 0 |
| SC191082 | 74 | 0 | 0 | 0 | 0 |
| JS18981 | 39 | 0 | ns | 0 | 0 |
| SM051273 | 0 | 0 | 0 | 0 | 0 |
| JM011178 | 0 | 0 | 0 | ns | ns |
| JL300578 | 312 | 0 | 0 | 0 | 0 |
| SD220384 | 0 | 0 | 0 | ns | ns |
| AC290980 | 0 | 0 | 0 | ns | 0 |
| PT020681 | 0 | 0 | 0 | 0 | 0 |
| DMcC51174 | 0 | 0 | 0 | 0 | 0 |

ns : no sample provided

Diazepam was found in 33 % (n= 30) of samples, with a mean and median of 369 and 65 ng/mL respectively. The concentrations found ranged from 5 – 4216 ng/mL. The number of positives was less than those found in oral fluid collected by the modified Omni-Sal® device (59 %) and less than those found in blood (92 %). The concentrations found in oral fluid collected by the Intercept® device were greater than those collected by the modified Omni-Sal® device. The median concentrations of diazepam and its metabolites are shown in Table 10-12 compared with the other oral fluid device and blood. From the table it is clear that median concentrations in oral fluid collected by the Intercept® device are greater than the Omni-Sal® device but still less than blood concentrations.

**Table 10-12 : Median concentrations of diazepam and its metabolites in blood, and oral fluid collected by two devices.**

| **Diazepam Median Concentration (ng/mL)** | | |
|---|---|---|
| *Oral Fluid (Omni-Sal®)* | *Oral Fluid (Intercept®)* | *Blood* |
| **22** | **65** | **353** |
| **N-desmethyldiazepam Median Concentration (ng/mL)** | | |
| *Oral Fluid (Omni-Sal®)* | *Oral Fluid (Intercept®)* | *Blood* |
| **26** | **261** | **380** |
| **Temazepam Median Concentration (ng/mL)** | | |
| *Oral Fluid (Omni-Sal®)* | *Oral Fluid (Intercept®)* | *Blood* |
| **3** | **6, 4272** | **55** |
| **Oxazepam Median Concentration (ng/mL)** | | |
| *Oral Fluid (Omni-Sal®)* | *Oral Fluid (Intercept®)* | *Blood* |
| **8** | **24** | **51** |

### 10.4.6 Discussion

Morphine, 6-MAM and codeine were found less frequently using the Intercept® device compared with the Omni-Sal® device. The concentrations found were also less. This can be explained by the stimulation effect produced by the salt impregnated in the Intercept® device. Drugs with a pKa close to the pH of saliva i.e. 8.5, are affected greatly by a change in saliva pH. The Intercept® device also only collects ~0.4 mL of oral fluid compared with 1mL using the modified Omni-Sal® device. Therefore samples close to the limit of detection using the modified Omni-Sal® device may be missed using the Intercept® device.

6-MAM was found in five cases using the Intercept® device compared with fifteen using the modified Omni-Sal® device and none in blood.

Dihydrocodeine has a slightly higher pKa (8.8) than morphine, and is therefore not as affected by the pH change caused by the device. The number of dihydrocodeine positives was less than using the Omni-Sal® device but the concentrations were much greater.

The number of benzodiazepine positives using the Intercept® device was less than using the Omni-Sal® device, but the concentrations found were greater.

### 10.4.7 Urine Results

87 urine samples were collected and analysed. Morphine was found in all patients on day 1, and in 90 % (n=78) of all samples analysed. The full set of results is shown in Table 10-13. Most patients are positive for morphine throughout the 5 days. This result is not surprising, and the rehabilitation centre use urine analysis to confirm opiate use before prescribing methadone.

**Table 10-13 : Morphine concentrations found in urine**

| Patient | Morphine Urine Concentrations (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| GY31290 | 425 | 496 | 293 | ns | 0 |
| MMG4167 | 730 | 2126 | 245 | 181 | 69 |
| SL16771 | 377 | 107 | 74 | ns | ns |
| MT171060 | 30 | 0 | 0 | 0 | 0 |
| BA3181 | 2334 | 737 | 163 | 68 | ns |
| HD180669 | 77 | 203 | 31 | 8 | 4 |
| CL180981 | 563 | 465 | 116 | ns | 0 |
| JC271273 | 265 | 521 | 63 | 12 | ns |
| CD221180 | 16254 | 968 | 508 | 653 | 190 |
| MM22676 | 5208 | 149 | 22 | 26 | 6 |
| DT4676 | 8 | ns | ns | 19 | 8 |
| SC191082 | 988 | ns | 589 | 451 | 63 |
| JS18981 | 1690 | 255 | 110 | 14 | 2 |
| SM051273 | 1051 | 414 | 188 | 71 | 52 |

| Patient | Morphine Urine Concentrations (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| JM011178 | 172 | 44 | ns | ns | ns |
| JL300578 | 3830 | 453 | 290 | 43 | 14 |
| SD220384 | 1119 | 392 | 192 | ns | 87 |
| AC290980 | 10428 | 1139 | 301 | 75 | 0 |
| PT020681 | 959 | 291 | 14 | 0 | 0 |
| DMcC51174 | 388 | 970 | 700 | 120 | 37 |

ns : no sample provided

6-MAM was found in 7 % (n=6) of samples. 6-MAM is not usually found in urine because it metabolises rapidly to morphine. The median concentration found for 6-MAM was 286 ng/mL with a range of 12 – 1080 ng/mL. In 5 cases where 6-MAM was found, the corresponding morphine level was greater than 2000 ng/mL.

Codeine was found in 38 % (n=33) of samples, with a median of 17 ng/mL, it was never found after day 4. Dihydrocodeine was found in 79 % (n=69) of samples, many of these were off the scale of the detector. Diazepam was found in 68 % (n=59) of samples, N-desmethyldiazepam in 36 % (n=31) of samples, temazepam in 54 % (n=47) of samples and oxazepam in 78 % (n=68) of samples. Many of these values were at very high concentrations and off the scale of the detector.

### 10.4.8 Discussion

Urine analysis can detect opiate use up to 5 days after admission to the centre. 6-MAM was rarely found unless the morphine level was very high. Codeine was also found up to day 4 in urine samples. Dihydrocodeine and diazepam and its metabolites were found at very high concentrations, most likely because they were given these drugs on admission in to the rehabilitation centre.

### 10.4.9 Conclusions for Detection of Opiates in Heroin Addicts

The table below, Table 10-14, shows the morphine concentrations found in samples taken on day 1 in the centre.

**Table 10-14 : Overall day 1 results for morphine in all matrices**

| Patient | Day 1 Concentrations (ng/mL) | | | |
|---|---|---|---|---|
| | Blood | Intercept® | Omni-Sal® | Urine |
| GY31290 | ns | 7 | 11 | 425 |
| MMG4167 | 12 | ns | 0 | 730 |
| SL16771 | 7 | 0 | 0 | 377 |
| MT171060 | ns | 0 | 21 | 30 |
| BA3181 | 19 | 0 | 0 | 2334 |
| HD180669 | 0 | 0 | 0 | 77 |
| CL180981 | 7 | 0 | 0 | 563 |
| JC271273 | 0 | 3 | 0 | 265 |
| CD221180 | 28 | 0 | 98 | 16254 |
| MM22676 | 13 | 20 | 54 | 5208 |
| DT4676 | 0 | 0 | 0 | 8 |
| SC191082 | ns | 25 | 52 | 988 |
| JS18981 | ns | 13 | 43 | 1690 |
| SM051273 | 3 | 0 | 4 | 1051 |
| JM011178 | 0 | 0 | 0 | 172 |
| JL300578 | 4 | 104 | 437 | 3830 |
| SD220384 | 3 | 0 | 0 | 1119 |
| AC290980 | 16 | 0 | 99 | 10428 |
| PT020681 | 0 | 0 | 0 | 959 |
| DMcC51174 | 3 | 0 | 6 | 388 |

ns : no sample provided

It is clear that if the object is to determine opiate use in the last 24 hours then urine analysis provides this evidence. Although oral fluid morphine concentrations were often higher then morphine blood concentrations, the number of positives was less for oral fluid than for blood.

It has been shown that diazepam and its metabolites do not diffuse into saliva easily, and concentrations were a magnitude less in oral fluid than in blood. The Intercept® device gave higher concentrations of diazepam and its metabolites because of salt present which stimulated saliva flow. This is in contrast to the opiates where concentrations were less using the Intercept® device than with the modified Omni-Sal® device. This can be

explained by the pKa of these analytes and their susceptibility of being affected by a change in saliva pH.

Most patients preferred the Intercept® device because it took less time than the modified Omni-Sal® device. The instructions for use of the Intercept® are to leave the device in the oral cavity for between 2 and 5 minutes. Most patients left the device in for 2 minutes only, which could have led to less sample being collected resulting in fewer positive results. Patients did not like the salty aftertaste when using the Intercept® device, but it seemed that the time taken was more important to them. The modified Omni-Sal® device took between 5 and 30 minutes to collect 1 mL of oral fluid. One of the side effects of opiate use is "dry mouth" resulting in a longer time to collect a specified volume.

The detection of 6-MAM in oral fluid where none was found in blood is significant in that it can be used as a specific marker for heroin use. 6-MAM was only found in 6 urine samples compared to 15 samples collected with the Omni-Sal® device and 5 with the Intercept® device.

Although oral fluid analysis proved unsuitable for the rehabilitation centre setting, it was generally accepted more than urine and blood. The morphine blood concentrations were very low, so it is unsurprising that there were many negative oral fluid samples.

# 11 Conclusions

The potential use of liquid chromatography – mass spectrometry has been investigated for its use in forensic toxicology. It has been shown that it is an invaluable tool and complements the use of gas chromatography – mass spectrometry.

The development of a sensitive and specific method for the detection of flunitrazepam in blood is essential for the increasing number of drug facilitated sexual assault cases submitted to the laboratory. Limits of quantitation of 0.2 and 0.5 ng/mL were achieved for flunitrazepam and its metabolite 7-aminoflunitrazepam respectively. It was also discovered that the use of very concentrated additives in the mobile phase frequently suppressed ionisation in the source, and lowered sensitivity.

In addition to flunitrazepam another method was developed for diazepam and its metabolites N-desmethyldiazepam, temazepam and oxazepam. This proved invaluable with limits of detection $\leq 1.1$ ng/mL for each analyte. The sensitivity of LC-MS is shown to be much greater than HPLC with UV detection and it doesn't suffer from the same interference from biological samples. With the use of the chlorine atom present in every analyte, a qualitative ion was found by looking for the pseudo-molecular ion containing $^{35}Cl$ and $^{37}Cl$. Not only the additives present in the mobile phase affect ionisation as does the organic solvent. Acetonitrile was found to be much more preferential to the benzodiazepines ionisation than methanol.

With the submission of a post-mortem blood sample requiring sildenafil analysis it was necessary to develop and validate a suitable instrumental method. LC-MS proved suitable, with its in-source collision capable of monitoring the pseudo-molecular ion and a specific daughter ion simultaneously. The method was validated and applied to post-mortem blood, eliminating sildenafil overdose as a cause of death.

The use of oral fluid as an alternative matrix to blood or urine, has come to the forefront of forensic toxicology in recent years. In the course of this project it was assessed as a roadside collection device and as an alternative to urine in a rehabilitation centre setting. A disadvantage of oral fluid testing is the lack of volume obtained from the subject. This need not be a problem if a unified extraction method is used to simultaneously extract multiple analytes. With the use of this method, analytes were analysed by using both LC

and GC-MS. Thermally labile drugs requiring derivatisation for GC-MS analysis were analysed on the LC-MS, this included 20 drugs of abuse and their metabolites. The LC-MS required less maintenance and consequently suffered from much less down-time than the GC-MS. In the first project involving oral fluid analysis (Chapter 7), the LC-MS was used and further qualitative information was obtained from the GC-MS. There was great correlation between the quantitative results from both instruments. Using LC-MS-MS in the second project (Chapter 8) made the acquisition of Full MS-MS data possible, negating the need for confirmation from the GC-MS. Although three injections were needed for each sample, to analyse all 21 analytes, many injections could be placed in a queue running the instrument almost continuously because of its reliability.

Finally the method developed in Chapter 8 was validated for 8 drugs extracted from urine, blood and an additional oral fluid collection device. This enabled the detection of analytes over various matrices collected at the same time. Unfortunately drugs of abuse do not seem to be suited to the prediction of blood concentrations from oral fluid concentrations. This is evident from the marked differences between the two oral fluid collection devices. Recent drug use can be assessed though; in the case of contamination of the oral cavity drug administration can be predicted within the last few hours.

With respect to benzodiazepine analysis in oral fluid, it is evident that concentrations found in oral fluid are considerably less than blood. The use of a collection device with salt stimulation resulted in higher concentrations, although these were still less than blood. This data provides valuable information on benzodiazepine concentrations found in subjects who are known abusers of Valium®.

In conclusion, the reliability, versatility and specificity of LC-MS and LC-MS-MS are unsurpassed and there is a definite need for its use in forensic toxicology. This is not to say that GC-MS has lost its place in the laboratory. The parallel use of these two instruments in the laboratory will provide a solution to most forensic toxicology problem.

# 12 Further Work

The use of LC-MS for the detection and quantitation for drugs of abuse in blood, urine and oral fluid has been shown. With some adaptation of the extraction methods, LC-MS analysis could be applied to different matrices including hair. The matrix effects would have to be monitored to ensure limits of detection were not compromised. Evaluating the matrix effects from different matrices would be useful in determining the optimum sample pre-treatment and extraction procedure. This may mean that for urine analysis a simple dilution would be sufficient because of the high drug concentrations expected.

Screening for unknown drugs in LC-MS is more difficult than in GC-MS as the spectra a variable with instrument, laboratory, chromatography conditions and voltages applied for fragmentation. To overcome this an in-house library would need to be built up with different collision energies for each drug. The use of two collision energies, a high and low, would give a molecular ion and a more detailed spectra with specific daughter ions. The use of data-dependent scanning enables the instrument to select the ion with the highest response and carry out an MS-MS scan on it.

With respect to oral fluid, there are many questions unanswered as to the concentrations obtained and their relevance. By doing more controlled research into the collection devices available, and their relationship with expectorate, a better understanding would be achieved. This could involve the measurement of the pH of oral fluid when it is sampled, as this is an important factor when analysing drugs of abuse. Also more work could be carried out with the Intercept® device to establish if leaving the pad in for a longer period of time greatly effects the final concentrations. Washing the pads from the collection devices and extracted the washes may be useful in determining if benzodiazepines are adsorbed in some way on the pad. By simultaneously collecting expectorate and oral fluid using collection devices, a better understanding of the effect of the collection device on drug concentrations could be achieved.

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# Appendix 1: Publications in Support of this Thesis

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# SIMULTANEOUS DETECTION AND QUANTITATION OF 21 DRUGS OF ABUSE AND THEIR METABOLITES IN ORAL FLUID USING LIQUID CHROMATOGRAPHY – ELECTROSPRAY MASS SPECTROMETRY

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**Aims:** An LC-MS method has been developed for drug analysis in oral fluid. The method was validated for 21 drugs of abuse and their metabolites. **Methods:** A single quadrupole instrument was used in Selected Ion Monitoring (SIM) mode with an electrospray interface. Skimmer cone voltages for each drug were optimised and defined, ranging from 5 V for amphetamine to 40 V for norbuprenorphine. The probe temperature was set at 350 °C with 5 kV applied. A Phenomenex Luna C18(2) 150 X 2.0 mm with 3 µm packing was used for separation with a guard column of identical packing material. The mobile phase composition was investigated to include the use of formic acid in combination with other buffers to monitor the effect on response. The use of a basic buffer, 10 mM ammonium bicarbonate, was also explored. The optimised mobile phase employed a gradient system of acetonitrile and 10 mM ammonium bicarbonate. Oral fluid samples were collected using the modified Omni-Sal® device supplied by Cozart and extracted using Bond Elut Certify solid phase extraction columns. The analytes were eluted using 4 mL acetone : chloroform (50:50) then 4 mL of ethyl acetate with 2 % ammonia. Both fractions were blown down under nitrogen, reconstituted in mobile phase and injected into the LC-MS in duplicate. **Results:** Recoveries for all drugs of interest were found to be over 80 %. Limits of detection were calculated as 3 times the standard error of the straight line + the intercept. They ranged from 0.1 ng/mL for buprenorphine to 1.5 ng/mL for cocaethylene. Limits of quantitation were calculated using 10 times the standard error, they were all found to be $\leq$ 5.0 ng/mL. This method was applied to 40 oral fluid samples. **Conclusions:** Ammonium bicarbonate was found to be favourable for the chromatography and ionisation of the compounds identified in this method. A single quadrupole LC-MS was successfully used to identify and quantify the major drugs of abuse and their metabolites from 1mL of oral fluid.

**Keywords: drugs of abuse, oral fluid, LC-MS**

# THE USE OF LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY IN FORENSIC TOXICOLOGY.

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Gas Chromatography – Mass Spectrometry (GC-MS) has been accepted as the gold standard technique for the qualitative and quantitative analysis of drugs in forensic toxicology. Unfortunately due to a number of factors including the high temperatures involved not all drugs chromatograph well by GC, especially thermally labile analytes. High Pressure Liquid Chromatography (HPLC) has proved useful in the analysis of such analytes. With only UV detection however it is not as sensitive or specific as MS. HPLC has its advantages because of the gentle separation and lack of high temperatures it employs. Using a UV detector does not give the structural information of MS spectra, and can suffer from interference if samples are especially dirty e.g. post-mortem blood extracts. To separate using liquid chromatography and detect with a mass spectrometer would be ideal. Only in the last two decades has LC been successfully interfaced to MS. With the use of LC-MS, sensitive and specific methods can be developed to analyse otherwise problematic drugs. For example Rohypnol®, the infamous "date rape" drug, is administered in very low doses and metabolises rapidly in the body. A very sensitive detection technique is required which LC-MS provides. Analysis of benzodiazepines by GC is not ideal because of thermal decomposition at the high temperatures involved in the separation. LC-MS uses gentle separation with no heat and with soft ionisation in the MS. Other forensic applications of LC-MS include the ability to quantitate 21 drugs of abuse and their metabolites. The advantages of this technique include no derivatisation, analysis of thermally labile compounds and little downtime of the instrument.

**Keywords: forensic toxicology, LC-MS**

# ANALYSIS OF SALIVA FOR DRUGS OF ABUSE USING LC-MS

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Oral fluid testing for drugs of abuse has become of great interest in a variety of areas such as roadside testing of potentially intoxicated drivers, testing of patients involved in drug maintenance programmes and drug testing in the workplace. Oral fluid samples can be collected using one of several commercially available devices. However, regardless of the device, the limitation of this specimen is often in the amount of sample available. In cases where the sample has to be tested for a wide variety of drugs ideally a single extraction method is preferable.

The use of Liquid Chromatography – Mass Spectrometry (LC-MS) for the separation and detection of drugs means that sensitive and specific methods can be developed to include drugs which may otherwise be problematic by Gas Chromatography-Mass Spectrometry.

This presentation will discuss the use of single quadrupole and ion trap LC-MS instruments with electrospray ionisation for the analysis of drugs of abuse in oral fluid samples. The drug findings in two projects involving drivers and the relative merits of each instrument will be addressed.