An Investigation into the Distribution and Concentration of Drugs of Abuse within Different Body Compartments and Relationship with Toxicity

A thesis submitted in part fulfilment of the requirements for admission to the Degree of

Doctor of Philosophy

By

Salah Ali Menshawi

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Forensic Pathology Unit
Department of Cancer Studies and Molecular Medicine
University of Leicester

Dedicated to my parents, wife and children

ABSTRACT

Femoral blood is preferable in post-mortem toxicological analysis but may not always be available. Alternative specimens such as cardiac blood, vitreous humour, stomach contents, bile, urine, hair, bone, liver and muscle may be used. However, only limited data are available for the interpretation of drug concentrations in these alternative specimens. The aim of the study was to investigate the distribution of drugs in different body compartments in relation to levels in femoral blood and their toxicity.

All post-mortem specimens from 95 subjects were provided by Professor M Tsokos, Institute of Legal Medicine, Germany. Ethanol was measured by gas chromatography, whilst other drugs of abuse were initially screened for by immunoassays (in blood, hair and bone by Cozart drug immunoassay kit; urine by cloned enzyme donor immunoassay). A general drug screen and confirmation was performed on all specimens using gas chromatography/mass spectrometry and drug quantification was by high-pressure liquid chromatography-tandem mass spectrometry.

Drug levels in femoral blood and median concentration ratios in relation to femoral blood (range) were as follows: ethanol 4-365mg/dL, median ratios for cardiac blood 1.9 (0.8:1-42.3:1), vitreous humour 1.6 (0.7:1-9.4:1), urine 6.5 (1.7:1-153:1) and bile 11.3 (1.0:1-34:1); for cocaine 5-2413ng/ml, median ratios for cardiac blood 1.2 (0.2:1-7.0:1), vitreous humour 1.4 (1.0:1-3.3:1), urine 4.9 (0.4:1->1000:1) and bile 7.0 (0.6:1-41.6:1); for methadone 7-1795 ng/ml, median ratios for cardiac blood 1.4 (0.6:1->3.9:1), vitreous humour 0.7 (0.04:1-0.99:1), urine 0.7 (0.3:1->3.9:1) and bile 2.9 (0.7:1->9.6:1); for morphine 7-1756 ng/ml, median ratios for cardiac blood 2.0 (0.9:1-11.5:1), vitreous humour 1.0 (0.4:1-18.5:1), urine 2.2 (0.1:1-56:1) and bile 24 (1.3:1 -> 195:1). Similarly, in liver and muscle for cocaine the median concentration ratio in ml/g was 0.2 (0.04:1-1.0:1) and 0.1 (0.1:1-2.5:1) respectively; for methadone 0.3 (0.2:1-<0.7:1) and 0.1 (0.05:1->0.7:1) respectively; and for morphine 0.6 (0.1:1-1.6:1) and 0.3 (0.1:1-1.6:1) respectively. In the majority of cases there was a good correlation between femoral blood levels and the concentration in other body compartments.

This study has added to our knowledge and understanding of the usefulness of using alternative post-mortem specimens to study the distribution of drugs of abuse in different body compartments and how the concentrations relate to femoral blood levels and toxicity.

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Finally many thanks to Dr Louay Al-alousi who with Dr Madira initially suggested this study and without those efforts, it would have been very difficult if not impossible for this study to go ahead.

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Abbreviations

ADME	Absorption, distribution, metabolism, excretion
6-MAM	6-monoacetyl morphine
ADH	Alcohol dehydrogenase
AEME	Anhydroecgonine methyl ester
Amph	Amphetamine
APCI	Atmospheric pressure chemical ionisation
BAC	Blood alcohol concentration
Barb	Barbiturate
BBB	Blood-brain barrier
BE	Benzoylecgonine
Benz	Benzodiazepines
BHB	Beta-hydroxybutyrate
Bup	Buprenorphine
BZDs	Benzodiazepines
C/P	Central to peripheral blood drug concentration ratio
cAMP	Cyclic adenosine monophosphate
Canna	Cannabinoids
CB	Cardiac blood
CE	Cocaethylene
CNS	Central nervous system
Coca	Cocaine
Cod	Codeine
CV%	Coefficient of variation percent
EC	Ecgonine
EDDP	2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine
EIA	Enzyme immunoassay
EME	Ecgonine methylester
ESI	Electrospray ionisation
EtG	Ethyl glucuronide
FB	Femoral blood
Flu	Flunitrazepam
GABA	Gamma-aminobutyric acid
GC/MS	Gas chromatography mass spectrometry
GHB	Gamma-hydroxy butrate
GIT	Gastrointestinal tract
HbA1c	Glycosylated haemoglobin
IV	Intravenous
IVC	Inferior vena cava
L	Liver
LC-MS/MS	High-Pressure Liquid Chromatography-Tandem Mass Spectrometry
LD_{50}	Lethal dose of the drug that kills 50% of subjects
LOD	Limit of detection
LOQ	Limit of quantification
LSD	Lysergic acid diethylamide
M	Muscle

MBTFA	N-methyl-bis trifluroacetamide
ME	Methylecgonine
MEOS	Microsomal ethanol oxidizing system
Metha	Methadone
Metham	Methamphetamine
mOHBE	m-hydroxybenzoylecgonine
Morph	Morphine
MSTFA	N-methyl-N-trimethylsilyl trifluroacetamide
NBE	Norbenzoylecgonine
Opi	Opiate
PM/AM	Post-mortem/ante-mortem blood drug concentration ratio
PMDR	Post-mortem drug redistribution
pOHBE	p-hydroxybenzoylecgonine
Pro	Promazine
SC	Stomach contents
$t_{1/2}$	Drug half-life
TCA	Tricyclic antidepressants
THC	Tetrahydrocannabinol
U	Urine
UAC	Urine alcohol concentration
V_d	Drug volume of distribution
VH	Vitreous humour
VHAC	Vitreous humour alcohol concentration

Posters and Presentations

1. S Menshawi, LM Al-Alousi, W Madira and M Tsokos title 'An investigation into the distribution and concentration of drugs of abuse within different body compartments and relationship with toxicity' oral presentation to the 1st Saudi Scientific Meeting, London, UK, May 2005.

2. S Menshawi, LM Al-Alousi, W Madira and M Tsokos title 'Detection of cocaine and methadone in different post-mortem fluid and tissue samples' Poster presentation to the 46th Conference of the Society of American Toxicologists, North Carolina, USA, March 2007. Poster number 2013.

3. S Menshawi, LM Al-Alousi, W Madira and M Tsokos title 'Detection of cocaine and methadone in different post-mortem fluid and tissue samples' Poster Presentation to the Saudi Innovation Conference, Newcastle upon Tyne, UK, May 2007.

4. S Menshawi, LM Al-Alousi, W Madira and M Tsokos title' Detection of Alcohol in Different Post-Mortem Samples' Oral presentation to the 1st International Conference for Forensic Science and Forensic Medicine, NUSS, Riyadh, KSA, November 2007.

CHAPTER ONE: INTRODUCTION

1: Introduction:

Post-mortem forensic toxicology deals with the detection of drugs/toxins in samples from the deceased and the contribution of the findings to the cause of death (Karch, 2007). Peripheral (femoral) blood is usually preferred over other post-mortem samples for forensic toxicology analysis (Hilberg et al., 1999; Moriya and Hashimoto, 2001). This is mainly because peripheral blood is affected to a lesser extent by the post-mortem drug redistribution process in comparison to cardiac blood (Pounder and Smith, 1995; Pounder et al., 1996).

However, blood is not always available for analysis in every case or the blood may not be suitable for analysis (e.g. where a blood transfusion took place shortly before death). In these situations where blood cannot be used for toxicological analysis, alternative tissue/fluid specimens such as urine, vitreous humour, stomach contents, liver, bile, muscle, hair, nails, bone, teeth, and even the larvae that feed on a human host can be very valuable (Uboh et al., 1995; Leikin and Watson, 2003; Drummer, 2004).

The body is not a single compartment and the concentrations and distributions of drugs throughout the body will depend on several factors, one of the most important being the hydrophilicity and hydrophobicity of the drug. This defines the degree to which a drug dissolves in the water compartment. As a result, the drug concentrations measured in blood samples do not necessarily equate to the exact concentration at the site of action. Therefore, interpretation of the concentration of a

drug in relation to toxicity requires a full understanding of the drug properties and metabolism (Milroy and Forrest, 2000; Flanagan et al., 2003; Crandall et al., 2006; Stimpfl, 2007).

In addition to normal physiological distribution of drugs in different body compartments, a phenomenon of post-mortem redistribution can also occur and further alters the tissue/specimen drug concentrations in the post-mortem period (Langford and Pounder, 1997; Hilberg et al., 1999; Cook et al., 2000; Yarema and Becker, 2005). The interpretation of post-mortem drug levels is further influenced by drug tolerance and drug interactions (Drummer et al., 2004; Gerevich et al., 2005).

Therefore, knowledge of the pharmacokinetics and pharmacodynamics of drugs is important in post-mortem forensic toxicology. Pharmacodynamics refers to the biochemical and physiological effects of drugs on the body, the mechanisms of drug action and the relationship between drug concentration and effect. The stages of the pharmacokinetic processes are absorption, distribution, metabolism and excretion, sometimes referred to as ADME (Neligan, 1999; Bourne, 2001; Greenblatt et al., 2002). See Figure 1.

1.1: General Pharmacodynamics and Pharmacokinetics Principles of Drugs:

1.1.1: Absorption:

Enteral and parenteral administrations are the two main routes by which drugs enter the bloodstream.

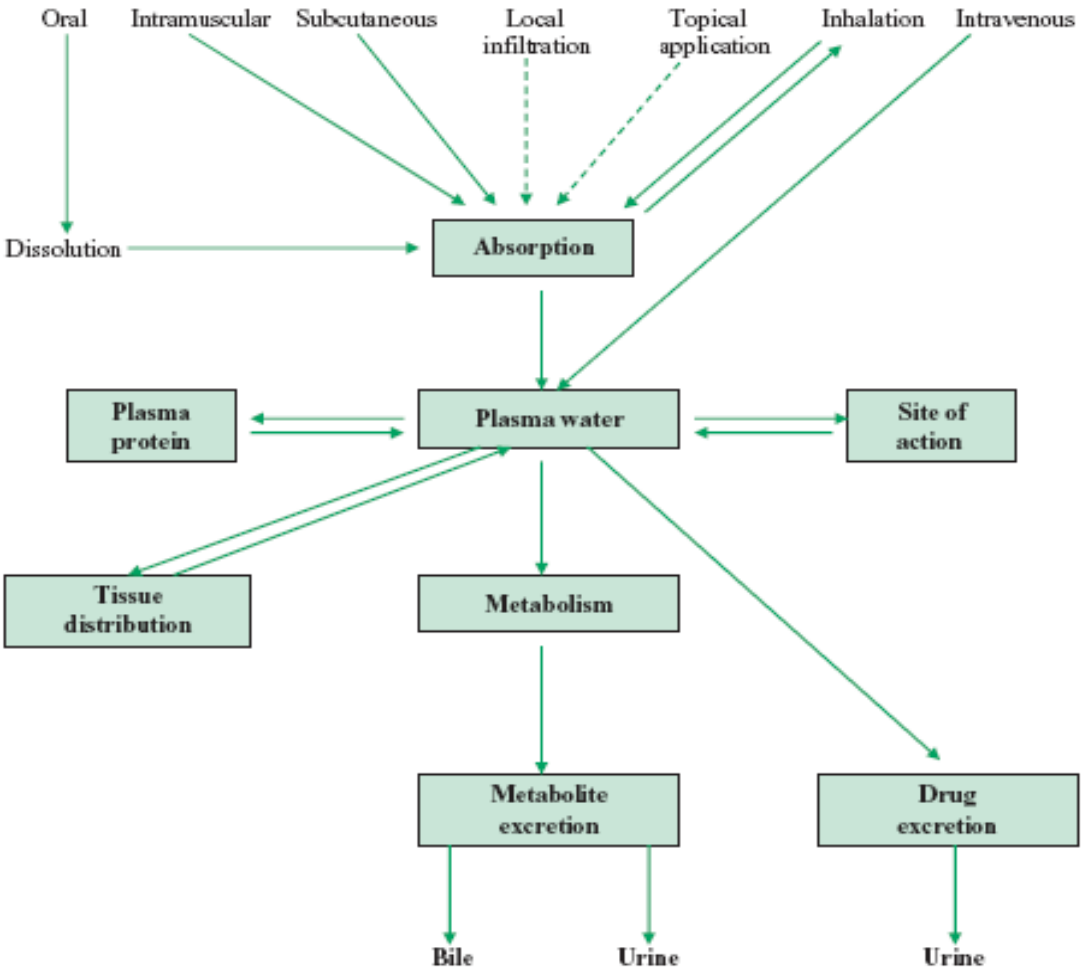


Figure 1: Drugs ADME, and concentration of drugs at their site of action (Calvey, 2007).

The enteral route includes oral, sublingual and rectal and the parenteral route includes intravenous, intramuscular, subcutaneous, dermal, vaginal and intraperitoneal administrations (DeBoer, 1994; Levine and Spiehler, 2003; Kopacek, 2007; Bourne, 2007; Klaassen, 2008). The route of drug administration will affect its bioavailability. For instance, when a drug is injected into the bloodstream it is

considered to have a bioavailability of 100% and the bioavailability of a drug given by any other route is compared to this value (Pleuvery, 2005).

Absorption refers to the movement of a drug from the mucous membrane of an organ/tissue, e.g. from small intestines or lungs, to the bloodstream. In general, due to the large surface area of the small intestines, rates of absorption for most drugs are predominantly higher in this region in comparison to any other part of the gastrointestinal tract (GIT). The mechanisms by which drugs are absorbed from the stomach and small intestines are:

1) Simple diffusion via a concentration gradient: The drug moves from a compartment where it is at a high concentration to compartment/s where the concentration of the drug is lower.
2) Non-ionic diffusion: Drugs are mostly weak acids or weak bases and once they dissolve in water they donate or accept hydrogen ions to produce an ionised base. Both ionised and non-ionised forms of the drug are at equilibrium. The ionised form of the drug is water-soluble and usually cannot cross the plasma membrane whilst the un-ionised form is lipid-soluble and can more readily cross the membrane.
3) Carrier transport: There are two carrier protein systems, facilitated diffusion which is similar to simple diffusion but requires a protein carrier and active transportation which is against the concentration gradient and requires energy in the form of adenosine triphosphate (ATP).

In addition to the surface area of the small intestine, other factors affecting drug absorption from the GIT include local blood flow rate, drug solubility and molecular

weight, drug permeability, drug-drug interaction, physical states, media pH, luminal and mucosal enzymes and intestinal motility (Burton et al., 2002; Martinez and Amidon, 2002; Klaassen, 2003). The presence of food in the gut may also influence the absorption of drugs, e.g. ethanol absorption and bioavailability is markedly reduced by the presence of food in the gut (Pawan, 1972; Anaise, 2002; Persson et al., 2008). Drug absorption through GIT is much slower than that through the lungs, but faster than that through the skin (Burton et al., 2002; Levine and Spiehler, 2003; Kopacek, 2007).

Drugs absorbed from the respiratory tract such as cocaine, gases and volatile liquids reach blood circulation very rapidly due to the large surface area of the lungs (Hickey, 1996). The lipophilicity of drugs is a less important factor in the rate of absorption through the lungs than that through the intestines because ionised molecules have very low volatility and do not achieve significant concentration in the inhaled air. In addition, the epithelial cell-lining of the alveoli is very thin and the capillaries are in close contact with the pneumocytes so that the distance for the drugs to diffuse is very short. Finally, the drugs absorbed by the lungs are removed very quickly by the blood due to the high blood flow through the lungs (Pawan, 1972; Craig and Stitzel, 2003; Klaassen, 2008).

Absorption through the skin is controlled by the ability of the drug to cross the epidermis before reaching the blood circulation. The skin is composed of three layers, subcutaneous tissue, dermis and epidermis (Déry, 2005). Drug absorption through the skin occurs mainly by passive diffusion from the stratum corneum or the upper layer of the epidermis (Klaassen, 2003; Simon and Loney, 2005). The extent

of drug absorption through the skin is variable from one site to another depending on the thickness and lipid content of the stratum corneum (Berti et al., 1995).

*1.1.2: **Distribution:***

Distribution refers to the movement of drugs from the blood into different body compartments. The distribution of drugs occurs more rapidly in organs with high blood flow e.g. heart, liver and brain compared with tissues with low blood flow e.g. bone and fat (Barker and Bromley, 2002; Levine and Spiehler, 2003; Gomella et al., 2004). After intravenous administration, the rate of decrease in plasma drug concentration is dose-dependent and its decline is exponential. This process is known as *first order kinetics* and is common with most drugs of abuse (Karch, 1997). Some drugs of abuse e.g. ethanol, follow *zero order kinetics*. This term is applied when drug distribution and elimination is not dose-dependent and requires degradation by liver enzymes (Bryson, 1996; Stringer, 2005).

In pharmacokinetics, the distribution of a drug in the body is described by the *compartment models* (Gibaldi and Perrier, 1982; Karch, 1997; Thomas, 2008). There are three main compartment models: In the *one-compartment model*, the body is considered as a single homogeneous compartment. This model is useful in describing the drugs that are presumably distributed rapidly and uniformly and are eliminated at rates and amounts that are proportional to drug concentrations left in the body (Figure 2). This model does not apply to most drugs of abuse but is applicable to water-soluble antibiotics given by an intravenous route e.g. gentamicin (Gibaldi and Perrier, 1982; Barker and BromLey, 2002).

(The rate of elimination of a drug which follows a one-compartment model is proportional to the amount of drug in the body). Only a few drugs actually follow the simple, first-order, one-compartment model (Arnikar, 1992; Thomas, 2008).

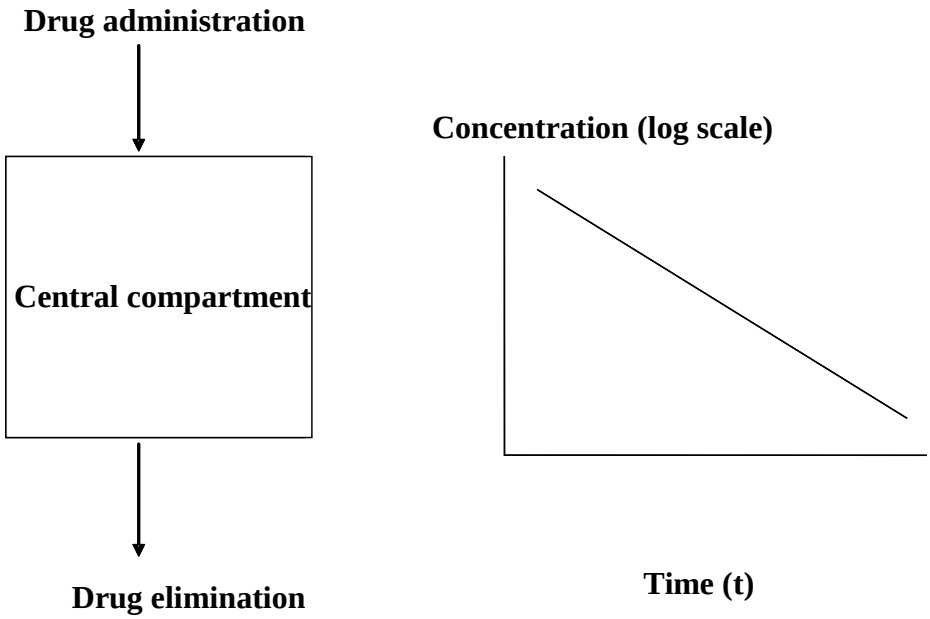


Figure 2: One-compartment model

In the *two-compartment model* (Figure 3), the drug enters the blood and it is then re-distributed to the second compartment from which it can be eliminated or it may return to the first compartment. The drug equilibrium between the two compartments indicates the end of the drug distribution phase (Karch, 1997; Nahas, 1997). The *two-compartment model* suggests that there is a decrease in the concentration of a drug in blood due to its re-distribution to a peripheral compartment/tissue (Barker and Bromley, 2002; Maher, 2006; Karch, 2008). A half-life of a chemical whose kinetic behaviour fits the two-compartment model is often referred to as the

"*biological half-life*". This is the most commonly used measure of the kinetic behaviour of drugs (Vallero, 2004).

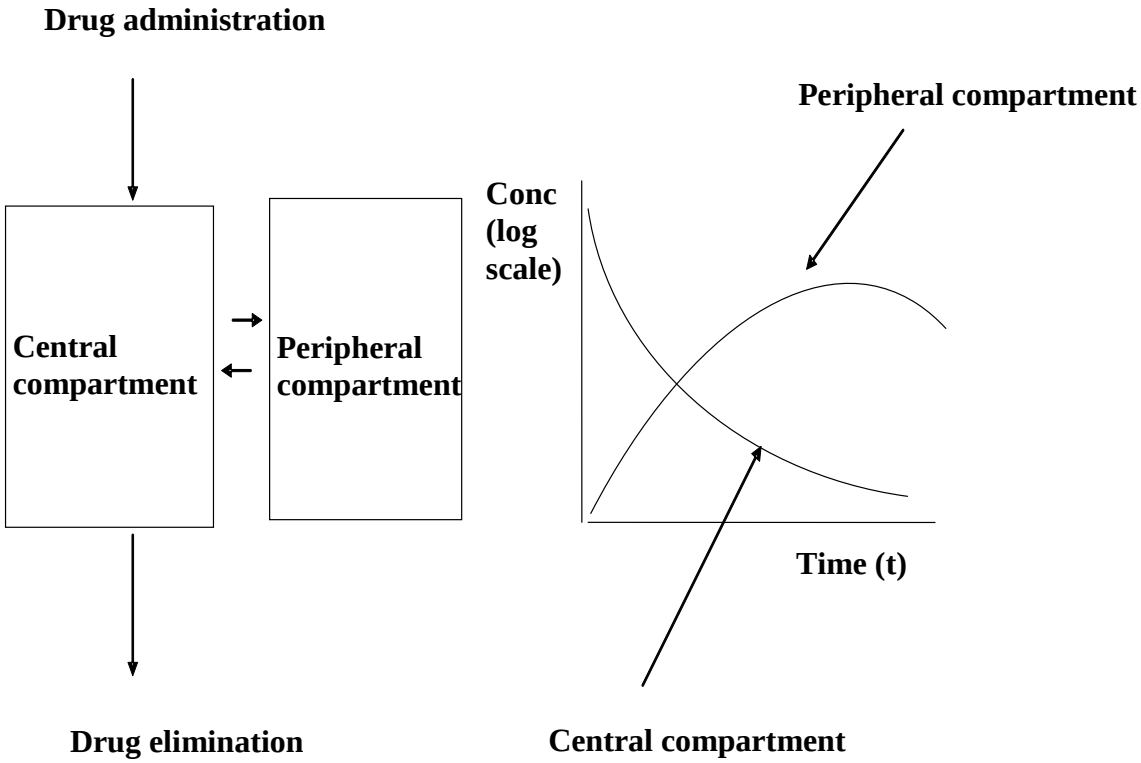


Figure 3: Two-compartment model

The *three or multiple-compartment model* (Figure 4) is similar to the two-compartment model, but with the addition of a third compartment or 'reservoir' e.g. adipose tissue, where a drug may remain for over a long period time. In this model there is multi-exponential decrease in drug concentration. An example of a drug of abuse which follows the multi-compartment model is tetrahydrocannabinol [THC] (Karch, 1997; Nahas, 1997; Guelcher, 2005; Maher, 2006).

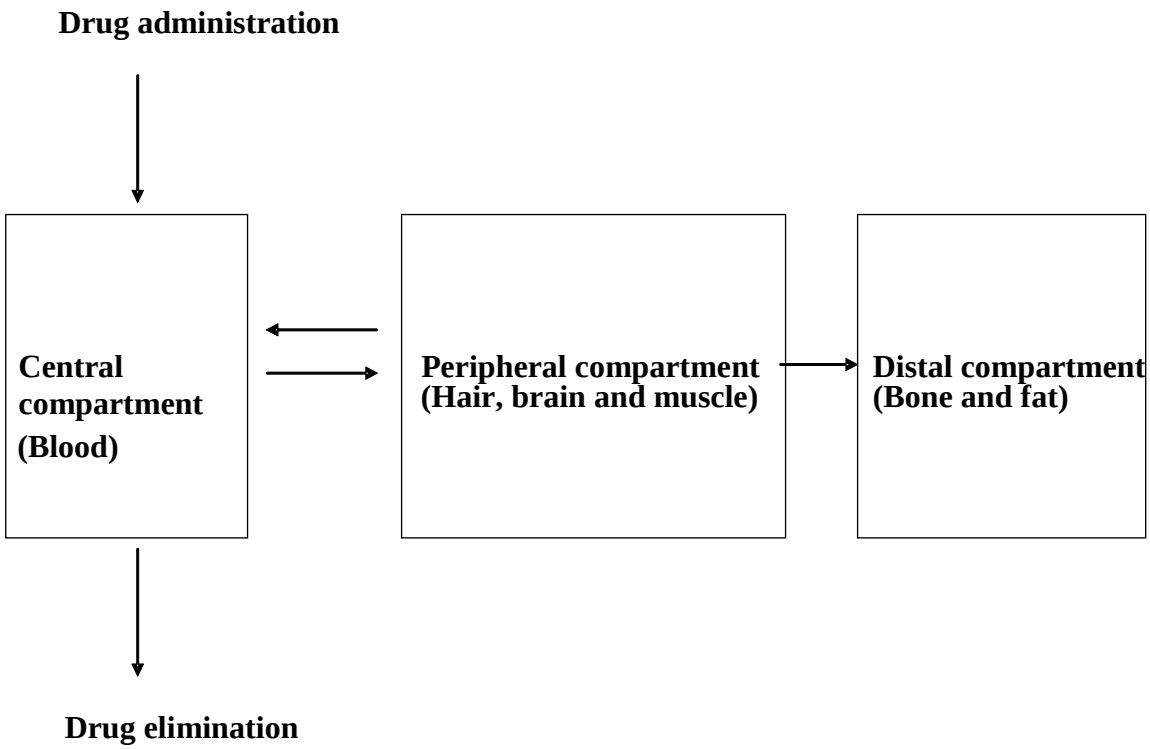


Figure 4: Three-compartment model

In addition to drug lipophilicity, factors affecting distribution include drug properties such as plasma-protein binding, molecular weight, volume of distribution, degree of ionisation, blood flow, fat storage, health state of the tissue e.g. liver or kidney disease, and the individual variables age, gender and race (Pleuvery, 2005; Greenstein, 2006). Organs with a high blood supply e.g. liver, kidney, heart and brain receive the majority of the absorbed drugs whilst those parts of the body with minor or less blood supply e.g. muscle, bone and fat tissue receive lesser amounts of the absorbed drugs (Neligan, 1999). Similarly, drug distribution from the skin and muscle increases with exercise due to increased blood supply (Somani 1995; Khazaeinia et al., 2000).

In general, hydrophilic drugs cannot readily enter the cells due to the relatively large molecular size (Klaassen, 2008). Thus, low molecular weight drugs were able to cross blood capillaries and distribute in the extra-cellular fluids and cross cell membranes and distribute in the intracellular fluids (Lüllmann, 2000). The amount of fat content in the body plays an important role in drug distribution. For instance women have a higher percentage of body fat than men, which affects the volume of distribution, rate of redistribution and clearance of lipophilic drugs (Bjorntorp et al., 1971). Thus, the lipophilic drugs such as diazepam are stored at higher concentrations in females than in males. By the same token, distribution of lipophilic drugs is different in slim versus obese individuals (Greenblatt et al., 1980; Ochs et al., 1981; Wilson, 1984).

The degree to which drugs bind to plasma-proteins also has a significant effect on drug distribution. This is due to the fact that the unbound drug, as opposed to a drug-protein-complex, can penetrate the wall of the blood vessel (Craig, 2003; Levine and Spiehler, 2003). In humans, the most important plasma proteins which bind many drugs, and thus affect their distribution into the body tissues or cells, are albumin and globulins (Levine and Spiehler, 2003). Albumin represents around 50% of the total plasma proteins that bind to drugs. The acidic drugs bind to albumin, while basic drugs often bind to alpha$_1$-acid glycoproteins and lipoproteins (Bourne, 2001). The protein binding process leads to an increased total drug concentration in blood and a decrease in the free drug level and may also prolong the elimination time and pharmacological effects of the drug (Lohman et al., 1986; Foss and McCormick, 2005).

Drug competition for the plasma-protein binding site or drug-drug interaction, increases the free drug and pharmacological effect, e.g. ethanol administration decreases the binding of warfarin to the plasma protein and increases the pharmacological effects of warfarin. Drugs which are highly plasma protein bound tend to be at lower concentrations in vitreous humour than in blood, e.g. diazepam and nordiazepam (Forrest, 1993). The free or unbound drug can be calculated from the following formula (Ansel and Prince, 2003)

$\alpha = C_U / (C_U + C_B) = C_B / C_T$ Equation 1

Where α is the free drug concentration, Cu is the plasma drug concentration, C_T is the total plasma drug concentration and C_B is the bound drug concentration.

α = 1- % Protein binding/100 Equation 2

The interpretation of the concentration of a drug measured in the post-mortem period also needs to take into account the drug volume of distribution (V_d) which will have an influence on its post-mortem redistribution. Drug V_d is defined as the volume of fluid required to contain the entire administered drug in the body at the same concentration as in the plasma. It can be calculated by *Equation 3* (Levine and Spiehler, 2003; Karch, 2006).

V_d = Administered dose/ blood drug concentration Equation 3

The total body water volume is approximately 0.55 L/kg. Therefore, drugs that have V_d of 0.55 L/kg or less are only distributed in the body fluids e.g. insulin and heparin (Drummer, 2001; Bean, 2007). In general, drugs with high V_d >1 L/kg tend to be distributed in the body tissue e.g. body fat as well as in body fluids. In other words, lipophilic drugs tend to have a high V_d. Therefore, drugs with a high V_d tend to be highly lipid soluble and can penetrate the blood-brain barrier [BBB] (Seydel and Wiese, 2002; Pleuvery, 2005). Table 1 shows the commonly abused drugs with their volume of distribution.

As stated earlier other body specimens, e.g. hair, can often be useful for toxicological analysis in the presence and/or absence of a blood sample. Some knowledge of hair anatomical morphology and physiology is useful. Hair consists of five components: cuticle, cortex, medulla, melanin granules and cell membrane complex (Nakahara and Kikura, 1994; Nakahara, 1999; Wei and Bhushan, 2006). In humans, there are around 80,000-100,000 hair follicles, which decrease with age (Potsch, 1995) and hair growth rate is 1.0-1.3 cm/month, which may vary according to age, gender and race (Kintz, 1998).

It is thought that the melanin content in pigmented hair leads to increased incorporation of drugs into the hair (Reid et al., 1994; Nakahara et al., 1995; Joseph et al., 1997; Kronstrand et al., 1999).

The exact mechanism/s by which drugs are incorporated into hair is unknown, but it appears that the incorporation into hair occurs during hair formation, from blood, sweat and sebum and from and from the external environment (Figure 5)

Table 1: V_d and half-life **(**$t_{1/2}$) of commonly abused drugs

Drug Name	**Mode of action**	**V_d(L/Kg)**	**$t_{1/2}$**
Cocaine	Stimulant	1-3	40 min -4 h
Amphetamine	Stimulant	3-5	4-30 h
Ecstasy(MDMA)	Stimulant	-	8 h
Methamphetamine	Stimulant	3-4	10-30 h
Diazepam	Tranquilizer, sedative and hypnotic	0.5-2.6	20-50 h
Nor-diazepam	Tranquilizer, sedative	0.9-1.3	50-99 h
Oxazepam	Hypnotic	0.5-2	4-15 h
Temazepam	Hypnotic	0.8-1.4	5-15 h
Flunitrazepam	Hypnotic	3.4-5.5	11-25 h
Ethanol	CNS depressant agent	0.43	15-30 mg/dL/h
GHB	Anaesthetic	-	<1 h
LSD	Hallucinogen	0.3	2-6 h
THC	Potent psychoactive agent	9-11	19-96 h
Methadone	Narcotic analgesic	3-5	15-72 h [190 h in rare cases]
Morphine	Narcotic analgesic	1-6	1-8 h
Heroin	Narcotic analgesic	-	2-7 min
6-MAM	Heroin metabolite	-	3-25 min
Codeine	Weak analgesic	3.5	2-4 h
Ketamine	Anaesthetic	3-5	2-4 h

(Henderson, 1993; Cone, 1996; Yinon, 2003; Kintz, 2004; Kintz, 2007).

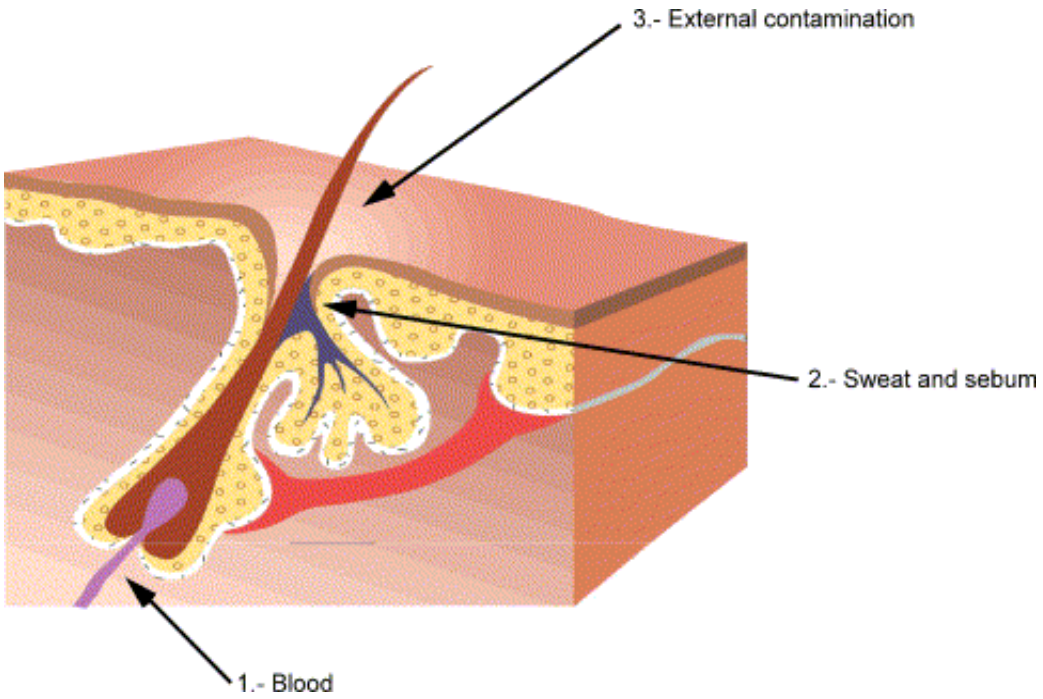


Figure 5: Possible methods for drug incorporation into hair

1.1.3: Drug metabolism and biotransformation:

During drug metabolism, the drugs are biochemically modified or degraded usually through specialised enzymatic reactions. This process often converts lipophillic compounds into more readily excreted polar products. Thus, the drug is converted to a more water-soluble compound by increasing its polarity to facilitate excretion in the urine, whilst non-polar drugs are excreted in bile. This process occurs mainly in the liver but also in the lungs, plasma and gastrointestinal tract (GIT) (Klaassen, 2003; Rolfe, 2004; Anderson, 2005). The degree of metabolism for some drugs is variable and some drugs are excreted in an unmodified state or after limited biotransformation.

The blood flow from the small intestines to the liver is through the portal circulation. Through this pathway, the liver is responsible for the first-pass metabolism. Therefore, the first-pass metabolism is the process whereby the concentration of the orally administrated drug is reduced before it reaches the entire systemic circulation. The first-pass can be avoided by the parenteral and sublingual route of administration (Barker and Bromley, 2002; Bath-Hextall, 2004; Vimala et al., 2004).

Phase I and Phase II biotransformation reactions take place during drug metabolism and Phase I reactions usually precede Phase II. Phase I reactions introduce a polar group to the parent drug to increase its water solubility and facilitate its elimination from the body. Phase I reactions are either oxidative or hydrolytic in nature including N- and O-dealkylation, aliphatic and aromatic hydroxylation, N- and S-oxidation and deamination (Karch, 1997; FDA, 2002; Karch, 2007). The two most important enzyme systems involved in Phase I metabolism are the cytochrome P-450 and the mixed function amine oxidases. The two most important reactions involved in Phase I are the oxidation (e.g. Cytochrome P-450 monooxygenase, flavin-containing monooxygenase, alcohol dehydrogenase and aldehyde dehydrogenase, monoamine oxidase, co-oxidation by perioxidases) and reduction (NADPH-cytochrome P-450 and reduced (ferrous) cytochrome P-450) systems (Ho Yo, 2000).

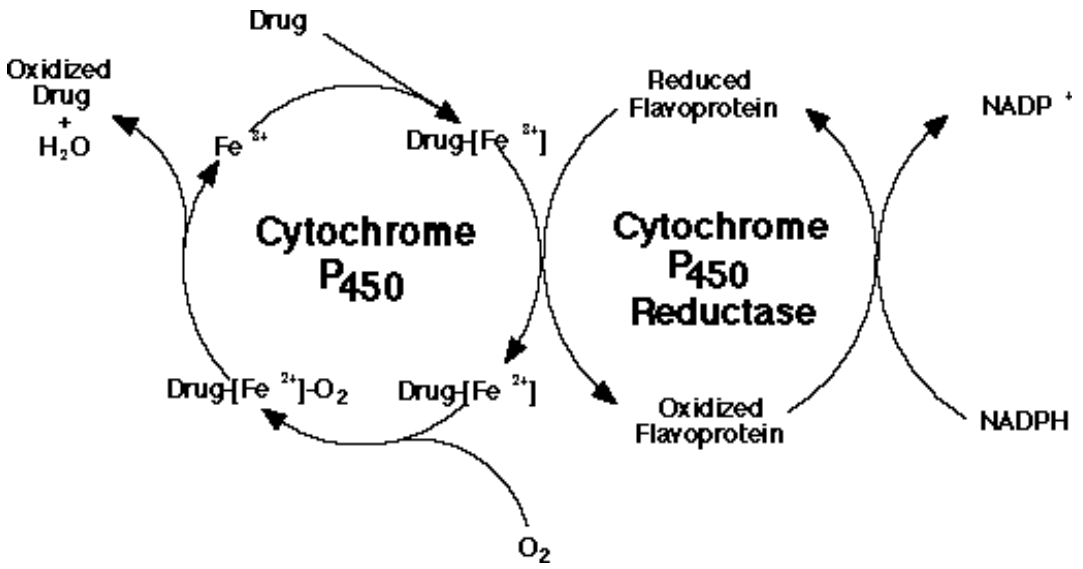


Figure 6: Cytochrome P-450 components and oxidation reaction (FDA, 2002)

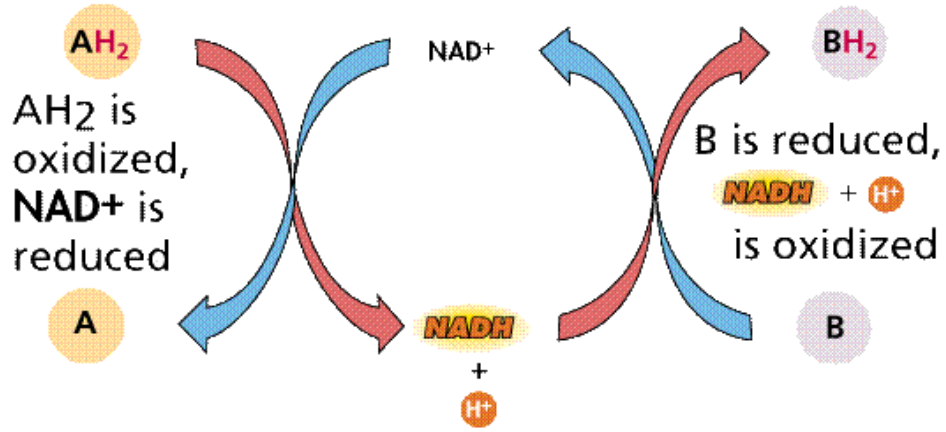


Figure 7: Oxidation reduction reactions (Farabee, 2001)

Cytochrome P-450 enzymes are responsible for the oxidative metabolism of more than 80% of commonly prescribed drugs. They are highly polymorphic and variable amongst individuals (Richelson, 1997; Drummer, 2001; Klaassen, 2003). For example the enzyme activity of these enzymes is higher in Ethiopians and

Caucasians than the Japanese, while Caucasian women have higher enzyme activities than Caucasian men (Shimada et al., 1994; Aklillu et al., 1996).

In individuals with low enzyme activity, the drug is present in the body for a longer period than normal and they require lower doses of normally prescribed drugs to prevent drug toxicity. Cytochrome P-450 2D6 (CYP2D6) is responsible for codeine metabolism to morphine, and is absent in 7% of Caucasians. Therefore, there may be limited pain relief from codeine administration in these individuals (Shimada et al., 1994; FDA, 2002; Clarke, 2006). In contrast, life-threatening morphine intoxication has been observed after the administration of small doses of codeine in ultra-rapid CYP2D6 metabolisers (Gasche et al., 2004; Reynolds et al., 2007). Table 2 shows cytochrome P-450 isoenzymes and their inducer and inhibitor drugs (Chang, 1999).

Table 2: Some inducer and inhibitor drugs for cytochrome P-450 isoenzymes (Chang and Kam 1999)

Isoenzyme	**Inducer**	**Inhibitor**
CYP1A1	Polycyclic hydrocarbons	Propofol
CYP1A2	Phenytoin	Quinolone
CYP2CB	Rifampicin	Cimetidine
CYP2C9/10	-	Sulphenazole
CYP2C19	Phenobarbitone	Sulphenazole
CYP2D6	Pregnancy	Cimetidine, Methadone
CYP2E1	Ethanol	Disulfiram
CYP3A4	Rifampicin, Phenobarbitone, Glucocorticoid, Carbamezepine	Cimetidine, Troleandomycin, Propofol
CYP3A5	Dexamethasone	Troleandomycin

Drug metabolites from Phase I reactions are either excreted from the body or go through Phase II metabolism. Phase II or conjugation reactions are the processes whereby the functional group of a Phase I substrate is bound to the endogenous compounds such as glucuronic acid, sulphate, glutathione, amino acids and acetate. This reaction is catalysed by the uridine diphosphate (UDP) glucuronosyltransferase (Karch, 2007). This yields conjugation metabolites that are highly water-soluble but with reduced pharmacological effect. However, the glucuronide products of Phase II do not always produce less active metabolites. In the case of morphine, Phase II produces morphine-6-glucuronide which has the same pharmacological effect as the parent morphine (Karch, 1997; Levine and Smialek, 2000).

Another Phase II conjugation reaction is *sulphation* of a hydroxyl group. The sulphotransferases are a group of soluble enzymes that transfer the inorganic sulphate to the hydroxyl moiety of phenol or aliphatic alcohols. Other important Phase II enzymes are glutathione-S-transferases which are present in both cytoplasm and the endoplasmic reticulum of cells. They catalyse the reaction between the sulphhydryl groups of the tripeptide glutathione with a xenobiotic containing electrophilic carbon atoms. The glutathione conjugates are cleaved to cysteine derivatives that are acetylated to produce mercapturic acid conjugates, which are excreted in the urine (Karch, 1997).

1.1.4: Excretion:

Excretion is a physical process whereby removal of a parent drug and/or its metabolites from the body to the external environment takes place (Levin and Spihler, 2003). The main routes of excretion are through the kidney, liver, lungs,

saliva, hair, stool, breast milk, sweat and semen. As mentioned earlier, most water-soluble drugs are excreted through the kidney and urine while lipid-soluble drugs are excreted through the liver and bile (Levin and Spihler, 2003).

The factors that affect drug excretion through the kidney include drug properties, pH of the urine, rate of the renal blood flow and concomitant kidney diseases. Drug properties such as water solubility, protein binding and half-life play an important role in drug excretion through the kidney. There are three main mechanisms by which drugs are excreted through the urine: by glomerular filtration of the unbound drug, transporter-mediated active excretion of the free and protein-bound drug including their glucuronide/sulphate conjugates, and by passive diffusion (Fliser et al., 1999; Drummer, 2001; Kullak-Ublick et al., 2001; Arakawa et al., 2005; Anderson, 2005; O'Callaghan, 2006; Kopacek, 2007; Karch, 2007).

In general, drugs with a short half-life tend to be excreted from the body more rapidly than those with longer half-life. The major route of excretion of cocaine and its metabolites is through the urine (Jeffcoat et al., 1989). This may explain why the cocaine metabolites methylecgonine and benzoylecgonine with half-life 3.5–6 and 5–8 hours respectively, can be detected in urine in some post-mortem cases in the absence of cocaine which has a half-life is 0.6-4 hours.

The intestine is not only a site of drug absorption but it is also involved in the excretion of drugs and their metabolites. Many drug metabolites formed in the liver are excreted through bile into the intestinal lumen where excretion in faeces and reabsorption back into the blood may occur.

Although drug excretion can also occur in hair, skin, sweat, saliva, tears and breast milk in small quantities, these routes of drug excretion may still have forensic significance. The drugs excreted into the saliva are usually swallowed and their fate thereafter is similar to those of drugs administered orally. However, for some drugs their concentration in saliva parallels that in plasma. Due to the greater acidic property of breast milk in comparison to blood plasma, basic drugs/metabolites may become slightly concentrated in breast milk (FORCON, 2004). The excretion of drugs in hair is covered in greater detail later.

1.1.5: Drug Toxicity:

Drug toxicity is related to the direct systemic effects of a drug on the body that usually occurs after overdose of the drug or its accumulation in the body as a result of disorders of organs e.g. kidney or liver disease which reduce the body's ability to eliminate the drug and thus increase its half life.

The toxic effects of drugs of abuse result from their interactions with neurotransmitters and their receptors by inhibiting or enhancing their function and responses. The common neurotransmitters include acetylcholine, dopamine, gamma-amino butyric acid, noradrenalin, opioids and serotonin. The signs and symptoms of intoxication can give a clue to which drug was administrated. For example, the toxic effects of stimulants are associated with an increase in body temperature, agitation, hypertension and intracerebral haemorrhage whilst CNS depressants (e.g. opiates, ethanol and benzodiazepines), toxicity is generally associated with respiratory depression (Giannini, 2000).

Acute toxicity results from a single exposure or multiple exposures to a toxicant usually within a 24 hour period. In general, the effects of acute toxicity occur within 14 days of the administration of the toxicant (Klaassen, 2001). On the other hand, chronic toxicity occurs after repetitive exposure to a toxicant over a longer period of time and often at lower amounts (Hodgeson and Levi, 1997).

In forensic toxicology, the toxic/lethal drug level information is compiled predominantly from published case reports (Repetto and Repetto, 1997; Winek et al., 2001; Schulz and Schmoldt, 2003; Musshoff, 2004) and the source of the post mortem blood (e.g. femoral, cardiac) is not always given. These reference data are used in most forensic laboratories around the world (Table 3). However, as case reports may differ in relation to gender, age, medical or drug history and state of health, this data must be used with caution when interpreting post mortem drug concentrations in relation to toxicity. This is also further complicated by post mortem drug metabolism and redistribution as explained later.

Drug tolerance is the decrease of the drug effect so that larger does are required to achieve the same desired effect (Nestler, 2001; Siegel, 2005). There are two types of tolerance: acute tolerance is the reduced effectiveness of a drug after a single administration whereas chronic tolerance is the reduced effectiveness of a drug after repeated or chronic exposure (Gordis, 1995). The mechanisms by which tolerance is induced are complex and may include impairment and down regulation of the dopamine reward system and regulation of some gene expression (Nestler, 2004; Nestler, 2005; Harvey, 2007).

Table 3: Therapeutic, toxic and lethal blood concentrations for most commonly abused drugs (modified fromWeink2001; Schulz, 2003; Musshoff, 2004)

Drug	Therapeutic	Toxic (ng/ml)	Lethal (ng/ml)
Codeine	30-250	500-1000	1800
Cocaine	50-300	500-1000	>1000
Diazepam	200-2500	3000-5000	-
Nor-diazepam	20-200(-800)	1500-2000	-
Oxazepam	200-1500	2000	3000-5000
Temazepam	20-150 (-900)	1000	8200, 14000
Ethanol	-	100-200 (mg/dl)	350-400 (mg/dl)
Methadone	50-750	200	>400
Methamphetamine	10-50	200-1000	10000-40000
MDA	400	1500	2000
MDEA	200	-	1000
Morphine	10-100	100	>100

The degree of tolerance to a drug plays an important role in drug toxicity. For instance, in individuals who do not have drug tolerance, low blood drug levels may be associated with severe toxic symptoms whilst in tolerant individuals, e.g. active chronic drug users, blood drug levels may reach potentially lethal concentrations without any symptoms of toxicity (Johnson, 1982; Karch, 2001; Brind, 2007). The toxicity of the drugs of abuse studied in detail in this thesis (ethanol, opiates, cocaine and benzodiazepines) is discussed later in this chapter.

1.2: Post-Mortem Drug Redistribution (PMDR):

PMDR refers to the changes in drug concentrations that occur after death. PMDR is the movement of a drug after death from sites or body tissues where its concentration is high to those sites where the level is low (Anderson and Prouty, 1989; Moriya and Hashimoto, 1999; Cook et al., 2000; Yarema and Becker, 2005; Drummer, 2001; Pélissier-Alicot, 2005). The main drug reservoir organs are the liver, lungs, heart and the GIT.

In PMDR, the drugs in these reservoirs diffuse to the adjacent compartments. For example, unabsorbed drugs in the stomach such as ethanol diffuse after death to the left cardiac chambers and Inferior vena cava (IVC) (Hilberg et al., 1993; Pounder and Smith, 1995). Studies of ethanol diffusion in post-mortem cases show that ethanol concentrations in aortic and vena cava blood are higher than the concentrations in the left and right heart chambers. This tends to suggest that ethanol directly diffuses to these sites from the stomach rather than diffusing from the cardiac and pulmonary blood (Pounder and Yonemitsu, 1991; Pounder, 1997).

In general, drug concentrations in the IVC following diffusion from the solid organs tend to be higher than those in the femoral vein. Therefore, in post mortem cases where a large volume of femoral blood is collected, ligating the femoral vein may be necessary to prevent collecting IVC blood where drug levels may have been influenced by PMDR. PMDR from the liver and lungs starts within a few hours after death leading to elevation of drug levels in the cardiac chambers and thoracic vessels (Pounder and Yonemitsu, 1991; Miyazaki et al., 1993; Hilberg et al., 1994).

Factors that influence the rate of PMDR include the drug's volume of distribution (V_d), lipophilicity and stability of the drug, post-mortem changes, amount of the drug in the stomach and the post-mortem interval. The contents of the body's cells become more acidic after death leading to more ionisation of the basic drugs, which enhances their PMDR (Yarema and Becker, 2005). Most drugs of abuse have a high V_d and therefore are more susceptible to PMDR. They tend to be concentrated in vital organs such as lungs, liver and heart leading to a concentration gradient for passive diffusion after death (Pelissier-Alicot et al., 2003).

Post-mortem body putrefaction affects drug levels via bacterial action. For example, post-mortem diazepam levels decrease as a result of bacterial metabolism and this can be reduced by refrigeration of the body at 4 °C (Drummer and Robertson, 1998). On the other hand, ethanol may increase in the body by up to 190 mg/dL after death due to bacterial action, depending on the storage conditions of the body (Collison, 2005; Rodda and Drummer, 2006).

Therefore, drugs with high central (cardiac) to peripheral (femoral) (C/P) ratios tend to have a high post-mortem to ante-mortem (PM/AM) ratio (Dalpe-Scott, 1995; Cook et al., 2000). For example, studies on morphine, dothiepin, dextropropoxyphene, amitriptyline and methadone have shown that high C/P drug ratios are associated with high post-mortem to ante mortem (PM/AM) drug ratios (Moriya and Hashimoto, 1999; Cook et al., 2000; Crandall, 2006) and thus increased PMDR. However, the research data in this area are limited.

The drugs studied in detail in this thesis in relation to their post-mortem concentrations in different body compartments and their relationships to toxicity, were cocaine, opioids, ethanol and benzodiazepines, which are further discussed below.

1.3: ***Cocaine***

Cocaine, which is a CNS stimulant, is a natural alkaloid found in the leaves of the *Erythroxylon coca* plant, which is found in South America. Cocaine is absorbed rapidly from mucous membranes and the pulmonary vasculature. The rate of cocaine absorption and its plasma peak are dependent on the route of administration. The administration of low intravenous (IV) cocaine doses produces a higher plasma peak in comparison to a higher dose administered by another route. The time order in which blood peak levels are reached after cocaine administration is intravenous>intranasal>smoked>oral as shown in Figure 8 (Feldman et al., 1997).

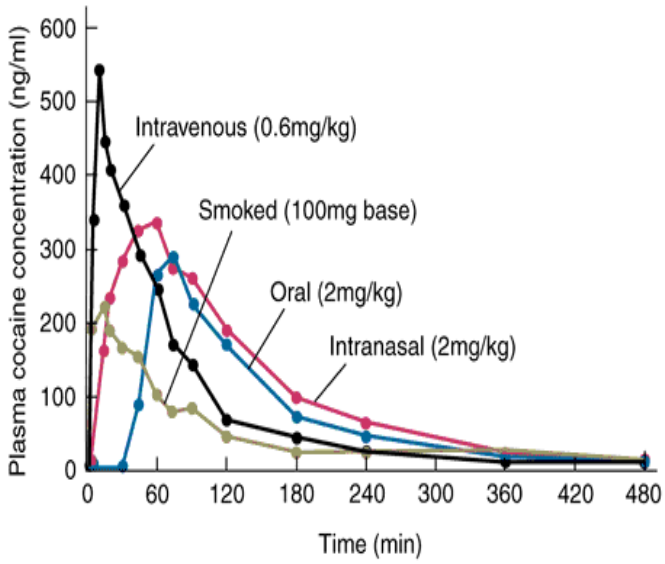


Figure 8: Effect of routes of administration on blood cocaine levels

(Feldman et al., 1997)

Pharmacokinetics and pharmacodynamics of cocaine have been studied according to the three most common routes of administration: IV, smoking and intranasal (Cone, 1995). The longest cocaine half-life shown after both oral and intranasal routes is 60-90 minutes, while IV and inhalation routes have a shorter half-life of 40-60 minutes. For the inhalation route, the peak level is reached in 1-5 minutes, for IV route in 3-5 minutes, for intranasal route in 15 minutes and for oral route in 60 minutes (Burnett, 2006). Intranasal cocaine administration has been characterised by lower cocaine plasma concentrations and slower onset of pharmacological effects and plasma peak within 35-160 minutes after intranasal administration 'snorting' (Wilkinson et al., 1980; Javaid et al., 1983; Cone, 1995, Burnett, 2006). The average cocaine bioavailability found was estimated to be 70% for smoking route and 93.7% for intranasal route (Cone, 1995).

Most of the administered cocaine is recovered in urine within a 24 hour period and benzoylecgonine is the major metabolite detected after all routes of cocaine administration (Jatlow, 1988; Cone et al., 1998; Huestis et al., 2007). However, a recent study found that ecgonine is the metabolite at the highest concentration detected after the smoking route of cocaine administration (Huestis et al., 2007). In the same study, cocaine and its metabolites benzoylecgonine (BE), ecgonine methylester (EME), m-hydroxybenzoylecgonine (mOHBE), p-hydroxybenzoylecgonine (pOHBE), norbenzoylecgonine (NBE) and ecgonine (EC) were detected in urine samples and all metabolite levels were dose related.

A study of eight cocaine metabolites (benzoylecgonine, ecgonine methylester, norcocaine, benzoylnorecgonine, m-hydroxy-BZE, p-hydroxy-BZE, m-hydroxy-COC, and p-hydroxy-COC) excreted in urine after intravenous, smoked and intranasal routes of cocaine administration showed that the concentrations of metabolites in urine were in the following order: benzoylecgonine>ecgonine methylester>cocaine>benzoylnorecgonine>p-hydroxy-BZE>m-hydroxy-BZE>m-hydroxy-COC>norcocaine>p-hydroxy-COC. The cocaine and metabolite half-lives are dependent on the route of administration in the following order: intranasal>intravenous>smoked route, with cocaine having the shortest half life (Cone et al., 2003).

As the main metabolite of cocaine is benzoylecgonine, it is also the main metabolite found in blood and urine. The other main cocaine metabolites are norcocaine, ecgonine methyl ester and ecogonine (Figure 9). When ethanol is consumed with

cocaine cocaethylene is formed (Hearn et al., 1991; Odeleye et al., 1992), which is a highly toxic metabolite whose plasma half-life is longer than that of cocaine (Jatlow, 1988; Bencharit, 2003; Harris et al., 2003; Burnett, 2006).

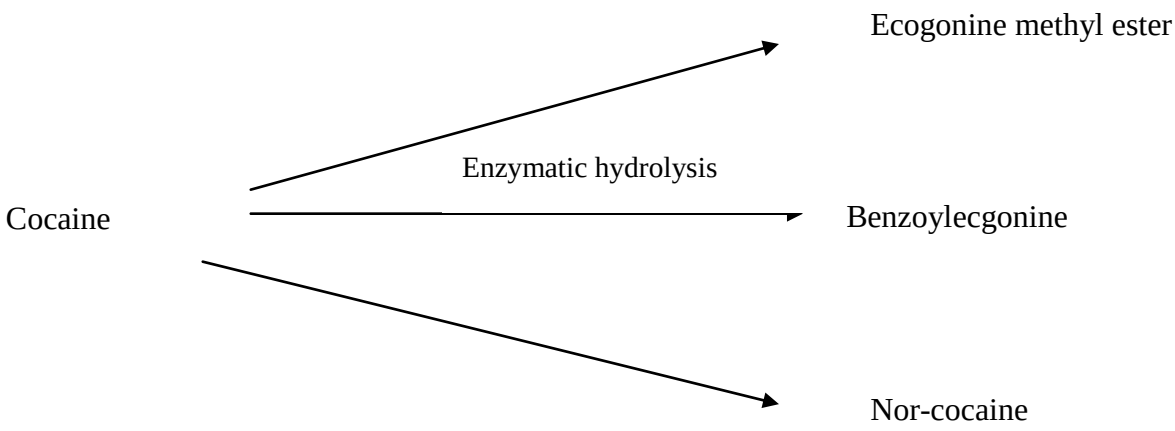


Figure 9: Cocaine metabolic pathway in humans (Feldman et al., 1997; Fowler, 2001)

Following different routes of administration, benzoylecgonine being the major cocaine metabolite, is detected in the blood of cocaine abusers ten times more frequently than cocaine (Clark and Hajar, 1987; Howell and Ezell, 1990; Karch, 2000). Cocaine smoking also produces a unique metabolite anhydroecgonine methyl ester (AEME), whose pharmacological and toxicological properties are still unknown (Karch, 2008).

Studies of cocaine pharmacokinetics in cocaine abusers show no gender differences in the pharmacokinetic properties such as peak plasma cocaine level and half-life (Mendelson et al., 1999). In the ante-mortem period, methylecgonine is converted to ecgonine. Furthermore, after death hydrolysis of cocaine continues but at a slower rate and methylecgonine accumulates, when its concentrations may become higher than those of benzoylecgonine (Logan and Peterson, 1994; McKinney et al., 1995).

A study of the radiolabelled cocaine distribution after intravenous injection has shown that cocaine is distributed to the following organs/body compartments: brain, liver, spleen, kidney, lungs, blood, heart and muscle (Busto et al., 1989). It has also been shown that there are strong cocaine and benzoylecgonine correlations between blood and vitreous humour (Duer et al., 2006). Cocaine and benzoylecgonine have also been detected in the brain, liver, kidney, heart, placenta, blood, and hair of foetuses whose mothers abuse cocaine.

Cocaine is highly distributed in the brain due to the high lipid content of this tissue (Spiehler and Reed, 1985; Moriya and Hashimoto, 1996). Pulmonary uptake for labelled cocaine was not demonstrated (Volkow et al., 1992). After intranasal administration, the distribution of cocaine is different in various brain regions and higher concentrations are found in the olfactory bulb. The high concentration in the brain was also observed after intranasal administration of benzoylecgonine (Chow et al., 1999; Chow, 2001).

Normally, when free cocaine is present in the blood, its concentration in urine is approximately 100 fold more than the level in blood. A recent study showed that cocaine and benzoylecgonine concentrations in urine were higher than in plasma by 15-100 fold, after a single dose of intravenous cocaine. After cocaine administration, benzoylecgonine is usually detectable in urine for 1-4 days. However, depending on the amount of cocaine administered and the sensitivity of the assay used, both benzoylecgonine and cocaine may be detectable in urine for up to 15 days (Karch, 2007).

Depending on the site of blood sampling, cocaine blood levels can be higher or lower than those in the ante-mortem period (Sylvester et al., 1998). Cocaine and its metabolite levels in post-mortem blood are affected by several factors which include drug stability, bacterial action, effect of endogenous enzymes, post-mortem drug redistribution processes, conditions of storage of body and site of specimen collection (Drummer, 2000; Leikin, 2003). There is limited information on cocaine in the literature but as it is known to be lipid-soluble, there is potential for its PMDR. It has been reported that post-mortem cocaine in vitreous humour is three times higher than its value in the ante-mortem sample (McKinney, 1995).

Cocaine is detectable in hair samples within 1-3 days after a single administration (Joseph et al., 1999; Scheidweiler et al., 2005), and it reaches maximum concentration three days after its disappearance from the plasma (Reid et al., 1994; Hubbard et al., 2000). After a single intravenous dose, cocaine can be detected in hair for 2-6 months, depending on the amount administered (Nakahara et al., 1992; Nakahara and Kikura, 1994; Nakahara et al., 1995; Henderson et al., 1996). Therefore, the distribution of cocaine and its metabolites in human hair is dose-dependent (Scheidweiler et al., 2005).

As with other drugs that are incorporated into hair, the factors that influence the incorporation of cocaine into hair include cosmetic treatments, hair dye, melanin contents, washing and genetic variations. Incorporation of cocaine into black hair has been found to be higher than into blonde hair within a 1 hour to 14 day period after the same dose has been administered (Henderson, 1998; Joseph et al., 1999;

Scheidweiler et al., 2005). When ethanol is consumed with cocaine the cocaethylene can also be detected in hair (Spiehler, 2000).

Environmental contamination of hair by cocaine is well recognised. Cocaine and benzoylecgonine in hair samples with levels of >1ng/mg and <0.5ng/mg respectively have been detected for up to 10 weeks after external contamination (Romano et al., 2001). In cocaine abusers, cocaine is detected at higher concentrations in hair than benzoylecgonines (Bermejo Barrera and Strano Rossi, 1995). Benzoylecgonine has also been detected in the hair samples of newborns whose mothers abuse cocaine (Katikaneni et al., 2002).

There is limited research about using human bone tissue to determine the concentration of cocaine and its metabolites. Most drugs are taken up in bone through blood and can be detectable in skeletonised remains with the exception of volatile drugs. There is some reference data available on toxicant levels in bone e.g. arsenic (Drummer, 2004; Elliott, 2004).

As there is very limited data available for bone tissue and drugs of abuse, this makes the interpretation of any drug concentration in bone difficult. Furthermore, drug incorporation into bone tissue is reversible, which means that a negative drug result in bone cannot entirely exclude the possibility of previous drug exposure, and a positive result does not provide any information about time of exposure (Karch, 2007). GC-MS analysis of drug levels extracted from teeth obtained from cocaine consumers over a 10-20 year period found that benzoylecgonine was the major metabolite present in most of the specimens (Pellegrini et al., 2006).

The clinical effects of cocaine are related to stress hormones and neurotransmitters. Cocaine prevents the re-uptake of the neurotransmitters dopamine, nor-epinephrine and serotonin at the synaptic clefts, which leads to prolongation of the nerve impulse and thus, prolongation of the central nervous system stimulant effects including euphoria, alertness, tachycardia, hyperthermia, hypertension and vasoconstriction (Warner, 1993; Smith et al., 2001). Cocaine also has local anaesthetic effects (Dunwiddie et al., 1988; Tuckley, 1994; Lennox, 1996).

The toxic effects of cocaine are well documented (Zhang et al., 1999; Ferdinand, 2000; McEvoy et al., 2000; Guiraudet et al., 2001; Hsue et al., 2002; Knuepfer, 2003; Gilbert et al., 2006; Haigney et al., 2006; Afonso et al., 2007; Aryana and Mooss, 2007; Glauser and Queen, 2007; Westover et al., 2007). It is also thought that females are more sensitive than males to the behavioural effects induced by cocaine due to the female hormone oestrogen (Carroll et al., 2002; Mello and Negus, 2007; Festa and Quinones-Jenab, 2004; Roth and Carroll, 2004). The administration of cocaine with ethanol is associated with a high fatality rate due to cardiovascular complications, hepatotoxicity, and extreme agitation behaviours. In around 74% of cocaine-related deaths in the United States, ethanol is the second most common drug ingested. This dangerous combination increases the risk of sudden death by 25 fold (Burnett, 2006).

In general, there is no agreeable safe concentration for cocaine or its metabolites, but any blood concentration can cause or contribute to the cause of death (Karch, 2002). Sometimes, post-mortem blood cocaine or metabolites show lower concentrations

than in the ante-mortem blood of some patients. This may be due to drug tolerance and could explain why small doses of cocaine can kill a drug naïve person while higher doses do not kill a chronic user. On some occasions, a person under a medical therapy programme dies immediately upon administration of the same dose he was accustomed to before treatment due to the decrease in the tolerance after the therapy programme (Drummer, 2001). Other drugs, such as aminophylline, can enhance the toxic effects of cocaine (Gasior, 2000).

In addition, hepatitis C and HIV infections are common in intravenous cocaine abusers (Roy et al., 2007; Volkow et al., 2007). Gangrene in the extremities is also a recognised complication in cocaine abusers (Dhawan, 2007) and foetal mental retardation has been reported in those whose mothers abuse cocaine (Paule, 2005).

1.3.2: Opioids Pharmacokinetics:

The natural and semi-synthetic opioids include morphine, diacetyl morphine (heroin), oxymorphone, codeine and oxycodone. Amongst the synthetic opioids are methadone, fentanyl, propoxyphene and pethidine. Morphine is a natural alkaloid derived from the opium poppy. Whilst most opioids have medicinal uses, heroin is an illicit drug derived from acetylating morphine and is the most commonly abused opioid (Karch, 2006).

The route of administration of opioids determines their bioavailability, e.g. rectal and intramuscular routes bypass the first-pass metabolism in the liver and therefore have higher bioavailabilities. The plasma level of morphine shows no significant

difference between intravenous, intramuscular and subcutaneous routes (Stuart-Harris et al., 2000). Before reaching the systemic circulation heroin is metabolised to 6-acetylmorphine (6-monacetyl morphine - 6-MAM) and then to morphine. The bioavailability of opioids is for intravenous 100%, oral 33-87%, transdermal 90%, subcutaneous 80%, snorting 80%, transmucosal 30-60%, smoking 20-40% and rectal 30-40%, depending on the type of opioid administered, individual variations and the state of health of the users (Lotsch, 1999; Cancer Control, 2000; Drummer, 2001). Due to degradation of morphine by heating, the concentration in blood after smoking heroin is only 20-40% of the amount administered intravenously.

The total morphine to free morphine ratio is useful in determining when morphine or heroin was administered. For example, the ratios of total to free morphine are typically 4:1 and 9:1 (after 15–60 minutes of administration) if given intravenously and 2:1 and 5:1 if given intramuscularly (Boerner, 1975).

Following an intravenous and intranasal heroin dose, the plasma peak levels are achieved at 2 and 5 minutes respectively. Whilst for morphine administered intravenously and intranasally, the peak plasma concentrations will be at 25 minutes and 48-69 minutes respectively. Morphine and heroin have different half-lives of 90-420 minutes and 2-6 minutes respectively. There is evidence that the half-lives will also vary depending on the gender of the individual (Jenkins, 1994). The half-life of 6-MAM is 6-25 minutes and both heroin and 6-MAM are more lipid soluble than morphine and therefore, enter the brain more readily (NHTSA, 2004). Vitreous humour appears to be a useful specimen for determining 6-MAM and establishing that the morphine is derived from heroin (Wyman and Bultman, 2004).

In healthy volunteers, the initial onset phase and duration of the pupillary and respiratory effects due to morphine do not differ whether administration is inhalation or intravenous (Dershwitz, 2000). However, for most opioids, there is a time delay between administration of the drug and the onset of the drug effect. This delay is due to the time taken for the drug concentrations in the blood and the CNS to equilibrate (Upton et al., 1997; Bouw, 2001).

The V_d of morphine is 2-5L/kg but may be >7L/kg in some cases (Sawe et al., 1981; Sawe et al., 1985; Osborne et al., 1990; Milne, 1996; Lotsch, 1996). This difference can be due to individual variations or state of health. Morphine is a moderately hydrophilic drug distributed in most body tissues and can be detected after death even in adipose tissue. Post-mortem studies of morphine in adipose tissue show that morphine deposition takes place during the real ante-mortem deposition and is not due to PMDR (Levisky, 2001).

Studies have found that incorporation of morphine into hair will also be influenced by race as is the case with cocaine. Caucasian hair shows lower morphine concentration than African American hair (Kidwell, 2000). However, this relationship was not confirmed by a previous study (Hoffman, 1999). It has also been found that the incorporation 6-MAM into hair is greater than for morphine (Tabernero, 1999).

As with cocaine, there is limited data available about the distribution of opioids in human bone. A study of morphine distribution in the thighbone, including its bone

marrow, found that morphine concentration dropped by 54.4% one year after burial (Raikos et al., 2001). 6-MAM was detected in teeth in 89% of morphine cases studied (Pellegrini, 2006).

Around 90% of a dose of morphine is excreted in urine within 72 hours from administration. Of the administered dose, about 10% is excreted as free morphine and 75% as morphine-3-glucuronide. The other 15% includes morphine-6-glucuronides, morphine-3-sulphate, nor-morphine and conjugates (Karch, 2007). As stated earlier, detection of 6-MAM indicates heroin use and 6-MAM is detectable in urine up to 24 hours after heroin uptake.

Morphine is distributed into high blood supply tissues/organs e.g. kidney, liver, muscle and lungs, and within a few minutes during Phase II biotransformations, 57% of administered morphine is converted to morphine 3-glucuronide and 10% to morphine 6-glucuronide before they are excreted through the kidney (Hasselstrom and Sawe, 1993). Thus, the major metabolic pathway for morphine is conjugation with glucuronic acid.

Codeine (methylmorphine) is an opioid widely used for its analgesic and anti-diarrheal properties. Approximately 5-10% of codeine will be converted to morphine in most people and codeine has 8-12% of the strength of morphine (Vree et al., 2000) and its half-life is 2.5 hours. Normally, codeine-glucuronide levels are 15 fold higher than those of codeine with a half-life of around 2 hours (Yue et al., 1991), and 3.5% of free codeine is excreted in urine, of which 70% is glucuronide-codeine (Milne, 1997). Due to its wide availability, codeine is often abused recreationally. In

addition, codeine is often present in the blood and urine of heroin abusers, as it is derived from acetyl-codeine, a contaminant present in street heroin.

Methadone is a potent anti-addictive opioid analgesic most commonly used to treat heroin addiction on "methadone maintenance programmes". However, it is well recognised that uncontrolled or inappropriate use of methadone also occurs. If the abused dose of heroin is known, 1mg of methadone is equivalent to 2-4mg of heroin. In cases of an unknown dose of heroin, usually 10-15mg of methadone is taken daily (Giannini, 2000). In the first two weeks of methadone treatment the risk of death is 7 fold higher than in non-treated heroin abusers while this risk increases to 98 fold for those who are treated for more than two weeks (Caplehorn, 1999). The $t_{1/2}$ of methadone is from 8.5-58 hour (Verebely, 1975; Meresaar, 1981). In general, opioids do not accumulate between doses; however, one daily dose of methadone leads to rapid accumulation (Drummer, 2001).

As the pharmacokinetics of methadone varies from one individual to another, different blood concentrations may be obtained from the same dose if given to different people. Methadone is metabolised through N-demethylation via CYP3A4 enzyme in the liver to yield its active metabolite 2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine (EDDP). CYP2D6 and CYP1A2 are also involved in the metabolism of methadone (Ferrari et al., 2004). Methadone has high lipid solubility and 98% of its blood concentration is rapidly transferred to the liver, kidneys, lungs and brain. 60–90% of the administered methadone is bound to the plasma proteins α1-globulins and 13.4–17.4% is bound to the γ-globulins (Dole, 1973; Olsen, 1973; Eap, 1990; Garrido, 2000)

It is noted that glycoproteins increase in chronic heroin abusers, which means that more methadone is bound to glycoproteins while a relatively small concentration is free and bioavailable (Olsen, 1973; Garrido, 1996).

PMDR of opioids in overdose cases has been widely reported (Drummer, 2001; Musshoff, 2004; Wyman, 2004). As mentioned earlier, morphine, methadone and codeine have volumes of distribution of 1-6, 3-5 and 3-5L/kg respectively, and so they would be expected to be susceptible to PMDR. A study of the cardiac blood morphine level during the 24-96 hours after death has shown that there is an increase in the cardiac blood morphine level in comparison to the ante-mortem concentration (Sawyer and Forney, 1988; Koren and Klein, 1992).

Morphine levels in blood collected from different sites (subclavian, heart and femoral) showed that heart blood morphine concentration was greater than that in the other two sites (review by Gerostamoulos and Drummer, 2000). However, other post-mortem studies showed no differences in concentration of morphine between the femoral and cardiac blood (Prouty and Anderson, 1990; Skopp et al., 1996; Bogusz, 1997; Moriya and Hashimoto, 1997).

The time lapse between death and sample collection for analysis may affect the measured drug concentration. In the studies reviewed above, this time interval varied from 3-144 hours (Logan and Smirnow 1996). In addition to the site of blood sample collection, the other factors that may affect post mortem drug concentrations are water content and haematocrit variability. For example, blood samples with high

water content and low haematocrit tend to have higher morphine levels and vice versa.

Opioids generally bind to three types of receptors μ (mu), κ (kappa), and δ (delta). These receptors are found in the brain and spinal cord (Stout and Farrell 2003). The μ receptor is the most important one due to its ability to bind both morphine and heroin. Binding to both μ and δ receptors reduces cyclic adenosine monophosphate (cAMP) and as a result increases the intracellular potassium ions and hyperpolarisation of the cell membrane (Milligan et al., 1985).

Opioids in general are known to cause respiratory depression even within their therapeutic levels and death is most commonly due to respiratory failure. However, each case is unique; even if two individuals have the same body weight and similar state of health and ingest the same dose, their responses and tolerance to the drug may be different (Wyman, 2004). Fatality rates are higher in people who also consume opioids with other drugs such as ethanol (Stephens, 1996; Taylor and Taylor, 2001). Co-abused drugs including ethanol, cocaine, benzodiazepines, cannabis and amphetamine lead to reduction in the lethal dose of morphine (Manzanares, 1999; Medsafe, 2006).

Ethanol blood concentrations of 100mg/dL may lead to an increased risk of morphine fatality by 22 fold (Ruttenber and Luke, 1984; Ruttenber et al., 1990). The clearance of methadone in naïve drug users may be prolonged which can also increase risk of overdose (Karch and Stephens, 2000). As with cocaine, there is an increased risk of HIV and other diseases in opioid abusers (Zhao and Ye, 2006).

1.3.3: ***Ethanol:***

Ethanol, also known as ethyl alcohol, is the most commonly abused psychoactive drug in the world and a leading cause of morbidity across cultures. Other types of alcohol are methanol, isopropanol and ethylene glycol.

Ethanol acts as a CNS depressant. It crosses cell membranes and changes cellular functions or neurotransmitter release. It affects the gamma-aminobutyric acid (GABA) inhibitory neurotransmitters and their receptors' functions. Ethanol also inhibits glutamate receptor functions which can cause memory loss or amnesia. Ethanol may also increase dopamine and serotonin release enhancing euphoric and sleep states respectively. This may explain the high blood serotonin levels found after excessive alcohol administration in the deceased (Weiss and Porrino, 2002).

Absorption of alcohol occurs mostly in the small intestines. Peak blood concentration of ethanol occurs within 30 minutes after administration, but if large doses are consumed this peak may not occur until 2 hours later. Bioavailability of ethanol after oral administration is around 80% and this increases with increasing ethanol uptake. Blood alcohol concentration (BAC) is affected by body weight and fat composition, blood flow rate at site of absorption and concomitant food intake. Hence, a slim or male person tends to have higher BAC than an obese or female person after consumption of the same amounts of ethanol (Cowan, 1996).

Ethanol is distributed throughout all the body fluids and tissues in proportion to their water content. Jones (2003) found that the ratio of urine alcohol concentration (UAC)

to BAC for new and casual ethanol users was about 1.25:1. Finding a mean UAC to BAC ratio of 1.3:1 or more suggests that the person has reached the post-absorptive phase. In deaths associated with chronic alcoholism, the UAC/BAC ratio has been found to be 1.3:1 suggesting that complete absorption and distribution of ethanol in all body fluids was complete. However, in those individuals who died from acute ethanol poisoning, the ratio was lower suggesting that absorption and distribution of ethanol was incomplete (Jones, 2003; Jones, 2006).

The presence of ethanol in vitreous humour and urine is a good indicator of ethanol consumption prior to death and its absence may be an indicator of an artefactual source in the matching blood sample (Forrest, 1993; Flanagan, 2005).

The vitreous humour alcohol concentration (VHAC) accurately reflects the femoral BAC prior to death and the BAC/VHAC ratio was 0.94 (Sylvester et al., 1998). Another study found VHAC/BAC ratios of 1.1–1.5, bile ethanol concentration / BAC ratios of 0.9–1.4 and UAC/BAC ratios were 1–2 (Kraut, 1992; Kraut, 1995). In post-mortem cases where a urine sample is unavailable, the BAC/VHAC ratio can be used reliably to estimate the ante mortem blood ethanol level (Yip and Shum, 1990). BAC / VHAC ratios have been found to be more consistent when blood ethanol level is > 100mg/dL (Honey, 2005). The mean BAC/bile ratio has been found to be 1.22 (Kass, 2006).

The biotransformation occurs in the liver (90%) while the remaining 10% takes place in extra-hepatic tissue such as stomach, intestines, kidneys and lungs. Conversion of ethanol to acetaldehyde in the liver is mediated by alcohol dehydrogenase (ADH).

Acetaldehyde dehydrogenase converts acetaldehyde to acetate (Gordon, 1980). Both enzymes utilize NAD in this process. The other enzymes in ethanol metabolism are catalase and the microsomal ethanol oxidising system (MEOS). MEOS is induced by regular alcohol consumption and plays an important role in ethanol metabolism in alcoholics (Gordon, 1980). A minor degree of ethanol metabolism can occur through UDP-glucouronyltransferease enzyme with formation of ethyl glucuronide (EtG); this has been used as ethanol biomarker in alcoholics (Foti, 2005).

The elimination rate for ethanol is usually around 15 mg/dL/h with a range of 10-40 mg/dL/h, which may increase above 30 mg/dL/h in chronic ethanol abusers (Winek and Murphy, 1984; Garriott, 1996). The elimination rate of ethanol is dose dependent and it is affected by many factors including genetic variations, age, body weight, sex, exercise and kidney and liver diseases (Andreasson, 1996; Lucey, 1999).

Alcohol overdose usually leads to a condition known as '*alcohol intoxication'*. This describes the state where physical and mental ability is impaired due to consumption of excessive ethanol. The degree of responses to ethanol consumption may vary in different individuals depending on age, weight, sex, previous exposure to ethanol, individual parameters of ethanol absorption, distribution and elimination. Because of individual variations in responses to ethanol some authors have questioned whether a fixed BAC is a valid measure of impairment due to ethanol (Andreasson, 1996).

The clinical and toxic effects of ethanol have been widely studied (Hansen, 1964; Hillbom, 1978; Lee, 1979; Jshley, 1980; Longstreth, 1985; Romm and Collins, 1987;

Kuna, 1992; Teunissen et al., 1996; Hillbom, 1998; Koski, 2005; Quertemont and Didone, 2006). Some of these effects are shown in Table 4.

Ethanol consumption can lead to respiratory depression and hypoxia, and sometimes hypoglycaemia, especially in patients with low glycogen stores who may have not eaten for several hours. Acute ethanol intoxication can cause hypothermia, tachycardia, myocardial depression, decreased inhibitions and loss of fine motor control skills. Higher doses of ethanol can lead to loss of gross muscle control (ataxia, slurred speech), acute pancreatitis, severe myocardial depression, pulmonary oedema and sudden death. Chronic ethanol abuse may cause foetal alcohol syndrome, hepatic dysfunction, haematologic disorders, hypertension, cardiomyopathy and malnutrition. As mentioned previously, ethanol can enhance the CNS depressant effect of other drugs. In addition, ethanol consumption is also associated with accidents, crime, violence and sexual assault.

Table 4: Stages of ethanol intoxication (Modified from Dubowski, 2006).

BAC (mg/dL)	**Symptoms**
10-50	Normal behaviour
30-120	Euphoria, sensory-motor impairment.
90-250	Excitation stage, emotional disturbances, drowsiness, reduces visual activity and response delay.
180-300	Confusion, dizziness, vision disturbance, slurred speech and deep lethargy
250-400	Stupor, vomiting, loss of control, deep lethargy
350-500	Coma and death

As mentioned earlier, ethanol may increase in the body by up to 190mg/dL after death due to bacterial action and depending on the storage conditions. If death occurs during the peri-absorptive phase for ethanol, thus may also influence BAC in particular if central blood is used for analysis. For the determination of BAC, post mortem blood should be collected into a bottle containing a preservative such as sodium fluoride in order to stop bacterial production or utilization of ethanol after the blood sample has been collected.

It has been reported that after blood has been collected at post mortem, ethanol may increase in blood (in vitro) by 150 – 250mg/dL or greater, depending on storage conditions. It has been reported that blood samples without preservative stored at 4°C for 96h showed an average increase in ethanol concentration of 147mg/dL. At 25°C, the same specimens showed an average increase of 143mg/dL after only 48h (O'Neal, 1996; Pounder, 1998; Richardson, 2000; Lewis, 2004; Athanaselis, 2005; Kugelberg, 2007).

*1.3.4: **Benzodiazepines:***

Benzodiazepines (BZDs) are a group of psychoactive drugs which have sedative, hypnotic, anxiolytic, anticonvulsant, muscle relaxant and amnesic properties. Although BZDs are normally used for the treatment of anxiety, insomnia, agitation, seizures, muscle spasms and alcohol withdrawal, they may cause respiratory depression (McKiernan et al., 2000; Charney, 2001). BZDs are also typically secondary drugs of abuse, misused in conjunction with other drugs. The following discussion has focused on diazepam and its metabolites (nordiazepam, temazepam, oxazepam) which were the main BZDs of interest in this study.

BZDs lead to inhibition of the CNS functions by binding to GABA receptors. They alter chloride movement, which inhibits the activity of neurons, e.g. in the treatment of anxiety. BZDs may also activate the dopaminergic reward pathways in the central nervous system (Soderpalm et al., 1991).

Benzodiazepines have a high degree of tolerance and this can explain their greater level of abuse than common illicit drugs (Chutka, 2004). Tolerance and dependence on benzodiazepines also develops very rapidly after just a few weeks of continuous use. Therefore, the US Federal Committee on Safety of Medicines has recommended that medical use of benzodiazepines must be limited to 2–4 weeks (Einarson, 1980; Priest, 1980; Kripke, 2000). BZDs are classified according to their half-lives, long-acting >24 hours (e.g. $t_{½}$ diazepam and nordiazepam 20-50 and 30-200 hours respectively), intermediate-acting 12-24 hours and short-acting (Mirski, 2007).

BZDs are administered intravenously, intramuscularly or orally, with the oral route being the most common one. BZDs absorption is not affected by the presence of food in the stomach but by gastric motility (Garzone, 1989; Gomersall, 1999).

BZDs plasma peak concentrations are reached in 30 minutes to 2 hours after oral administration. However, when diazepam is administered intramuscularly, absorption can be slow and unpredictable. BZDs have a V_d ranging from 1-3L/Kg body weight. The protein binding of diazepam is around 98.5%. BZDs bioavailability is from 40-100% of administered dose (Gomersall, 1999; Karch, 2007). The distribution of most BZDs follows the two-compartment model

described earlier. In the two-compartment model the distribution of BZDs occurs rapidly from the central compartment (blood) and more slowly from the second (peripheral) compartment (Bailey, 1994).

Diazepam having high lipophilic properties is widely distributed throughout the body after administration. It crosses the blood brain barrier with ease and it is found in high concentrations in muscle and in particular in adipose tissue. There is limited information on the PMDR of BZDs. In general, BZDs are thought to show less PMDR than other drugs. However, animal PMDR studies have shown that diazepam levels increase in the liver and kidney, with small increases in cardiac blood (Drummer, 2007)

1.3.4.4: Biotransformation of Benzodiazepines:

BZDs biotransformation occurs through different metabolic processes including hydroxylation, demethylation and glucuronidation. For example, diazepam is metabolised through N-demethylation to produce nordiazepam. Both diazepam and nordiazepam are hydroxylated to temazepam and oxazepam, and these metabolites are active but they are highly water-soluble so therefore tend not to accumulate inside the body. Oxazepam conjugates with glucuronic acid to form an inactive metabolite (Karch, 2007).

Only small amounts of diazepam and nordiazepam are excreted in the urine. Therefore, diazepam is excreted in urine mainly as oxazepam glucuronides (about 33%) and other conjugates (20%) (Baselt, 1995).

The symptoms of BZDs toxicity are similar to those of other CNS depressants. Death due to BZDs toxicity alone is very rare; for instance, fatalities due to BZDs alone or in combination with ethanol represent 5.9 cases per million (Serfaty and Masterton, 1993) compared to 118 cases per million for barbiturate (Johns, 1977). Most fatalities involving BZDs are associated with ethanol consumption (Tanaka, 2002, Tanaka, 2003).

Whilst the case studies in this thesis involved the drugs discussed above, the table below shows the toxic effects of the commonly abused drugs, which are often taken alone or in combination with the above drugs.

Table 5: A summary of other common drugs of abuse and their toxicity (Drummer 2001; Gahlinger 2004).

Drug	**Mode of action**	**V_d** (L/kg)	**$t_{1/2}$** (h)	**Route**	**Toxicity**
Amphetamine	Stimulant	3-5	4-30	Oral	Tachycardia, tremor, hypertension and arrhythmias
Ecstasy (MDMA)	Stimulant	-	8	Oral and smoked	
Methamphetamine	Stimulant	3-4	10-30	Oral and smoked	
GHB	Anaesthetic	-	<1	Oral	Insomnia, anxiety, bradycardia, respiratory arrest, seizures, coma and death
LSD	Hallucinogen	0.3	2-6	Oral	Anxiety, hypertension, hyperthermia and rhabdomyolysis
THC	Psychoactive agent	9-11	19-96	Oral and smoked	Tachycardia and angina
Ketamine	Anaesthetic	3-5	2-4	Oral, IV and IM	Memory loss, impaired vision and attention and respiratory depression

MDMA: 3, 4 methylenedioxymethamphetamine, IV: intravenous, IM: intramuscular

The Aims of This Study:

In post mortem cases of suspected drug toxicity, blood samples are the preferred specimens for toxicological analysis. As stated earlier, blood may not be available or suitable for analysis in which case other specimens such as urine, vitreous humour, stomach contents, liver, bile, muscle, hair, nails, bone and teeth, may be invaluable for qualitative and quantitative toxicological investigations and their contribution to the cause of death.

In the method section, the analytical performances of the assays used for quantitative analysis in this thesis were validated in terms of co-efficient of variation (CV), detection limits and linearality. The specimens for toxicological analysis (856 from 95 post-mortem cases) were obtained from Professor Michael Tosokos, Hamburg University, Germany.

The specimens provided for qualitative and quantitative analysis were femoral blood, cardiac blood, vitreous humour, urine, bile, stomach content, liver, muscle, bone and hair. Drug concentrations in these different specimens were compared in relation to toxicity and cause of death. It is hoped that the findings in this study will aid our understanding of drug toxicity in particular in those cases where a blood sample is unavailable or unsuitable for analysis.

CHAPTER TWO: MATERIALS AND METHODS

2.1: Samples:

The ninety-five post-mortem samples used in this study were provided by Professor Michael Tosokos of the Institute of Legal Medicine, Hamburg University, Germany (now of Berlin University, Germany). All the cases studied were adults and their deaths were suspected to be associated with drugs and/or alcohol. Ethical requirements were fulfilled (Appendix I) and the demography of the cases is detailed in Appendix II. The following post-mortem samples were provided for the study: femoral vein blood (95 samples), cardiac blood (90), urine (65), vitreous humour (75), stomach contents (85), bile (82), thigh muscle tissue (93), liver tissue 88), scalp hair (88) and sections of rib bone (95). The total number of post-mortem samples studied was 856. All samples were received from Germany during a one year period and were stored at 4°C until analysis, which was performed within a maximum of one week.

2.2: Sample Analysis:

All samples were screened for drugs of abuse including ethanol. Fluid samples were screened for volatile solvents (ethanol, acetone, methanol and isopropanol) by gas chromatography–flame ionisation detection (GC-FID). Drugs of abuse screening in urine and blood samples was performed by specific immunoassay and the results confirmed by gas chromatography/mass spectrometry (GC/MS). Drugs of abuse detected in all samples were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The distribution of drugs of abuse in different post-mortem body compartments was studied in detail.

2.3: Instrumentation, methods and reagents:

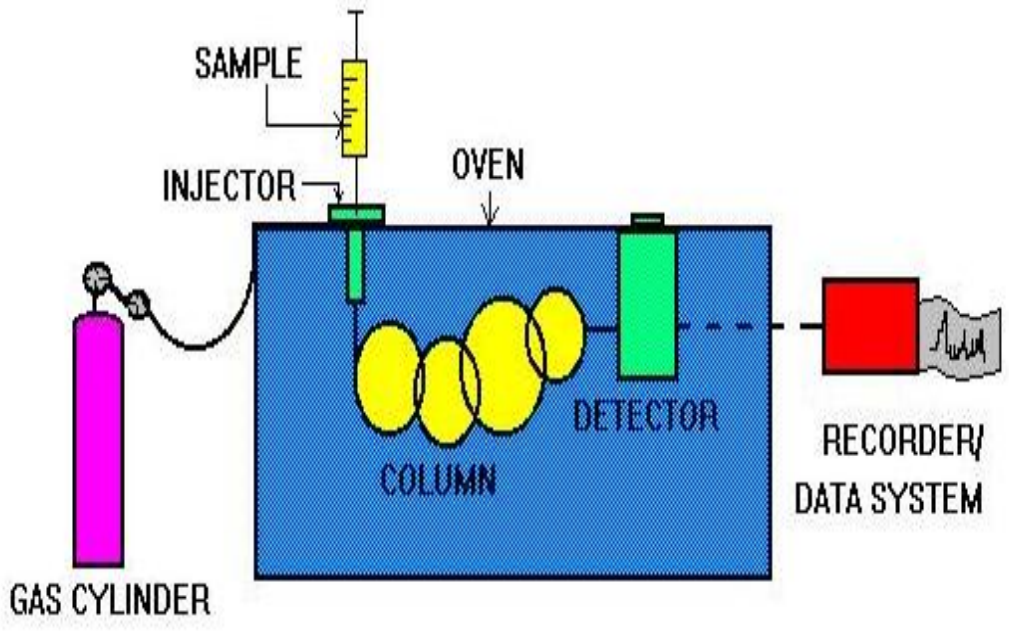
2.3.1: *Principles of gas chromatography-flame ionisation detection (GC-FID):*

Gas chromatography is regarded as a reference method for the measurement of volatile solvents. Flame ionisation detection is widely used due to its sensitivity, broad applicability and large linear dynamic range. The gas chromatography instrument used was an Agilent 6890 series GC with auto-sampler and a fused silica capillary column (Agilent INNOWAX, 15 metre length, 0.32 mm I.D. and film thickness 0.50μm) with a flame ionisation detector (FID). The instrument details are given in Figures 10-a and 10-b.

The sample is injected through the injector inlet and heated to 250°C causing the volatile solvents (ethanol, methanol, acetone and isopropanol) to vaporise. This vapour is transported by a carrier gas (hydrogen) into the fused silica capillary column heated to 80°C. The volatile solvents are separated according to their interaction with the silica coated with the stationary phase over a run-time of three minutes. The column effluent exits into a hydrogen/air flame, where the analyte molecules are ionised in addition to some of the carrier gas. The amplified current resulting from carrier gas alone exiting the column is monitored as a baseline. As analyte molecules are ionised in the flame, more current flows and this amplified current is the detector response. Signal size is recorded and plotted against elapsed time to produce a chromatogram. Quantitative results were obtained by measuring the peak area ratios, which were calculated as a ratio of analyte to internal standard concentration in the test sample to the ratio of analyte to internal standard concentration in the calibrator (or standard sample). As results are calculated as

ratios any loss of recovery during the extraction process is compensated for. Another advantage when using an internal standard is that it controls the variations in both the detector response and the injected volume. Quantitative analysis for the drug of interest is made through calculating the area under the peak by computer system.

Figure 10-a: Gas Chromatography



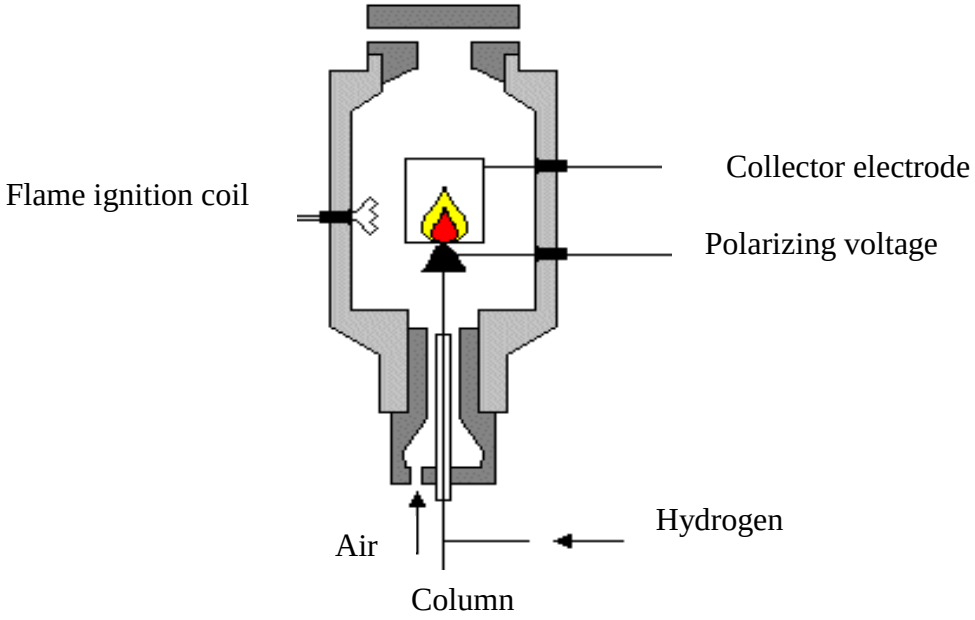


Figure 10-b: Flame ionisation detector

2.3.2: ***Gas chromatography reagents:***

The following reagents were used:

Solution A (stock internal standard): 1-propanol (1g) added to deionised water (1L)

Solution B (ethanol/methanol mixed standard): ethanol (100mg/100mL) and methanol (100mg/100mL) were diluted to 1 litre with deionised water

Solution C (internal standard): 1-propanol diluents (250mg/100mL) were prepared by adding 25mL of solution A to deionised water (75mL)

Ethanol quality control samples at 80 and 200mg/100mL

Serum volatile standard 1 (STD1, 48mg/100mL) and standard 2 (STD2, 140mg/100mL).

Solutions A and B were purchased from BDH-UK, ethanol quality control samples from LGC-Promochem-UK and standards 1 and 2 from Biorad-UK.

As stated above, the working internal standard used was n-propanol. The internal standard has similar chromatographic characteristics to the components in the sample but elutes at a different retention time and therefore does not interfere with any of the peaks of interest.

2.3.3: Assay protocol for quantitation of ethanol by GC-FID:

The detection of ethanol using GC has been described by several authors (Ohshima et al., 1997; De Martinis et al., 2006; Pelissier-Alicot et al., 2006). In our method fluid samples were centrifuged at 10800 rpm (revolutions per minute) for 10 minutes and 50µL of supernatant analysed as per protocol assay (Table 6). The tissue samples (liver and muscle) were homogenised, using a Type T 25 BS2 tissue homogeniser (GMBH & CO.KG-Germany), with deionised water (1/4 dilution). The homogenate was then centrifuged at 3800 rpm for 10 minutes and analysed as per the assay protocol.

Table 6: Ethanol Assay Protocol (μL)

Ethanol STD2 (140mg/dL)	**Ethanol STD1 (48mg/dL)**	**Centrifuged supernatant/s samples (In triplicate)**	**n-propanol internal standard (250mg/dL)**	**Ethanol QC 200mg/dL**	**Ethanol QC 80mg/dL**	**Negative blood control**
50	-	-	500	-	-	
-	50	-	500	-	-	
-	-	50	500	-	-	
-	-	-	500	50	-	
-	-	-	500	-	50	
-	-	-	500	-	-	50
All tubes were centrifuged at 3800 rpm for 5 minutes 200μL supernatant aliquoted into vials and analysed by GC (1μL injected)						

2.3.4: Detection of β-hydroxybutyrate (BHB) in blood samples:

Analysis was performed using BHB Liqicolor kit reagent (STANBIO, USA). The kit consists of the following reagents:

Enzyme (R1): β-hydroxybutyrate dehydrogenase and diaphorase enzyme.

Catalyst (R2): contains NAD, INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium cation) and oxalate.

Standard: 1mM sodium D-3-hydroxybutyrate.

The method principle is based on enzymatic quantification of β-hydroxybutyrate by using β-hydroxybutyrate dehydrogenase enzyme (Figure 11). β-hydroxybutyrate (D-3-hydroxybutyrate**)** in the presence of NAD is converted to acetoacetate and NADH

by β-hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate dehydrogenase) at pH 8.5. The NADH produced reacts with INT in the presence of diaphorase to produce colour that is measured at a wavelength of 505nm.

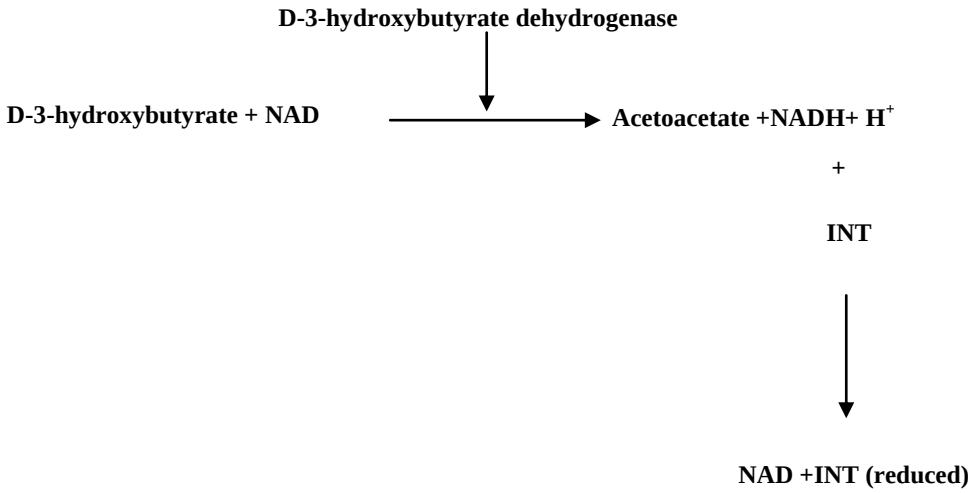


Figure 11: BHB method

Method protocol:

Enzyme (R1) was equilibrated at 37°C for 3 minutes. R1 (2.15mL) was added into two cuvettes (cuvette 1 and 2). Blood sample (60μL) was added to cuvette 1 and the baseline absorbance measured at 505nm (zero time A1) using a UNICAM UV 500 spectrophotometer (serial number 090901, model UV530, Thermo Fisher Scientific-UK). R2 (0.36mL) was then added to cuvette 1 and the absorbance again measured after 5 minutes (A2). Standard (60μL) was pipetted into cuvette 2 and the baseline absorbance measured (zero time A1). R2 (0.36mL) was then added to cuvette 2 and absorbance again measured after 5 minutes incubation (A2). The BHB concentration was calculated using the equation:

$$\text{BHB concentration (mM)} = : \frac{\text{(A2-A1) sample x 1.0mmol/l}}{\text{(A2-A1)STD}}$$

2.3.5: Principles of Cozart microplate enzyme immunoassay (EIA) kits used for drugs of abuse screening in blood samples:

Cozart microplate enzyme immunoassay kits are used for forensic and clinical toxicology analysis. The assay is based on competitive enzyme immunoassay techniques (EIA). The principle of EIA is that a known amount of enzyme labeled drug from the test kit competes for antibody binding sites with the variable amount of unlabeled drug present in the blood sample. The antibody binding sites are limited, which may lead to the saturation of the antibody binding sites. The absorption reading is compared to the drug cut-off. A negative result is indicated if the sample absorption is above the cut off reading, whilst a positive result is indicated if the sample absorption is below the cut-off (Figure 12).

The enzyme catalyzes a colour reaction when exposed to substrate. The process is divided into four steps. Step (a): competition between a pre-determined concentration of an enzyme labelled antigen present in the kit and free drug present in the sample for antibody binding. Step (b): saturation of antibody binding sites with enzyme labelled antigen and free drug occurs. Any remaining unbound free drug is removed by a wash step. According to the drug level present in the sample the possibilities are either step (c): saturation of antibodies with enzyme labelled antigen producing a high signal, which indicates a low drug concentration and a negative

result, or step (d): saturation of antibodies with a high concentration of unlabeled drug present in the sample, which produces a low signal, indicating a positive result.

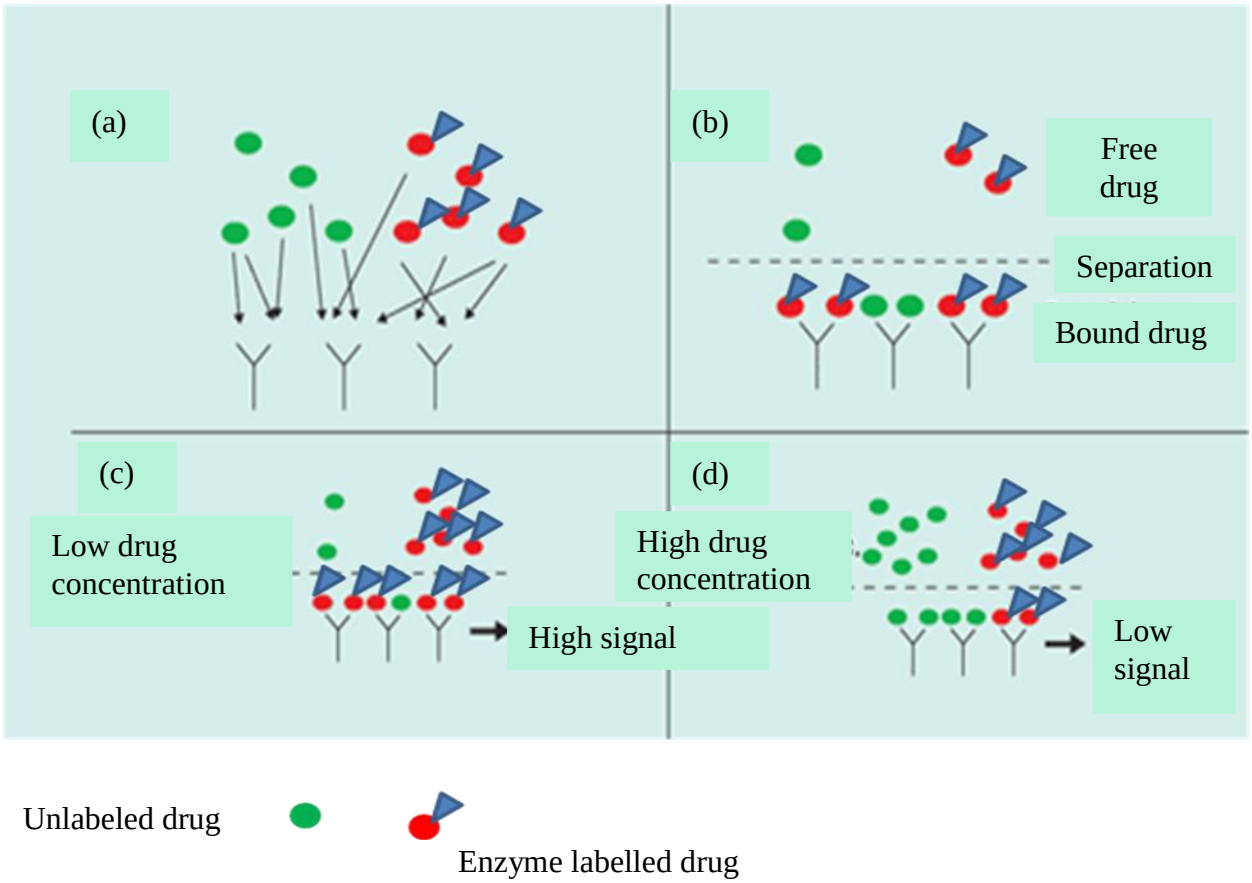


Figure 12: Competitive enzyme immunoassay techniques

2.3.6: Cozart microplate kit reagents:

Cozart microplate kits contain: 96 well microplates coated with anti-drug polyclonal antibodies (5 microplates, each consisting of 12x8 wells), enzyme conjugate (55mL), wash buffer (50mL), substrate solution (55mL), stop solution (55mL), negative drug standard (zero calibrator) and four different positive drug standards with different drugs levels (cut-off standards).

Each microplate has 12 break-apart strips, which allow the simultaneous screening for the following 12 drug groups: cocaine and metabolites (including benzoylecgonine, methylecgonine and cocaethylene), opiates (including morphine, codeine, dihydrocodeine), amphetamine, methamphetamine, benzodiazepines (including diazepam and its metabolites nordiazepam, temazepam and oxazepam), flunitrazepam, barbiturates, methadone and metabolites (including EDDP), cannabinoids, buprenorphine, promazines and tricyclic antidepressants (TCA).

Drug standards were already provided with the kits except for promazine and TCA. The cut-off calibrator concentrations used were: 100ng/mL for amphetamine, methamphetamine, benzodiazepines and opiates, 10ng/mL for cannabinoids, 50ng/mL for cocaine, 25ng/mL for methadone, 20ng/mL for barbiturates, 1ng/mL for buprenorphine and 5ng/mL for flunitrazepam. For promazine, the cut-off calibrator was prepared in the laboratory by adding promazine enzyme diluent (1mL) to promazine enzyme conjugate (20μL). For TCA, the cut-off calibrator was also prepared in the laboratory by adding TCA enzyme diluent (2mL) to TCA enzyme conjugate (10μL). The wash solution was prepared by adding the wash-solution reagent (10mL) to deionised water (500mL).

Cozart microplate kits were purchased from Cozart Bioscience Ltd-UK. The microplates were read at 450nm using a Multiskan MCC/340 plate reader (Life Science International-UK).

2.3.7: *Drug immunoassay screening in blood samples using Cozart microplate kits:*

This method has been validated for drugs of abuse in oral fluids in a previous study (Cooper, Wilson et al. 2004). Cozart kits were used to screen for the presence of the following drug groups in blood samples: cocaine and metabolites, opiates, amphetamines, methamphetamine, benzodiazepines, flunitrazepam, barbiturates, methadone and metabolites, cannabinoids, buprenorphine, promazine and tricyclic antidepressants.

Each blood sample (1mL) was centrifuged at 10800 rpm for 10 minutes and 25μL of the supernatant was used for the analysis for each drug group. Table 7 summarises the immunoassay method used in blood samples.

2.3.8: *Principles of cloned enzyme donor immunoassay (CEDIA) for drugs of abuse screening in urine:*

The CEDIA assay uses recombinant DNA technology to produce a unique homogeneous enzyme immunoassay system. This assay is based on the Escherichia coli generated β-

Table 7: Drugs of abuse immunoassay protocol in blood samples (µL)

Reagent and sample used	Coca	Opi	Amph	Metham	Benz	Flu	Barb	Metha	Canna	Buprine	Pro	TCA
Zero drug STD	25	25	25	25	25	25	25	25	25	25	-	-
Drug cut-off calibrator (ng/ml)	25 (50)	25 (100)	25 (100)	25 (100)	25 (100)	25 (5)	25 (20)	25 (25)	25 (10)	25 (20)	-	-
Deionised water	-	-	-	-	-	-	-	-	-	-	25	25
Positive control urine or serum	-	-	-	-	-	-	-	-	-	-	5	5
Deionised water											20	20
Centrifuged blood sample	25	25	25	25	25	25	25	25	25	25	25	25
Enzyme conjugate	100	100	100	100	100	100	100	100	100	100	100	200
Incubated at room temperature for 2h												
Washed three times and dried for 5min												
Substrate solution	100	100	100	100	100	100	100	100	100	100	100	100
Incubated for 30min												
Stop solution	100	100	100	100	100	100	100	100	100	100	100	100
Reading taken at 450nm wavelength												

Coca = cocaine, Opi = opiate, Amph = amphetamine, Metham = methamphetamine, Benz = benzodiazepines, Flu = flunitrazepam, Barb = barbiturate, Metha = methadone, Canna = cannabinoids, Bup = buprenorphine, Pro = promazine and TCA = tricyclic antidepressant

D-galactosidase enzyme, which has been genetically engineered into two inactive fragments, termed enzyme acceptor (EA) and enzyme donor (ED) (Levine and Spiehler, 2003). In the assay, drug in the sample competes with drug conjugated to one inactive fragment of β-galactosidase (ED-drug conjugate) for the antibody binding site. If drug is not present in the sample, antibody binds to drug conjugated on the inactive ED fragment, inhibiting the reassociation of inactive β-galactosidase fragments, and no active enzyme will be formed (figure 13, step 1). However, if drug is present in the sample, it binds to the antibody, leaving the inactive enzyme fragments (EA and ED) free to form the active enzyme (figure 13, step 2).The amount of active enzyme formed and resultant absorbance change are proportional to the amount of drug present in the sample.

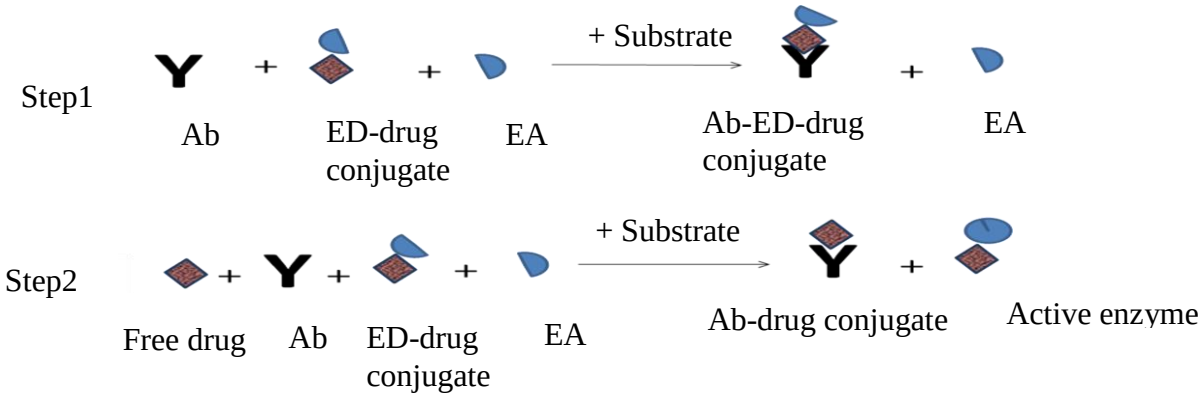


Figure 13: Cloned enzyme donor immunoassay (CEDIA)

2.3.9: ***CEDIA* reagents:**

For each drug screened four reagent bottles were provided. Bottle 1 contained monoclonal antibody reagents, bottle 1a contained enzyme acceptor reagent, bottle 2 contained a buffer salt and preservative and bottle 2a contained an enzyme donor conjugate. Enzyme acceptor and enzyme donor reagents were prepared as follows:

Enzyme acceptor solution (R1):

Bottle 1a (reagent) was connected to bottle 1 (reconstitution buffer) using one of the enclosed adapters and mixed. Bottle 1a was separated from bottle 1 and discarded. Bottle 1 was capped, left for 5 minutes at room temperature (15-25°C) and then mixed.

Enzyme donor solution (R2):

Bottle 2a (reagent) was connected to bottle 2 (reconstitution buffer) using one of the enclosed adapters and mixed. Bottle 2a was disconnected from bottle 2 and discarded. Bottle 2 was capped and left to stand for approximately 5 minutes at room temperature (15-25°C) and then mixed.

2.3.10: CEDIA urine immunoassay method:

Urine samples (2mL) were analysed using an Olympus AU 400 instrument and screened for the following drugs: amphetamine, MDMA (ecstasy) and its metabolite MDA, barbiturates, cocaine and metabolites (benzoylecgonine, methylecgonine and cocaethylene), benzodiazepines including diazepam and metabolites (nordiazepam, temazepam and oxazepam), methadone and metabolites (EDDP), opiates including morphine, codeine and dihydrocodeine, heroin metabolite 6-monoacetylmophine (6-MAM) and cannabinoids. Different volumes of the urine sample were used for each drug screen. For amphetamine, MDMA, barbiturates, benzodiazepines and opiates 2μL of urine was required. For cocaine, 6-MAM and cannabinoids 4μL of urine was required, whilst for methadone 8μL of urine was required. From reagents R1 and R2, 87μL was used for each drug and was added automatically. Samples were mixed

automatically by the instrument and the change in absorbance measured at a primary wavelength of 570nm and secondary wavelength of 660nm following a period of incubation. The method summary is given in Table 8.

Table 8: CEDIA immunoassay

Reagent and sample used (μL)	Amph	Benzo	Opi	Coc	6-MAM	THC	Meth/ EDDP	MDMA	Barb
Urine sample	2	2	2	4	4	4	8	2	2
Enzyme acceptor solution (R1)	87								
Enzyme donor solution (R2)	87								
Mixed and substrate automatically added									
Developed colour read at 570 and 660nm									

Amph = amphetamine, Benz = benzodiazepines, Opi = opiate, Coca = cocaine, 6-MAM = 6-monacetylmorphine, THC = tetrahydrocannabinol, Meth = methadone, EDDP = 2-ethylidene-1,5-dimethyl3,3-diphenylpyrrolidine, MDMA = 3,4-methylenedioxymethamphetamine and Barb = barbiturate.

2.3.11: *Principles of gas chromatography/mass spectrometry (GC/MS):*

The GC/MS apparatus is outlined in Figure 14-a. The extracted and derivatised drug is injected (1µL) onto the gas chromatography column through the injection port and transported by a carrier gas (helium) along the heated silica column (30m). The drugs and other compounds present on the column are separated depending on their volatility. Highly volatile compounds travel faster than less volatile molecules and are subsequently transferred from the GC column into the mass spectrometer.

Molecules that enter the mass spectrometer are fragmented into small positively charged particles (ions) by the ion source (Figure 14-b). The ions pass through an electromagnetic field that separates ions according to their mass (m) to charge (z) ratios (m/z). The ions are then directed through a mass analyser to a detector system, where a signal is generated to represent the ions that impinge on the detector. The system requires a vacuum to allow for transfer of ions form one place to another while virtually eliminating the chance for the ions to collide with other ions or molecules. Manipulation of the electrical and magnetic fields allows for the isolation of ions of a single m/z ratio. Data is sent to the computer and a mass spectrum is created (Figure 14-b). The most common ionisation techniques used are electron and chemical ionisation.

Electron ionisation (EI) technique uses an electron beam which is generated from a tungsten filament to ionise gas-phase molecules and create ions (Figure 15). This technique is commonly used for the analysis of small molecular weight compounds that can be converted to gas by heat without decomposition. The reaction between the electron beam and gas-phase molecules results in the loss of an electron from the analyte according to the equation: $\mathbf{M + e^- \rightarrow M^+ + 2e^-}$

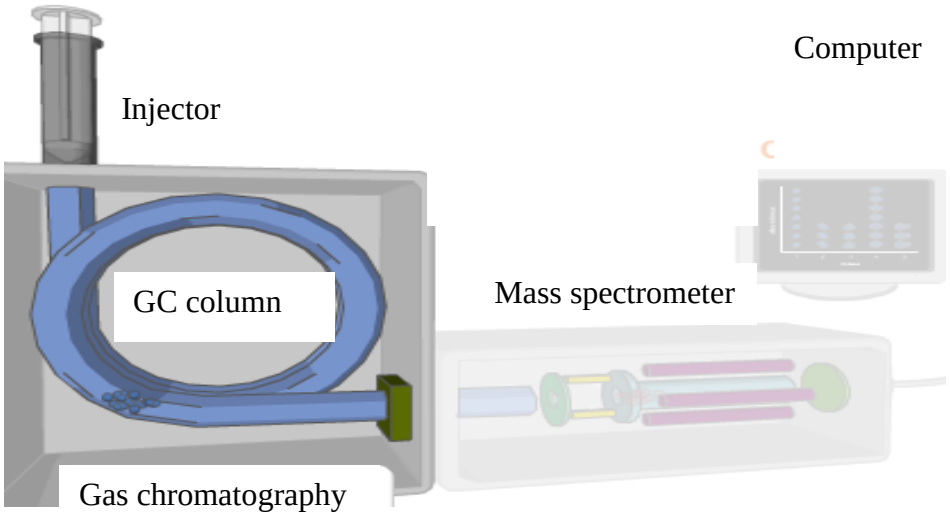


Figure 14-a: Gas chromatography mass spectrometry structure

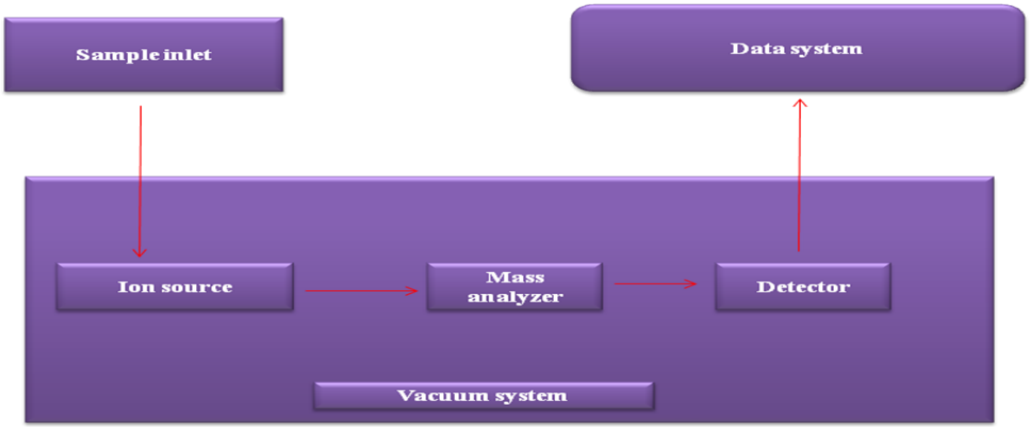


Figure 14-b: Mass spectrometry components

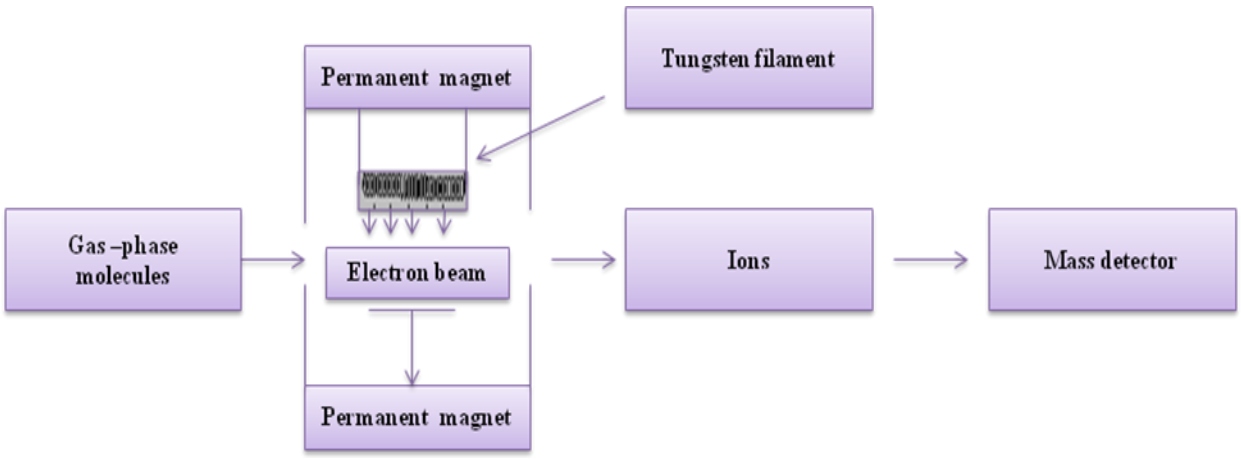


Figure 15: Electron ionisation technique (EI)

Chemical ionisation (CI) technique also depends on electrons as the primary ion source. However, the electrons ionise a reagent gas rather than directly ionising analyte molecules. The reagent gas and analyte molecules are ionised by high energy electrons upon entering the ion source. The chemical ionisation process yields less analyte fragmentation as compared to electron ionisation. Chemical ionisation also differs from electron ionisation in that it is a more gas dependent process, allowing high concentrations of gas (e.g. methane, ammonia, isobutane) into the ion source to allow ion molecule reactions to take place.

Drug identification in GC/MS depends on the retention time and the mass spectral library match. The GC/MS is highly sensitive and can be used for the quantification of drug levels as low as 1ng/mL. This sensitivity can be improved by the use of selected ion monitoring (SIM) for target compounds such as cocaine. However, there are limitations for GC/MS in that the drug needs to be extracted from the sample matrix before analysis by solvent or solid phase extraction. Derivatisation of unstable compounds increases the number of drugs that can be screened for by

GC/MS, but sample preparation time is long and drug identification is dependent upon its presence in the reference library.

The GC/MS used in this study was a 6890N series in EI mode connected to a 7683B series injector and a 5975 inert mass selective detector provided by Agilent Technologies - UK. The silica column used was also provided by Agilent Technologies - UK.

2.3.12: *Gas chromatography/mass spectrometry (GC/MS) reagents:*

Nalorphine (LGC Promochem-UK), hexabarbitone (2mg/mL, Sigma-UK), methanol (BDH-UK), phosphate buffer (0.1M, pH 6) (BDH-UK), acetic acid (10mM, BDH-UK), eluent A (80mL chloroform, 20mL isopropanol) (BDH-UK), eluent B (80mL chloroform, 20mL isopropanol, 3mL ammonia solution 35%) (BDH-UK), ethyl acetate (BDH-UK), pyridine (BDH-UK), MSTFA (N-methyl-N-trimethylsilyl trifluroacetamide), MBTFA (N-methyl bis-trifluroacetamide) (Sigma-UK), beta-glucuronidase (STANBIO Laboratory-USA), sodium acetate buffer (5M, pH 5.0) and sodium hydroxide (BDH-UK).

2.3.13: *General drug screen in blood by GC/MS*:

Each blood sample (2mL) was added to phosphate buffer (2mL), hexabarbitone (20μL) and nalorphine (40μL) and placed in a water bath at 37 °C for 10 minutes, sonicated for 10 minutes and then centrifuged at 3800 rpm for a further 10 minutes. Narc-2 columns (JT Baker – Holland) were placed into a vacuum manifold for solid phase extraction. Each column was conditioned by adding methanol, deionised water and phosphate buffer (0.1M, pH 6). The supernatant (3mL) from each sample was

added to the Narc-2 column and eluted. This was followed by a deionised water wash and acidification of the column with glacial acetic acid (10mM, 250µL). The waste was removed from the manifold and a glass tube placed under each column. Eluent A (2mL) was added to each column and eluted into the glass tubes, followed by eluent B (3mL). The tubes were then removed from the manifold and placed into a sample concentrator under nitrogen until dry. Ethyl acetate (200µL) was added to each tube. After mixing, 200µL from each tube was transferred into an appropriate GC/MS glass vial and were again placed into the sampler concentrator under nitrogen until dry. Derivatisation agent pyridine (10µL), MSTFA (25µL) and MBTFA (5µL) were then added to each vial, which was then capped, mixed and placed into a Dri-Block DB-3 sand bath at 70 °C for 30 minutes. The samples were then analysed by GC/MS.

2.3.14: ***General drug screen in urine by GC/MS:***

Each urine sample (5mL) was placed into a Toxi-lab tube (Varian-Netherland), hexabarbitone (20µL) and nalorphine (25µL) were added. The resulting solution was then mixed using a rotating mixer (Ecomcat-UK) for 1 hour and then centrifuged for 5 minutes at 3800 rpm. The supernatant was removed by a glass pipette and the samples placed into a sample concentrator (Techne-Ltd-UK) under nitrogen until dry. Ethyl acetate (200µL) was added to the dry sample tubes, mixed and then transferred to an appropriate GC/MS glass vial using a glass pipette and the vials again dried under nitrogen in the sample concentrator. Derivatisation agent pyridine (10µL), MSTFA (25µL) and MBTFA (5µL) were then added to each vial. The vials

were capped, mixed and placed into the Dri-block DB-3 (Techne-Ltd-UK) sand bath for 30 minutes. The samples were then analysed by GC/MS.

2.3.15: *General drug screen in hydrolysed urine by GC/MS*:

This method is used when screening for conjugated drugs such as opiates. A disadvantage of this method is that the concentration of some drugs or their metabolites may be affected by the separation process.

Deionised water (2mL), beta-glucuronidase (50μL) (for hydrolysis), sodium acetate buffer (200μL), and the internal standards hexabarbitone (20μL) and nalorphine (40μL) were added to the urine sample (3mL). The mixture was placed into a water bath at 70 °C for 2 hours; samples were cooled and centrifuged at 3800 rpm for 10 minutes. Narc-2 columns (J.T.Baker- Holland) were placed into a vacuum manifold for solid phase extraction. The columns were conditioned by eluting deionised water (3mL) through each column, followed by glacial acetic acid (250μL, 10mM, pH 3.3).

The supernatant was added to the conditioned Narc-2 columns and eluted. The waste was removed from the manifold and eluent A (2mL) eluted followed by eluent B (3mL) into glass tubes which were dried under nitrogen. Ethyl acetate (250μL) was then added to each tube and mixed for 1 minute. The resulting solution was then transferred into an appropriate GC/MS glass vial and again dried under nitrogen. Derivatisation agent's pyridine (10μL), MSTFA (25μL) and MBTFA (5μL) were then added to each vial, the vials capped and placed in the Dri-Block DB-3 sand bath at 70 °C for 30 minutes. The samples were then analysed by GC/MS.

2.3.16: ***Drugs of abuse screen in tissue samples by GC/MS (qualitative analysis):***

Tissue samples were weighed, diluted 1:4 (w/v) with deionised water and homogenised using a Type T 25 BS2 homogeniser (GMBH & CO.KG-Germany). Homogenised tissue (20mL) was centrifuged at 3800 rpm for 10 minutes. The supernatant (10mL) was transferred to a 25mL centrifuge tube and the pH adjusted to 1.0 by the addition of sulphate buffer. Nalorphine (40µL) and ethyl acetate (10mL) were added and the tubes centrifuged at 3800 rpm for 10 minutes. The lower aqueous layer was transferred to a glass tube, pH was adjusted to 7.0 by the addition of sodium hydroxide (10M, Sigma-UK), and equal volumes transferred into two Toxi-lab tubes, which were mixed for 10 minutes and then centrifuged at 3800 rpm for 10 minutes. The top layers were removed, placed into a glass tube and dried under nitrogen. Ethyl acetate (200µL) was then added. The resulting solution was then transferred into an appropriate GC/MS glass vial and again dried under nitrogen. Pyridine (10µL), MBTFA (5µL) and MSTFA (100µL) were added for sample derivatisation, the vials capped and placed in the Dri-Block DB-3 sand bath at 70 °C for 30 minutes. The samples were then analysed by GC/MS.

2.3.17: ***Qualitative analysis of drugs of abuse in hair and bone samples by GC/MS:***

Portions of hair and bone were weighed and ground by Pulverisette *7* (Planetary Micro Mill) at 800 rpm for 30 minutes. Methanol (4mL) was added to the ground material and the mixture sonicated for 30 – 60 minutes. Samples were then incubated in a water bath at 45 °C for 24 hours. The hair or bone extracts were

divided into 2 tubes: the first tube for GC/MS (qualitative method), and the second for LC-MS/MS (quantitative method).

Nalorphine internal standard (20μL) was added to 1mL of the methanolic extract and dried at 60 °C under nitrogen. Ethyl acetate (200μL) was added and the mixture transferred to a GC/MS vial and dried at 60 °C under nitrogen. Derivatisation agents pyridine (10μL), MBTFA (5μL) and MSTFA (100μL) were added, the vials capped and placed in the Dri-Block DB-3 sand bath at 70 °C for 30 minutes. The samples were then analysed by GC/MS.

2.3.18: *Principles of liquid chromatography-tandem mass spectrometry (LC-MS/MS):*

The instrument consists of a liquid chromatograph (LC) attached to a tandem mass spectrometer (MS/MS). Compounds are initially separated by LC according to their interaction with the chemical coating (silica) of the stationary phase and the solvent eluting through the column (mobile phase). LC offers some advantages as a separation technique over GC. Extraction procedures can be less extensive than required for GC, derivatisation is often not used thus saving time and expense, and many compounds that are not stable at high temperature fare much better in LC.

Molecules elute from the LC column and enter the MS where they are ionised by an ion source in the first mass spectrometer (MS1). A single ionised mass from the analyte mixture is selected, which enters into the gas-collision chamber where it is fragmented by a collision-induced dissociation process (Figure 16). The mass of a single ion is measured in MS2.

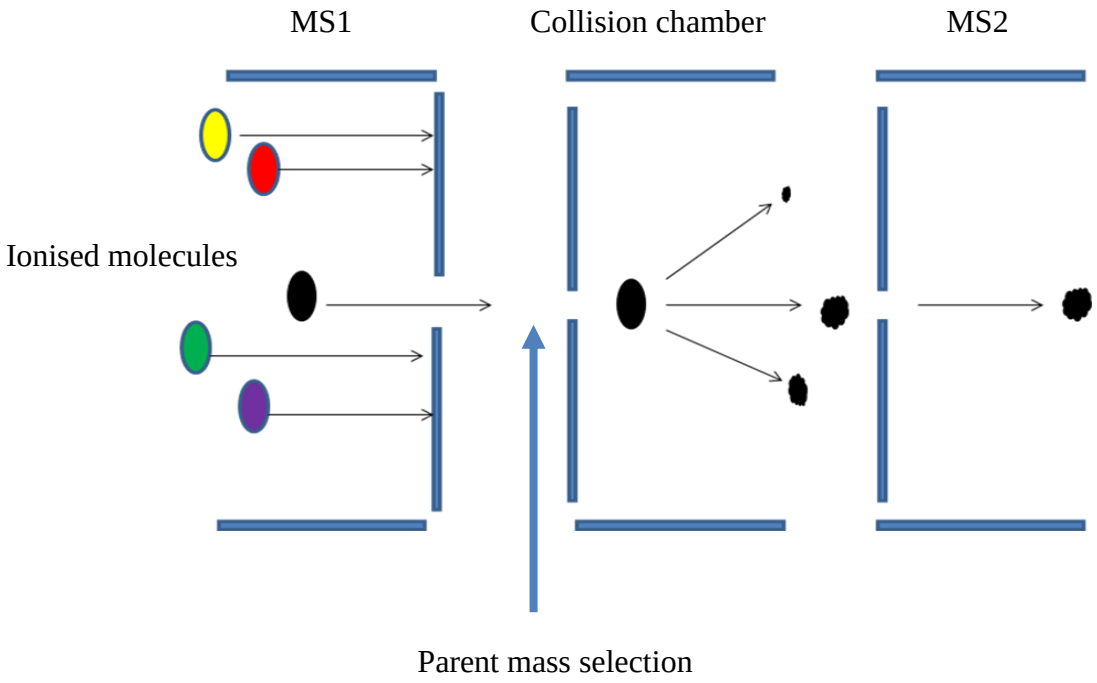


Figure 16: Tandem mass spectrometry technique.

There are two main methods for ionisation: electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) interfaces. Electrospray ionisation allows for large, non-volatile molecules to be analyzed directly from the liquid phase. An advantage of APCI is that its gives molecular weight information for volatile substances at a flow rate up to 2mL/min. It also provides increased separation for less polar compounds compared to ESI. Many drugs can be analysed by either APCI or ESI (Yinon, 2003).

Advantages of tandem mass spectrometry include the ability to analyse a wider range of compounds, it is more selective and specific for drugs compared to GC/MS. It can also measure very small quantities of analytes with excellent precision. The MS/MS mode is not used for general drug screening but for specific analyte quantitation. The drug of interest must be ionised and the fragmentation of the parent ion must

produce a stable fragmentation pattern of qualifier ions, which are used to identify and confirm the presence of an individual drug.

The LC-MS/MS used in this study was a LCQ Dexa XP MAX Plus in ESI mode with a surveyor auto-sampler provided by Thermo Scientific, UK. The LC-MS/MS Hypersil BDH (base deactivated silica) columns were also provided by Thermo Scientific-UK.

2.3.19: *Reagents for LC-MS/MS:*

Methanol, chloroform, isopropanol, ammonia solution (35%), glacial acetic acid, sodium acetate buffer (5M, pH 5.0), hydrochloric acid (0.1M, BDH-UK), beta-glucuronidase (100,000units/mL, Sigma-UK), potassium dihydrogen orthophosphate (Sigma-UK) and phosphate buffer (0.1M, pH 6.0).

Stock standards for opiate quantitation were obtained from LGC Promochem-UK. These stock standards consist of 100μg/mL of morphine, codeine, norcodeine and dihydrocodeine, and 50μg/mL of heroin, normorphine and 6-MAM. Calibration curve standards were prepared from the above stock standard solutions by dilution with methanol to concentrations of 2000, 5000 and 10000ng/mL.

Stock deuterated opiate internal standards (1mL) were provided in Cerilliant™ vials (LGC Promochem-UK). A working opiate internal standard was prepared by adding 1mL of deuterated morphine, codeine, dihydrocodeine and 6-MAM standards to methanol up to a total volume of 50mL. Similarly, deuterated cocaine and metabolites (benzoylecgonine, methylecgonine and cocaethylene) were added to

acetonitrile (total volume 50mL). Methadone and metabolite (EDDP) deuterated internal standard and diazepam and metabolites (nordiazepam, temazepam and oxazepam) Cerilliant^{TM} internal standards were also prepared using acetonitrile (total volumes 50mL).

Whole blood toxicology quality control for drugs of abuse (DOA-QC, drugs of abuse level 1, product number 98818; UTAK laboratories-UK) were used for morphine, codeine and dihydrocodeine quantitation. In house QC pool 1 and pool 2 were used for norcodeine, normorphine and 6-MAM. Pool 1 and pool 2 in-house QC's were prepared using commercially available stock solutions of all standards (1mg/mL) and their metabolites. From this stock solution a working solution was for each opiate standard were prepared by diluting the stock solution (1mL) horse serum (100mL, TCS Bioscience - HA002) to give a final working solution concentration of 10µg/mL. Each working internal standard solution was then spiked into pool 1 and pool 2 to give concentrations of 200ng/mL and 400ng/mL respectively. Aliquots (1mL) of pools 1 and 2 were stored at -20°C.

Hair QC for drugs of abuse (Medidrug DHF 1/01-B H-plus) was provided by Medichem-Germany. It contained 260mg of drugs of abuse hair QC for cocaine, benzoylecgonine, morphine and 6-MAM. As no bone QC material for drugs of abuse was found to be available, the hair QC material was used.

2.3.20: ***LC-MS/MS reagent preparation:***

Sodium acetate buffer (5M):

Sodium acetate trihydrate (214.5g, BDH-UK) was dissolved in deionised water (400mL). Glacial acetic acid (10.4mL) was added and the solution pH adjusted to 5.0 by the drop wise addition of glacial acetic acid. Deionised water was added to a final volume of 500mL. The prepared buffer was stable for use within a 12 month period.

Phosphate buffer (0.1M, pH 6.0):

Potassium dihydrogen orthophosphate (27.22g, Sigma-UK) was dissolved in deionised water (1900mL) and the pH of the solution adjusted to 6.0 by the dropwise addition of potassium hydroxide (1M, Sigma-UK). Deionised water was added to give a final volume of 2000mL.

Hydrochloric acid (HCl, 0.1M):

This was prepared by adding HCl (2.2mL, specific gravity 1.18, BDH-UK) to deionised water (250mL).

Elution buffer (eluent A):

This was as prepared by adding chloroform (800mL) to isopropanol (200mL) and stored at room temperature.

Elution buffer (eluent B):

Eluent B was freshly prepared for each extraction by adding ammonia solution (35%, 3mL) to eluent A (100mL).

Mobile Phase Solvents A, B, C:

Solvent A was prepared by dissolving ammonium acetate (1.56g, BDH-UK) in deionised water (1000mL) and the pH adjusted to 4 by the drop wise addition of glacial acetic acid. Solvent B was prepared by the addition of glacial acetic acid (1mL) to methanol (999mL). Solvent C was prepared by the addition of glacial acetic acid (1mL) to deionised water (999mL).

2.3.21: ***Drug quantitation by LC-MS/MS:***

Quantitation methods for opioids, cocaine and diazepam by LC-MS/MS have been described by many authors (Tyrefors et al., 1996; Marquet and Lachatre, 1999; Pichini et al., 1999; Van Bocxlaer et al., 2000; Dams et al., 2003; Johansen and Bhatia, 2007).

Opiate Quantitation

The quantitation method used is given in Table 9. Mobile phase solvents B and C were used for opiate quantitation. A similar extraction procedure was performed for total opiate quantitation except that each sample was hydrolysed using beta-glucuronidase (50μL) and sodium acetate buffer (200μL, 5M, pH 5) in deionised water (2mL). The method used is outlined in Table 10.

Cocaine, methadone and diazepam quantitation

The procedure for cocaine and metabolites (benzoylecgonine, methylecgonine and cocaethylene), methadone and metabolite (EDDP), and diazepam and metabolites (nordiazepam, temazepam and oxazepam) quantitation was the same as used for free opiate extraction as described in Table 9. Mobile phase solvents A and B were used.

Table 9: Free opiates quantification method using LC/MS

Reagent	Tube Number											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Opiate standard (2000ng/mL)	100	-	-	-	-	-	-	-	-	-	-	-
Opiate standard (5000ng/mL)	-	100	-	-	-	-	-	-	-	-	-	-
Opiate external standard (10000ng/mL)	-	-	100	-	-	-	-	-	-	-	-	-
Horse serum	900	900	900	1000	-	-	-	-	-	-	-	-
Drugs of abuse low control	-	-	-	-	1000	-	-	-	-	-	-	-
Drugs of abuse high control	-	-	-	-	-	1000	-	-	-	-	-	-
Positive samples	-	-	-	-	-	-	1000	1000	1000	1000	1000	1000
Opiate internal (2000 ng/mL)	200	200	200	200	200	200	200	200	200	200	200	200
Deionised water	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000
Phosphate buffer 0.1M, pH 6	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Mixed and sonicated in water bath at 37 °C for 10min Centrifuged for 5 min at 3800 rpm and supernatants were added to all Narc-2 columns 2000µL deionised water, 1000µL HCL (0.1M) and 1000µL Methanol were added to all Narc-2 columns and waste was removed Eluent B was added to all Narc-2 columns and extracted fluids were collected and dried at 60-70 °C for 10-15 min 300µL methanol were added and run by LC/MS												

Table 10: Total opiates quantification method using LC/MS

Reagent	Tube Number											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Opiate standard (2000ng/mL)	100	-	-	-	-	-	-	-	-	-	-	-
Opiate standard (5000ng/mL)	-	100	-	-	-	-	-	-	-	-	-	-
Opiate external standard (10000ng/mL)	-	-	100	-	-	-	-	-	-	-	-	-
Horse serum	900	900	900	1000	-	-	-	-	-	-	-	-
Drugs of abuse low control	-	-	-	-	1000	-	-	-	-	-	-	-
Drugs of abuse high control	-	-	-	-	-	1000	-	-	-	-	-	-
Positive samples	-	-	-	-	-	-	1000	1000	1000	1000	1000	1000
Opiate internal standard for hydrolysis (2000ng/mL)	200	200	200	200	200	200	200	200	200	200	200	200
Deionised water	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000
5 M Na acetate buffer pH 5	200	200	200	200	200	200	200	200	200	200	200	200
Beta-glucuronidase	50	50	50	50	50	50	50	50	50	50	50	50
Mixed and sonicated in water bath at 70 °C for 2-3 h Centrifuged for 5min at 3800 rpm and supernatants were added to all Narc-2 columns 2000µL deionised water, 1000µL HCL (0.1 M) and 1000µL Methanol were added to all Narc-2 columns and waste was removed Eluent B was added to all Narc-2 columns and extracted fluids were collected and dried at 60-70 °C for 10-15 min 300µL methanol were added and analysed by LC/MS												

2.3.22: *Quantitation method for drugs of abuse in tissue samples by LC-MS/MS:*

Liver and muscle tissue samples were weighed, diluted 1:4 (w/v) with deionised water and homogenised using a Type T 25 BS2 homogeniser (GMBH & CO.KG-Germany). The drug extraction protocol outlined in Table 9 was used.

2.3.23: *Cocaine quantitation method in hair and bone using LC-MS/MS:*

A hair QC was prepared for extraction by adding 40mg of hair QC (Medidrug DHF 3/01-A H-plus) to methanol (4mL) and cocaine internal standard (100μL). The mixture was incubated at 45 °C for 24 hours.

From each cocaine-positive case, portions of hair and rib bone were weighed and ground. Each hair sample was cut into 3 segments, each 3cm long. Rib bones were divided into three sections each 1cm in length. Methanol was added to the ground materials (1:4 w/v). The mixture was incubated at 45 °C for 24 hours. The supernatant was then prepared for analysis as outlined in Table 11.

2.3.24: *Opiate quantitation in hair and bone by LC-MS/MS:*

For opiate positive hair and bone samples, a similar method to that of cocaine (as described above) was implemented using opiate standards for calibration and opiate deuterated internal standards.

Table 11: Cocaine quantification method in hair and bone samples by using LC/MS

Reagent and samples	**Tube Number**						
	1	2	3	4	5	6	7
Cocaine external standard 2000ng/mL	25	50	100	-	-	-	-
Cocaine internal standard 2000ng/mL	-	-	-	100			
Extracted hair/ or bone	-	-	-	-	1000	-	-
Hair QC low	-	-	-	-	-	1000	-
Hair QC high	-	-	-	-	-	1000	-
Cocaine internal standard 2000ng/mL	100	100	100	100	100	100	100
All tubes were mixed and dried							
Methanol	300	300	300	300	300	300	300
Injected to LC/MS							

Volumes in µL

2.3.25: ***Other instruments used*:**

The tissue homogeniser used in this study was a Type T 25 BS2 (serial number 00.249096, GMBH & CO.KG-Germany). The hair and bone grinder used was a *FRITSH* pulverisette *7* (serial number 07.4000/00544, Planetary Micro Mill, Fritsch Gmbh-Germany). A Dri-Block DB-3 was used for drying samples under nitrogen (Techne-Ltd-UK). A rotating mixer (Ecomcat-UK) and a Hearaeus Sepatech centrifuge (model Megafuge 1.0, serial number 160939, DJB Lab Care-UK) were also used.

2.4: Validation of the analytical method:

The validation of analytical methods is the process of establishing whether or not the method is acceptable for its purpose. Before analyzing the post mortem samples the quantitation methods for ethanol, cocaine, morphine, codeine, methadone, EDDP, diazepam and its metabolites were validated.

Parameters used for validation included selectivity/specificity, accuracy, precision by obtaining the coefficient of variation (CV) at different drug concentrations, recovery, linearity, analytical range, limit of quantitation (LOQ) and limit of detection (LOD). The theoretical aspects of these parameters will be discussed later.

In this study, selectivity/specificity of the quantitative methods was determined for each of the 6 drugs at 6 different concentrations using 12 replicates for each concentration. For determining accuracy and recovery of the analytical method, 12 measurements with 6 different concentrations for opiates, cocaine, methadone and

benzodiazepines methods were performed. In this study, acceptable linearity and calibration curves were achieved. All precision values were obtained within batch.

2.4.1. ***Validation method and tissue recovery of opioids:***

Linearity

Several authors have reported a validation method for morphine (Naidong et al., 1999; Slawson et al., 1999; Concheiro et al., 2006). Calibration curves for opioids were obtained using different concentrations of opiate standards (i.e., 2000ng/mL, 5000ng/mL, and 10000ng/mL) diluted 1/10 (v:v) in methanol to give the calibration curve points 200, 500 and 1000ng/mL respectively. Thus a curve for each opiate studied was obtained. Linearity for all calibration curves was achieved (Appendix III).

Precision

Opiate concentrations of 25, 50, 100, 200, 250 and 500ng/mL were prepared. The percentage CV for opiates at different concentrations was calculated within batch (Table 12). International guidelines state that a reasonable CV for biological samples is less than 20% (Shah et al., 2004; Chandran, 2007). In our study, all percentage CV's were less than 20%. Six replicates of a blank measurement were used to determine the limit of detection (LOD) and limit of quantification (LOQ) for each opiate studied (note: a blank sample was analysed directly following a high standard concentration to avoid the possibility of any sample carry over).

Recovery

Recovery for morphine, codeine and dihydrocodeine from post-mortem tissue

Table 12: Precision values for opiates at different concentrations and limit of detection LOD and limit of quantification LOQ values

Concentration ng/mL	CV%							
	6-MAM	**Morphine**	**Nor-morphine**	**Nor-codeine**	**Codeine**	**Dihydrocodeine**	**Methadone**	**EDDP**
25	11.4	13.2	18.4	13.8	15.5	9.3	10.6	15.9
50	10.4	10.7	12.4	12.0	10.2	8.7	8.8	13.9
100	9.2	10.1	11.3	9.9	9.4	7.6	7.2	12.1
200	8.2	9.4	8.2	9.2	9.3	6.8	6.0	9.1
250	7.6	7.4	6.9	8.4	8.7	5.8	5.7	7.9
500	4.9	6.4	5.3	6.1	5.0	3.5	4.8	4.0

	6-MAM	**Morphine**	**Nor-morphine**	**Nor-codeine**	**Codeine**	**Dihydrocodeine**	**Methadone**	**EDDP**
LOD (ng/mL)	0.57	0.36	0.72	0.51	0.81	0.36	0.6	1.1
LOQ (ng/mL)	1.9	1.2	2.4	1.7	2.7	1.2	2.0	3.7

samples was studied. Liver and muscle samples were obtained from opiate negative cases before the method was applied to the positive cases. The mass of each sample was measured and the sample homogenised following a 1:4 (w:v) dilution with deionised water. The final volumes for the homogenized liver and muscle samples were measured (89 and 82mL respectively). Morphine, codeine and dihydrocodeine stock standards (500µL of 100000ng/mL, i.e. 50000ng/mL) were added.

Samples were incubated at 4°C for 8 hours. The tissue homogenates were then mixed for 4 hours. The homogenate samples (1mL) were then extracted using the protocol for opiate quantification as previously outlined. The recovery for morphine, codeine and dihydrocodeine in liver was 101.1%, 99.8% and 103%, whilst in muscle the recovery was 93.6%, 89.8% and 95.3% respectively. The recovery of morphine from the liver tissue was higher than that reported by Cingolani in liver tissue fixed with formalin (36.3%) and in formalin solution used for fixation (74.9%) (Cingolani et al., 2001). For methadone the total volumes of liver and muscle used were both 130mL. Methadone recovery was 85.8% from liver and 79.5% for the muscle tissue.

2.4.2: ***Validation method for cocaine:***

A recovery study for cocaine and benzoylecgonine from liver and muscle samples was also performed. Similar to opiates (Paragraph 2.4.1), the CV obtained for cocaine and its metabolites were obtained within batch (Table 13). Liver and muscle samples were obtained from cocaine negative cases. The final volumes for the homogenized liver and muscle samples were measured (140mL and 120mL respectively). The recovery of cocaine and benzoylecgonine from liver was 94.2%

Table 13: Precision values for cocaine and its metabolites at different concentrations and limit of detection (LOD) and limit of quantification (LOQ)

Concentration ng/mL	CV% Cocaine	Benzoylecgonine	Methylecgonine	Cocaethylene
25	8.5	17.3	17.2	10.2
50	8.2	13.2	14.7	6.2
100	6.8	8.2	9.4	6.0
200	4.6	6.8	6.3	5.7
250	3.3	6.5	6.0	3.6
500	3.0	5.0	4.0	3.5

	Cocaine	Benzoylecgonine	Methylecgonine	Cocaethylene
LOD (ng/mL)	1.7	0.6	5.4	2.13
LOQ (ng/mL)	5.94	2.0	18.2	7.09

and 97.9%, and from muscle tissue was 98.4% and 99.4% respectively. Calibration curves are given in Appendix III, whilst QC material used is given in Appendix IV.

2.4.3: ***Validation method for diazepam and metabolites:***

Similar to opiate validation (paragraph 2.4.1), the CV for diazepam and its metabolites were obtained within batch (Table 14). Recovery of diazepam and nordiazepam was studied in liver and muscle tissues from negative diazepam cases. Each diazepam and nordiazepam standard concentration was 50000ng/mL. The total volume of homogenised liver was 110mL and muscle 130mL. The expected level of diazepam and nordiazepam in the liver is 454ng/mL and in the muscle 384ng/mL. The mean concentrations for diazepam in the liver was 314.8 and for the muscle 320.5 respectively. The recovery for diazepam in the liver was 69% and for the muscle was 83.3%. Nordiazepam recovery from liver was 89.2% and 97% from muscle.

2.4.4: ***Validation method for ethanol:***

The method for ethanol detection was validated and the precision of the method was obtained through calculating the CV within batch from ethanol QC concentrations of 17, 50, 80, 100 and 200mg/dL. The percentage CV values obtained were 3.1, 2.7, 2.3, 2.1 and 1.7% respectively. Ethanol recovery values with different ethanol QC values (17, 50, 80, 100 and 200mg/dL) were 95.2, 96.6, 94, 96.6 and 95.5% respectively.

Table 14: Precision values for diazepam and its metabolites at different concentrations and limit of detection (LOD) and limit of quantification (LOQ)

Concentration ng/ml	CV%				
	Diazepam	Nor-diazepam	Oxazepam	Temazepam	Chlordiazepoxide
25	11.2	14.5	17.1	13.4	15.6
50	10.6	11.2	12.7	7.4	14.4
100	9.0	8.9	10.9	6.0	6.0
200	7.0	7.4	9.9	6.0	5.3
500	6.1	5.2	5.7	5.0	3.7

	Diazepam	Nor-diazepam	Oxazepam	Temazepam	Chlordiazepoxide
LOD (ng/ml)	0.01	0.009	0.01	0.014	0.009
LOQ (ng/ml)	0.03	0.03	0.03	0.048	0.03

2.5: Statistical analysis:

The statistical analysis, which included correlation and regression analysis and calculation of median ratios to femoral blood drug level, were obtained by using SPSS version 14 provided through the University of Leicester.

CHAPTER THREE: RESULTS AND DISCUSSION

As mentioned in Chapter Two the specimens used in this study were collected from 95 forensic cases autopsied at the Hamburg Institute of Legal Medicine. The preliminary suspected causes of death or the major contributors to the cause of death were thought to be drugs and/or alcohol. All samples were screened for ethanol by GC and for drugs by GC/MS. Urine and femoral blood samples were also screened for drugs of abuse by immunoassays. Ethanol was detected in 11 cases, cocaine and metabolites in 10 cases, methadone and metabolite in 8 cases, morphine and codeine in 15 cases and diazepam and its metabolites in 8 cases. The distribution and concentration of each of the drugs of abuse was determined in different post-mortem body compartments. Although the study focused mainly on drugs of abuse the other drugs detected at screening by GC/MS included dothiepin (6 cases), mirtazapine (8 cases), loxapine (3 cases), orphenadrine (5 cases), tramadol (3 cases) and paracetamol (23 cases).

3.1: Distribution of ethanol in post-mortem samples:

The ethanol positive cases are described in Table 15. All were male except one female; the mean age was 42 years (range 28-61). All cases were known to be ethanol abusers and histological findings in 5 cases showed signs of chronic ethanol abuse represented by fatty liver and liver cirrhosis. In 5 cases brain and lung oedema was found while in one case (the only female) no pathological findings were reported. The preliminary causes or suspected contributors to the causes of death were intoxication in 7 cases while in 4 cases were pneumonia, drowning, acute respiratory infection and gastrointestinal bleeding.

Table 15: Ethanol abuse cases

Case	Age (year)	Gender	Medical history	Post-mortem findings	The cause or suspected contributor to cause of death
1	30	M	Alcohol abuse	Brain and lung oedema	Pneumonia
2	61	M	Alcohol abuse	Fatty liver	Drowning
3	52	M	Alcohol abuse	Brain and lung oedema	Intoxication
4	47	M	Alcohol abuse	Brain and lung oedema	Intoxication and myocardial infarction
5	28	F	Alcohol abuse	No pathological finding	Intoxication
6	41	M	Alcohol abuse	Fatty liver	Acute infection of the respiratory system
7	42	M	Alcohol abuse	Fatty liver	Intoxication
8	42	M	Alcohol abuse	Brain and lung oedema	Intoxication
9	41	M	Alcohol abuse	Brain and lung oedema	Intoxication
10	42	M	Alcohol abuse	Liver cirrhosis.	Intoxication
11	44	M	Alcohol abuse	Liver cirrhosis	Gastrointestinal bleeding

M = male, F = female

Post-mortem ethanol concentrations in different post-mortem body fluid compartments as well as other associated drugs together with beta-hyroxybutyrate (BHB) and glycosylated haemoglobin (HbA1c) levels are given in Table 16 (a). The presence of alcoholic ketoacidosis was investigated in all ethanol abuse cases by determining BHB levels in the femoral blood. Hb1Ac levels were measured in each case to try and distinguish alcoholic ketoacidosis from diabetic ketoacidosis. The concentrations of the other associated drugs of abuse found in the ethanol positive cases are given in Table 16 (b).

In general, in cases 2, 4, 6, 7, 8, 9 and 10 the femoral blood ethanol concentrations (365, 99, 180, 168, 6, 9 and 20 mg/dL respectively) were comparable to cardiac blood levels (323, 80, 204, 194, 16, 19 and 32mg/dL respectively) [Table 16 (a)]. Where available (in 6 cases), vitreous humour ethanol concentrations were also similar to the cardiac blood levels even for case 6 as the vitreous humour to cardiac blood ethanol ratio in these cases ranged 0.3-1.6 (see page 93). Whilst in cases 1, 3 and 5 the femoral blood ethanol concentrations were relatively low (4, 7, 6mg/dL respectively) as compared to cardiac blood levels (169, 100 and 168mg/dL respectively) the levels in urine and bile, where available, as in cases 1 and 3 the ethanol levels were comparable to those in cardiac blood [Table 16 (a)].

Therefore, the low femoral blood ethanol concentrations in cases 1, 3 and 5 are most likely unreliable. One of the possible explanation discrepant femoral and cardiac blood ethanol levels is evaporation of ethanol from the blood tubes on repeated tube opening during sample analysis.

Table 16 (a): Ethanol concentrations in different fluid body compartments (mg/dL) and associated other drugs.

Case	FB	CB	VH	U	Bile	SC	HbA1c (%)	BHB (mmol/L)	Drug screen by GC/MS
1	4	169	NA	205	130	84	NA	0.36	Methadone and EDDP
2	365	323	346	NA	NA	124	4.4	0.43	Paracetamol
3	7	100	66	373	241	265	NA	0.34	Morphine, codeine, diazepam, nor-diazepam
4	99	80	NA	173	NA	168	NA	1.17	Paracetamol,mirtazapine, amitriptyline
5	6	168	NA	NA	NA	40	NA	0.25	Diazepam and its metabolites
6	180	204	330	300	192	490	6.5	0.91	Diazepam and its metabolites
7	168	194	NA	526	196	227	9.3	0.98	No other drug found
8	6	16	4	920	NA	280	15.7	0.47	Cocaine, methadone and their metabolites
9	9	19	26	77	174	139	8.3	0.19	Cocaine and its metabolites
10	20	32	16	88	65	59	6.2	0.18	Morphine, codeine, Ibuprofen, diazepam and its metabolites
11	217	NA	NA	NA	NA	198	NA	0.14	No other drug found

FB = femoral blood, CB = cardiac blood, VH = vitreous humour, U = urine, SC = stomach contents, NA = not available, BHB = beta-hydroxybutyrate normal reference range <0.5mmol/L, HbA1c = glycosylated haemoglobin and normal reference range 4-6.1%. The blood quoted reference ranges for ethanol are: toxic 100-200mg/dL and lethal >350mg/dL (Weink et al., 2001; Schulz, 2003; Musshoff, 2004). Femoral blood ethanol level >300mg/dL may be associated with fatalities.

Table 16 (b): Femoral blood concentrations for other associated drugs of abuse.

Case	Associated drugs of abuse concentration in femoral blood (ng/mL)
1	Methadone 300
2	-
3	Morphine 86, codeine 20 and diazepam 27
4	-
5	Diazepam 220
6	Diazepam 51
7	-
8	Cocaine 6 and methadone 603
9	Cocaine 2413
10	Morphine 56, codeine 9 and diazepam 219
11	-

**Quoted reference ranges*

(Weink et al., 2001; Schulz and Schmoldt, 2003; Musshoff et al., 2004)

Drug	*Therapeutic (ng/mL)*	*Toxic (ng/mL)*	*Lethal (ng/mL)*
Methadone	*50-750*	*200-2000*	*>400*
Morphine	*10-100*	*50-400*	*>100*
Codeine	*30-250*	*500-1000*	*>1600*
Diazepam	*20-4000*	*3000-5000*	*>30000*
Cocaine	*50-300*	*500-1000*	*>4000*

**These references ranges are for guidance alone and drug interpretation should take in account the patients full clinical and drug history.*

Ethanol concentration may decrease in the collecting tube depending on the duration and temperature of storage conditions, the presence of large amount of air space present in the blood container and escape of ethanol during multiple opening of femoral blood tube container as the sample was used for multiple analysis. The femoral blood samples were used for multiple analyses which would enhance the escape of ethanol as the tubes are opened more frequently. The loss of ethanol has been reported up to 50% at room temperature and up to 20% if the sample is stored at 5 °C when the blood sample is unsealed (Kloppel and Weiler, 1982).

However, there are also other factors which may affect the ethanol concentration in post-mortem blood samples. These factors may cause formation or degradation of ethanol. The formation of ethanol in post-mortem samples by microorganisms requires substrates such as glucose, amino acids, fatty acids and lactate. Ethanol formation was shown to increase with prolonged storage period and the amount of glucose present in the sample (Yajima et al, 2006). Formation of ethanol can be prevented by sample preservation with sodium or potassium fluoride. This will inhibit ethanol formation by many microorganisms including Candida albicans. Ethanol concentration in unpreserved blood samples has been shown to increase in vitro by 150 to 250 mg/dL (Blume and Lakatua, 1973). Contamination by bacteria which utilise ethanol, prior to sample collection, will cause ethanol levels to decrease, even with the subsequent use of preservative to inhibit the bacterial ethanol utilization. This will not reverse the ethanol degradation which occurs prior to the addition of the preservative. In this study, the possibility of post-mortem bacterial contamination was unlikely as the post-mortem interval period was short and all the samples used for ethanol determination had been collected into preservative.

The urine ethanol concentration were within the quoted ratio range to the femoral blood ethanol concentration in most cases except in cases 7 and 8 were the levels were highly elevated (526 and 920mg/dL respectively) further explanation is given. Bile ethanol concentrations were above the cardiac blood levels in three cases (3, 9 and 10) and similar to the cardiac blood in two cases (6 and 7). An explanation for bile ethanol variation is given. Stomach contents ethanol concentration range was 40-490mg/dL.

HbA1c level is normally 4.0-6.2% and the level is elevated in cases of diabetes and glucose intolerance. HbA1c levels were elevated in cases 6, 7, 8 and 9 (6.5, 9.3, 15.7 and 8.3% respectively) and within the borderline for alcoholic ketoacidosis level in case 10 (6.2%). These results suggesting that the cases (6, 7, 8 and 9) were diabetics or glucose intolerant in case 10. In cases 6 and 7 the HbA1c was also elevated but the urine to femoral blood ethanol ratios were within the quoted post absorptive range for non diabetics. It is possible that in some diabetic patients in this study the urine to femoral or cardiac blood ethanol ratios may have been influenced by the prevailing ethanol absorptive phase, glucosuria, bacteriuria and the a typical re-distribution of ethanol in the postmortem period. No sufficient sample was available for measuring HbA1c levels in cases 1, 3, 4, 5 and 11.

The normal level of blood BHB is < 0.5mmol/l whilst this level ranged from 1.26 to 47.2mmol/l (Iten and Meier, 2000). However, alcoholic ketoacidosis should be considered in cases where the deceased has a history of alcoholism, and post-mortem investigations show little or absent alcohol, elevated beta-hydroxybutyrate and

acetone, and neither autopsy, histology, microbiology nor toxicology reveal the cause of death (Iten and Meier, 2000; Karch, 2006). BHB levels were borderline in case 8 (0.47mmol/l) and elevated in three cases 4, 6 and 7 (1.17, 0.91 and 0.98% respectively) [Table 16 (a)]. Usually BHB is elevated in starvation, diabetic and alcoholic cases (Denmark, 1993; Garriot, 2003). Ketone bodies, acetone and BHB are sometimes measured in post-mortem blood in order to determine ketoacidosis as the cause or contributing to the cause of death. Acetone may be formed in the post-mortem period but BHB is unlikely to result from post-mortem changes and thus is useful for determining ketoacidosis (Iten and Meier, 2000). In a study by Thomson et al 1995, it was reported that alcoholic ketoacidosis should be suspected when BHB level is > 0.53mmol/l (Thomson et al., 1995) and in another study ketoacidosis was suspected to cause or contribute to the cause of death when BHB is > 1.0mmol/l (Kenetake et al., 2005). Diabetic ketoacidosis was suspected to be the cause of death when BHB level was equal to or above 3.5mmol/l (Sheikh-Ali et al., 2008). In the above three cases (4, 6 and 7) the BHB was marginally elevated and case 6 and 7 were diabetic and in case 4 the HbA1c was not available. In these cases where HbA1c is elevated it may not be possible to distinguish alcoholic ketoacidosis from diabetic ketoacidosis.

The ratios to femoral and cardiac blood ethanol concentration in different fluids are given in Table17 (a) and (b). As femoral blood ethanol in cases 1, 3 and 5 may be unreliable the ethanol concentration ratios were expressed in relation to cardiac blood.

Table 17: Ratios of ethanol concentration in different fluids to femoral and cardiac blood with median and ratio ranges.

(a) Ratio to FB					
Case	**CB/FB**	**VH/FB**	**U/FB**	**Bile/FB**	**SC/FB**
1	*(42.3)	NA	*(51)	*(32)	*(21)
2	0.9	0.9	NA	NA	0.3
3	*(14.3)	*(9.4)	*(53)	*(34)	*(37.9)
4	0.8	NA	1.7	NA	1.7
5	*(28)	NA	NA	NA	*(6.7)
6	1.1	1.8	1.7	1.0	2.7
7	1.2	NA	3.1	1.2	1.3
8	2.7	0.7	*(153)	NA	46.7
9	2.1	2.9	*(8.6)	19.3	15.4
10	1.6	0.8	*(4.4)	3.3	2.9
11	NA	NA	NA	NA	0.9
***Median**	1.2	0.9	1.7	2.3	2.2
Ratio range	0.8-2.7	0.7-2.9	1.7-3.1	1.0-19.3	0.3-46.7
(b) Ratio to CB					
Case	**VH/CB**	**U/CB**	**Bile/CB**	**SC/CB**	
1	NA	1.2	0.8	0.49	
2	1.1	NA	NA	0.4	
3	0.7	3.7	2.4	2.6	
4	NA	2.2	NA	2.1	
5	NA	NA	NA	0.2	
6	1.6	1.5	0.9	2.4	
7	NA	2.7	1.0	1.2	
8	0.3	57.5	NA	17.5	
9	1.4	4.1	9.2	7.3	
10	0.5	2.7	2.0	1.8	
11	NA	NA	NA	NA	
Median	0.9	2.7	1.5	1.9	
Ratio range	0.3-1.6	1.2-57.5	0.8-9.2	0.2-17.5	

NA = Not available, FB = femoral blood, CB = cardiac blood, VH = vitreous humour, U = urine and SC = stomach contents. *Median: obtained after ignoring the unreliable femoral blood ethanol results and diabetic and glucose intolerance urine ethanol results.

The cardiac to femoral blood ethanol concentration ratios ranged from 0.8 to 2.7. Variations in cardiac to femoral blood ethanol concentration have been studied in post-mortem cases. It has been reported that cardiac blood ethanol levels can be more than 400% compared to the femoral blood (Briglia et al., 1992; Pounder and Smith, 1995). However, in a study of 100 post-mortem cases the cardiac to femoral blood ethanol ratio was near to unity (0.98) in the vitreous humour (Prouty and Anderson, 1987). Some studies have attributed the elevation of ethanol in the cardiac blood as being due to diffusion from stomach or from lungs contaminated with vomit from stomach contents (Chikasue et al., 1988; Pounder and Yonemitsu 1991; Pounder and Smith, 1995; Iwasaki et al., 1998). A previous study suggested that diffusion from stomach to the cardiac blood occurs when ethanol in the stomach is > 500mg/dL (Briglia et al., 1991). In this study all stomach contents ethanol levels were below 300mg/dL except in case 6 were ethanol level was 490mg/dL.

Vitreous humour samples were provided in six cases. Vitreous humour is a preferable sample for ethanol detection in post-mortem cases because it is easily obtained and there is less chance for contamination by bacteria unlike most other post-mortem samples (O'Neal and Polkis, 1996; Garriott, 2003). In a moderately decomposed body few bacteria were detected in the vitreous humour which indicates that any ethanol found in vitreous humour is more likely to have resulted from ethanol administration prior to death rather than due to bacterial action in the post-mortem period (Zumwalt et al., 1982). If ethanol is detected in the blood sample and not in the corresponding vitreous humour this is most likely to indicate post-mortem ethanol production rather than its ante-mortem administration (Caplan and Levin, 1990).

In case 3 the vitreous to femoral blood ethanol ratio was probably unreliable for the reason given earlier and no vitreous humour samples were available for cases 1 and 5. In the remaining cases the vitreous humour to femoral blood ethanol concentration ratios ranged from 0.7 to 2.9 whereas the vitreous to cardiac blood ethanol concentration ratios ranged from 0.3 to 1.6.

Several published studies, considering the analysis of 1047 post-mortem samples gave vitreous humour: femoral blood ethanol ratios ranging from 0.86:1 - 2.20:1, with an overall mean value from these studies of 1.08:1 (Backer et al., 1980; Honey et al., 2005; Jones and Holmgren, 2003; Sylvester et al., 1998). Therefore, the ratios of vitreous humour to femoral and cardiac blood in this thesis are similar to those observed in other studies

The vitreous humour/femoral blood ethanol concentration ratio can be used to estimate the ante-mortem ethanol concentration when blood samples are unavailable. However, this ratio cannot be applied to all cases as each case has different circumstances. For example, it was found that the vitreous humour/cardiac blood ethanol concentration ratio was 0.28 in a victim of a motor vehicle crash which was explained by the diffusion of ethanol from the stomach to the cardiac blood, contamination of blood sample prior to specimen collection and ingestion of large amount of ethanol prior to death (Hardin, 2002). It was suggested that vitreous humour ethanol concentration may be more representative of the level in the femoral blood if the death occurred at least two hours after ethanol consumption and when

stomach contents ethanol concentration is likely to be less than 500mg/dL as discussed earlier (Garriott, 2003).

In case 2 the suspected cause of death was drowning but the vitreous humour to femoral blood ethanol ratio was 0.9 which suggest that the body was probably removed from water relatively shortly after drowning. Drowning cases may have low vitreous humour/blood ethanol concentration ratio as ethanol may diffuse from the vitreous humour to the water resulting in a decreased level. The rate of this diffusion may depend on the time of the body exposure to water. In two drowning cases where the bodies were in water for 2-4 weeks the cardiac blood ethanol concentrations were 260 and 280mg/dL and the vitreous humour to cardiac blood ethanol concentration ratios were 0.19 and 0.28 respectively which is much lower than in case 2 (Singer et al, 2007).

Urine samples were provided in 8 cases of suspected ethanol intoxication. Similar to vitreous humour the urine samples especially when collected with preservative, are also preferred to other post-mortem samples in ethanol interpretation because ethanol formation in urine is thought to be rare except in some cases with glycosuria (e.g. in cases of uncontrolled diabetes mellitus). In general, presence of ethanol in post-mortem urine is usually an indication of its pre-mortem administration (Levine et al., 1993; Zumwalt et al., 1998; Athanaselis, 2005). A study of urine samples from two rape victims who were diabetic and did not drink alcohol showed presence of ethanol in urine with levels of 82 and 102 mg/dL. Glycosuria of 1 % w/v (56mmol/L) may produce urine ethanol concentration of 500 mg/dL (Jones et al., 1999; Jones et al., 2000).

The urine to femoral blood ethanol concentration ratios may have been unreliable in cases 1 and 3 for the reason given earlier and there was no urine sample provided for case 5. In cases 4, 6 and 7 the urine to femoral blood ethanol ratio ranged from 1.7 to 3.1 and the ratios in urine to cardiac blood ranged from 1.5 to 2.7. Urine to femoral blood ethanol concentration ratio can be divided into two phases: an absorptive phase and post absorptive phase. It was found that in the absorptive phase the ratios of urine to femoral blood ethanol concentration ranged <0.1->1.3:1 whilst in the post absorptive phase this ratio is usually >1.2:1 but <3.1:1 (Jones, 1992; Jones and Holmgren, 2003; Garriott, 2003).

In cases 8, 9 and 10 the urine to femoral blood ethanol concentration ratios ranged from 4.4 to 153 [Table 17 (a)] and the urine to cardiac blood ethanol concentration ratios ranged from 2.7 to 57.5 [Table 17 (b)]. In cases 8, 9 and 10 the ratios were much higher than the rest. The urine to femoral blood ethanol ratio may be misleading when obtained from poorly controlled diabetics. Urine samples from some diabetic patients may contain glucose these patients are more prone to urinary tract infection. These circumstances may result in the production and elevation of urine ethanol in poorly controlled diabetic patients who may not have consumed ethanol.

Bile samples were provided in six ethanol cases. Bile is stored in the gallbladder and is not normally used for the estimation of blood ethanol in post-mortem cases when blood is not available or not suitable for analysis. However, bile is thought to be protected from bacterial contamination in severe trauma compared to other body

fluids. It can be used as an alternative sample to femoral blood or vitreous humour for ethanol determination (Karch, 2007). The presence of ethanol in the bile in addition to stomach and the urine may indicate pre-mortem ethanol administration (Athanaselis, 2005). It was found that the presence of high concentrations of ethanol in the femoral blood and it's absence in vitreous humour, urine and bile is an indication of its post-mortem production and not for ethanol consumption prior to death (Kugelberg and Jones, 2007). Bile could be used as an alternative to vitreous humour and femoral blood in determining blood ethanol level in post-mortem cases (Stone and Rooney, 1984).

The ratios of bile to cardiac blood ethanol concentration ratios ranged from 0.8 to 2.4 in five cases and 9.2 in one of the diabetic cases (case 9). The ratio of bile to femoral blood ethanol concentration ratio ranged from 1.0-19.3. Many authors studied the bile to femoral ethanol concentration ratio in post-mortem cases. Bile to femoral blood ethanol concentration mean ratio was 0.99:1 and ranged from 0.48 to 2.04 in 89 post-mortem cases where femoral blood ethanol concentration ranged from 46 to 697mg/dL (Stone and Rooney, 1984). In another study the mean blood ethanol (origin not mentioned)/bile ethanol concentration ratio was found 1.22:1 in 115 post-mortem cases (Kass, 2006). The ratio was similar in one case of drowning (Pélissier-Alicot et al., 2005). It was suggested that the lipid content of bile my lead to variation in its ethanol concentration as an increase in lipid content compared to water content will lead to lower ethanol level in bile (Winek et al., 1983). In this study no determination of bile lipid contents was investigated.

The stomach contents were provided for all ethanol cases. As stated earlier the amount of ethanol present in the stomach varies according to the phase of absorption of ethanol, peri or post-absorptive. It has been shown that most of administered ethanol is absorbed from the stomach within two hours of administration while in some cases this occurs after one hour as mentioned in Chapter One. Ethanol absorption from the stomach is affected by the presence of food in the stomach (Jones and Jonsson, 1994). It is also known that due to bacterial metabolism ethanol may be formed in the stomach (Karch, 2006). As stated earlier that possibility of post-mortem bacterial contamination is excluded in this study due to the short post-mortem interval. However, in general, the presence of ethanol in the stomach usually indicates its administration prior to death (Petkovic, 2005; Kugelberg, 2007).

The cardiac blood ethanol concentrations were elevated in 7 cases (1-7) and in femoral blood sample for cases 2 and 11 [Table 16 (a)]. It is well known that LD_{50} for ethanol is 450mg/dL while levels > 300mg/dL may be associated with fatalities (Drummer, 2001; Koski et al., 2002; Dubowski, 2006). In a study of 693 acute ethanol poisoning cases, the mean ethanol concentration was 360mg/dL and ranged from 74 to 680mg/dL while for chronic alcohol poisoning cases (825 cases) the mean ethanol concentration was 172mg/dL and ranged from 10 to 560mg/dL (Jones and Holmgren, 2003). In 35% of traffic fatalities in United States where ethanol is involved the blood ethanol concentration was found to be equal to or above 80mg/dL (NHTSA, 2000). There is no clear evidence at what blood level ethanol intoxication occurs, but intoxication symptoms become clear at blood ethanol concentrations of >100mg/dL (Drummer, 2001).

As mentioned in Chapter One, ethanol intoxication describes the state where physical and mental ability is impaired due to excessive consumption of ethanol which may vary in different individuals depending on age, weight, sex, previous exposure to ethanol, individual parameters of ethanol absorption, distribution and elimination. Because of individual variations in responses to ethanol some authors have questioned whether a fixed BAC is a valid measure of impairment due to ethanol (Andreasson, 1996). In this study the blood ethanol concentration was >100mg/dL in five cardiac and two femoral blood samples. The cardiac blood ethanol level ranged from 80 to 323mg/dL in 7 cases (1-7) while the femoral blood levels in cases 2 and 11 were 365 and 217mg/dL respectively.

The interactions between ethanol and many drugs are complex, and the likelihood of significant side-effects or toxicity may be enhanced. Ethanol is known to interact with a number of drugs to enhance their toxic effects, which include opioids, cocaine and diazepam. The mechanism of interaction between CNS depressant drugs may be competitive, additive or synergistic (Schoener, 1986).

Femoral blood ethanol concentrations for other drugs of abuse were given in Table 16 (b). Methadone was measured in the femoral blood in cases 1 and 8 with levels of 300 and 603ng/mL respectively. There is a lack of studies in humans on the interaction of ethanol and methadone. A study in rats found that excessive administration of ethanol will inhibit the N-demethylation of methadone to from its inactive product EDDP and this will increase the blood level of methadone and enhance its pharmacological effect (Borowsky and Lieber, 1978). A recent study on opioid-treated patients (methadone, levo-α-acetylmethadol and buprenorphine) found

that opioid administration reduces the blood ethanol concentration compared to non-opioid treated control patients. This was suggested to be due to the delay in gastric emptying due to opioids which slows the absorption of ethanol and reduces its blood level (Clark et al., 2006).

There is overlap between the methadone therapeutic (75–1100ng/mL), toxic (200-2000ng/mL) and lethal range (400-1800ng/mL). Methadone may cause respiratory depression even at therapeutic level and the combination of ethanol and methadone (both are CNS suppressant drugs) will enhance the ethanol toxic effect on respiration and death may occur. Studies of opioid drug-related deaths in combination with ethanol showed a decrease in the minimum fatal concentration of opioids up to 50 % when ethanol concentration was > 200mg/dL, whilst another study suggested that when ethanol >100mg/dL toxicity of opioids may be enhanced (Ruttenber et al., 1990; Drummer, 2001). Other study found that small amount of ethanol may enhance the opioids toxicity and this risk increase when blood ethanol concentration is >300mg/dL (Levine et al., 1995).

The cardiac blood ethanol in case 1 was 169mg/dL and femoral blood methadone was 300ng/mL. As mentioned above, opioid toxicity is enhanced when blood ethanol level >100mg/dL. It is well known that methadone toxicity leads to fluid accumulation in the lung and may cause pneumonia (Allan and Roberts, 2009). This may apply in case 3 where cardiac blood ethanol was 100mg/dL and femoral blood morphine was 86ng/mL. In case 4 the femoral blood ethanol was 99mg/dL and no other drugs of abuse were found. The cause or suspected contributor to cause of death in this case was intoxication and myocardial infarction.

In case 8 femoral blood ethanol level was 6mg/dL, blood cocaine level was 6ng/mL while methadone level was 603ng/mL which may explain the presence of lung oedema due to methadone effect as mentioned above. In case 9 the femoral blood ethanol was 9mg/dL but cocaine level was above the quoted therapeutic range (50-300ng/mL) 2413ng/mL. It was reported that stimulants similar to cocaine can cause lung congestion and oedema and death may be occur (Kostakis and Byard, 2009). It was reported that co-administration of cocaine and ethanol is associated with a high fatality rate due to cardiovascular complications, hepatotoxicity, and extreme agitation behaviours and this dangerous co-administration increases the risk of sudden death by 25 fold (Burnett, 2006).

Morphine was detected in the femoral blood in case 10 with femoral blood concentration 56ng/mL and femoral blood ethanol 20mg/dL. Similar to methadone there is overlap between the therapeutic (10-100ng/dL) and toxic (50-4000ng/mL) morphine concentration. Morphine co-administration with ethanol will enhance the toxic effect on respiration with different ethanol concentrations as mentioned above and death may occur (Ruttenber et al., 1990; Levine et al., 1995). Blood ethanol concentrations of 100mg/dL may lead to an increased risk of morphine fatality by 22 fold (Ruttenber, 1984; Ruttenber et al., 1990). However, femoral blood ethanol (20mg/dL) in this case is unlikely to produce any toxic effect on respiration.

Codeine is a mild analgesic drug and was detected in two cases, 3 and 10, within the therapeutic range (30 - 340ng/mL). Diazepam, a benzodiazepine usually used for

anxiety and insomnia treatment, was detected in therapeutic level (20 - 4000ng/mL) in four cases 3, 5, 6 and 10 [Table 16 (b)].

When excluding the unreliable femoral blood ethanol results in cases 1, 3 and 5 the correlation analysis showed a significant correlation between cardiac blood and femoral blood ethanol with P value < 0.001 and Pearson correlation coefficient (r) = 0.98. There was a significant correlation between vitreous humour and femoral blood ethanol concentrations with P value = 0.03 and Pearson correlation coefficient (r) = 0.92. When excluding the urine ethanol concentration results for cases 7 and 8 (diabetic cases) the results showed a significant correlation between urine and femoral blood ethanol concentrations with P value = 0.005 and Pearson correlation coefficient (r) = 0.99 (Table 18).

From all cases studied it was found that interpretation of ethanol concentration in post-mortem femoral blood may be very difficult as many factors may change its level. The presence of more than one sample from different body compartments may clarify the interpretation of results. From this study the ratios of vitreous humour and urine ethanol to cardiac blood ethanol were near to those obtained in the previous studies but urine ethanol concentration to cardiac blood ethanol concentration ratios showed elevation in diabetic and glucose intolerance cases.

Table 18: Correlations to the femoral blood ethanol concentrations and levels in different post-mortem samples

	Correlations to femoral blood ethanol levels	
Cardiac blood	Pearson Correlation (r)	0.98**
	P value	<0.001
Vitreous humour	Pearson Correlation (r)	0.92*
	P value	0.03
Urine	Pearson Correlation (r)	0.99**
	P value	0.005
Bile	Pearson Correlation (r)	0.66
	P value	0.3
Stomach contents	Pearson Correlation (r)	0.09
	P value	0.82

P value is significant <0.05

3.1.1 Summary of ethanol results

In three cases, the femoral blood ethanol concentrations were much lower compared with levels in other body compartments. This was likely to have been due to post-mortem ethanol formation and redistribution/diffusion but may also have occurred due to evaporation of ethanol from the femoral blood tube. In the remaining cases the ethanol concentrations ratios to femoral blood ranged: cardiac blood 0.8-2.7, vitreous humour 0.7-2.9, bile 1.0-3.3 and urine 1.7-3.1. Ethanol was below detectable limit in both liver and muscle tissues.

3.2: Distribution of cocaine and its metabolites in post-mortem samples:

The ten cocaine positive cases are described in Table 19. All were male except one female; the mean age was 41 years (range 38-47). All the cases were known to be drug abusers and histological findings showed brain and lung oedema (7 cases one of which also had endocarditis), fatty liver (1), gastrointestinal haemorrhage (1) and pneumonia (1). The causes or suspected contributors to the causes of death were drug toxicity (7), pneumonia (2) and exsanguinations (1).

The major cocaine metabolites are benzoylecgonine and methylecgonine which are inactive and the active metabolite cocaethylene (Bencharit et al., 2003; Harris et al., 2003; Burnett, 2006). The elimination half-life of cocaine is 0.6-4 hours, methylecgonine 3.5-6 hours and for benzoylecgonine is 5-8 hours. The concentrations of post-mortem cocaine and its metabolites in different post-mortem body fluids compartments are given in Table 20 (a). Femoral blood concentrations for other associated drugs of abuse found in these cases are given in Table 20 (b).

The femoral blood cocaine levels were above the quoted therapeutic range (50-300ng/mL) in cases 9, 13 and 19 with concentrations of 2413, 1200 and 420ng/mL respectively. The remaining cases had femoral blood cocaine levels <50ng/mL (ranged from 5-28ng/mL). The blood cocaine level >1000ng/mL may be associated with acute fatality (Moffat et al., 2004). The cause of death in such cases is usually due to arrhythmias and cardiac arrest (Chakko and Myerburg, 1995; Karila et al., 2009; Phillip et al., 2009). However, toxicity due to cocaine use is not necessarily related to blood cocaine level (Bertol et al., 2008). Cocaine use may induce

Table 19: Cocaine abuse cases

Case	Age (year)	Gender	Medical history	Post-mortem findings	The cause or suspected contributor to cause of death
8	42	M	Drug abuser	Brain and lung oedema	Drug toxicity
9	41	M	Drug abuser	Brain and lung oedema	Drug toxicity
12	43	M	Drug abuser	Brain oedema and endocarditis	Pneumonia
13	41	M	Drug abuser	Fatty liver	Exsanguinations
14	40	M	Drug abuser	Brain and lung oedema	Drug toxicity
15	39	F	Drug abuser	Pneumonia, enlargement of liver and spleen	Pneumonia
16	43	M	Drug abuser	Brain and lung oedema	Drug toxicity
17	41	M	Drug abuser	Gastrointestinal haemorrhagic, spleen rupture	Drug toxicity
18	47	M	Drug abuser	Brain and lung oedema	Drug toxicity
19	38	M	Drug abuser	Brain and lung oedema	Drug toxicity

M = male and F = female

Table 20 (a): Concentrations of cocaine and its metabolites in different fluid body compartments (ng/mL).

Case	Cocaine /metabolites	FB	CB	VH	S	Bile	U
8	Coca	6	42	20	NA	106	47
	ME	9	15	11	NA	125	378
	BE	155	135	127	NA	915	1180
	CE	6	14	20	NA	113	46
9	Coca	2413	3905	>5000	>5000	>5000	>5000
	ME	4033	>5000	4287	>5000	>5000	>5000
	BE	4445	>5000	3578	>5000	>5000	>5000
	CE	253	197	274	2740	956	>5000
12	Coca	28	56	70	2005	694	1295
	ME	390	450	97	>5000	>5000	1854
	BE	1462	3042	3716	>5000	>5000	>5000
	CE	<5	<5	<5	<5	<5	<5
13	Coca	1200	1203	NA	NA	1209	NA
	ME	769	985	NA	NA	1895	NA
	BE	2760	1941	NA	NA	2925	NA
	CE	<5	<5	NA	NA	<5	NA
14	Coca	5	5	5	NA	5	>5000
	ME	78	551	43	NA	45	3575
	BE	5	11	39	NA	24	>5000
	CE	<5	<5	<5	NA	<5	<5
15	Coca	14	25	17	103	9	6
	ME	5	5	5	30	417	145
	BE	98	170	805	593	1590	741
	CE	<5	<5	<5	<5	<5	<5
16	Coca	5	5	5	5	NA	>5000
	ME	135	82	30	66	NA	>5000
	BE	35	9	15	5	NA	>5000
	CE	<5	<5	<5	<5	NA	<5
17	Coca	5	5	5	5	5	5
	ME	20	192	35	995	66	145
	BE	27	48	140	236	103	741
	CE	<5	<5	<5	<5	<5	<5
18	Coca	5	6	8	244	208	NA
	ME	5	6	5	30	417	NA
	BE	5	6	8	593	1590	NA
	CE	<5	<5	<5	<5	<5	NA
19	Coca	420	NA	600	NA	>5000	>5000
	ME	389	NA	430	NA	1952	>5000
	BE	600	NA	1018	NA	2701	>5000
	CE	<5	NA	<5	NA	<5	<5

The blood quoted reference ranges for cocaine are: therapeutic 50-300ng/mL, toxic 500-1000ng/mL and lethal 4000ng/mL (Weink et al., 2001; Schulz, 2003; Musshoff, 2004). Coca = cocaine, ME = methylecgonine, BE = benzoylecgonine, CE = cocaethylene, FB = femoral blood, CB = cardiac blood, VH = vitreous humour, S = stomach contents and U = urine.

Table 20 (b): Femoral blood concentrations for other associated drugs of abuse

Case	Associated drugs of abuse concentration (ng/mL) in femoral blood
8	Methadone 603
9	No other drugs found
12	Methadone 520
13	Methadone 7
14	Methadone 1266
15	Methadone 366
16	Methadone 18, morphine 48 and diazepam 219
17	Methadone 1795
18	No other drugs found
19	No other drugs found

**Quoted reference ranges*

(Weink et al., 2001; Schulz and Schmoldt, 2003; Musshoff et al., 2004)

Drug	*Therapeutic (ng/mL)*	*Toxic (ng/mL)*	*Lethal (ng/mL)*
Methadone	*50-750*	*200-2000*	*>400*
Morphine	*10-100*	*50-400*	*>100*
Diazepam	*20-4000*	*3000-5000*	*>30000*

**These references ranges are for guidance alone and drug interpretation should take in account the patients full clinical and drug history.*

hyperthermia which may lead to dehydration, rhabdomyolysis and ultimately renal failure due to accumulation of myoglobin in kidney tubules (Lombard et al., 1988). Cocaine use may also lead to hypertension which may cause stroke, aortic dissection and acute pulmonary oedema (Egred and Davis, 2005; Singh et al., 2007; Daniel et al., 2007; Pollak and Rees, 2008).

In the remaining cases the femoral blood cocaine concentration was <50ng/mL. There is some evidence that blood cocaine levels less 50ng/mL are unlikely to produce symptomatic toxicity (Vinci et al., 1999). There is also evidence that cocaine may have contributed to the cause of death even if its concentration in blood is zero. In these situations cocaine use has been associated with myocardial infarction, which has been reported in young cocaine abusers (Karch, 2008; Wood and Vega, 2008).

A study of 111 cocaine abusers who were admitted to emergency departments (ED) found that the mean blood cocaine level was 260± 500ng/mL. From all cases two patients died (one from excited delirium and the second from brain stem haemorrhage) with mean blood cocaine level 200±250ng/mL. The majority of cases (88 with mean blood cocaine 290±500ng/mL) were discharged from the ED (Blaho et al., 2000).

Several studies mentioned the blood cocaine levels in cocaine related deaths. In one study, the data collected from 140 cocaine related deaths showed that the median femoral blood cocaine concentration was 100ng/mL and ranged from 10-800ng/mL (Darke et al., 2005). Blood cocaine concentrations were reported for 11 cases with

excited delirium who died in police custody. In one case only a trace amount of cocaine was detected in blood whilst in the remaining 10 cases the blood cocaine levels ranged from 10-6940ng/mL (DiMaio and DiMaio, 2005). Other case reports of individuals with hyperthermia and excited delirium showed the blood cocaine levels to range between 260-400ng/mL (Hime et al., 1991; Blaho et al., 2000). Therefore, there is no minimum blood cocaine level which can be considered to be safe.

In some reported cases the blood cocaine levels may be extremely high. Blood cocaine was reported for different body packer's cases (individuals who are ingesting multiple packets of drugs of abuse for smuggling purposes) with concentrations of 4000ng/mL (Furnari et al., 2002), 98100ng/mL (Fineschi et al., 2002) and 104000ng/mL (Patel, 1996). Even in one case who was thought to be a recreational user of cocaine, the blood cocaine level was 330000ng/mL, which was explained as massive administration of cocaine (Peretti et al., 1990).

Furthermore, due to drug tolerance a blood cocaine level alone without full drug and clinical history should ideally not be used to explain the cause of death (Bertol et al., 2008). Chronic users may be requiring large doses of cocaine to achieve the same pharmacological effect (Drummer, 2001). It has been reported that LD_{50} for cocaine ranged from 1.2-1.5g and doses of 0.15g and above can kill naïve individuals (Cohen, 1985; Rawson and Urban, 1999) whilst a chronic cocaine user can tolerate 3-10g of cocaine per day (Claustre et al., 1993; Schuckit, 1999).

In many situations cocaine is abused in combination with other drugs. These commonly include ethanol, opioids and cannabis (Cohen, 1989). Cocaine

administration in combination with methadone, heroin and alcohol has been rising in European countries and heroin related deaths involving cocaine increased from 1.9% to 5.4% in the period 1992-2002 (Polettini et al., 2005). In methadone related death cases cocaine and its metabolites were present in almost 50% of all cases (Karch and Stephens, 2000).

Methadone was detected in 7 cases [Table 20 (b)] with femoral blood methadone concentrations ranging from 7-1795ng/mL. In addition to cocaine and methadone, morphine and diazepam were also detected in case 16. Cocaine and heroin are often used together and named as "speedball" (Cornish et al., 2005).

When methadone is administered with cocaine, toxicity due to cocaine is enhanced (Preston et al, 1996). The interaction between cocaine and methadone is very complex and it is not clear weather methadone enhances or inhibits cocaine toxicity. It has been suggested that cocaine increases the elimination rate of methadone resulting in decreased blood methadone concentration (Moolchan et al., 2001). This may suggest that individuals under methadone therapy, who also use cocaine, may administer higher doses of methadone to obtain the required pharmacological response and in such cases this leads to methadone overdose. It is interesting that a previous study on monkeys concluded that combination use of cocaine-heroin reinforces the effects of both drugs compared to individual administration (Negus et al., 1998). Similar to methadone the cocaine–heroin interaction is complex and it is not clear which drug enhances the toxicity for the other.

The cocaine levels in femoral blood, cardiac blood and vitreous humour were relatively similar in magnitude [Table 20 (a)]. The median concentrations ratio of vitreous humour to femoral blood cocaine was 1.4:1 (range 1.0:1-3.3:1) whilst for ME and BE were 1.0:1 (range 0.2:1-1.8:1) and 1.7:1 (range 0.4:1 to 8.2:1) respectively (Table 21).

A study of 62 post-mortem cases found that mean vitreous humour to blood cocaine and BE concentration ratios were 1.3:1 and 0.5:1 respectively (Mackey-Bojack et al., 2000). Another study of 28 post-mortem cases found that the mean concentration ratio of vitreous humour to blood cocaine was 1.61:1 and ranged from 0.1:1-2.6:1 (Logan and Stafford, 1990). This ratio was found to be 0.44:1, 1.3:1 and 1.8:1 in three different cases respectively (Sturner and Garriott, 1975; Polkis et al., 1985; Furnari et al., 2002). The site of the blood sampling is not always defined in these other studies.

The cocaine level in brain is thought to have influence on vitreous humour to femoral blood cocaine concentration ratios. The brain contains a high concentration of cocaine due to the presence of a large number of cocaine receptors (Calligaro and Eldefrawi, 1987). Cocaine diffuses out of the brain into the vitreous humour and thus its concentration may rise in the vitreous humour in the post-mortem period (Spiehler and Reed, 1985; Moriya and Hashimoto, 1996). Another possible explanation why cocaine concentration may be higher in the vitreous humour than blood is that cocaine continues to be hydrolysed in blood in the post-mortem period due to the presence of hydrolytic enzymes. This process is highly unlikely to occur

Table 21: Concentration ratios of cocaine and its metabolites in different body fluid compartments to the femoral blood levels (ng/ml)

Case	Cocaine /metabolites	FB (ng/ml)	VH/FB	CB/FB	S/FB	Bile/FB	U/FB
8	Coca	6	3.3	7.0	NA	17.7	7.8
	ME	9	1.2	1.7	NA	13.9	42.0
	BE	155	0.8	0.9	NA	5.9	7.6
	CE	6	3.3	2.3	NA	18.8	7.7
9	Coca	2413	>2.1	1.6	>2.1	>2.1	>2.1
	ME	4033	1.1	>1.2	>1.2	>1.2	>1.2
	BE	4445	0.8	>1.1	>1.1	>1.1	>1.1
	CE	253	1.1	0.8	10.8	3.8	>19.8
12	Coca	28	2.5	2.0	71.6	24.8	46.3
	ME	390	0.2	1.2	>12.8	>12.8	4.8
	BE	1462	2.5	2.1	>3.4	>3.4	>3.4
	CE	<5	<1	<1	<1	<1	<1
13	Coca	1200	NA	1.0	NA	1.0	NA
	ME	769	NA	1.3	NA	2.5	NA
	BE	2760	NA	0.7	NA	1.1	NA
	CE	<5	NA	<1	NA	<1	NA
14	Coca	5	1.0	1.0	NA	1.0	>1000
	ME	78	0.6	7.1	NA	0.6	45.8
	BE	5	7.8	2.2	NA	4.8	>1000
	CE	<5	<1	<1	NA	<1	<1
15	Coca	14	1.2	1.8	7.4	0.6	0.4
	ME	5	1	1	6	83.4	29
	BE	98	8.2	1.7	6.1	16.2	7.6
	CE	<5	<1	<1	<1	<1	<1
16	Coca	5	1.0	1.0	1.0	NA	>1000
	ME	135	0.2	0.6	0.5	NA	>37.0
	BE	35	0.4	0.3	0.1	NA	>142.8
	CE	<5	<1	<1	<1	NA	<1
17	Coca	5	1.0	1.0	1.0	1.0	1.0
	ME	20	1.8	9.6	49.8	3.3	7.3
	BE	27	5.2	1.8	8.7	3.8	27.4
	CE	<5	<1	<1	<1	<1	<1
18	Coca	5	1.6	1.2	48.8	41.6	NA
	ME	5	1.0	1.2	6.0	83.4	NA
	BE	5	1.6	1.2	118.6	318	NA
	CE	<5	<1	<1	<1	<1	NA
19	Coca	420	1.4	NA	NA	>11.9	>11.9
	ME	389	1.1	NA	NA	5.0	>12.8
	BE	600	1.7	NA	NA	4.5	>8.3
	CE	<5	<1	NA	NA	<1	<1

Coca = cocaine, ME = methylecgonine, BE = benzoylecgonine, CE = cocaethylene, FB = femoral blood, CB = cardiac blood, VH = vitreous humour, S = stomach contents and U = urine

or is severely limited in vitreous humour (Logan and Peterson, 1994; McKinney et al., 1995).

The median concentration ratio of cardiac to femoral blood cocaine was 1.2:1 (range 1.0:1-2.0:1 except for case 8 where the ratio was 7.0:1). The concentration ratio for BE was 1.2:1 (range 0.6:1-1.7:1 except for cases 14 and 17 were the ratios were 7.1:1 and 9.6:1 respectively) (Table 21).

In a previous study the cardiac to femoral blood cocaine concentration ratios ranged from 1.5:1 to 2.3:1 (Leikin and Watson, 2003). This result is in agreement with the finding in this study.

The median stomach to femoral blood cocaine concentration ratios varied greatly (range 1.0:1-71.6:1) (Table 21). This may be due to different routes of cocaine administration: intranasal, intravenous, smoking or oral route (Cone, 1995). In addition, it has been reported that a finding of a high cocaine concentration in stomach does not necessarily indicate an oral overdose of cocaine and conversely absence of cocaine in stomach does not exclude oral ingestion as death may occur long after cocaine overdose (Jenkins, 2008).

The median bile to femoral blood for cocaine concentration ratio was 2.1:1 (range 0.6:1-41.6:1), for BE 4.5:1 (range 1.1:1-318:1) and for ME was 5.0:1 (range 0.6:1-83.4:1) (Table 21). It can be seen that these concentration ratios also varied greatly. Bile usually contains higher concentrations of cocaine and its metabolites as compared to blood levels (Agrawal and Lemos, 1996). The variations in these ratios

may also be due to different routes of cocaine administration as orally administered drugs are susceptible to first-pass metabolism by the liver which will increase the concentration of cocaine in bile (Karch, 2007).

The median urine to femoral blood cocaine concentration ratio was 9.8:1 (range 0.4->1000:1), BE 7.9:1 (range 1.1:1->1000:1) and for ME was 20.9:1 (range 1.2:1-45.8:1) (Table 21). Except for cases 14 and 16 where concentration ratios were greater than 1000:1, the ratios in the remaining cases ranged from 0.4:1-46.3:1. One of the possible explanations for high ratios in cases 14 and 16 is the finding that cocaine being a stimulant drug may cause hyperthermia resulting in the loss of body water through the skin leading to decreased urine volume and an increase in the concentration of drugs excreted in urine (Cary, 2004).

It is worth noting that in cases 15 and 17 where the ratios of urine to femoral blood cocaine concentrations were 0.4:1 and 1.0:1 respectively only trace amounts of cocaine were detected in both urine and blood. The urine to femoral blood cocaine ratio is also influenced by the route of cocaine administration. It was reported that about 1-9% of intravenous cocaine is excreted unchanged in urine with 35-55% as BE and the excretion of cocaine is increased when urine is acidic. The urinary excretion for cocaine represents 80-95% of administered cocaine dose. Of this amount 1-5% is cocaine while the remainder consists of benzoylecgonine and methylecgonine (Galloway and White, 1991). Urine excretory percentages for cocaine of 1-14%, methylecgonine 12-60% and 14-55% for benzoylecgonine from an administered dose of cocaine were also found (Takekawa, 2005). During intranasal administration the amount of cocaine excreted in urine represents 4% and

BE 16-36% of the administered dose (Moffat et al., 2004). In general it has been estimated that cocaine concentration in the urine is higher than in the plasma by 15-100 fold (Karch, 2007), most likely reflecting different routes of its administration.

Cocaine and its metabolites were detected in liver and muscle specimens provided and the concentration ratios to femoral blood were determined (Table 22). The concentrations of cocaine and its metabolites in liver and muscle were highest in cases 9, 13 and 19 (no liver sample was available for case 19) corresponding to femoral blood levels. In this study cocaine and its metabolites appear to be more abundant in the liver as compared to concentrations in muscle

The median concentration ratio for muscle to the corresponding femoral blood cocaine was 0.7:1 (range <0.1:1-1:1), for BE 0.2:1 (range <0.1:1-1.4:1) and for ME was 0.3:1 (range 0.1:1-<1:1) (Table 22).

There is very limited cocaine and metabolites data available for both tissues in relation to blood levels. The median concentration ratio for liver to femoral blood cocaine was 1:1 (range 0.2:1-1.4:1), for BE 0.2:1 (range <0.1:1-1.5:1) and for ME was 0.3 (range 0.1:1-<1.0:1) (Table 22).

A study of 66 cocaine related deaths found that the mean blood cocaine and BE concentrations were 460ng/mL and 2750ng/mL (range 0.0-7400ng/mL and 0.0-14400ng/mL respectively) and the mean liver cocaine and BE concentrations were 960ng/g and 5920ng/g (range 0.0-33000ng/g and 0.0-41300ng/g respectively) (Bailey and Shaw, 1989). This would make the mean liver to blood cocaine

Table 22: Concentrations ratios of cocaine and its metabolites in different post-mortem tissue body compartments to the femoral blood levels (ng/mL).

Case	Cocaine /metabolites	FB (ng/mL)	L (ng/g)	M (ng/g)	L/FB (ml/g)	M/FB (ml/g)
9	Coca	2413	392	296	0.2	0.1
	ME	4033	572	404	0.1	0.1
	BE	4445	1032	564	0.2	0.1
	CE	253	32	24	0.1	0.1
12	Coca	28	20	<5	0.7	<0.1
	ME	390	56	24	0.1	0.1
	BE	1462	360	108	0.2	0.1
	CE	<5	<5	<5	<1	<1
13	Coca	1200	1660	856	1.4	0.7
	ME	769	720	580	0.9	0.7
	BE	2760	4100	3918	1.5	1.4
	CE	<5	<5	<5	<1	<1
14	Coca	5	<5	<5	<1	<1
	ME	78	28	20	0.4	0.3
	BE	5	<5	<5	<1	<1
	CE	<5	<5	<5	<1	<1
15	Coca	14	<5	<5	<0.3	<0.3
	ME	5	<5	<5	<1	<1
	BE	98	24	<5	0.2	<0.1
	CE	<5	<5	<5	<1	<1
16	Coca	5	<5	<5	<1	<1
	ME	135	32	20	0.2	0.1
	BE	35	<5	<5	<0.1	<0.1
	CE	<5	<5	<5	<1	<1
17	Coca	5	<5	<5	<1	<1
	ME	20	<5	<5	<0.2	<0.2
	BE	27	<5	<5	<0.2	<0.2
	CE	<5	<5	<5	<1	<1
18	Coca	5	<5	<5	<1	<1
	ME	5	<5	<5	<1	<1
	BE	5	<5	<5	<1	<1
	CE	<5	<5	<5	<1	<1
19	Coca	420	NA	84	NA	0.2
	ME	389	NA	144	NA	0.4
	BE	600	NA	248	NA	0.4
	CE	<5	NA	<5	NA	<1

FB = femoral blood, L = liver and M = muscle.

concentration ratio of 2.0:1 (ranged 0.0-2.8:1) and for BE 2.1:1 (ranged 0.0-4.4:1). Another study on 19 post-mortem cases the liver cocaine concentrations ranged from 100 to 20000ng/g (mean 4200) and the corresponding blood cocaine levels ranged from 900 to 21000ng/mL (mean 5300) (Baselt, 2004). The mean liver to blood cocaine concentration ratio in this study was 0.7:1 (ranged 0.1:1-0.9:1). The ratios are similar to those observed in this thesis.

Understandably the major difficulty for the interpretation of liver and muscle cocaine concentrations is that there is no reference data in published literature in terms of therapeutic, toxic and lethal concentration similar to those levels in the blood. In addition, drugs with V_d of greater than 3L/kg are more susceptible to PMDR and become more stored in deep tissues such as liver. Cocaine has a V_d of 1-3L/kg which indicates that it is unlikely to be significantly sequestered into the liver (Kapur, 2009). However, cocaine metabolism may also be influenced by age, gender and health states which will affect its distribution into the tissues (Drummer, 2001). Liver is the main site for cocaine metabolism and in cases of liver disease the metabolism of the cocaine will be impaired and this may change the level in the liver tissue (Kapur, 2009).

The concentration ratios of cocaine to its metabolites in different body compartments were also studied (Table 23). There was a significant correlation between cocaine and its metabolites in femoral blood, cardiac blood, vitreous humour, liver and muscle. There was no correlation between cocaine and its metabolites in bile and the correlation with concentrations in urine was weak.

Table 23: Post-mortem cocaine to its metabolites concentrations ratios in different body compartments
The concentrations for cocaine and its metabolites in all fluid samples were ng/ml and for tissue samples ng/g.

Case	Femoral blood		Cardiac blood		Vitreous humour		Bile		Urine		Liver		Muscle	
	Coc/ ME	Coc/ BE	Coc/ ME	Coc/ BE	Coc/ ME	Coc/ BE	Coc/ ME	Coc/ BE	Coc/ ME	Coc/ BE	Coc/ ME	Coc/ BE	Coc/ ME	Coc/ BE
8	0.7	0.04	2.8	0.3	1.8	0.16	0.85	0.1	0.1	0.04	NA	NA	NA	NA
9	0.6	0.5	0.8	0.8	1.2	1.4	1	1	1	1	0.7	0.4	0.7	0.5
12	0.1	0.02	0.1	0.02	0.7	0.02	0.1	0.1	0.7	0.3	0.3	0.05	0.2	0.04
13	1.6	0.43	0.2	0.1	NA	NA	0.64	0.41	NA	NA	2.3	0.4	1.5	0.2
14	0.1	1.0	0.01	0.5	0.12	0.13	0.1	0.2	1.4	1	0.2	1.0	0.3	1.0
15	2.8	0.1	5	0.2	3.4	0.02	0.02	0.01	0.04	0.01	1.0	0.2	1.0	1.0
16	0.04	0.1	0.1	0.6	0.17	0.33	NA	NA	1.4	1	0.1	1.0	0.2	1.0
17	0.3	0.2	0.03	0.1	0.14	0.04	0.07	0.05	0.03	0.01	1.0	1.0	1.0	1.0
18	1.0	1.0	1	1	1.6	1.0	0.5	0.1	NA	NA	1.0	1.0	1.0	1.0
19	1.1	0.7	NA	NA	1.4	0.59	2.6	1.8	1	1	NA	NA	0.6	0.3
Median	0.64	0.31	0.2	0.3	1.2	0.6	0.5	0.1	0.8	0.6	0.8	0.7	0.7	1.0
Range	0.04-2.8	0.02-1.0	0.01-5	0.02-1	0.12-3.4	0.02-1.4	0.02-2.6	0.01-1.8	0.03-1.4	0.01-1	0.1-2.3	0.05-1.0	0.2-1.5	0.04-1.0
Correlations to cocaine levels														
r	0.95	0.95	0.98	0.81	0.99	0.99	0.55	0.54	0.82	0.67	0.88	0.99	0.95	0.98
P	<0.001	<0.001	<0.001	0.007	<0.001	<0.001	0.16	0.17	0.01	0.07	0.004	<0.001	<0.001	<0.001

Coc/ME = cocaine/methylecgonine concentration ratio, Coc/BE = cocaine to benzoylecgonine concentration ratio, Pearson Correlation Coefficient = r and P value <0.05 indicates significant correlation.

Bile and urine represent the excretory fluids which contain the excreted drugs and their metabolites. The amount of drug excreted in urine or bile will to some extent depend on the hydrophobicity or hydrophilicity of the drug. As mentioned earlier, most water-soluble drugs are excreted through the kidney into urine whilst lipid-soluble drugs are excreted through the liver into bile (Levin and Spihler, 2003). Cocaine is a lipid soluble drug and its biotransformation process occurs in the liver to form more hydrophilic compounds BE and ME (Peterson et al., 1995). In 7out of 9 cases the BE and ME levels in bile were higher than cocaine concentration. One possible explanation is that as both these metabolites are water soluble and bile contains water, the concentrations of these metabolites will be higher in bile as explained earlier in ethanol cases (Winek et al., 1983).

Correlations of cocaine and its metabolites in different post-mortem compartments in relation to femoral blood concentrations are given in Table 24. It was shown that there were significant correlations between the cocaine level in femoral blood and that in cardiac blood, vitreous humour and muscle samples, BE in femoral blood also correlated with that in cardiac blood, vitreous humour and bile samples and that of the femoral blood and ME with cardiac blood, vitreous humour, bile and muscle.

Table 24: Correlations of cocaine and its metabolites in different post-mortem compartments in relation to femoral blood concentrations

Sample	Cocaine		Benzoylecgonine		Methylecgonine	
	r	P	R	P	r	P
Cardiac blood	0.91	0.001	0.94	<0.001	0.99	<0.001
Vitreous humour	0.99	<0.001	0.84	0.005	0.99	<0.001
Urine	0.57	0.14	0.41	0.31	0.55	0.16
Bile	0.64	0.09	0.79	0.01	0.70	0.03
Liver	0.59	0.12	0.67	0.1	0.67	0.07
Muscle	0.99	<0.001	0.37	0.32	0.72	0.03

r = Pearson correlation coefficient. P value <0.05 indicates significant correlation.

Hair specimens were provided for all cocaine cases except cases 9, 14 and 18. The cocaine and BE concentrations in different hair segments ranged 1.3-107ng/mg and 3.3-19ng/mg respectively (Table 25). Cocaine was at higher concentrations in hair compared to BE levels and this agrees with previous observations (Henderson, 1992; Nakahara et al., 1992; Henderson, 1993).

Hair cocaine and BE levels in all segments were above 0.5ng/mg. Hair cocaine concentrations ≥0.5ng/mg and BE ≥0.05ng/mg indicate positive results (Kintz, 2007). It has been shown that cocaine and BE concentrations in hair obtained from chronic cocaine abusers ranged 6.4-19.2ng/mg and 0.3-2.5ng/mg respectively (Cone et al., 1991). However, there is also variation in cocaine concentration in hair as the level was 160ng/mg in one fatal cocaine overdose case (Giroud et al., 2004).

The cocaine to BE concentration ratios in hair ranged 3.3:1-19:1. The concentration ratios of cocaine to BE in hair have been reported in previous studies. In a previous study of 20 cocaine chewers the ratio ranged from 1.3:1-4.7:1 (Moller et al., 1992) and in a study of 10 chronic users the ratio ranged from 5:1-10:1 (Cone et al., 1991). Another study of 5 coca chewers the ratio ranged from 2.1:1-8.6:1 (Henderson et al., 1992). The variations in these ratios may be due to different factors which affect cocaine incorporation and retention in hair. These factors include the route of administration of cocaine, administered dose, cosmetic treatments, hair dye, melanin contents, washing and genetic variations (Kintz, 2007). Cocaine incorporation into black hair is higher than into blonde hair after the same dose has been administered (Henderson, 1998; Joseph et al., 1999; Scheidweiler et al., 2005).

Table 25: Cocaine and benzoylecgonine concentrations in different hair segments (ng/mg)

Case	Hrcoc	H2coc	H3coc	Mean coc	HrBE	H2BE	H3BE	Mean BE	Mean coc/BE
8	10	14	9.7	11.4±2.4	1.7	2.6	1.9	2.1±0.5	5.4
12	5.1	5.1	6.1	5.4±0.6	0.5	0.7	1.4	0.9±0.5	6.0
13	6.4	6.8	6.2	6.5±0.3	0.4	0.4	0.3	0.4±0.1	16.3
15	1.1	1.5	1.3	1.3±0.2	0.1	0.3	0.3	0.4±0.1	3.3
16	3.6	4.5	3.2	3.8±0.7	0.3	0.4	0.3	0.2±0.1	19
17	80	153	90	107±39.6	23	12	11	15±6.6	7.1
19	56	61	109	75±29.3	5.7	11	30	15±12.8	5.0

Hrcoc = hair cocaine root segment, H2coc = hair cocaine middle segment, H3coc = hair cocaine peripheral segment, HrBE = hair benzoylecgonine root segment, H2BE = hair benzoylecgonine middle segment, H3BE = hair benzoylecgonine peripheral segment, Mean coc = mean cocaine concentrations in different hair segments for each case, Mean BE = mean benzoylecgonine concentrations in different hair segments for each case and Mean coc/BE = mean cocaine/benzoylecgonine concentration ratio.

The distribution of cocaine and BE was studied in different rib bone sections (Table 26). Rib bones were cut into three sections each 2cm long and from each section 1cm was cut (B1, B2 and B3) and used for analysis. Therefore, B1 represents the section nearest to the sternum. The cocaine and BE concentrations in different rib bone sections ranged from 0.9-67ng/mg to 5.2-75ng/mg respectively. There appeared not to be any correlation between femoral blood cocaine and its metabolites and the levels in bone. In all rib bone sections BE concentrations were higher than cocaine levels except for case 9. A pervious study on teeth obtained from a cocaine abuser also showed that BE was the major metabolite present in most of the specimens (Pellegrini et al., 2006).

The mechanism by which cocaine or other drugs are incorporated into bone is unknown, but drug incorporation into bone tissue is thought to be reversible, which means that a negative drug result cannot entirely exclude the possibility of previous drug exposure and a positive result does not provide any information about time of exposure (Karch, 2007). In fact, there is very limited data available for bone tissue and drugs of abuse and this makes the interpretation of any drug concentration in bone very difficult. This study attempted to address this issue.

Table 26: Cocaine and benzoylecgonine concentrations in different rib bones sections (ng/mg) with corresponding femoral blood cocaine and benzoylecgonine

Case	FBcoc	B1coc	B2coc	B3coc	Mean coc	FBBE	B1BE	B2BE	B3BE	Mean BE
9	(2413)	57	77	67	67±10	(4445)	32	58	42	44±13.1
12	(28)	0.9	0.9	0.9	0.9±0.0	(1462)	17	8.2	17	14±5.0
13	(1200)	26	32	27	28±3.2	(2760)	49	124	54	75±41.9
14	(5)	2.1	0.9	1.4	1.5±0.6	(5)	4.4	5.4	5.7	5.2±0.7
15	(14)	4.6	3.6	2.6	3.6±1.0	(98)	20	9.9	5.9	12±7.3
16	(5)	2.6	3.2	2.7	2.8±0.3	(35)	4.9	12.4	5.4	7.6±4.2
17	(5)	3.8	4.5	5.4	4.6±0.8	(27)	7.8	8.0	9.9	8.6±1.2
19	(420)	2.8	1.4	1.2	1.8±0.9	(600)	25	14	21	20±5.6

FBcoc = femoral blood cocaine concentration (ng/ml), B1coc = rib bone cocaine first section, B2coc = rib bone cocaine middle section and B3coc = rib bone cocaine end section, Meancoc = mean cocaine concentration in different bone sections (ng/mg), FBBE = femoral blood benzoylecgonine concentraion (ng/ml), B1BE = rib bone benzoylecgonine first section, B2 BE = rib bone benzoylecgonine middle section and B3BE = rib bone benzoylecgonine end section and MeanBE = mean benzoylecgonine concentrations in different bone sections (ng/mg).

3.2.1 Summary of cocaine results

The concentration ratios of cocaine in different body compartments are affected by several factors which may include post-mortem diffusion/redistribution as well as its continued hydrolysis in the post-mortem period. Nevertheless, the cocaine concentration ratios to femoral blood were: cardiac blood 1.0-7.0, vitreous humour 1.0-3.3, bile 0.6-41.6, urine in six cases 0.4- 46.3 and in two remaining cases >1000. The median liver tissue to femoral blood cocaine concentration ratio was 1.0 and for muscle to femoral blood cocaine was 0.7 in ml/g.

3.3: Distribution of opioids:

3.3.1: Distribution of methadone and its metabolite in post-mortem samples:

The methadone positive cases are described in Table 27. All cases were male except one female; the mean age was 39 years (range 30-43). All were known to be drug abusers and histological findings showed brain and lung oedema (5 cases one of which also had endocarditis), fatty liver (1), pneumonia (1) and gastrointestinal haemorrhage (1). The causes or suspected contributors to the causes of death were drug toxicity (4), pneumonia (3) and exsanguination (1).

Methadone is a potent anti-addictive opioid analgesic most commonly used to treat heroin addiction of patients on "methadone maintenance programmes". Uncontrolled or inappropriate use of methadone also occurs. The elimination half-life for methadone is about 8.5-58 hours and V_d is 3-5L/kg (Verebely, 1975; Meresaar, 1981; Karch, 2007). The major inactive methadone metabolite is 2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine (EDDP) (Tober et al., 2003). The half-life and the volume of distribution for EDDP are unknown (Sullivan, 2009). The concentrations of methadone and EDDP in different post-mortem body fluids and tissue compartments are given in Table 28 (a). Associated drugs of abuse and their concentrations in femoral blood are given in Table 28 (b).

The femoral blood methadone concentrations ranged from 7-1795ng/mL. In cases 8, 12, 14 and 17 the femoral blood methadone levels were above the potentially lethal range of 400ng/mL. The femoral blood methadone concentrations in cases 13 and 16 (7 and 18ng/mL respectively) were within the sub-therapeutic range whilst in cases 1 and 15 (300 and 366ng/mL respectively) the levels were within the therapeutic to

Table 27: Methadone abuse cases

Case	Gender	Age (year)	Medical history	Post-mortem findings	The cause or suspected contributor to cause of death
1	M	30	Drug abuser	Brain and lung oedema	Pneumonia
8	M	42	Drug abuser	Brain and lung oedema	Drug toxicity
12	M	43	Drug abuser	Brain oedema and endocarditis	Pneumonia
13	M	41	Drug abuser	Fatty liver	Exsanguination
14	M	40	Drug abuser	Brain and lung oedema	Drug toxicity
15	F	39	Drug abuser	Pneumonia	Pneumonia
16	M	43	Drug abuser	Brain and lung oedema	Drug toxicity
17	M	41	Drug abuser	Gastrointestinal haemorrhagic	Drug toxicity

Table 28 (a): Methadone and its metabolite concentrations in different post-mortem body fluids (ng/mL) and tissue compartments (ng/g).

Case	Drug	FB (ng/mL)	CB (ng/mL)	VH (ng/mL)	S (ng/mL)	Bile (ng/mL)	U (ng/mL)	L (ng/g)	M (ng/g)
1	Methadone	300	1121	NA	3434	767	97	119	32
	EDDP	36	29	NA	65	1730	25	52	28
8	Methadone	603	1636	408	3700	1719	1960	232	128
	EDDP	464	357	460	2826	>5000	>5000	110	58
12	Methadone	520	736	518	>5000	>5000	279	211	24
	EDDP	432	607	352	2006	>5000	>5000	56	32
13	Methadone	7	8	5	5	5	5	<5	<5
	EDDP	2703	3076	795	5	936	63	32	24
14	Methadone	1266	>5000	547	NA	>5000	>5000	346	81
	EDDP	1494	>5000	495	NA	>5000	>5000	36	20
15	Methadone	366	225	13	>5000	1234	NA	120	95
	EDDP	14	357	260	2826	>5000	NA	44	28
16	Methadone	18	13	17	5	NA	15	<5	<5
	EDDP	132	28	10	405	NA	26	<5	<5
17	Methadone	1795	2378	1673	>5000	>5000	901	384	98
	EDDP	3197	>5000	191	325	>5000	>5000	75	24

The blood quoted reference ranges for methadone are: therapeutic 50-750ng/mL, toxic 200-2000ng/mL and lethal >400ng/mL (Weink et al., 2001; Schulz and Schmoldt, 2003; Musshoff et al., 2004). FB = femoral blood, CB = cardiac blood, VH = vitreous humour, S = stomach contents, U = urine, L = liver and M = muscle

Table 28 (b): Femoral blood concentrations for other associated drugs of abuse

Case	Associated drugs of abuse concentration (ng/mL) in femoral blood
1	No other drug found
8	Cocaine 6
12	Cocaine 28
13	Cocaine 1200
14	Cocaine 5
15	Cocaine 14
16	Cocaine 5, morphine 48 and diazepam 219
17	Cocaine 5

**Quoted reference ranges*

(Weink et al., 2001; Schulz and Schmoldt, 2003; Musshoff et al., 2004)

Drug	*Therapeutic (ng/mL)*	*Toxic (ng/mL)*	*Lethal (ng/mL)*
Diazepam	*20-4000*	*3000-5000*	*>30000*
Cocaine	*50-300*	*500-1000*	*>4000*
Morphine	*10-100*	*50-400*	*>100*

**These references ranges are for guidance alone and drug interpretation should take in account the patients full clinical and drug history.*

toxic ranges which overlap. However, case 13 also had a potentially fatal cocaine concentration in the femoral blood (1200ng/mL) but the cause of death was exsanguinations.

A study of 104 methadone therapy cases found variations in blood methadone levels with mean concentration of 451± 306ng/mL (range 20-1308ng/mL) (Loimer and Schmid, 1992). Another study of 55 methadone related deaths found that the mean blood methadone level was 584ng/mL (range 84-2700ng/mL) (Milroy and Forrest, 2000). Post-mortem blood methadone levels are helpful but not always a conclusive cause of death, as fatal concentrations vary widely probably due to tolerance (Segal and Catherman, 1974; Worm et al., 1993). The elimination half-life for methadone also varies greatly (8.5-58h) (Verebely, 1975; Meresaar, 1981; Karch, 2007), which may partly account for the varying concentrations observed.

Toxicity due to methadone is similar to that by other opioids and other CNS suppressant drugs in that it causes respiratory arrest even at the therapeutic doses and this toxic effect is enhanced by the presence of other CNS depressant drugs (Drummer, 2001; Wolff, 2002; Wolf et al., 2004; Shields et al, 2007). It has been reported that methadone related deaths are more likely to occur during the first few weeks of treatment due to fatal respiratory depression (Drummer et al., 1992; Coleridge et al., 1992). The risk of fatal respiratory depression in the first two weeks of methadone therapy is 7 fold higher than in non-treated heroin abusers and 98 fold higher than for patients who have been on methadone maintenance for more than two weeks (Dole et al, 1969; Wu and Henry, 1990; Ceplehorn and Drummer, 1999). The overall sequence of events in methadone related deaths is respiratory depression,

airway obstruction, pulmonary oedema and pneumonia leading to death (John et al., 2004).

In addition and as said above, there are individual variations to methadone effects and pharmacokinetics (Ferrari et al, 2004), which may in part related to tolerance and different rates of drug metabolism. A dose of 50-70 mg methadone can kill an opiate-naïve individual (Roche et al., 2008) whilst patients undergoing methadone maintenance therapy can tolerate 80-120mg/day (Leavitt, 2006).

Cocaine was present in all methadone cases except case 1, and case 16 also had morphine and diazepam. It has previously been reported that in all cases of methadone related deaths, cocaine and morphine were found in 42% and 34% respectively (Karch and Stephens, 2000). It was also reported that fatality due to methadone alone represents 6.3% of deaths where methadone was involved but this value increased to 21.9% when cocaine was co-administrated with methadone (Shields et al., 2007).

The interaction of methadone with other CNS drugs is complex and when it is administered in combination with other drugs which affect cytochrome P450 enzymes its metabolism and clearance is altered. The lethal dose of methadone was found to be decreased in individuals who consumed methadone with other drugs including ethanol, cocaine, benzodiazepines, cannabis and amphetamine (Stephens, 1996; Manzanares, 1999: Taylor, 2001; Medsafe, 2006). Nevertheless, co-administration of cocaine with methadone was also shown to increase methadone excretion (Tennant and Shannon, 1995; Moolchan et al., 2001). This may imply that methadone treated individuals who administered cocaine may ingest higher doses of

methadone to attain the same benefit which may in some cases lead to methadone overdose as mentioned earlier. Two studies have reported that methadone increased sensitivity to cocaine (Foltin et al., 1995; Preston et al., 1996). There is also some evidence that methadone may even encourage cocaine abuse in some patients (Kosten et al., 1990).

It is also known that co-administration of benzodiazepines with methadone will increase the free methadone level in the blood due to competition of benzodiazepines binding to the plasma proteins which may lead to overdose and death due to high free methadone levels. In general, high blood free methadone concentrations were observed in chronic methadone abusers who co-administered benzodiazepines (Mikolaenko et al, 2002). It has been reported that opioid effects of methadone were enhanced by concurrent administration of diazepam in methadone treated individuals (Preston et al., 1986). An *in vitro* study found that diazepam inhibits the N-demethylation of methadone in methadone dependent rats (Spaulding et al., 1974).

Except for case 15, the concentrations of methadone in samples of stomach contents were in direct proportion to the cardiac blood levels i.e. high concentration in the stomach was associated with high concentration in cardiac blood. Whilst the concentration of methadone in stomach contents in case 15 was very high, the vitreous humour methadone level in this case was relatively very low which may indicate that death may have occurred soon after ingestion methadone [Table 28 (a)]. Except for cases 13, 15 and 16 the cardiac blood methadone was higher than the femoral blood levels with median ratio of 1.4 (range 0.6:1- >3.9:1) (Table 29). In cases 13 and 16 [Table 28 (a)] the results showed low methadone levels in femoral

Table 29: Methadone concentration ratios to femoral blood levels in different fluid and tissue body compartments

Case	FB (ng/ml)	CB/ FB	VH/ FB	Bile/ FB	U/ FB	L/FB (ml/g)	M/FB (ml/g)
1	300	3.7	NA	2.6	0.3	0.4	0.1
8	603	2.7	0.7	2.9	3.3	0.4	0.2
12	520	1.4	1.0	>9.6	0.54	0.4	0.05
13	7	1.1	0.7	0.7	0.7	<0.7	<0.7
14	1266	>3.9	0.4	>3.9	>3.9	0.3	0.1
15	366	0.6	0.04	3.4	NA	0.3	0.3
16	18	0.7	1.0	NA	0.8	<0.3	<0.3
17	1795	1.3	0.9	>2.8	0.5	0.2	0.05
Median ratio		1.4	0.7	2.9	0.7	0.3	0.1
Range ratio		(0.6->3.9)	(0.04-0.99)	(0.7->9.6)	(0.3->3.9)	(0.2-0.4)	(0.05-0.3)

FB = femoral blood, CB = cardiac blood, VH = vitreous humour, S = stomach contents, U = urine, L = liver and M = muscle

and cardiac blood which may indicate that methadone was administered a long time before death. In case 15 both cardiac blood and vitreous humour methadone concentrations were lower than femoral blood level which may indicate that death occurred rapidly after methadone administration [Table 28 (a)].

The high cardiac blood opioid level in relation to femoral blood concentrations were found in previous studies (Sawyer and Forney, 1988; Prouty and Anderson, 1990; Koren and Klein, 1992; Gerostamoulos and Drummer, 2000). As mentioned earlier, the concentration of drugs with volume of distribution >1L/kg tend to be high in the central blood compared to the femoral blood (Drummer, 2000; Leikin and Watson, 2003; Yarema and Becker, 2005). Methadone V_d is 3-5L/kg and it would be expected to be susceptible PMDR. Diffusion of drugs from stomach to the cardiac blood in the post-mortem period was observed in previous studies (Pounder et al., 1996; Pelissier-Alicot et al., 2003). The possibility of methadone diffusion from the stomach into the central blood cannot be excluded (especially in cases 1, 8, 12, 15 and 17) and this phenomenon was described earlier with the ethanol cases (Pounder and Smith, 1995; Pounder et al, 1996; Iwasaki et al, 1998).

Vitreous humour samples were provided for 7 cases [Table 28 (a)]. The methadone concentration in vitreous humour was of the same magnitude as that in femoral blood (median 0.7 and ratio ranged 0.7:1-1.0:1) except for cases 14 and 15 (ratios were 0.4 and 0.04 respectively (Table 29). Although a stomach contents sample was not available for case 14, it appears that death may have occurred soon after ingestion of methadone, similar to case 15.

In a previous study of 47 post-mortem cases, the mean vitreous humour to femoral blood methadone concentration ratio was 0.29 and range 0.08-0.98 (Jennings et al., 2005). The less than unity vitreous humour to femoral blood methadone concentration ratio was observed in a previous study of two methadone overdose fatalities where the blood methadone levels were above the vitreous humour concentrations in both cases (Sturner and Garriott, 1975). The main advantage of the current study is that we know the site from which the blood samples were collected, the post-mortem interval and the drug concentrations in other body compartments.

Bile samples were available for all but case 16 [Table 28 (a)]. Except for cases 13 where femoral blood methadone was very low (7ng/mL), in the remaining cases the methadone levels in bile were at least two to three fold higher than in femoral blood with ratios ranging from 2.6->9.6 (Table 29). Methadone is a lipid soluble drug and previous studies found that lipid soluble drugs were present at high concentrations in bile compared with blood (Vanbinst et al., 2002; Giroud et al., 2004; Tassoni et al, 2007).

Methadone concentrations in urine in relation to the femoral blood levels were variable. The urine to femoral blood methadone concentration ratios ranged from 0.3-0.8 except for cases 8 and 14 where the ratios were 3.3 and >3.9 respectively. There are many factors which may influence methadone levels in urine. These are urine pH, ingested dose, urine volume and administration of methadone with other drugs (e.g. cocaine).

A study of 1539 methadone urine samples found that high methadone concentration in urine samples was associated with low urine pH (Bernard et al., 2007). The methadone and EDDP concentrations in urine represent 5% of administered oral dose and this proportion increases from 5% to 22% in acidic urine (Chamberlain, 1997). Another study of 1023 urine samples collected from methadone treated patients found that the excretion of methadone and it's metabolite was dose dependent i.e. high dose of ingested methadone will be associated with increased methadone and EDDP concentrations in urine (Preston et al., 2003). Urine volume is an important factor that influences the drug level as mentioned in cocaine cases that low urine volume (dehydration) will result in high urine drug concentration (Cary, 2004).

Excluding cases 13 and 16 were the methadone concentrations in liver and muscle were <5ng/g, in the remaining cases, the concentrations in the liver was about 1.5 to 8 fold that in muscle [Table 28 (a)]. Similarly the EDDP concentration was 1.3-2 fold higher in the liver than in muscle. Again excluding cases 13 and 16, the concentration ratios in relation to femoral blood were median 0.3 (range 0.2-0.4) for liver and 0.1 (range 0.05-0.3) for muscle (Table 29). In general, organs with high blood flow like liver will contain more drug compared to low blood flow organs such as muscle (Barker and Bromley, 2002; Levine and Spiehler, 2003; Gomella et al., 2004). In addition, liver is more lipophilic (Winstanley and Walley, 2002) and these lipophilic drugs will be concentrated in this organ.

Understandably, no reference data is available for therapeutic or toxic drug concentrations in the liver or muscle. There is only limited data on the postmortem concentration of methadone in liver and muscle. Liver methadone concentrations

have been measured in three methadone overdose cases and the levels were 1900, 1300 and 700ng/g but there were no corresponding blood levels (Nelson and Selkirk, 1975).

The distribution of EDDP in different body compartments in relation to femoral blood levels is also summarised in Table 30. The cardiac blood EDDP concentration was higher than that in femoral blood levels in all cases except cases 1, 8 and 16 [Table 28 (a)]. The cardiac to femoral blood EDDP concentrations ratios ranged 0.2:1->3.3:1 except for case 15 the ratio was 38.4:1 (Table 30). The vitreous humour EDDP concentrations were lower than that in femoral blood in all cases except for case 8 where the concentrations were similar. The vitreous humour to femoral blood EDDP concentrations ratios ranged 0.1:1-0.9:1 except for case 15 where the ratio was 18.6:1. The stomach contents EDDP concentrations were higher than those in femoral blood in all cases except for cases 13 and 17. The stomach to femoral blood EDDP concentrations ratios ranged 0.002:1-6.1:1. Except for case 15 the ratio was 201.8:1. The bile EDDP levels were highly elevated compared to femoral blood values in all cases except case 13. Excluding case 13 the bile to femoral blood EDDP concentrations ratios ranged >1.6:1:-48:1 whilst for case 15 the ratio was >357.1:1. The urine EDDP concentrations were above the femoral blood levels in four cases (8, 12, 14 and 17) and below the femoral blood level in three cases (cases 1, 13 and 16). The urine to femoral blood EDDP concentrations ratios ranged 0.02:1-20.3:1 (no sample available for case 15) (Table 30). Except for case 16 the liver EDDP concentration was higher than that in muscle [Table 28 (a)]. The liver to femoral blood EDDP concentrations ratios ranged 0.01:1-1.4:1 except for case 15 where the ratio was 3.1 (Table 30). The muscle to femoral blood EDDP

Table 30: EDDP concentration ratios to femoral blood levels in different fluid and tissue body compartments

Case	FB (ng/ml)	CB/FB	VH/FB	S/FB	Bile/FB	U/FB	L/FB (ml/g)	M/FB (ml/g)
1	36	0.8	NA	1.8	48	0.6	1.4	0.8
8	464	0.8	0.9	6.1	>10.8	>10.8	0.2	0.1
12	432	1.4	0.8	4.6	>11.6	>11.6	0.1	0.07
13	2703	1.1	0.3	0.002	0.3	0.02	0.01	0.01
14	1494	>3.3	0.3	NA	>3.3	>3.3	0.02	0.01
15	14	38.4	18.6	201.8	>357.1	NA	3.1	2.0
16	132	0.2	0.1	3.1	NA	20.3	0.04	0.04
17	3197	>1.6	0.1	0.002	>1.6	>1.6	0.02	0.01
Median		1.3	0.3	3.1	10.8	3.3	0.1	0.1
Range		(0.2-38.4)	(0.1-18.6)	(0.002-201.8)	(0.3->357.1)	(0.02-20.3)	(0.01-3.1)	(0.01-2.0)

NA = not available, FB = femoral blood, CB = cardiac blood, VH = vitreous humour, S = stomach contents, U = urine, L = liver and M = muscle

concentrations ratios ranged 0.01:1-0.8:1 except for case 15 where the ratio was 2.0. As far as I am aware there are no previous studies comparing the EDDP levels in different body compartments and those in the femoral blood. This is probably the first time such a study has been carried out

The correlations coefficient of methadone concentrations in different body compartments in relation to femoral blood are given in Table 31. There were significant correlations between femoral blood methadone concentrations and its levels in cardiac blood, vitreous humour, bile and liver but there was no correlation between femoral blood methadone concentrations and both urine and muscle samples.

Table 31: Correlation coefficients of methadone concentrations in different body compartments and those in the femoral blood

Sample	(r)	P
Cardiac blood	0.77	0.02
Vitreous humour	0.92	0.004
Bile	0.77	0.03
Urine	0.55	0.2
Liver	0.95	<0.01
Muscle	0.61	0.1

Pearson Correlation Coefficient = r and P value is significant <0.05

The concentration ratios of methadone to its metabolite in different post-mortem body compartments is summarised in Table 32. The median and ratio ranges for methadone to EDDP were as follows: femoral blood 1.0 (range 0.03:1-26.1:1), cardiac blood 0.8 (range 0.003:1-38.6:1), vitreous humour 1.1 (range 0.01:1-8.8:1),

Table 32: Methadone to its metabolite concentration ratios in different post-mortem body compartments
Meth/EDDP = methadone to EDDP concentration ratio, r = Pearson Correlation Coefficient and P value < 0.05 indicates significant correlation.

Case	Femoral blood	Cardiac blood	Vitreous humour	Bile	Urine	Liver	Muscle
	Meth/ EDDP	Meth/ EDDP	Meth/ EDDP	Meth/ EDDP	Meth/ EDDP	Meth/ EDDP	Meth/ EDDP
1	8.3	38.6	NA	0.44	3.8	2.3	1.1
8	1.3	4.6	0.89	0.34	0.39	2.1	2.2
12	1.2	1.2	1.5	1.0	0.06	3.8	0.7
13	0.03	0.003	0.01	0.01	0.08	0.1	0.2
14	0.85	1.0	1.1	1.0	1.0	9.6	4.0
15	26.1	0.63	0.1	0.25	NA	2.7	3.4
16	0.14	0.46	1.7	NA	0.01	1.0	1.0
17	0.56	0.48	8.8	1.0	0.18	5.1	4.0
Median	1.0	0.8	1.1	0.4	0.2	2.5	1.6
Range	0.03-26.1	0.003-38.6	0.01-8.8	0.01-1.0	0.01-3.8	0.1-9.6	0.2-4.0
Correlations of methadone to EDDP levels in different body compartments							
r	0.55	0.69	-0.22	0.71	0.58	0.56	0.29
P	0.16	0.06	0.63	0.08	0.2	0.1	0.04

bile 0.4 (range 0.01:1-1.0:1), urine 0.2 (range 0.01:1-3.8:1), liver 2.5 (range 0.1:1-9.6:1) and for muscle was 1.6 (range 0.2:1-4.0:1) (Table 32). Correlation coefficients of methadone to EDDP in different post-mortem samples are given in Table 32. Generally there was no significant correlation between methadone and EDDP in different post-mortem specimens (Table 32).

In a previous study, blood methadone to EDDP concentration ratio ranged from 18:1 to 22:1 in methadone treated individuals (de Vos et al., 1998). However, this ratio was found to be lower in post-mortem cases. In a study of 38 methadone related deaths the mean methadone to EDDP concentration ratio was 13.6:1 (range 0.57:1 to 60:1) (Karch and Stephens, 2000). In this thesis, the ratios of methadone to EDDP in both femoral and cardiac blood samples (Table 32) varied greatly, similar to previous observation (Karch and Stephens, 2000). In this study it was concluded that the ratios determined had no diagnostic value in terms of the therapeutic, toxic or lethal dose of methadone. No other studies determining methadone to EDDP in different postmortem specimens were found.

3.3.2 Summary of methadone results

The methadone concentration ratios to femoral blood were: cardiac blood 0.6->3.9, vitreous humour 0.04-1.0, bile 0.7->9.6 and for urine 0.2-0.4. The median liver tissue to femoral blood methadone concentration ratio was 0.3 and for muscle was 0.1 in ml/g.

3.3.3: Distribution of 6-MAM, morphine and codeine in post-mortem samples:

The 15 morphine positive cases are described in Table 33. All cases were male except one female; the mean age was 44 years (range 27-61). All the cases were known to be drug abusers (except cases 21, 22 and 23) and histological findings were brain and lung oedema (10 cases), brain oedema (1), myocardial infarction (1), liver cirrhosis (1), coronary fibrosis (1) and cerebral haemorrhage (1). The causes or suspected contributors to the causes of death were drug toxicity (12), coronary thrombosis (1), cerebral haemorrhage (1) and myocardial infarction (1).

The half-life of heroin is 2-7 minutes, 6-MAM 3-27 minutes and morphine 1-8 hours (Drummer, 2001; Rook et al., 2006). This explains why in heroin related deaths heroin may sometimes not be detected whilst 6-MAM or morphine are present. The volume of distribution for 6-MAM is unknown but for heroin and morphine is 0.52L/kg and 1-6L/kg respectively (Karch, 2008).

The concentrations of 6-MAM, total morphine and free morphine in different fluid and tissue body compartments together with concentration ratios in relation to femoral blood are given in Table 34 (a). Other associated drugs of abuse found in these cases are given in Table 34 (b). Free and total codeine concentrations in femoral blood with concentration ratios of total to free codeine levels are given in Table 35.

Table 33: Morphine positive cases

Case	Gender	Age (year)	Medical history	Post-mortem findings	The cause or suspected contributor to cause of death
3	M	52	Drug abuser	Brain and lung oedema	Drug toxicity
10	M	42	Drug abuser	Liver cirrhosis	Drug toxicity
16	M	43	Drug abuser	Brain and lung oedema	Drug toxicity
20	M	27	Drug abuser	Brain and lung oedema	Drug toxicity
21	M	80	Sudden death	Coronary fibrosis	Coronary thrombosis
22	M	61	Sudden death	Cerebral haemorrhage	Cerebral haemorrhage
23	M	61	Sudden death	Myocardial infarction	Myocardial infarction
24	F	33	Drug abuser	Brain and lung oedema	Drug toxicity
25	M	30	Drug abuser	Brain and lung oedema	Drug toxicity
26	M	44	Drug abuser	Brain oedema	Drug toxicity
27	M	43	Drug abuser	Brain and lung oedema	Drug toxicity
28	M	43	Drug abuser	Brain and lung oedema	Drug toxicity
29	M	38	Drug abuser	Brain and lung oedema	Drug toxicity
30	M	33	Drug abuser	Brain and lung oedema	Drug toxicity
33	M	41	Drug abuser	Brain and lung oedema	Drug toxicity

Table 34 (a): 6-MAM, total morphine and free morphine concentrations in different post-mortem samples.

Case	Drug/ metabolite	FB (ng/mL)	FBtmorp/ FBfmorph	CB	VH	S	Bile	U	L (ng/g)	M (ng/g)
3	Tmorph	165	1.9	-	-	-	-	-	-	-
	Fmorph	86	-	91	NA	1465	1712	NA	60	24
	6-MAM	-	-	-	NA	386	-	NA	-	-
10	Tmorph	128	2.3	-	-	-	-	-	-	-
	Fmorph	56	-	177	42	376	198	122	NA	28
	6-MAM		-	-	-	245	-	80	NA	-
16	Tmorph	158	3.2	-	-	-	-	-	-	-
	Fmorph	48	-	89	65	122	>5000	1303	32	24
	6-MAM	-	-	-	-	-	-	1953	-	-
20	Tmorph	75	1.6	-	-	-	-	-	-	-
	Fmorph	46	-	130	NA	683	3841	2619	64	28
	6-MAM	-	-	-	NA	-	-	946		
21	Tmorph	229	1.0	-	-	-	-	-	-	-
	Fmorph	212	-	184	NA	NA	602	146	80	20
22	Tmorph	13	1.9	-	-	-	-	-	-	-
	Fmorph	7	-	14	NA	23	486	27	<5	<5
23	Tmorph	1965	1.1	-	-	-	-	-	-	-
	Fmorph	1756	-	1942	610	2529	4314	1595	2800	356
	6-MAM	-	-	-	-	156	-	-	-	-
24	Tmorph	323	1.3	-	-	-	-	-	-	-
	Fmorph	250	-	NA	90	NA	NA	51	NA	20
25	Tmorph	432	1.4	-	-	-	-	-	-	-
	Fmorph	300	-	NA	688	NA	385	556	NA	68
	6-MAM	-	-	NA	-	NA	-	240	NA	-
26	Tmorph	180	1.6	-	-	-	-	-	-	-
	Fmorph	110	-	1268	340	NA	NA	1790	NA	NA
	6-MAM	-	-	-	-	NA	NA	1038	NA	NA
27	Tmorph	28	1.4	-	-	-	-	-	-	-
	Fmorph	20	-	40	NA	578	263	145	<5	<5
	6-MAM	-	-	-	NA	1406	-	147	-	-
28	Tmorph	111	2.8	-	-	-	-	-	-	-
	Fmorph	40	--	104	740	974	>5000	NA	24	64
29	Tmorph	300	1.7	-	-	-	-	-	-	-
	Fmorph	177	-	445	NA	1245	>5000	NA	20	NA
30	Tmorph	304	1.1	-	-	-	-	-	-	-
	Fmorph	265	-	NA	128	NA	NA	24	68	52
33	Tmorph	129	2.2	-	-	-	-	-	-	-
	Fmorph	60	-	NA	NA	NA	>5000	NA	28	24

Blood quoted reference ranges for morphine are: therapeutic 10-100ng/mL, toxic 50-400ng/mL and lethal >100ng/mL (Weink et al., 2001; Schulz and Schmoldt, 2003; Musshoff et al., 2004). Morph = morphine, Cod = codeine and 6-MAM = 6-monacetylmorphine. FBtmorph/FBfmorph = femoral blood total to free morphine concentration ratio.

Table 34 (b): Femoral blood concentrations for other associated drugs of abuse

Case	Associated drugs of abuse concentration (ng/mL) in femoral blood. Codeine was present in all cases see Table 35
3	Diazepam 27 and ethanol 100mg/dl (cardiac blood)
10	Diazepam 219 and ethanol 20mg/dl
16	Cocaine 5, methadone 18 and diazepam 9
20	Diazepam 51
21-33	No other drug was found

**Quoted reference ranges*

(Weink et al., 2001; Schulz and Schmoldt, 2003; Musshoff et al., 2004)

Drug	*Therapeutic (ng/mL)*	*Toxic (ng/mL)*	*Lethal (ng/mL)*
Methadone	*50-750*	*200-2000*	*>400*
Diazepam	*20-4000*	*3000-5000*	*>30000*
Cocaine	*50-300*	*500-1000*	*>4000*

**These references for guidance alone and drug interpretation should take in account with patient full drug history.*

Table 35: Total and free femoral blood codeine concentrations (ng/mL) and concentration ratio of total to free morphine.

Case	Total codeine	Free codeine	Codt/ Codf
3	16	9	1.8
10	23	9	2.6
16	32	28	1.1
20	14	13	1.1
21	28	16	1.7
22	85	74	1.1
23	35	28	1.3
24	109	90	1.2
25	126	47	2.7
26	90	35	2.6
27	10	8	1.3
28	13	8	1.6
29	14	13	1.1
30	27	25	1.1
33	25	16	1.6

Blood quoted reference ranges for codeine are: therapeutic 30-250ng/mL, toxic 500-1000ng/mL and lethal >1600ng/mL (Weink et al., 2001; Schulz and Schmoldt, 2003; Musshoff et al., 2004). Codt/Codf = total to free codeine concentrations ratio.

Seven cases had femoral blood free morphine concentration within the quoted potentially lethal range (>100ng/ml) a range which overlaps with the toxic range. In the remaining cases the femoral blood free morphine concentration ranged from 7-86ng/ml. Femoral blood free morphine levels greater than 50ng/ml are thought to be within the quoted toxic range. However, as shall be discussed later, the interpretation of blood morphine levels required a knowledge of the subject's drug history as toxicity depends upon the degree of tolerance that has been required (Drummer, 2001). The wide range of femoral blood morphine was observed in a previous study of 21 heroin related deaths where free morphine concentrations ranged from 8 to 1539ng/mL (Bogusz et al, 1997). Heroin related deaths are not necessarily restricted to intravenous users where very high blood morphine levels may be achieved but may also occur after both intranasal and oral administration (Darke and Ross, 2000). The different routes of morphine administration together with the degree of tolerance may explain the wide range of morphine concentrations associated with fatalities in heroin overdose cases (< 25 to 4700ng/mL) (Logan et al., 1987; Levine et al, 1995), concentrations similar to those found in this thesis. There are even cases of heroin related deaths where no morphine was detected in the blood and this thought be due to sudden death after heroin administration and before heroin had time to circulate and distribute in blood (White, 2004).

In addition to morphine and codeine, the other drugs found included diazepam (4 cases), cocaine and methadone (1 case) and ethanol (2 cases) [Table 34 (b)]. It has been reported that administration of morphine with diazepam enhances the pharmacological effect of morphine which was also found in methadone treated individuals who were administered diazepam (Stitzer et al., 1981). Co-

administration of diazepam with opioids such as methadone and morphine particularly enhances their toxic effect on respiration As mentioned earlier that cocaine and heroin are often used together and named 'speedball', this combination enhances the effects of both drugs than when administered individually (Negus et al., 1998). When ethanol is administered with heroin the respiratory depression effects are enhanced. Studies of opioids drug related deaths in combination with ethanol showed a decrease in the minimum fatal dose of opioids by up to 50% when ethanol concentration was >200mg/dL while other studies suggested that when blood ethanol level is >100mg/dL the toxicity of opioids may be enhanced (Ruttenber et al., 1990; Drummer, 2001). Another study found that even small amounts of ethanol may enhance opioid toxicity and this risk increases when ethanol concentration is >300mg/dL (Levine et al., 1995).

The concentration ratios of femoral blood total to free morphine ranged from 1.1:1 to 3.2:1 [Table 34 (a)]. The advantage of using blood total morphine (total morphine = free morphine + morphine glucuronide) to free morphine ratio is that it gives an idea of the time interval between administration of heroin and time of death or blood sampling. Usually 15 and 60 minutes after administration the concentration ratios of total morphine to free morphine are typically 4:1 and 9:1 if given intravenously while this ratio was found to be 2:1 and 5:1 respectively if given intramuscularly (Boerner, 1975).

A previous study of 43 heroin related deaths found the concentration ratio of total to free morphine ranged from 1:1 to 2:1 when death occurred in a relatively short period after heroin administrations (3 hours) while this ratio ranged from 2.5:1 to

4.6:1 when death occurred after long period since heroin administration (Staub et al., 1990). In this study, the ratio of femoral blood total to free morphine ranged from 1:1-3.2:1 in all cases [Table 34 (a)]. This implies deaths relatively soon after administration of morphine/heroin. Usually 6-MAM can be detected in the urine sample within 2-8 hours following heroin administration and remains detectable for 12-24 hours also depending on the dose administered (Cone et al., 1991; O'Neal and Polkis, 1997; Karch, 2002).

6-MAM was present in stomach contents samples for cases 3, 10, 23 and 27 at concentrations ranging from 156 to 1406ng/mL while it was present in the urine samples for cases 10, 16, 20, 26 and 27 at concentrations ranging from 80 to 1953ng/mL [Table 34 (a)]. The presence of 6-MAM in urine indicates recent administration of heroin/diacetylmorphine. However, due to the short half-life of 6-MAM in blood it may be detected in stomach contents and urine (Moriya and Hashimoto., 1997). It was found that 6-MAM can be measured in stomach contents samples stored in a freezer for 6 years. It was also suggested that the presence of 6-MAM in stomach contents may be due to oral administration of heroin or due to early gastric bleeding after heroin administration (Collins, 2007). However, no previous studies were found to give an indication as to the stability of 6-MAM in both stomach contents and urine at the storage conditions normally used for forensic cases.

Cardiac blood free morphine concentrations were above the femoral blood levels in most cases and the cardiac to femoral blood morphine concentration ratios ranged between 0.9:1-3.2:1 (Table 36). Diffusion/redistribution of drugs from stomach to

Table 36: Concentration ratios to femoral blood morphine in all samples

Case	FBmorph	CB/FB	VH/FB	S/FB	Bile/FB	U/FB	L/FB ml/g	M/FB ml/g
3	86	1.1	NA	17	19.9	NA	0.7	0.3
10	56	3.2	0.8	6.7	3.5	2.2	NA	0.5
16	48	1.8	1.3	2.5	>104	27	0.7	0.5
20	46	2.8	NA	14	83.5	56	1.4	0.6
21	212	0.9	NA	NA	2.8	0.7	0.4	0.1
22	7	2	NA	3.3	69	3.9	<0.7	<0.7
23	1756	1.1	0.4	1.4	2.5	0.9	1.6	0.2
24	250	NA	0.4	NA	NA	0.2	NA	0.1
25	300	NA	2.3	NA	1.3	1.9	NA	0.2
26	110	11.5	3	NA	NA	16.3	NA	NA
27	20	2	NA	28	13	7.3	<0.3	<0.3
28	40	2.6	18.5	24	>195	NA	0.6	1.6
29	177	2.5	NA	7	>28.2	NA	0.1	NA
30	265	NA	0.5	NA	NA	0.1	0.3	0.2
33	60	NA	NA	NA	>83.3	NA	0.5	0.4

FBmorph = femoral blood morphine concentration (ng/ml)

the cardiac blood has been observed in previous studies (Pounder et al., 1996; Pelissier-Alicot et al., 2003).

Vitreous humour free morphine levels varied in relation to its femoral blood concentration. The vitreous humour to femoral blood morphine concentration ratios ranged from 0.4:1-3.0:1 except in one case (28) where the ratio was 18.5 (Table 36). It was suggested that the free morphine concentration in vitreous humour depends on when morphine was administered. It has been suggested that a high concentration ratio of vitreous humour to femoral blood morphine is due to the short survival time after heroin/diamorphine administration (Pragst et al., 1995). A previous study of 154 post-mortem cases found that survival time significantly influenced vitreous humour to blood morphine concentration. When the survival time was >5 hours the free morphine concentration in the vitreous humour was lower than in the blood whilst the concentration was higher in the vitreous humour when death occurred rapidly after its administration (Pragst et al., 1995). However, the possibility of drug top-up leading to accumulation of free morphine in the vitreous humour cannot entirely be discounted. Another study of 11 post-mortem cases found that the vitreous humour to the femoral blood morphine concentration ratios ranged from 0.03:1-6:1 (Ziminski et al., 1984).

Stomach contents morphine concentrations were higher than femoral blood levels in all the cases. The stomach contents to femoral blood morphine concentration ratios ranged from 1.4:1-28:1 (Table 36). As mentioned earlier, the presence of drugs in the stomach contents may indicate oral administration or gastric secretion. However, low stomach contents to femoral blood drug concentration ratios may indicate

passive diffusion from the blood to the stomach content and may not indicate ante-mortem oral ingestion (Kerrigan et al., 2004; Karch, 2007).

In general, morphine concentrations were much higher in bile compared to femoral blood morphine levels and the bile to femoral blood morphine ratios ranged from 1.3:1-195:1 (Table 36). The high drug level in bile compared to femoral blood levels were observed in previous studies. The mean concentration ratio of bile to femoral blood morphine was 159 in 7 post-mortem cases (Vanbinst et al., 2002). Morphine metabolism occurs in the liver through a glucuronidation process where morphine is conjugated to glucuronic acid to form morphine 6-glucuronide and morphine 3-glucuronide which all accumulate in the bile at high concentrations. There is even a case report of a heroin related death where the individual had survived for 144 hours; heroin was detected in bile but not in urine (Tassoni et al., 2007).

The urine to femoral blood morphine concentration ratios ranged from 0.1:1 to 56:1 (Table 36). Morphine can be detected in a urine sample from a few hours to several days after administration (Mitchel et al., 1991; Cone et al., 1996; Karch, 2007). As mentioned earlier in Chapter One, 90% of the administered dose of morphine is excreted in urine within 72 hours from administration. Of the administered dose, about 10% is excreted as free morphine. The wide concentration ratios in this study may be due to different administered doses of heroin/diacetylmorphine and the survival times. The varied concentration ratios of urine to femoral blood morphine were observed in previous studies (Levine et al., 2002; Cengiz et al., 2006).

In general, the free morphine concentrations in the liver tissue were higher than those in muscle [Table 34 (a)]. The liver to femoral blood free morphine concentration ratios ranged from 0.1:1-1.6:1 and for muscle the ratios were 0.1:1-1.6:1 (Table 36). Interpretation of drug level in liver and muscle samples is very difficult as published lethal concentrations are limited. A study of 10 heroin deaths found that the mean liver morphine concentration was 3000ng/g whilst the corresponding blood morphine mean concentration was 700ng/mL (Felby et al., 1974). A previous study of two morphine related fatalities where morphine was administered by oral and intravenous routes the liver morphine concentrations were 700ng/g and 290ng/g and blood levels were 350ng/mL and 70ng/mL ml respectively (Chan et al., 1986). A previous study of a fatal morphine overdose found that blood morphine level was 8000ng/mL with corresponding liver level of 6000ng/g but it was not clear whether it was total or free morphine which had been measured (Cravey, 1985). However, the relationship of liver to blood morphine concentration was estimated in a study of 200 morphine overdose cases and it was shown that the femoral blood morphine levels were >250ng/mL and the concentrations in the liver were >500ng/g (Spiehler, 1989). Except for case 23 where the liver free morphine concentration was 2800ng/g, in the remaining cases where liver morphine was measured, the concentrations were less than 80ng/g [Table 34 (a)]. However, the suspected contributor to the cause of death in case 23 was given as myocardial infarction and was not due to drug toxicity.

The correlations of free morphine concentrations in different body compartments in relation to femoral blood levels are given in Table 37. There was a significant correlation between the femoral blood morphine concentrations and the levels in cardiac blood, stomach contents, liver and muscle samples.

Table 37: Correlations of free morphine concentrations in different body compartments in relation to femoral blood levels.

Sample	Pearson correlation coefficient (r)	P value
Cardiac blood	0.84	0.001
Vitreous humour	0.37	0.36
Stomach contents	0.82	0.007
Bile	0.20	0.53
Urine	0.23	0.49
Liver	0.99	<0.001
Muscle	0.98	<0.001

P value is significant <0.05

Codeine was measured in all cases (Table 35). The femoral blood total to free codeine concentration ratios ranged from 1.1 to 2.7 (Table 35).

The total codeine levels ranged from 10 to 126ng/ml and the free codeine concentrations ranged from 8 to 90ng/ml. Codeine is a weak opiate analgesic and usually detected in the urine and blood samples in heroin abusers as it is derived from acetylcodeine, a contaminant present in street heroin. The presence of codeine is common in heroin abuser cases and it was present in 96% of heroin related deaths (Meadway et al., 2002; Fugelstad et al., 2003; Drummer, 2004). Codeine may also

be used therapeutically as a pain killer, and so not be present due to heroin abuse; such as in three cases (21, 22 and 23).

Codeine may enhance the opioid effects of morphine. A previous study of 39 fatalities who had taken a combination of codeine and other drugs found that total blood codeine ranged from 100 to 8800ng/mL (Nakamura et al., 1976). Another study of 8 codeine overdose cases found that total blood codeine levels ranged from 1400 to 5600ng/mL (Wright et al., 1975). Another study of 107 cases, in six cases where codeine was considered to have contributed to the causes of death, the concentration ratio of total to free codeine ranged from 2.9 to 5.3 whilst in the remaining cases (101) where codeine was involved, showed the ratio to be 2.0 to 2.9 (Gerostamoulos et al., 1996). Therefore, the ratios found in this thesis are similar to those reported previously.

Free morphine and 6-MAM were also measured in hair samples (Table 38). It was clear that 6-MAM concentrations in different hair segments were higher than morphine levels. This observation was found in previous studies (Goldberger et al., 1991; Moeller et al., 1993). It is well known that more lipophilic drugs are readily incorporated into hair; 6-MAM is more lipid soluble than its metabolite morphine which may explain its high levels in different hair segments (Brunton et al, 2007).

The mean 6-MAM to free morphine concentration ratios ranged from 2.7:1-13.7:1 (Table 38). It was suggested that when the 6-MAM to morphine concentration ratio in hair is >7:1, this indicates chronic heroin abuse (Goldberger et al., 1991). Another

study found that 6-MAM to morphine concentration ratios ranged from 1.3:1 to 10:1 (Moeller et al., 1993).

Table 38: 6-MAM and morphine levels in the different hair segments (ng/mg).

Case	Drug	Hr	H2	H3	Mean 6-MAM/Morph
	6-MAM	32	64	48	
3	Morph	5	7	6	7.8
	6-MAM/Morph	6.4	9.1	8	
	6-MAM	37	75	52	
10	Morph	16	27	18	2.7
	6-MAM/Morph	2.3	2.8	2.9	
	6-MAM	40	52	49	
16	Morph	4	7	5	7.5
	6-MAM/Morph	10	7.4	9.8	
	6-MAM	43	37	68	
20	Morph	5	5	13	7.1
	6-MAM/Morph	8.6	7.4	5.2	
	6-MAM	80	53	47	
24	Morph	3	9	7	13.1
	6-MAM/Morph	26.7	5.9	6.7	
	6-MAM	32	47	51	
25	Morph	3	3	6	11.6
	6-MAM/Morph	10.7	15.7	8.5	
	6-MAM	23	39	31	
26	Morph	3	4	3	9.2
	6-MAM/Morph	7.7	9.7	10.3	
	6-MAM	40	48	60	
27	Morph	3	3	5	13.8
	6-MAM/Morph	13.3	16	12	
	6-MAM	62	144	135	
28	Morph	21	33	39	3.6
	6-MAM/Morph	2.9	4.4	3.5	
	6-MAM	36	29	68	
33	Morph	8	9	5	7.1
	6-MAM/Morph	4.5	3.2	13.6	

Hr = hair root segment, H2 = hair middle segment, H3 = hair peripheral segment.

The concentrations of 6-MAM in different hair segments ranged from 23-144ng/mg. The level of 6-MAM in hair is thought to give an idea of the heroin abuse habit. For instance, when hair 6-MAM level <2ng/mg this indicates low heroin abuse and for moderate heroin abuser this level ranged from 2 to 10ng/mg hair whilst levels >10ng/mg indicate high heroin abusers (Pepin and Gaillard, 1997).

In all cases morphine and 6-MAM were below detectable limits in different rib bone sections. Again, there is limited data available about 6-MAM and morphine distribution in human bone. However, morphine (concentration range 155-340ng/g) was detected in human thigh bone including its marrow (Raikos, 2001) whilst 6-MAM (range 36.5-570ng/g) and morphine (range 8.7-155ng/g) were detected in teeth of heroin abuse cases (Pellegrini, 2006).

3.3.4 Summary of morphine results

Morphine concentration in different body compartments may also affected by post-mortem diffusion/redistribution as well as the survival time after heroin/diamorphine administration. Except for one case where the cardiac blood morphine concentration was 1268ng/ml with corresponding femoral blood morphine level of 110ng/ml the morphine concentration ratios to femoral blood in the remaining cases ranged: cardiac blood 0.9-3.3, vitreous humour 0.4-18.5, bile 1.3->195 and for urine 0.1-56. The median liver tissue to femoral blood morphine concentration ratio was 0.6 and for muscle was 0.3 in ml/g.

3.4 Summary of the Findings

In this study, among the 95 suspected drugs related death cases, ethanol was found in 11%, cocaine 10%, methadone 8%, and 6-MAM and morphine in 15%. In addition, the other drugs which were detected by GC/MS included: diazepam (8%), paracetamol (24%), codeine (15%), dothiepin (6%), mirtazapine (8%), loxapine (3%), orphenadrine (5%) and tramadol (3%). The diazepam concentrations (range 9-220ng/mL) were either sub-therapeutic or at the lower limit of the quoted therapeutic range (therapeutic range 20-400ng/mL).

This study aimed to investigate the distribution of drugs of abuse in the different body compartments and their relationship to toxicity. The median concentration ratios and ranges of drugs of abuse in different post-mortem compartments in relation to femoral blood levels are given in Table 39.

The cardiac to femoral blood ethanol median concentration ratio was 1.9:1 and ranged from 0.8:1-2.7:1 in most cases, except in three where the ratio was highly elevated (14.3:1-42.3:1). Some of the high cardiac to femoral blood ratios could be explained by post-mortem ethanol formation and redistribution/diffusion but it is also likely that evaporation of ethanol from the femoral blood tube could in part account for the high ratios. Vitreous humour to femoral blood ethanol median concentration was 1.6:1 and range 0.2:1-2.9:1 except for one case where the ratio was 9.4:1. Again, evaporation of ethanol from the femoral blood tube could explain this high ratio. The femoral blood tube was the one most used and to have been opened more often which would allow ethanol to escape/evaporate. The median ethanol ratio in

Table 39: A summary of the median concentration ratios and ranges of drugs of abuse in different post-mortem body compartments in relation to femoral blood (for liver and muscle the units are mL/g)

Drug	Cardiac blood	Vitreous humour	Bile	Urine	Liver	Muscle
Ethanol	1.9:1 (0.8:1-42.3:1)	1.6:1 (0.7:1-9.4:1)	11.3:1 (1.0:1-34:1)	6.5:1 (1.7:1-153:1)	*BDL	*BDL
Cocaine	1.2:1 (1.0:1-7.0:1)	1.4:1 (1.0:1-3.3:1)	7.0:1 (0.6:1-41.6:1)	4.9:1 (0.4:1->1000:1)	1.0:1 (0.2:1-1.4:1)	0.7:1 (<0.1:1-<1.0:1)
Benzoylecgonine	1.2:1 (0.3:1-2.3:1)	1.7:1 (0.4:1-8.2:1)	4.5:1 (1.1:1-318:1)	7.9:1 (1.1:1->1000:1)	0.2:1 (<0.1:1-1.5:1)	0.2:1 (<0.1:1-1.4:1)
Methylecgonine	1.2:1 (0.6:1-9.6:1)	1.0:1 (0.2:1-1.8:1)	5.0:1 (0.6:1-83.4:1)	19.7:1 (1.2:1-45.8:1)	0.3:1 (0.1:1-<1.0:1)	0.3:1 (0.1:1-<1.0:1)
Methadone	1.4:1 (0.6:1->3.9:1)	0.7:1 (0.04:1-0.99:1)	2.9:1 (0.7:1->9.6:1)	0.7:1 (0.3:1->3.9:1)	0.3:1 (0.2:1-0.4:1)	0.1:1 (0.05:1-0.3:1)
EDDP	1.3:1 (0.2:1-38.4:1)	0.3:1 (0.1:1-18.6:1)	10.8:1 (0.3:1->357.1:1)	3.3:1 (0.002:1-20.3:1)	0.1:1 (0.01:1-3.1:1)	0.1:1 (0.01:1-2.0:1)
Morphine	2.0:1 (0.9:1-11.5:1)	1.0:1 (0.4:1-18.5:1)	24:1 (1.3:1->195:1)	2.2:1 (0.1:1-56:1)	0.6:1 (0.1:1-1.6:1)	0.3:1 (0.1:1-1.6:1)

*BDL = below detectable limit

bile was highest (11.3:1) but ranged 1.0:1-34:1. The high water content in bile may be associated with high ethanol concentration in this compartment.

Urine to femoral blood ethanol ratios varied widely (1.7:1-153:1:1). In 4 of the subjects where urine samples were available the ratios ranged 1.7:1-3.1:1. In the remaining 5 cases the ratios ranged from 4.4:1-153:1. These remaining 5 cases included two poorly controlled diabetics and one subject with glucose intolerance. The high urine to femoral blood ethanol ratios in this study (>4.4:1) may in part be attributed to diabetes (i.e. glucose in urine may be converted to ethanol by bacteria), delayed emptying of urinary bladder whilst blood ethanol levels decline and as mentioned above, evaporation of ethanol from the femoral blood tube.

Ethanol concentrations were below detectable limits in both liver and muscle tissues. This was not entirely unexpected, as both samples are known to have low water content and are thus unsuitable for the determination of ethanol levels in post-mortem cases.

The cardiac to femoral blood cocaine median concentration ratio was 1.2:1 and except for one case with a ratio of 7.0:1, the ratios for the remaining cases ranged from 1.0:1-2.0:1. It is worth noting that in 50% of subjects, the femoral blood cocaine was <10ng/mL. Hydrolysis of cocaine in blood to its metabolites continues in the post-mortem period. The vitreous humour to femoral blood cocaine median concentration ratio was 1.4:1 (range 1.0:1-3.3:1). As stated above, continued hydrolysis of cocaine in blood but not in vitreous humour, may continue in the post-mortem period which may lead to high ratios.

Bile to femoral blood concentration ratios also varied widely (0.6:1-41.6:1). In addition to the continued metabolism of cocaine in blood in the post-mortem period, cocaine is known to be hydrophobic and therefore its concentration in bile will also be influenced by the fat content of this compartment. The cocaine level in urine in relation to femoral blood showed the greatest variation (0.4:1->1000:1), compared to other fluid compartments. Urine is the major route of excretion of cocaine and its concentration in urine will also be influenced by urine concentration/volume.

The concentration ratios of cocaine in liver were higher than in muscle, median 1.0:1 (range 0.2:1-1.4:1) and 0.7:1 (range <0.1:1-<1.0:1) respectively. In general cocaine and its metabolites are known to be at higher concentration in liver compared to muscle which has been explained by high blood volume in the liver.

In most cases, the concentrations of cocaine metabolites benzoylecgonine and methylecgonine were higher than that of cocaine due to their longer half lives and the continued hydrolysis of cocaine in blood in post-mortem period. The median ratios for benzoylecgonine were 1.2:1, 1.4:1, 7.0:1 and 4.9:1 and for methylecgonine were 1.2:1, 1.0:1, 5.0:1 and 19.7:1 for cardiac blood, vitreous humour, bile and urine respectively, in relation to femoral blood.

The cardiac to femoral blood methadone median concentration ratio was 1.4:1 (range 0.6:1-3.7:1) except for a single case with a ratio >3.9:1. The median concentration ratio of vitreous humour methadone in relation to femoral blood was 0.7:1 and

except for a single case with a ratio of 0.04:1 in the remaining cases the ratios ranged from 0.4:1-1.0:1.

The concentration of methadone in bile also tends to be higher than that in blood or vitreous humour with a median concentration ratio of 2.9:1 (range 0.7:1->9.6:1) in relation to femoral blood. Similar to cocaine, methadone is also lipid soluble and its concentrations will be influenced by the lipid content of bile. The median ratio for urine was 0.7:1 (range 0.3:1->3.9:1). The concentrations of methadone in urine are influenced by several factors including urine pH, urine volume and other co-administered drugs such as cocaine which enhances methadone excretion.

The concentrations of methadone in liver were much higher than that in muscle (1.5-8 folds higher in most cases). The median concentration ratio in liver was 0.3:1 (range 0.2:1-0.4:1) and for muscle 0.1:1 (range 0.05:1-0.3:1) in relation to femoral blood respectively. Again this has been explained as to be due to the increased blood flow to the liver. The median concentration ratios for EDDP were for cardiac blood 1.3:1, vitreous humour 0.3:1, bile 10.8:1 and for urine 3.3:1.

For the subjects studied in this thesis, the concentrations of morphine in cardiac blood tended to be higher than those in vitreous humour with median concentration of 2.0:1 (range 0.9:1-11.5:1) and 1.0:1 (range 0.4:1-18.5:1) respectively in relation to femoral blood. Morphine has V_d of 1-6L/kg which implies that it is concentrated in solid organs and is more liable to PMDR and together with diffusion from stomach may lead to its increased concentration in cardiac compared to femoral blood. Low vitreous humour to femoral blood morphine concentration ratios are also known to

occur if the survival time is long (>5 hours) after administration of morphine. Bile has the highest concentrations of morphine with ratios ranging from 1.3:1->195:1 and similarly the urine has the second highest concentrations (range 0.1:1-56:1).

Again, the concentration of morphine tends to be higher in liver compared to muscle with median concentration ratios of 0.6:1 and 0.3:1 respectively in relation to femoral blood.

In the majority of cases there was a good correlation between femoral blood levels and the concentration in other body compartments. This study has added to our knowledge and understanding of the usefulness of using alternative post-mortem specimens to study the distribution of drugs of abuse in different body compartments and how the concentrations relate to femoral blood levels and toxicity.

It is worth noting that the reference ranges quoted in this thesis are for guidance only and they are compiled from published case reports. However, the sources of the post mortem blood samples used in the literature are not always defined. The use of femoral blood samples will reduce but not eliminate post-mortem drug metabolism and redistribution. Samples taken from other sites of the body, as demonstrated in this study, may give variable results. Therefore, interpretation of post-mortem drug levels ideally should take into account the possible effects of drug metabolism, redistribution, tolerance and drug-drug interaction. Conclusions on drug toxicity should not be based on post-mortem drug levels alone.

3.5 Future Work

This study has added to our knowledge and understanding of the usefulness of using alternative post-mortem specimens to study drugs of abuse distribution in different body compartments and know how the concentrations relate to femoral blood levels and toxicity.

Due to constraints of time and resources, it was not possible to investigate many other areas of research which would have added more to the understanding and the interpretation of post-mortem drug levels and toxicity.

1. Although the post-mortem intervals were relatively short in the study, the exact times were not recorded and without this information it is not possible to fully assess PMDR. The other factors that would influence PMDR e.g. body tolerance, storage conditions, route of administration of drugs etc, were not assessed in this study.
2. The study would have benefited from increased number of subjects in order to ensure statistical validation of the results.
3. This study focused on drugs of abuse but it would be useful to use a similar approach to investigate other classes of drugs known to cause toxicity and death.
4. Much of our understanding of drug metabolism and toxicity has been on animal studies. Notwithstanding the ethical considerations, using animals would allow for more controlled research in terms of drug/s route used, duration of administration and assessment of the post-mortem conditions.

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APPENDIX II

Details of the cases with medical history, post-mortem findings and suspected causes of death

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Case	Suspected cause of death	Gender	Age (year)	Medical History	Post-mortem finding	Missing samples
1	Pneumonia	M	30	Drug abuser, sudden death in public path	Brain oedema, lung oedema, fresh injection right vein	NO
2	Drowning	M	61	Abuse of alcohol	Sign of chronic alcoholism[fatty liver]	U
3	Acute intoxication	M	52	Sudden death, he sniffed heroin	Brain and lung oedema, bronchitis	NO
4	Suspicion of intoxication, Myocardial infarction	M	47	History of IV Drug Abuse	Arterio/coronary sclerosis, COP and lung oedema.	VH, Bile
5	Suspicion of intoxication	F	28	IV-drug abuser, under methadone therapy	No pathological finding	U, VH
6	Acute infection of respiratory system	M	41	Alcoholism	Acute haemorrhagic inflammation of the respiratory system, fatty liver.	NO
7	Suspicion of intoxication	M	42	Alcohol and drug abuser	Brain oedema, fatty liver, fibrosis of pancreas and COPD	NO
8	Acute intoxication	M	42	Drug abuser found dead after leaving drug therapy	Old and fresh needle punctures, lung and brain oedema and acute bronchitis.	Bile
9	Suspicion of intoxication	M	41	Drug abuser, history of depression	Old and fresh needle punctures, lung and brain oedema	NO
10	Suspicion of overdose, acute intoxication	M	42	Drug abuser	Needle punctures, scared veins, liver cirrhosis.	NO
11	Gastrointestinal bleeding	M	44	Alcoholism, epileptic seizures	Liver cirrhosis, gastrointestinal bleeding	CB, Bile

Case	Suspected cause of death	Gender	Age (year)	Medical History	Post-mortem finding	Missing samples
12	Pneumonia	M	43	Sudden death no medical history	Oedema of brain, endocarditis	NO
13	Exsanguinations	M	41	Drug abuser	Signs of chronic IV- drug abuse [needle puncture, scars], open apses in right inguinal region and fatty liver	NO
14	Suspicion of intoxication	M	40	Found dead at home known as drug abuser	Brain oedema and lung oedema	SC
15	Pneumonia	F	39	Drug abuser	Pneumonia, enlargement of liver and spleen.	VH
16	Suspicion of acute intoxication	M	43	Drug abuser	Brain and lung oedema	NO
17	Suspicion of intoxication	M	41	Drug abuser	Gastrointestinal haemorrhagic, spleen rupture.	VH, SC, U
18	Suspicion of intoxication	M	47	Drug abuser	Signs of intoxication, brain and lung oedema, fresh needle puncture	SC, L, M
19	Suspicion of intoxication	M	38	Drug abuser	Needle puncture, brain and lung oedema	CB, SC, L
20	Acute intoxication	M	27	Drug abuser	Signs of intoxication, brain and lung oedema, fresh needle puncture	VH

Case	Suspected cause of death	Gender	Age (year)	Medical History	Post-mortem finding	Missing samples
21	Coronary thrombosis	M	80	Diabetic, COPD	Middle grade arteriosclerosis, coronary fibrosis, lung emphysema.	NO
22	Trachea bronchitis and cerebral haemorrhage	M	61	History of dyspnoea.	Craniocerebral trauma, subarachnoid bleeding	NO
23	Myocardial infarction	M	61	Bronchogenic carcinoma.	Middle-high grade arterio/coronary sclerosis, old and fresh MI	NO
24	Suspicion of epileptic seizure, intoxication	F	33	Known multiple sclerosis	Brain and lung oedema.	U
25	Acute intoxication	M	30	IV-Drug abuser, hepatitis C	Signs of acute intoxication, fresh needle punctures, brain oedema and enlargement of liver and spleen	VH, H, Bile
26	Suspicion of intoxication	M	44	HIV +, drug abuser	Needle puncture, scars, lung and brain oedema	NO
27	Suspicion of intoxication	M	43	Drug abuser	Needle puncture, scars, lung and brain oedema	CB,SC,BIL
28	Suspicion of intoxication	M	39	Drug abuser	Signs of intoxication	CB, VH, SC, U
29	Suspicion of intoxication	M	38	Drug abuser	Needle puncture, brain and lung oedema	CB, SC, L
30	Suspicion of intoxication	M	33	IV- drug abuser	Fatty liver, lung and brain oedema	SC, Bile, L, M
31	Coronary fibrosis	M	80	Lung emphysema Pneumonia, enlargement of liver and spleen.	Middle grade arteriosclerosis, coronary fibrosis,	NO

Case	Suspected cause of death	Gender	Age (year)	Medical History	Post-mortem finding	Missing samples
32	Insufficiency of right heart due to COPD	F	80	History of COPD, carcinoma of breast	COPD, acute Bronchitis, gastric ulcer.	U
33	Suspicion of intoxication	M	41	Drug abuser	Needle puncture, fatty liver	CB, SC, BILE, L
34	Subarachnoid bleeding	M	47	Alcoholism	Fatty liver, fracture of the skull	U
35	Poly trauma [suicide]	F	64	Depression	Multiple fractures of extremities and ribs, skull fracture.	NO
36	Cardiac decompensation/ suspicion of arrhythmia	M	46	Hypertension, strong smoker	Heart hypertrophy and dilatation, lung and brain oedema, COPD, goitre.	H, L, VH
37	Strangulation/hanging	M	24	No further history known	Hanging, Petechia in skin of face/ tongue haemorrhage, lung oedema.	NO
38	Cerebral haemorrhage	M	66	Found dead at home, History of diabetes & arterial fibrillation.	Arterio and coronary sclerosis, heart hyper trophy, cerebral haemorrhage, COPD, lung and brain oedema.	VH
39	Bronchial Carcinoma	M	76	Found dead at home, Larynx carcinoma.	Bronchial carcinoma, Infiltration of thorax, no metastasis, COPD.	VH
40	Myocardial infarction	M	39	Died during dialysis, History of chronic renal disease, HT.	Arterio/coronary sclerosis, fresh MI, COPD, nephron-cirrhosis	NO

Case	Suspected cause of death	Gender	Age (year)	Medical History	Post-mortem finding	Missing samples
41	Suspicion of arrhythmia	F	67	Abuse alcohol	Fatty liver, beginning cirrhosis.	Bile
42	Strangulation/hanging	M	46	Electrical cable around neck.	Strangulation with petechiae in skin of face, acute lung emphysema	NO
43	Myocardial infarction	F	66	Possible homicide	old and fresh MI, COPD	U, Bile
44	Myocardial infarction	F	83	Hypertension	COPD, brain atrophy, fibrosis of pancreas, liver congestion.	U
45	Myocardial infarction	M	40	Drug abuser	Fatty liver, enlargement LN and spleen, brain oedema, haemorrhages in brain and MI	NO
46	Coronary thrombosis	M	49	Breast pain prior to death	Coronary thrombosis, middle grade sclerosis, old MI, liver hypertrophy.	H
47	Myocardial infarction	M	65	Collapsed dead shortly after visit medical doctor.	Fresh myocardial infarction, carcinoma of pancreas	U
48	Gastrointestinal bleeding	M	40	Found dead, near body 10 bottles of vodka.	Fatty liver, sign of blood loss, fibrosis of pancreas.	NO

Case	Suspected cause of death	Gender	Age (year)	Medical History	Post-mortem finding	Missing samples
49	Myocardial infarction	F	83	History of Angina Pectoris	High grade of arterio/ coronary sclerosis & fresh MI	NO
50	Suspicion of Myocardial infarction	M	59	Abuse of alcohol and nicotine	Fatty liver, liver cirrhosis and suspicion of MI	NO
51	Myocardial decompensation	F	93	Found dead in bathroom	MI, COPD, high grade arteriosclerosis	VH
52	COR pulmonare	F	72	Found dead at home	COPD, cystic goitre	VH
53	Shock due to illness/ peritonitis	M	70	Endoscopic surgery due to inguinal hernia	Peritonitis, COPD	NO
54	Craniocerebral trauma	M	85	Suicidal leap from Balcony	COPD, high grade arteriosclerosis, multiple injuries, fracture of the skull, ribs and spine	VH, L
55	Suspicion of heart/lung disease, intoxication	M	78	No medical history known.	High grade arterio/coronary sclerosis	NO
56	Pneumonia	F	59	Known COPD	Pneumonia, COPD, hypertrophy of heart	VH
57	Gastrointestinal bleeding	F	64	History of stomach pain	Gastric ulcer, COPD, lung emphysema.	U, Bile
58	Epileptic seizure, intoxication	M	32	History of epilepsy	Brain and lung oedema.	VH, H
59	Heart decompensation	F	96	Depression	High grade arterio/coronary sclerosis, COPD.	U

Case	Suspected cause of death	Gender	Age (year)	Medical History	Post-mortem finding	Missing samples
60	Myocardial infarction	M	61	Hepatitis, collapse while plying Tennis.	Middle grade arterio /coronary sclerosis, COPD, liver necrosis	NO
61	Coronary thrombosis	F	86	Alzheimer disease	COPD, hypertrophy of heart, small coronary thrombosis	U, SC
62	Suspicion arrhythmia	M	64	Found dead at home, No medical History	COPD, middle- high grade arterio/coronary sclerosis.	NO
63	Cardiac decompensation /suspicion of angina	F	39	Nicotine abuse	lung oedema	NO
64	Coronary thrombosis	M	88	Found dead in bathroom in old people home.	COPD, cardiac hypertrophy.	U, Bile
65	Myocardial decompensation	M	69	No medical history known.	COPD, fatty liver, cyst of thyroid.	U
66	Myocardial infarction	M	85	Renal failure	COPD, Nephrocirrhosis	U, Bile
67	Subdural haematoma and brain oedema	M	89	Fall at home	Brain oedema, liver cirrhosis and lung oedema	U
68	Myocardial infarction	M	68	History of hypertension and alcoholism	COPD, old and fresh MI, heart hypertrophy.	H

Case	Suspected cause of death	Gender	Age (year)	Medical History	Post-mortem finding	Missing samples
69	Myocardial infarction	M	53	History of nicotine abuse	High grade arterio and coronary sclerosis., COPD	NO
70	Myocardial infarction	M	51	Died at home	Fresh MI, brain and lung oedema	NO
71	Acute coronary thrombosis	M	61	Traffic accident	Heart hypertrophy, high grade arteriosclerosis	U, Bile
72	Pulmonary embolism	F	38	Back pain days prior to death	Pulmonary embolism, arteriosclerosis	U, Bile
73	Subarachnoid bleeding SAH	M	47	Epilepsy and alcoholic	High-grade arteriosclerosis, chronic bronchitis, fatty liver.	U
74	Pneumonia	M	41	Car accident prior to death.	Fracture of the Skull, sub arachnoids	U
75	Gastrointestinal bleeding	F	79	Stomach pain	Duodenal ulcer, intestinal bleeding	U

Case	Suspected cause of death	Gender	Age (year)	Medical History	Post-mortem finding	Missing samples
76	Asphyxiation	M	61	Collapse with dyspnoea	Asphyxiation due to laryngostenosis due to laryngeal cyst.	VH
77	Sepsis	M	50	Drug abuser and under methadone therapy	Chronic pneumonia, liver cirrhosis, gastritis	SC
78	Suspicion of acute intoxication	F	44	Drug abuser, alcoholism	Fatty liver and COPD.	U
79	Myocardial infarction	M	31	Drug abuser	Low grade coronary arteriosclerosis, MI	U
80	Pneumonia	M	69	Car accident prior to death.	Pneumonia, chronic bronchitis, arteriosclerosis	U
81	Pneumonia	F	66	Known pulmonary and circulatory disease	High grade arteriosclerosis, COPD, pneumonia.	NO
82	Cardiac arrest	M	74	Heart problem, kidney problems, gastric ulcer amputation of right lower leg, pain therapy with morphine	COPD, cardiac hypertrophy and dilatation, high grade arteriosclerosis	NO

Case	Suspected cause of death	Gender	Age (year)	Medical History	Post-mortem finding	Missing samples
83	Intracerebral haemorrhage	M	45	Surgical trepanation, death occurs after operation.	Sever head trauma, brain oedema.	U, H
84	Myocardial infarction	F	54	Neck pain	High grade arteriosclerosis, heart hypertrophy, congested liver/spleen, fresh myocardial infarction	NO
85	Epileptic seizure	M	26	Alcoholism and epileptic seizure	Brain oedema, acute bronchitis, lung oedema	VH, U, H
86	Cardiac arrest	M	41	Fall from Bicycle	Coronary sclerosis, COPD, suspicion of acute Pancreatitis.	NO
87	Urosepsis	F	41	History of alcoholism	Fatty liver, purulent Infection of right kidney and urinary bladder and urosepsis	VH
88	Myocardial infarction	M	61	History of coronary sclerosis, diabetes, hypercholesterolemi a, bypass surgery	High grade arterio/ coronary- sclerosis heart hypertrophy, old MI, COPD.	U
89	Myocardial decompensation	M	76	History of MI	High grade arteriosclerosis/ coronary sclerosis, COPD.	NO
90	Myocardial infarction	M	82	History of COPD, MI	High grade arteriosclerosis/ scar tissue of old MI, COPD.	NO

Case	Suspected cause of death	Gender	Age (year)	Medical History	Post-mortem finding	Missing samples
91	Cardiomyopathy	M	76	History of heart weakness	High grade arteriosclerosis/ coronary sclerosis cardiomyopathy and hypertrophy, COPD.	NO
92	Septic multiple organ failure	F	35	Sudden death after delivery	Purulent infection of upper female genital tract.	VH, U
93	Myocardial infarction	M	79	History of COPD	High grade of Arteriosclerosis, COPD and MI	VH
94	Myocardial decompensation	M	66	Known liver cirrhosis	High grade of arterio/ coronary thrombosis, fatty liver and cirrhosis.	No
95	Suffocation	F	90	Parkinson	Middle grade arteriosclerosis and COPD	U

APPENDIX III

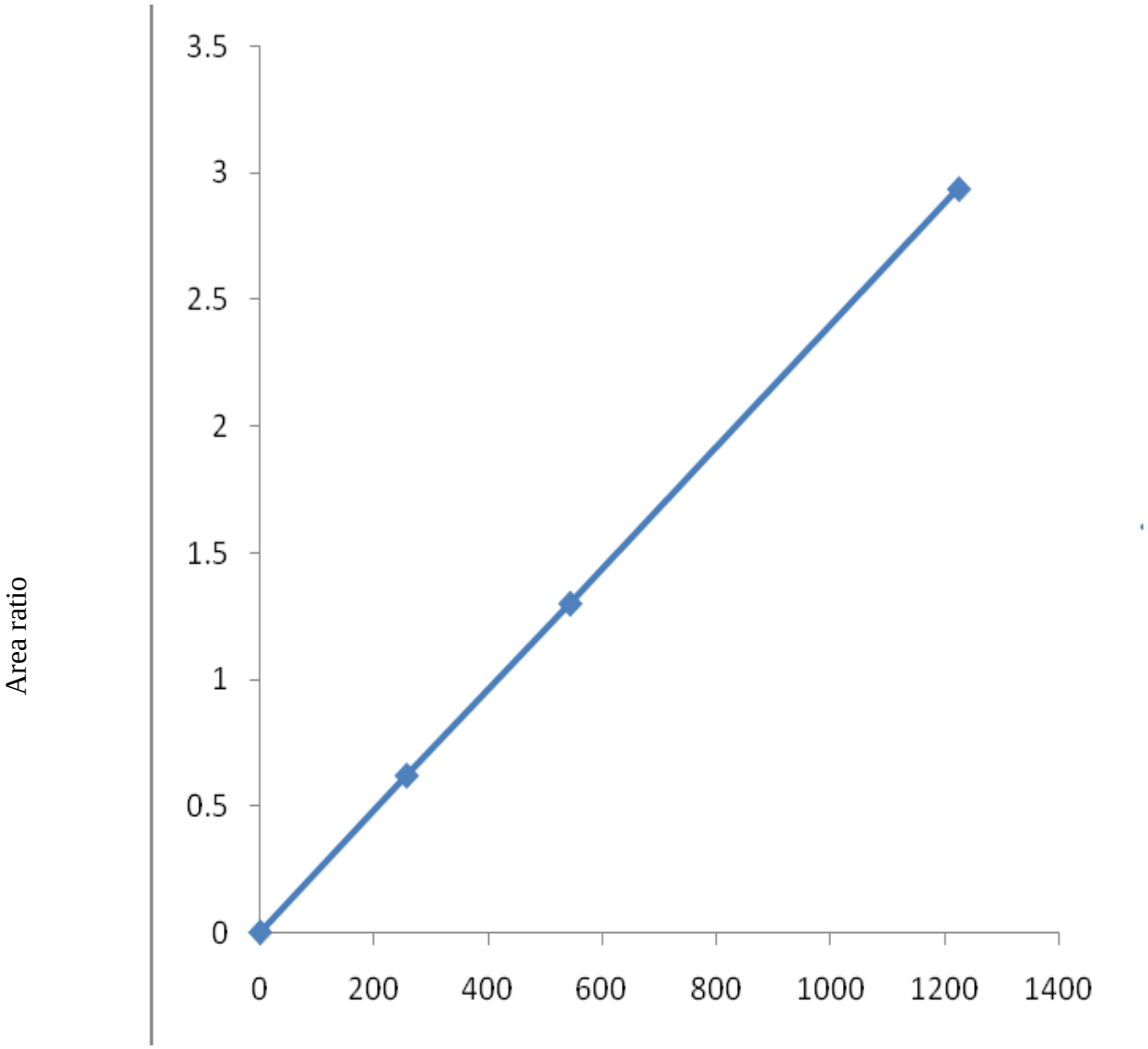

Figure 17: Morphine calibration curve

Morphine Std concentration (ng/ml)	Area ratio
0	0
257	0.6
544	1.3
1226	2.9

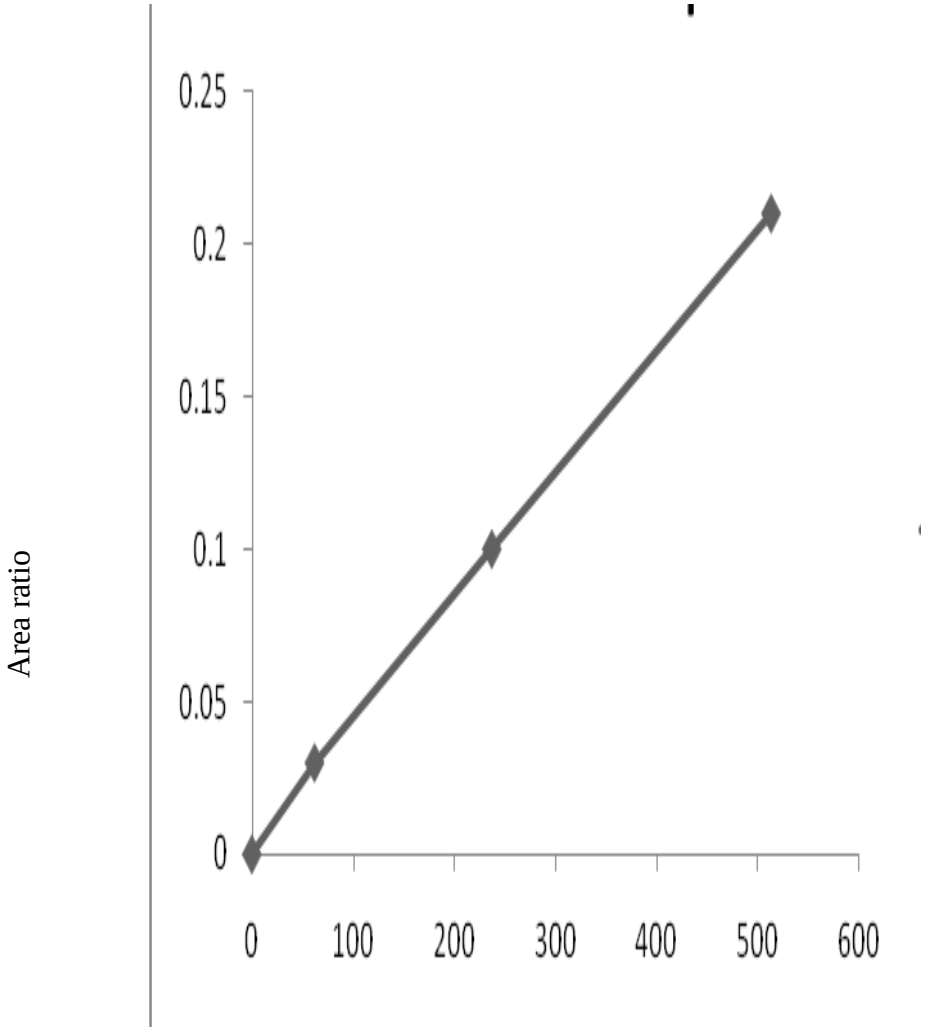

Figure 18: Nor-morphine calibration curve

Nor-morphine Std concentration (ng/ml)	Area ratio
0	0
62	0.03
237	0.1
513	0.2

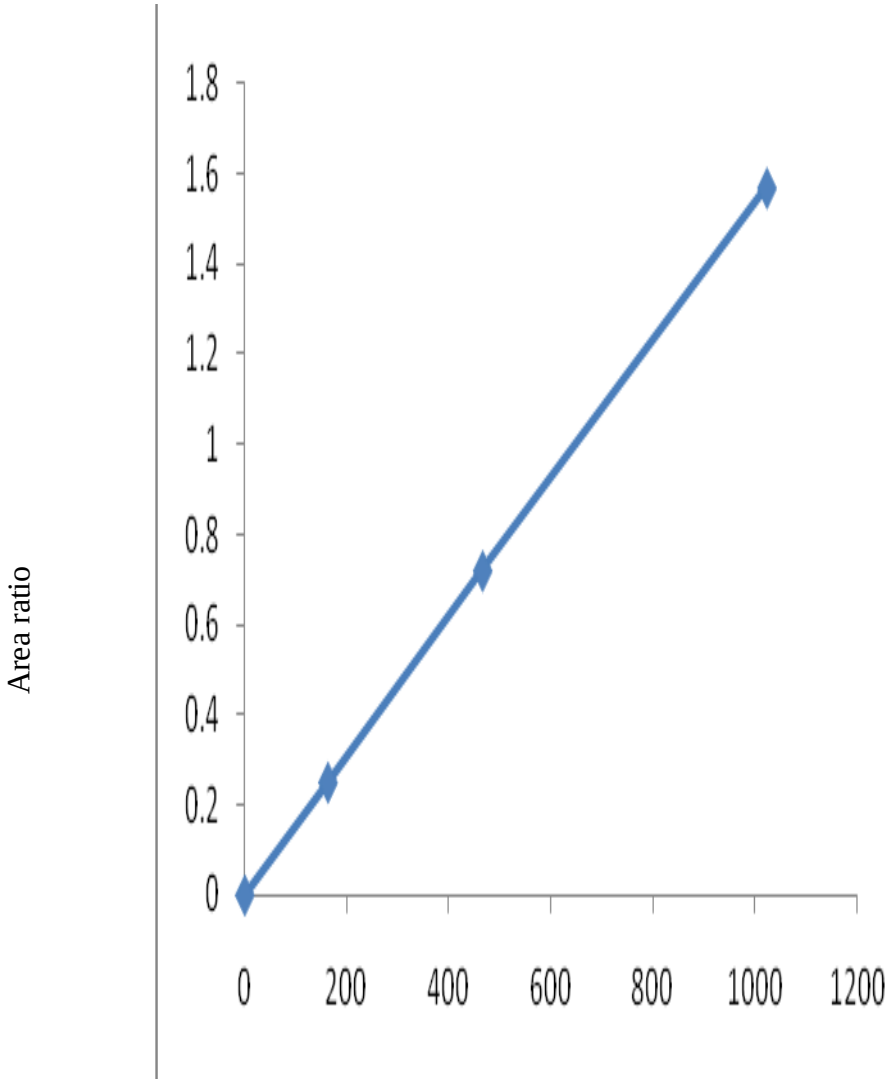

Figure 19: Codeine calibration curve

Codeine Std concentration (ng/ml)	Area ratio
0	0
163	0.3
466	0.7
1023	1.6

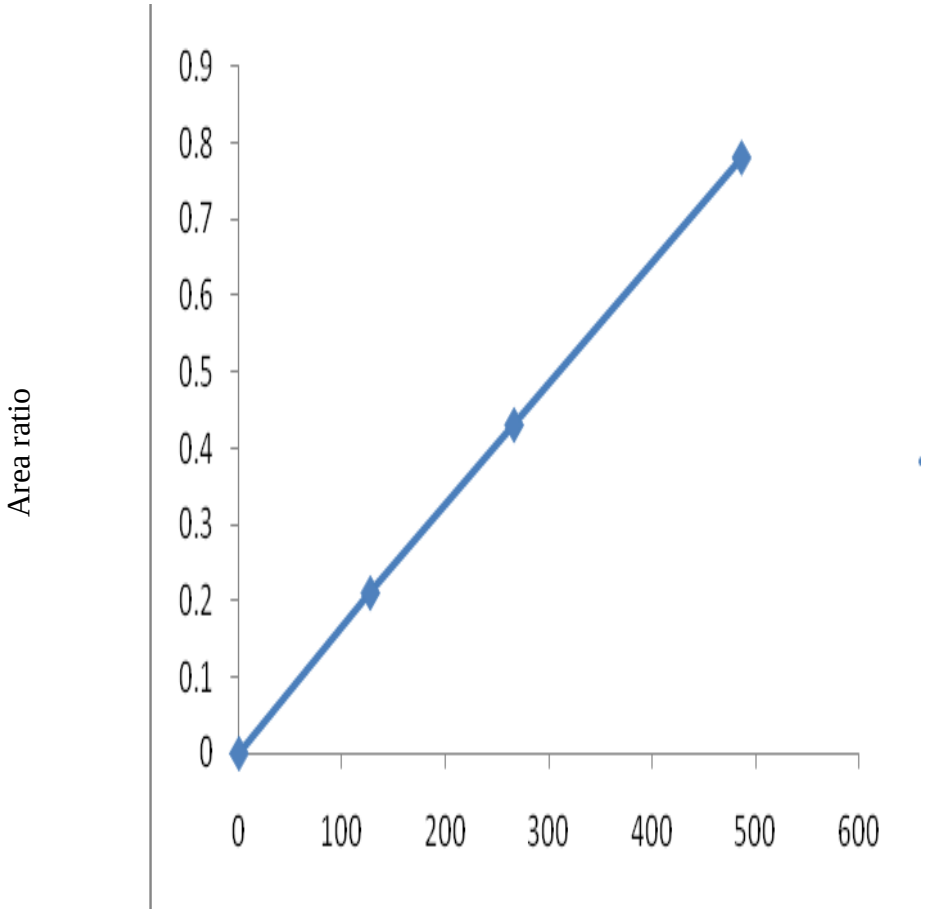

Figure 20: 6-MAM calibration curve

6MAM Std concentration (ng/ml)	Area ratio
0	0
127	0.2
266	0.4
486	0.8

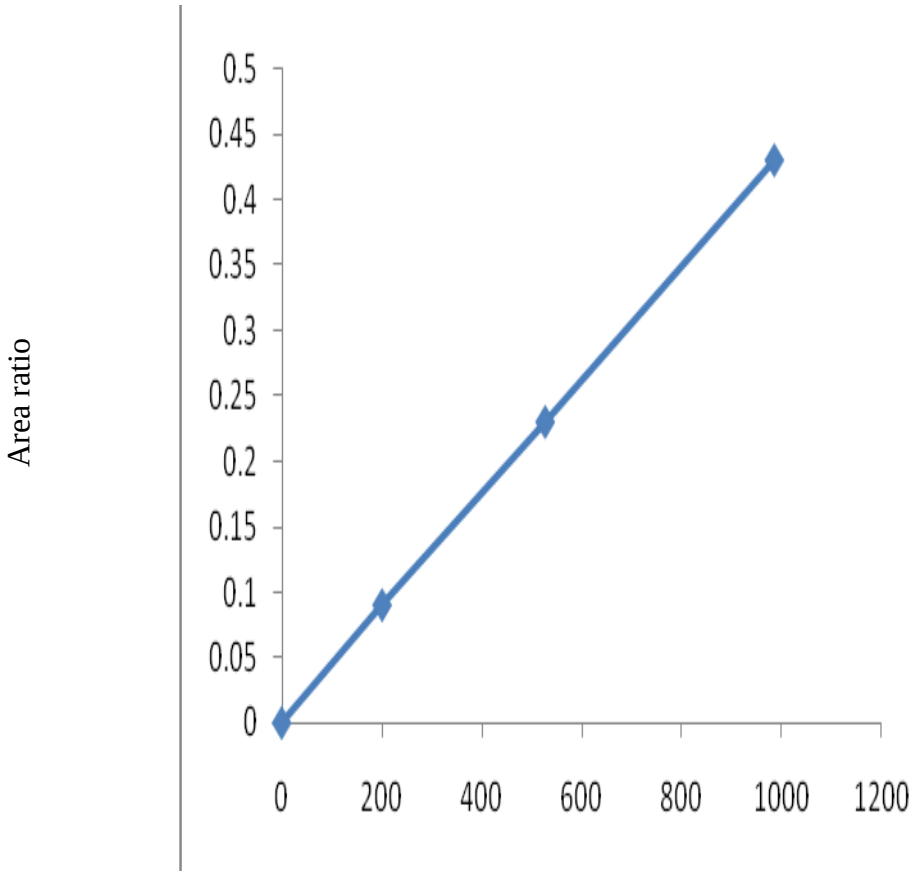

Figure 21: Nor-codeine calibration curve

Nor-codeine Std concentration (ng/ml)	Area ratio
0	0
201	0.1
527	0.2
985	0.4

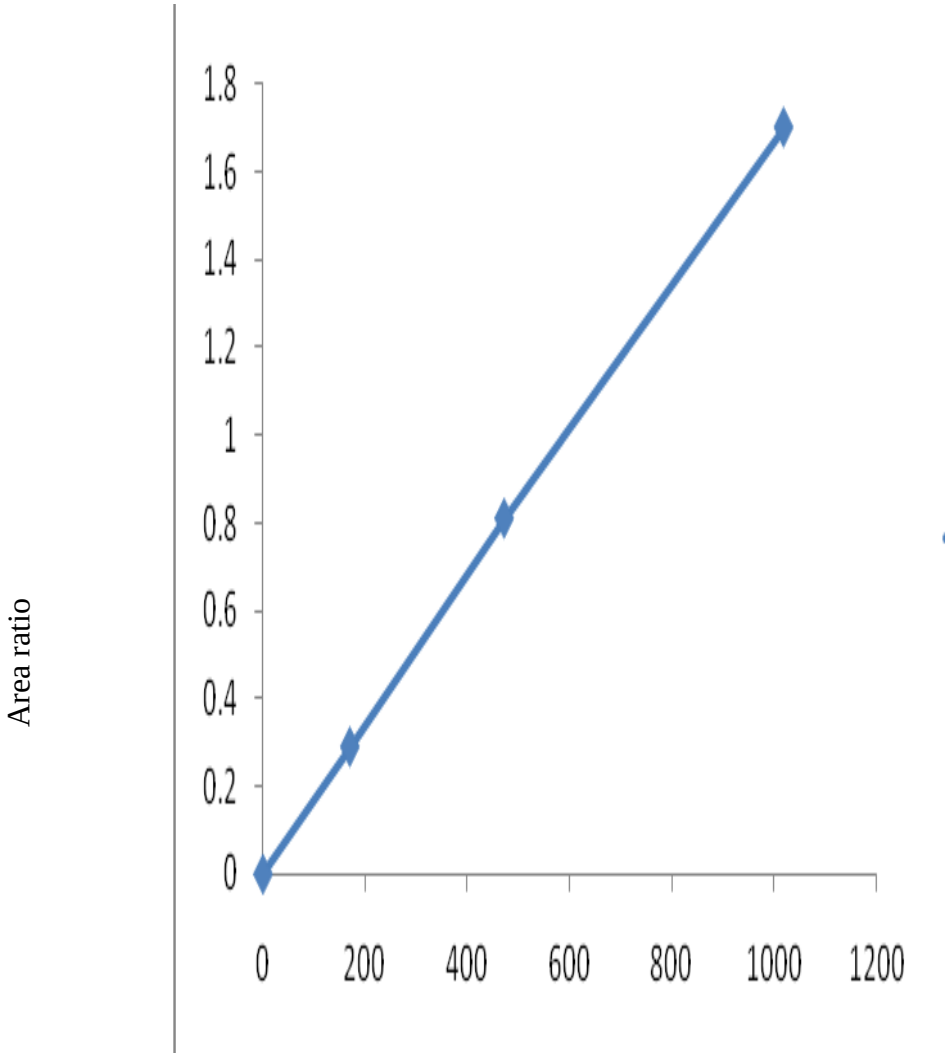

Figure 22: Dihydrocodeine calibration curve

Dihydrocodeine Std concentration (ng/ml)	Area ratio
0	0
170	0.3
472	0.8
1019	1.7

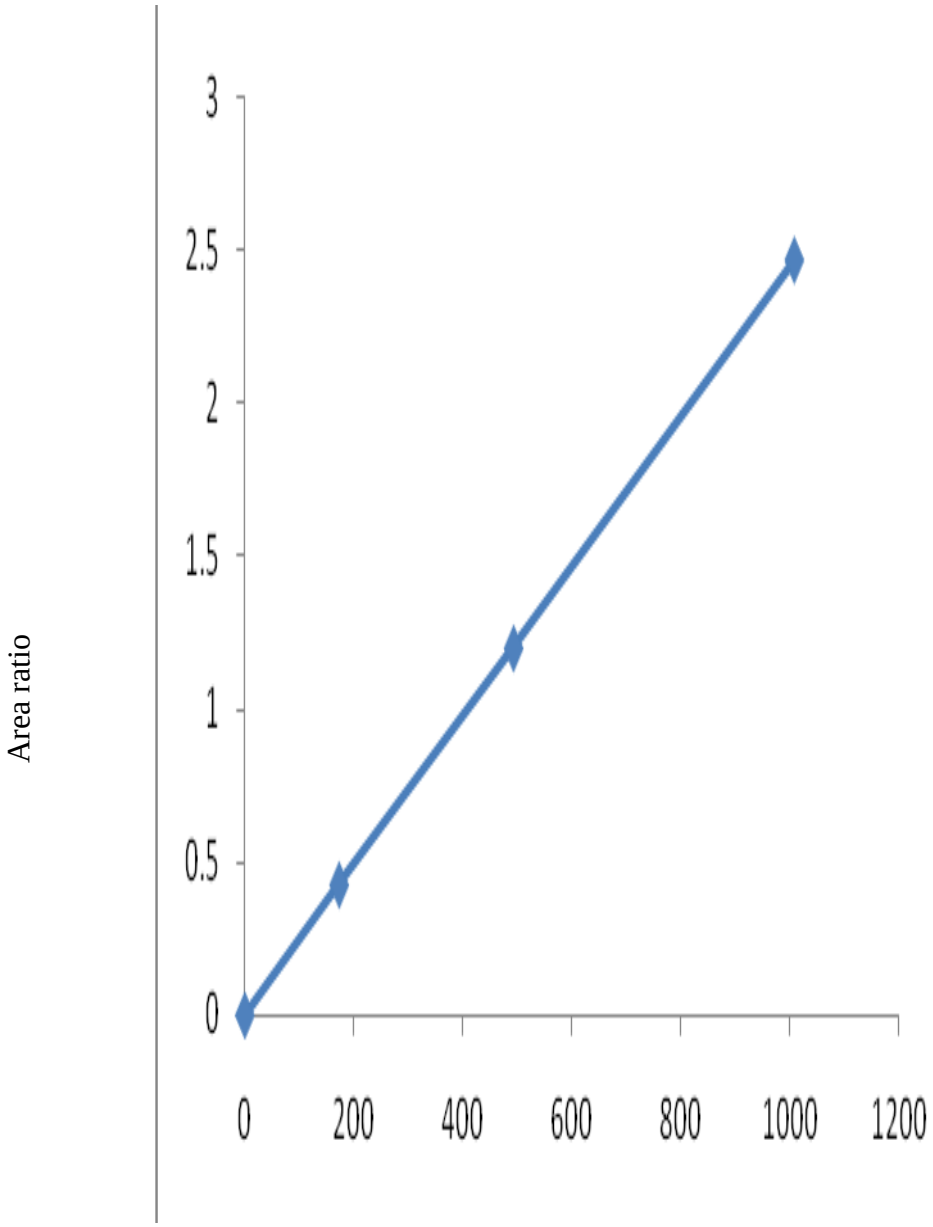

Figure 23: Methadone calibration curve

Methadone Std concentration (ng/ml)	Area ratio
0	0
173	0.4
493	1.2
1008	2.5

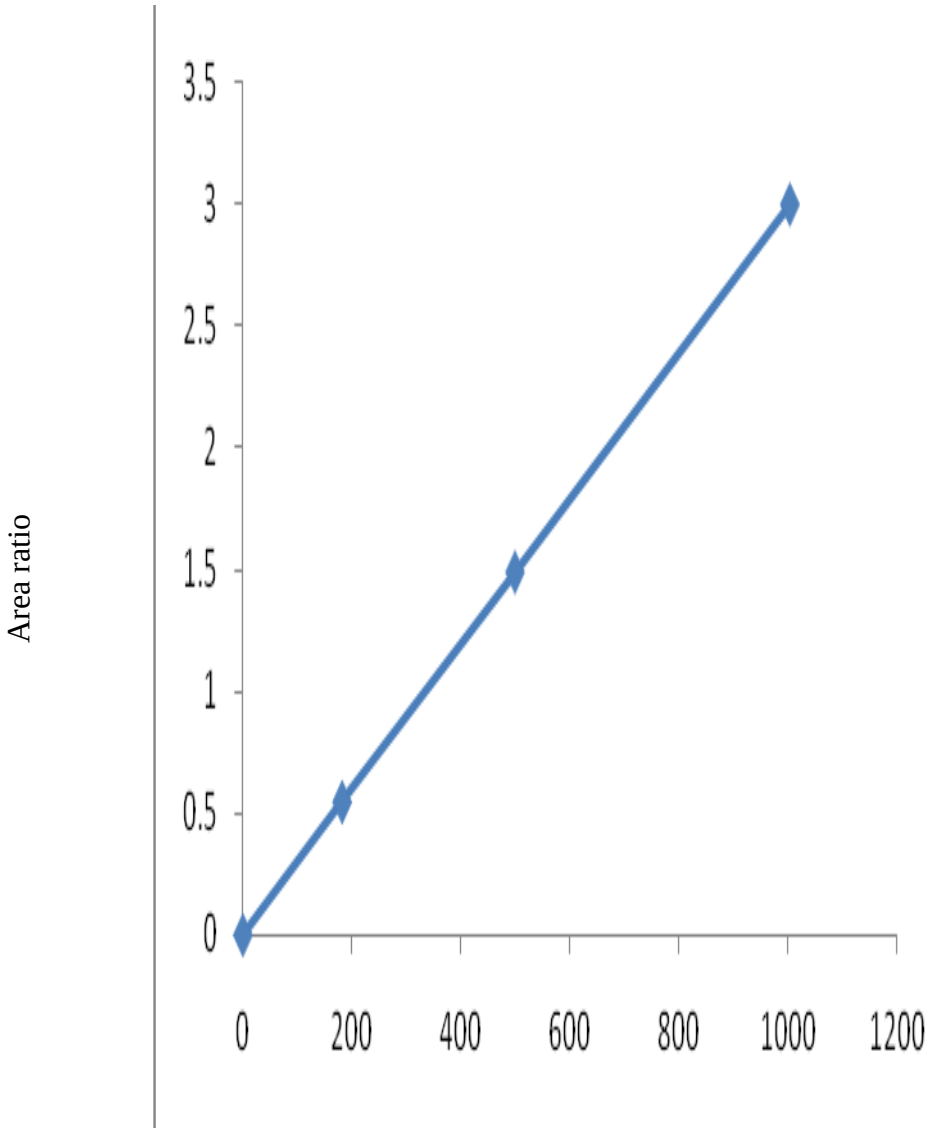

Figure 24: EDDP calibration curve

EDDP Std concentration (ng/ml)	Area ratio
0	0
182	0.5
499	1.5
1003	3

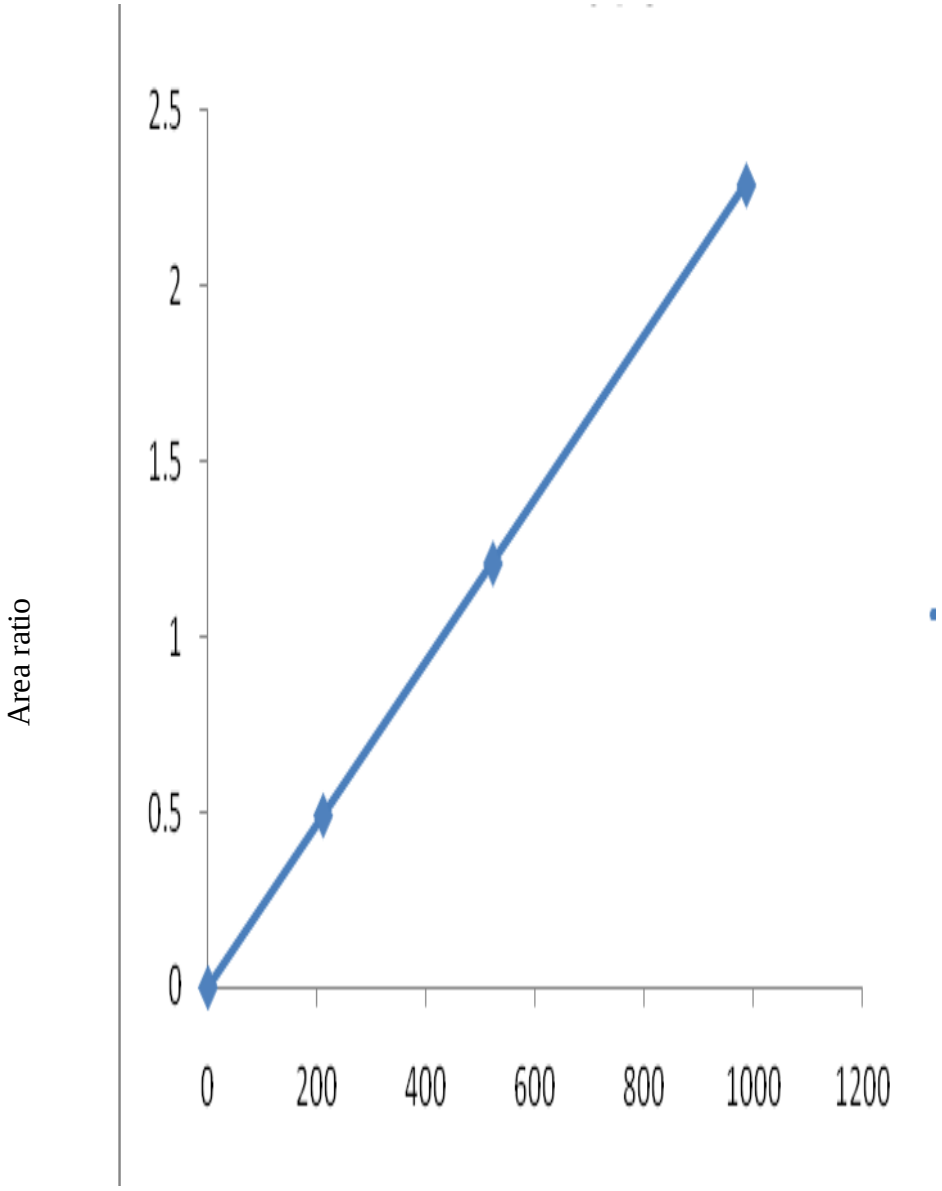

Figure 25: Cocaine calibration curve

Cocaine Std concentration (ng/ml)	Area ratio
0	0
211	0.5
522	1.2
986	2.3

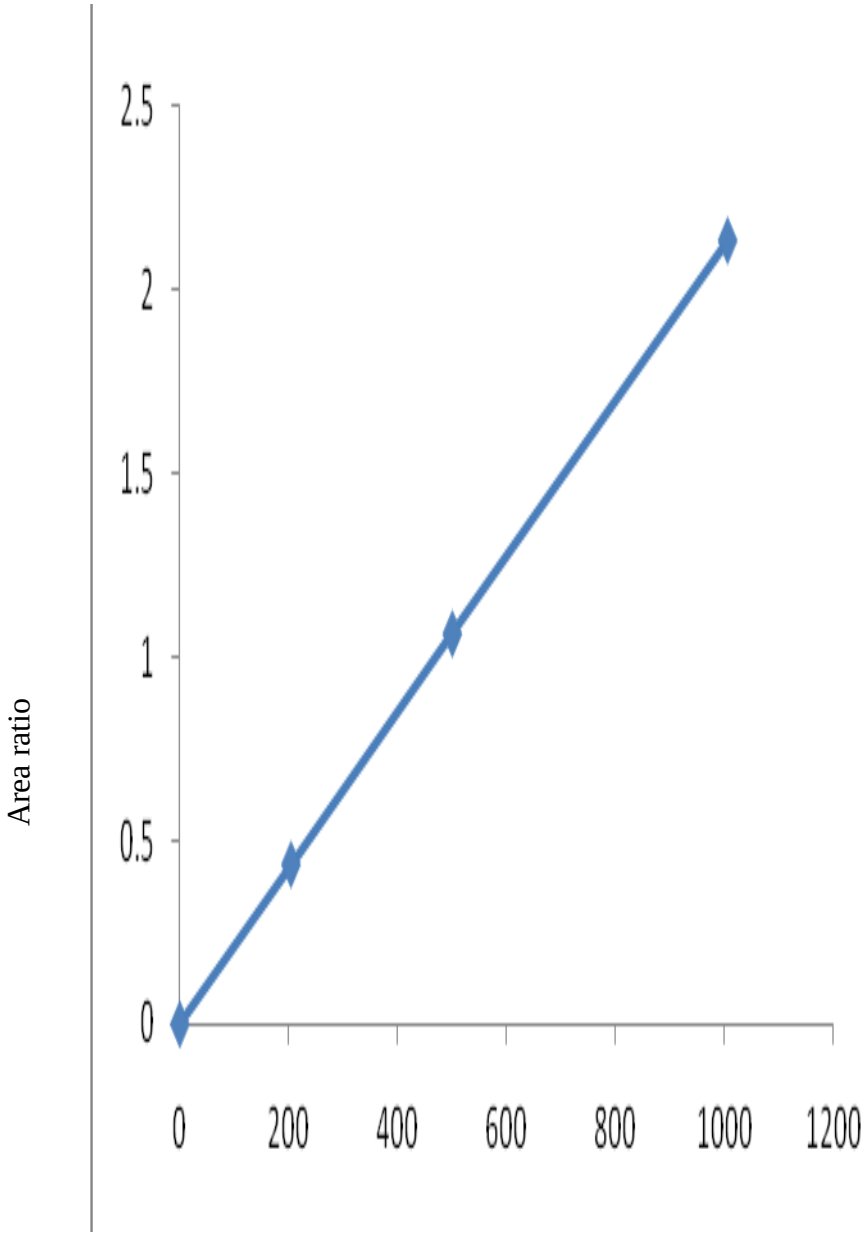

Figure 26: Methylecgonine calibration curve

Methylecgonine Std concentration (ng/ml)	Area ratio
0	0
204	0.4
500	1.1
1005	2.1

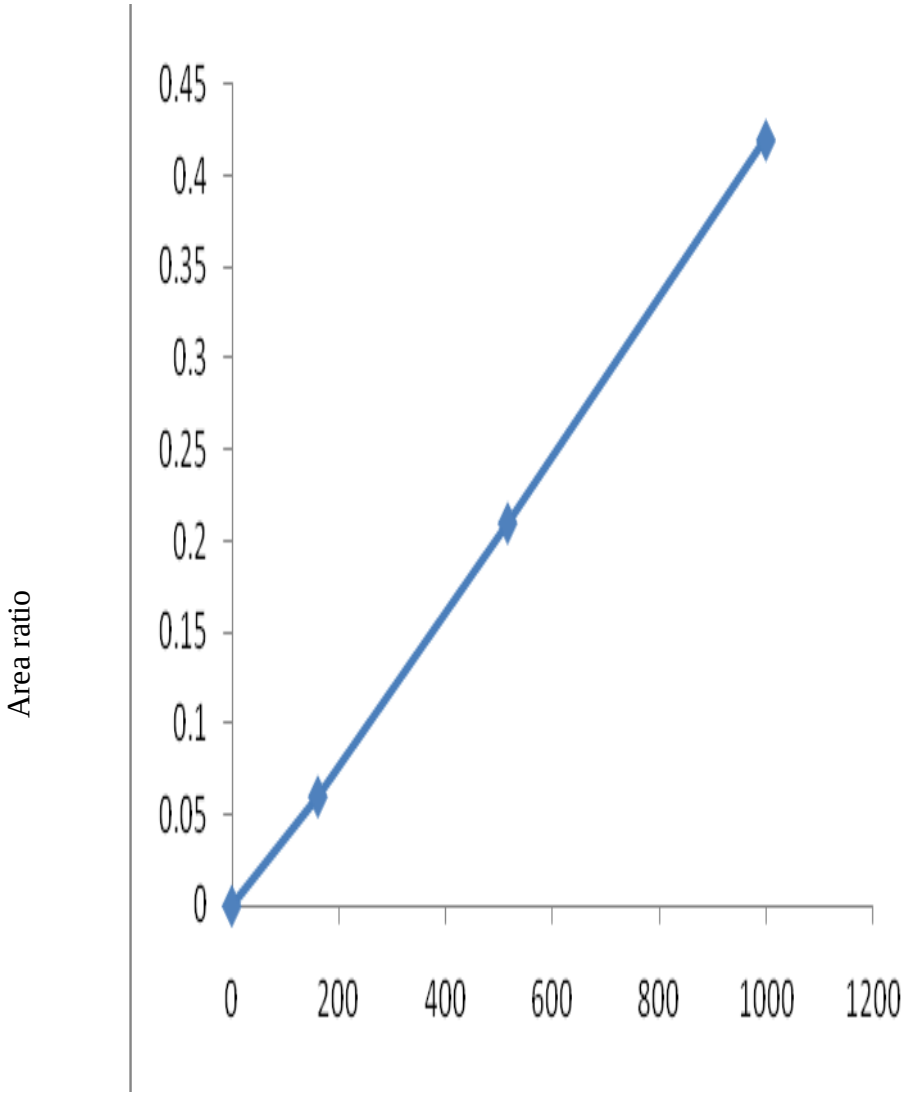

Figure 27: Benzoylecgonine calibration curve

Benzoylecgonine Std concentration (ng/ml)	Area ratio
0	0
161	0.1
516	0.2
999	0.4

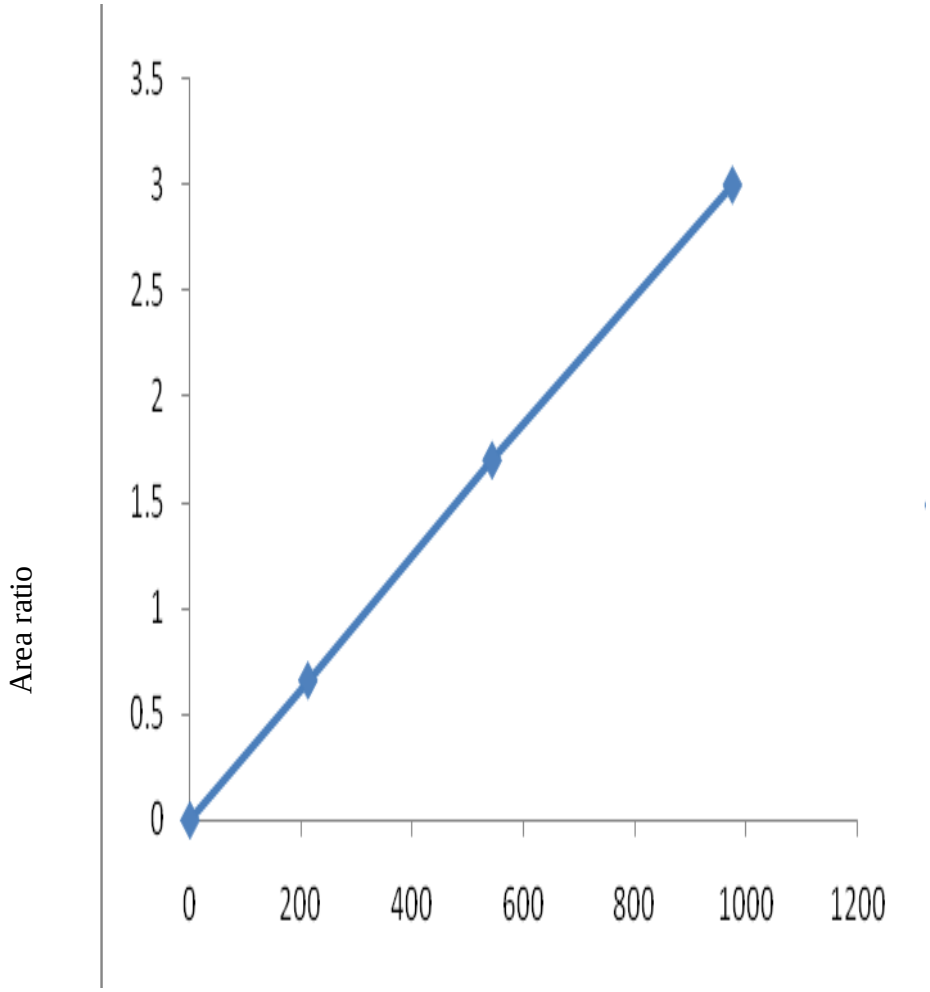

Figure 28: Cocaethylene calibration curve

Cocaethylene Std concentration (ng/ml)	Area ratio
0	0
212	0.7
543	1.7
975	3

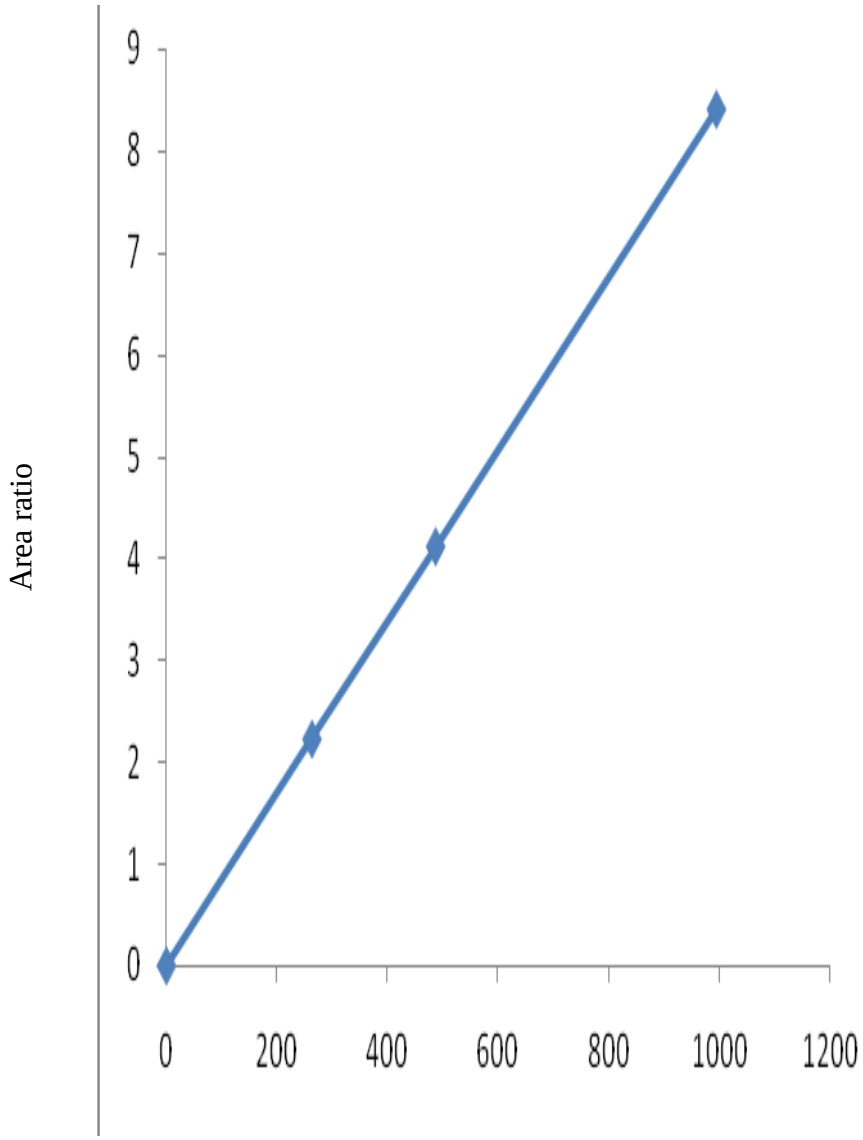

Figure 29: Diazepam calibration curve

Diazepam Std concentration (ng/ml)	Area ratio
0	0
263.1	2.2
486.6	4.1
994.1	8.4

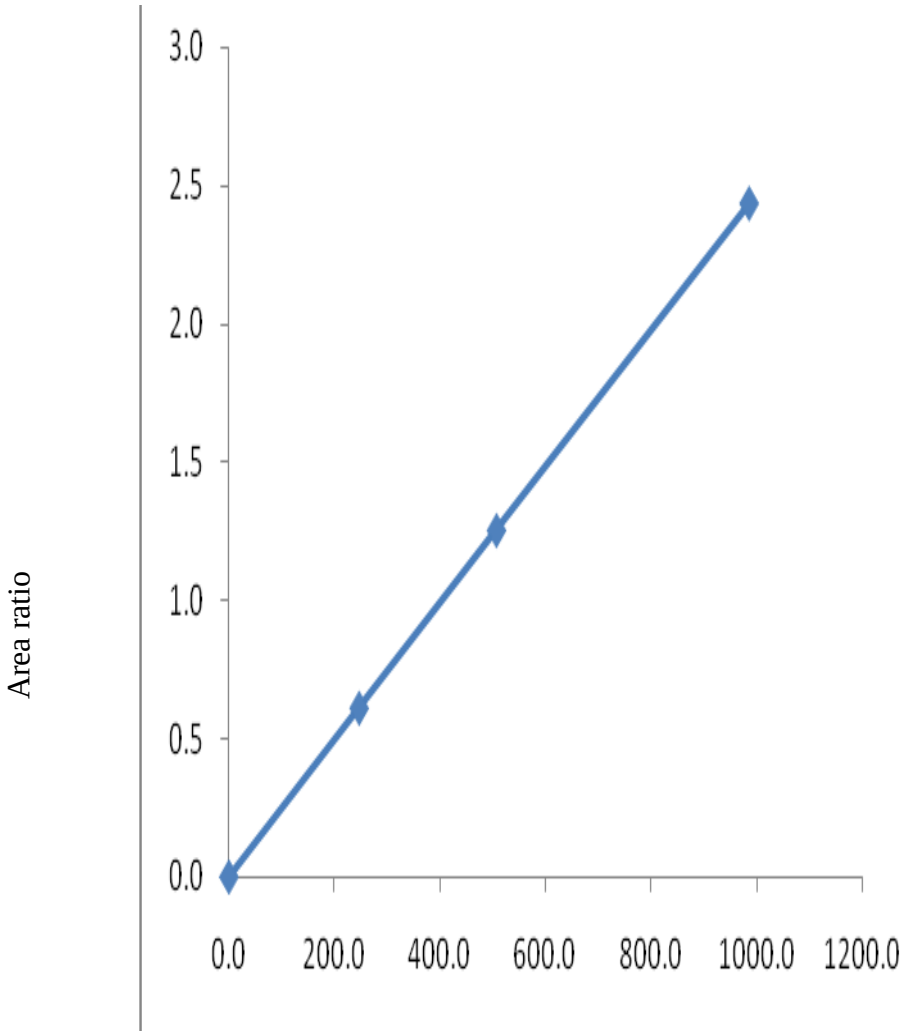

Figure 30: Nor-diazepam calibration curve

Nordiazepam Std concentration (ng/ml)	Area ratio
0	0
247.1	0.6
507.6	1.3
986.8	2.4

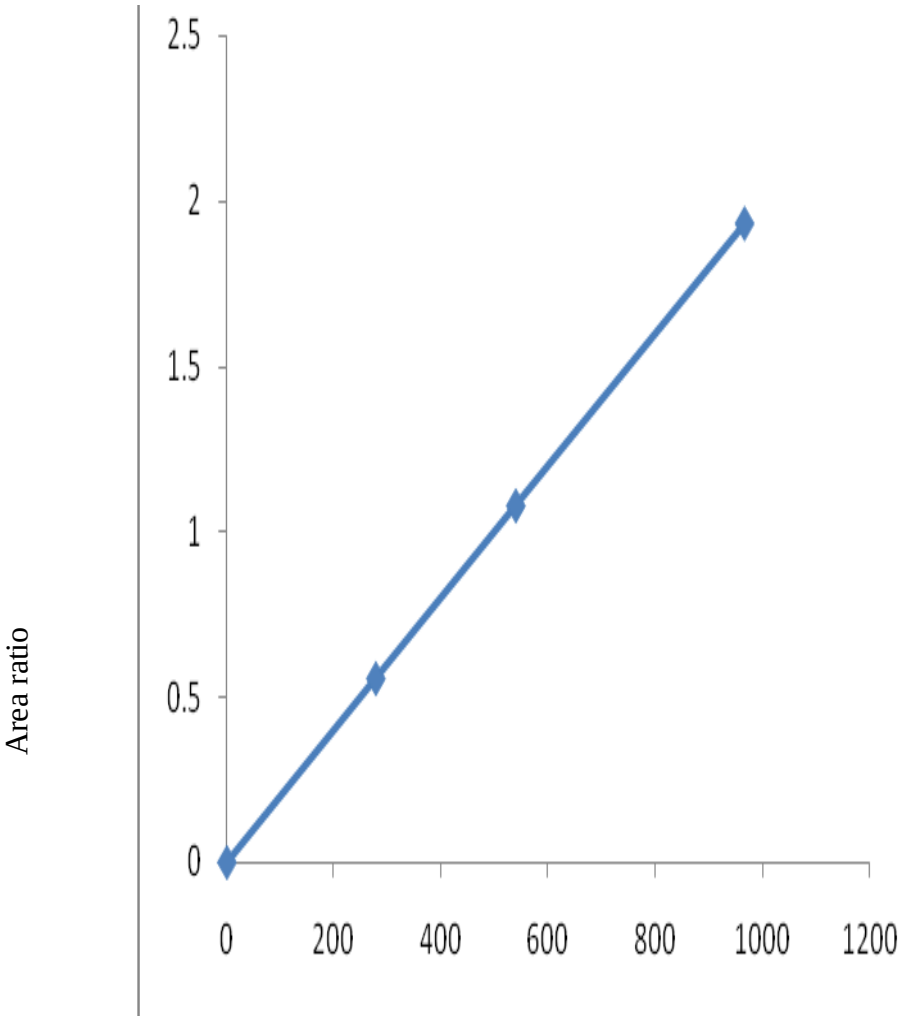

Figure 31: Temazepam calibration curve

Temazepam Std concentration (ng/ml)	Area ratio
0	0
277.7	0.6
538.7	1.1
965.1	1.9

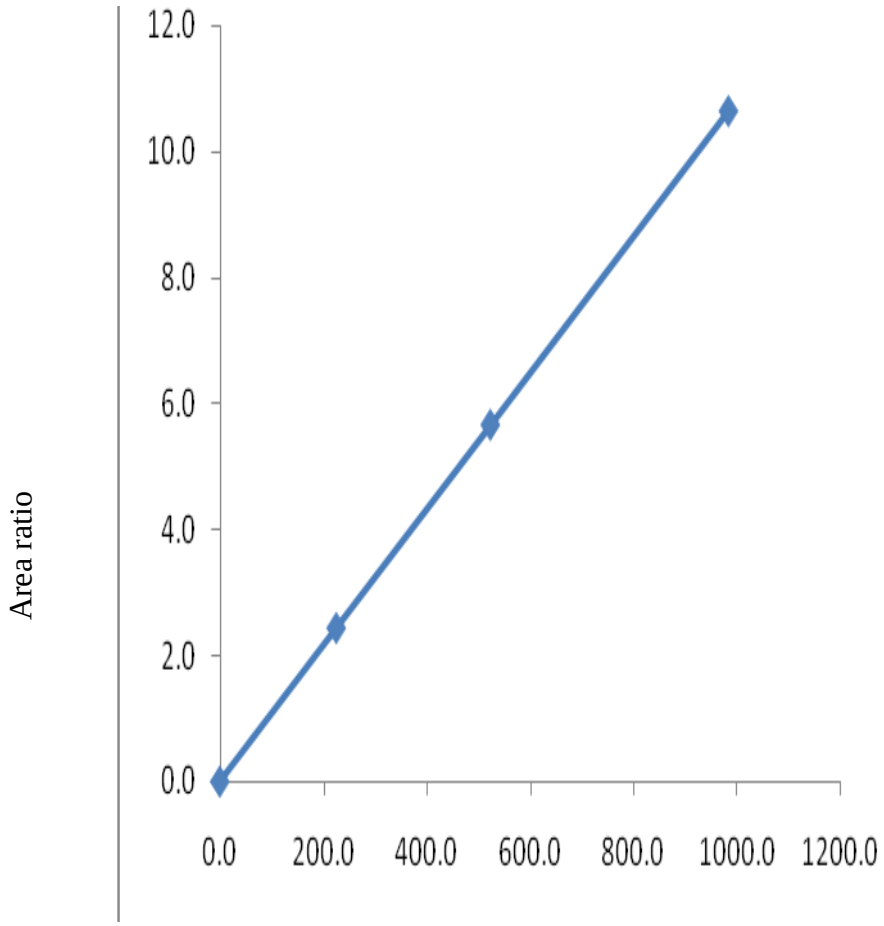

Figure 32: Chlordiazepoxide calibration curve

Chlordiazepoxide Std concentration (ng/ml)	Area ratio
0	0
225.1	2.4
523.2	5.7
983.4	10.6

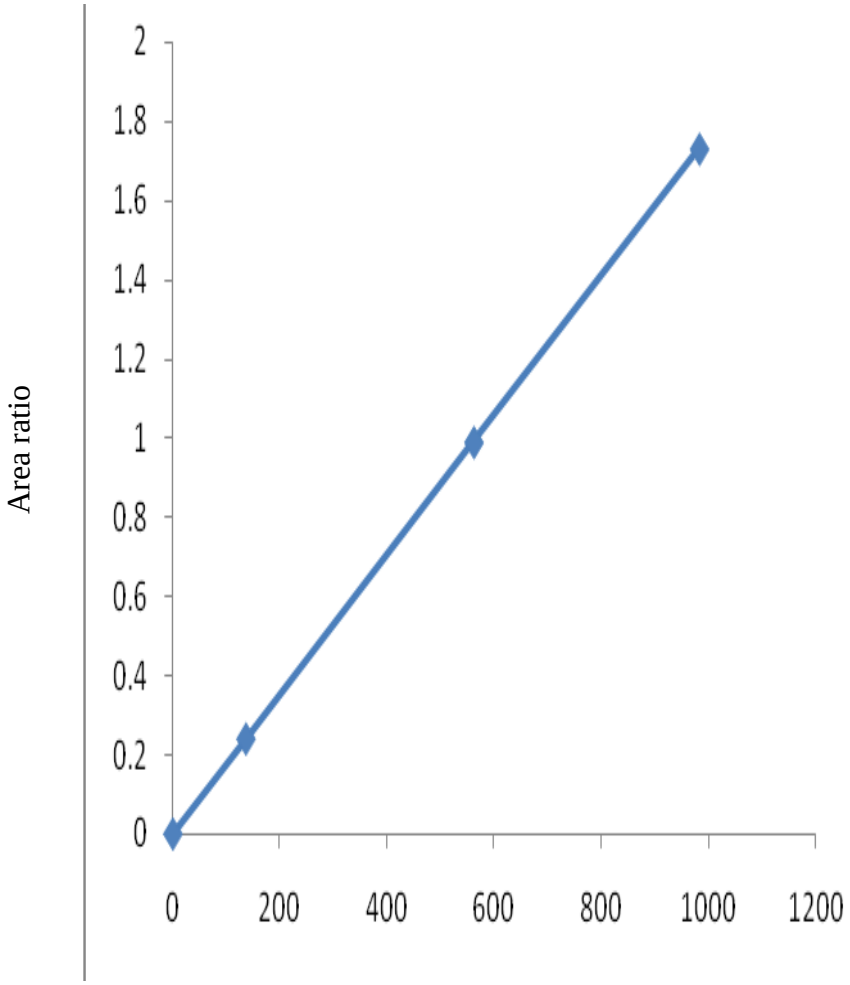

Figure 33: Oxazepam calibration curve

Oxazepam Std concentration (ng/ml)	Area ratio
0	0
136.3	0.2
561.5	1
982	1.7

APPENDIX IV

QC level used for different drugs

Drug	POOL1 (QC in ng/ml)	POOL2 (QC in ng/ml)
Cocaine	200	400
Benzoylecgonine	200	400
Morphine	200	400
Codeine	200	400
Methadone	200	400
Diazepam	200	400
Nor-diazepam	200	400

Drug	QC (mg/dl)	QC (mg/dl)
Ethanol	80	200