An Investigation into the Postmortem Redistribution of Drugs

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

By

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Author's Declaration

"I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or at any other institution"

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Definitions/Abbreviations

% CV	Coefficient of Variation percentage
% ME	Matrix Effect
% PE	Process Efficiency
% R	Percent Recovery
°C	Degree Celsius
<	Less than
>	More than
δ	Delta
κ	Kappa
μ	Mu
μg	Micrograms
-d	Deuterated compound
5-HT	Serotonin
6MAM	6-monoacetylmorphine
ADME	Absorption, distribution, metabolism and excretion
ACN	Acetonitrile
AChE	**Acetylcholinesterase**
BuChE	**Butyrylcholinesterase**
BSTFA	N, O-Bis(trimethylsilyl)trifluoroacetamide
c and m	The constants
C6G	Codeine-6-glucuronide
Ca^{2+}	Calcium
CE	Collision energy
CID	Collision induced dissociation
CO_2	Carbon dioxide
COD	Codeine
CNS	Central nervous system
CYP 450	Cytochrome P450
DBS	dried blood spot
DCM	Dichloromethane
dH2O	Deionised water
DHC	Dihydrocodeine
DHC6G	Dihydrocodeine-6-glucuronide
DRD	Drug Related Death
EDDP	2-ethylidene-1, 5-dimethyl-3, 3 diphenylpyrrolidine
EMDP	2-ethyl-5-methyl-3, 3-diphenyl-1-pyrrolidine
EI	Electron Ionisation
ESI	Electron Spray Ionisation
EtOAc	Ethyl acetate
eV	Electron voltage
FMS	Forensic Medicine and Science

FM	Free morphine
g	Gram(s)
GABA	Gamma amino butyric acid
GBP	Gabapentin
GC-	Gas Chromatography
HB	Heart Blood
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
i.d.	Internal diameter
IPA	Isopropanol
IS	Internal standard
K_2EDTA	dipotassium ethylene diamine tetra-acetic acid
kg	Kilogram
kV	Kilovoltage
L	Litre
LC	Liquid Chromatography
LLE	Liquid-liquid extraction
LLOA	Lower Limit of Argument
LLOQ	Lower Limit of Quantitation
LOD	Limit of Detection
LOQ	Limit of Quantitation
m	Metre
M	Molar
m/z	Mass to charge ratio
M3G	Morphine-3-glucuronide
M3G-d3	Morphine-3-glucuronide-d3
M6G	Morphine-6-glucuronide
M6G-d3	Morphine-6-glucuronide-d3
MeOH	Methanol
MF	Matrix Factor
mg	Milligram (s)
mL	Millilitre (s)
MMP	Methadone maintenance programme
MOR	Morphine
MOR-d3	Morphine-d3
MP(A)	Mobile Phase (A)
MP(B)	Mobile Phase (B)
MRM	Multiple reaction monitoring
MSD	Mass Selective Detector
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)- trifluoroacetamide
N_2	Nitrogen gas

N6G	Norcodeine-6-glucuronide
NaF	Sodium fluoride
NaOH	Sodium hydroxide
$Na_2C_2O_4$	Sodium oxalate
$NaH_2PO_4.H_2O$	Sodium dihydrogen orthophosphate monohydrate
Na_2HPO_4	Disodium hydrogen orthophosphate anhydrous
NH_4OH	Ammonium hydroxide
NE	norepinephrine
NPS	Novel psychoactive substances
(NMDA) receptors	N-methyl-D-aspartate receptors
OH	Hydroxyl group
OST	Opioid Substitution Treatment
PAR	Peak Area Ratio
PFPA	Pentafluoropropionic anhydride
pKa	Logarithmic acid dissociation constant
PB	Peripheral Blood
PM	Postmortem
PMI	Postmortem Interval
PMR	Postmortem Redistribution
PPT	Protein precipitation
QC	Quality Control
R^2	Linear correlation coefficient
RSD	Relative standard deviation
rpm	Revolutions per minute
S/N	Signal to Noise Ratio
SCAT	separated clot activator tube
SIM	Selected ion monitoring
SLE	Supported Liquid Extraction
SNBTS	Scottish National Blood Transfusion Service
SPE	Solid Phase Extraction
SSRIs	selective serotonin reuptake inhibitors
StDev/SD	Standard deviation
SWGTOX	Scientific Working Group for Forensic Toxicology
TCA	Tricyclic antidepressant
TIAFT	The International Association of Forensic Toxicologists
TDM	Therapeutic Drug Monitoring
TIC	Total ion chromatogram
TM	Total Morphine
TMCS	Trimethylchlorosilane
TMS	trimethylsilyl
UCT	United Chemical Technologies
ULOA	Upper Limit Of Argument
ULOQ	Upper Limit of Quantitation

UNODC	United Nations Office on Drugs and Crime
Vd	volume of distribution
v/v	Volume to Volume

Abstract

Postmortem blood drug concentrations change over time as a consequence of postmortem changes and therefore may not reflect the drug concentration at the time of death. In order to reduce the effect of postmortem redistribution on drug concentrations, early collection of samples for analysis is preferable. In addition, many other factors should be considered in the evaluation of drug concentrations in postmortem samples, such as stability of drugs during sample storage as this may be an additional source of variation.

The aim of this study was to develop a method for quantifying drug concentrations in postmortem blood samples taken in drug-related deaths. A gas chromatography mass spectrometry (GC-MS) method with solid phase extraction (SPE) was developed and validated for the simultaneous determination of opioids and antidepressant drugs in whole blood. In addition, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method with protein precipitation was developed and validated for simple and accurate analysis of gabapentin, morphine, morphine 3-glucuronide (M3G) and morphine 6-glucuronide (M6G) in whole blood samples. The methods were successfully verified using authentic postmortem blood samples.

This study was designed to understand the stability of morphine and its glucuronides, in real postmortem blood samples, after storage in different conditions for short and long periods of time. The stability study revealed that, the concentrations of free and total morphine were stable during storage in the freezer for ~10 years and no significant losses were observed. The stability study of authentic case samples with and without preservative stored in the freezer for three years revealed that, sodium fluoride and potassium oxalate as a preservative for postmortem blood samples does not affect morphine, M3G and M6G stability under storage temperatures of -20°C for three years.

This study also aimed to investigate morphine and morphine metabolites, together with their ratios, in order to achieve a comprehensive interpretation of time since death. The investigation of morphine to the respective glucuronide concentration ratio to estimate the survival times after administration of heroin revealed that, the lower ratios of these conjugates to total morphine (TM), are suggestive of a more rapid death, as there has been less time for the metabolism

of morphine to occur. Similarly, the presence of 6MAM in the blood of a heroin toxicity death suggests a more rapid death. More specifically, when M3G/TM is less than 0.40 could show a quick death in the absence of 6MAM in the blood.

The purpose of this study was to investigate changes in the concentration of specific drugs (gabapentin, morphine, M3G and M6G) in postmortem blood samples between death and autopsy, and to identify any patterns of these changes. The sampling technique and site of sampling (peripheral and central) were considered to further define the extent of PMR of drugs and identify a possible mechanism of PMR. Concerning sampling site, for all substances, femoral blood concentrations were significantly lower than those found in cardiac blood, indicating that femoral blood is probably less prone to PMR. In addition, the evaluation of drug concentrations in postmortem samples collected using different techniques of blood sampling revealed that, needle puncture in the upper thigh (blind stick) as opposed to dissection of the same vein, also appeared to have an effect on femoral drug concentrations, since femoral concentrations tended to be closer to cardiac concentrations with the dissection sampling than the blind stick sampling technique. Finally, the analytical results derived from sampling (blind stick) as soon as possible to assess whether it is necessary to obtain blood samples prior to autopsy and prevent contamination by PMR, suggest that PMR is a continuous phenomenon in central as well as in peripheral compartments, but also that femoral blood appears more resistant to it.

Generally, the results conclude that PMR is an ongoing phenomenon in central as well as in peripheral compartments, but also that femoral blood seems more resistant to it. Therefore, to avoid the effect of the pre-autopsy interval on drug concentrations it is always preferable for early collection of samples for analysis, which are collected closer to the time of death, and would enable a better assessment of the likely contribution of drugs to the death.

Chapter 1 Introduction

Opioids are one of the most widely abused drug groups in the world. The majority of drug related deaths in the UK relate to opiate use, chiefly heroin/morphine [1]. The acute toxicity of heroin, especially after intravenous administration, is high compared with other drugs of abuse [2]. There is considerable evidence that many instances of drug related deaths are due to the combined effects of opioids with other respiratory depressants drugs [3-10] and this kind of poly-drug use is highly prevalent among illicit drug users in the UK [11, 12].

“Drug-related death” means deaths happening shortly after consumption of one or more psychoactive drugs, and directly related to this consumption. The National Records of Scotland’s National Statistics on DRDs show that, almost all (97% in 2016) DRDs occurred after the consumption of multiple substances. In Scotland 2016, opioids (methadone, diamorphine/ morphine or buprenorphine) were implicated in over three quarters of DRDs. Morphine, alcohol, anti-depressants, diazepam, etizolam and gabapentin were the most common substances found at post mortem in Scotland 2016 – all have increased in prevalence since 2011, with the exception of diazepam replaced by etizolam [1, 13].

There are a number of challenges in the interpretation of drug and metabolite blood concentrations. Drug concentrations vary greatly depending on the dosage, route of administration, individual tolerance, age, state of health, concomitant use of other drugs, the period of survival after drug intake [14], and the manner of sample storage [15]. Correlating a specific drug concentration in blood to toxicity is difficult, even in a living individual [16, 17]. The situation after death is even more complex because the concentrations determined in samples collected at autopsy do not necessarily, accurately reflect those at the time of death.

In general, the large variability and lack of appreciable patterns in opioid related death data may be due to inherent properties of the postmortem material. It may also possibly be due to variable toxicological significance of opioid intake and bioconversion to active metabolites in these materials. A significant amount of data suggests that the bioconversion of morphine

glucuronides to morphine is responsible, but the precise mechanism behind the bioconversion is not known. Residual metabolic enzyme activity, known to occur in the early postmortem period, can contribute significantly to changes in drug concentration.

It is known that weakly basic drugs (e.g. antidepressant drugs) may be subject to a process known as postmortem redistribution (PMR), where drugs move from areas of high concentration in the central viscera to areas of lower concentration after death [18]. Residual metabolic enzyme activity, known to occur in the early postmortem period, can contribute significantly to changes in drug concentrations [19]. As time between death and sampling increases so does the likelihood that site- and time-dependent changes in blood drug concentrations will have occurred [14, 20].

It is widely recognised that PMR is particularly important for drugs with high lipid solubility or high tissue concentrations relative to blood. Particular drug groups such as tricyclic antidepressants have been reported to be subject to PMR [21-25]. When comparing concentrations of drugs in central samples to peripheral samples, a significant PMR has been reported for blood sertraline [26] and amitriptyline [27] indicating that dependence on the site of sampling was an important factor. Another study showed that some drugs exhibit particularly large changes in drug concentration when femoral blood was collected on mortuary admission compared to similar blood taken in autopsy a few days later [28].

The pre-autopsy interval while storing bodies for long periods of time can cause greater changes in blood drug concentrations [14]. Therefore, to avoid the effect of the pre-autopsy interval on drug concentrations early collection of specimens for analysis is preferable, to enable a better assessment of the likely contribution of drugs to the death [28-30]. Many other factors must be considered in the evaluation of drug concentrations in a postmortem sample, such as techniques of blood sampling [31-33]. A limited number of studies have shown that needle puncture in the upper thigh (blind stick) did not affect the blood concentration as opposed to blood samples during autopsy from upper thigh [28, 34]. Therefore, it is important to not only understand the effect of how they are stored, but also how postmortem blood samples are collected at

autopsy and what interpretative value the measurements of drugs and their metabolites have on the circumstances of death.

After administration, diamorphine is rapidly converted to its proximate metabolite 6-monoacetylmorphine (6MAM), which is quickly transformed into morphine, which is the target analyte when diamorphine-related deaths are investigated [35, 36]. The presence of 6MAM in blood and its short elimination half-life (15–30 min) suggest that the individual has died within 1–2 h after they last used diamorphine [7, 37]. In addition, some laboratories analyse further conjugated metabolites of morphine, reporting either a “total morphine” concentration or identifying the specific metabolite, for example morphine-3-glucuronide or morphine-6-glucuronide [36, 38, 39].

Several analytical methods for morphine and its glucuronide metabolites have enabled the direct and specific analysis of opioids and their glucuronide metabolites [40-64]. These metabolites potentially play an important role in the interpretation of deaths involving diamorphine, for at least two reasons: first, morphine-6-glucuronide (M6G) is pharmacologically active and has even been advocated to have a slightly different, and maybe more respiratory depressant action than morphine; secondly, the ratio of free morphine over its metabolites can help evaluate the time elapsed between diamorphine injection and death [65-67]. Therefore, investigations of opioid-related deaths should include quantification of morphine and morphine metabolites together, in order to achieve a comprehensive interpretation of postmortem opioid findings.

As previously mentioned, a great majority of drug related deaths involving diamorphine, also include other respiratory depressant drugs, one of these is gabapentin. Mao *et al*. (2011) have reported that gabapentin had analgesic and opioid pairing effects when used in conjunction with opioids in pre- or postoperative pain management, thus improving the analgesic efficacy of opioids and decreasing cumulative morphine consumption. Information on the synergistic effects of gabapentin and opioids and the increased risk for addiction and overdose has recently emerged in the literature [68].

Knowledge of the stability of drugs in biological samples is of great importance in interpreting results of analysis after a significant time delay. It may be necessary to re-analyse forensic samples in criminal cases months/years after

initial analysis (for example, when new evidence emerges, or defence legal teams challenge original results). It is therefore important to know to what extent the concentration of the drug changes when blood is stored for very long periods of time. A decrease in drug concentration would follow drug degradation, which could be spontaneous, caused by endogenous enzymes present in or released into blood, or happen as a consequence of microbial activity. In the latter case, a more general decomposition of the sample would be expected with degradation of most, if not all drugs present. Increased free drug concentrations can also occur as a consequence of deconjugation of conjugated drug metabolites (e.g. morphine glucuronides hydrolysed to morphine) [69]. In addition, the differences between the initial and subsequent drug analyses could be due to the heterogeneous nature of postmortem blood samples due to clotting and separation of components following cell lysis.

Whereas limited attention has been paid to the influence of the pre-analytical factors on the analysis of opioids, some data has been reported on the thermal stability of morphine in blood [70, 71]. An additional determination of morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) was published in a number of studies [72-75]. Even less attention has been paid to the stability of morphine metabolites especially long-term storage real-life (not spiked), although conclusions may be drawn from parent drug concentrations in forensic cases.

The modern analytical instrument has made reliable and highly sensitive systems for determinations based on individual analyte parameters. Among these characteristic parameters of the compound is its mass spectrum, which can be used for its identification. Combining various versions of chromatography with the mass-selective detection of the separated components is one of the promising trends in the analysis of composite mixtures of unknown composition.

Several analytical methods for opioids and antidepressant drugs have been reported [40-51, 53-64, 76-90]. The use of GC–MS for the identification and measurement of drugs of abuse is currently believed to be crucial to acceptance of evidence in legal proceedings because of its sensitivity and specificity. In recent years, the coupling of liquid chromatography with mass spectrometry (LC-MS) and atmospheric pressure ionisation (ESI) has enabled the direct and specific

analysis of opioids and their glucuronide metabolites [40-51, 53-64, 76-85, 91-101]. Consequently, the evaluation of this analytical technique in the determination of morphine, M3G, and M6G has been pushed forward.

The previously published methods were primarily intended for plasma or serum matrix solely, and the extraction techniques were mostly focused on solid-phase extraction, which contained multiple steps of sample extraction and were time-consuming. There is, until now, a lack of an analytical method for the simultaneous extraction of opioids and antidepressants in biological samples.

Chapter 2 Literature review

2.1 Drug Related Death

The National Records of Scotland's National Statistics on DRDs show that, in each year since 2014, the numbers of both drug-related and opioid-related deaths were at their highest recorded levels in Scotland. Opioids were implicated in 77% of deaths and an increasing percentage of those who died from opioid-related death were prescribed an Opioid Substitution Treatment (OST) at the time of death (46% in 2016). Drug-related deaths in Scotland, 3- and 5-year moving averages, and likely range of values around 5-year moving average are illustrated in Figure 2-1 below [13]. `

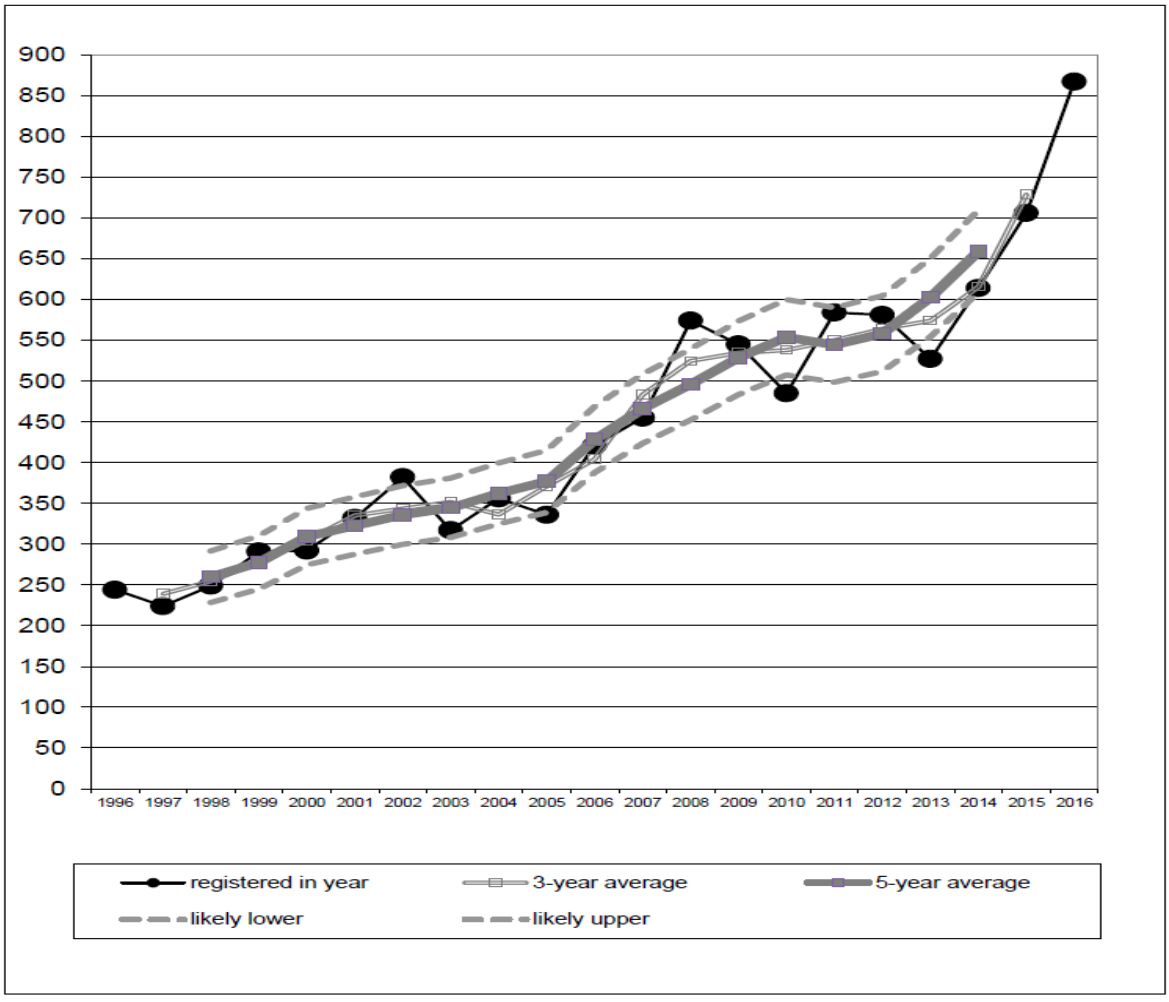


Figure 2-1: Drug-related deaths in Scotland, 3- and 5-year moving averages, and likely range of values around 5-year moving average

In 2016, almost all (796, 97%) DRDs occurred after the consumption of multiple substances. Opioids (methadone, diamorphine/morphine or buprenorphine) were

implicated in over three quarters (632,77%) of DRDs. Diamorphine/morphine (502, 61%), alcohol (398, 49%) anti-depressants (385, 47%), diazepam (376, 46%), etizolam ((269, 33%) and gabapentin (117, 15%) were the most common substances found at post mortem in 2016 – all have increased in prevalence since 2011, except diazepam [102].

In 2016, antidepressants were prescribed in the three months prior to death. Although there was an overall increasing trend from 2009 (27%) onwards, recent anti-depressant prescribing appears to have peaked in 2013 (44%). Around three-quarters (238/328, 73%) of those recently prescribed anti-depressants had them present at post mortem. Among individuals prescribed an anti-depressant in the 90 days before death, mirtazapine (145, 44%) was the most commonly prescribed drug, followed by sertraline (49, 15%) and amitriptyline (33, 10%). For other antidepressant drugs there was no clear trend over this time period [102].

The report on 2015 and 2016 DRDs highlights some new findings and emerging trends. High levels of diamorphine/morphine presence among individuals on OST demonstrated the extent of non-compliance with specialist drug treatment using methadone, antidepressants and gabapentin. These specific drugs consumed alongside opioids increase risk of overdose and (at high dose) are associated with respiratory depression. Further research on the effects of these substances in such circumstances would be beneficial [102].

Complex analgesic regimens pose risks for adverse drug interactions, since they frequently include different classes of analgesics, such as non-opioids, opioids, and/or adjuvants (antidepressants, anticonvulsants, etc.). On the one hand, medications modulating serotonergic or noradrenergic pathways, such as tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) or serotonin–noradrenaline reuptake inhibitors (SNRIs), are frequently used both in the treatment of depression [68, 103] and of pain [104]. However, it is less well known that opioids may significantly affect serotonin kinetics in the presence of other serotonergic agents, causing increased intra-synaptic serotonin levels [105]. On the other hand, some antidepressant medications have been shown to augment and prolong the effects of opioids [106-109].

2.2 Opioids

2.2.1 Background

The term opioid refers to all natural and synthetic drugs with morphine-like properties and they are one of the largest of the drug families, consisting of more than 30 drugs. Opioids are defined as compounds which have similar pharmacological effects to those of morphine, and they can be divided into three main groups: naturally occurring (morphine and codeine); semi-synthetic or derived from morphine by chemical modification (dihydrocodeine); and obtained synthetically (methadone) [110].

Diamorphine (Aceto-morphine; Diacetylmorphine) is a semisynthetic morphine derivative [111, 112]. It was synthesised for the first time in 1874 by the simple modification of morphine - acetylation of the hydroxyl groups at positions 3- and 6- of the phenanthrene ring of morphine using acetic anhydride [113]. It was intended to be used in medication as an antitussive agent, but unfortunately, the misuse of diamorphine has become a major cause of death in the world today [114, 115].

Morphine, the first “alkaloid” isolated from the opium poppy (*Papaver somniferum*), is well-known and an indispensable pain-relief medication. In addition to the distinctive pharmacological features, morphine has a unique pentacyclic skeleton, including a benzylic quaternary carbon. Over the years, extensive synthetic studies of morphine and related natural products have been conducted and more than 30 products as well as formal syntheses have been reported to date.

Codeine (Codeinum; Methylmorphine; Metilmorfina; Morphine Methyl Ether) is another natural opioid obtained from opium. This naturally occurring opioid can also be prepared by methylation of morphine. It has been prescribed widely for the relief of mild to moderate acute pain as an analgesic and antitussive agent. Codeine is often combined with non-opioid painkillers such as acetaminophen (paracetamol) and aspirin. Although codeine has been extensively used as a medication, illicit use of codeine has been reported [98, 116].

Dihydrocodeine (Dihydroneopine, Drocode, hydrocodeine, 6- α-hydrocodol, drocol, DHC-plus, Synalgos-DC) is a semisynthetic analgesic opioid [117]. DHC was prepared for the first time in 1920 by hydrogenating the double bond between carbon atoms 7 and 8 in codeine. It has been used for the relief of moderate to severe pain and has also been commonly used as an antitussive and analgesic [118]. Since 1961, it has been widely used in some countries for the treatment of opiate addicts as an alternative to methadone. However, the use of DHC has increased sharply in recent years and fatal poisoning has been reported due to the abuse of DHC [119].

2.2.2 Pharmacokinetics and Metabolism

Analysis of drugs of abuse is a common feature of forensic investigations and correct interpretation of the measured concentrations is important in both postmortem and human performance toxicology. Accurate estimation of the time of drug intake and expected drug effects from a certain dose or concentration are also frequent issues in drug-facilitated crimes. The four fundamental processes which influence the *in vivo* pharmacokinetics of a compound are absorption, distribution, metabolism and excretion (ADME). These are distinct, although in many respects, interrelated processes which occur between the administration and elimination of a compound from the body.

The proportion of active drug—whether given intravenously or absorbed from the gastrointestinal, respiratory, or cutaneous system—that enters the systemic circulation is defined as bioavailability. Wide bioavailability range among different opioids is partially attributable to differences in first-pass metabolism, when the drug is metabolized directly by the liver from the gastrointestinal tract before it reaches systemic circulation.

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 (Vd) which will have an influence on its post-mortem redistribution. The volume of distribution of a drug is the proportionality constant between the amount of drug in the body and the plasma concentration of the drug. It can be calculated by Equation 2-1 [120].

Equation 2-1 Volume of distribution

$$\text{Volume of distribution (Vd)} = \frac{\text{Amount of drug in the body at a particular time}}{\text{Plasma drug concentration at that time}}$$

The total body water volume is approximately 0.55 L/kg. Therefore, drugs that have Vd of 0.55 L/kg or less are only distributed in the body fluids [121]. In general, drugs with high Vd >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 Vd. Therefore, drugs with a high Vd tend to be highly lipid soluble and can penetrate the blood-brain barrier [BBB] [122]. Those opioids with a higher Vd are usually more lipophilic, and more likely to distribute faster and more strongly both into and out of the blood-brain barrier.

After oral administration, morphine and codeine are rapidly and almost completely absorbed. The bioavailability of morphine and codeine are (<40 % and 53%, respectively) with Vd of (2 – 5 L/kg and 3 – 6 L/kg, respectively). The vast majority of opioids are excreted as metabolites through the kidneys [119, 123, 124], morphine and codeine are mainly excreted through the kidneys 90%, as summarised in Table 2-1.

The most important area of opioid pharmacokinetics is metabolism, which converts the parent drug to a metabolite via phase I reactions, eg, oxidation, reduction, hydrolysis, and/or phase II reactions, eg, glucuronidation, methylation, acetylation, or sulfonation [124, 125]. The metabolism process may involve the cytochrome P-450 (CYP) enzymes, particularly CYP 2D6 and CYP 3A4, or other enzymes, such as UDP-glucuronyl transferase [123].

After administration, diamorphine is rapidly converted to its proximate metabolite 6-monoacetylmorphine (6MAM), which is quickly transformed into morphine. Through hepatic metabolism, morphine undergoes extensive hepatic first-pass metabolism, and is predominantly metabolised through glucuronidation in the liver into the conjugates morphine-3-glucuronide (M3G; 45–55%) and morphine-6- glucuronide (M6G; 10–15%) [124-127]. The biotransformation of diamorphine is illustrated in Figure 2-2.

6MAM is the target analyte when diamorphine-related deaths are interpreted [128]. In this case series, the presence of 6MAM in blood and its short elimination half-life (15–30 min) means that the individuals died within 1–2 h after they last

used diamorphine [129-131]. When 6MAM is detected in another matrix (e.g. urine or vitreous humour), but not in blood, a longer time span between diamorphine intake and death is likely [67, 132-135]. The plasma half-life of free morphine has been reported to be between 2 to 4 hours, but its glucuronide can be determined in urine more than 5 days after diamorphine use [136].

Codeine is mainly metabolised in the liver, although some intestinal and CNS metabolism probably occurs. A major part (50–70%) of a codeine dose is glucuronidated to codeine-6-glucuronide (C6G), while 10–15% is N-demethylated to norcodeine via the cytochrome P450 isoenzyme 3A4 (CYP3A4) [137]. Norcodeine is in turn glucuronidated to norcodeine-6-glucuronide (N6G), and a minor part is O-demethylated to normorphine [138]. Of an ingested codeine dose, 0–15% is O-demethylated to morphine by the polymorphic cytochrome P450 isoenzyme 2D6 (CYP2D6), and further glucuronidated to the inactive metabolite morphine3-glucuronide (M3G; approximately 45-55% of morphine formed) and the active metabolite morphine-6-glucuronide (M6G; 10–15%) [139].

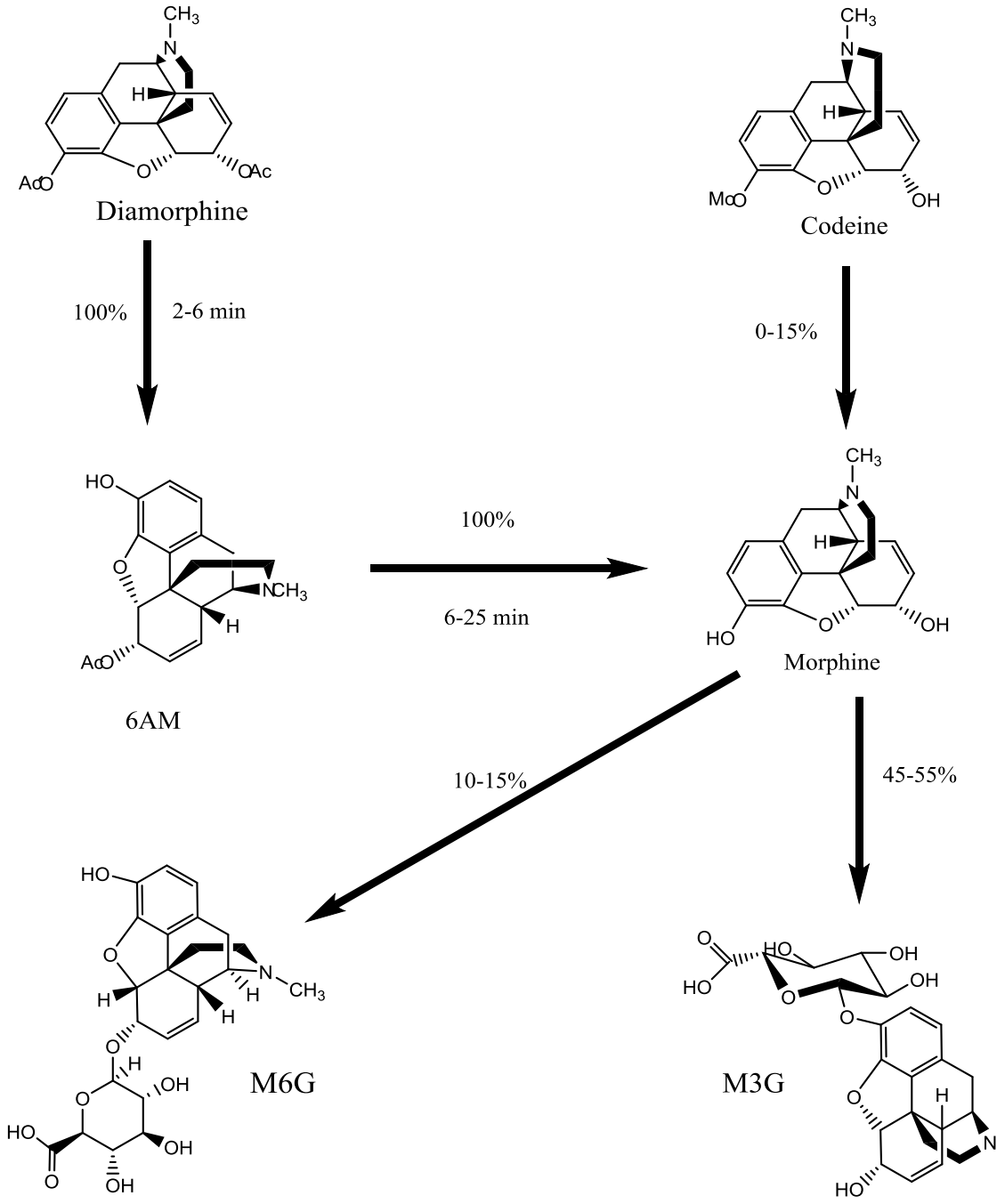


Figure 2-2: The biotransformation of Diamorphine

DHC is metabolised by N-and O-demethylation, and it is conjugated with glucuronic acid at the 6-hydroxy group to produce dihydrocodeine-6-glucuronide (DHC6G). O-demethylation is responsible for metabolising DHC to its active metabolite dihydromorphone (DHM) which is then conjugated with glucuronic acid to form dihydromorphine 3- and 6-glucuronide. Cytochrome P-450 enzyme CYP2D6 has been found responsible for O-demethylation of DHC [119].

2.2.3 Mechanism of action

Opioids produce their analgesic effects through activity at three major receptor subtypes: mu (μ), kappa (κ) and delta (δ). Morphine and most other clinically significant opioids produce their effects primarily at μ receptors which are widely distributed throughout the CNS and also in the gastrointestinal tract. Of all the opioid agonists, μ receptor agonists display the strongest analgesic action and have the highest abuse liability [140-142].

Diamorphine is two to three times more potent than morphine, although it exhibits relatively low affinity for μ receptors [143, 144]. Binding to μ receptors requires a free phenolic hydroxyl group in the morphinian structure (3-OH) which diamorphine does not possess. Thus, diamorphine is considered to be a pro-drug with its prolonged effects mediated by its more stable agonistic metabolites, 6MAM and morphine [145]. 6MAM has been found to be more potent at the μ receptor than morphine [146]. Morphine's major metabolite, M3G is a highly water-soluble metabolite and the predominant metabolite. M3G has a low affinity to opioid receptors; thus, no opioid reaction will be produced, but it seems to produce the side effects of morphine. On the other hand, M6G is a pharmacologically active metabolite being an agonist at μ and δ receptors. It has also been shown to have greater analgesic potency than morphine in humans [147, 148].

Codeine is also considered a prodrug, it binds weakly to μ-opioid receptors and exerts its clinical effect by conversion to morphine which has a much higher affinity for the μ-receptor [149].

2.2.4 Drug Interaction

Opioids undergo phase 1 metabolism by the CYP pathway, phase 2 metabolism by conjugation, or both. Phase 1 metabolism of opioids mainly involves the CYP3A4 and CYP2D6 enzymes. The CYP3A4 enzyme metabolises more than 50% of all drugs; consequently, opioids metabolised by this enzyme have a high risk of drug-drug interactions. The CYP2D6 enzyme metabolises fewer drugs and therefore is associated with an intermediate risk of drug-drug interactions. Drugs that undergo phase 2 conjugation, and therefore have little or no involvement with the CYP system, have minimal interaction potential [150].

2.2.5 Toxicity

Diamorphine is known as the drug most likely to kill as a result of an overdose and is two times more potent than its precursor morphine. Death attributed to diamorphine may occur with a dosage as low as 200 mg but also highly depends on the tolerance of the deceased. The fatal dose of diamorphine may be much more, up to ten-fold, for chronic drug abusers. However, deaths attributed to diamorphine have been reported following doses of 10 mg [143].

There are several reasons why diamorphine addicts overdose. Some may simply take too much drug, especially when the heroin is particularly high purity diamorphine, or it is enriched with further potent opioids [151]. Others may suffer from synergism between the opiate and other, concomitantly administered depressive drugs (e.g., alcohol). Another risk factor is the drug-administration environment. Addicts are at risk of overdose if they take the drug when they have no tolerance. On the occasion of the overdose, such victims do not make the preparatory conditional responses that mediate chronic tolerance, and thus are not sufficiently tolerant to the drug to survive [152].

As indicated earlier, diamorphine has a very short half-life and is rarely detected in post mortem blood samples. As a result, concentrations of its active metabolite morphine have been employed for the interpretation of cause of death and elapsed time after diamorphine administration. The presence of 6MAM in blood has been used as death occurring shortly after the administration of heroin because 6MAM has a short half-life of less than 40 minutes after administration [7]. Morphine concentrations determined in diamorphine fatalities vary between cases and there is a large overlap between deaths attributed and not attributed to diamorphine [153, 154]. The effects of diamorphine toxicity is not fully understood, and many deaths are attributed to respiratory depression [155].

The typical triad of opioid intoxication consists of coma, pinpoint pupils and respiratory depression [156]. Additional toxic effects of morphine include apathy, cold and clammy skin, confusion, constipation, dizziness, drowsiness, hypotension, hypothermia, nausea, urinary retention and vomiting [157, 158]. In healthy volunteers, impairment of cognition and motor control was observable at plasma morphine concentrations at or above 0.04 mg/L [159]. While small doses

of morphine merely depress the respiratory rate, large doses cause respiratory arrest. Profound respiratory depression and the need for assisted ventilation corresponded with peak plasma concentrations of 0.8 – 2.6 mg/L following the intravenous infusion of 55 - 66 mg/kg of morphine in surgical patients [160]. Suppression of respiratory drive accounts for the mechanism of death in most instances of opiate overdose [161].

Deaths involving diamorphine can be classified in three categories: intoxication with diamorphine alone, in combination with other centrally acting drugs and non-diamorphine related. Diamorphine users are known to build their tolerance with chronic use and can administer high doses without leading to fatal toxic effects. Diamorphine overdose deaths due to high doses would be expected to result in high concentrations of diamorphine metabolites such as morphine in postmortem blood. However, relatively low concentrations of morphine are observed in many diamorphine deaths. These can be explained due to a lack of or loss of tolerance or a long time may have elapsed between injection and death (delayed death). However, low concentrations of morphine can still be considered toxic, especially in the presence of other centrally-acting drugs and may contribute to death [153, 154, 162-164].

Overdose of codeine leads to unconsciousness and convulsions, with death likely to happen as a result of respiratory failure within 2-4 hours. A single dose of 120mg of codeine in an adult produced a peak codeine concentration averaging 0.47 mg/L [165]. A detailed Australian study on codeine related deaths considered free codeine concentrations >0.4 mg/L and a total codeine concentration of >2.0 mg/L sufficient to cause death in the absence of other contributing factors [166]. Other compilations of toxic and fatal concentrations of drugs in blood indicate codeine concentrations of 0.5 – 1.0 mg/L as potentially toxic and concentrations of 0.6 – 2.1 mg/L as potentially fatal [167]. However, codeine deaths are dependent upon the tolerance of users in which fatal concentrations can be detected in living subjects after codeine administration [168].

DHC toxicity involves dizziness, drowsiness, light-headedness, nausea and constipation. In severe exposure respiratory depression occurs followed by coma, convulsion, cardiovascular collapse and death [169]. Concentrations found at

autopsy overlapped between toxic and therapeutic concentrations due to the presence of other harmful substances while death can occur with concentrations below fatal concentrations [170]. Toxic concentrations reported were 0.8 mg/L or higher, and therapeutic concentrations were suggested to be 0.03–0.25 mg/L. In a previous study in 54 living subjects involving DHC, the mean concentration was 0.7 mg/L (range 0.1–3.3 mg/L). However, concentrations of DHC that caused death may be lower in polydrug intoxication, as reported below 1.0 mg/L, whereas most cases involving DHC alone have DHC concentrations higher than 1.0 mg/L, with the exception of naive users who had no tolerance to DHC, or in the case of delayed death [119].

2.3 Tramadol

2.3.1 Background

Tramadol is a synthetic analogue of codeine and it is a widely used therapeutic alternative to other opioid analgesics [171]. Like other opioids it can be liable to misuse. Therefore, the number of cases reporting dependence, abuse, intentional overdose or intoxication by tramadol is increasing. The administration of toxic doses of tramadol concomitantly with other central nervous system depressants is one of the most common causes of severe or fatal acute intoxication [172, 173]. In 2014, The Advisory Council on the Misuse of Drugs recommended that tramadol should be re-classified as a Class C Schedule 3 drug; prompted by increasing reports of misuse and harm. However, changes in the classification of drugs that occurred in the years up to and including 2013 had little effect on the figures [174].

2.3.2 Pharmacokinetics and Metabolism

After oral administration, tramadol is rapidly and almost completely absorbed. Plasma protein binding is ~20% and is rapidly distributed in the body with distribution volume of 3 L/kg. Tramadol is mainly excreted through the kidneys (90%) [123, 172, 175]. Pharmacokinetic properties are summarized in Table 2-1 and the chemical structure is shown in Figure 2-3.

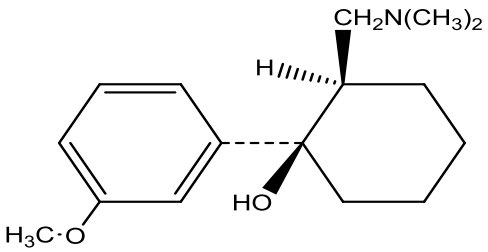


Figure 2-3 Chemical Structure of Tramadol

Tramadol is mainly metabolised in the liver by O- and N-demethylation, catalysed by the cytochrome P450 (mainly isoenzyme CYP2D6) and followed by conjugation with glucuronic acid and sulfate. O-demethylation of tramadol to O-desmethyltramadol (M1) is mediated by cytochrome P450 (CYP) 2D6 and possibly by 2B6 enzymes [176-178]. N-demethylation via CYP3A4 and CYP2B6 yields N-desmethyltramadol (M2) [179, 180]. Tramadol may be further metabolised to three additional secondary metabolites (M3, M4 and M5) [176, 181, 182].

2.3.3 Mechanism of action

Tramadol is a centrally acting opioid analgesic. It has a dual mechanism of action, which is a partial agonist of μ-opioid receptors and inhibits serotonin and noradrenaline reuptake at the synapses of the spinal cord, acting on the pain transmission mechanism [172].

2.3.4 Drug Interaction

The risk of interactions is elevated in patients who use multiple medications for pain control or antidepressants for the treatment of comorbid depression. These interactions increase the risk of serotonin syndrome, which may occur with a high dose of a single drug but appears more common when serotonergic agents are used together. Serotonin syndrome results from excessive central nervous system and peripheral serotonergic activity [183].

In a Finnish study, every fifth inpatient using tramadol took concomitant medication with potential to inhibit the conversion of tramadol to its pharmacologically active O-desmethyl metabolite [184], which may therefore increase blood concentrations and in turn increase risk of adverse effects.

2.3.5 Toxicity

Tramadol is a widely used therapeutic alternative to other opioid analgesics since it was thought to have a low potential for abuse, dependence and tolerance, and low probability to cause adverse effects, including respiratory depression. However, the number of cases reporting dependence, abuse, intentional overdose or intoxication by tramadol is increasing. Fatal intoxications due to tramadol alone also exist but are not common. The administration of toxic doses of tramadol concomitantly with other central nervous system depressants is one of the most common causes of severe or fatal acute intoxication [172].

2.4 Methadone

2.4.1 Background

Methadone, a very useful analgesic and the most utilised drug for replacement therapy in patients with opioid-related addiction, is now also widely used for the management of chronic pain. Methadone was synthesized in Germany before World War 2 and imported to the USA by Lilly after the war and was utilised for several years as an opioid analgesic but lost popularity in the 1950s. In the early 1960s, Dole and Nyswander proposed that patients abused opioids to compensate for an endogenous opioid deficiency, and it was introduced as a maintenance medication to control craving in patients treated for drug addiction [185, 186].

2.4.2 Pharmacokinetics and Metabolism

After oral administration, methadone is rapidly and almost completely absorbed and distributed in the body with distribution volume (Vd) of 1 - 8 L/kg. The bioavailability of methadone is 36 – 100 % and it is primarily excreted via bile. Pharmacokinetic properties are illustrated in Table 2-1. [123, 187]

Methadone is extensively metabolised in the liver, methadone is converted into its primary metabolites 2-ethylidene-1, 5-dimethyl-3, 3 diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3, 3-diphenyl-1-pyrrolidine (EMDP) by the cytochrome P450 enzymes CYP3A4, CYP2B6 and to a lesser extent CYP2D6 [188, 189]. The biotransformation of methadone to EDDP is illustrated in Figure 2-4.

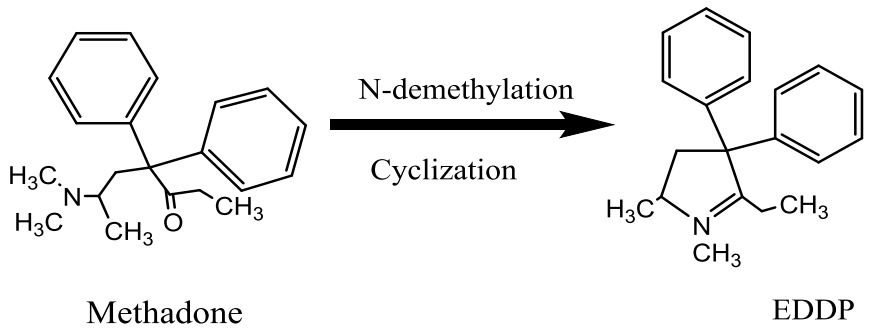


Figure 2-4: The biotransformation of methadone to EDDP

2.4.3 Mechanism of action

Methadone has a number of unique characteristics when compared to other opioids. It has agonist affinity for both the μ and δ opioid receptors, it acts as an inhibitor at the presynaptic N-methyl-D-aspartate (NMDA) receptors, and it blocks the reuptake of noradrenaline and serotonin in the peri-aqua-ductal grey matter [190].

2.4.4 Drug Interaction

Combined methadone and antidepressants are frequently prescribed in different settings, and data on possible interactions of different regimens of methadone with different antidepressant drugs are sparse. The interaction of acute and chronic methadone with antidepressants is a complex phenomenon, and further studies are needed in order to assess the interactions of chronic antidepressant medications with single-dose and chronically administered methadone in greater depth. See also section 2.5.4.

2.4.5 Toxicity

A recommended serum therapeutic range of between 0.15 and 0.6 mg/L has been reported when monitoring methadone maintenance patients [191] and a therapeutic window for dosage ranging from 60 mg to 120 mg suggested [192]. In methadone toxicity, respiratory depression after treatment with methadone and other μ opioid agonists results from decreased chemoreceptor sensitivity to circulating CO_2 concentrations in the medullary brain stem. Thus, relatively low doses of methadone in opiate-naive individuals and sufficiently high doses of methadone in opioid-tolerant individuals can lead to fatal overdose consequent to respiratory depression and cardiopulmonary failure. Of 176 methadone-

related fatalities, methadone was the only drug detected in postmortem blood and urine toxicological analyses in 11 (6.25%) cases. The mean methadone blood concentration of all 176 cases was 0.535 mg/L (0.02–4.0 mg/L) [193]. There is large overlap between therapeutic concentrations and concentrations reported in fatal methadone cases. The risk for methadone (and opioid) overdose is exacerbated when used in combination with other opioids and/or sedatives, including alcohol and benzodiazepines [194].

2.5 Antidepressants

2.5.1 Background

Over the past three decades, tricyclic antidepressants (TCAs) were the first line agents to treat depression such as amitriptyline introduced in the 1960s as a tricyclic compound [195, 196]. On the other hand, selective serotonin reuptake inhibitors (SSRIs) are the most widely prescribed antidepressants worldwide. By the early 1990s, the SSRIs became first-line antidepressants in clinical practice and accounted for more than half of all antidepressant prescriptions such as sertraline and citalopram [197, 198]. Among the antidepressants of the newer generations, mirtazapine is one antidepressant which is used mainly in the treatment of depression and sometimes used in the treatment of anxiety disorders, insomnia, nausea, and vomiting, and to produce weight gain when desirable [199].

2.5.2 Pharmacokinetics and Metabolism

Amitriptyline is completely but slowly absorbed from the gastrointestinal tract after its oral uptake such that the peak plasma concentrations are achieved within 4 to 8 h of its administration with distribution volume of 6-10 L/kg. It has a systemic bioavailability ranging from 33 to 62% and is subject to extensive hepatic pre-systemic elimination [200, 201].

After oral administration, citalopram is rapidly absorbed, with peak plasma levels observed approximately after 1–4 h and it has a plasma half-life of approximately 35h. Citalopram is highly lipophilic and has one chiral centre, its lipophilicity results in high bioavailability (approximately 80%) after oral administration. Approximately 12–23% of orally dosed citalopram is excreted

unchanged in the urine, and approximately 10% is excreted in the faeces [28, 201, 202].

Sertraline is slowly absorbed from the gastrointestinal tract after its oral uptake such that the peak plasma concentrations are achieved within 4.5 to 8.4 h of its administration. It distributed in the body with distribution volume of 20-50 L/kg, and protein binding 98% [201, 203, 204].

After oral administration, mirtazapine is rapidly and completely absorbed with peak plasma concentrations achieved within 2 h of dosing, and distribution volume of 10-14 L/kg. Mirtazapine has an elimination half-life of 20-40 h, is approximately 85% plasma protein bound, and is 75% excreted in the urine [201, 205, 206].

Antidepressants are primarily metabolised through the hepatic isoenzyme CYP2D6 with 1A2 and 3A4 as secondary routes [201]. Pharmacokinetic properties are illustrated in Table 2-1 and chemical structures in Figure 2-5.

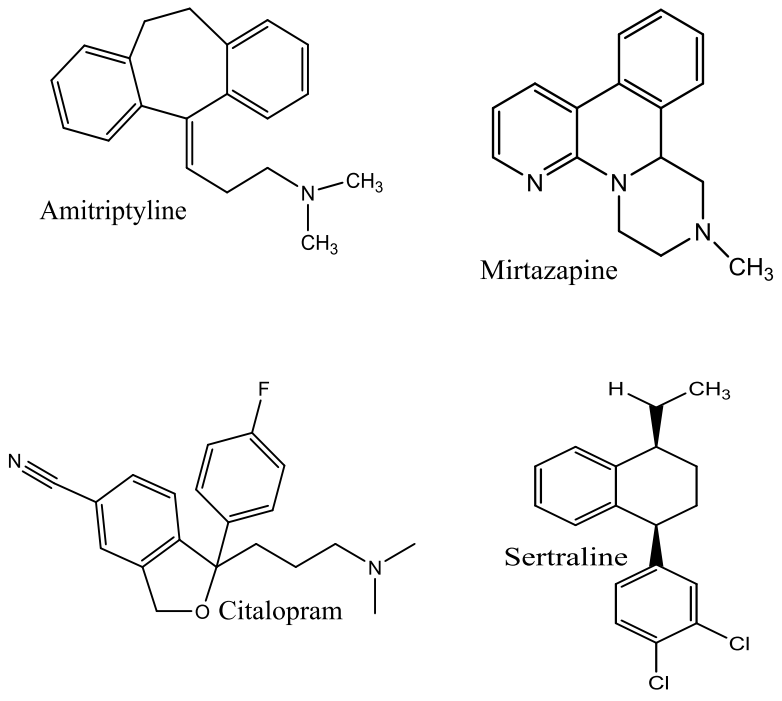


Figure 2-5 Chemical Structures of Mirtazapine, Amitriptyline, Citalopram and Sertraline.

2.5.3 Mechanism of action

The antidepressant action of TCAs is thought to be due to their inhibition of norepinephrine (NE) and serotonin (5-HT) reuptake, thus leading to increased

concentrations of these monoamines in the synaptic cleft, down-regulation of postsynaptic receptors and subsequent changes in gene expression [207].

All SSRIs, although differ structurally, have the same mechanisms of action: as the name implies, these compounds selectively inhibit the serotonin transporter, all agents are potent inhibitors of the serotonin reuptake transporter. In addition, sertraline may have relatively greater inhibitory potential at the dopamine transporter [208].

Mirtazapine is a postsynaptic drug which enhances noradrenergic and 5-HT1A-mediated serotonergic neurotransmission via antagonism of central α2-auto- and hetero-adrenoreceptors [209].

2.5.4 Drug Interaction

TCAs, the majority of significant pharmacokinetic interactions with antidepressants involve drug induced changes in hepatic metabolism, which predominantly involve the cytochrome P450 isoenzymes. In some cases, increased TCA toxicity with methadone co-administration have been reported [210, 211]. In a retrospective study, decreased methadone clearance was found in patients receiving amitriptyline [212]. Further studies are required to better understand the underlying mechanism of these interactions.

SSRIs that inhibit the CYP 450 systems will impair metabolism of other medications (P450 enzyme substrates). Enzyme inhibition occurs within two to three days, due to the offending drug binding to the metabolising enzyme preventing it functioning [213], thus prolonging their elimination half-life and increasing their blood concentration. The SSRI inhibition of cytochrome P450 activity may lead to elevated concentrations of concurrently administered TCAs which are metabolised by CYP 2D6 and 3A4 isoenzymes. This may lead to adverse effects such as seizure and death [214, 215].

The interaction of mirtazapine with opioid, noradrenergic and serotonergic agonists and antagonists found that, the antinociceptive effect of mirtazapine mainly involves μ- and κ3-opioid mechanisms. This opioid profile of mirtazapine may be one of the explanations to its efficacy in severe depression, unlike the SSRIs and other antidepressants which lack opioid activity [209].

2.5.5 Toxicity

TCAs have a relatively low therapeutic index and serious consequences in overdose. For most TCAs, the therapeutic dose is about 3–4 mg/kg/day and a potentially fatal dose is 15–20 mg/kg/day. Postmortem femoral blood levels of mirtazapine concentration ranged from 0.01-2.7 mg/L [206, 216], citalopram in the range of 0.014 – 11.6 mg/L, sertraline in the range of 0.09 – 0.88 mg/L [217] and amitriptyline in the range of 1.0 – 39 mg/L [218]. Several studies have published maximum toxic serum concentrations for amitriptyline <0.6 mg/L, citalopram <0.4 mg/L, sertraline <0.6 mg/L and mirtazapine <0.3 mg/L [219].

TCAs have strong anticholinergic (antimuscarinic) activity, which may cause constipation, dry mouth, urinary hesitancy/retention, blurred vision, dyspepsia, and confusion [198, 220]. In elderly patients, more severe side effects, such as tachycardia, confusion, agitation, or even delirium may occur at therapeutic doses [221]. Although rare, these severe complications may occur when a patient has been taking another anticholinergic drug concomitantly with a TCA; neuroleptics, anti-Parkinsonian agents, antihistamines, antispasmodics and over the counter sleeping pills are commonly involved.

SSRIs, although generally well tolerated, may produce anxiety, sleep disturbances, and gastrointestinal discomfort, especially at the initiation of therapy. There have been fatalities with overdoses of more than 150 times the usual daily dose. Almost all fatalities occurred in patients who took SSRIs and other substances, usually alcohol, benzodiazepines, morphine or other drugs [222, 223].

Mirtazapine in a study of 30 patients found that, a patient developed slurred speech, mood swings, depression, drowsiness and fatigue during treatment for gastroparesis with oral mirtazapine 15mg once a day [224].

2.6 Gabapentin

2.6.1 Background

Gabapentin is an antiepileptic drug shown to be effective as add-on therapy for patients with drug-resistant partial seizures with or without secondary generalization [225, 226]. Due to its tolerability, and its broad and complex

mode of action, gabapentin has also been studied for other indications. The largest area of nonepileptic use of gabapentin is neuropathic pain [227], but it appears to be effective in other types of pain as well. In addition, it has been reported to be a useful pharmacological agent for several psychiatric disorders, such as bipolar disorder, social phobia and other anxiety disorders [228, 229]. A number of studies have also suggested the potential effectiveness of gabapentin in the treatment of alcohol withdrawal [230, 231], and cocaine dependence [232].

2.6.2 Pharmacokinetics and Metabolism

Gabapentin is a gamma amino butyric acid (GABA) analogue, 1- (amino methyl) cyclohexane acetic acid. It is absorbed from the gastrointestinal tract through an active transport system whose efficiency decreases at higher doses. Gabapentin is distributed in the body with distribution volume of 0.8–1.3 L/kg. The time to reach peak concentration in blood is 4–5 hrs. The drug does not bind to plasma proteins and is not metabolized and eliminated unchanged. Elimination is solely by renal excretion (76 – 81%) [117, 233, 234]. Pharmacokinetic properties are illustrated in Table 2-1 and chemical structure in Figure 2-6.

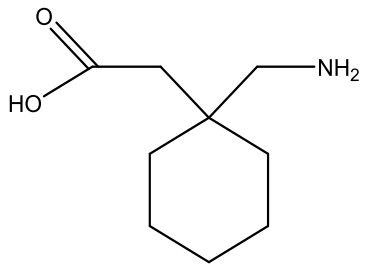


Figure 2-6 Chemical structure of Gabapentin

2.6.3 Mechanism of action

Gabapentin interacts at binding site of alpha$_2$ delta subunit of voltage-dependent Ca^{2+} channels, correlates with decreased Ca^{2+} channel function and release of neurotransmitters and decreased neurotransmitter release is associated with reduced neuronal hyperexcitability [225].

2.6.4 Drug interaction

The mechanism by which gabapentin may increase the risk of death in opioid users likely reflects both a pharmacodynamic and pharmacokinetic interaction [235]. More specifically, it likely reflects additive respiratory depression as well as increased gabapentin concentrations with concomitant opioid use [236]. A pharmacokinetic interaction most likely reflects increased gabapentin absorption, which occurs primarily in the upper small intestine [235]. Thus, opioid induced slowing of gastrointestinal transit could prolong the time spent within this narrow absorption window and increase gabapentin bioavailability [235].

2.6.5 Toxicity

The therapeutic range for gabapentin in blood is about 2 to 20 mg/L, with toxicity at >25 mg/L, but toxicity has been reported at concentrations as low as 15 mg/L, and many patients with concentrations >25 mg/L have no manifestations of toxicity [237, 238]. To date, reported cases of death attributed to self-poisoning with gabapentin have been associated with postmortem peripheral blood concentrations of 37 to 88 mg/L [239]. In another study (n=14), blood concentrations ranged from 30 to 82 mg/L in cases of mixed drug toxicity as the cause of death [240]. Manifestations of toxicity include dizziness, confusion, lethargy, myoclonus, ataxia, and tremulousness [237]. Although gabapentin is widely perceived as safe, drug-induced respiratory depression has been described when gabapentin is used alone or in combination with other medications. Because gabapentin and opioids are both commonly prescribed for pain, the likelihood of co-prescription is high, concomitant treatment with gabapentin was associated with a substantial increase in the risk of opioid-related death [241-243].

Table 2-1: Pharmacokinetic parameters

Analytes	Ref.	T½ (hours)	Bioavail-ability (%)	Vd (L/kg)	Protein Binding (%)	Time to peak conc. in bl. (Hrs)	Blood conc. in chronic therapy (mg/L)	Major metabolic enzyme(s)	Active metabolite (s)	Main organ excretion (%)
Diamorphine	[123]	>0.1	<1%	-	-	-	0.01-0.1	CYP 2D6, 3A4, UGT	Morphine & 6-monoacetyl - morphine	-
MOR	[123, 124]	2–4	<40%	2-5	-	-	0.01-0.1	UGT	M6G	Urine 90% Feces 10%
COD	[123]	3	53%	3–6	-	-	0.01-0.25	CYP 3A4, 2D6	Morphine & Hydrocodone	Urine 90%
DHC	[119]	2-4	High	-	-	-	-	UGT CYP 2D6	-	-
Tramadol	[123, 172, 175]	6.3 - 7.4	75%	2.6- 3	20%	-	-	CYP 3A4, 2D6,	M1, (ODT) O-desmethyltramadol	Urine 90% Feces 10%
Methadone	[123, 187]	7–59	36–100%	1–8	-	-	0.1-0.5	CYP 3A4, 2D6, 2B6, 2C19	None	Bile
Sertraline	[201, 203, 204]	22–36	-	20–50	98%	4.5 - 8.4	20 - 309	CYP 2D6, 1A2, 3A4	Desmethylsertraline	-
Citalopram	[28, 201, 202]	33	80%	12-16	50%	2 - 4 or 4 - 8	-	CYP 2D6, 1A2, 3A4	Desmethylcitalopram	Urine 12–23 %
Mirtazapine	[201, 205, 206]	20-40	-	10-14	85%	2	0.02 - 0.075	CYP 2D6, 1A2, 3A4	Desmethylmirtazapine	Urine 75%
Amitriptyline	[200, 201]	9–25	33 - 62%	6-10	95%	-	-	CYP 2D6, 1A2, 3A4	Nortriptyline, 10-OH nortriptyline, 10-OH amitriptyline	-
GBP	[117, 233, 234]	5-9	-	0.8-1.3	<0.03%	4 - 5	1.9-2.6	-	-	Urine 76-81%, Feces 10-32%

2.7 Interpreting postmortem drug and metabolite concentrations

There are several challenges in the interpretation of postmortem drug and metabolite concentrations. The difficulty associated with correlating a specific blood concentration with toxicity is well known. In the first instance, drugs may affect different people in different ways and may even affect the same person differently on different occasions. The existence of tolerance introduces further complications in the interpretation of blood drug concentrations because a particular drug concentration may be associated with death in a naive user yet produce minimal symptoms in a tolerant individual. In addition, changes in drug concentration occurring during the postmortem interval can grossly complicate the interpretation of toxicology findings [244].

In the determination of cause of death and likely level of drug impairment prior to death, toxicological measurements can never be considered in isolation. Autopsy findings and additional information such as that obtained from the scene, eye witness reports and the individuals medical/drug history, where available, must also be taken into consideration.

2.7.1 Relating postmortem blood morphine concentrations to toxicity

One of the major problems in the interpretation of morphine concentrations is that the fatal concentrations reported in the literature often overlap with the stated therapeutic (0.0003 – 0.73 mg/L) and toxic concentrations (0.001 – 15.7 mg/L) [245]. Tolerance to both the pharmacological and respiratory depressant effects of morphine occurs rapidly, and morphine concentrations obtained at autopsy may be misinterpreted if concentrations presumed to be fatal in non-tolerant individuals are applied to active diamorphine or morphine users or to individuals undergoing chronic pain treatment with opioids who have built up tolerance to the drug [245]. In cases of acute overdose, blood morphine concentrations have ranged anywhere between 0.02 to 3.7 mg/L [245-257]. However, blood morphine concentrations in excess of 1 mg/L have been reported in drug impaired drivers apprehended in Sweden [258]. In patients receiving adequate opiate therapy for chronic pain, morphine concentrations as high as 2.1 mg/L have been observed [259].

Heroin overdose as the cause of death may be evident in cases involving heroin body packing, where blood morphine concentrations as high as 120 mg/L have been reported [260]. In a great majority of heroin overdose cases the morphine concentrations recorded at autopsy are, in fact, lower than or similar to those recorded in living intoxicated addicts or heroin users who have died of causes other than overdose [3, 5]. Darke *et al*. (1997) observed substantial overlap in the blood morphine concentrations measured in heroin overdose fatalities (averaged: 0.35 mg/L; ranged: 0.08 – 3.2 mg/L; n = 39) with those measured in living addicts receiving maintenance diamorphine (averaged: 0.09 mg/L; ranged: 0. 05 – 1.45 mg/L; n = 100). Only four of the 39 heroin fatalities had morphine concentrations exceeding the highest concentration measured in the current users. Low blood morphine concentrations in cases of heroin overdose has been largely attributed to periods of abstinence resulting in loss of tolerance, delayed death and/or the concomitant use of other drugs [163].

2.7.2 Estimating survival time in diamorphine fatalities

The interpretation of toxicological analyses in diamorphine-related deaths is complicated because many factors affect the concentration of morphine measured following autopsy, and its potential significance, including [261] the time interval between last dose and death, [135] the postmortem interval, [262] an individual's drug tolerance level, [254] the concomitant use of other drugs and [263] the site of sampling. As a consequence of the rapid metabolism of diamorphine and its unique metabolic marker 6-monoacetylmorphine (6MAM), distinguishing diamorphine from morphine use may be difficult. Esterase mediated conversion of 6MAM to morphine occurs quickly in blood ($T_{1/2}$ circa 15 min) and 6MAM may only be detectable in this matrix for 2–3 h following diamorphine exposure [261]. Urine is generally thought to be the best specimen for 6MAM detection owing to its limited esterase activity and comparatively high 6MAM concentrations [135]. One advantage of 6MAM's rapid metabolism in blood is that its detection in this matrix provides an indicator of very recent diamorphine exposure [145, 254, 264].

Some toxicology laboratories report the concentration of free-morphine in blood when investigating heroin-related deaths whereas others report total-morphine (free + conjugated), which is the sum of free-morphine along with the amounts

released after hydrolysis of the morphine-3-glucuronide and morphine-6-glucuronide metabolites [254, 258]. The use of free morphine (FM) to total morphine (TM) concentration ratios as a means of evaluating the time of survival following heroin or morphine injection has been advocated in several studies [134, 250, 254, 256, 264-267] and may be of use in cases in which 6MAM is not detectable in the blood (a high FM/TM ratio is thought to reflect insufficient time for morphine metabolism to occur and thus be indicative of a relatively rapid death) [135, 268, 269].

In an examination of 57 medical examiner cases Burt *et al*. (2001) [36] reported lower femoral blood TM and FM concentrations in cases where 6MAM was detectable in the blood. Comparing blood concentrations for cases with (n = 23) and without (n = 34) detectable 6MAM demonstrated a mean TM concentration of 0.9 and 2.1 mg/L respectively with higher FM/TM ratios in individuals with detectable 6MAM (0.35 mg/L) compared to cases where the metabolite could not be detected (0.14 mg/L) [36].

The relative amount of FM to TM in blood was suggested as a way to distinguish a rapid heroin death from a delayed death [256, 267]. The ratio of FM/TM should be closer to unity (1.0) in a rapid death because less time is available for transformation of morphine into its glucuronide conjugates. However, this rests on the assumption that these metabolites were not measurable in blood before the fatal dose of heroin was taken, which is potentially not the case in a chronic heroin user. Morphine and its metabolites undergo enterohepatic recirculation and after chronic usage the glucuronide metabolites might persist in the body for a long time [270]. Moreover, the analytical conditions used to cleave the glucuronides, whether by enzymes or acid, and the relative rates of conversion back to morphine might differ between laboratories [69]. Another aspect to consider is the stability of morphine glucuronides in blood after death and the possibility that they undergo re-distribution and conversion in storage [50, 147, 161, 271-275].

2.7.3 Postmortem change and redistribution

In addition to tolerance and poly-drug use, the interpretation of postmortem blood concentrations is further complicated by the fact that drug concentrations measured at autopsy do not necessarily reflect the concentration at the time of

death. It is known that after death there is a movement of drugs around the body which takes place via a process known as postmortem redistribution (PMR) [67, 276, 277]. These changes are still not entirely understood but a large volume of distribution (Vd), pH of the blood and/or pka of the drug, protein binding, how lipophilic the compound is, as well as putrefactive processes and bacterial breakdown, all seem to play a role. [19, 278]

PMR has been most associated with a large volume of distribution (Vd) >3 L/kg and a high degree of lipophilicity [25, 172, 276, 279, 280]. Basic drugs are more susceptible to PMR as their ionised fraction increases with the mainly aqueous content of cells as they become more acidic postmortem. During postmortem lysis of cells basic drugs diffuse more easily into hydrophilic body fluids, which can potentially cause increases in drug concentrations in blood [281]. Since opioids and antidepressant drugs are basic and generally lipophilic with a large Vd they are likely to be susceptible to PMR, however, this has not been studied in detail. The high Vd of morphine (3 – 5 L/kg) would indicate that it undergoes PMR and it has been shown to do so in animal models [20, 251, 282]. There are, however, conflicting reports on its redistribution in humans and PMR was found not to be a factor in diamorphine fatalities [255]. Considering the low Vd of M3G (0.14 L/kg) and M6G (0.15 L/kg) (Hunt *et al*. 1999), these metabolites would not necessarily be expected to undergo PMR. However, Skopp (1996) [273] observed two to three folds differences in the molar concentrations of both glucuronides in blood sampled from different sites in four diamorphine overdose deaths. Some of the variation was attributed to variations in water content (65 - 83%) and haematocrit (25 – 75%). According to Carrupt *et al*. (1991), morphine glucuronides can exist in two conformational forms, the folded one being more lipophilic than the unfolded one. The site- to site-variations in the concentrations of these metabolites could be associated with this peculiarity.

Frost *et al* [238] investigated the postmortem redistribution of morphine, M3G, M6G, codeine, nor-codeine and C6G in different biological matrices. Principal component analysis was used to investigate possible correlations between free morphine in the various tissue samples and to compare ante-mortem with postmortem samples. Some correlations were observed but gave poor predictions (>20 % error) when back calculating. In matrices other than blood, the concentration pattern was similar, although in a less systematic fashion. The

different blood/tissue concentration ratios showed no systematic relationship with the postmortem interval. No coherent degradation or formation patterns for codeine, morphine, M3G and M6G were observed upon reanalysis in peripheral blood after storage. [282, 283]

Earlier studies have proved that for some, but not all drugs, there are significant differences in concentrations between peripheral and corresponding heart blood. The drug concentrations in the heart blood are often higher than in the peripheral blood [284] and do not reflect concentration at the time of death [276]. The underlying mechanisms include passive drug release from drug reservoirs such as the gastrointestinal tract, liver, lungs, and myocardium at once after death and, later, cell autolysis and the putrefactive process [285]. In general, PM blood from a femoral vein, showing less PMR than central blood, should be used for quantitative determinations on a routine basis.

Logan and Smirnow examined 40 heroin-related deaths where the mean postmortem interval was 59 hours and found no significant difference between morphine concentrations in admission and autopsy blood. The cardiac to femoral blood concentration ratios averaged 1.1 for morphine, 1.3 for M6G and 1.1 for M3G. They found no evidence of time-dependent changes in morphine concentrations at either central or peripheral blood sites in 32 cases. They did, however, report consistently higher morphine concentrations in ventricular compared with femoral blood, with the greatest differences observed in cases where the ventricular morphine concentration exceeded 0.3 mg/L [249]. In ten deaths involving morphine, Dalpe-Scott *et al* (1995) found central to peripheral blood morphine concentration ratios ranging from 1.0 – 5.8 with a mean of 2.2. Other authors have also reported obvious differences between central and peripheral blood concentrations of morphine, M3G and M6G in humans [25, 60, 61, 273, 274]. Concentration differences within the heart have also been reported for morphine with left ventricle concentrations two to three times higher than in the right ventricle [274]. It is thought that PMR from tissue to blood may easily double the morphine concentration in the latter [60, 61, 273] and since the glucuronides are predominantly distributed in plasma this occurrence would invalidate the use of morphine to metabolite ratios in estimating survival time [161, 273].

The liver to peripheral blood (L/PB) ratio, based upon review of previously published works, was evaluated as a marker of PMR. Literature supported the proposed model that drugs exhibiting an L/PB ratio of less than 5 are prone to little or no PMR, while those with an L/PB ratio greater than 20–30 have propensity for significant redistribution. Many antidepressants, including both tricyclic antidepressants and selective serotonin re-uptake inhibitors, were markedly differentiated from drugs previously verified to be free from, or exhibit little, PMR. The magnitude of the liver to blood concentrations also appeared to provide an advantage over the conventional central to peripheral blood ratio model of PMR by demonstrating a wide range of values (1.6–97) for interpretation of drugs' potential for, and variations in, redistribution [286]. The average HB/PB and L/PB ratios that were generated from previously published data are listed in Table 2-2 [25, 167, 203, 206, 216, 240, 249, 287-292].

Table 2-2: Central to peripheral blood (HB/PB) and liver to peripheral blood (L/PB) ratios

Drug	HB/PB	L/PB	References
Morphine	1.8 (n=44) (range 0.1-11.0)	-	[167, 249, 287]
M3G	2.7 (n = 44) (range 0.0-8.7)	-	[167, 287]
M6G	2.6 (n = 44) (range 0.5-8.8)	-	[167, 287]
Codeine	1.0	-	[167]
DHC	No data available		
Tramadol	1.1 ± 0.3, (n = 6)	1.6 ± 1.3, (n = 8)	[288]
Methadone	1.3 ± 0.2, (n = 6)	6.8 ± 0.9, (n = 5)	[25]
Amitriptyline	3.0 ± 2.8, (n = 30)	25 ± 18, (n = 8)	[289]
Citalopram	1.2 ± 0.5, (n = 20)	9.9 ± 5.9, (n= 22)	[290]
Sertraline	1.3 ± 0.3, (n = 11)	97 ± 40, (n = 10)	[203, 291]
Mirtazapine	1.1 ± 0.3, (n = 19)	5.8 ± 2.4, (n= 19)	[206, 216, 292]
Gabapentin	0.84 ± 0.25 (n=14)	0.61 ± 0.19 (n=14)	[240]

Currently published data on the PMR for drugs of interest has been obtained from animal studies, targeting one or a few analytes [20, 282], or from human tissue distribution studies in postmortem cases. These studies focused predominantly on the impact of sampling site on a postmortem drug concentration, rather than the influence of the postmortem time interval (PMI). This is probably due to the difficulty in obtaining relevant specimens for testing and ethical restrictions on human experimentation on deceased persons. Since

an autopsy is unlikely to be carried out immediately following admission of a body to a mortuary, a PMI of several days is common, increasing the likelihood of substantial postmortem changes in concentrations. The Victorian Institute of Forensic Medicine was able to obtain a peripheral blood specimen on admission to the mortuary as part of its ability to conduct preliminary examinations prior to a coroner order on whether an autopsy should be conducted. The order to conduct an autopsy can take several days. This allows an opportunity to compare the blood concentrations on admission and the subsequent concentrations [255].

The postmortem phenomena of pre-autopsy interval during storing bodies for long periods of time will cause greater changes in blood drug concentrations [14]. Therefore, to avoid the effect of the pre-autopsy interval on drug concentrations it is always preferable for early collection of specimens for analysis, which are collected closer to the time of death, and would enable a better assessment of the likely contribution of drugs to the death [28-30].

However, many other factors must be considered in the evaluation of drug concentrations in a postmortem sample, such as techniques of blood sampling [31-33]. Several reviews have considered the factors that can influence the concentration of drugs measured postmortem including techniques of blood sampling. However, this may not be consistent for all techniques, a limited number of studies have shown that needle puncture in the upper thigh (blind stick) did not affect the blood concentration compared to dissection of the vein in the upper thigh [28, 34].

Further, residual metabolic enzyme activity, variable with the nature of the enzyme involved, occurs in the early postmortem period [19]. Continuing drug metabolism and metabolite deconjugation during the postmortem interval is an important consideration when interpreting parent drug to metabolite ratios. The concentrations of free morphine can increase significantly in the postmortem period due to hydrolysis of the morphine glucuronides [274]. Escherichia coli, one of the most predominant bacteria present in intestinal flora, is an important source of β-glucuronidase [293], a hydrolase known to deconjugate morphine glucuronides, particularly M3G [271], in putrefying blood and tissues [262, 274, 294]. The hydrolysis of morphine glucuronides back to free morphine during the postmortem interval can alter the ratios significantly as a function of time.

2.7.4 Diamorphine stability during sample storage

Degradation of diamorphine to 6-monoacetylmorphine (6MAM) and then morphine happens rapidly *in vivo* and *in vitro*. The rates of diamorphine and 6MAM degradation depends on the type of biological samples, and the duration and conditions of storage. Thus, the potential for drug concentrations to change following specimen collection is a further factor to be considered when interpreting results.

In whole blood, plasma Butyryl-cholinesterase (BuChE), as well as erythrocyte Acetylcholinesterase (AChE), has been shown to hydrolyse diamorphine to 6MAM with further hydrolysis to morphine mediated by AChE but not BuChE [204, 295]. Owing to the absence of AChE in plasma, deacetylation of 6MAM to morphine is not observed in this matrix [296-299]. It is known that 6MAM degrades in a blood sample at room temperature with a half-life of 8 hours [300]. At 4°C, 6MAM was reported to be unstable in blood with only 20% of the initial concentration detected after storage for 7 days [301].

Regarding spiked samples from living subjects, three separate studies found that morphine with their metabolites remained stable at least 6 days at room temperature (n = 6, n = 5 and n = 10, respectively) [72-74]. In another separate study (n = 5), they found excellent stability for morphine and their metabolites during 6 months of storage at 4°C and -20°C in blood and plasma samples. On the other hand, blood samples from living subjects (not spiked) showed morphine concentrations decreased significantly after two years of storage in tubes containing sodium fluoride and potassium oxalate; the tubes were stored at ambient temperature, but with further storage, increased concentrations were observed [75].

The stability of morphine, codeine, and 6MAM in blood was studied after different sampling conditions: (i) in glass, polypropylene or polystyrene tubes, (ii) with addition of dipotassium ethylene diamine tetra-acetic acid (K_2EDTA) or sodium oxalate ($Na_2C_2O_4$), and (iii) with or without the addition of sodium fluoride (NaF). Spiked blood samples were stored at two different temperatures (4 and -20°C), analysed after different storage times and after three freeze–thaw cycles. Opiate concentrations decreased in all conditions, with the most unstable being 6MAM. The addition of NaF as preservative improved the stability

of opioids at all conditions studied, whereas the type of anticoagulant did not affect the stability of opioids. It was concluded that blood samples should be stored at -20°C in glass tubes containing oxalate and NaF for maximum stability [302]. Inconsistent with other studies, Rees, K.A., *et al*. (2011) reported that the addition of NaF slowed but did not prevent the breakdown of 6MAM in blood stored at room temperature for 84 days [262]. Other studies have shown a considerable decrease in 6MAM concentrations, even with the addition of NaF, hydrolytic activity may persist for months [294, 303].

In a previous study, total morphine, total codeine, free morphine, and free codeine were stored in glass culture tubes without preservatives at room, refrigerator, and freezer. They were then analysed each month for 11 months. Total morphine and total codeine concentration decreases (10 to 40%) were observed for all specimens in all storage conditions. Free morphine and free codeine showed slight but steady increases [70]. Another separate study showed increased stability of opioids (morphine, codeine, oxycodone, hydrocodone) in dried blood spot (DBS) matrix compared to blood/plasma. This method was successfully used to measure hydrocodone and its major metabolite nor-hydrocodone [71].

It has been documented that the rate of deacetylation of diamorphine to 6MAM in aqueous solution is dependent on pH and temperature, increasing with higher pH and temperature. The breakdown of the drug is significantly inhibited in aqueous solution at pH 4 and at a temperature of 4°C. 6MAM is also unstable in aqueous solutions, but its degradation to morphine is limited under acidic conditions [299]. The rate of deacetylation of diamorphine and 6MAM has been shown to be pH and temperature dependent in aqueous solution with the rate of degradation substantially increased at higher pH, oxygen and temperature [296, 299, 304-307].

The stability profile of morphine across the physiologically relevant pH range of 1.2–7.4 was studied in rat plasma and rat brain homogenate, or in simulated rat gastric and intestinal fluids. This study demonstrated that, morphine is highly stable and resilient to either enzymatic- or pH-dependent hydrolysis in *vitro* [308]. Another separate study investigated the stability of morphine in saline at pH 5.5-7.5 over a period of 4 days. This showed that, at a clinically relevant

concentration it seems to be stable at room temperature at a wide range of pH values for at least 4 days [309].

The stability of total morphine in urine stored under various conditions was studied using the pH adjusted to 5.5, 6.5, and 7.5. Samples were stored for 6, 12, 18, and 24 months following storage at −20, 4, 25, and 35°C. Effects of sample treatment (azide addition and precipitate removal), pH, and storage temperature and length were evaluated by examining the percentage of total morphine remaining at the four-time intervals following the initial determination. Major findings were total morphine decomposition was minimal when stored for 12 months at −20°C, which is a common current practice; moreover, samples with lower initial sample pH had slower total morphine decomposition rates; and azide addition appeared to have no detectable effect, whereas precipitate removal appeared to marginally reduce the decomposition rate, especially for samples with lower pH [310].

It may be speculated that, the increase in pH value with increasing storage time and temperature might have promoted the pH-dependent degradation of morphine. It is known that the two glucuronide metabolites are hydrolysed back to morphine in unpreserved tissues, and that this is expedited by factors such as temperature and bacterial contamination of the specimens.

It has been reported that morphine can undergo oxidative degradation when exposed to light for lengthy periods of time. The deterioration of the analytes in plasma samples exposed to light through window glass suggests that an oxidation reaction was involved in their degradation. As there was no gain in the concentration of free morphine resulting from the hydrolysis of M3G and M6G, photodegradation of M3G and oxidation may be the rate-limiting step in the decomposition of morphine glucuronides. This assumption was supported by the investigations on the stability of the analytes in whole blood and plasma [69].

Holmgren *et al*. have investigated long-time storage in post mortem blood samples (not spiked). They found excellent stability both for morphine (n = 12) and codeine (n = 5) during 12 months of storage at -20°C [268]. Two other separate studies found that morphine remained stable at -20°C for three months (n = 2 and n = 4, respectively) [69, 311]. Another separate study found that, in spiked post mortem samples (n = 25) analytes were stable for 11 months of

storage in both 4°C and -20°C, some decrease in the concentration of M3G and M6G and an apparent increase in morphine concentration was observed at room temperature which could be the breakdown of M3G and M6G to morphine over time.[312]

The long-term stability of opioids in authentic postmortem blood samples was studied. 73 samples were reanalysed after storage at -20°C for 16-18 years, samples containing morphine and codeine showed the results within acceptance criteria ±30% of the initial concentration [313]. In addition, in spiked post mortem samples (n = 4), the analytes were stable only when stored at -20°C [69]. In highly putrefied blood samples, hydrolysis of morphine glucuronides may occur during sample storage due to residual glucuronidase (GCR) activity following postmortem bacterial invasion. The residual activity of endogenous GCR over time has been demonstrated in serum and plasma [314]. GCR still had approximately 100% activity in serum and plasma following storage at -20°C and 4°C for 20 and 2 days respectively. The postmortem stability of this enzyme may account for increases in the concentration of free morphine observed in blood and tissues in *vitro* [274, 294, 315].

Carroll *et al*. have demonstrated further that the hydrolysis of M3G to free morphine in *vitro* occurs and may persist for months in antemortem and postmortem specimens (n=9) under various conditions, despite using tubes containing EDTA and other tubes containing sodium fluoride and potassium oxalate for inhibition of bacterial growth [294]. In another study, they investigated the time course of degradation of diamorphine, 6MAM, and morphine in four biological matrices: rat blood, rat brain homogenate, bovine serum, and human plasma under various conditions, which were ice-cold solvents, sodium fluoride (NaF) and a low pH (3.0) maintained sample stability. Diamorphine degradation to 6MAM was faster in rat whole blood than in plasma, and faster in rat plasma than in rat brain homogenate. Maintaining NaF at 4 mg/mL throughout processing enhanced stability; higher NaF concentrations added to whole blood caused haemolysis. Samples processed through solid phase extraction and stored as dried pellets at – 80°C constituted the most stable environment for diamorphine and was superior to the storing of samples in solution prior to or after extraction [316].

One study highlighted the importance of preserving postmortem blood samples to inhibit bacterial growth. Spiehler and Brown (1987) found the ratio of free morphine to total morphine to be stable in postmortem blood preserved with 1% NaF and potassium oxalate after more than a year of storage at room temperature [250]. In another study, the long-time stability of real-life post mortem blood samples (n = 37) and living person blood samples (n = 22) was investigated. All samples contained fluoride and were initially analysed and stored in normal conditions (-20^{o}C) for 4–9 years. This study showed that, the concentrations of morphine and codeine are relatively stable during long-term storage at -20^{o}C [303]. Low pH, addition of NaF and freezing at -20°C is only partially effective in stabilising diamorphine in blood [317].

The method of sample storage and preservation is vital, particularly when analyses cannot be performed promptly. Very little data on the stability of morphine glucuronide in blood has been reported. The stability of morphine, M3G and M6G in blood constitutes a major gap in the published literature. Where information is available, it is based on small sample numbers and limited study conditions. To date, there is no data pertaining to the stability of morphine, M3G and M6G in real postmortem blood samples (not spiked). Further investigations in this area are thus required.

2.8 Analytical Methodology

A variety of analytical methods for the quantification of opioids and other basic drugs have been reported, including gas chromatography–mass spectrometry and liquid chromatography–tandem mass spectrometry.

GC–MS is the reference technique for the determination of opioids and other basic drugs in different biological samples [76-87]. Considering that routine analytical applications must be less expensive, and since GC is, in fact, less expensive than LC coupled to mass spectrometry, GC methods seem more suitable within the forensic/clinical toxicology context, however, there is still a concern over the direct determination of morphine glucuronides. It is not possible to measure glucuronides directly with GCMS, which is why classically, glucuronides have been hydrolysed to morphine and the term “total morphine” used to describe the morphine plus the conjugated metabolites. Clearly there is error involved in this analysis, not least in postmortem samples when the

heterogeneous nature of the blood makes it difficult to analyse precise aliquots. In this way, LC–MS/MS has become the technique of choice for simultaneous analysis of MOR, M3G and M6G because of its greater sensitivity and selectivity as previously published in literature [40-51, 53-64, 76-85, 91-101].

2.8.1 Sample Preparation and Extraction Techniques

Before introducing any samples to chromatographic techniques such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-tandem mass spectrometry (LCMS/MS), the analytes first need to be extracted from either the product or matrix and concentrated.

Sample preparation is an essential stage of the analytical process to convert the biological specimen into a form that is suitable for analytical investigation. The extraction step is the main part of the procedure of sample preparation and presents two major advantages for the analysis process. Firstly, it removes interfering matrix compounds (such as proteins, salts and phospholipids) which reduces background noise. Secondly, it concentrates the target drugs, increasing sensitivity and achieving lower limits of detection [318].

Protein precipitation (PPT), solid phase extraction (SPE) and liquid-liquid extraction (LLE) have been used for the extraction of opioids and other basic drugs [43, 56, 77, 79-82, 84, 95, 319-321]. Protein precipitation has been found to be more useful when analysing protein rich matrices, such as serum, plasma and whole blood. An organic solvent, commonly acetonitrile or methanol, is added to the specimen to reduce the solubility of the solute and precipitate the protein, and then it can be removed from the specimen by centrifugation or filtration. Protein precipitation is a rapid and simple extraction technique; however, it does not remove many of the matrix interferences [321].

A review of the most recent literature has revealed an increasing trend towards using SPE in place of LLE. The advantages of using SPE include increased selectivity, cleaner extracts, reduced volume of solvent used, and better reproducibility. On the other hand, LLE has been used for the extraction of opioids in the following matrices: blood [81, 319], plasma [94], urine [81], bile [319], cerebrospinal fluid [94], and vitreous humour [81]. The extraction of analytes from the aqueous phase depends on several factors including solubility

of the analyte in the organic solvent, pH of the aqueous phase and polarity of the organic solvent. The ideal solvent should be, highly selective and allow only the active agent to be extracted, have a high capacity in order to reduce the amount of solvent necessary, and a high positive difference in density. There are also practical concerns when selecting extraction solvents.

In recent years, SPE has become more popular as an extraction technique for the analysis of illicit drugs. Different types of SPE columns can be used depending on the cost, availability and the nature of the analytes of interest. The sample matrix is allowed to pass via the sorbent to waste, with the target analytes being retained. A series of washing steps is essential to remove matrix interferences, and then target analytes are eluted off the sorbent and collected in a clean vial [322]. There are several published methods detailing the extraction of opioids using SPE on a variety of body fluids including whole blood [43, 56, 77, 79, 80, 82, 84, 95, 320], plasma [40-42, 44, 46, 47, 49-51, 54, 56, 58, 64, 77-79, 95, 98, 99, 298, 323, 324], serum [53, 59, 80], urine [50, 64, 78, 83, 93, 95, 325], cerebrospinal fluid [41, 47, 64], and oral fluid [78, 326].

The isolation of substances from postmortem matrices is in general more difficult than that from clinical specimens primarily owing to the range of specimens encountered and the inferior quality of many specimens received in the laboratory resulting from putrefaction or trauma of the body during the death process, or both. For example, it is almost impossible to obtain serum or plasma because of postmortem haemolysis; therefore, whole blood is the most common specimen. SPE offers better clean up than PPT because SPE removes many of the matrix interferences. In addition, it can be optimised for different compound classes. In addition, mixed-mode SPE columns have been used successfully for the analysis of different illicit drugs. Some published procedures adopt extraction methods that enable acidic, neutral, and basic drugs to be present in one chromatograph. This can occur most economically by combining two SPE-based eluates [327] or using polar solvents such as ethyl acetate at pH 4.6 [328] or acetone precipitation [329]. Clearly chromatography of separate extracts can also be performed, but this lengthens the analysis time for the case [330].

Following extraction, and when using GC, derivatisation is often necessary to achieve satisfactory chromatography and to improve detection of an analyte. Polar analytes are generally derivatised to improve chromatographic properties. Compounds containing hydroxyl groups are not usually amenable to GC because of their polarity. Three of the fourteen analytes studied (morphine, codeine and 6MAM) contain hydroxyl groups and thus required derivatisation. The most commonly used derivatisation method for opioids and their respective metabolites is via silylation, where OH groups are replaced by a trimethylsilyl (TMS) group. Silylation can be achieved by addition of bis(trimethylsilyl)-trifluoroacetamide (BSTFA), either alone or with 1% trimethylchlorosilane (TMCS) added as catalyst, or N-methyl-N-(trimethylsilyl)- trifluoroacetamide (MSTFA), each followed by heating. The addition of BSTFA+ 1% TMCS followed by heating at 90°C for 15 minutes is commonly used for the drugs of interest [331, 332] and thus was chosen for use in the current method.

2.8.2 Principles and Applications of Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry is an analytical procedure that separates and identifies different components within a given sample. Because of its high reliability and versatility, GC-MS is applied in the analysis of various classes of compounds (combined, if necessary, with derivatisation), to identify and quantitatively determine different compounds in a wide range of concentrations.

GC-MS analyses gas-phase ions formed from a sample in terms of their mass-to-charge ratios (m/z) and their relative abundances in the resulting spectra. The mass spectrum is a graphical representation of the ion intensities versus the m/z. Under constant, hard electron ionisation conditions (typically 70eV) a sample molecule will fragment into smaller ions of characteristic and highly reproducible m/z values and relative abundances. This fragmentation pattern is a molecular fingerprint for a given analyte, which can be compared to the mass spectra of drug standards for ultimate identification. Identification of a compound is also achieved by retention time data. The separation of a mixture of compounds in a sample is based on 1) the affinity of the molecules for the column stationary phase and; 2) the boiling point of the molecule. Molecules having a greater affinity for the stationary phase will spend more time on the

column and finally elute when the oven temperature reaches the molecules boiling point. Oven temperature is often gradually increased throughout the analysis so that compounds may be separated and elute in order of increasing boiling point.

2.8.2.1 Analyte identification and confirmation

Analyte identification in full scan mode and in SIM is achieved by comparison of retention times and by comparison of the relative abundance of major fragment ions to those values obtained for standards assayed in the same run. Typically, three ions are monitored and utilised in the confirmation of compound identity. As a result of matrix interferences, ion ratios are necessary for analyte confirmation in full scan analysis. They are also necessary in SIM with quadrupole instruments because only a few ions are monitored at one time and thus a full MS profile is not available. Then its confirmation is based on the MS spectrum and the retention time matched to that of the reference standards.

2.8.2.2 Analyte quantification

Analyte quantification is often achieved by means of an internal standard (IS) calibration graph plotting analyte response divided by IS response versus concentration in calibration standards. Because of the degree of ionisation in MS and the potential for drug loss during sample preparation, the use of an internal standard is vital when performing quantitative measurements. Even with analyte losses during sample preparation the ratio of the sample to the IS will remain constant and the measured concentration should reflect more accurately that of the original biological sample. The concentration of an analyte in an unknown sample may be calculated against the corresponding calibration curve providing the detector response is proportional to analyte concentration in the calibration standards.

2.8.2.3 Optimising GC-MS Parameters

Several software and dissociation parameters have to be optimised so that suitable MS product ion spectra can be obtained for each analyte. Prior to MS method development the oven temperature programme has to be optimised to obtain the best analyte separation possible within an acceptable run time.

2.8.3 Principles and Applications of Liquid Chromatography-Mass Spectrometry

In recent years, liquid chromatography– tandem mass spectrometry (LC–MS/MS) has largely supplanted GC–MS as the gold standard for opioid confirmatory testing [88, 89]. Unlike LC–MS/MS, GC–MS is not amenable to the detection of polar and non-volatile compounds without performing a time-consuming sample preparation, including hydrolysis, derivatisation and sample clean-up. Moreover, LC–MS/MS has enabled the direct and specific analysis of opioids and their glucuronide metabolites [40-51, 53-64, 90].

2.8.3.1 Mass Spectrometry Instrumentation

Coupling of MS to chromatographic techniques has always been desirable due to the sensitive and highly specific nature of MS compared to other chromatographic detectors. Mass spectrometers operate by converting the analyte molecules to a charged (ionised) state, with subsequent analysis of the ions and any fragment ions that are produced during the ionisation process, on the basis of their mass to charge ratio (m/z). Several different technologies are available for both ionisation and ion analysis, resulting in many different types of mass spectrometers with different combinations of these two processes. In practice, some configurations are far more versatile than others and the following descriptions focus on the common type of ion source and mass analyser likely to be used in LC-MS systems within toxicological laboratories.

2.8.3.2 Electrospray Ionisation Source

Liquid samples are pumped through a metal capillary maintained at 3 to 5 kV and nebulised at the tip of the capillary to form a fine spray of charged droplets. The capillary is usually orthogonal to, or off-axis from, the entrance to the mass spectrometer in order to minimise contamination. The ionised analytes are then transferred into the high vacuum of the mass spectrometer via a series of small apertures and focusing voltages. The ion source and subsequent ion optics can be operated to detect positive or negative ions and switching between these two modes within an analytical run can be performed.

2.8.3.3 Quadrupole Mass Analysers

The quadrupole analyser consists of a set of four parallel metal rods. A combination of constant and varying (radio frequency) voltages allows the transmission of a narrow band of m/z values along the axis of the rods. By varying the voltages with time, it is possible to scan across a range of m/z values, resulting in a mass spectrum. Most quadrupole analysers operate at <4000 m/z and scan speed up to 1000 m/z per sec or more are common. They usually operate at unit mass resolution meaning that the mass accuracy is seldom better than 0.1 m/z [333].

As an alternative to scanning, the quadrupoles can be set to monitor a specific m/z value, then set to monitor another m/z value, and so on. This is achieved by stepping the voltages. This technique is useful in improving the detection limits of targeted analytes because more detector time can be devoted to detecting specific ions instead of scanning across ions that are not produced by the analyte. Stepping can be carried out in a few milliseconds and a panel of m/z values can be stepped through for the detection of several analytes [334].

Ions can be induced to undergo fragmentation by collisions with an inert gas such as nitrogen or argon, a process called collision induced dissociation. One type of collision cell is a quadrupole that has been designed to maintain the low pressure of the collision gas required for dissociation and transmit most of the fragment ions that are produced. A particularly useful mass spectrometer configuration is obtained by placing a collision cell between two quadrupole mass analysers. This combination is called a triple quadrupole mass spectrometer and is an example of tandem MS in which two stages of mass analysis are independently applied [335].

The first and third quadrupoles can also be simultaneously stepped to different m/z values, and panels of precursor/ product ion pairs can be created to specifically detect a large number of targeted analytes. This process, called multiple reaction monitoring (MRM), is commonly used in LC-MS assays [335].

2.8.3.4 Liquid Chromatography Considerations

Current ion sources are capable of handling a wide range of flow rates and mobile phase compositions so existing LC separations can often be directly

coupled to the mass spectrometer. However, a number of factors can affect the quality of MS data and some modification of the LC separation may be desirable to improve assay performance.

1) **Flow Rate**

While standard Electron Spray Ionisation (ESI) sources can generally handle flow rates up to 1 mL/min, lower flow rates in the range 0.05 to 0.2 mL/min result in improved sensitivity. The ideal is to have a small drop that forms over a relative long period of time. This will ensure that the droplet contains many charged species and it will allow optimal sampling of gas phase analyte ions.

Columns with 1.0 or 2.1 mm diameters are therefore well suited to direct coupling with these ion sources. The conversion from 4.6 mm to 1.0 or 2.1 mm columns is generally easily managed and predictable, and the resulting decrease in mobile phase consumption is a useful side benefit. Even higher sensitivities and resolutions can be obtained with capillary columns and sub µL/min flow rates. These separations are generally more demanding as different ion sources and LC pumps are required, and care is required in making column connections. These separations are useful in the proteomics area where high sensitivity and resolution are required to identify as many components as possible [336].

2) **Mobile Phase**

Typical solvents used in reverse and normal phase LC (e.g. water, acetonitrile, methanol, ethanol) are compatible with ESI. It should be noted that a grade of solvent (including water) that is suitable for a conventional LC separation may not always be suitable for an LC-MS based separation. A good electrospray solvent will easily support ions in solution; this generally requires that the solvent has some dipole moment (polarity). Although water is an excellent solvent for ions its high surface tension makes de-solvation and ion desorption more difficult often requiring higher drying gas temperatures, higher nebulising gas flow rates and/or higher nebulising voltages [337].

The use of buffers containing inorganic ions such as phosphate and sodium acetate should be avoided. They cause significant ion suppression, can create MS adducts of sodium and potassium, and can quickly contaminate the ion source. Substitution of buffers that are more compatible with MS such as those based on

ammonium acetate, ammonium formate or ammonium bicarbonate is generally possible. Even so, these buffers still cause ion suppression, so the concentration of buffer used should be the minimum required to produce satisfactory chromatography. Ion pairing reagents, such as trifluoroacetic acid and other fluorinated carboxylic acids, also result in ion suppression so their concentrations should also be minimised as much as is practical [338].

Higher organic content of the mobile phase can result in improvements in ionisation efficiency in ESI, and this may affect the choice of separation mode. For example, polar molecules are poorly retained on reverse phase columns and elute at the beginning of the chromatogram with a low organic content. This will result in relatively poor ionisation efficiency, and polar interferences are more likely to co-elute in this region causing ion suppression [339, 340].

3) **Resolution and Throughput**

Baseline separation of peaks, if the peaks have independent MS signals, is not required in LC-MS. Therefore, lower resolution, shorter columns are often used in LC-MS assays with consequently shorter run times, although ion suppression effects may be a limitation. Guard columns can sometimes help to provide sufficient separation from interferences. Another approach used to speed up the analytical process is to use short columns with small particle sizes (<2 μm) and consequently even higher pressures, (so-called ultrahigh performance LC) [341-343]. These columns are also capable of generating highly resolved chromatograms with better signal: noise ratios than columns with larger particle sizes [344].

Chromatographic resolution also impacts on the scanning speed of the mass spectrometer. To achieve accurate integration, it is desirable to have at least 10 scans across the chromatographic peak. It may therefore be necessary to use fast scan speeds with highly resolved peaks, and this may compromise sensitivity when several ions are being monitored. It is possible to minimise this to some extent by dividing the chromatogram into smaller windows and using smaller panels of ions specific for the analytes eluting within these windows.

2.9 Project Aims and Objectives

2.9.1 The aims were:

The aims of this study were chosen to help investigate the distribution of drugs (mainly opioids) after death.

1) To investigate if there is a change in the concentration of opioids, antidepressants and gabapentin in blood samples over time in bodies stored prior to autopsy and if this change is consistent.

2) To determine how the sampling technique and site affect the measured drug concentration.

3) To investigate the stability of drugs within postmortem samples and how storage conditions affect this.

2.9.2 The objectives were:

1) To review relevant published scientific literature to assist in the interpretation of the toxicological findings (Chapter 2), including:

- the pharmacokinetic properties of the investigated drugs,

- factors affecting postmortem drug concentrations and their subsequent interpretation,

- the current utility of pre-autopsy blood sampling in toxicological determinations

- and the stability of the drugs of interest in blood during sample storage.

2) To develop and validate analytical procedures for the simultaneous extraction, confirmation and quantification of investigated drugs and their respective metabolites (Chapters 3, 4 and 5).

3) To examine the stability of morphine and its conjugated metabolites in real postmortem blood stored in both short and long-term storage conditions as an aid to the interpretation of free and total morphine blood concentrations. And, to examine the effect of storage temperature, different sampling tubes and

preservation on drug stability in the various samples with the aim of making recommendations for optimal storage conditions for postmortem blood (Chapters 3, 4, 5 and 6).

4) To examine the ratio between the blood morphine concentration and the respective glucuronide concentrations in addition to the presence of 6MAM following heroin administration - in order to achieve a comprehensive interpretation of time since death (Chapter 6).

5) To examine the analytical findings derived from new techniques (needle puncture in the upper thigh) for blood collection before autopsy to prevent contamination by PMR - determining the correlation between drugs concentration from blood drawn on the day prior to autopsy and in blood drawn at the time of autopsy. To compare peripheral and central blood samples - comparing drug to drug metabolite ratios in different samples over a period of time after death with particular focus on survival time (Chapter 7).

Chapter 3 Method Development and Validation for Opioids and Antidepressant Drugs in Whole Blood using GC/MS

3.1 Introduction

Considering the increasing occurrence of polydrug deaths involving both, drugs of abuse and medications, and given that the available sample volume is usually small in forensic cases it is important to maximise the information gained from any test. Therefore, a method for the simultaneous extraction and quantification of opioids and antidepressant drugs in biological matrices was developed. The optimised assay involved solid phase extraction of the analytes from whole blood followed by derivatisation and quantification using GC/MS.

This chapter discusses the principals and the development of the analytical methods used to test the drugs of interest. It will begin by giving a description of the method used to obtain mass spectrometric data and a brief overview of analyte confirmation and quantification. The optimisation of the mass spectrometric assay is also discussed followed by an assessment of instrument performance using the optimised MS assay. Development of sample preparation procedures is discussed thereafter.

Full details of the final optimised assay and its validation are discussed thereafter. In order to determine whether an analytical method is fit for purpose, it should be thoroughly tested and validated. This is extremely important in the context of forensic toxicology where the results will have significant impact on individuals coming into contact with the criminal justice system.

The performance parameters and statistical protocols followed throughout a validation study vary with the source of guidelines. A number of guidelines to validate the new method developed are, for example, published by the FDA Guidance for industry-bioanalytical method validation (2001)[345], SOFT/AAFS forensic toxicology laboratory guidelines (2006)[346], the United Kingdom and Ireland Association of Forensic Toxicologist's forensic toxicology laboratory guidelines–(2018) [347] and the standard practices for method validation in forensic toxicology which was published by the Scientific Working Group for

Forensic Toxicology (SWGTOX) in May 2013 [348]. All these protocols and many others aim to improve method development and validation procedures.

Method validation of the following studies were performed using the 'SWGTOX Standard Practices for Method Validation in Forensic Toxicology' revision draft 003 (32) as a guide: selectivity, calibration model, precision and accuracy, limit of quantification (LOQ), limit of detection (LOD), carryover and stability study of analytes during specimen processing.

3.2 Aim and Objectives

The aim of this study was to develop a method for quantitation of morphine, codeine, dihydrocodeine, 6-monocetylmorphine, tramadol, methadone, EDDP, amitriptyline, citalopram, sertraline and mirtazapine in biological matrices collected postmortem. The focus was development of a single extraction method for all these compounds of interest, and a chromatographic system for separation and detection of all the targets with acceptable recoveries and accuracies. A gas chromatography-mass spectrometry method with solid-phase extraction (SPE) that accomplishes these goals is reported.

The goal of validation is to confirm by examination and the provision of objective evidence that the method developed for a specific intended use is fulfilled. It is important as it defines whether it will produce reliable results and identifies the method's limitations under normal operating conditions.

3.3 Chemicals and Reagents

Standard solutions of morphine, codeine and dihydrocodeine, 6-monocetylmorphine, tramadol, methadone, EDDP, amitriptyline, citalopram, sertraline and mirtazapine were manufactured by Cerilliant (Texas, USA) and purchased as solutions with the concentration of 1 mg/mL from Sigma Aldrich (Basingstoke, UK). Deuterated standards; morphine-d3, codeine-d3, 6-monocetylmorphine-d3 and methadone-d3 were manufactured by Cerilliant (Texas, USA) and purchased as solutions with the concentration of 1 mg/mL from Sigma Aldrich (Basingstoke, UK).

UCT® Clean Screen ZSDAU020 cartridges were purchased from Chromatography Direct (Cheshire, UK). N, O-Bis (trimethylsilyl) trifluoroacetamide containing 1%

trimethylchlorosilane (BSTFA + 1 % TMCS) was manufactured by Cerilliant (Texas, USA) and purchased from Sigma Aldrich (Basingstoke, UK). Methanol (MeOH), ethyl acetate, acetic acid, acetonitrile (ACN), isopropanol and dichloromethane (DCM) were obtained from VWR International Ltd, (Lutterworth, UK). Disodium hydrogen orthophosphate anhydrous and sodium dihydrogen orthophosphate monohydrate were supplied by Sigma Aldrich (Basingstoke, UK). Deionised water was obtained from the in-house Merck Millipore system.

The two types of sampling tubes used were separator clot activator tubes (SCAT) and plain tubes (PT), purchased from Goods Wagon (Glasgow/Scotland) and VWR (Glasgow/Scotland), respectively.

3.4 Preparation of Materials and Solutions

3.4.1 Preparation of Drug Standard and Internal Standard Solutions

3.4.1.1 Preparation of Stock Solutions

Stock solutions were prepared in methanol for each standard and internal standard individually giving a concentration of 100 μg/mL. This was achieved by transferring 1 mL of each drug standard solution (1mg/mL) into a single 10 mL volumetric flask and making up to the mark with methanol. The entire amount was transferred to individual amber glass bottles and stored at −20°C for 6 months.

3.4.1.2 Preparation of Mixed Standard Solutions

Three different working solutions were prepared by dilution of stock solutions in methanol, then transferring to individual amber glass bottles and storing at −20°C for 6 months.

1) **Opioids Working Solution**

Firstly, to obtain 5 μg/mL mixture solution containing (Morphine, Codeine, DHC) was prepared in methanol by adding 500 μL of each drug solution (stock solution at 100 μg/mL) to a 10 mL volumetric flask and making up to volume with methanol.

2) 6MAM Working Solution

Secondly, to obtain 1 µg/mL for 6MAM. This was achieved by adding 100 µL of the stock solution to a 10 mL volumetric flask and making up to volume with methanol.

3) Basic Drug Working Solution

Lastly, 100µL of mirtazapine and EDDP stock solutions (100µg/mL) and 1mL of amitriptyline, citalopram, sertraline, tramadol and methadone stock solutions (100µg/mL) were all added to a 10mL volumetric flask and made up to volume with methanol to obtain a mixture containing 1µg/mL (mirtazapine and EDDP) and 10µg/mL (amitriptyline, citalopram, sertraline, tramadol and methadone).

3.4.1.3 Preparation of Mixed Internal Standard Solutions

Two different internal standard solutions in methanol were prepared which were transferred to individual amber glass bottles and stored at −20°C for 6 months.

Firstly, to obtain 10 µg/mL for Methadone-d3. This was achieved by adding 1 mL of the stock solution (100 µg/mL) to a 10 mL volumetric flask and making up to volume with methanol.

Secondly, to obtain 1 µg/mL for each drug (Morphine-d3, Codeine-d3 and 6-monocetylmorphine-d3). This was achieved by adding 100 µL of the stock solution (100 µg/mL) to a 10 mL volumetric flask and making up to volume with methanol.

3.4.1.4 Preparation of Quality Control Samples

Quality control samples (QCs) were prepared at 3 concentrations for each drug in whole blood. The working solutions used for preparation of the QCs were prepared as in section 3.4.1.2 but using different lot numbers (or prepared on different days). The QCs were made in 50 mL volumetric flasks, after addition of drug standard solutions detailed in Table 3-1, and evaporation of solvent, these were made up to the mark with appropriate blank matrix.

For each QC, 1mL aliquots were placed into cap tubes. All QCs were stored at -20°C for 12 months.

Table 3-1 Preparation of QCs in biological matrices

Drugs	Amount Added (mL)		
	QC1	QC2	QC3
Opioids Working Solution	0.5	1.0	4.0
6MAM Working Solution	0.5	2.5	8.0
Antidepressant Working Solution	1.25	5.0	17.5
Drugs	QC Concentration (mg/L)		
	QC1	QC2	QC3
Morphine, Codeine and DHC.	0.05	0.1	0.4
6MAM	0.01	0.05	0.16
Mirtazapine and EDDP	0.025	0.1	0.35
Methadone, Amitriptyline, Citalopram, Sertraline and Tramadol.	0.25	1.0	3.5

3.4.1.5 LOD & LOQ Solutions

In order to assess the methods LOD and LOQ further solutions were made.

1) **Opioids Solution**

To a new 10 mL volumetric flask, 1 mL of opioids working solution in section 1)3.4.1 was added. This was then made up to the mark using MeOH and inverted several times producing a final concentration of 0.5 mg/L.

2) **6MAM Solution**

To another clean 10 mL volumetric flask, 1 mL of 6MAM working solution in section 3.4.1 was added. This was again made up to the mark using MeOH and inverted several times producing a final concentration of 0.1mg/L.

3) **Basic drug Solution**

1 mL of antidepressant working solution in section 3.4.1 was added to another clean 10 mL volumetric flask. This was again made up to the mark using MeOH and inverted several times to give a final concentration of 1 mg/L (methadone, amitriptyline, citalopram, sertraline and tramadol) and 0.1 mg/L (mirtazapine and EDDP).

3.4.2 Preparation of Buffers

3.4.2.1 0.1 M, pH 6.0 Phosphate Buffer

1.7 g of disodium hydrogen orthophosphate anhydrous (Na_2HPO_4; MW 141.96) was weighed accurately into a 1 L beaker. 12.14 g of sodium dihydrogen orthophosphate monohydrate ($NaH_2PO_4.H_2O$; MW 137.99) was then added and this was dissolved in 800 mL of deionised water and the pH was adjusted to 6 with 0.1 M dibasic sodium phosphate (raises pH) or 0.1 M monobasic sodium phosphate (lowers pH). The entire amount was placed in a 1 L volumetric flask and made up to volume with deionised water. Mixed well and stored at 4°C for up to 1 month.

3.4.2.2 pH6 Buffer/deionised water (1:2)

200 mL of pH 6 Buffer and 100 mL of deionised water was measured and transferred to a reagent bottle. This was sonicated for 5 minutes to mix and prepared daily.

3.4.2.3 Acetate buffer 0.1M pH 4.5

5.86 g of sodium acetate tri-hydrate was weighed into a beaker and dissolved in 800 mL of DI water. 3.24 mL of glacial acetic acid was then added, and pH adjusted to 4.5 with 0.1 M acetic acid (lowers pH) or 0.1 M sodium acetate (raises pH). The buffer was transferred to a 1 L volumetric flask with DI water, made up to volume with DI water, mixed well and stored at room temperature for up to 1 month.

3.4.2.4 1M Acetic acid

Pipette 28.6mL of glacial acetic acid into a 500mL volumetric flask that is approximately half filled with deionised water Mix, and then bring the total volume up to 500mL with deionised water. Store at room temperature, discard after two months.

3.4.2.5 Dichloromethane: isopropanol: ammonia (78:20:2)

Measure 78ml of dichloromethane, 20ml of isopropanol and transfer to a reagent bottle. Add 2mL of concentrated ammonia. Sonicate for 5 minutes or shake vigorously to mix. Prepare fresh daily.

3.4.3 Blank Blood

Expired packed red blood cell pouches are used in toxicology laboratories for preparing matrix-matched biological standards. Cell pouches were provided from the Scottish National Blood Transfusion Service (SNBTS) (submission reference number 14-07) based at Gartnaval General Hospital (Glasgow, UK). They were stored at −20°C on receipt within Forensic Medicine and Science. To prepare blank blood, packed red blood cells were defrosted and suspended in a ratio of 1:1 with 1% saline solution which was prepared by dissolving 9.5 g of sodium chloride in 1 L of deionised water.

3.5 Gas chromatography-mass spectrometry

GCMS method optimisation was carried out on an Agilent Technologies 7890 GC System with 5975C inert XL MSD Triple-Axis Detector equipped with an equivalent stationary phase capillary column (Agilent 19091S-433:325C:30m×250µm×0.25µm) using an automatic injector in split mode. Pure helium (99.9999%) was used as the carrier gas at a flow rate of 1.5 mL/min.

The mass spectrometer was initially operated in the full mass scanning mode, however this proved problematic when analysing postmortem biological samples because of endogenous compounds interfering with the identification and quantification of the analytes of interest. SIM mode was then used as this effectively removed matrix interference by isolating a single ion.

3.6 Optimisation of the GC-MS assays

Several tests were performed to optimise the chromatographic separation of the peaks to obtain six-time windows based on the retention time of each compound. Within the windows, the mass spectrometer identified and selected only the specified ions, thus reducing the background noise and increasing sensitivity.

Table 3-2 summarises the final GC-MS procedure which included 2 injections, one to analyse opioids and a second for the antidepressant drugs.

Table 3-2 GC-MS Procedure for Opioids and Basic Drugs

GC-MS Parameters	Setting for Opioids	Setting for Basic drugs
Inlet temperature	225 °C	250 °C
Injection volume	1 µL	
Injection port type	Split less mode	
Septum purge flow	3 mL/min	
Column HP-1	30 m x 0.25 mm id x 0.25 µm	
Oven programme	150 °C initial & ramped to 300 °C @ 10 °C/min. Hold 5 minutes	100 °C initial for 1 min & ramped to 240 °C @ 10 °C/min. Hold 10 minutes, then ramped to 300 °C @ 10 °C/min. Hold 5 minutes
Interface temperature	230 °C	200 °C
Ionisation mode	EI mode, ionisation energy 70 eV.	
Detection mode	Full Scan over the range m/z 40-600	
Run time	20 minutes	36 minutes

3.6.1 Analyte identification and confirmation

The identification of compounds was performed using the full scan acquisition mode, which allowed the analysis of the total ion chromatogram (TIC), extrapolating retention times and characteristic ions. Quantifier and qualifier ions used for each analyte were selected based on their abundance and m/z values. Because of their reproducibility and lack of interference, high mass ions were selected when possible. This was not possible when there were ions in common with those of the deuterium labelled internal standard. Upon selection of unique ions as detailed in Table 3-5 the MS was run in selected ion monitoring (SIM) mode due to the high sensitivity required with the low concentrations used.

Firstly, identification and confirmation experiments of opioids were carried out using unextracted standards of each analyte (morphine, codeine, DHC, 6MAM, morphine-d_3, codeine-d_3 and 6MAM-d_3). 100 µL of standard at concentration 50 mg/L was pipetted into a glass vial, evaporated under nitrogen at ≤ 37 °C until dry and derivatised with 50 µL BSTFA+1% TMCS at 90 °C for 15±2 min. After cooling to room temperature, this was transferred to a clean GC autosampler vial and 1 µL was injected on to the GC-MS to identify and confirm each drug individually.

Secondly, identification and confirmation experiments of antidepressants and other opioid drugs were carried out using unextracted standards of each analyte (amitriptyline, citalopram, sertraline, tramadol, methadone, mirtazapine, EDDP and methadone-d_3). 100 μL of standard at concentration 50 mg/L was pipetted into a glass vial and evaporated under nitrogen at room temperature until dry. Then 50 μL of ethyl acetate was added to each tube, the tubes were vortex mixed for 5 s and the mixture transferred to clean GC auto-sampler vials and 1 μL injected on to the GC-MS to identify and confirm each drug individually.

3.6.2 Initial Sensitivity and Linearity Assessments

Following development of the instrument method, sensitivity and linearity assessments were carried out to evaluate the suitability of the analytical method before starting the development of sample preparation. The Limit of Detection (LOD) of a method is considered the lowest concentration of analyte that gives a reproducible instrument response with a signal greater than or equal to three times the noise level of the background signal from the negative samples [348]. Acceptable LODs should achieve the purpose of the method.

The linearity of the method was evaluated by preparing calibration curves for all compounds. The range considered was established to include values from the therapeutic and toxic levels of each compound. The calibration curves were plotted using the ratio of the observed peak areas of reference standards and internal standards against concentration. Linear regression analysis of the calibration data was performed using the equation $y = mx + c$ without weighting, where y is the peak area ratio, x is the concentration of calibrators and m and c are constants. The linearity of extracted calibrators investigated in this study was evaluated over the concentration range for both opioid and basic drug groups.

Firstly, for linearity experiments of opioids, five calibrators of each analyte were prepared at the following concentrations as detailed in Table 3-3.

Table 3-3: Standard Calibrators Preparation of opioids

Calibrator levels	Opioids working solution Volume(µL)	Morphine, Codeine, Dihydrocodeine Conc. (mg/L)	6MAM working solution Volume(µL)	6MAM Conc. (mg/L)
Cal. 1	5	0.025	5	0.005
Cal. 2	10	0.050	10	0.010
Cal. 3	20	0.100	50	0.050
Cal. 4	40	0.200	100	0.100
Cal. 5	100	0.500	200	0.200

After addition of standard solutions to all calibrator tubes, 100 µL of deuterated internal standard mix (IS) at 1 µg/mL (morphine-d_3, codeine-d_3 and 6-monocetylmorphine-d_3) was added to each calibrator. All calibrators were evaporated under nitrogen at ≤ 37 ^{o}C until dry and derivatised with 50 µL BSTFA+1% TMCS at 90 ^{o}C for 15±2 min. After cooling at room temperature, these were transferred to clean GC autosampler vials and analysed to establish a linear calibration model for each drug individually.

Lastly, for linearity experiments of antidepressant and other opioid drugs, seven concentration points of each analyte were prepared at the following concentrations as detailed in Table 3-4.

Table 3-4: Standard Calibrators Preparation of antidepressant and other opioid drugs

Calibrator levels	Antidepressant and another Opioids working solution Volume(µL)	Amitriptyline, Citalopram, Sertraline, Tramadol, and Methadone Conc. (mg/L)	Mirtazapine and EDDP Conc. (mg/L)
Cal. 1	5	0.05	0.005
Cal. 2	10	0.10	0.010
Cal. 3	25	0.25	0.025
Cal. 4	50	0.50	0.050
Cal. 5	100	1.0	0.100
Cal. 6	200	2.0	0.200
Cal. 7	400	4.0	0.400

After addition of standard solutions to all calibrator tubes, 100 µL of deuterated internal standard of 10 µg/mL methadone-d_3 was added to each calibrator. All calibrators were evaporated under nitrogen at room temperature until dry then 50 µL of ethyl acetate was added to each tube, the tubes were vortex mixed for

5 s and the mixture was transferred to clean GC auto-sampler vials and analysed to establish a linear calibration model for each drug individually.

3.7 Optimisation of Analyte Extraction

Following the optimisation of the GC-MS method, extraction experiments were performed to develop and optimise the best conditions and best possible drug recovery. Additionally, the LOD and linearity range were evaluated when the extraction methods were optimised. Two in-house FMS extraction methods were evaluated; an opioids extraction method and basic drugs extraction method. The focus was the development of a single extraction method for all the compounds of interest. After deciding to use SPE as a method of choice to extract all analytes simultaneously, further investigation was conducted on different types of pH adjustment used for washing in SPE. Both 1M Acetic acid and 0.1 M acetate buffer were tested for the simultaneous extraction. The opioids extraction method uses an acetate buffer 0.1 M pH 4.5 during wash stages; whereas the basic drugs extraction method uses 1M acetic acid. However, the literature reported that the sample and column may also be adjusted to pH 4.5 with 0.1 M acetate buffer for basic drugs extraction [349, 350]. Therefore, steps evaluated in this study are the pH adjustment during wash stages used for extraction.

3.7.1 Recovery of drugs using Acetate Buffer or Acetic Acid as a wash step

Two sets of 3 replicates of spiked whole blood were prepared as follows; all drugs of interest were added using working solutions prepared as in section 3.4.1.2. Volumes of 80, 160 and 350 µL were added for opioids, 6MAM and antidepressant working solutions, respectively to obtain concentrations 0.4 mg/L for morphine, codeine and DHC; 0.16 mg/L for 6MAM; 0.35 mg/L for mirtazapine and EDDP and 3.5 mg/L for methadone, amitriptyline, citalopram, sertraline and tramadol. After addition of drug standard solutions to tubes, the solvent was evaporated, and 1 mL of blank whole blood was added and diluted in 5 mL of pH 6 Buffer/deionised water (1:2) mixed solution. The tubes were vortex mixed for 5 s and centrifuged for 10 min at 2500 to 3000 rpm. Each set was extracted identically except the one wash stage which was either pH 4.5, 0.1 M acetate buffer or 1M Acetic acid as shown in Figure 3-1.

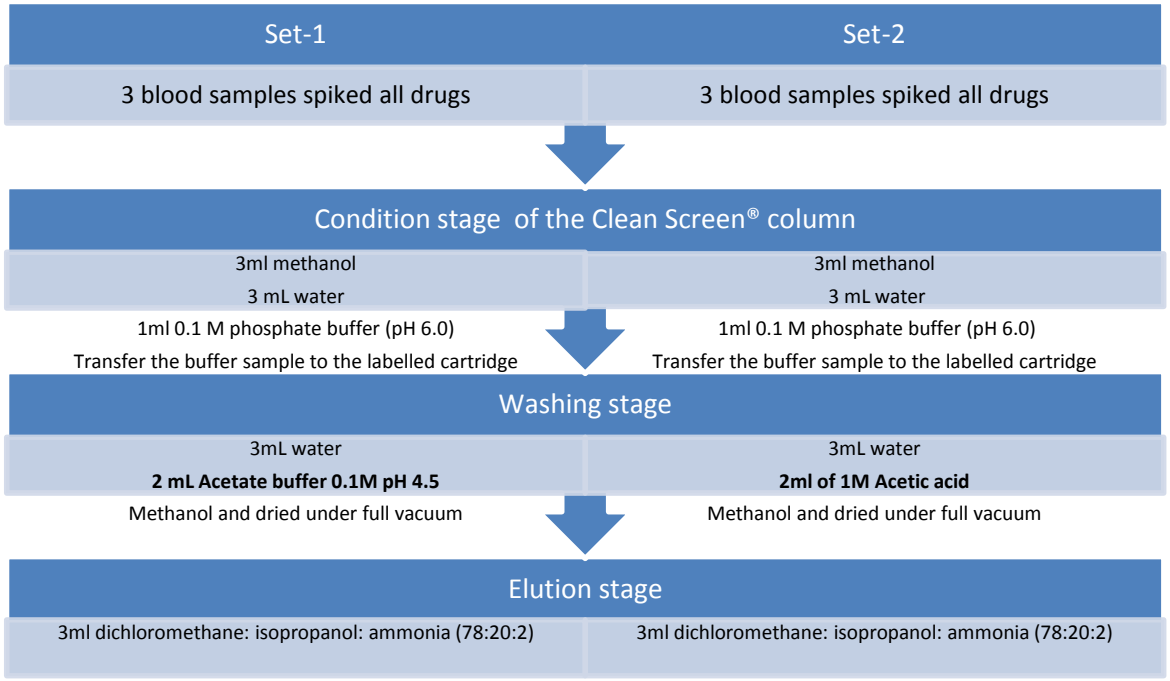


Figure 3-1: Flow chart of extraction using Acetate Buffer or Acetic Acid as a wash step

After extraction, 100 µL of deuterated internal standard mix (IS) [10 µg/mL (Methadone-d3) and 1 µg/mL (Morphine-d3, Codeine-d3 and 6-monocetylmorphine-d3)] was added to each sample. Each replicate was then split in 2 to test for opioids in one and basic drugs in the other. The first group were evaporated under nitrogen at ≤ 37 °C until dry and derivatised with 50 µL BSTFA+1% TMCS at 90 °C for 15±2 min. After cooling at room temperature, they were transferred to clean GC autosampler vials. The second set were evaporated under nitrogen at room temperature until dry then 50 µL of ethyl acetate was added to each tube, the tubes were mixed with vortex mixing for 5 second and the mixture transferred to clean GC auto-sampler vials.

In order to calculate the recovery, an unextracted standard at the same concentration was also prepared at the same time in triplicate. Internal standard solution was added to the unextracted standard at the same time as the extracted samples. The peak area ratio of the analyte to its respective deuterated IS was calculated. This method can calculate recovery (RE) according to the equation below (Equation 3-1).

Equation 3-1 Recovery

$$\mathbf{Recovery}(\%) = \frac{\mathbf{Peak\ Area\ Ratio\ of\ Extracted\ Standards}}{\mathbf{Peak\ Area\ Ratio\ of\ Unextracted\ Standards}} * \mathbf{100}$$

3.8 Method Validation

3.8.1 Selectivity and Specificity

Selectivity is the ability of the bioanalytical method to determine the analyte(s) in the analysed matrices without interference. Specificity is the ability of the bioanalytical method to differentiate the analyte(s) in the presence of other components, which may be expected to be present. Typically, these might include metabolites, impurities, degradants and matrix components. [351, 352]

The selectivity of the method was carried out by comparing the chromatograms of potential interferences from blank matrix (n = 10) with those of corresponding standards spiked at a concentration of 1 mg/L. Whereas, specificity was assessed by spiking a number of analytes, at a concentration of 10 mg/L, which not included in the method to evaluate the extent to which the method is specific for each drug and whether there is any interference. Interferences from common drugs of abuse and common prescription medication were assessed as detailed in Appendix 1.

3.8.2 Limits of Detection and Limits of Quantification

The limit of detection (LOD) was assessed by determining the lowest concentration at which the drug could be detected with a signal to noise (S/N) ratio greater than 3. The concentrations that yielded a reproducible instrument response with S/N ratio ≥ 3, was selected as LOD.

The LOD was determined by spiking pooled blank blood with decreasing concentrations of mixed working solution within the expected range of LODs; over the concentration range 0.0025, 0.0050, 0.0100, 0.0250 and 0.0500 mg/L for Amitriptyline, Citalopram, Sertraline, Tramadol and Methadone; and 0.00025, 0.0005, 0.0010, 0.0025 and 0.0050 mg/L for Mirtazapine and EDDP. Similarly, LOD was determined over the range; 0.0010, 0.0025, 0.0050, 0.0100 and 0.0250 mg/L for Morphine, Codeine and DHC and; 0.00025, 0.00050, 0.00100, 0.00250 and 0.00500 mg/L for 6MAM. All LODs were extracted in duplicate using three different sources of fortified matrix and then analysed in three separate runs. These were run alongside a set of calibrators and QC's. The

signal to noise was calculated manually using the following equation (Equation 3-2).

The lower limit of quantification (LLOQ) has been assigned as the lower concentration of calibrator and should have a %CV less than 20% and S/N ≥ 10. The LLOQ was verified by spiking three samples per run of a fortified matrix at the concentration of the decision point and analysed over three runs to demonstrate that all detection, identification, bias, and precision criteria are met.

Equation 3-2 Signal to Noise Ratio Calculation.

$$Signal\ to\ Noise\ = \frac{height\ of\ analyte}{height\ of\ noise}$$

3.8.3 Linearity

For this method, linearity was assessed using 7 points on the curve over the concentration range 0.05, 0.10, 0.25, 0.50, 1.00, 2.00 and 4.00 mg/L for Amitriptyline, Citalopram, Sertraline, Tramadol and Methadone; and 0.005, 0.010, 0.025, 0.050, 0.100, 0.200 and 0.400 mg/L for Mirtazapine and EDDP. On the other hand, five calibrators were prepared and analysed over the concentration range 0.025, 0.050, 0.100, 0.200 and 0.500 mg/L for Morphine, Codeine and DHC and; 0.005, 0.010, 0.050, 0.100 and 0.200 mg/L for 6MAM.

All calibrators were prepared freshly every day in duplicate over 5 different days. The calibrations were prepared by spiking blank biological samples with working mixture solutions and extracted in accordance with the method reported in section 3.7.1. A blank extract containing internal standard only was ran with each batch but not included in the calibration curve. Calibration curves were obtained by plotting SIM PAR of analyte to internal standard (calculated as per Equation 3-3) against concentration using the linear regression model.

Equation 3-3 Peak Area Ratio Equation

$$PAR\ = \frac{Anayte\ Peak\ Area}{I.S.\ Peak\ Area}$$

In order for calibration curves to comply with SWGTOX guidelines, R^2 values must be greater than 0.99 and QCs when plotted should not give accuracy values more than ±20%.

Although it has become widespread practice, it is emphasized that a calibration model cannot be evaluated simply via its correlation coefficient (r). Instead, a calibration model shall be visually evaluated using standardized residual plots. These allow one to check for outliers that must be eliminated if found to be statistically significant (outside ±3 standard deviations).

3.8.4 Precision and accuracy

Precision and accuracy for intra-day and inter-day assay were examined by analysing blood quality control samples (QCs) containing the analytes at three concentrations; low (QC1), medium (QC2) and high (QC3). A calibration curve was prepared with each batch of QCs using the optimised method to calculate the concentrations. Intra-day (within) precision and accuracy were calculated from 6 replicates per QC in one batch. Inter-day precision and bias were determined from 3 replicates per QC over 5 different runs.

Accuracy (Bias) was assessed as a percentage of the nominal concentration to determine how close the measured concentration was to the accepted reference value. To follow SWGTOX guidelines the mean value should not deviate by more than 20% from the true value (±20%). Their values were calculated using the following equations (Equation 3-4, Equation 3-5).

Equation 3-4 Mean Measured Concentration

$$Mean\ measured\ concentration \quad \bar{X} = {\sum xi}/{n}$$

$$(\sum xi = sum\ of\ all\ observed\ concentrations)$$

$$(n = sample\ size)$$

Equation 3-5 Bias

$$Bias\ (\%) = \frac{\bar{X}}{X} \times\ 100\ (X\colon nominal\ concentration)$$

The precision of the method was assessed as the percentage of the coefficient of variation (%CV) to express the degree of scatter between a series of measurements. This was obtained from multiple sampling of the same homogeneous samples under the prescribed conditions. Their values were calculated using the following equations (Equation 3-6, Equation 3-7, Equation 3-8). The results are considered acceptable if %CV is less than 20% [348].

Equation 3-6 Standard deviation

$$Standard\ deviation\ (SD) = \frac{\sqrt{\sum(x\ -\ \bar{x})2}}{n\ -\ 1}$$

Equation 3-7 Intra-day run

$$\boldsymbol{Intra\ -\ day\ run\ (\%CV) = \frac{SD\ for\ single\ run\ of\ samples}{\bar{X} for\ single\ run\ of\ samples} \times 100}$$

Equation 3-8 Inter-day run

$$Inter\ -\ day\ run\ (\%CV) = \frac{SD\ for\ each\ concentration\ over\ 5\ runs}{\bar{X} for\ each\ concentration} \times 100$$

3.8.5 Recoveries

Recovery is defined as 'the extraction efficiency of an analytical process, reported as percentages of the known amount of analytes of interest which are extracted and analysed by an optimised method' [353]. The loss of analyte during extraction should be investigated with at least three replicates at two quality control (QC) levels. This method can calculate recovery (RE) according to Equation 3-1.

3.8.6 Carryover

As part of method validation requirements, carryover has to be investigated under analysis conditions. Carryover can be defined as the appearance of unintended analyte signal in samples transferred from a previously run positive sample. This signal will, subsequently, lead to inaccurate quantitation.[348] Analyte carryover was assessed for 2 different batches by injecting three blank controls after two injections of an extract of blood containing all analytes at a high concentration (QC4) double the upper limit of quantification in the calibration curve. To establish if there is carryover, the chromatograms were

analysed visually. The method was considered free of carryover if no interfering signal for all analytes was observed in the blank solution.

3.8.7 Stability

The validation of the analytical method should demonstrate the extent to which the analytes in a given matrix under specific conditions are stable for certain periods of time in order to ensure accurate quantitative results, particularly if no information is available from previous work [30]. Therefore, the assessment of analyte stability in matrix during the validation process is required for reliable quantification. Storage conditions evaluated were: auto sampler temperature stability, fridge stability and stability after 3 freeze-thaw cycles at -20±2 ºC.

In order to evaluate auto sampler, the HQC samples were prepared and then divided into three different autosampler vials. The first vials were immediately analysed in triplicate to establish the time zero responses. All remaining vials were stored at room temperature on autosampler, then analysed in triplicate at three different time intervals.

In order to evaluate fridge stability, the (low and high) QC samples were prepared and then divided into three different fridge stability vials. The first (low and high) QC vials were immediately analysed in triplicate to establish the time zero responses. All remaining vials were stored at -20 ºC, then analysed in triplicate at three different time intervals.

It is part of a laboratory's standard practice to freeze samples prior to analysis, analyte stability was determined after three freeze and thaw cycles. The prepared (low and high) QC samples were aliquoted into three separate storage containers per concentration and then frozen at -20±2 ºC for 24 hours. This was followed by an unassisted thaw at room temperature. When completely thawed, the first set of samples was analysed in triplicate, while the others were refrozen for 24 hours under the same conditions. The freeze/thaw cycle and analysis were repeated three more times.

Subsequently, samples were extracted and processed in triplicate along with freshly spiked calibration standards and analysed using the regression equation obtained. The recovery of each analyte was then calculated using Equation 3-9.

Equation 3-9 Stability recovery equation

$$Recovery\ = \frac{Time\ Final\ Concentrations}{Time\ Initial\ Concentrations}\ X\ 100$$

Analytes were identified as being unstable if their recovery fell out with the acceptable criteria of ±20%.

3.9 Stability study of drugs stored in different tubes

The present study was designed to determine the stability of opioids and antidepressant drugs in spiked blood stored in two types of sampling tubes at -20 ^{o}C, 4 ^{o}C and 20 ^{o}C for a time interval of up to three months. The two types of sampling tubes used were separator clot activator tubes (SCAT) and plain tubes (PT).

Opioids and antidepressant drugs were subjected to stability experiments in order to investigate the effect of the sampling tubes and the storage temperature. The analytes of interest were spiked at low and high concentrations for each analyte (0.05 and 0.4mg/L for Morphine, Codeine and DHC; 0.01 and 0.16mg/L for 6MAM; 0.025 and 0.35mg/L for Mirtazapine and EDDP; and 0.25 and 3.5mg/L for Methadone, Amitriptyline, Citalopram, Sertraline and Tramadol), respectively. All samples were stored at three different storage temperatures (-20 °C, 4 °C and 20 ^{o}C), as well as being subjected to three freeze–thaw cycles (-20 °C for 24 h/room temperature for 4 h). The time points selected for -20 ^{o}C and 4 ^{o}C were day 1 (day zero), 7, 14, 21, 30, 60 and 90 days and room temperature at day 1 (day zero), 2 and 14 days.

One set of spiked blood was stored in separator clot activator tubes, which contain a separation gel in the base of the tube; during centrifugation, this gel forms a stable barrier between the serum and the blood cells. The inner wall of the tube is also coated with microscopic silica particles, which activate the coagulation process. The other set of spiked blood was stored in plain tubes. The method used for extraction and analysis was the SPE and GC-MS validated method for extraction of opioid and antidepressant drugs from blood as detailed in section 3.6 and 3.7. Determination of each analyte concentration was based on the calibration curve of each day of analysis. The concentration found was compared with the expected concentration, with the latter determined by analysing freshly prepared blood spiked samples at the same concentration using the GC/MS method on the same day. The percent concentration change in each analyte concentration was calculated. In addition, differences with respect to the reference value (percentage change) were determined for each storage condition.

3.10 Results and Discussion

3.10.1 Results of Method Development and Optimisation

3.10.1.1 Analyte identification and confirmation

Unextracted standards of each analyte were used for injection on to the GC-MS to obtain their mass spectra.; Table 3-5 shows the selected ions and retention times for morphine, codeine, DHC and 6MAM.

Table 3-5 Selected Ions and Retention Times for Opioids

Drug	Quantitative Ion (m/z)	Qualitative Ion 1 (m/z)	Qualitative Ion 2 (m/z)	Retention Time (min)
Morphine	429	414	236	13.8
Morphine-d3	432	-	-	13.6
6-monocetylmorphine	399	340	287	14.4
6-monocetylmorphine-d3	402	-	-	14.2
Codeine	371	196	146	13.4
Codeine-d3*	374	-	-	13.2
Dihydrocodeine	373	236	178	12.9

* Codeine-d3 is used as the internal standard for codeine and dihydrocodeine quantitation.

Table 3-6 shows the selected ions and retention times for tramadol, methadone, EDDP, amitriptyline, citalopram, sertraline and mirtazapine.

Table 3-6 Selected Ions and Retention Times for Opioids and Antidepressant Drugs

Drug	Quantitative Ion (m/z)	Qualitative Ion 1 (m/z)	Qualitative Ion 2 (m/z)	Retention Time (min)
Tramadol	58	263	135	16.0
Methadone	223	165	294	18.4
EDDP	277	262	220	17.0
Amitriptyline	232	217	202	19.4
Mirtazapine	195	208	180	20.6
Sertraline	274	159	262	23.19
Citalopram	58	324	208	24.12
Methadone-d3*	226	-	-	18.2

*Methadone-d3 is used as the internal standard for all analytes.

3.10.1.2 Initial Sensitivity and Linearity Assessments

The assay can quantify Morphine, Codeine and DHC over a range of 0.025–0.5 mg/L, 6MAM over a range of 0.005–0.2 mg/L, Amitriptyline, Citalopram, Sertraline, Tramadol, and Methadone over a range of 0.05–4.0 mg/L, and

Mirtazapine and EDDP over a range of 0.005–0.4 mg/L in a single run achieving good linearity and sensitivity based on correlation coefficients (R2) higher than 0.995 and acceptable LODs for all substances.

3.10.1.3 Optimisation of Analyte Extraction

When choosing extraction methods analyte recovery and cleanliness of extract must be weighed against sample preparation time and cost. Although the SPE methods involve many steps and this technique takes time from start to finish, the focus was on developing one extraction method for all compounds of interest.

Opioid extraction method usually uses 0.1 M acetate buffer during pH adjustment, however, during the method development for the current assay in recovery experiments comparing the use of Acetate buffer 0.1M pH 4.5 and 1M Acetic acid, Acetate buffer gave consistently better results, particularly for Morphine and 6MAM where recovery was 25 - 40% greater for Acetate buffer as seen in Figure 3-2.

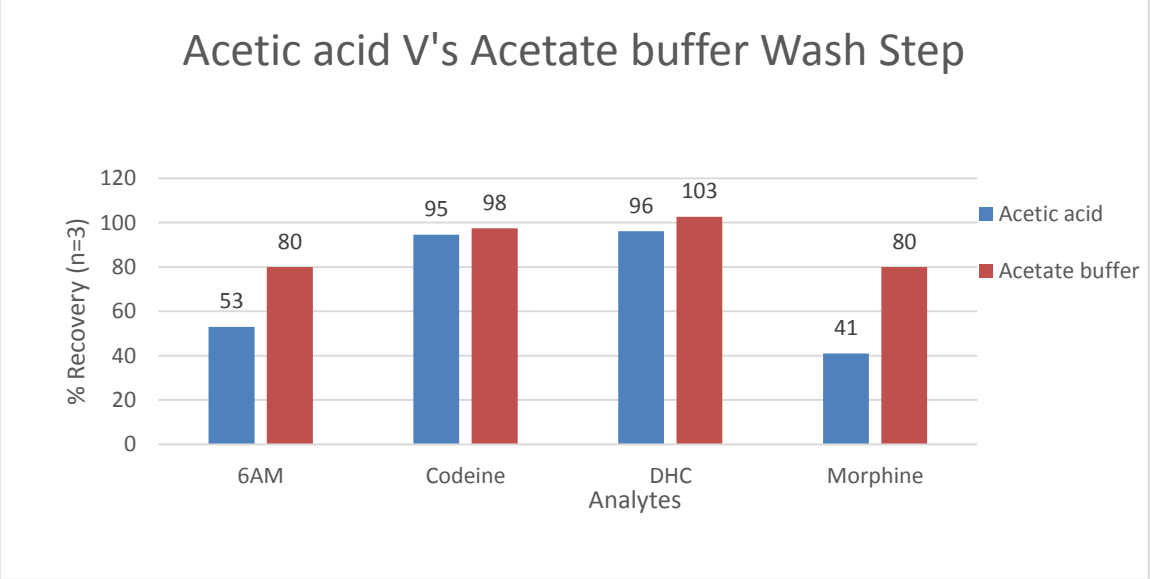


Figure 3-2 Recovery of Opiates in blood using Acetate Buffer or Acetic Acid as a wash step

Typically, the antidepressant drug extraction method uses 1M Acetic acid. For recovery experiments comparing the use of 1M Acetic acid and Acetate buffer 0.1M pH 4.5, acetate buffer consistently gave similar results, particularly for Methadone, EDDP, Citalopram, Amitriptyline and Mirtazapine as seen in Figure 3-3.

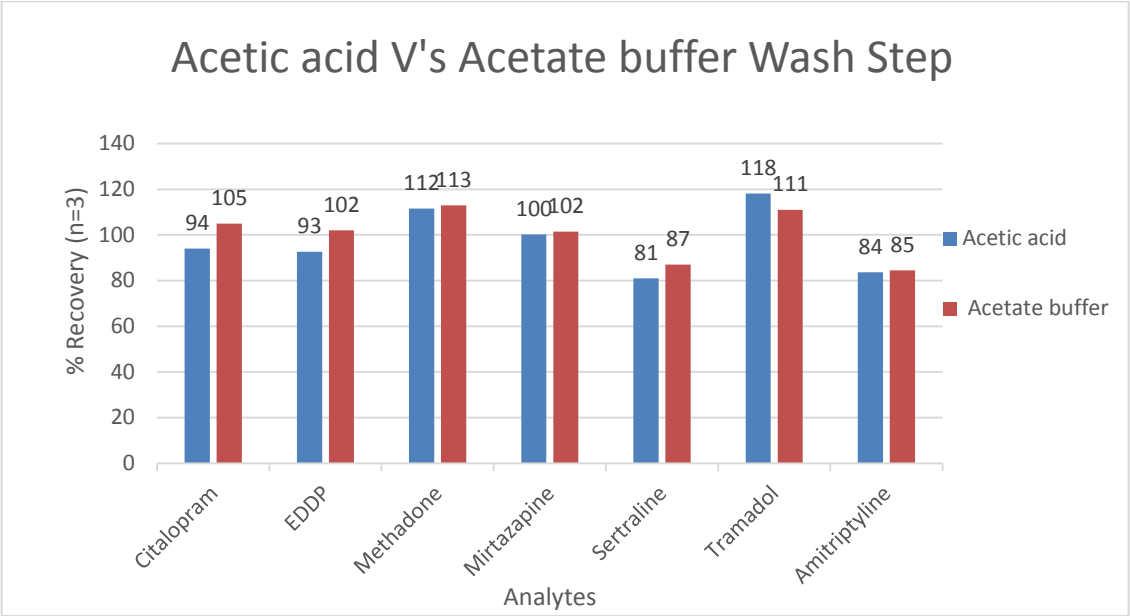


Figure 3-3 Recovery of Antidepressant drugs in blood using Acetate Buffer or Acetic Acid as a wash step

Based on these results, it was decided to use the Acetate buffer 0.1M (pH 4.5) method because it gave higher recoveries for most of the analytes compared with the other methods and for its ease in practice.

3.10.2 Results of Method validation

3.10.2.1 Selectivity and Specificity

No inferences were found from assessed common drugs of abuse and common prescription medications in Appendix 1. Clean baselines with negligible matrix components were found. No interfering peaks were detected at the retention times of any of the analytes used.

3.10.2.2 LOD and LLOQ

Table 3-7 shows (LOD and LLOQ) results.

Table 3-7 LOD and LOQ of drugs of interest in whole blood samples

Drugs	LOD (mg/L)	LLOQ (mg/L)
Methadone	0.005	0.05
Amitriptyline	0.005	0.05
Citalopram	0.010	0.05
Tramadol	0.005	0.05
Sertraline	0.005	0.05
Mirtazapine	0.001	0.005
EDDP	0.001	0.005
Morphine	0.005	0.025
Codeine	0.005	0.025
DHC	0.005	0.025
6MAM	0.001	0.005

The lowest calibrator chosen for each analyte was verified, and therefore can be quantified with the greatest confidence.

3.10.2.3 Linearity

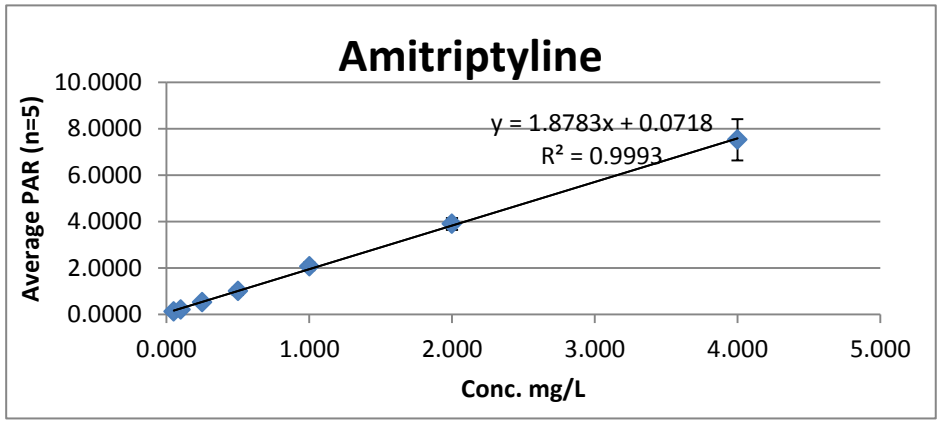
All analytes followed an unweighted linear calibration model with R^2 values greater than 0.996 over 5 days as shown in (Table 3-8, Figure 3-4, Figure 3-5 and Figure 3-6).

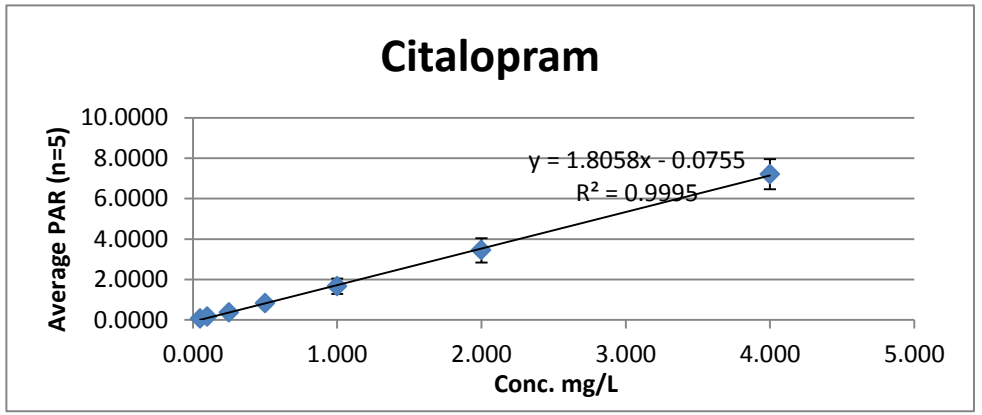
All the calibration points presented accuracy in the range of 90 to 110% for all runs. Further, residual plots to determine if the variances appear to be equal across the calibration range had a similar degree of scatter at each concentration. They also give an indication that, the chosen model adequately fits the data and random distribution of individual residuals around the zero line

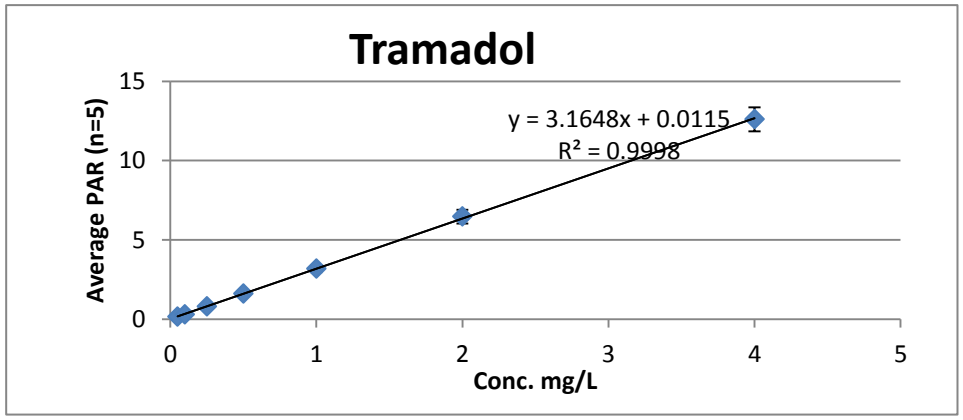
(homoscedasticity) suggests that a linear model is appropriate for all analytes, as shown in Figure 3-7, Figure 3-8 and Figure 3-9.

Table 3-8 Calibration range and average R^2 of Opioids and Antidepressants in Whole Blood

Drugs	Calibration Range (mg/L)	R^2 (n=5)	Internal Standard
Methadone	0.05-4.0	0.999	Methadone-d3
Amitriptyline	0.05-4.0	0.999	Methadone-d3
Citalopram	0.05-4.0	0.999	Methadone-d3
Tramadol	0.05-4.0	0.999	Methadone-d3
Sertraline	0.05-4.0	0.999	Methadone-d3
Mirtazapine	0.005-0.4	0.999	Methadone-d3
EDDP	0.005-0.4	0.999	Methadone-d3
Morphine	0.025-0.5	0.999	Morphine- D3
Codeine	0.025-0.5	0.997	Codeine-d3
DHC	0.025-0.5	0.997	Codeine-d3
6MAM	0.005-0.2	0.999	6MAM-d3







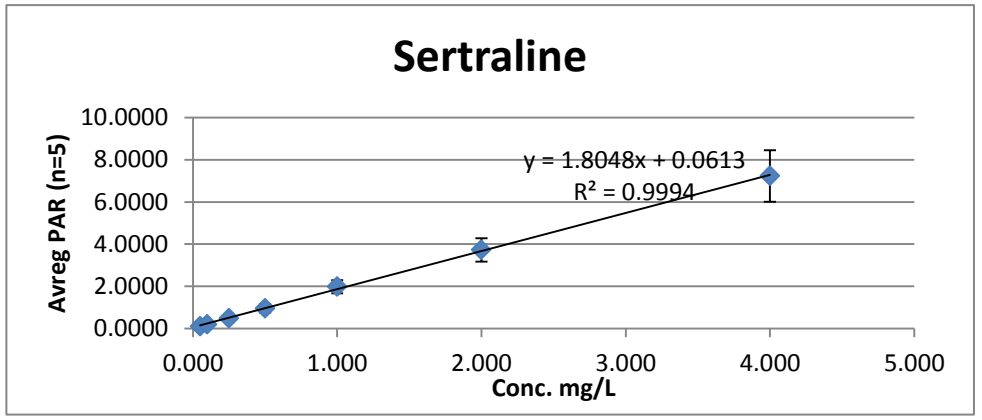
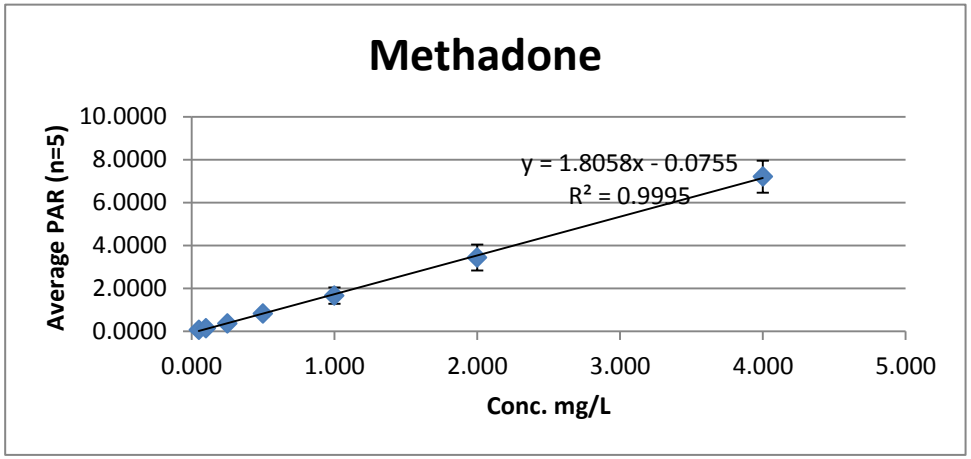
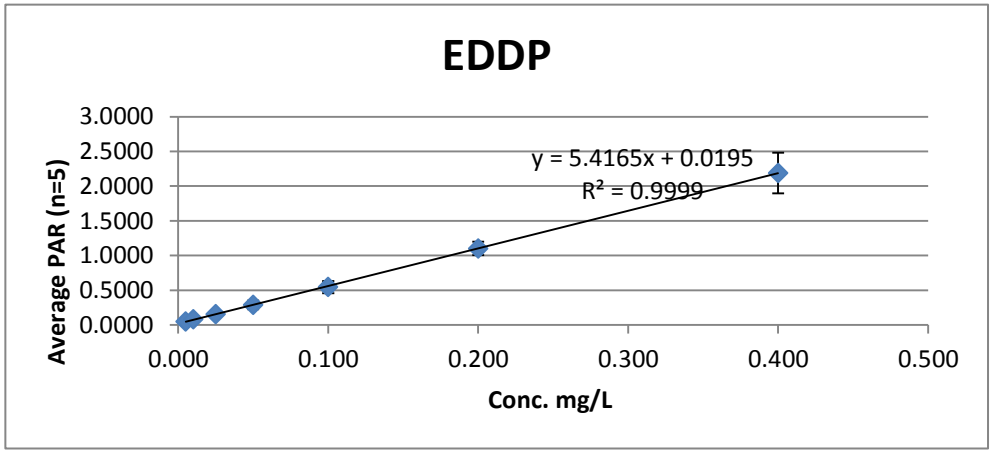


Figure 3-4 Linearity Graphs of Amitriptyline, Citalopram, Tramadol and Sertraline





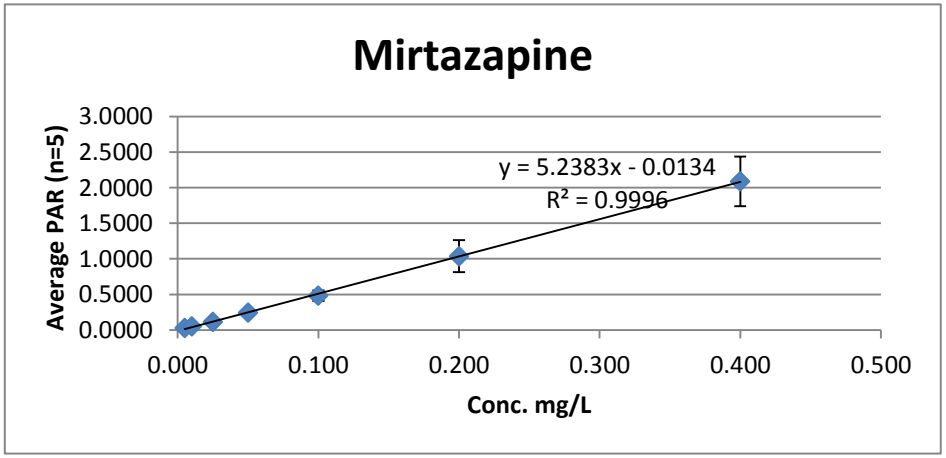
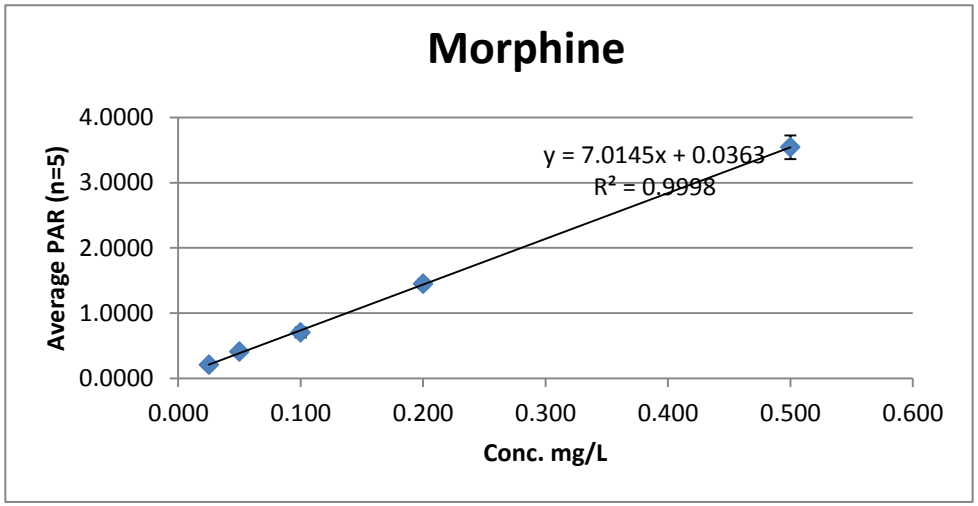
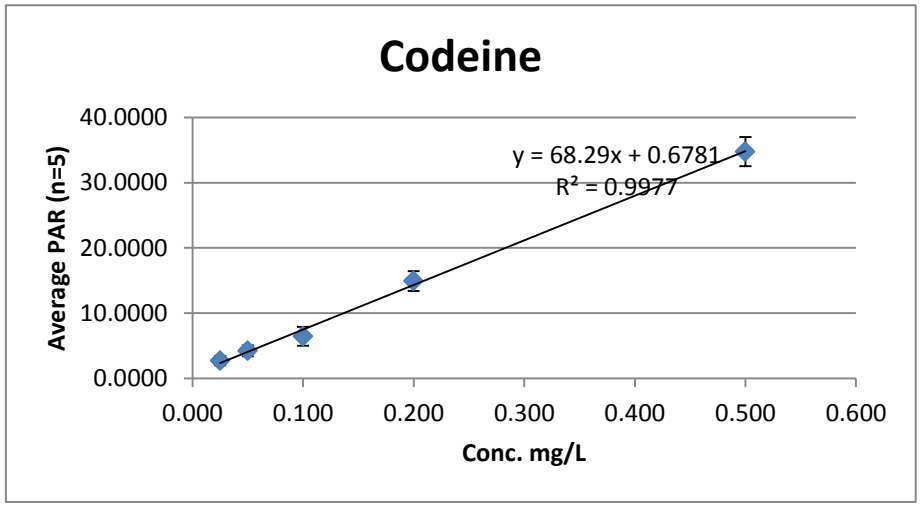
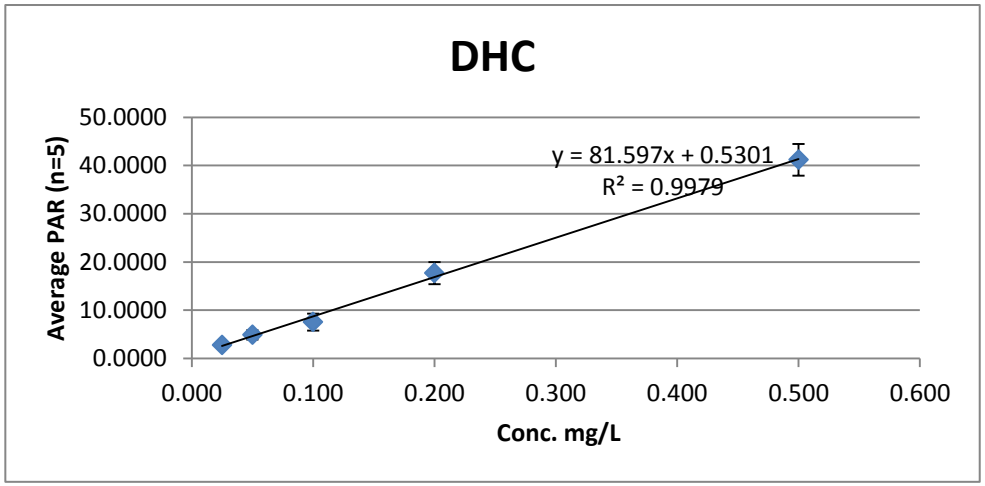


Figure 3-5 Linearity Graphs of Methadone, EDDP and Mirtazapine







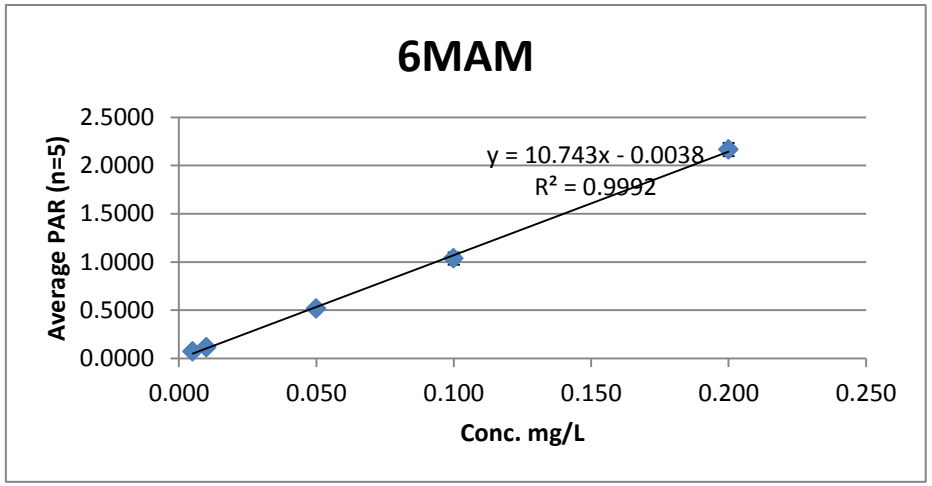
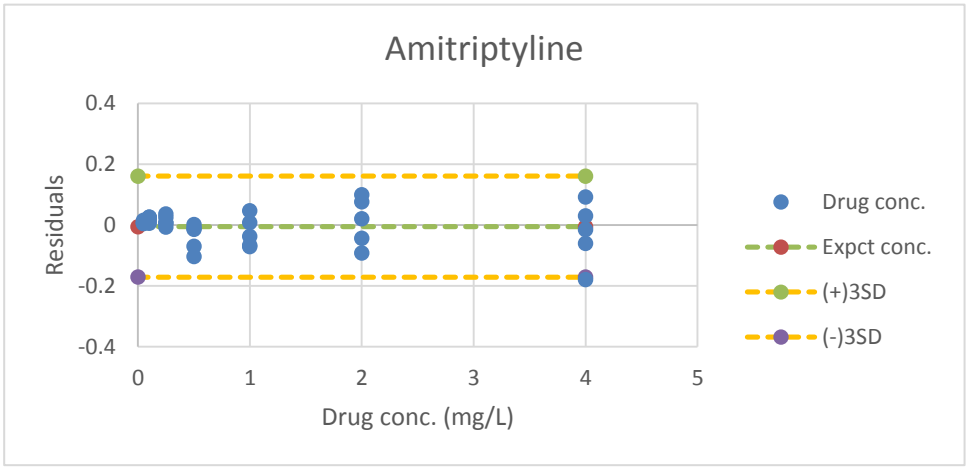
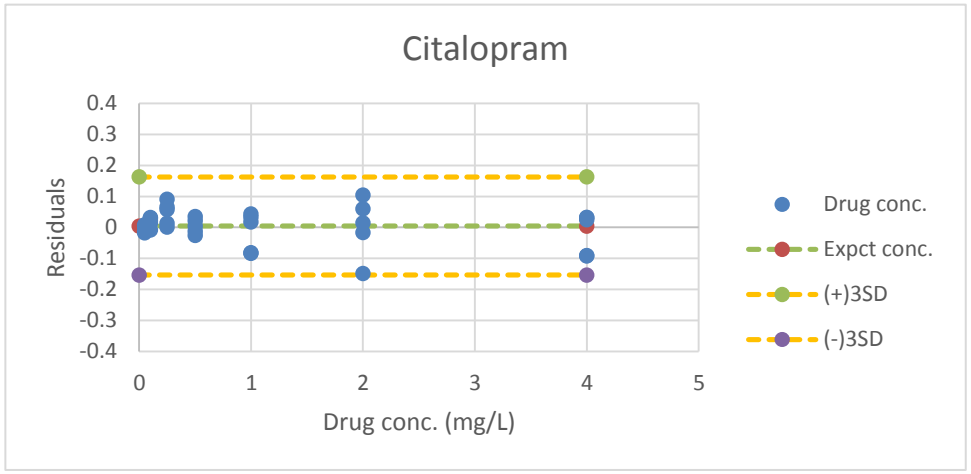
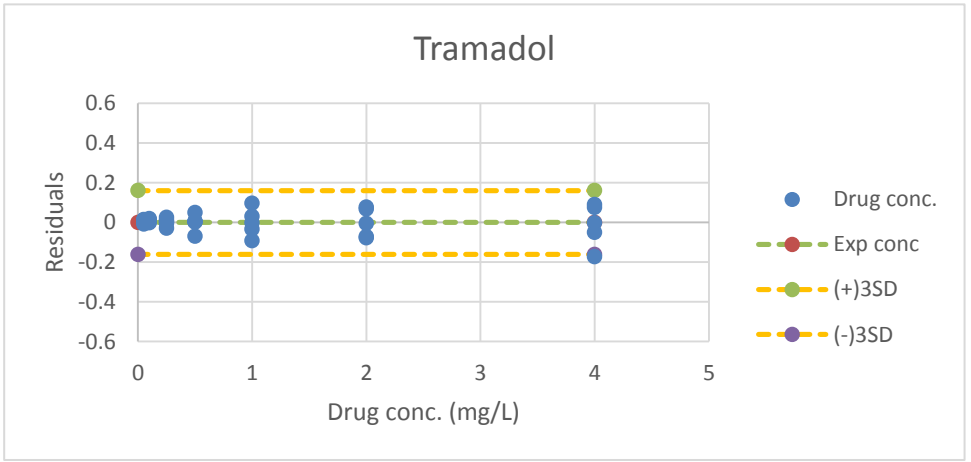


Figure 3-6 Linearity Graphs of Morphine Codeine, DHC and 6MAM







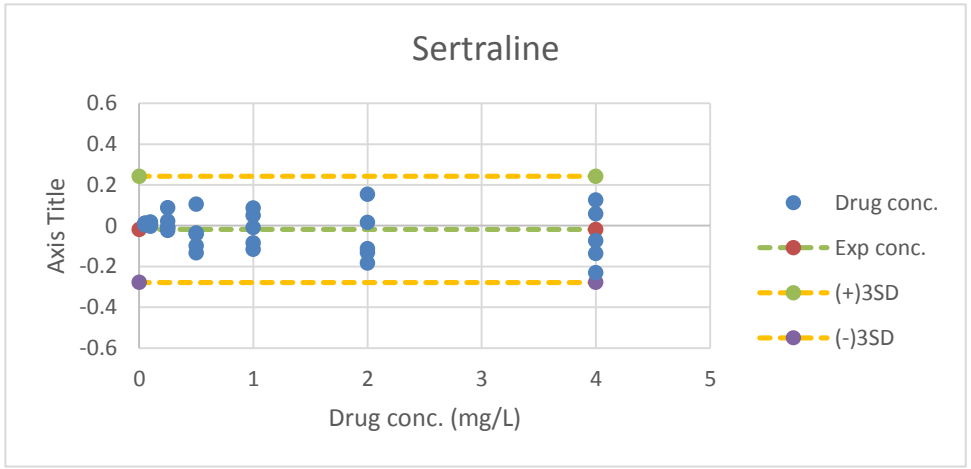


Figure 3-7 Standardised Residual Plot Graphs of Amitriptyline, Citalopram, Tramadol and Sertraline

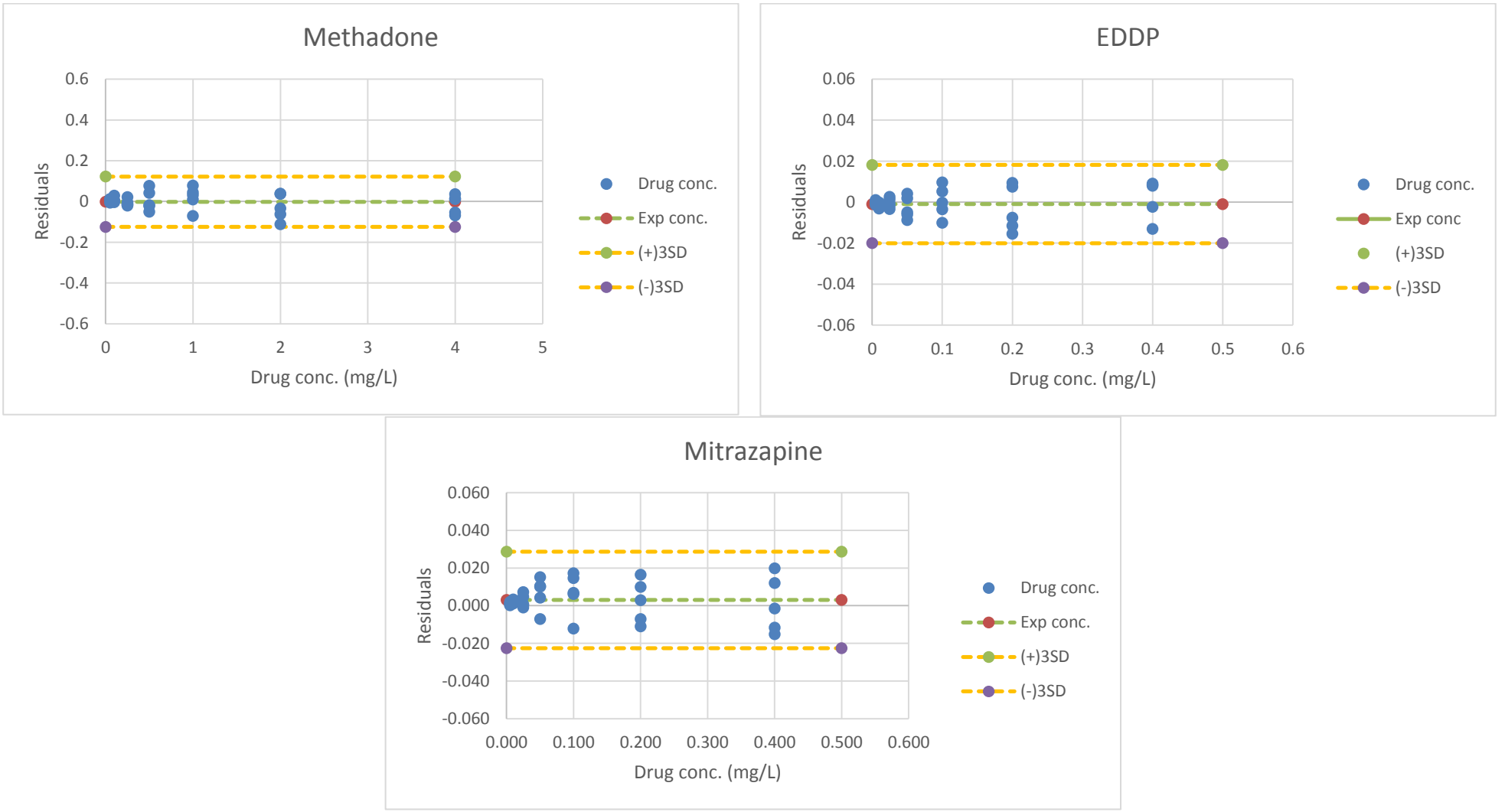
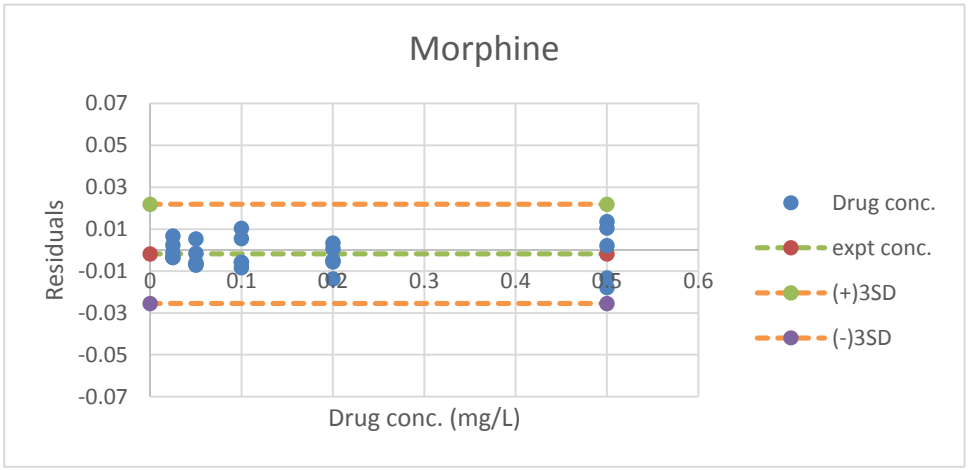
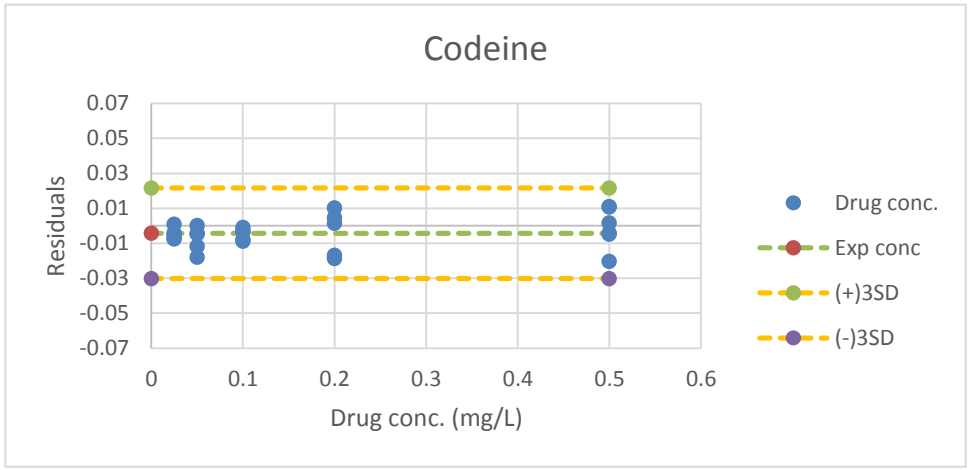
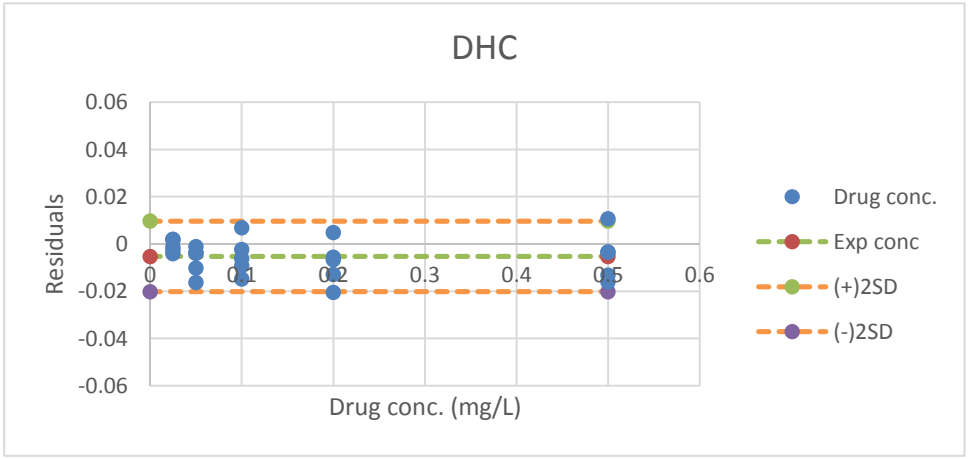


Figure 3-8 Standardised Residual Plot Graphs of Methadone, EDDP and Mirtazapine







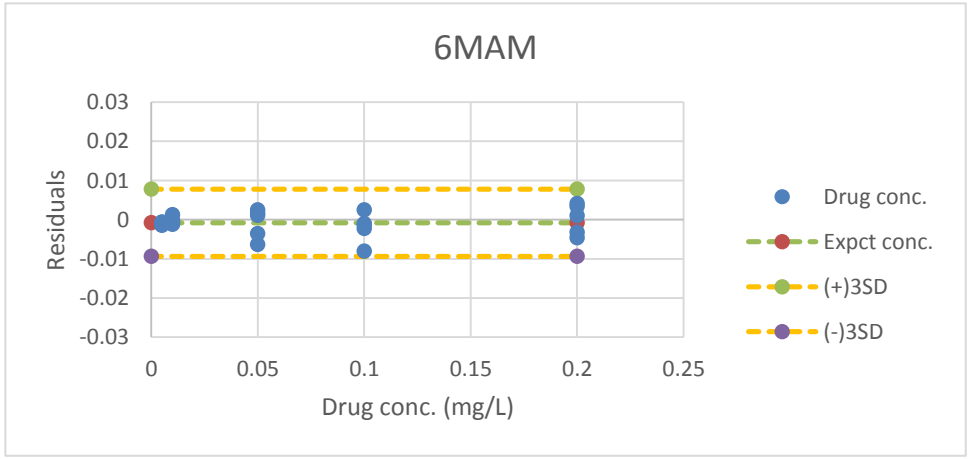


Figure 3-9 Standardised Residual Plot Graphs of Morphine Codeine, DHC and 6MAM

3.10.2.4 Bias and Precision

The average accuracy (Bias) for the analytes across all analytes for the low, medium and high QCs (as detailed in Table 3-1) were within the acceptable range of ± 20% of the nominal concentrations. The intra-day accuracy was from 80.27- 114.07%. The inter-day accuracy ranged from 84.02 - 110.24%. The intra-day precision values were less than 19%. The inter-day precision values were less than 20%. Accuracy and precision results for all analytes are listed in Table 3-9 and Table 3-10.

Table 3-9 Intra-Day Assay Precision and Accuracy

	LQC					MQC					HQC				
	Expected Conc. mg/L	Average Conc. mg/L (n=6)	SD	% CV	Bias	Expected Conc. mg/L	Average Conc. mg/L (n=6)	SD	% CV	Bias	Expected Conc. mg/L	Average Conc. mg/L (n=6)	SD	% CV	Bias
Methadone	0.25	0.24	0.03	11.2	-4.3	1.00	1.06	0.1	9.1	6.3	3.50	3.79	0.33	8.6	8.3
EDDP	0.025	0.02	0	17.0	-14.1	0.10	0.08	0.01	11.2	-18.0	0.35	0.31	0.02	7.7	-10.9
Amitriptyline	0.25	0.18	0.01	7.8	-6.9	1.00	1.03	0.07	6.9	2.6	3.50	3.52	0.14	4.1	0.6
Mirtazapine	0.025	0.02	0	8.2	-9.0	0.10	0.1	0.01	9.7	-1.4	0.35	0.36	0.02	4.9	2.1
Sertraline	0.25	0.19	0.03	14.7	-3.3	1.00	0.94	0.11	11.2	-5.9	3.50	2.66	0.11	4.2	-8.9
Tramadol	0.25	0.24	0.03	11.2	-4.3	1.00	1.06	0.1	9.1	6.3	3.50	3.79	0.33	8.6	8.3
Citalopram	0.25	0.15	0.03	17.5	-4.5	1.00	0.94	0.07	8.0	-6.0	3.50	3.99	0.18	4.6	14.1
MOR	0.05	0.052	0.003	5.8	3.4	0.10	0.11	0.01	5.4	10.9	0.40	0.44	0.01	1.7	9.0
COD	0.05	0.05	0.009	19.0	-0.8	0.10	0.08	0.01	15.0	-16.6	0.40	0.3	0.04	13.1	-19.8
DHC	0.05	0.05	0.009	18.7	-0.4	0.10	0.08	0.01	14.2	-18.7	0.40	0.31	0.03	9.0	-19.8
6MAM	0.010	0.011	0	4.0	6.1	0.05	0.05	0	1.0	4.3	0.16	0.17	0	1.0	6.5

Table 3-10 Inter-Day Assay Precision and Accuracy

	LQC					MQC					HQC				
	Expected Conc. mg/L	Average Conc. mg/L (n=5)	SD	% CV	Bias	Average Conc. mg/L (n=5)	Expected Conc. mg/L	SD	% CV	Bias	Expected Conc. mg/L	Average Conc. mg/L (n=5)	SD	% CV	Bias
Methadone	0.25	0.25	0.01	5.8	1.1	0.93	1.00	0.02	6.1	-5.9	3.50	0.93	0.09	10.2	-7.5
EDDP	0.025	0.02	0	6.2	-4.2	0.33	0.10	0.01	7.6	-6.9	0.35	0.33	0.03	8.3	-5.7
Amitriptyline	0.25	0.24	0.05	3.2	-5.9	3.7	1.00	0.13	12.2	6.1	3.50	3.7	0.72	19.4	5.8
Mirtazapine	0.025	0.02	0	15.0	-15.9	0.34	0.10	0.01	11.7	-9.7	0.35	0.34	0.02	7.2	-2.4
Sertraline	0.25	0.21	0.07	3.6	-14.9	3.4	1.00	0.3	19.1	-4.8	3.50	3.4	0.95	18.0	-2.9
Tramadol	0.25	0.23	0.02	8.1	-8.8	3.41	1.00	0.1	10.0	-1.5	3.50	3.41	0.33	9.8	-2.6
Citalopram	0.25	0.15	0.05	3.7	-4.3	3.59	1.00	0.1	11.6	-12.7	3.50	3.59	0.34	9.5	2.6
MOR	0.05	0.05	0.01	13.1	7.5	0.42	0.10	0	4.5	10.2	0.40	0.42	0.02	5.7	3.9
COD	0.05	0.05	0.01	19.5	-1.9	0.42	0.10	0.03	19.8	4.3	0.40	0.42	0.17	19.2	3.8
DHC	0.05	0.05	0.01	19.9	-4.0	0.42	0.10	0.03	19.6	2.7	0.40	0.42	0.16	18.6	4.8
6MAM	0.010	0.01	0	8.6	0.4	0.17	0.05	0	3.5	1.2	0.16	0.17	0.01	3.4	3.3

3.10.2.5 Recoveries

Recovery (%) is shown below in Figure 3-10. From this information it can be shown that the solid phase extraction of 11 analytes from whole blood was extremely efficient with percentage recoveries ranging from 80 – 118 %.

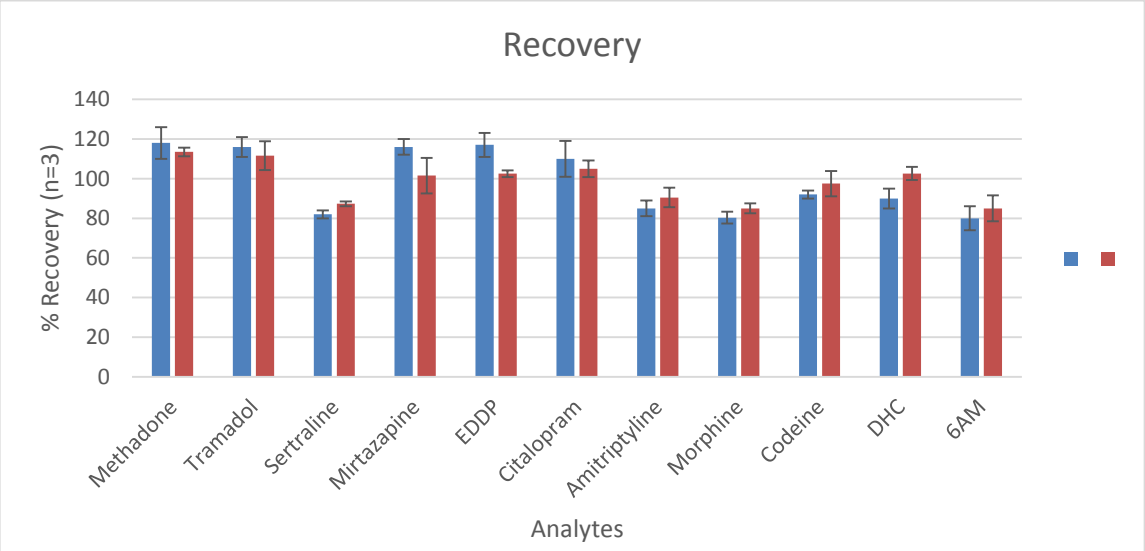


Figure 3-10 Recovery Values for LQCs & HQCs from Whole Blood.

3.10.2.6 Carryover

After two consequent injections of the highest analyte concentration no signal was observed in the blank QC samples. Carryover was therefore not considered to be a problem for all analytes.

3.10.2.7 Stability study

1) Autosampler stability:

Table 3-11 showed that all the drugs were stable in the whole blood samples in the autosampler temperature for up to 5 days. This is further illustrated by the number of QC's which had recovery results ranging ±20%, except for 6MAM which was less stable (-21%).

Table 3-11 Autosampler Stability of drugs of interest for 5 Days

	% Recovery of QC3 (n=3)			
	*D0	*D3	*D4	*D5
Amitriptyline	100	102	101	101
Sertraline	100	104	101	103
Tramadol	100	97	96	98
Citalopram	100	94	95	96
Methadone	100	102	100	98
EDDP	100	99	97	98
Mirtazapine	100	100	101	104
MOR	100	113	108	121
COD	100	98	102	102
DHC	100	87	104	100
6MAM	100	79	84	79

*D refers to time in days.

2) Refrigerator stability:

Refrigerator stability results for each analyte are shown in Table 3-12. The extracted samples were stable at 4ºC for 5 days. All analytes tested had % recoveries within the acceptable criteria of ±20%, except for mirtazapine (+22%) and 6MAM (+27%), this may be because of analytical variation/error.

Table 3-12 Refrigerator stability of drugs of interest for 5 Days

	% Recovery of QC1 (n=3)				% Recovery of QC3 (n=3)			
	*D0	*D3	*D4	*D5	*D0	*D3	*D4	*D5
Tramadol	100	92	82	92	100	98	99	99
Amitriptyline	100	104	115	103	100	96	99	98
Sertraline	100	83	91	100	100	103	101	99
Citalopram	100	84	82	92	100	106	102	101
Methadone	100	90	90	89	100	98	97	99
EDDP	100	83	108	100	100	101	100	98
Mirtazapine	100	100	122	112	100	100	104	101
MOR	100	97	99	119	100	89	107	95
COD	100	85	85	92	100	102	105	105
DHC	100	109	90	90	100	115	115	119
6MAM	100	106	104	100	100	127	100	107

*D = refers to time in days.

3) Freeze-thaw cycles stability:

Table 3-13 shows that, drugs of interest were stable in whole blood after 3 freeze-thaw cycles at - 20ºC for 5 days. All analytes tested had % recoveries within the acceptable criteria of ±20%, apart from 6MAM (+27%), this may be because of analytical variation/error.

Table 3-13 Freezer-Thaw Stability of drugs of interest for 25 days

	% Recovery of QC1 (n=3)				% Recovery of QC3 (n=3)			
	*D0	*D3	*D4	*D5	*D0	*D3	*D4	*D5
Methadone	100	100	89	109	100	101	100	99
Tramadol	100	101	121	97	100	101	99	97
Amitriptyline	100	83	92	100	100	99	98	102
Mirtazapine	100	91	89	108	100	97	99	104
Sertraline	100	101	101	112	100	103	102	102
EDDP	100	83	108	100	100	100	97	100
Citalopram	100	89	109	89	100	97	100	97
MOR	100	80	82	83	100	94	89	83
COD	100	92	92	108	100	95	100	98
DHC	100	120	100	110	100	87	104	100
6MAM	100	106	104	100	100	100	107	127

*D refers to time in days. **% R refers to percent recovery

3.10.3 Stability study of Drugs in Different Tubes

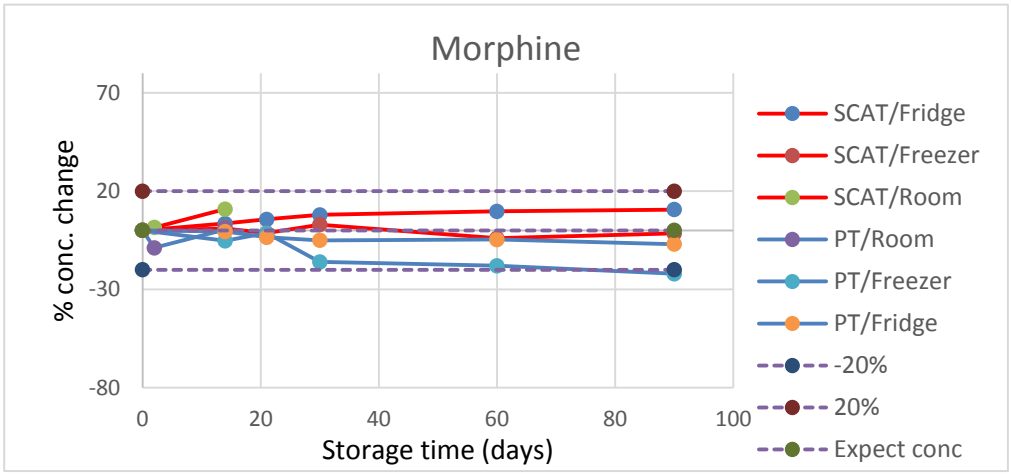
A) The stability results of opioids

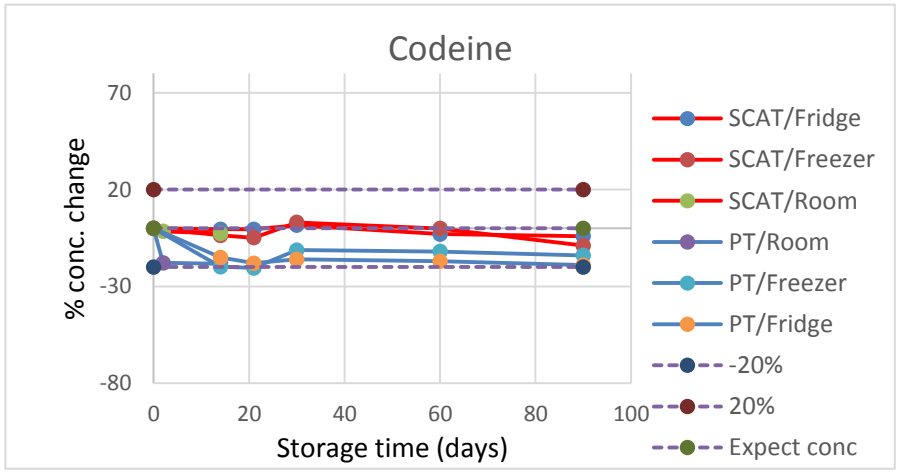
The stability results from the experiments for morphine, codeine, DHC and 6MAM are shown in Figure 3-11. All analytes in two types of sampling tube were found to be stable up to 3 months at -20°C. The percentage change after 3 months of storage at -20°C was less than 20 % for all analytes in both sampling tubes. The percentage of the concentration changes for morphine, codeine, DHC and 6MAM after three Freeze–thaw cycles were found to be -1.5%, -8.9%, -8.9% and -19.8% in separated clot activator tubes, and -20%, -14.0%, 19% and -9% in plain tubes, respectively.

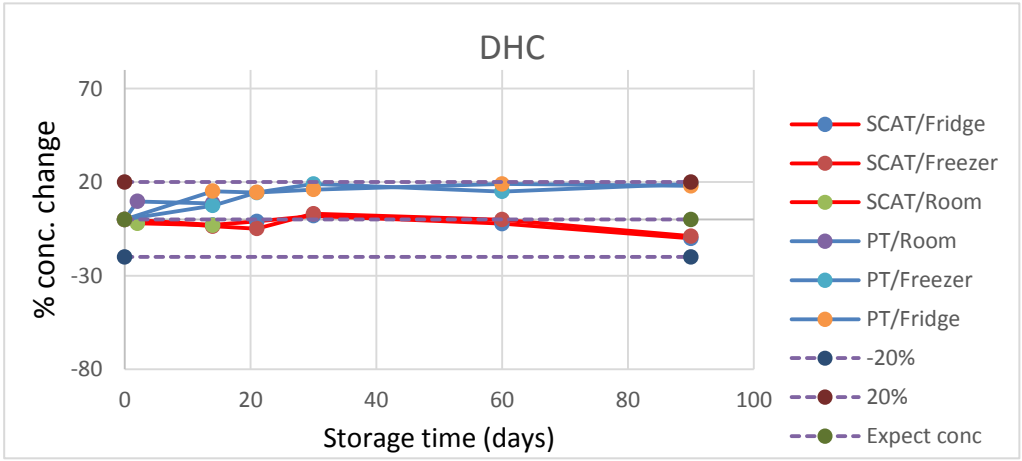
In plain tubes, 6MAM was stable when stored in all conditions. While in separated clot activator tubes, the 6MAM concentration was significantly decreased when stored at refrigerator and room temperature. In separated clot activator tubes, the 6MAM was decreased not only after 14 days of storage at room temperature (64%) but also at 4°C (26% decreased) and continuously decreased up to 78.5% during 3 months of storage at 4°C.

In summary, morphine, codeine, and DHC concentrations were stable in all conditions, while, 6-monoacetylmorphine was unstable under certain conditions. The separated clot activator tube affected the stability of 6MAM, especially when it was stored in room and refrigerator temperature. It may be the gel in the tubes absorbed 6MAM preferentially compared to other tubes.

The results of our stability study are in agreement with those reported by other respective studies of morphine and codeine [69, 302, 311] in blood. Some limited data concerning the stability of these four opiates in blood are also published, but during a method validation process [354-356]. Specifically, published stability studies of 6MAM (not in the framework of a method validation) were referred only in other biological matrices such as urine [274], hair [357], and oral fluid [358]. To our knowledge, it is the first stability study of morphine, codeine, DHC and 6MAM in blood concerning the effect of the type of sampling tubes.







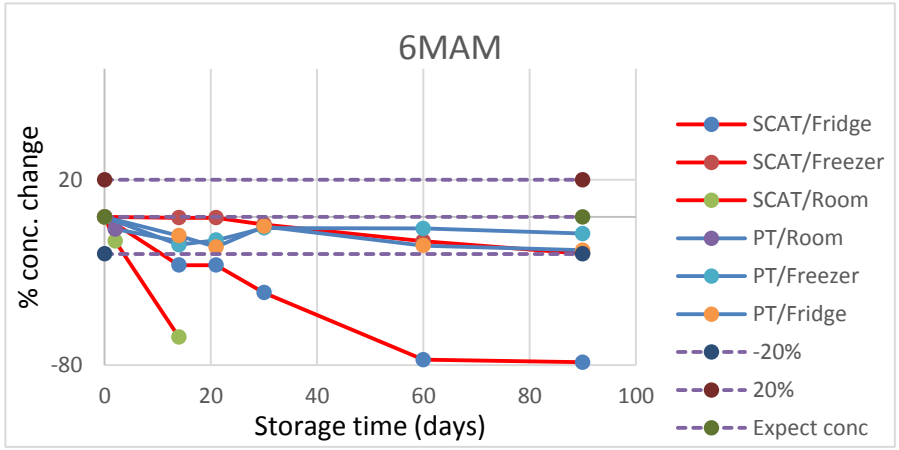


Figure 3-11: Stability of Morphine, Codeine, DHC and 6MAM by using separated clot activator tubes versus plain tubes

B) The stability results of antidepressant drugs

Figure 3-12 and Figure 3-13 show that, all analytes appeared to be stable in both type of sampling tubes at -20 °C, 4 °C and 20 °C for a minimum of 30 days. Methadone and tramadol were found to be the most stable analyte throughout the observation period under all conditions. In plain tubes, all analytes were found to be stable up to 3 months at -20°C and 4 °C storage, except mirtazapine concentrations decreased 25 % of the initial concentrations in fridge.

In separated clot activator tubes, the stability pattern totally differed from that of plain tubes. After one-month storage at -20 °C and 4 °C changes in analyte concentrations were observed for all analytes except methadone and tramadol. In separated clot activator tubes, a strong influence of the sampling tube on EDDP was obvious under all conditions. However, the concentrations of mirtazapine and citalopram decreased after 30 days of storage at -20 °C and 4 °C. The decrease in mirtazapine and citalopram concentration was smaller compared to amitriptyline or sertraline. Amitriptyline was found to be the most unstable analyte in separated clot activator tubes, at a storage temperature of 4 °C, its concentration decreased by as much as 60% from the initial concentration.

In summary, methadone and tramadol concentrations were stable under all conditions in both tubes. While, EDDP was less stable in separated clot activator tubes than in plain tubes under all conditions. The separated clot activator tube affected the stability of citalopram, sertraline, amitriptyline and mirtazapine especially when they were stored more than one-month in the fridge and freezer. It may be the gel in the tubes absorbed these analytes preferentially compared to other tubes.

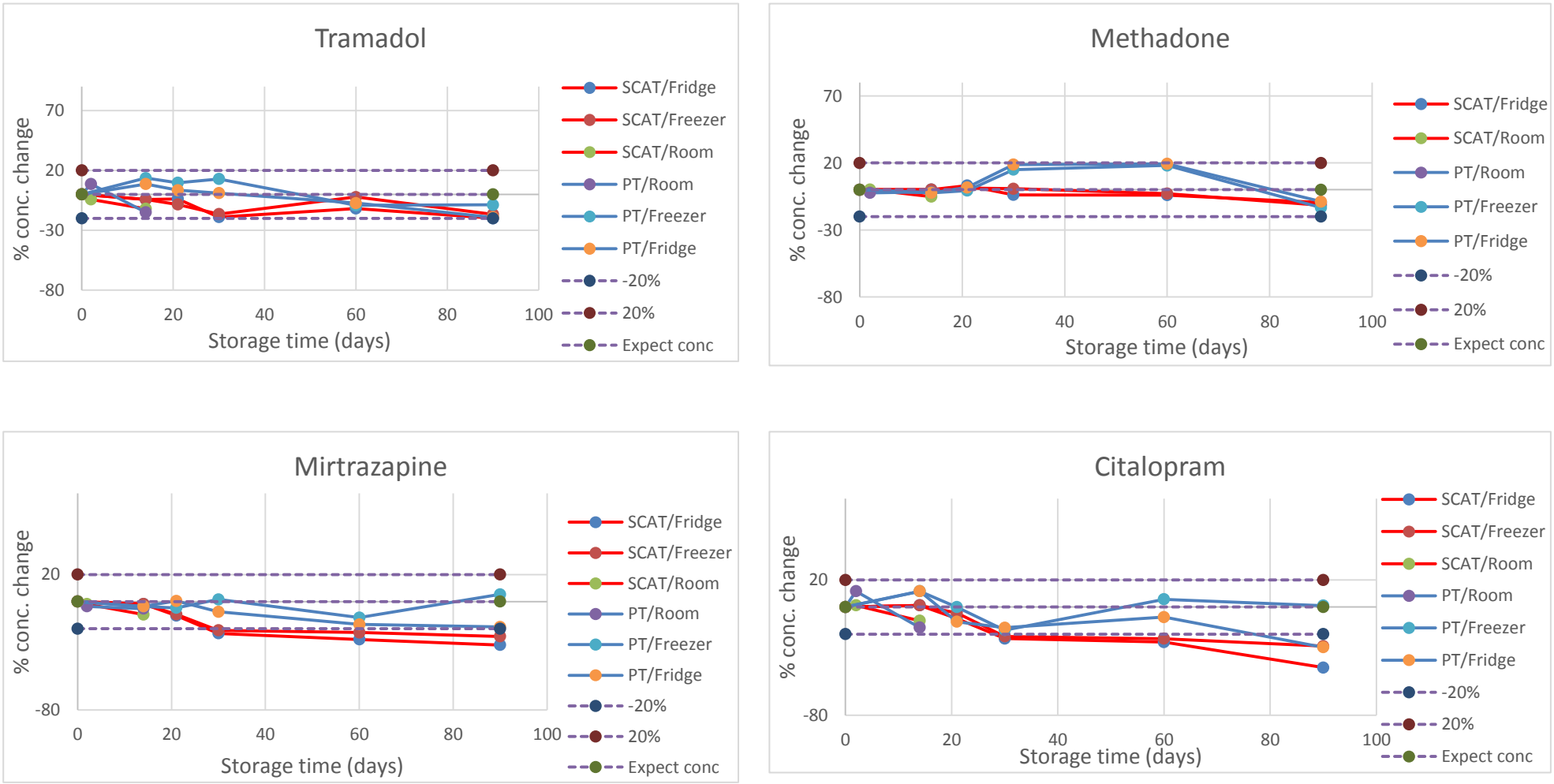


Figure 3-12: Stability of Tramadol, Methadone, EDDP and Mirtazapine by using separated clot activator tubes versus plain tubes.

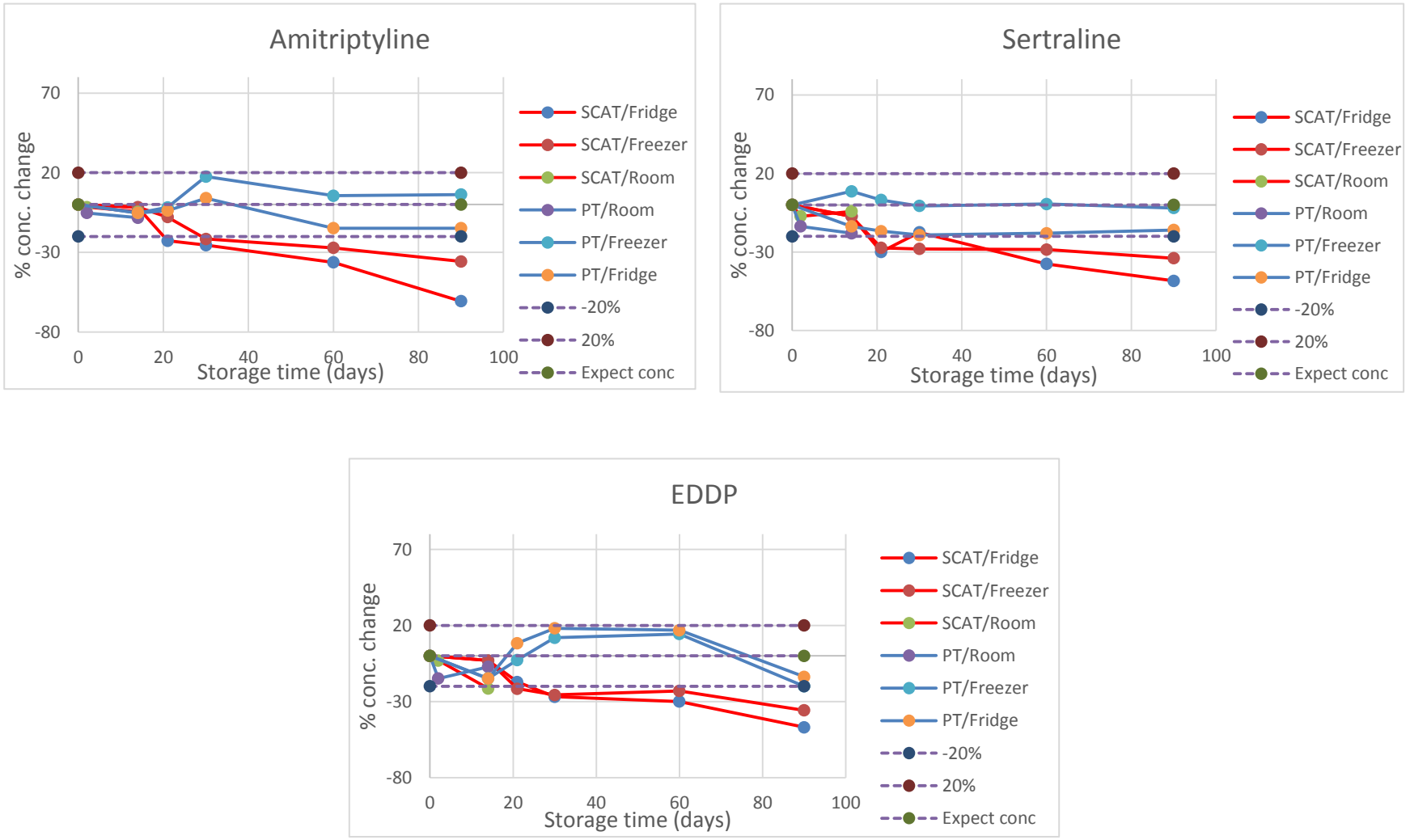


Figure 3-13: Stability of Citalopram, Amitriptyline and Sertraline by using separated clot activator tubes versus plain tubes.

3.11 Conclusions

The GC- MS method with SPE has been successfully developed and validated according to Standard Practices for Method Validation in Forensic Toxicology (SWGTOX) guidelines [348] for the simultaneous determination of Morphine, Codeine, DHC, 6MAM , Tramadol, Methadone, EDDP, Amitriptyline, Citalopram, Sertraline and Mirtazapine in whole blood. Although a large number of analytes were included in the method, acceptance criteria for linearity, accuracy, precision, and recovery were achieved for all analytes. There were no endogenous or exogenous interferences and all analytes showed satisfactory stability in freezer as well as fridge and autosampler.

The stability of opioids and antidepressant drugs in spiked blood was studied under different sampling and storage conditions. In both separated clot activator tubes and plain tubes, morphine, codeine, and DHC concentrations were stable under all conditions, while, 6MAM was unstable under certain conditions. The separated clot activator tube affected the stability of 6MAM, especially when it was stored in room and refrigerator temperature. It may be the gel in the tubes absorbed 6-monoacetylmorphine preferentially compared to the other tubes. On the other hand, methadone and tramadol concentrations were stable under all conditions in both separated clot activator tubes and plain tubes. While, EDDP was less stable in separated clot activator tubes than in plain tubes under all conditions. The separated clot activator tube also affected the stability of citalopram, sertraline, amitriptyline and mirtazapine especially when they were stored more than one-month in fridge and freezer. This is possibly due to absorption of the drugs to the gel.

The stability of drugs in different tubes is vital, particularly when analyses cannot be performed promptly. Also, forensic laboratories are required to store biological samples for months or years and re-testing or further analysis may be necessary after a period of time. Following the results of this study, it is recommended that, biological samples collected for the analysis of drugs are collected in plain tubes. Furthermore, stability of other drugs, especially stored in separated clot activator tube, requires further investigation under different storage conditions and time periods to ensure quantitative analysis reflects the actual drug concentration in the biological matrix.

Chapter 4 Method Development for Testing of Morphine, its glucuronides and Gabapentin in Whole Blood Using LC-MS/MS

4.1 Introduction

Considering the increasing occurrence of poly-drug deaths involving both, drugs of abuse and medications, and given that the available sample volume is usually small in forensic cases it is important to maximise the information gained from any test. Therefore, a method for the simultaneous extraction and quantification of Gabapentin and Morphine, as well as the detection of M3G and M6G was developed.

In addition, morphine metabolites detection is an important issue in forensic toxicology and a number of specific reviews describing analytical methodologies for its detection in blood have been published [359-361]. Gas chromatography-mass spectrometry (GC–MS) has been widely used for the quantification of morphine in biological samples [362, 363]. Despite its excellent sensitivity and selectivity, the major drawbacks related to this technique are the required de-conjugation step of the glucuronides and extraction and derivatisation prior to analysis. Additionally, enzymatic hydrolysis of morphine glucuronides has been shown to be problematic.[364-366] Liquid chromatography-mass spectrometry has advantages over gas chromatography-mass spectrometry as the conjugated metabolites can be measured directly without the required hydrolysis and derivatization [72, 367, 368].

4.2 Aims and Objectives

The purpose of this study was to develop and validate a quantitative method for the analysis of morphine, M3G, M6G and gabapentin in postmortem blood. This would be appropriate to determine the correlation ratios between morphine, M3G and M6G concentrations measured in these samples; and to evaluate the patterns of gabapentin prescribing, misuse, and diversion among opioids user, as well as among the general population to inform prescribing practices and policies.

4.3 Materials and Methods

4.3.1 Materials

Morphine, morphine-3-glucuronide and morphine-6-glucuronide and gabapentin and their corresponding deuterates (MOR-d3, M3G-d3, M6G-d3 and GBP-d10) were purchased from Sigma Aldrich (Basingstoke, UK). All of these drugs were purchased as solutions at 1mg/mL in methanol, except Gabapentin which was a 99% pure powder.

Methanol (HPLC Grade), acetonitrile (HPLC LC-MS Grade) and isopropanol (HPLC Grade) were supplied by VWR International Ltd, (Lutterworth, UK). Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, formic acid (reagent grade ≥95%), ammonium acetate and glacial acetic acid (HPLC LC-MS Grade), and ammonium acetate (reagent grade ≥98%) were purchased from Sigma Aldrich (Basingstoke, UK). Deionised water was obtained from a Millipore® system (Direct-Q®3UV-R).

The two sampling tubes used were separated clot activator tubes (SCAT) and Eppendorf tubes (EPNT), purchased from Goods Wagon (Glasgow/Scotland) and VWR (Glasgow/Scotland), respectively.

4.3.2 Solutions Preparation

4.3.2.1 Preparation of Stock Solutions

All stock solutions were prepared in methanol and stored at -20°C for 6 months.

1) **Gabapentin Stock Solution**

GBP stock solution was prepared at 1000 mg/L by adding 5000 mg drug powder to a 5 mL volumetric flask and making up to volume with methanol.

2) **Opioids Stock Solutions**

For MOR, M3G and M6G stock solutions, each drug was prepared individually at 100 mg/L by adding 1mL drug at concentration 1mg/mL to a 10mL volumetric flask and making up to volume with methanol.

4.3.2.2 Preparation of 10 mg/L Standard Solutions for Method Development

All 10 mg/L standard solutions were prepared and stored at 4°C.

1) **Gabapentin 10 mg/L Solution**

A 10 mg/L GBP solution was achieved by adding 100 µL of GBP stock solution (1000 mg/L) to a 10 mL volumetric flask and making up to volume with mobile phase mixture (deionised water/ methanol: 50/50).

2) **Opioids 10 mg/L Solution**

For MOR, M3G and M6G solutions, each drug was prepared individually at 10 mg/L by adding 1mL of stock solutions (100 mg/L) of each drug to a 10 mL volumetric flask and made up to volume with mobile phase mixture (deionised water/ methanol: 50/50).

4.3.2.3 Preparation of Working Solutions for Analysis

All working solutions were prepared in methanol and stored at 4°C for 6 months.

1) **Gabapentin Working Solution**

A 100 mg/L GBP working solution was achieved by adding a 1 mL of GBP stock solutions (1000 mg/L) to a 10 mL volumetric flask and making up to volume with methanol.

2) **Opioids Working Solution**

Opioids working solution containing MOR and M3G at 1 mg/L and M6G at 0.4 mg/L was prepared. This was achieved by adding stock solutions (100 mg/L) of each drug (100 µL for MOR and M3G and 40 µL for M6G) into one 10 mL volumetric flask and making up to volume with methanol.

4.3.2.4 LOD and LLOQ Solution

In order to evaluate the LOD and LLOQ 10 times more dilute solutions were prepared as follows:

1) **Gabapentin Solution**

A 10 mg/L GBP solution was achieved by adding a 1 mL of GBP working solution to a 10 mL volumetric flask and making up to volume with methanol.

2) **Opioids Solution**

LOD & LLOQ opioids (MOR, M3G and M6G) solution was achieved by adding 1 mL of mixed opioids working solution to a 10 mL volumetric flask and making up to volume with methanol to give a final concentration of 0.1, 0.1 and 0.04 mg/L for MOR, M3G and M6G, respectively.

4.3.2.5 Preparation of Internal Standards

Stock solutions were prepared for each internal standard individually in methanol. For GBP-d10, the stock was prepared at 1000 mg/L by dissolving 1 mg drug powder in 1 mL methanol. For all other stock solutions, MOR-d3, M3G-d3 and M6G-d3 were individually prepared at 100 mg/L by adding 1mL of drugs at a concentration 1mg/mL to a 10mL volumetric flask and made up to volume with methanol. All internal standard stock solutions were stored at -20°C for 6 months.

Four internal standards, GBP-d10, MOR-d3, M3G-d3 and M6G-d3 were prepared in a mixed working solution. This was achieved by adding varying amounts of each internal standard stock solution to a 10 mL volumetric flask and making up to volume with methanol as outlined in Table 4-1. The working solution was stored at 4°C for no longer than 6 months.

Table 4-1: Internal Standard Working Solution Preparation

Internal Standard	Abbreviations	Stock Conc. (mg/L)	Amount Added (µL)	Final Conc. mg/L
Gabapentin-d10	GBP-d10	1000	100	10.0
Morphine-d3	MOR-d3	100	100	1.0
Morphine -3-Glucuronide-d3	M3G-d3	100	40	0.4
Morphine -6-Glucuronide-d3	M6G-d3	100	40	0.4

4.3.2.6 Preparation of Calibrators

The concentration range for calibration curves was chosen to include therapeutic and toxic concentrations of each compound. Two working solutions as previously described in section 4.3.2.3 were used to prepare calibrators. A seven-point calibration curve was prepared by combining certain volumes of the two working solutions in test tube of each calibrator as illustrated in Table 4-2 and Table 4-3. To this, 50 µL of combined internal standard solution and 800 µL of methanol were added. A 200 µL aliquot of the blood sample was transferred, vortex mixed for 30 seconds and centrifuged for 10 minutes at 3000 rpm. Following centrifugation, the supernatant was transferred to a 3.5 mL vial using a glass pasteur pipette. Samples were evaporated under nitrogen gas, with the heating block set at 25°C. Once the samples had been evaporated, these were reconstituted with 200 µL mobile phase (90/10: A/B). This was then transferred into correctly labelled LC autosampler vials and then a 10 µL volume was injected into LC/MS/MS.

Table 4-2 Preparation of GBP Calibration curve

	Amount Added (µL) of Gabapentin Working Solution	Final Concentration of GBP (mg/L)
Cal 1	5	2.5
Cal 2	10	5
Cal 3	15	7.5
Cal 4	20	10
Cal 5	25	12.5
Cal 6	50	25
Cal 7	100	50

Table 4-3 Preparation of MOR, M3G and M6G Calibration curve

	Amount Added (µL) of Opioids Working Solution	Final Concentration of MOR & M3G (mg/L)	Final Concentration of M6G (mg/L)
Cal 1	10	0.050	0.020
Cal 2	15	0.075	0.030
Cal 3	20	0.100	0.040
Cal 4	25	0.125	0.050
Cal 5	50	0.250	0.100
Cal 6	100	0.500	0.200
Cal 7	150	0.750	0.300

4.3.2.7 Preparation of Quality Controls

Quality control standards (QCs) have been prepared and analysed in addition to the calibration curves and samples to ensure accurate sample results. Four quality control samples (QC1, QC2, QC3 and QC4) were prepared in whole blank blood to examine the analytes of interest. Two solutions were prepared using the same method as previously described in section 4.3.2.2.

Four QCs were prepared in whole blood by adding the appropriate volume of the stock solutions into 10 mL volumetric flasks. Then, to avoid precipitation in the blood, the methanol solvent was evaporated under nitrogen at room temperature. The dry volumetric flasks were then made up to volume with blank blood as mentioned in section 3.4.3 to achieve target concentrations as detailed in Table 4-4. Aliquots of 0.2mL were stored in the freezer for use individually.

Table 4-4 Preparation of QCs in Whole Blood

	Amount Added (µL)			Final Concentration (mg/L)		
QC No.	GBP	MOR & M3G	M6G	GBP	MOR & M3G	M6G
QC 1	40	6	2.5	4	0.06	0.025
QC 2	200	30	12.5	20	0.30	0.125
QC 3	400	60	25.0	40	0.60	0.250
QC 4	1000	150	60.0	100	1.50	0.600

4.3.2.8 Preparation of Blank Blood

The preparation of blank blood was described in section 3.4.3

4.3.2.9 Preparation of Formic Acid 0.1%

900 mL of deionised water was transferred to a 1 L volumetric flask, then 1 mL of concentrated formic acid was added, made up to the mark with deionised water and stored at 4°C for up to 1 month.

4.3.2.10 Preparation of Formic Acid 0.01%

900 mL of deionised water was transferred to a 1 L volumetric flask, then 100 µL of concentrated formic acid was added, made up to the mark with deionised water and stored at 4°C for up to 1 month.

4.3.2.11 Preparation of 10 mM Ammonium Formate (pH 3)

Ammonium formate (0.631g) was transferred to a 1 L volumetric flask and made up to the mark with deionised water, then mixed and stored at 4°C for up to 1 month.

4.3.2.12 Preparation of 10 mM Ammonium Acetate (pH 5)

900 mL deionised water was transferred to a 1 L volumetric flask, then ammonium acetate (0.77g) and 200 µL of concentrated acetic acid were added, made up to the mark with deionised water and stored at 4°C for up to 1 month.

4.3.2.13 Preparation of 10 mM Ammonium Carbonate (pH 9.3)

Ammonium carbonate (0.48 g) was added to a 500 mL volumetric flask and made up to the mark with deionised water, mixed well and stored at 4°C for up to 1 month.

4.3.2.14 Preparation of 2 M Ammonium Acetate

15.42 g of ammonium acetate was weighed out into a 100 mL volumetric flask and made up to the mark with deionised water and stored at 4°C for up to 1 month.

4.3.2.15 Preparation of 2 mM Ammonium Acetate (pH = 6.8)

1 mL of 2 M ammonium acetate was added to a 1 L volumetric flask and made up to the mark with deionised water and stored at 4°C for up to 1 month.

4.3.2.16 Preparation of 0.5 mM Ammonium Acetate ~~(pH = 6.8)~~

250 µL of 2 M ammonium acetate was added to a 1 L volumetric flask and made up to the mark with deionised water and stored at 4°C for up to 1 month.

4.3.2.17 Preparation of Infusion Solution

100 mL of methanol, 0.25 mL of 2 mM ammonium acetate and 0.1 mL of formic acid were added to a 1 L volumetric flask and made up to the mark with deionised water.

4.3.2.18 Preparation of 0.1 M monobasic sodium phosphate

Weigh 2.76 g of sodium dihydrogen orthophosphate in a beaker and dissolved in 100 mL of dH_2O. Transfer to a 200 mL volumetric flask and make up to the mark with dH_2O. Mix well. Store in the fridge and discard after 6 months.

4.3.2.19 Preparation of 0.1 M dibasic sodium phosphate

Weigh 2.76 g of disodium hydrogen orthophosphate in a beaker and dissolved in 100 mL of dH_2O. Transfer to a 200 mL volumetric flask and make up to the mark with dH_2O. Mix well. Store in the fridge and discard after 6 months.

4.3.2.20 Preparation of 0.1 M, pH 6.0 Phosphate Buffer

0.1 M phosphate buffer was prepared by weighing 1.7 g of Na_2HPO_4 into a 1 L beaker. To that, 12.14 g of $NaH_2PO_4 \bullet H_2O$ was added. The mixture was dissolved in 800 mL of dH_2O. The pH was adjusted to 6 (± 0.1) with 0.1 M monobasic sodium or 0.1 M dibasic sodium to lower or raise the pH, respectively. This was then transferred to a 1 L volumetric flask, then made up to the mark with deionised water and mixed thoroughly. The buffer was then stored at approx. 4°C for a maximum of 6 months.

4.3.2.21 0.1 M Acetate buffer pH 4.5

2.93 mg of sodium acetate trihydrate was dissolved in 400 mL of deionised water in a 500 mL volumetric flask. In addition, 1.62 mL of glacial acetic acid was added and diluted to 500 mL with deionised water. The buffer was adjusted to pH 4.5 with acetic acid (to lower pH) or sodium acetate (to lower pH). This was stored at approx. 4°C and discarded after 30 days.

4.3.3 Instrumentation

The analysis was carried out using an Agilent LC-MS/MS triple quadruple G6420A mass spectrometer equipped with an Agilent 1200 Series Auto sampler SL, Agilent 1200 Series Binary Pump SL with degasser and Agilent 1200 Series Thermo-Statted Column Compartment SL. Positive electrospray ionisation (+ESI) was used and the MS operated in multiple reaction monitoring mode (MRM). The turbo ion-spray interface was operated in positive-ion mode with nitrogen as the collision gas. The Agilent Mass-Hunter Workstation software (version: B.01.05)

was used for system control and data acquisition. Optimiser software was used to optimise the product ions and their fragmentor voltages and collision energies.

4.3.4 Optimisation of the Fragmentor Voltage and Collision Energy

All compounds of interest and internal standards were individually tuned using an optimiser software for detecting the precursor ions and optimising their product ions, fragmentor voltage and collision energy. The solutions were prepared for each drug at concentration 10 mg/L as detailed in section 4.3.2.2, and directly infused in the mass spectrometer at a flow rate of 1800-3000 µL/hour (30-50 µL/min). The ions were continuously monitored in the scanning, SIM and MRM modes using Agilent Mass-Hunter Acquisition software.

The tunings were performed using the MS1 scan method to determine the precursor ions for each analyte. The second step, after identifying the precursor ions, the fragmentor voltages (V) were optimised by altering the voltage over 40-400V using step sizes of 5V. This was achieved by building 7 individual methods with different values of fragmentor. After that, the fragmentation of the precursor ions was investigated by altering the collision energies (eV) from 0-240eV using a step size of 5eV.

Finally, for each analyte, the MS2 scan method was used to determine the product ion profiles using the results from steps 1 and 2; two MRM transitions were identified and optimised for each drug and one for the IS. One of the ion transitions was selected as the quantifier and the other as the qualifier.

4.3.5 Optimisation of Nebuliser Gas Pressure, Temperature and flow

Nebuliser pressure (15, 20, 25, and 30psi), drying gas temperature (300, 310, 320, 330, 340 and 350°C) and flow (8, 9, 10 and 11L/min) were optimised by measuring drug and IS peak areas produced for each MRM transition after the injection of a standard solution with all the analytes and IS at 1mg/L in mobile phase (MeOH:dH_2O 1:1 (v/v)) and transferred to an LC vial. Preliminary LC conditions were used to perform these tests: mobile phases A and B 50:50 (v/v) at a flow rate of 0.3mL/min in isocratic elution mode. The column initially used

to perform these tests was a Gemini C18 column (150 x 2.1 mm, 5 µm) with guard column of the same packing material.

The results of this method were saved as data files as shown in Table 4-5 and Table 4-6 and were processed using the Mass-Hunter Qualitative program by overlapping analyte chromatograms to compare peak height and area.

Table 4-5 Summary of LC and Ion Source Parameters Used During Method Development

LC Parameters	
Column	Gemini C18 column (150 x 2.1 mm, 5 µm) with guard column of the same packing material
Mobile phase	50:50 A/B → A: dH_2O with 0.1% FA and B: methanol with 0.1% FA.
Column temperature	25 ºC
Flow rate	0.3 mL/min
Run Time	30 minutes
Mass Spectrometry Parameters	
Operating mode	ESI-in positive and negative mode
Gas temperature	300 ºC
Gas flow	11 L/min
Nebuliser pressure	15 PSI
Capillary voltage	4000 V
Scan mode	MRM

Table 4-6 MRM transitions of MOR, M3G, M6G and GBP

Drugs	Precursor (m/z)	Optimiser Software			
		Quantifier (m/z)	Qualifier (m/z)	Fragmentor voltage	Collision energy
MOR	286	201.1	229	200	25
M3G	462.4	286	268	160	30
M6G	462.4	286	268	160	30
GBP	172.1	154.1	137	160	30
Internal Standards					
MOR-d3	289.3	201	n/a	140	25
M3G-d3	465.4	289.1	n/a	200	30
M6G-d3	465.4	289.1	n/a	200	30
GBP-d10	164.3	147.2	n/a	175	10

4.3.6 Optimisation of Mobile Phase

The mobile phase is an important component for both the separation and ionisation of compounds. It often consists of two parts, the aqueous phase and the organic phase. The optimal conditions were achieved using an organic modifier (acetonitrile or methanol) and a volatile buffer [71, 72, 354]. To determine the most suitable mobile phase composition for drugs of interest, an investigation into its aqueous and organic components was carried out. The concentration of which can be critical; concentrations that are too high may result in the suppression of the analyte signal, while concentrations that are too low may lead to poor peak shape and efficiency.

4.3.6.1 Organic Phase

In morphine, M3G, M6G and gabapentin analysis, as with most LC-MS methods, methanol or acetonitrile are commonly used for the organic solvent of the mobile phase [71, 72, 354]. A comparison was carried out between methanol and acetonitrile to investigate their effects on the chromatograms of the analytes, their ion abundances and peak shape.

4.3.6.2 Aqueous Phase Additives

Chromatography analysis has been reported with the addition of volatile buffers, which are commonly used in the mobile phase to improve the ionisation of compounds as well as the separation of molecules and the peak shape of the chromatograms. It can also stabilise pH in the mobile phase, which in turn helps to develop reproducible chromatography. However, the pH of the mobile phase should be at least two units below the pKa. This was a bit challenging to apply because of a wide range of drugs of interest. Morphine has a pKa from 9 to 10, and its glucuronides have a pKa ranging from 3 to 4. Large differences in pK values of morphine and its glucuronides often cause difficulties in developing a combined method [369, 370].

For this investigation, four buffers with different additives were tested for each drug separately under the same operating conditions to select the buffer that produced good chromatograms and high abundances for all the analytes. The four buffers were tested each time; 0.1% Formic acid (pH=2.8), 10mM

Ammonium formate (pH=3.0), 10mM Ammonium acetate (pH=5.0) and 10mM Ammonium carbonate (pH=9.3).

Four separate unextracted standards of each analyte were prepared using 200 µL aliquot of the 10 mg/L working solution, dried under nitrogen. Each sample was reconstituted with 100 µL of one selected mobile phase (buffer/methanol, 80:20). A 10 µL volume was injected in triplicate for each mobile phase.

4.3.6.3 Formic Acid addition

Formic acid is volatile organic acid. It is mainly chosen as a buffer additive, which are often added to mobile phases, and will evaporate readily in the LC interphase [312]. Formic acid acts as a stabilising buffer and aids the chromatographic resolution. Due to its nature, it also donates protons during positive ionisation mode, as well as non-suppression of ionisation in the mass spectrometer [371]. In order to determine which concentration of formic acid gave the best results, ten concentrations from 0.0% to 0.2% were investigated by altering formic acid from 0 to 2000 µL in 1 L using a step size of 200 µL in 1 L. Ionisation and resolution were evaluated for each analyte.

4.3.6.4 Molarity of Ammonium Acetate

The reports published in the literature use a range of molarities, and it has been reported that increased concentration of the additive leads to a significant reduction in the sensitivity of the analyte due to its suppressive effect on electrospray ionisation [372]. In order to investigate the various molarity that gives the best overall chromatography molarities investigated were: 0.5, 2, 3, 4, and 5 mM of ammonium acetate.

4.3.7 Optimisation of Stationary Phase

4.3.7.1 Optimisation of LC Column

Due to the wide range of pKa and different polarities of the analytes of interest a simple and general column which can tolerate a wide range of pH in order to elute all the compounds was needed. In addition, small particle size columns are not recommended for postmortem blood analysis because of the complex nature of the sample and clotted sample that can cause a rapid accumulation in the column if the extraction procedure is not sufficiently clean. A typical C18

column was used as a starting point [373]. In order to determine the best chromatographic performance, two different columns were compared with the previous Gemini C18 column, Synergi Fusion-RP column and Synergi Polar-RP Phenomenex [354] were used, details of which are below in Table 4-7.

Table 4-7 Chromatographic Column Properties

Column	Length (mm)	Internal Diameter (mm)	Particle Size (µm)	Recommended Use
Gemini C18 Pheomenex	150	2.0	5	multiple separation modes (ion exchange, reverse and normal phase)
Synergi Fusion-RP Pheomenex	150	2.0	4	Separation of mixtures with both polar and non-polar compounds
Synergi Polar-RP Pheomenex	150	2.0	4	Separation of polar and aromatic compounds

4.3.7.2 Optimisation of LC Column Temperature

After choosing the best mobile phase and column system to analyse all the analytes simultaneously, the column temperature was tested at 25, 30, 35, 40, 45 and 50˚C.

4.3.8 Optimisation of Chromatographic Separation

The separation of the sample components is greatly enhanced by the use of a gradient system, which changes the mobile phase composition during the chromatographic run [374, 375]. Reflecting the large polarity range of the major opium alkaloids, most applications apply reversed-phase liquid chromatography with gradient elution and ion-pairing agents. Such methods can be very sensitive to minor changes in chromatographic conditions, contribute to prolonged duration of analysis, and narrow the columns lifetime [376]. As a result, the gradient was employed using a mobile phase consisting of 0.01% formic acid (FA) and 0.5mM of ammonium acetate in both (A: deionised water and B: Methanol). In order to achieve optimal separation of all analytes, ten experiments were performed by changing the mobile phase gradient system.

In system (9) for instance, the gradient mobile phase system started at 90:10 A/B and maintained for 5 minutes before the organic content was increased to 10:90 A/B for 2 minutes. The organic percentage was decreased finally to 90:10 A/B for 5 minutes in order to condition the column before the next injection as illustrated in Table 4-8.

Table 4-8: Gradient System Used to Improve the Separation of 4 Drugs of interest

Tim (min)	System									
	1	2	3	4	5	6	7	8	9	10
	*B%	*B%	*B%	*B%	*B%	*B%	*B%	*B%	*B%	*B%
0.00	90	80	70	60	50	40	30	20	10	5
5.00	90	80	70	60	50	40	30	20	10	5
6.00	90	90	90	90	90	90	90	90	90	90
8.00	90	90	90	90	90	90	90	90	90	90
8.10	90	80	70	60	50	40	30	20	10	5
13.00	90	80	70	60	50	40	30	20	10	5

*B: Methanol.

After choosing the best gradient system to analyse all drugs of interest simultaneously, the flow rate was optimised at 0.1, 0.2 and 0.3 mL/min.

4.3.9 Extraction Optimisation - Solid Phase Extraction vs Protein Precipitation:

Following the development of the LC-MS/MS method, extraction experiments were performed to optimise the best conditions and best possible drug recovery. At the same time as investigating the extraction efficiencies as different extraction methods, the Effect of the Matrix (ME) was also assessed. Additionally, the linearity range, the cleanliness of the extracts and extraction time were evaluated when the extraction methods were optimised. It has been decided to evaluate two extraction methods; solid phase extraction (SPE) and protein precipitation (PP).

4.3.9.1 Solid Phase Extraction

Two types of SPE cartridge were evaluated to determine which cartridges would have an advantageous effect on the recovery; type I (Bond Elut C18 cartridge) and type II (DAU® CleanScreen cartridge). Type I, Bond Elut C18 cartridge has been used for opioids in previous studies [71, 359, 377-382]. Type II, the DAU® CleanScreen cartridge used in this study is currently used for routine analysis of postmortem blood samples in-house. This extraction is validated for the analysis of morphine and other opioids but does not currently include M3G, M6G and gabapentin. Drug recovery was assessed for each cartridge to determine which was most effective for extracting drugs from whole blood.

To achieve this, spiked whole blood samples were extracted using the two sets of cartridges, 3 replicates each. The samples were prepared as follow; 1 mL of blank whole blood was diluted in 5 mL of pH 6 Buffer/deionised water (1:2) mixed solution and spiked with all drugs in different concentration ranges. Deuterated internal standards were used by adding 100 µL of 1 µg/mL solution to all the samples examined. The tubes were vortexed for 5 s and centrifuged for 10 min at 2500 to 3000 rpm.

After SPE columns were conditioned by sequentially adding 3 mL methanol, 3 mL water and 1 mL pH6 phosphate buffer, the prepared samples were poured onto the conditioned column and allowed to drain. Each column was then washed by the sequential addition and elution of 3 mL deionised water, 2 mL acetate buffer 0.1M pH 4.5 and 3 mL methanol and dried under full vacuum for 10 minutes. Elution was performed by adding 3 mL dichloromethane: isopropanol: ammonia (78:20:2) to collect the compounds. After solvent evaporation by using nitrogen evaporator at ≤ 37 ^{o}C until dry, the residue was reconstituted with 200 µL mobile phase (90/10: A/B) and transferred to a LC vial. A 10 µL volume was injected into the LC/MS/MS.

In order to calculate the recovery, another set of unextracted samples with the same concentration were prepared at the same time in triplicate as follows; 100 µL of the standard solution and 100 µL of internal standard evaporated under nitrogen gas at 37 ^{o}C, then reconstituted with 200 µL mobile phase (90/10: A/B) and transferred to LC autosampler vials.

The internal standard was added to the extracted and unextracted samples at the same time after extraction. Drug recoveries were calculated as described in Equation 4-1.

4.3.9.2 Protein Precipitation Extraction

Protein precipitation was used in literature [72, 381, 383-385]; therefore, it was chosen as a sample preparation method for comparison with SPE. The length of time spent on SPE development was kept to a minimum and only more promising results pursued. Although protein precipitation would have led to the simultaneous detection of all the analytes due to its nonselective nature.

Protein precipitation extraction was evaluated to optimise the effect of sample preparation steps on the extraction and to create an efficient method. Steps evaluated in this study are the solvents used for extraction. Methanol and acetonitrile were evaluated for the reference method, as both of these solvents were used in the literature.[72, 381, 383-386] Drug recoveries were assessed for both sets of solvents to determine which was the most effective at extracting drug of interesting from the blood.

Two sets; 3 samples each, of spiked whole blood samples were prepared. Each set was extracted with one of the two solvents as follows; 800 µL of solvent was added to 200 µL of blood spiked with 100 µL of 10 mg/L standard working solution. Samples were vortex mixed before being centrifuged at 2000-3000 rpm for 10 minutes. Following centrifugation, the supernatant was transferred to a 3.5 mL vial using a glass pasteur pipette, spiked with 100 µL of the internal standard. Samples were evaporated under nitrogen gas, with the heating block set at 25 °C. Once the samples had been evaporated they were reconstituted with 200 µL mobile phase (90/10: A/B). This was then transferred into correctly labelled LC autosampler vials and 10 µL injected into LC/MS/MS.

In order to calculate the recovery, two sets of unextracted samples at the same concentration were also prepared at the same time in triplicate as follows; 100µL of 10 mg/L standard solution and 100 µL of 10 mg/L internal standard were evaporated under nitrogen gas at 25°C. Then reconstituted with 200 µL mobile phase (90/10: A/B) and transferred to LC autosampler vials.

Finally, after analysing the samples, the peak area was used to calculate the recovery using the Equation 4-1 to determine whether methanol or acetonitrile would have an advantageous effect.

4.3.9.3 Matrix Effect Evaluation

The change in response observed for a given concentration of a target analyte in the presence of other sample components can be defined as matrix effect. These sample components can cause suppression or enhancement of the target analyte response [386-388].

The matrix effects were evaluated for all the drugs and the internal standards using the post-extraction addition approach, which was achieved as follows;

three sets of QCs at low and high concentrations were used. The concentrations are detailed in section 4.3.2.7 and the 3 sets will now be referred to as “neat”, “post” and “pre”. Six sources of blank blood were used.

Set one “neat”: Unextracted standards (QCs) and internal standards injected six times to establish a mean peak area for each concentration.

Set two “post”: Blank blood was extracted and spiked with QC solutions and internal standards after extraction.

Set three “pre”: Blank blood was spiked with QC solutions and internal standards before extraction and extracted.

Finally, different extraction approaches were compared regarding matrix effect, which was achieved as follows; each matrix source was extracted in triplicate and the extract spiked with either methanol or acetonitrile. After analysing the samples, the peak area was used to calculate the recovery, process efficiency and matrix effect using the following Equation 4-1, Equation 4-2 and Equation 4-3.

Equation 4-1 Equation of Recovery

$$\boldsymbol{Recovery\ RE}\ (\%)\ =\ \boldsymbol{Pre}\ (\mathbf{3})\ /\ \boldsymbol{Post}\ (\mathbf{2})\ \boldsymbol{x}\ \mathbf{100}$$

Equation 4-2 Equation of Process Efficiency

$$\boldsymbol{Process\ Efficiency\ PE}\ (\%)\ =\ \boldsymbol{Pre}\ (\mathbf{3})\ /\ \boldsymbol{Neat}\ (\mathbf{1})\ \boldsymbol{x}\ \mathbf{100}$$

Equation 4-3 Equation of Matrix Factor

$$\boldsymbol{Matrix\ Factor}\ (\boldsymbol{MF})\ =\ \boldsymbol{Post}\ (\mathbf{2})\ /\ \boldsymbol{Neat}\ (\mathbf{1})$$

MF is acceptable if the value is within 1±0.25.

If MF = 1, there is no matrix effects.

If MF <1, there is an ionisation suppression effect.

If MF >1, there is ionisation enhancement and/or analyte loss in the absence of matrix.

4.4 Results and Discussion

4.4.1 Optimisation of the Fragmentor Voltage and Collision Energy

The fragmentor voltage, parent and product ions, and subsequently the collision energies for each individual analyte were determined using the sample injection program. All the analytes investigated in this study generated the prominent protonated molecular ion in positive-ion mode. All drugs had an optimum fragmentor voltage in the range of 160–200 V, except M3G-d_3 and M6G-d_3 as illustrated in Figure 4-1 and Figure 4-2. The optimum collision energy for most of the product ions is 20 eV as illustrated in Figure 4-3 and Figure 4-4. The optimum fragmentor voltages, collision energies as well as the ions monitored for each of the analytes are summarised in Table 4-9.

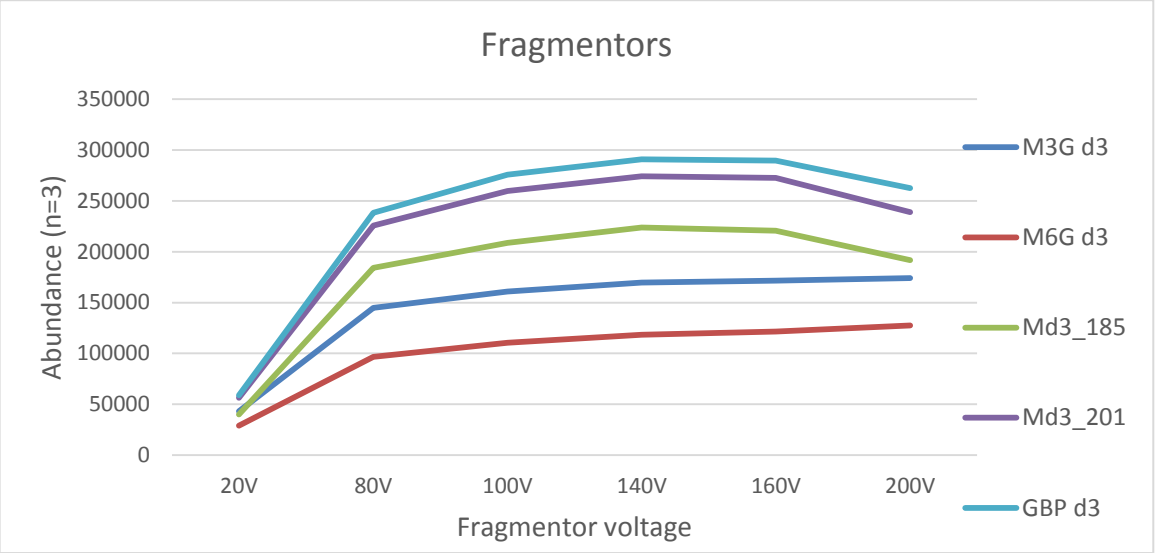


Figure 4-1 Fragmentor Voltage Optimisation for all internal standards

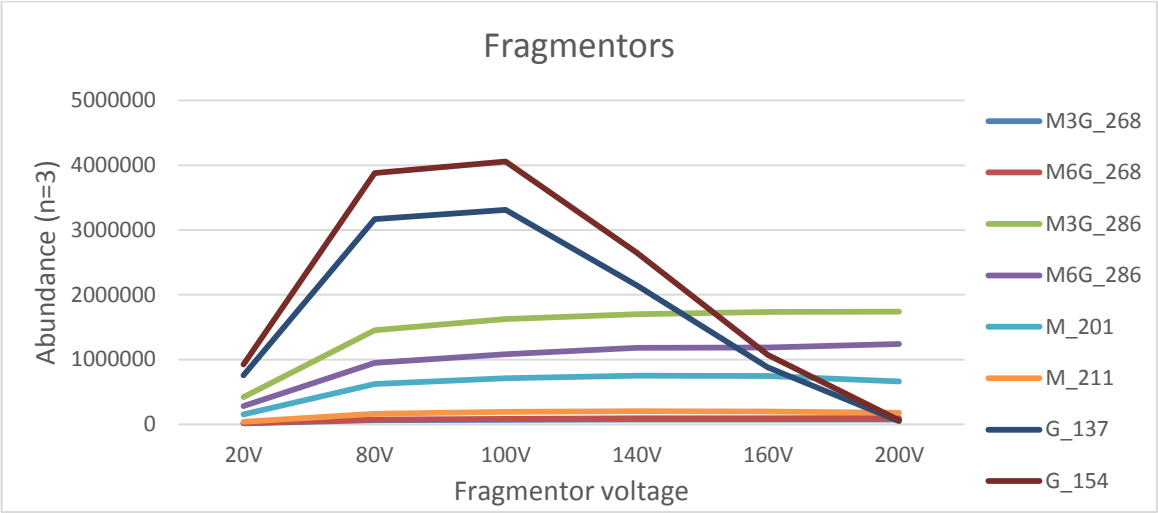


Figure 4-2 Fragmentor Voltage Optimisation for all standards and their transitions

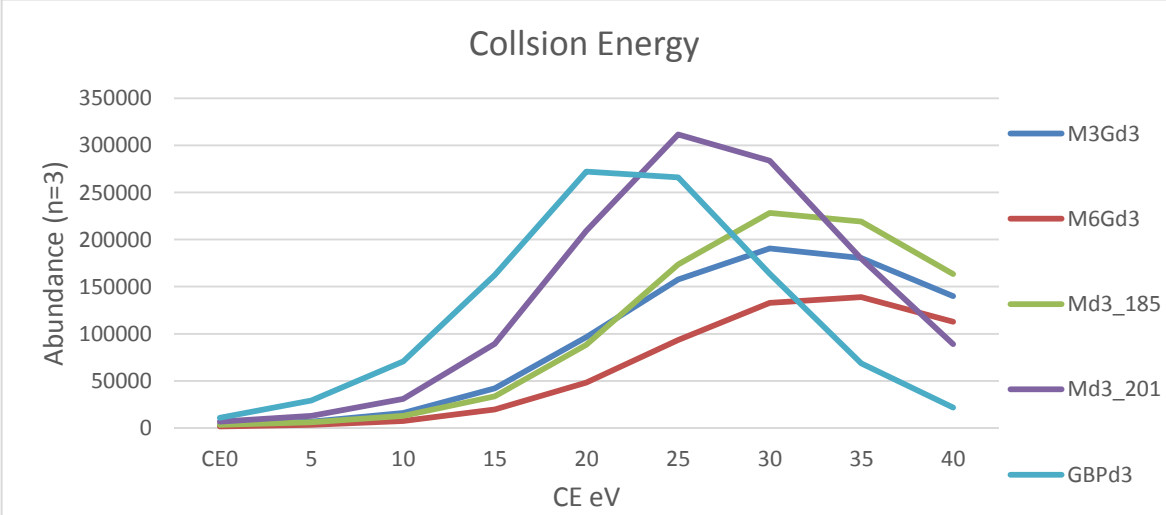


Figure 4-3 Collision Energy Optimisation for all internal standards

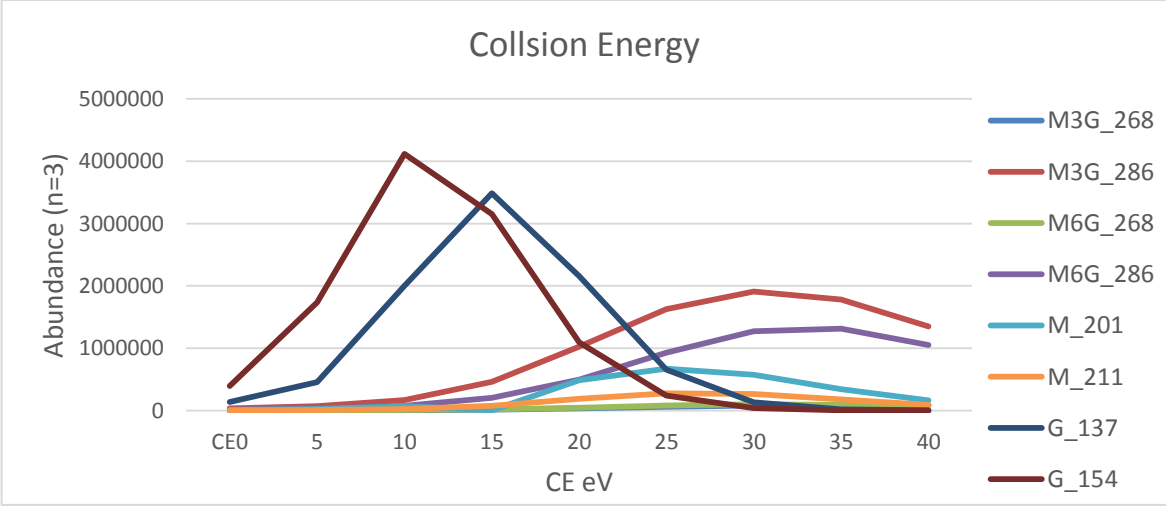


Figure 4-4 Collision Energy Optimisation for all standards and their transitions

Table 4-9 Summarises the Optimisation Parameters of Tune Method

Drugs	Precursor (m/z)	Optimizer Software			
		Quantifier (m/z)	Qualifier (m/z)	Fragmentor voltage	Collision energy
MOR	286	201.1	229	200	25
M3G	462.4	286	268	160	30
M6G	462.4	286	268	160	30
GBP	172.1	154.1	137	160	30
Internal Standards					
MOR-d3	289.3	201	N/A	140	25
M3G-d3	465.4	289.1	N/A	200	30
M6G-d3	465.4	289.1	N/A	200	30
GBP-d3	164.3	147.2	N/A	175	10

N/A: Not Available

4.4.2 Optimisation of Nebuliser Gas Pressure, Temperature and Flow Rate

4.4.2.1 Optimisation of Nebuliser Gas Pressure

Based on the results obtained from varying the ion source nebuliser gas pressure showed an increase in abundance response when increasing the nebuliser pressure from 15 to 35, the optimum condition adopted for all analytes was 35 psi as illustrated in Figure 4-5 and Figure 4-6.

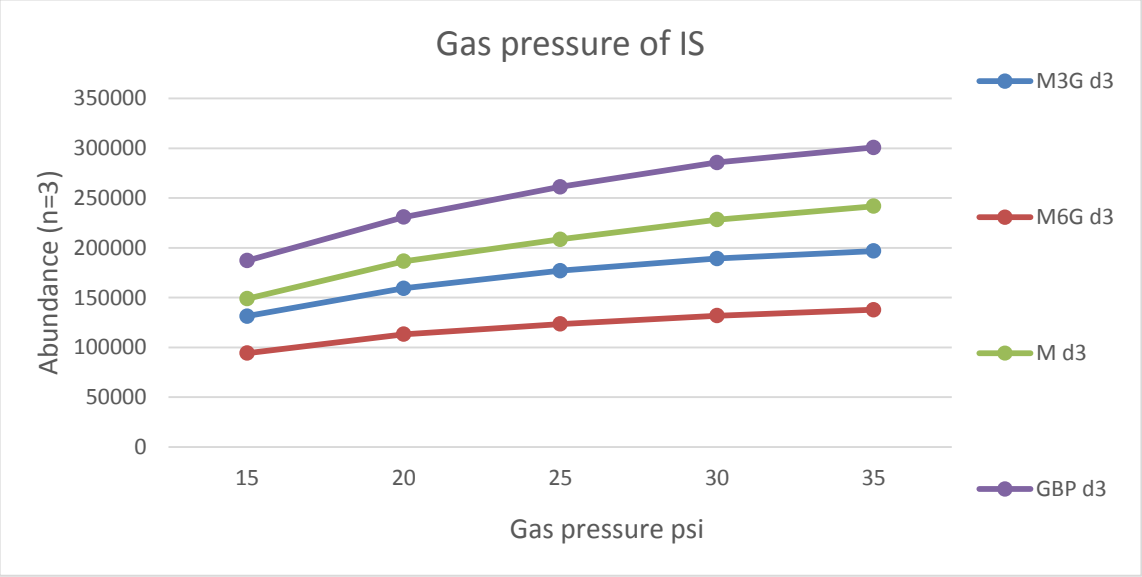


Figure 4-5 Nebuliser Gas Pressure Optimisation of MOR-d3, M3G-d3, M6G-d3 and GBP-d3

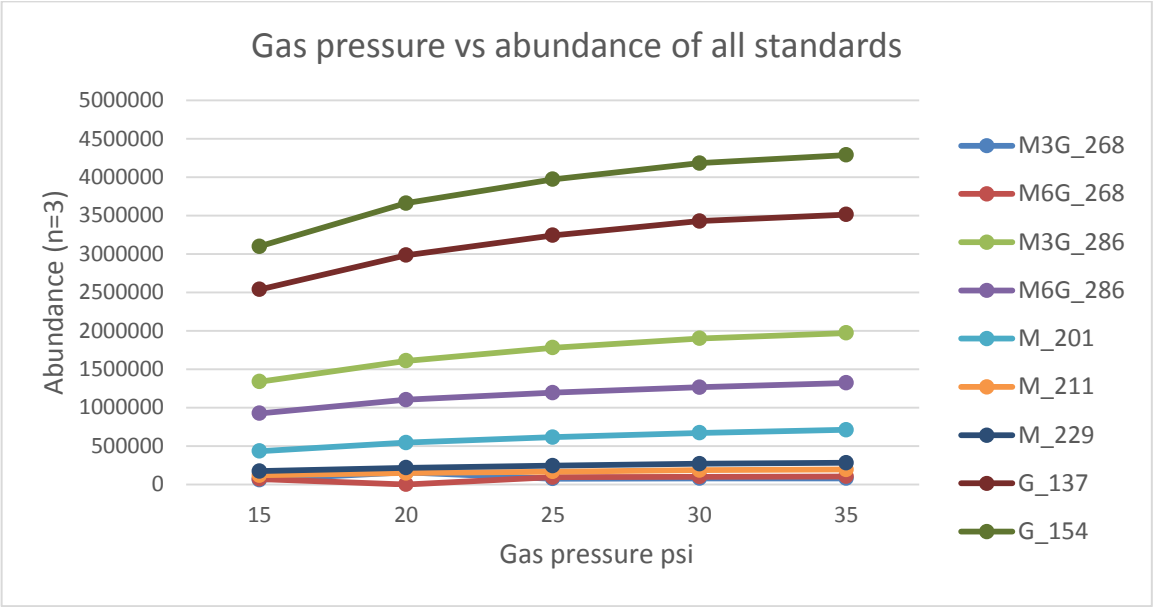


Figure 4-6 Nebuliser Gas Pressure Optimisation of Morphine, M3G M6G and Gabapentin

During the development period of the method, the 30 psi gas pressure was used, in order to obtain a good sensitivity to all drugs. However, at a later stage, nebuliser pressure dropped to 25 psi, which helped to prevent MS source saturation due to the high concentration of gabapentin calibrator.

4.4.2.2 Optimisation of Gas Temperature

Figure 4-7 and Figure 4-8 show a slight increase in analytes abundance when increasing the nebuliser temperature from 300 to 350 °C, the optimum condition adopted for all analytes was 350 °C.

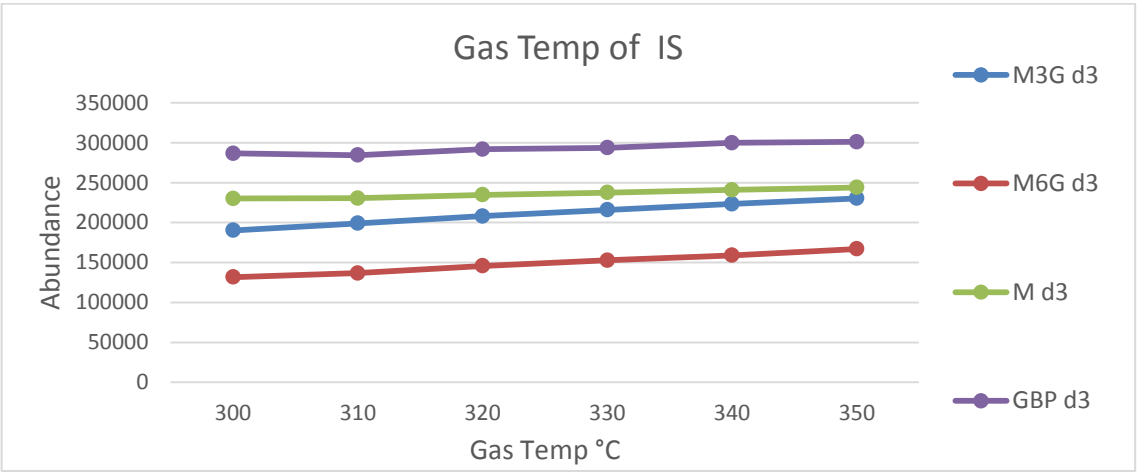


Figure 4-7 Nebuliser Gas Temperature Optimisation for Internal Standards

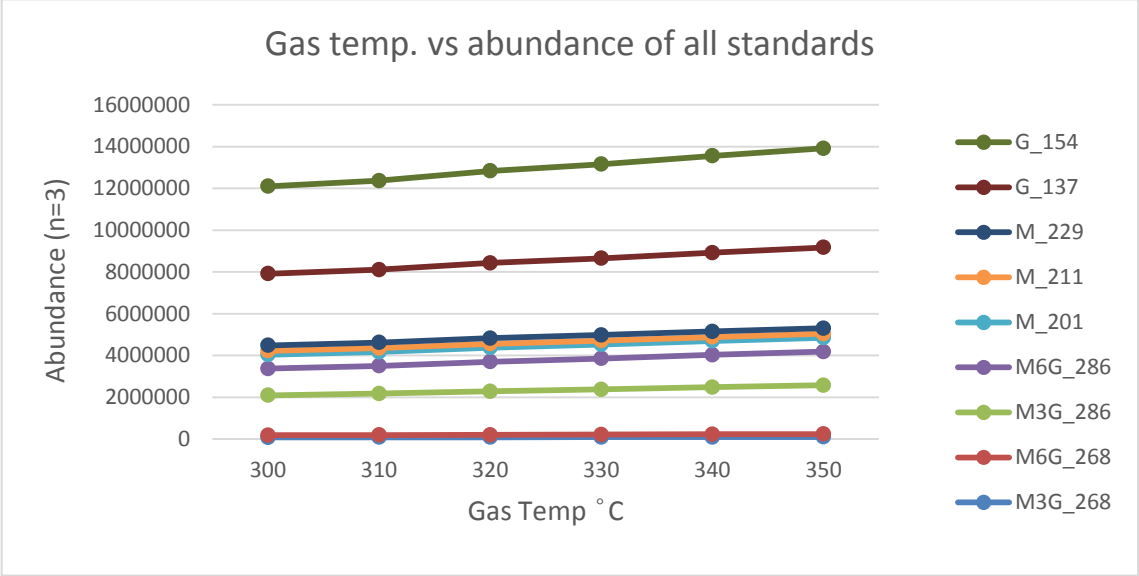


Figure 4-8 Nebuliser Gas Temperature Optimisation for Analytes of Interest

4.4.2.3 Optimisation of Nebuliser Gas Flow

Figure 4-9 and Figure 4-10 show a slight increase in analytes abundance when increasing the nebuliser pressure from 8 to 11 L/min, the optimum condition adopted for all analytes was 11 L/min.

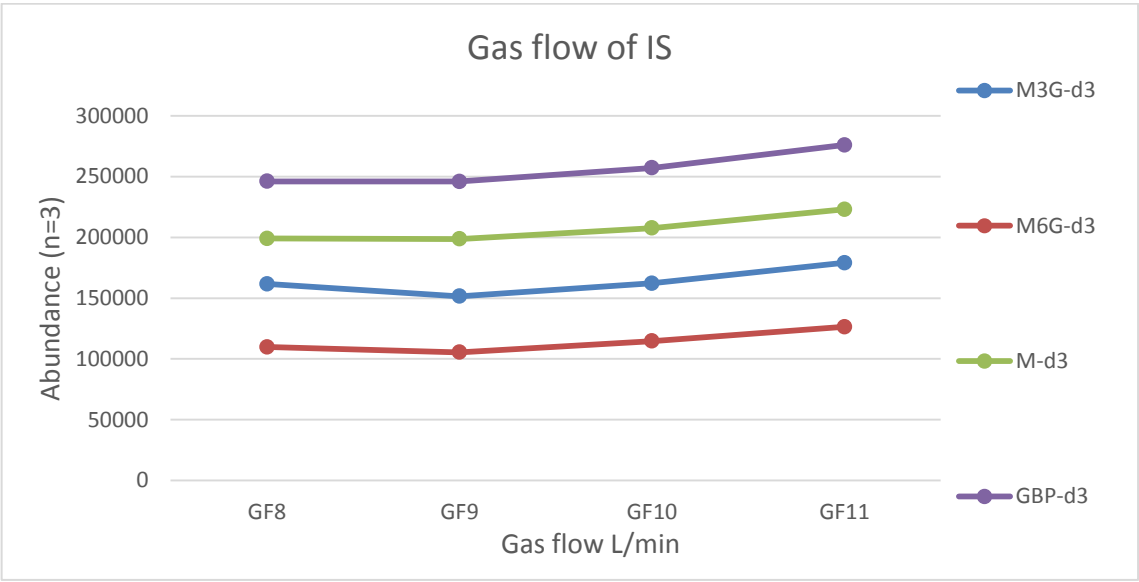


Figure 4-9 Nebuliser Gas Flow Optimisation of Internal Standards

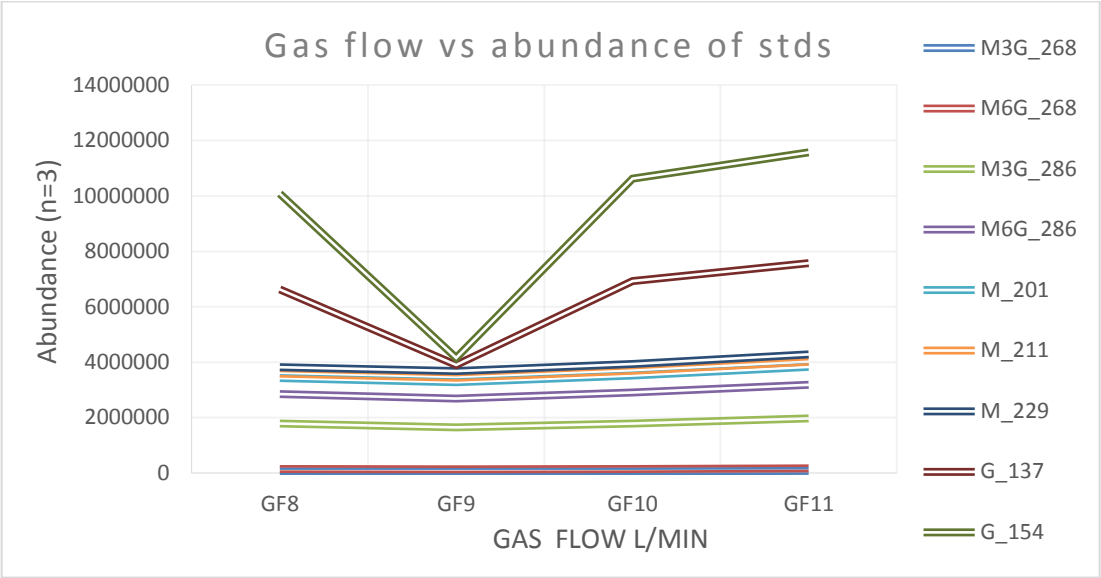


Figure 4-10 Nebuliser Gas Flow Optimisation of Analytes of Interest

4.4.3 Optimisation of Mobile Phase

4.4.3.1 Organic Phase

It has been shown that the substitution of methanol to acetonitrile in the LC mobile phase leads to a significant difference in electrospray ionisation for a variety of compounds. Figure 4-11 and Figure 4-12 show the effect of methanol and acetonitrile on the abundance of all drugs with their internal standards. Methanol gives greater abundance and gives a better peak shape compared to acetonitrile. Therefore, MeOH was used as an organic phase for all the investigations.

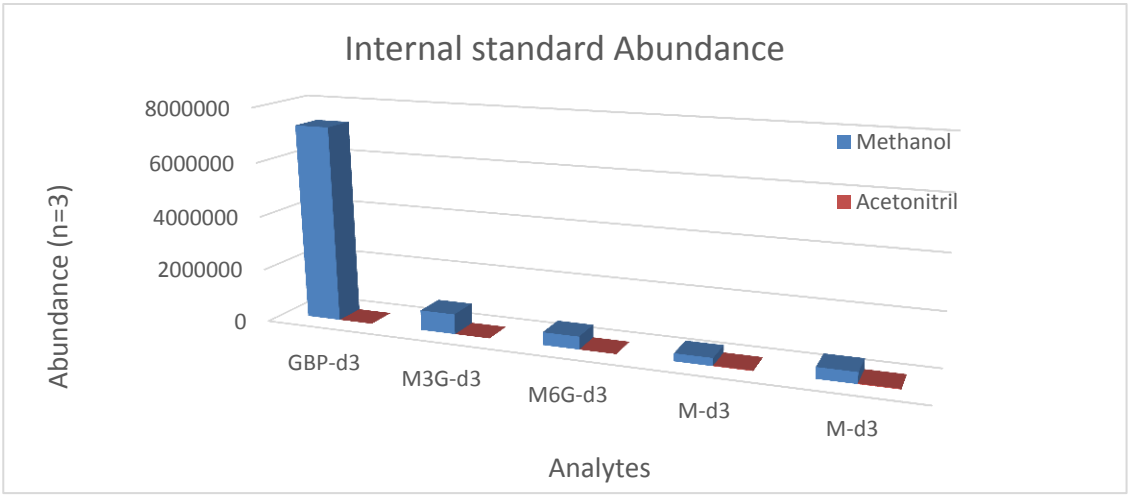


Figure 4-11 Response of Internal Standards with varying organic solvent in Mobile Phase

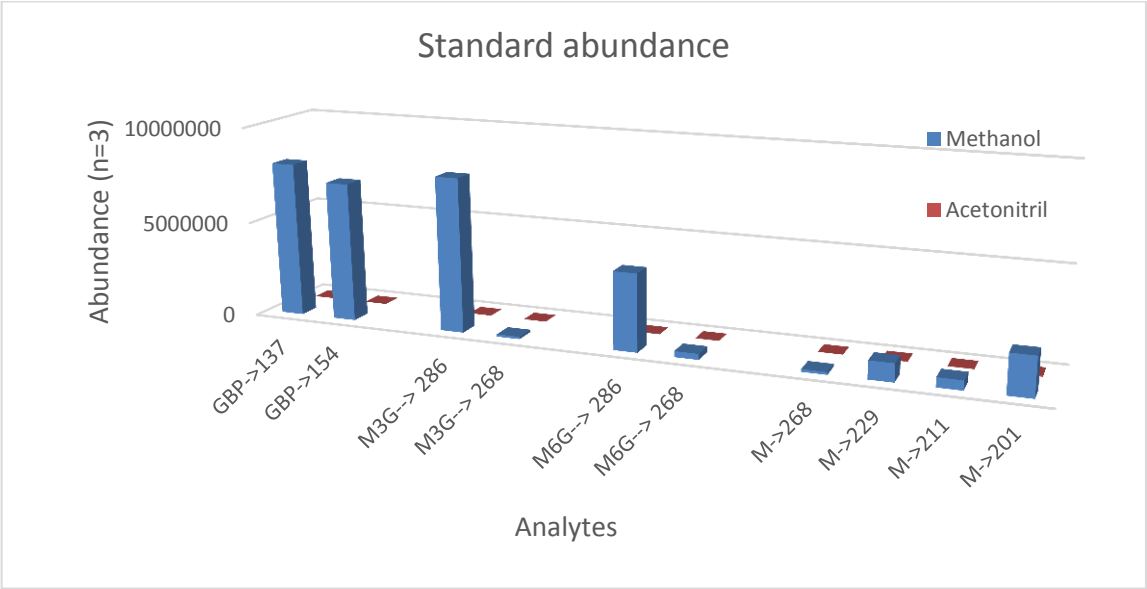


Figure 4-12 Response of analytes with varying organic solvent in Mobile Phase

4.4.3.2 Aqueous Phase Additives

The composition of the mobile phase additives was investigated with four different buffers and pH ranging from 2.8 to 9 as illustrated in Figure 4-13 and Figure 4-14. The resulting chromatographic peak shape and abundance was considered when assessing suitability. It was clear that all additives with mobile phase had a suppression effect on product ion formation of all analytes. However, formic acid (pH= 2.8) and ammonium acetate (pH= 5) gave the optimum resolution for most of the drugs and their internal standards under the same conditions.

The concentrations of the buffer are very important because concentrations that are too low could lead to poor efficiency and peak shape, while concentrations that are too high could cause ion suppression of the compound. The pH of the additives is an important factor that must be considered as well. The pH measured for 0.1 % formic acid was 2.8 and 10 mM ammonium acetate was 5.0, compared to a pH of 3 for 10mM ammonium formate and a pH of 9.3 for 10 mM ammonium carbonate. When considering the pKa values of the analytes, it is understandable that a lower pH would help the ionisation of the molecules and helps to explain why relative peak shapes were better with the addition of formic acid and ammonium acetate. Therefore, formic acid and ammonium acetate were the additives of choice for future work.

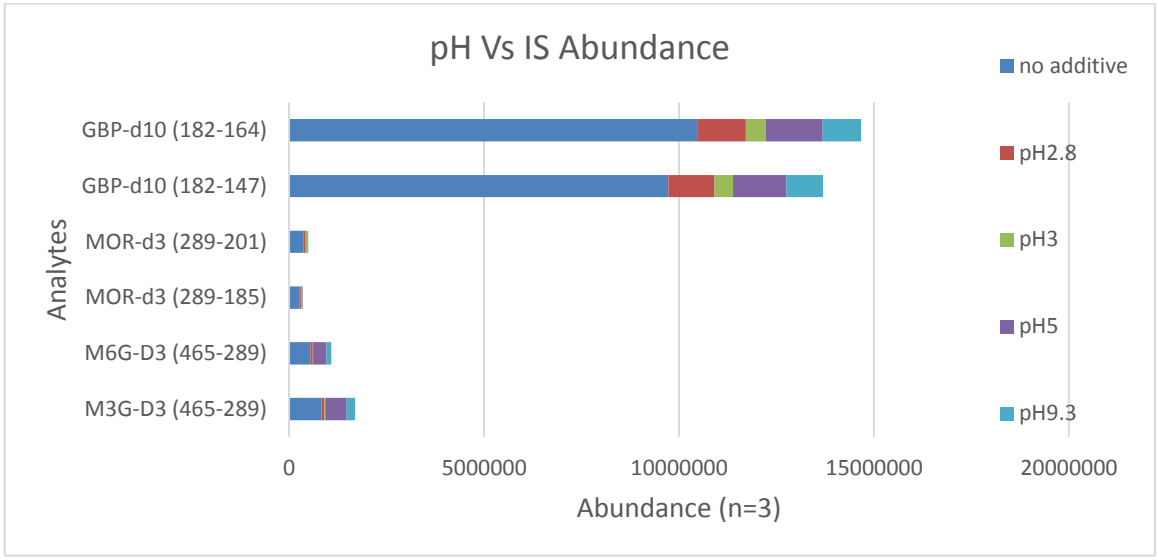


Figure 4-13 Effect of Mobile Phase Additives on internal standard Abundance

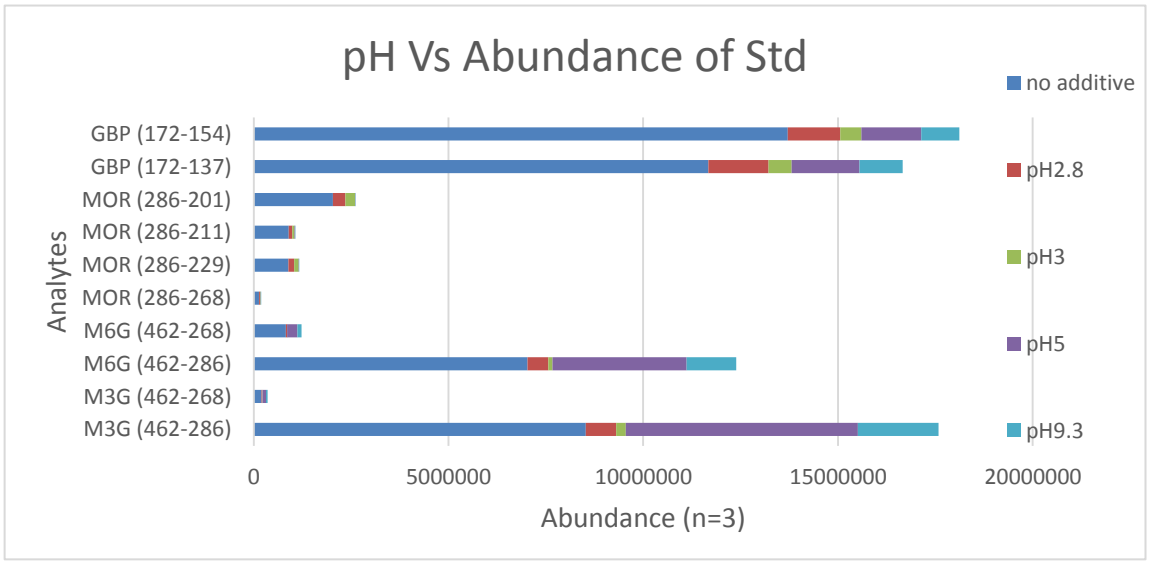


Figure 4-14 Effect of Mobile Phase Additives on Analyte Abundance

With LC-MS applications there are special considerations that must be taken into account when choosing a buffer. For ESI volatile buffers are required to avoid fouling of the API interface. In ESI an increase in buffer concentration can lead to a decrease in the analyte signal, however, this effect is compound dependent, with some analytes showing only a small loss of response.

4.4.3.3 Formic Acid addition

Figure 4-15 shows that, aqueous mobile phase was adjusted with different concentrations of formic acid to give a higher response and improve the sensitivity of all analytes. Formic acid concentrations from 0.0 % to 0.02 % were

evaluated and a concentration of 0.01% formic acid gave a better resolution with the highest responses for all analytes, except M3G. Therefore, this concentration was chosen for future work.

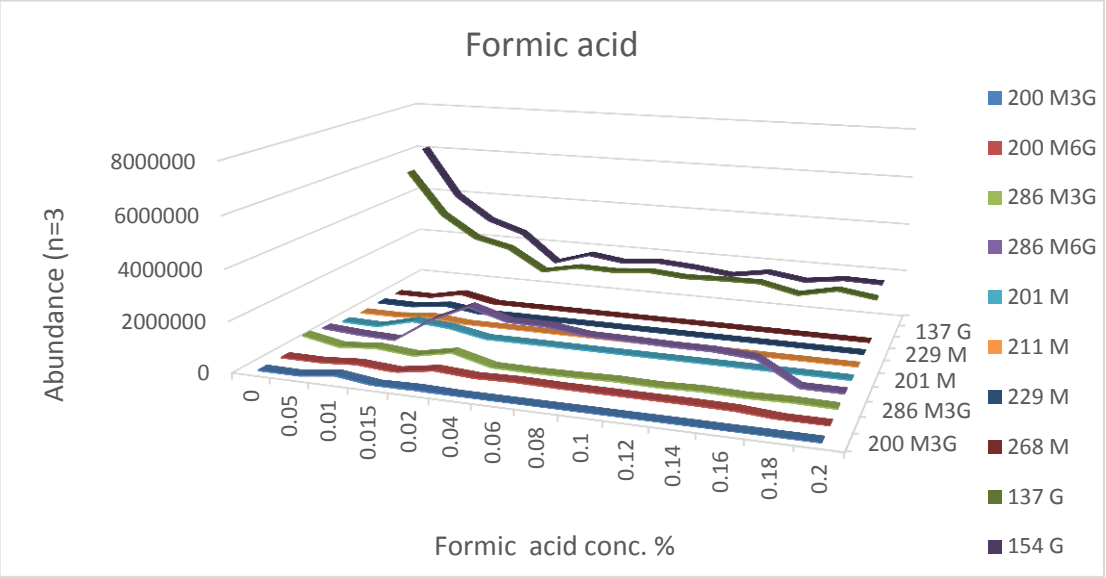


Figure 4-15 Effect of Formic Acid as an Additive in Mobile Phase on Analytes Response

4.4.3.4 Molarity of Ammonium Acetate

It was clear that increasing the concentration of ammonium acetate from 0.5 mM to 5 mM showed no improvement or inhibition in abundance except for gabapentin which decreased in abundance with increasing molarity, as shown in Figure 4-16. However, the concentration of 0 mM ammonium acetate gave the highest responses to M6G and gabapentin, but 0.5 mM gave a better resolution for all analytes, and was therefore chosen for future work.

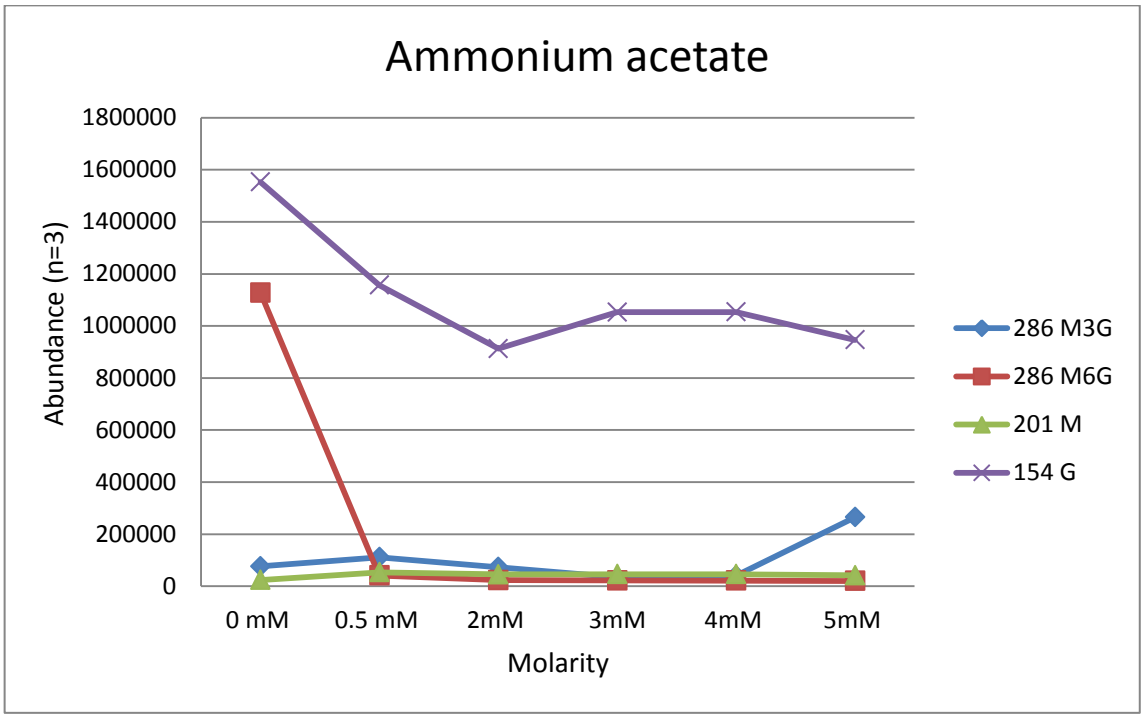


Figure 4-16 Effect of Ammonium Acetate Concentration on Morphine, M3G, M6G and Gabapentin Responses

Finally, combined 0.01% formic acid and 0.5mM ammonium acetate in aqueous mobile phase were evaluated and gave a better response with a good resolution for all analytes. Therefore, they were used as the mobile phase of choice for all drugs of interest and their internal standards.

4.4.4 Optimisation of Stationary Phase

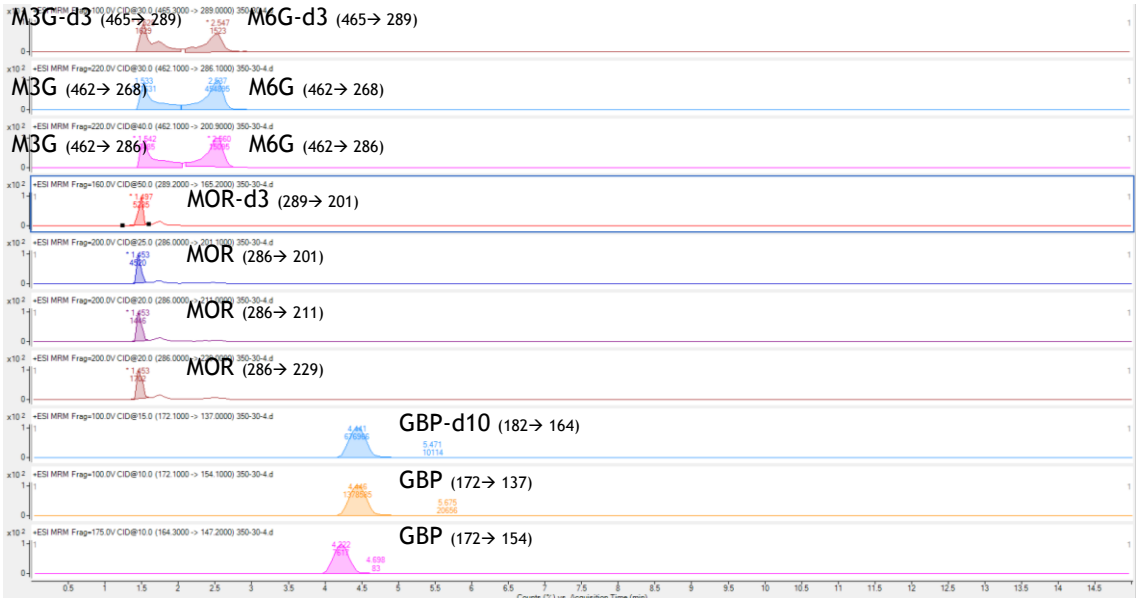
4.4.4.1 Optimisation of LC Column

Method development started by using a reversed-phase (RP) Phenomenex Gemini C18 (150 x 2 mm, 5 µm) column as it is one of the most commonly employed separation columns in forensic toxicology. An isocratic system with a high organic solvent mobile phase content (90%B) was used first. The aim of this was to check how long the analytes would be retained on the column. As the organic solvent-rich mobile phase has high elution strength, all drugs were eluted less than one minute after injection. A better retention time was obtained when a higher aqueous mobile phase content was used (90%A). This is because the reversed-phase column contains a non-polar stationary phase and, when using mobile phase with higher organic content, analytes would elute more quickly. Despite the improved retention of drugs of interest by this column, peak

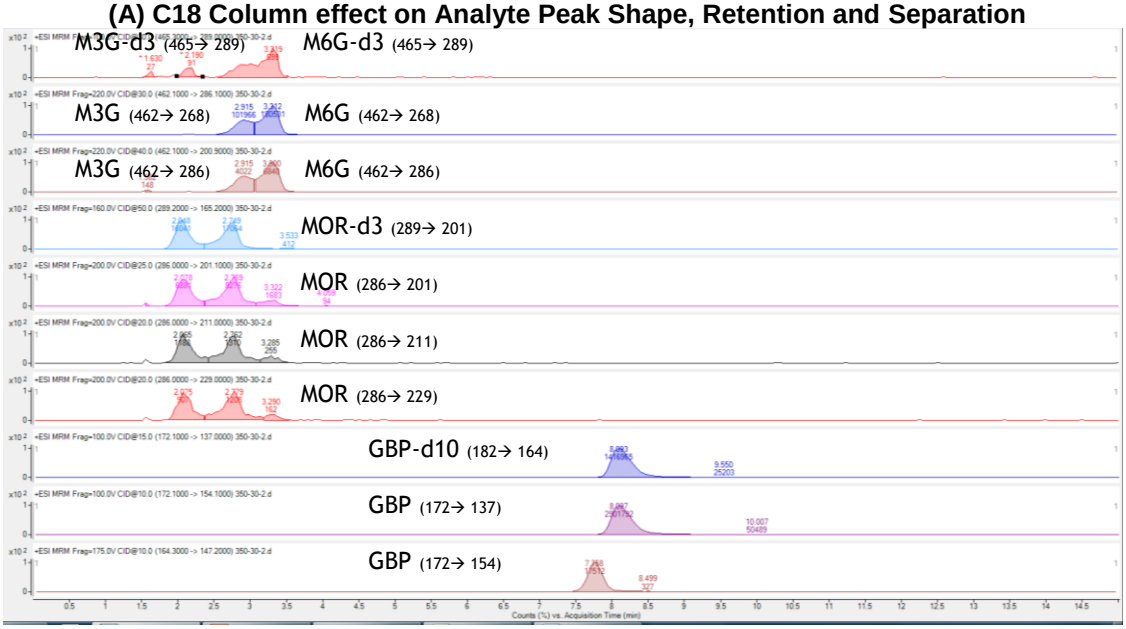
broadening was a problem and the column was unable to achieve acceptable chromatographic separation as illustrated in Figure 4-17 (A).

Synergi Fusion-RP column (150 x 2 mm, 4 µm) also failed to achieve the minimally acceptable criteria for the peak shape chromatography. Furthermore, did not give good separation results with morphine, M3G and M6G. The same issue was seen when using C18 column due to its a non-polar stationary phase as well shown in Figure 4-17 (B).

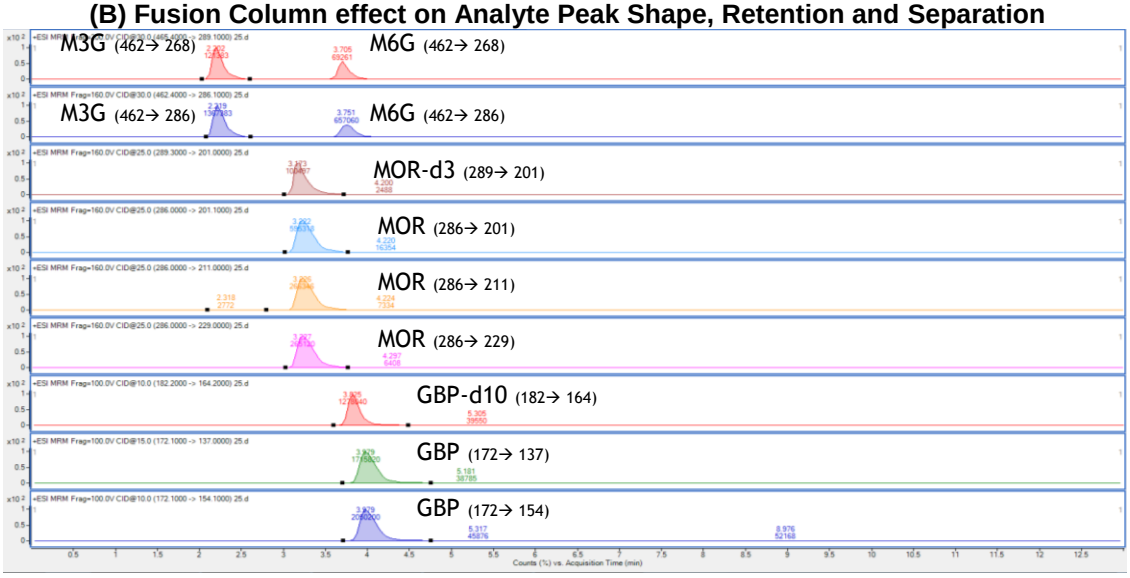
As the drugs of interest are basic polar drugs, the Synergi Polar-RP Phenomenex (150 x 2 mm, 4 µm) was installed and tested. Column testing was started using an isocratic system with a high aqueous content (90% A). The goal of this was to assess the ability of the column to retain the drugs. It was found that all drugs were interacting with the stationary phase and eluted 2 minutes (M3G), 3 minutes (morphine), 3.75 minutes (M6G) and 3.75 min (GBP) after injection. The observed improvement in the retention time when using this column compared to a C18 column is believed to be due to the elution using a low percentage of solvent. Thus, it is compatible with a wide range of buffers and organic solvents for injection. It was clear that using the Synergi Polar-RP column offers additional retention, better peak shape and a greater separation for every analyte with a run time of 13 min as illustrated in Figure 4-17 (C).



(A) C18 Column effect on Analyte Peak Shape, Retention and Separation



(B) Fusion Column effect on Analyte Peak Shape, Retention and Separation



(C) Polar Column effect on Analyte Peak Shape, Retention and Separation

Figure 4-17 Stationary Phase Effect on Analyte Peak Shape, Retention and separation

4.4.4.2 Optimisation of LC Column Temperature

The optimal conditions were achieved using a Synergi Polar-RP column (150 mm x 2.0 mm, 4 µm) and an isocratic system with a high aqueous mobile phase content (90% A) mixed with 0.01% formic acid and 0.5mM ammonium acetate at a flow rate of 0.3 mL/min. It was found that increasing the column temperature from 25 to 50°C showed no significant change in chromatography resolution. While, a slight decrease in the method sensitivity by increasing the temperature at 30 °C (Figure 4-18).

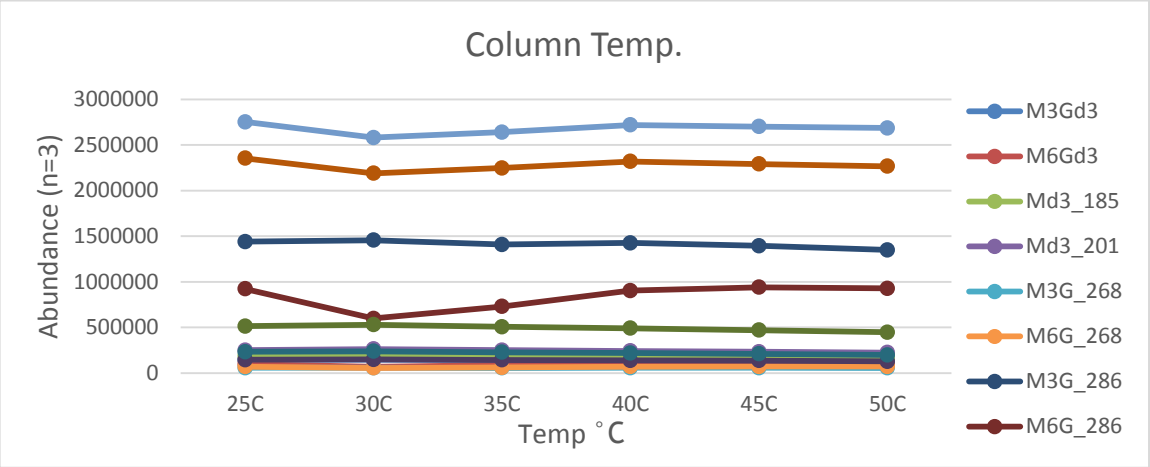


Figure 4-18 Effect of Chromatographic Column Temperature on Standards and Internal Standards Sensitivity

Finally, the column temperature was maintained at 40 °C to obtain optimum sensitivity and decreasing the turbo pump pressure as a result of decreasing the mobile phase viscosity.

4.4.5 Optimisation of Chromatographic Separation

Error! Reference source not found. shows the M3G and M6G are structurally imilar and have identical fragmentation patterns, their product ions are 286 and 268 m/z.

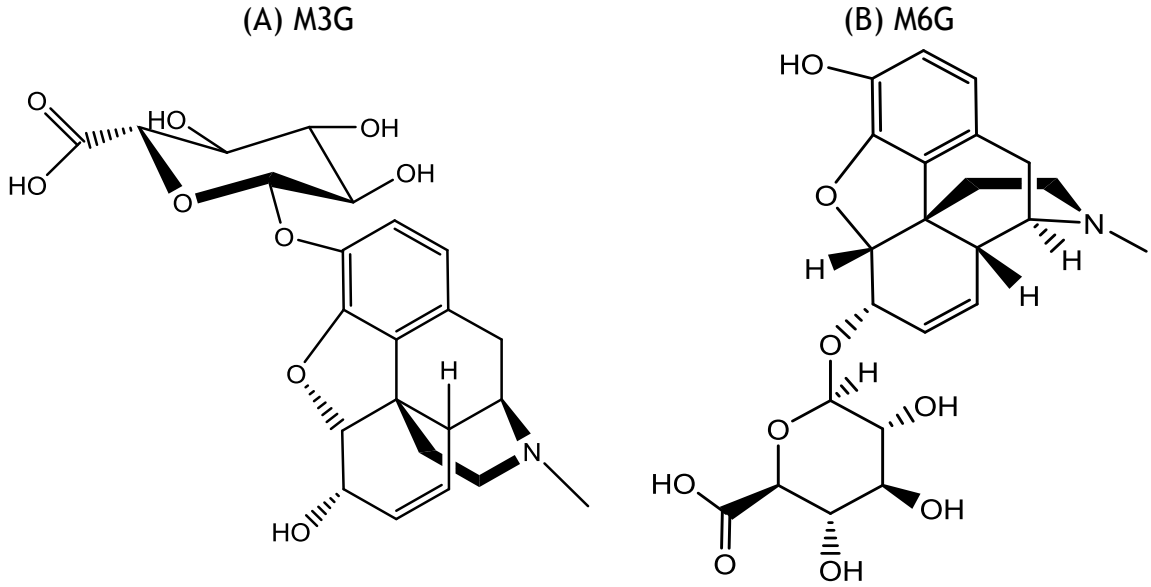
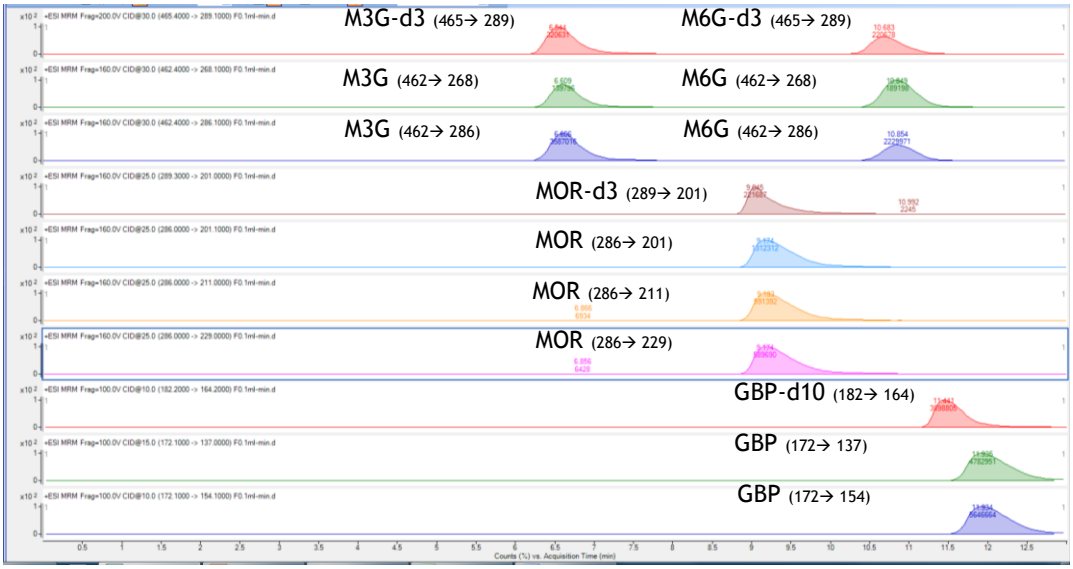


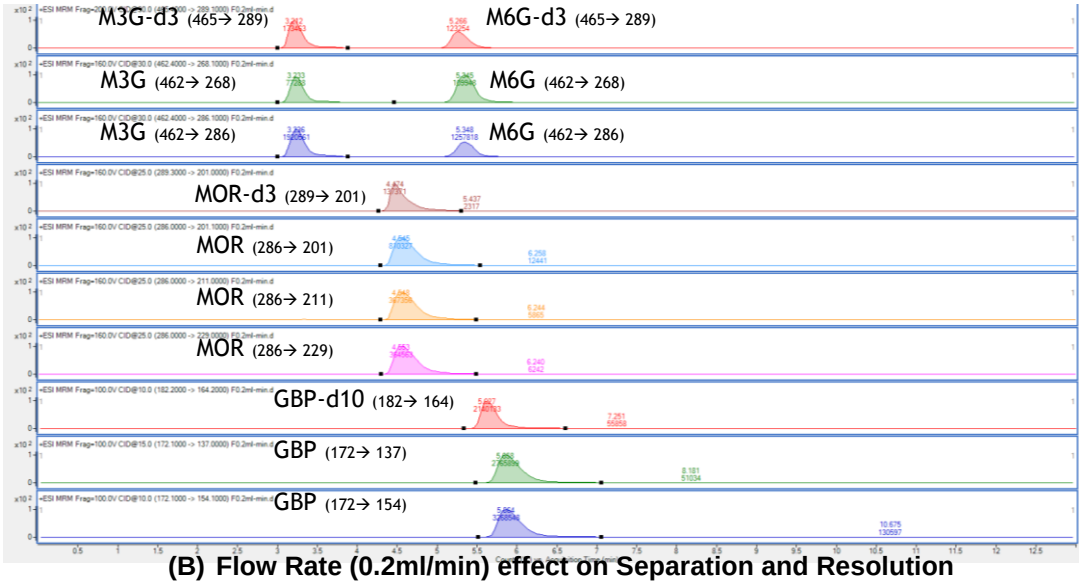
Figure 4-19 (A) M3G and (B) M6G Chemical Structure

Although the Synergi Polar-RP column provided a good separation between these two metabolites, the separation was very sensitive to any change in the composition of the mobile phase or the flow rate, resulting in the merging of the peaks again.

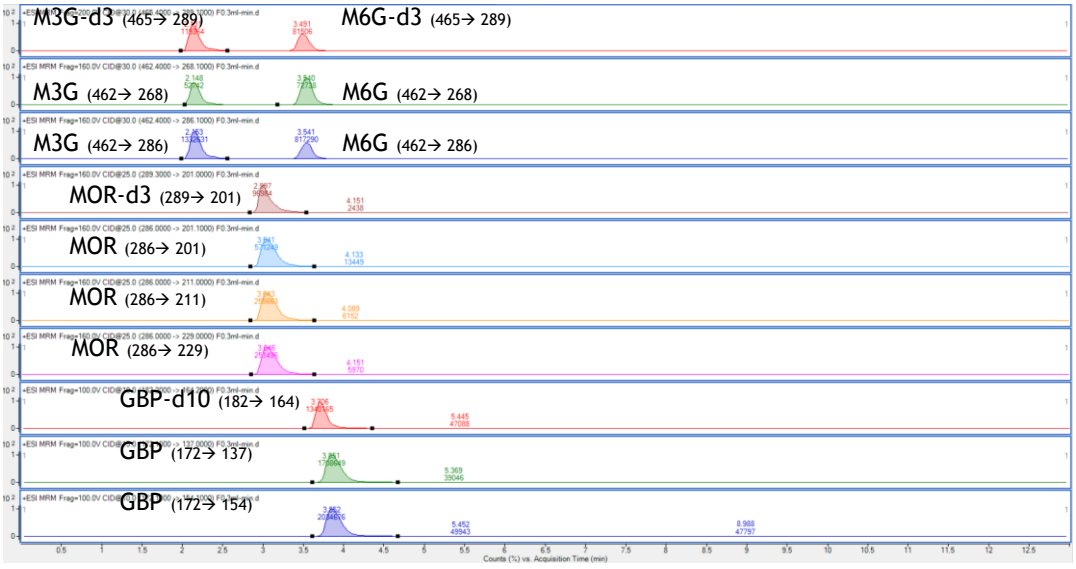
Decreasing the flow rate from 0.3 to 0.1 mL/min gave a partial separation of M3G and M6G. On the other hand, reducing the flow reduced resolution and increased the run time of the method from 13 to 20 minutes in order to elute all drugs as shown in **Error! Reference source not found.**.



(A) Flow Rate (0.1ml/min) effect on Separation and Resolution



(B) Flow Rate (0.2ml/min) effect on Separation and Resolution

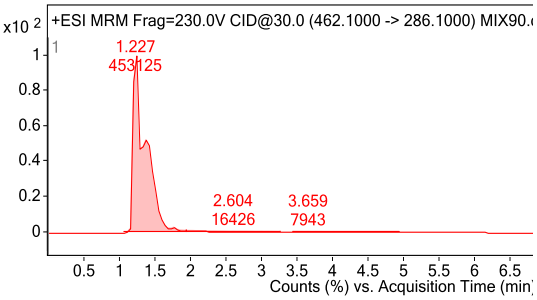


(C) Flow Rate (0.3ml/min) effect on Separation and Resolution

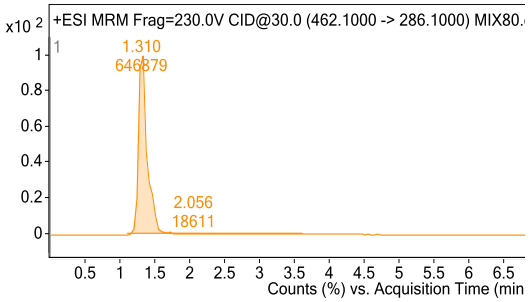
Figure 4-20 Flow Rate Effect on Gabapentin and morphine Derivatives Separation and resolution

It was decided to use flow rate 0.3 mL/min to achieve a balance between the analytes separation and good chromatography.

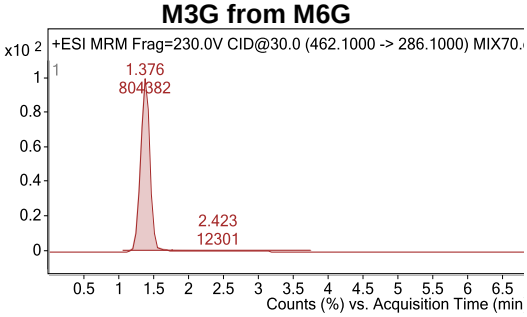
Several gradient systems were applied to obtain better separation and improve resolution for all analytes with a satisfactory runtime in a single analysis. This was achieved by adjusting the aqueous/organic ratio, which was tested in 10 different systems ranging from 10% A to 95% A, as shown in Table 4-8. As a result, the increase of the aqueous mobile phase to 90% showed a good separation between the two morphine glucuronides as shown in Figure 4-21.



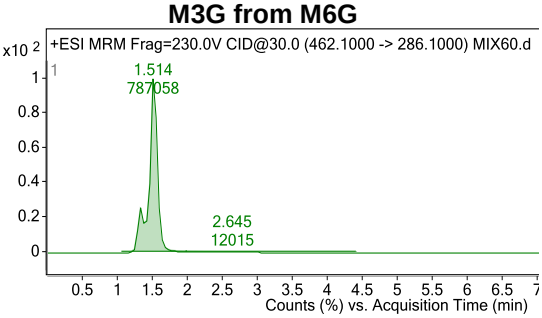
Aqueous mobile phase (10%) to separate M3G from M6G



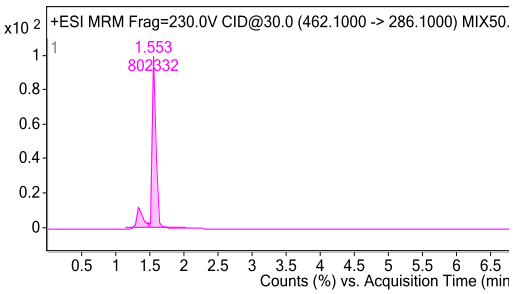
Aqueous mobile phase (20%) to separate M3G from M6G



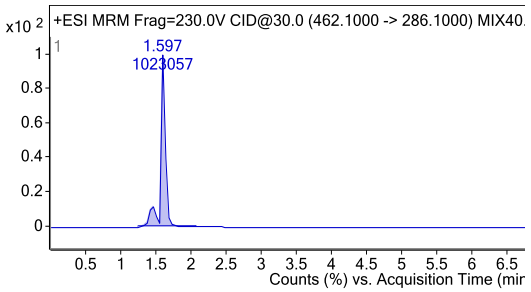
Aqueous mobile phase (30%) to separate M3G from M6G



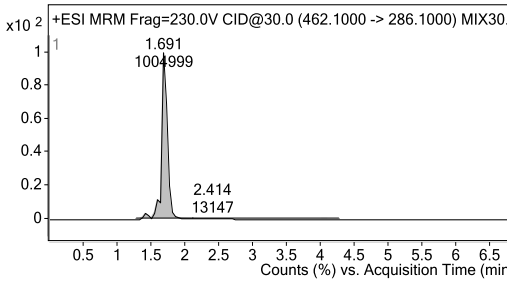
Aqueous mobile phase (40%) to separate M3G from M6G



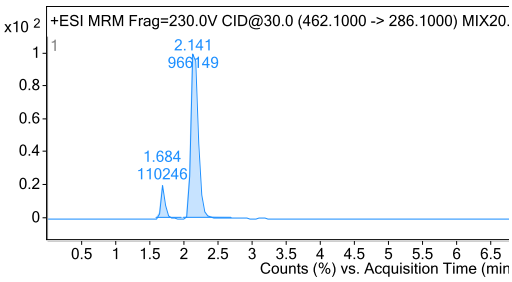
Aqueous mobile phase (50%) to separate M3G from M6G



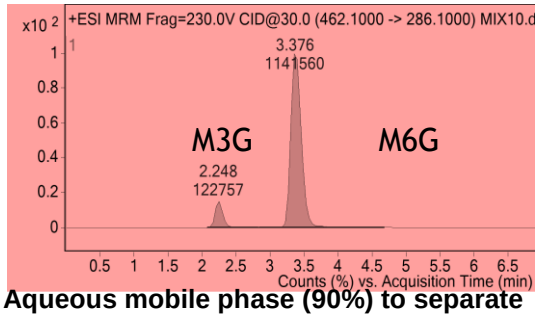
Aqueous mobile phase (60%) to separate M3G from M6G



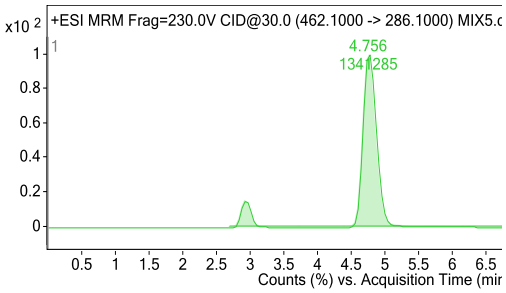
Aqueous mobile phase (70%) to separate M3G from M6G



Aqueous mobile phase (80%) to separate M3G from M6G



Aqueous mobile phase (90%) to separate M3G from M6G



Aqueous mobile phase (95%) to separate M3G from M6G

Figure 4-21 The aqueous/organic phase percentage tested at 10 different gradient systems ranged from (10% A to 95% A) to separate M3G from M6G

Finally, it was decided to use the aqueous mobile phase to 90% to achieve a good separation.

Figure 4-22 illustrates the chromatogram with all analytes obtained using dynamic multiple reaction monitoring mode and using a mixture of unextracted standards at a concentration of 10 mg / L.

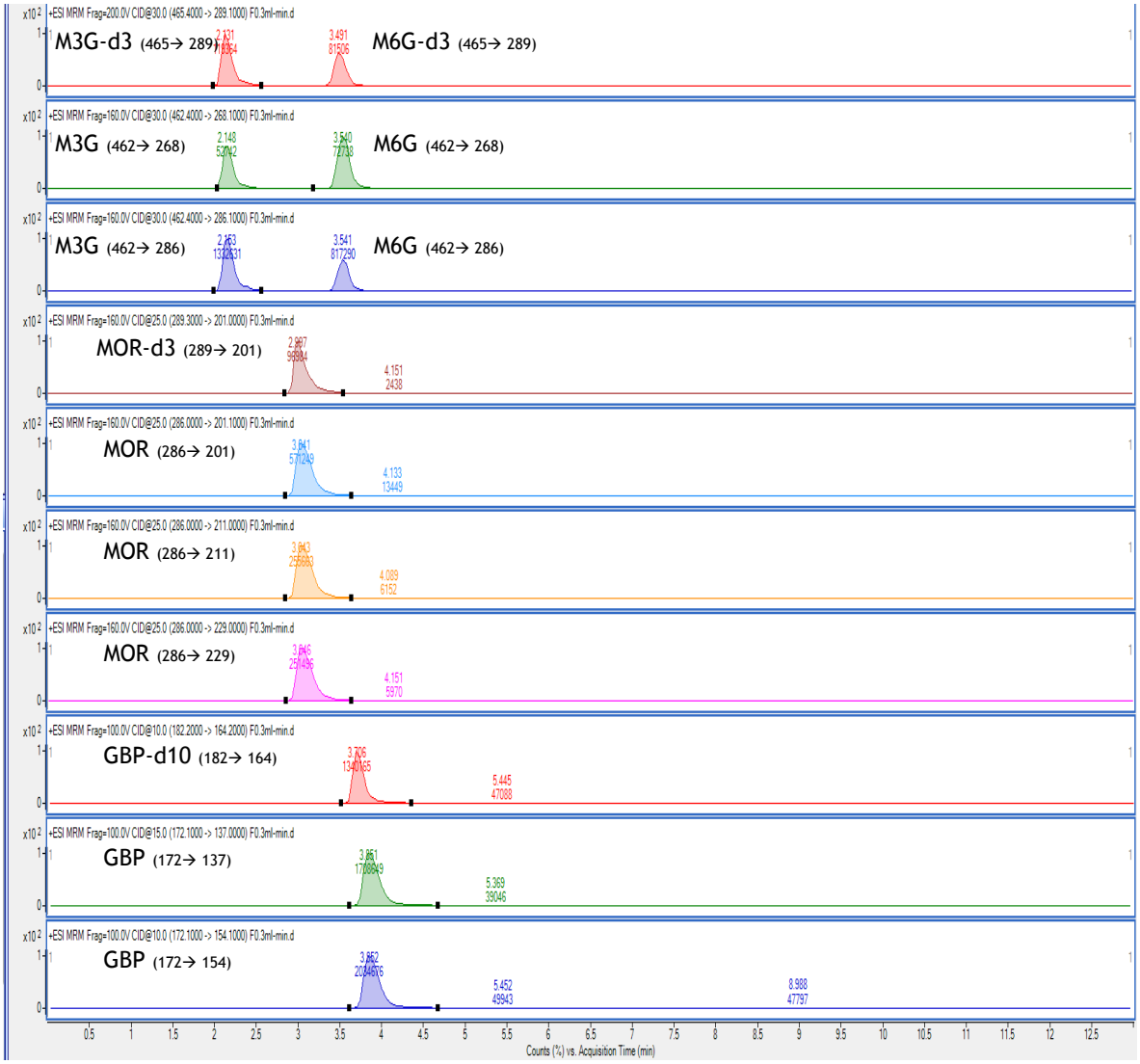


Figure 4-22 The Chromatogram of 4 drugs and 4 Internal Standards

4.4.6 Extraction Optimisation - Solid Phase Extraction V's Protein Precipitation

A commercial cartridge was evaluated for use in a method for the quantitation of morphine, M3G, M6G and GBP in postmortem blood based on SPE and LC-MS/MS analysis.

The following cartridges were evaluated: UCT's Clean Screen® DAU and Bond Elut C18, where the cartridges used to extract opioids and gabapentin mixtures were designed. Clean Screen® cartridge showed poor recovery for GBP, while Bond Elut C18 cartridge showed poor recovery for all the analytes compared to protein precipitation which gave higher recoveries for all analytes as illustrated in Figure 4-23. The R^2 values were not acceptable for some drugs using SPE due to their poor recoveries as illustrated in Table 4-10. Moreover, achieving the upper limit of quantification was not acceptable for some drugs using SPE compared to protein precipitation. Furthermore, SPE was found to be time-consuming due to the number of steps involved in sample preparation.

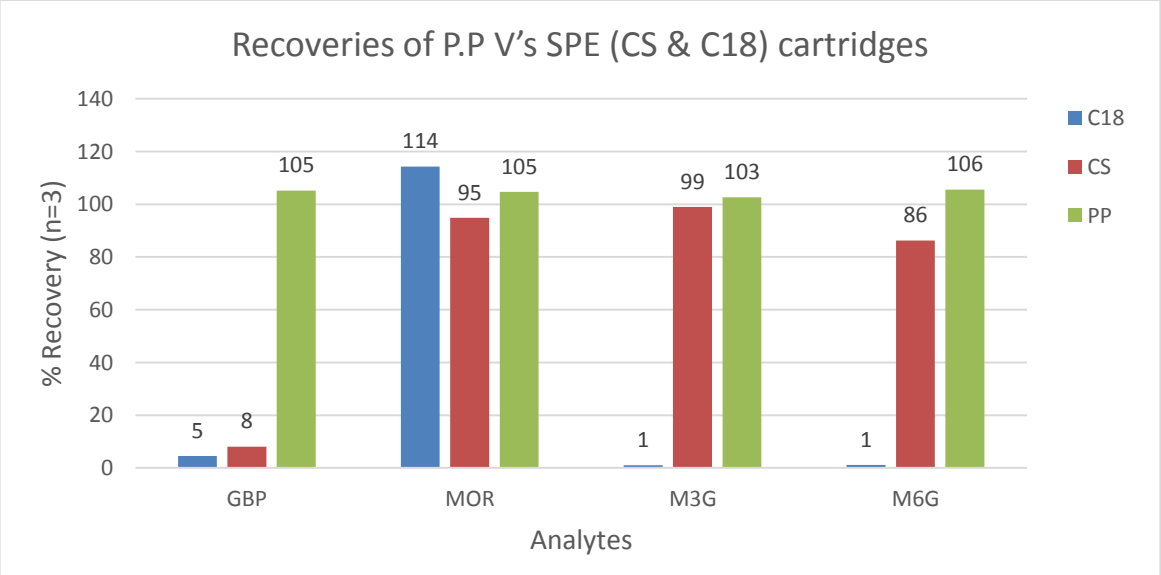


Figure 4-23 Recovery for SPE (UCT's Clean Screen® DAU and Bond Elut C18) V's Protein Precipitation (P.P).

Therefore, the protein precipitation method was suitable for routine application. The recovery (>95%) and R^2 values (> 0.997) are detailed in (Table 4-10).

Table 4-10 Recoveries, Calibration Ranges and Linearity Values for Protein Precipitation (P.P) V's Two Cartridges of SPE (Clean Screen® and Bond Elut C18)

Analytes	Recovery (%) (n=3)			Calibration Range (mg/L)	R^2 (n=3)		
	SPE (C18)	SPE (CS)	PP		SPE (C18)	SPE (CS)	PP
MOR	164.3	94.8	104.7	0.05-0.75	0.966	0.999	0.999
M3G	1.0	99.0	102.6	0.05-0.75	0.939	0.994	0.997
M6G	1.2	86.2	105.5	0.02-0.30	0.958	0.730	0.999
GBP	4.5	8.1	105.2	2.5-50	0.919	0.937	0.999

The sample preparation of the protein precipitation compared with the SPE method was faster since it contained fewer and shorter steps and used less solvent. Thus, it was decided to use protein precipitation as the method of choice to extract all the analytes simultaneously.

4.4.7 Investigation into Protein Precipitation Extraction Conditions

4.4.7.1 Effect of Extraction Solvent

Two solvents were evaluated in order to determine which one gave the best extraction for all analytes. Acetonitrile gave recoveries ranging from 73-87% for all analytes. While methanol achieved recoveries ranging from 89-104% for all the analytes as illustrated in Figure 4-24.

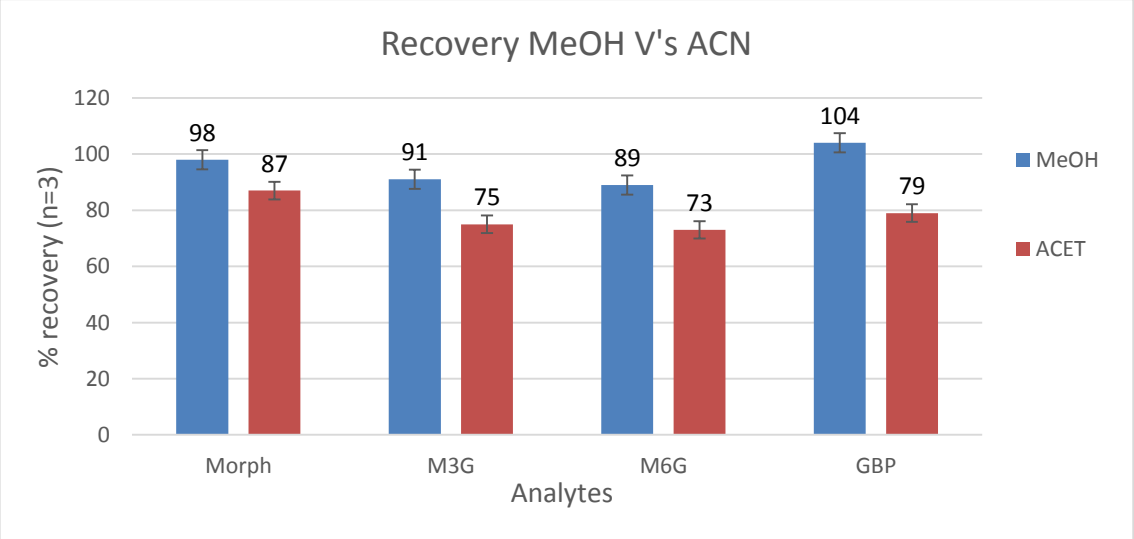


Figure 4-24 Comparison of the Recovery for Methanol and Acetonitrile Extractions

Extraction recovery was also calculated using the Matuszewski strategy, which uses the mean peak area only, while the recovery was calculated previously using the analyte/internal peak area ratios. Both methods presented equivalent results as shown in Figure 4-25.

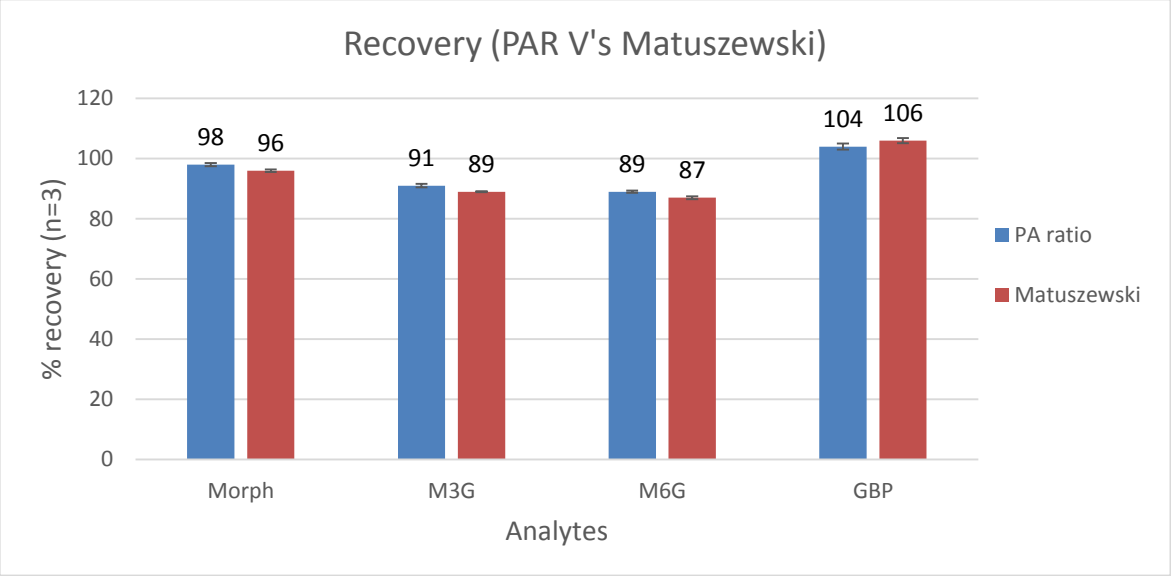


Figure 4-25 Recovery Comparison for Analyte/IS Peak Area Ratio and Matuszewski Strategy

4.4.7.2 Matrix Effect Evaluation

The matrix effect was assessed for both methanol and acetonitrile extraction. No significant ion suppression or enhancement was observed for both extraction methods. Figure 4-26 shows an acceptable matrix effect with both extractions (within ± 25%). However, the matrix effect is slightly better with MeOH extraction.

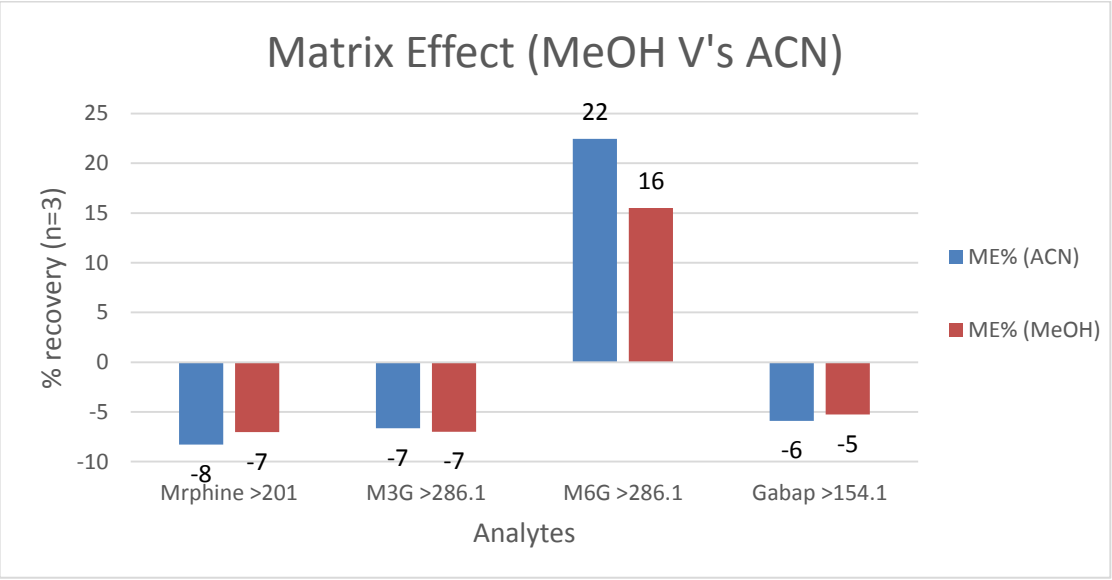


Figure 4-26 Comparison of the Matrix Effect for Methanol and Acetonitrile Extractions

4.5 Conclusion

A sensitive method for the simultaneous detection and quantification of Morphine, Morphine-3-glucuronide, Morphine-6-glucuronide, Gabapentin was developed using an Agilent LC-MS/MS triple quadruple coupled with a Synergi Polar-RP column (150 mm x 2.0 mm, 4µm) maintained at 40°C. Electrospray ionisation was used, and the MS operated in multiple reaction monitoring mode (MRM) with ion mode switching. The optimal MS conditions were achieved using a nebuliser pressure of 30 psi, a capillary voltage of 4,000 V, nitrogen gas heated to 350 °C and delivered at 11 mL/min.

The mobile phase system was developed and optimised using a mobile phase consisting of A: 0.01 % formic acid and 0.5 mM ammonium acetate in water, and methanol at a rate of 0.3 mL/min. The total run time was 13 minutes.

Protein precipitation with methanol was the most suitable extraction protocol for routine application as recovery >95% and R2 values > 0.997 were achieved. Moreover, the sample preparation of protein precipitation compared with the SPE method was faster since it contained fewer and shorter steps and used less solvent.

Chapter 5 Method Validation of GBP, MOR, M3G and M6G in Whole Blood Using LC-MS/MS

5.1 Introduction

The validation of new analytical methods prior to their use in casework is a prerequisite to prove that an accurate, precise and rugged method has been developed to yield reliable results which can be satisfactorily interpreted.[348, 389].
The method validation was conducted using the Standard Practices for Method Validation in Forensic Toxicology (SWGTOX), revision draft 003 (32) as a guide: selectivity, calibration model, precision and accuracy, limit of quantification (LOQ), limit of detection (LOD), carryover, a matrix effect assessment and stability study of analytics during specimen processing.

The purpose of validation is to confirm through the examination and provide objective evidence that the method developed in chapter 4 for a specific intended use are fulfilled. It is important as it defines whether it will produce reliable results and to identify the method's limitations under normal operating conditions.

5.2 Materials and Methods

5.2.1 Materials

5.2.2 Chemicals & Reagents

All reference standards, blood and other analytical grade chemicals were purchased from the same suppliers as listed in section 4.3.1.

5.2.3 Solution preparation

All reference for solutions preparation were the same as those described in Section 4.3.2.

5.2.4 Instrumentation

An Agilent LC-MS/MS triple quadruple G6420A mass spectrometer equipped with an electrospray ionisation (ESI) source, Agilent 1200 Series Auto sampler SL,

Agilent 1200 Series Binary Pump SL with degasser and Agilent 1200 Series Thermostatted Column Compartment SL was used. The turbo ion-spray interface was operated in positive-ion mode with nitrogen as the collision gas. The Agilent Mass-Hunter Workstation software (version: B.01.05) was used for system control and data acquisition. A Synergi Polar-RP column (150 mm x 2.0 mm, 4µm) maintained at 25°C. The MS was operated in multiple reaction monitoring mode (MRM) with ion mode switching. A nebuliser pressure of 30 psi, a capillary voltage of 4,000 V, nitrogen gas heated to 350 °C and delivered at 11 mL/min was used.

Gradient elution was employed using a mobile phase consisting of 0.01% Formic acid and 0.5 mM ammonium acetate in water / methanol at a flow rate of 0.3 mL/min. The total run time was 13 min in Figure 5-1. The gradient mobile phase system started at 90:10 A/B and this percentage was maintained for 5 min before being increased to 10:90 A/B within 1 min and maintained for 2 min. The percentage was finally decreased to 90:10 A/B for 5 min in order to condition the column before the next injection.

5.2.5 Sample Preparation

The samples were extracted using the protein precipitation as follows; 800 µL of MeOH was added to 200 µL of blood and spiked with 50 µl of internal standard solution. Samples were vortex mixed before being centrifuged at 2000-3000 rpm for 10 minutes. Following centrifugation, the supernatant was transferred to a 3.5 mL vial using a glass pasteur pipette. The supernatant was evaporated under nitrogen gas, with the heating block set at 25°C. Once the samples were dried, they were reconstituted in 200 µL of mobile phase (90/10: A / B). This was then transferred to the LC autosampler vials and 10 µL volume was injected into LC-MS/MS.

5.2.6 Selectivity and Specificity

Method validation acceptability criteria for selectivity and specificity were the same as those described in section 3.8.1.

5.2.7 Limits of Detection and Limits of Quantification

In order to determine LODs for each compound, blood was spiked with decreasing concentrations of mixed working solution within the expected range of LODs; over the concentration range 0.05, 0.10, 0.25, 0.50, 1.0 and 2.5 mg/L for GBP; 0.0025, 0.0050, 0.0100, 0.0250, and 0.0500 mg/L for MOR and M3G; and 0.0025, 0.0050, 0.0075, 0.0100, 0.0200 mg/L for M6G. All LODs were extracted as detailed in section 5.2.5 and analysed in duplicate in three separate runs using three different sources (donors) of blood. These were run alongside a set of calibrators and QC's. Mass-Hunter Workstation program was used to calculate the S/N ratio. The acceptability criteria of the limits of detection and limits of quantification were the same as those described in section 3.8.2.

5.2.8 Linearity

Linearity was determined by preparation and analysis of seven calibrator points over the concentration range 2.5, 5.0, 7.5, 10, 12.5, 25 and 50 mg/L for gabapentin; 0.05, 0.075, 0.100, 0.125, 0.250, 0.500 and 0.750 mg/L for Morphine and M3G and; 0.02, 0.03, 0.04, 0.5, 0.10, 0.20 and 0.30 mg/L for M6G.

Five fresh calibrations were prepared in duplicate by spiking blank blood with different volumes of working solutions 1 and 2 as detailed in Section 4.3.2 and extracted according to the method reported in Section 5.2.5 over 5 different days. The acceptability criteria for linearity were the same as those described in section 3.8.3.

5.2.9 Accuracy and Precision

Accuracy (Bias) and precision were calculated by running 3 replicates for each quality control sample (LQC, MQC and HQC). A calibration curve was prepared with each batch to calculate the concentrations.

Intra-day (within) precision and bias were calculated from 6 replicates per QC in one batch. Inter-day precision and bias were determined over 5 different runs. Their values were calculated using equations (Equation 3-4 to Equation 3-8). The acceptability criteria for accuracy and precision were the same as those described in section 3.8.3 3.8.4.

5.2.10 Recoveries and Matrix Effects

Recoveries and matrix effects for all drugs of interest and internal standards were evaluated using the post-extraction addition approach. This method examined the peak areas of analyte in three different sets of samples as described previously in (section 4.3.9).

5.2.11 Carryover

Analyte carryover was assessed by injecting three blank blood extracts after two injections of QC4 over different batches. QC4 was double the upper limit of quantification in the calibration curve (100 mg/L for GBP; 1.5 mg/L for Morphine and M3G, and 0.6 mg/L for M6G), as detailed in section 4.3.2.7. Carryover was evaluated by examining the chromatograms visually.

5.2.12 Stability

For reliable quantitation, the analytes stability in the matrix over different storage conditions was required during the validation process. The storage conditions were evaluated: room temperature stability, auto-sampler stability and stability after 3 freeze-thaw cycles at -20±2 ºC.

In order to evaluate this, the same protocol described in section 3.8.7 was followed. Subsequently, samples were extracted and processed in triplicate along with freshly spiked calibration standards and analysed using the regression equation obtained. The recovery of each analyte was then calculated using Equation 3-9. Analytes were identified as being unstable if their recovery fell out with the acceptable criteria of ±20%.

5.2.13 Stability study using Separated Clot Activator tubes and Eppendorf tubes

The present study was designed to determine the stability of morphine and its glucuronides, and gabapentin in spiked blood by using two types of sampling tube. The two sampling tubes used were separated clot activator tubes (SCAT) and Eppendorf tubes (EPNT).

The present study was designed to investigate the effect of the sampling tubes and storage temperature during the period between sampling and analysis. The

samples were stored at -20°C, 4°C and 20°C for a time interval of up to four months. Blank blood was spiked at low and high concentrations of each analyte separately (0.06 and 0.60 mg/L for morphine, 0.06 and 0.60 mg/L for M3G, 0.025 and 0.25 mg/L for M6G and 4 and 40 mg/L for gabapentin, respectively), and then mixed on a roller for 1 hour to ensure that all compounds were distributed equally in the blood. The prepared samples were divided into three groups and were then stored at three different temperatures (-20 ºC, 4 ºC, and 20 ºC). The times selected for analysis were at day 1 (day zero), 3, 7, 14, 21 and 28 for 20ºC and day zero, 7, 14, 21, 28 and 120 for -20ºC and 4ºC.

Three samples from the prepared blood of each group (SCAT and EPNT) were analysed and found to be homogeneous, and the mean concentration of each analyte was considered to be the day zero concentration. Each time point samples were analysed; three replicates were taken for analysis from the storage racks for each of the different storage conditions. The internal standards were added into each sample and then extracted by protein precipitation as detailed in section 5.2.5. A calibration curve and QCs were extracted with each set of samples.

5.3 Results and Discussion

5.3.1 Chromatography

Good chromatography was achieved for all analytes

Figure 5-1 shows an example of the chromatographic profiles of gabapentin, morphine, M3G and M6G and the four internal standards in whole blood.

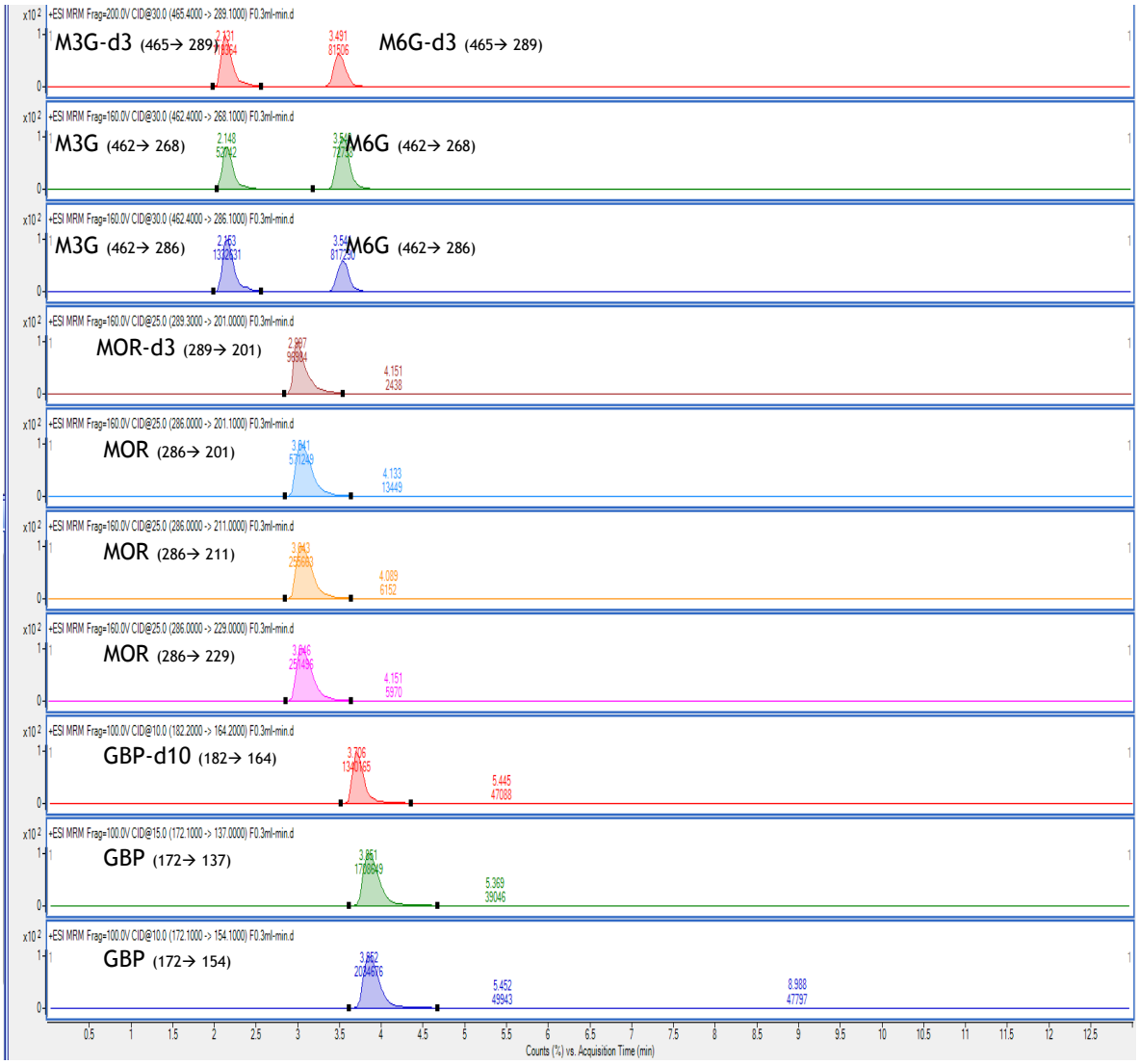


Figure 5-1 Chromatographic profiles of analytes in whole blood at concentration 10 mg/L.

5.3.2 Selectivity and Specificity

There were no endogenous interferences identified at the GBP, MOR, M3G and M6G retention times. Therefore, the results of selectivity confirmed the ability of the method to distinguish target analytes in a complex matrix without any potential interference from other matrix components of similar behaviour.

There were no exogenous interferences identified at the analyte retention times from structurally-related analytes as detailed in Appendix 1. In addition, no contribution was observed from the internal standards to the analytes or vice versa.

5.3.3 LOD and LLOQ

Table 5-1 shows the results of LOD and LLOQ. In general, the lowest calibrator chosen for each analyte was verified, and therefore can be quantified with the greatest confidence.

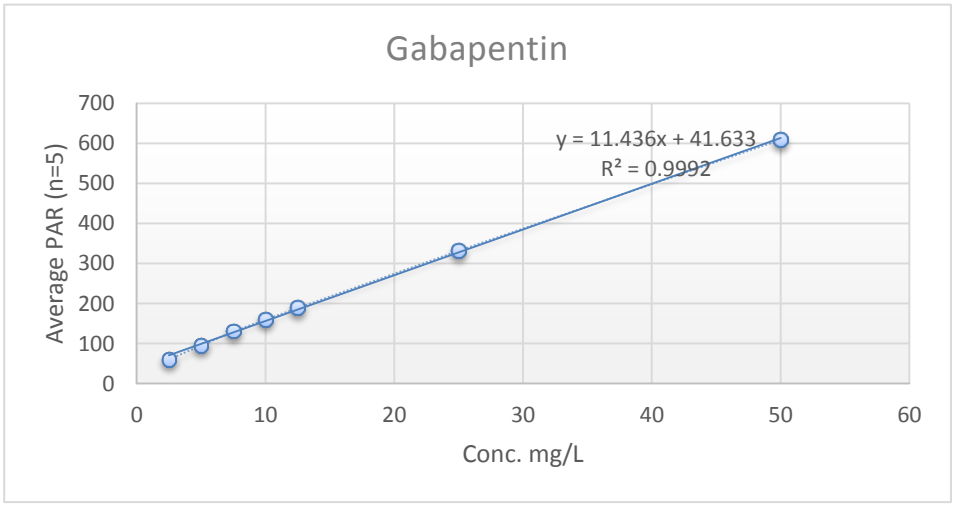
5.3.4 Linearity

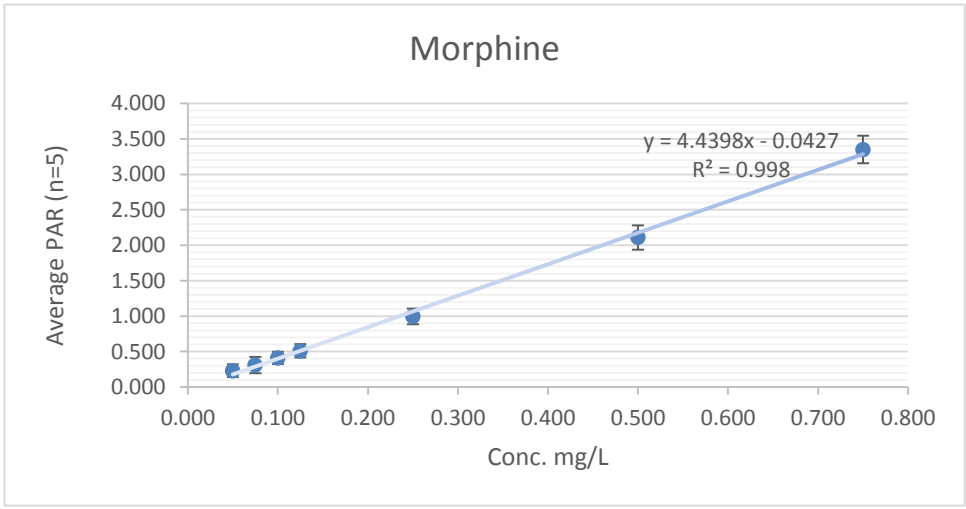
All analytes followed an unweighted calibration model and were linear for all analytes over the wide range of concentrations in blood; all the calibration graphs passed all the acceptance criteria and R2 was greater than 0.995 in the 5 validations as shown in Table 5-1 and Figure 5-2.

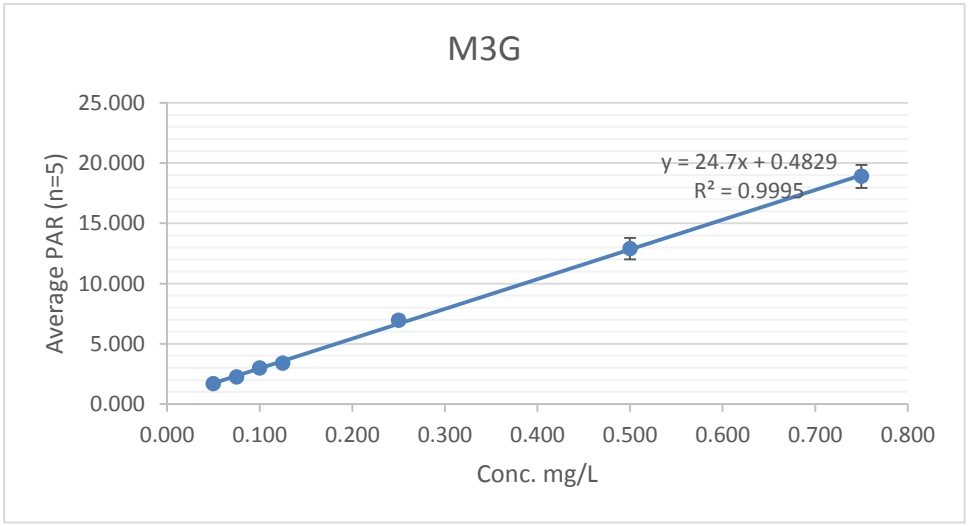
Table 5-1 LOD, LOQ, Calibration Model and Linearity of GBP, MOR, M3G and M6G in Blood

Drugs	LOD (mg/L)	LLOQ (mg/L)	Calibration Range (mg/L)	Internal Standard	Blood R^2 (n=5)
GBP	0.10	2.50	2.5-50	MOR-d3	0.999
MOR	0.01	0.05	0.05-0.75	MOR-d3	0.998
M3G	0.01	0.05	0.05-0.75	M3G-d3	0.999
M6G	0.005	0.02	0.02-0.30	M6G-d3	0.995

Residual plots were used to determine the variance across the calibration range. They show random variance across the target values and also give an indication that the chosen model adequately fits the data. Plots are displayed in Figure 5-3 below.







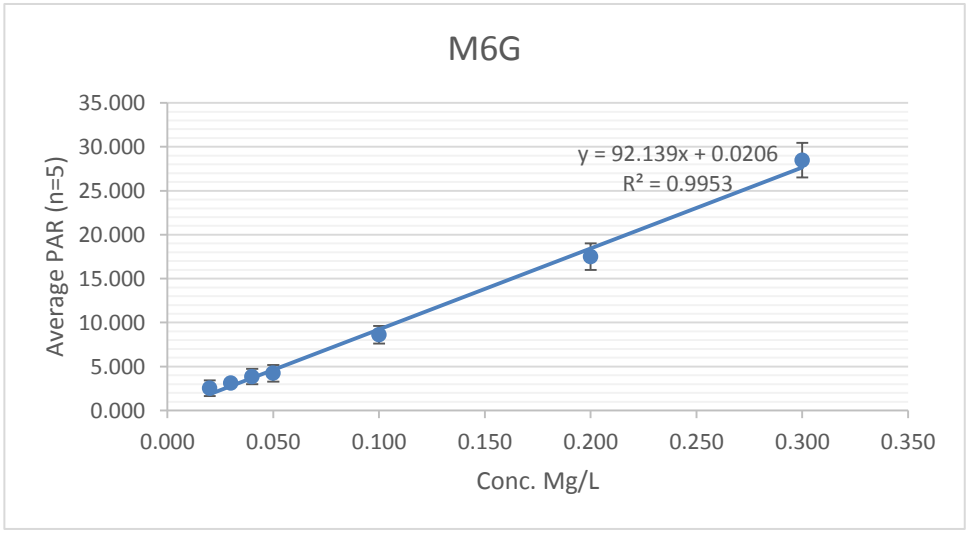


Figure 5-2 Linearity Graphs of Drugs of interest

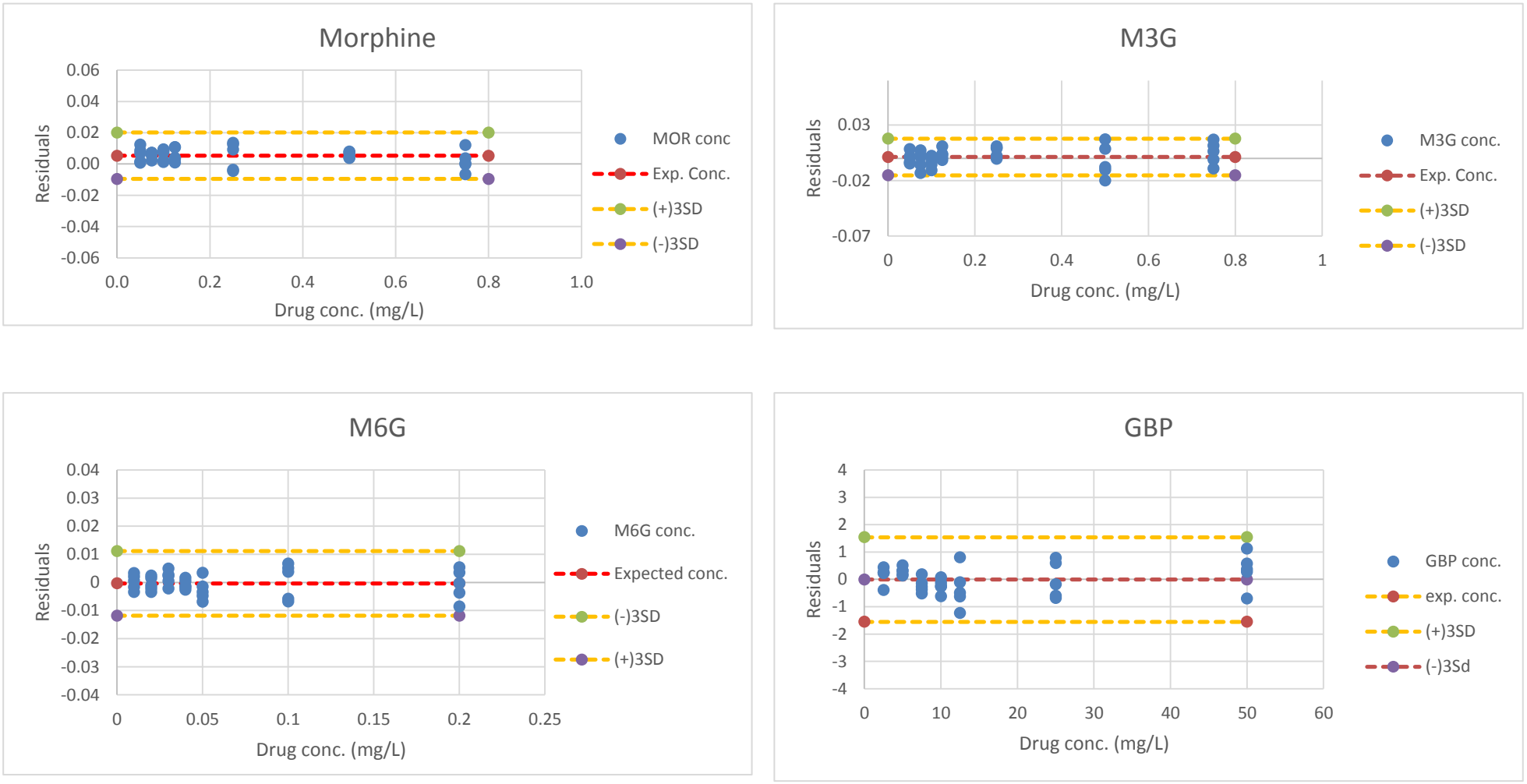


Figure 5-3 Standardised Residual Plot Graphs of Drugs of interest

5.3.5 Bias and Precision

The accuracy (bias) of each analyte in blood fell within the SWGTOX criteria. The average accuracy for the analytes across all QCs was within the acceptable range of ± 20 % of the nominal concentrations. The intra-day accuracy was from 84-118 %. The inter-day accuracy ranged from 83-115 %.

Intra-day and inter-day precision results of all analytes fell within the <20% criteria across all control samples of low, medium and high. The intra-day precision values were less than 19 %. The inter-day precision values were less than 19 %. Accuracy and precision results for all drugs of interest are presented in Table 5-2.

Table 5-2 Intra- and Inter-Day Accuracy Results of GBP, MOR, M3G and M6G Results

Analyte	Expected conc. (mg/L)	Intra-assay (n = 6)				Inter-assay (n = 5)			
		Mean Conc. (mg/L)	Std. Dev (mg/L)	Bias (%)	CV (%)	Mean Conc. (mg/L)	Std. Dev (mg/L)	Bias (%)	CV (%)
GBP	4	4.7	0.555	17.5	11.8	4.6	0.437	15.0	9.5
	20	19.5	0.663	-2.5	3.4	20.5	0.554	2.5	2.7
	40	42.9	3.604	7.3	8.4	44.2	2.033	10.5	4.6
MOR	0.06	0.070	0.008	16.7	11.9	0.067	0.011	11.7	16.4
	0.30	0.273	0.009	-9.0	3.4	0.297	0.007	-0.9	2.3
	0.60	0.690	0.089	15.0	12.9	0.512	0.073	-14.7	14.2
M3G	0.06	0.069	0.009	14.8	13.5	0.052	0.010	-13.3	18.6
	0.30	0.313	0.022	4.3	6.9	0.310	0.026	3.3	8.3
	0.60	0.690	0.101	15.0	14.6	0.500	0.067	-16.7	13.4
M6G	0.025	0.021	0.004	-16.0	18.7	0.022	0.004	-12.8	17.8
	0.125	0.128	0.005	2.4	3.6	0.124	0.009	-0.8	6.9
	0.250	0.222	0.038	-11.2	16.9	0.278	0.026	11.2	9.4

5.3.6 Recoveries and Matrix Effects

Extraction of all drugs from whole blood by using protein precipitation was highly effective with recovery ranging from 102.6 to 109 %. The matrix factor values for all drugs were within the acceptable range of 1± 0.25 and standard deviations were less than 10%. From this information, it was observed that there is no significant ion suppression or enhancement of the matrix. Table 5-3 below shows the recovery and matrix effect values for drugs of interest using Low and High QCs and 6 different whole blood sources (n=6 per QC per Matrix).

Table 5-3: Recovery and Matrix Factor Values

	QC1 (n=3)		QC3 (n=3)	
Drugs	Recovery (%)	Matrix Factor	Recovery (%)	Matrix Factor
GBP	109.0±3.6	0.99±0.08	105.0±8.2	1.01±0.03
MOR	107.4±2.2	0.97±0.09	104.7±0.08	1.07±0.02
M3G	105.3±3.25	0.87±0.05	102.6±1.25	1.06±0.06
M6G	108.2±6.02	0.92±0.05	105.5±4.12	1.09±0.07

5.3.7 Carryover

No carry over was observed in the blank samples after two injections of the highest standards for MOR, M3G, M6G and GBP in whole blood. Carryover after first blank injection was lower than the LOD for all drugs.

5.3.8 Stability study

5.3.8.1 Room temperature stability:

The results showed that all the drugs were stable in the whole blood at room temperature for up to 5 days. This indicates no significant decomposition of drugs of interest. This is further illustrated by the number of QC's which had recovery results ranging ±20% in Table 5-4.

Table 5-4 Room temperature Stability of Drugs of Interest for 5 Days

	% Recovery of QC1 (n=3)				% Recovery of QC3 (n=3)			
	*D0	*D3	*D4	*D5	*D0	*D3	*D4	*D5
GBP	100	93	93	93	100	95	95	104
MOR	100	97	96	94	100	108	107	104
M3G	100	91	88	90	100	98	89	91
M6G	100	88	84	108	100	94	81	83

*D refers to time in days.

5.3.8.2 Autosampler stability

Autosampler stability results for each analyte are shown in Table 5-5. The extracted samples were stable in the autosampler (approximately 20ºC) for up to 5 days. All analytes tested had % recoveries within the acceptable criteria of ±20%.

Table 5-5 Autosampler Stability of drugs of interest for 5 Days

	% Recovery of QC3 (n=3)			
	D 0	D 3	D 4	D 5
GBP	100	98	96	94
MOR	100	104	108	106
M3G	100	94	90	90
M6G	100	86	83	105

5.3.8.3 Freeze-thaw cycles stability:

All drugs of interest were stable in whole blood after 3 freeze-thaw cycles at –20 ºC as shown in Table 5-6.

Table 5-6 Freezer-Thaw Stability of drugs of interest

	% Recovery of QC1 (n=3)				% Recovery of QC3 (n=3)			
	*D0	*D3	*D4	*D5	*D0	*D3	*D4	*D5
GBP	100	96	96	96	100	94	85	93
MOR	100	102	113	99	100	119	102	107
M3G	100	103	97	93	100	85	85	92
M6G	100	86	89	84	100	88	89	85

* D refers to time in days.

5.3.9 Stability results of Separated Clot Activator tubes and Eppendorf tubes

The stability results for M, M3G, M6G and GBP in spiked blood stored at different conditions using separated clot activator tubes (SCAT) and eppendorf tubes (EPNT) are presented as percentage concentration change from day zero concentrations.

A) Morphine

In all three storage temperatures and both tubes, morphine showed no statistically significant change over the time period, as illustrated in Figure 5-4.

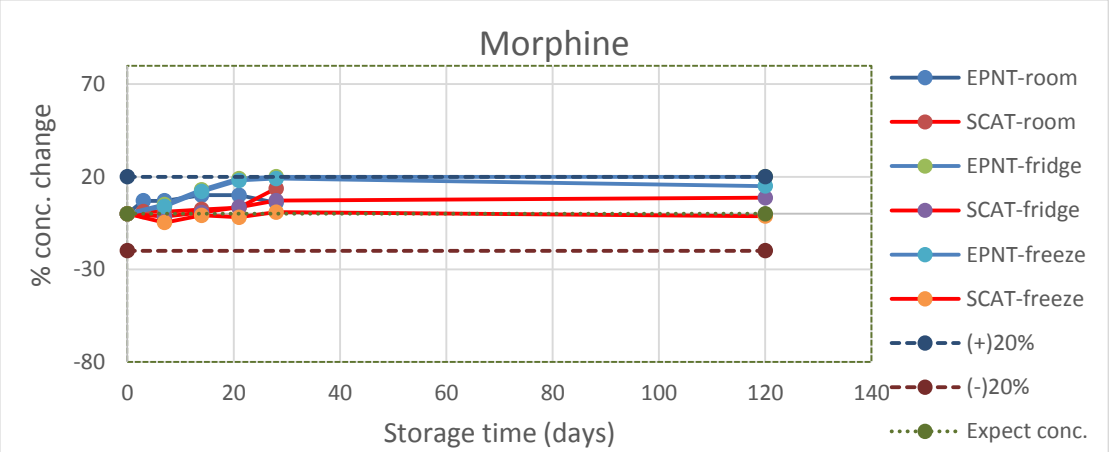


Figure 5-4: Stability of morphine by using SCAT and EPNT tubes.

B) M3G

In all three storage temperatures and in both tubes, M3G showed no significant changes over the time period studied, as illustrated in Figure 5-5.

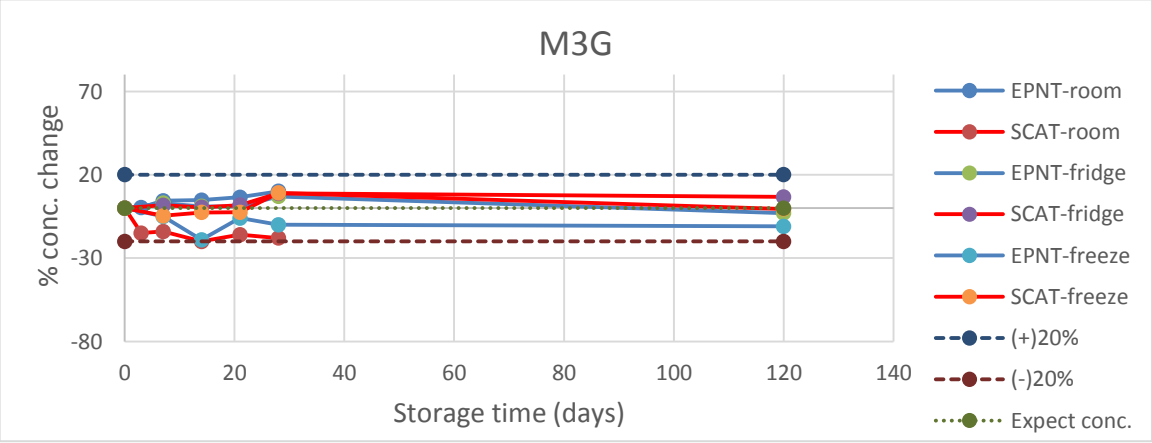


Figure 5-5: Stability of M3G by using SCAT and EPNT tubes.

C) M6G

In all three storage temperatures and in both tubes, M6G showed no significant changes over the time period studied, as illustrated in Figure 5-6

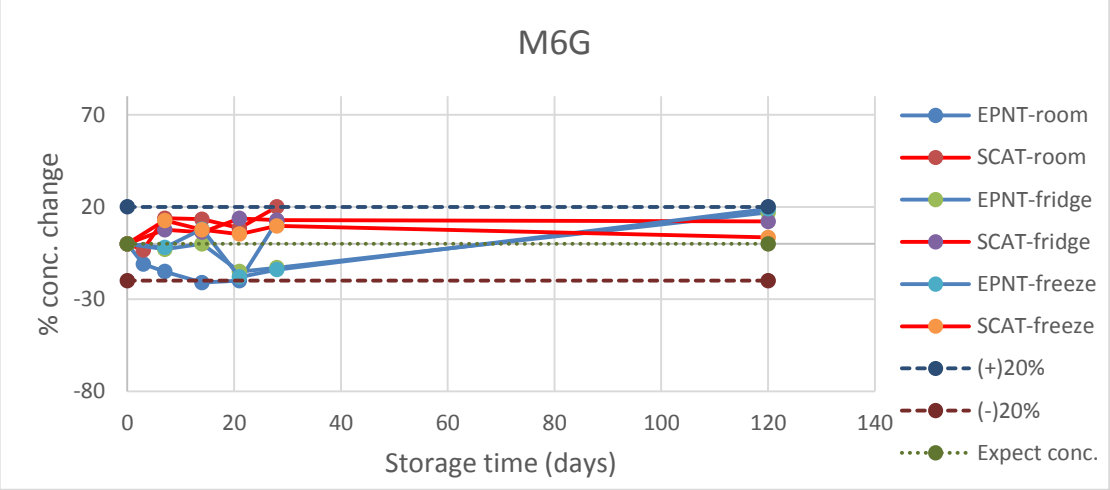


Figure 5-6: Stability of M6G by using SCAT and EPNT tubes.

D) Gabapentin

In all three storage temperatures and in SCAT, gabapentin showed significant decreases in concentration (23 %) after one month at 20 °C and decreases in concentration of 34 % and 47 % after four months at -20 °C and 4 °C, respectively. However, in EPNT, gabapentin showed no significant changes over the time period studied, as illustrated in Figure 5-7.

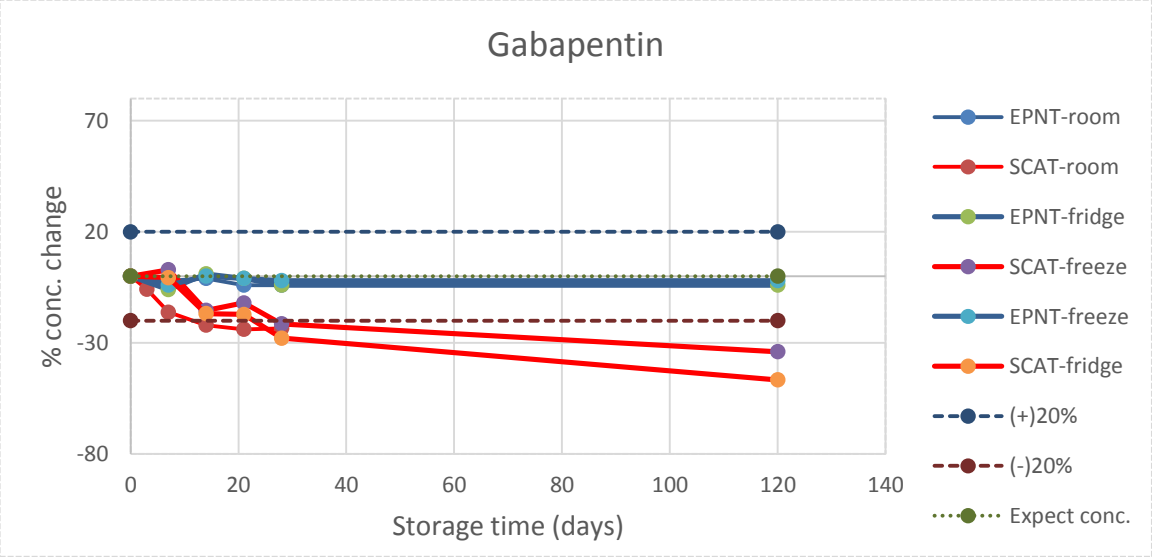


Figure 5-7: Stability of Gabapentin stored in SCAT and EPNT tubes.

In summary, the stability of morphine, M3G, M6G, and gabapentin in blood was studied after different storage conditions in the SCAT and EPNT. In both tubes, the results for all drugs present were within our acceptance criteria ±20% of the initial concentration except gabapentin. The stability of gabapentin stored in SCAT was significantly adversely affected even when stored in the fridge and freezer. It may be the gel in the tubes absorbed gabapentin preferentially compared to morphine and its glucuronides, this might be to do with the relative concentrations. The summary of stability data of morphine, M3G, M6G and gabapentin under different storage conditions is summarised Table 5-7.

Table 5-7: Summary of stability data of separated clot activator tubes and Eppendorf tubes.

Compound	Storage conditions	Tubes	Results
Morphine	-20 °C, 4 Months	SCAT	1% decreased
		EPNT	15% increased
	4 °C, 4 Months	SCAT	9% increased
		EPNT	20% increased
	Room temperature, 1 Month	SCAT	14% increased
		EPNT	6% increased
M3G	-20 °C, 4 Months	SCAT	1% decreased
		EPNT	11% decreased
	4 °C, 4 Months	SCAT	7% increased
		EPNT	3% decreased
	Room temperature, 1 Month	SCAT	18% decreased
		EPNT	10% increased
M6G	-20 °C, 4 Months	SCAT	3% increased
		EPNT	19% increased
	4 °C, 4 Months	SCAT	12% increased
		EPNT	17% increased
	Room temperature, 1 Month	SCAT	20% increased
		EPNT	12% increased
Gabapentin	-20 °C, 4 Months	SCAT	34% decreased
		EPNT	2% decreased
	4 °C, 4 Months	SCAT	47% decreased
		EPNT	4% decreased
	Room temperature, 1 Month	SCAT	23% decreased
		EPNT	3% decreased

5.4 Conclusion

The LC-MS / MS with protein precipitation method was developed and validated according to SWGTOX guidelines for simple and accurate analysis of gabapentin, morphine, M3G and M6G in whole blood samples. The developed method achieved recovery rates greater than 95% for all drugs with acceptable matrix effect (± 25%). Good precision, accuracy and linearity were obtained for all drugs. On a long-term basis, the method would save on the cost of consumables and analyst time as it requires small volumes of solvent and sample. The simultaneous analysis of morphine, M3G and M6G in whole blood samples can be a great advantage for forensic cases as a complementary test to estimate time since death, especially in the cases of heroin intoxication with no 6MAM detection.

The stability of morphine, M3G, M6G, and gabapentin in spiked blood using separated clot activator tubes and Eppendorf tubes was studied after different storage conditions for 4-months. Overall in Eppendorf tubes, the results demonstrated that morphine, M3G, M6G and gabapentin are very stable under all conditions of storage. While in separated clot activator tubes, gabapentin tended to decrease under all conditions especially when the sample was stored for more than one-month. In comparison to morphine, M3G and M6G were stable under all conditions of storage.

Chapter 6 Stability of Opioids in Postmortem Blood Samples

6.1 Introduction

The stability of drugs in biological specimens is a major concern for forensic toxicology laboratories [75]. Frequently, there is a delay of days between sampling and drug testing in biological samples [311]. In forensic circumstances, it is further complicated by the possibility of more than one laboratory doing testing at different times and on different aliquots of the same sample. The knowledge of the stability of a drug assists in the evaluation of the veracity of the toxicological result [75] and helps to define the optimal sampling and storage conditions [69, 70, 358]. This is of particular significance to forensic laboratories who are required to keep all biological samples for a time period of months or years (depending on the relevant law) to enable re-analysis if requested.

The changes in drug concentrations can be caused by diverse mechanisms, such as substance degradation, adsorption to the collection tubes and desiccation [390]. As a result, re-analysis of drug concentrations may differ from the initial results, and sometimes the validity of toxicological test results may be disputed by interested parties [75, 391].

As indicated earlier, diamorphine has a very short half-life and is rarely detected. As a result, concentrations of its active metabolite morphine have been employed for the interpretation of cause of death and elapsed time after diamorphine administration. The presence of 6MAM in blood has been used as evidence of a short-elapsed time after administration because 6MAM has a short half-life of less than 40 minutes after administration. However, monitoring blood concentrations of morphine and its metabolites is important for the understanding of time since death.

The purpose of this study was to evaluate the stability of opioids in blood samples after storage.

6.2 Comparing GCMS analysis with LC-MS/MS analysis of Morphine and its Glucuronides

6.2.1 Introduction

This study involved the re-analysis of blood samples, taken at autopsy, from cases previously investigated by the Procurator Fiscal as a potential "drug-related death". These samples were originally analysed by the Forensic Toxicology Service laboratory within FMS at the University of Glasgow and stored in the freezer until authorisation for destruction had been obtained. The laboratory is accredited by UKAS to the international standard ISO/IEC 17025 and the analysis of opiates is within their scope of accreditation.

In order to establish that the LC-MS/MS method described in sections 5.2.4 and 5.2.5 was comparable to the method used by the FMS laboratory, blood samples of current cases were selected for analysis, before being put into long term storage. The same sample was analysed by the accredited FMS labora4.5tory method using GCMS and the previously described LC-MS/MS method.

6.2.2 Ethical Considerations

For this study it was vital that real postmortem blood samples were used. Therefore, consideration was given to the ethics surrounding testing individual samples which were taken for the purposes of determining the cause of death. The Head of the Scottish Fatalities Investigation Unit at the Crown Office and Procurator Fiscal Service gave approval in principle, assuming the case was either closed or there was sufficient sample to complete all potential investigations.

The study protocol was approved by the West of Scotland Research Ethics Service (WoSRES), (reference: 17/WS/0102) in Appendix 2.

6.2.3 Methodology

During the period from July 2017 to June 2018, thirty-one postmortem femoral blood samples were analysed by both methods. The FMS laboratory GC-MS method analysed for free morphine and total morphine, after hydrolysis. The LC-

MS/MS method detected morphine, M3G and M6G without hydrolysis. Samples were extracted in batches with appropriate QCs, blanks and calibrators.

A) GC-MS method:

The FMS laboratory GC-MS method analysed for free morphine and total morphine, after using a hydrolysis step. Total opiates (TM) hydrolysis step was achieved as follows; 0.4 ml ß-glucuronidase was added to 0.5 mL of each blood sample, the test-tube capped and vortex mixed for at least 5 seconds and then placed in the incubator oven at 37±2 °C overnight (15-24 hours).

The blood samples were extracted using SPE. To achieve this; all samples were diluted in 5 mL of pH 6 Buffer/deionised water (1:2) mixed solution. Opiate internal standards were used by adding 100 µL of 1 µg/mL solution to all the samples examined. The tubes were vortexed for 5 s and centrifuged for 10 min at 2500 to 3000 rpm.

SPE columns (UCT® Clean Screen ZSDAU020 cartridges) were conditioned by sequentially adding 3 mL methanol, 3 mL water and 1 mL pH6 phosphate buffer. The prepared samples were poured onto the conditioned column and allowed to drain. Each column was then washed by the sequential addition and elution of 3 mL deionised water, 2 mL acetate buffer 0.1M pH 4.5, and 3 mL methanol and dried under full vacuum for 10 minutes. Elution was performed by adding 3 mL dichloromethane: isopropanol: ammonia (78:20:2).

The solvent was evaporated under nitrogen at ≤ 37 ^{o}C until dry and the residue was derivatised with 50 µL BSTFA+1% TMCS at 90 ^{o}C for 15±2 min. After cooling at room temperature, they were transferred to clean GC autosampler vials.

B) LC-MS/MS method:

The LC-MS/MS method detected morphine, M3G and M6G without hydrolysis. To achieve this, whole blood samples were extracted using protein precipitation as follows; 800 µL of methanol was added to 200 µL of blood spiked with 100 µL of mixed internal standard solution, as detailed in section 4.3.2.5. Samples were vortex mixed before being centrifuged at 2000-3000 rpm for 10 minutes. Following centrifugation, the supernatant was transferred to a 3.5 mL vial using a glass pasteur pipette. Samples were evaporated under nitrogen gas, with the

heating block set at 25 °C. Once the samples had been evaporated they were reconstituted with 200 µL mobile phase (90/10: DH_2O/Methanol). This was then transferred into correctly labelled LC autosampler vials and 10 µL injected into LC/MS/MS.

6.2.3.1 Statistical Method Comparison

Free morphine concentrations from the original FMS results were compared to the free morphine concentrations of the repeat analyses.

Since the true concentrations of each morphine glucuronide were not known by the FMS method, the total morphine concentrations from the original FMS results were compared to the calculated total morphine concentrations of the repeat results (Morphine + M3G + M6G).

The comparative data was used to evaluate the performance of the transferred LC/MS/MS method. Pearson correlation, an estimated 95% confidence interval and a regression equation (Equation 6-1) describing the line of best fit between the results of two methods was calculated and the standard error of this regression slope determined for each drug.

Several authors have agreed that the Pearson correlation and the test of significance (95% confidence interval) may be misleading and do not reflect the actual agreement between two methods. In the case of the Pearson correlation, the results obtained by the two labs could be highly correlated with a systematic difference between them. It has also been discussed how a high correlation may be associated with a considerable lack of agreement between two instruments. In addition, the range of the results significantly affects the value of the correlation coefficient: the higher the range, the higher the value of the correlation coefficient [392-397].

Equation 6-1: Pearson's correlation coefficients (r)

$$(\mathrm{r}) = \frac{n(\sum xy)(\sum x)(\sum y)}{\sqrt{(n\sum x^2 - (\sum x)^2) - (n\sum y^2 - (\sum y)^2)}}$$

Bland and Altman suggested using a plot, with bias and precision statistics, to determine agreement between methods. The Bland-Altman plot considers the proportion between the magnitude of measurements and the error graphically,

but not quantitatively. The plot uses the difference between the two methods against their means. This allows investigation of any possible relationship between the measurement error and the true value. Since the true value is not known, the mean of the two measurements is the best estimate available. Consequently, agreement between the two measurements was tested by calculating the systemic error (bias), and the 95% limits of agreement as bias ± 2 SD, as described by Bland and Altman (2010) [395].

6.2.4 Results and Discussion

A summary comparing the two methods used by the FMS laboratory GC-MS method and the repeat LC-MS/MS method are given in Table 6-1.

Table 6-1: Method parameters used by the FMS Lab and the new LC-MS/MS Method.

Drugs	FMS Lab			LC-MS/MS Method		
	LOD mg/L	LLOQ mg/L	Calibration Ranges (mg/L)	LOD (mg/L)	LLOQ (mg/L)	Calibration Ranges (mg/L)
Free Morphine	0.010	0.025	0.025 – 0.50	0.010	0.050	0.05 – 0.75
Total Morphine	0.010	0.025	0.025 – 0.50	-	-	-
M3G	-	-	-	0.010	0.050	0.05 – 0.75
M6G	-	-	-	0.005	0.020	0.02 – 0.30

Medians, means, standard deviations, Pearson correlations and regression equations were calculated for free and total morphine, these are shown in the table below Table 6-2.

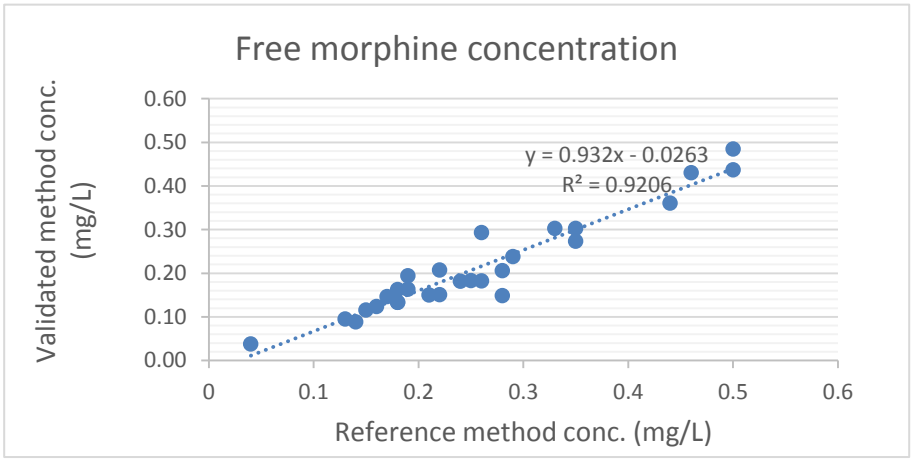
Table 6-2: Overall comparison of results for both methods on 31 samples.

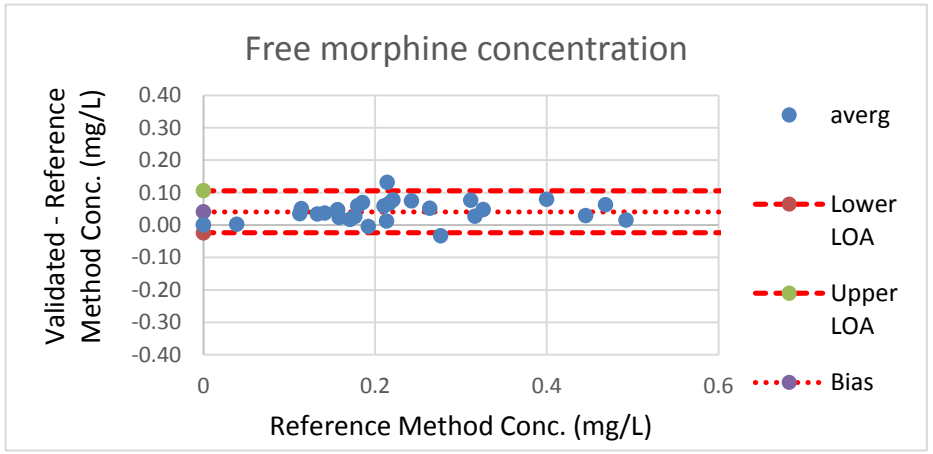
	Free Morphine		Total Morphine	
	FMS Results	LC-MS/MS Results	FMS Results	LC-MS/MS Results
Conc. Range (mg/L)	0.040-0.500	0.048-0.485	0.060-0.950	0.088-0.955
Median (mg/L)	0.230	0.181	0.510	0.459
Mean (mg/L)	0.255	0.211	0.531	0.494
SD (mg/L)	0.114	0.111	0.204	0.219

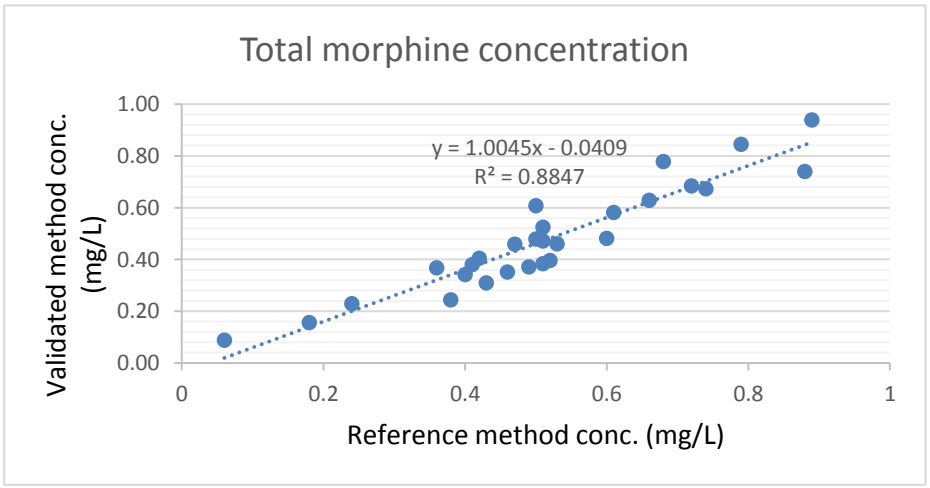
In Figure 6-1, paired results of current cases (n=31) were used to create a regression equation describing the line of best fit between two methods. The correlation coefficient for comparison of the quantitation results with the FMS laboratory methods was good, giving an R^2 of >0.92 and >0.88 for FM and TM, respectively.

Moreover, Bland-Altman plots in the current study showed that the mean difference between free morphine of the transferred method and the reference laboratory was 0.043 ± 0.032 mg/L indicating that the transferred method measured slightly higher concentrations than the reference lab method. The lower and upper levels of agreement were -0.019 and 0.107. Out of 31 samples, only 2 samples were considered outliers. The mean difference between total morphine of the transferred method and the reference laboratory was 0.037 ± 0.068 mg/L indicating that the transferred method measured slightly higher concentrations than the reference lab method and that the scatter increases when the concentration is greater than 0.5 mg/L. The lower and upper levels of agreement were -0.097 and 0.171. Out of 31 samples, only 1 sample was considered an outlier.

Direct comparisons of the two technologies have hitherto not been performed in previous studies. However, Bland-Altman plots in the current study indicated that, the results were very similar for both methods; there was an agreement between the morphine concentrations measured by the new LC-MS/MS method and the FMS laboratory GC-MS method. The results were very similar for both concentrations values; there was an agreement between the initial concentration values and the current concentration values. No differences in the morphine concentrations were observed between samples with low (0.06 mg/L) and high (0.60 mg/L) morphine concentrations, after reanalysis. Bland-Altman Plots for free and total morphine are illustrated in Figure 6-1.







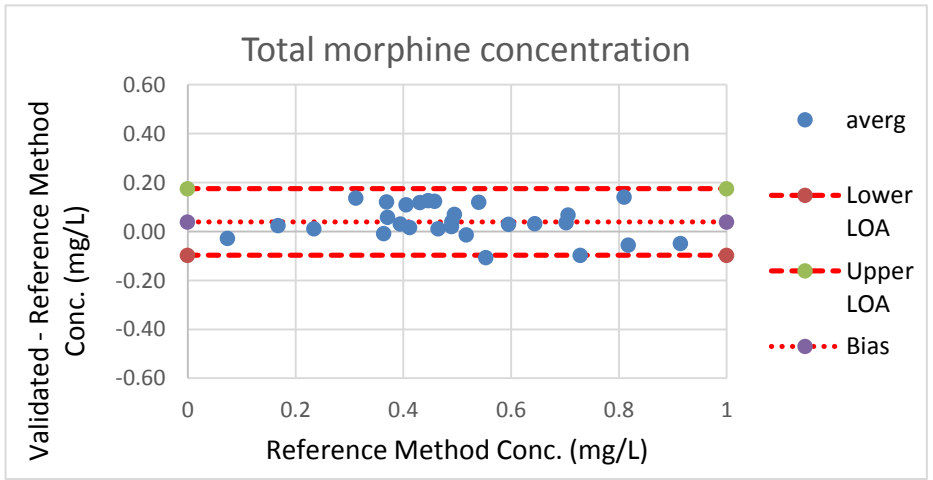


Figure 6-1: Correlation and Bland-Altman plots evaluation of the Validated method versus the Reference method of analysis for FM and TM

6.2.5 Conclusion

The statistical evaluation of these two methods of analysis for morphine and its glucuronides show that they are comparable. There is no difference statistically between the methods of measurement and consequently the LC-MS/MS method described in Chapter 4.5 can be used to analyse samples in long-term storage and these results can be compared to the original FMS laboratory results.

6.3 Stability of Morphine and its Glucuronides in Samples Stored Long-Term

6.3.1 Introduction

Postmortem examinations carried out in the West of Scotland requiring toxicological investigation are routinely tested for illicit drugs and stored in the refrigerator for approximately 3 months while investigations are on-going before being moved to long-term storage at less than -8^{o}C. This study was designed to understand the stability of morphine and its glucuronides, in real postmortem blood samples, after storage in the freezer for a long period of time.

6.3.2 Ethical Considerations

The same ethical considerations were given as in section 6.2.2 and was approved by the West of Scotland Research Ethics Service (WoSRES), (reference: 17/WS/0102) in Appendix 2. The only difference to this study was the length of time the samples have been in storage, however the ethical considerations concerning testing were the same.

6.3.3 Methodology

Two hundred and twenty-six postmortem femoral blood samples were selected for analysis. They all had to be positive for 6MAM either in the blood and/or urine to make sure of heroin use. Although the use of additional codeine or morphine could not be excluded. This is significant when talking about the ratio of free/total morphine and suggesting a timescale between injection and death. These were all currently being stored in the freezer over a period of 1-8.5 years. The initial analysis was carried out by FMS laboratory using their accredited GC-MS method. The reanalysis of the blood was carried out using the validated LC-MS/MS method described in Chapter 4.

Free morphine concentrations from the original FMS results were compared to the free morphine concentrations of the repeat analyses. Since the true concentrations of each morphine glucuronide were not known by the FMS method, the total morphine concentrations from the original FMS results were compared to the calculated total morphine concentrations of the repeat results (Morphine + M3G + M6G).

The statistical data of Pearson correlations and regression equations were calculated for both free and total morphine and their ratio. The closeness of agreement between the initial analysis and the reanalysis in the present study was examined in each case by comparison of the free and total morphine concentrations measured at each time. The agreement between the two groups was tested by calculating the systemic error (bias), and the 95% limits of agreement as bias ± 2 SD, as described by Bland and Altman.

For analyte stability compared to time scale, stability of the analytes was measured as change in concentration from the time of the original analysis to the time of the reanalysis and expressed as the concentrations were within upper and lower limit of agreement. Changes within upper and lower limit of agreement were considered to be within the analytical variation for any of the analytes.

6.3.4 Results and Discussion

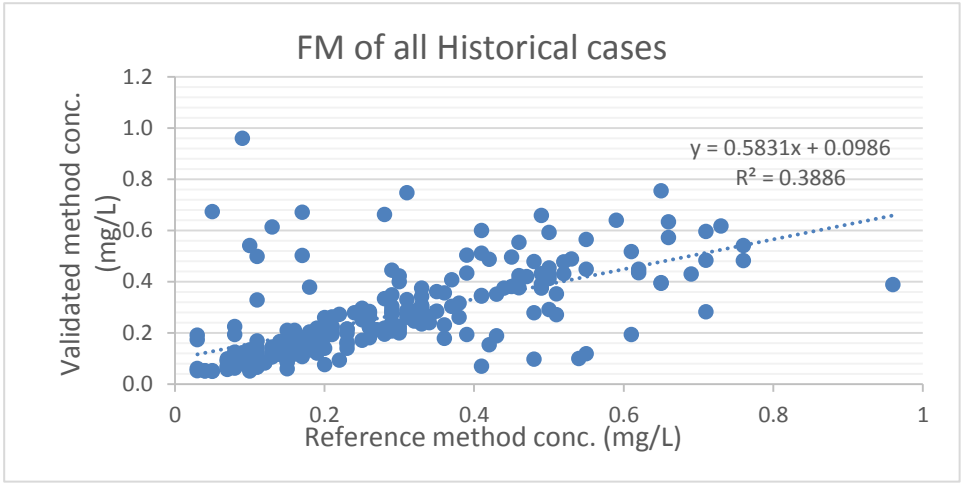
The range of storage time for the 226 femoral blood samples analysed was 666 to 3092 days with a median of 1160 days. The statistical analysis of Pearson correlations and closeness of agreement between the initial analysis and the reanalysis were calculated for both free and total morphine and their ratio as follows:

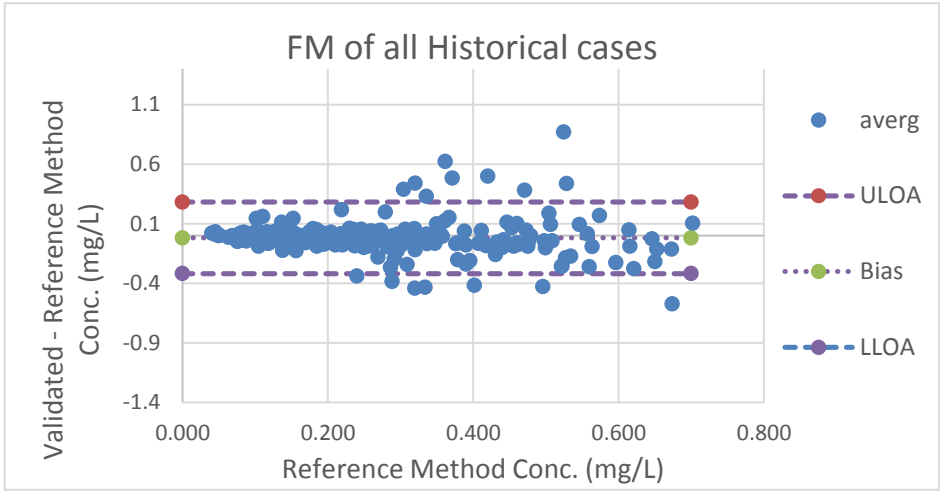
A) Free and Total Morphine

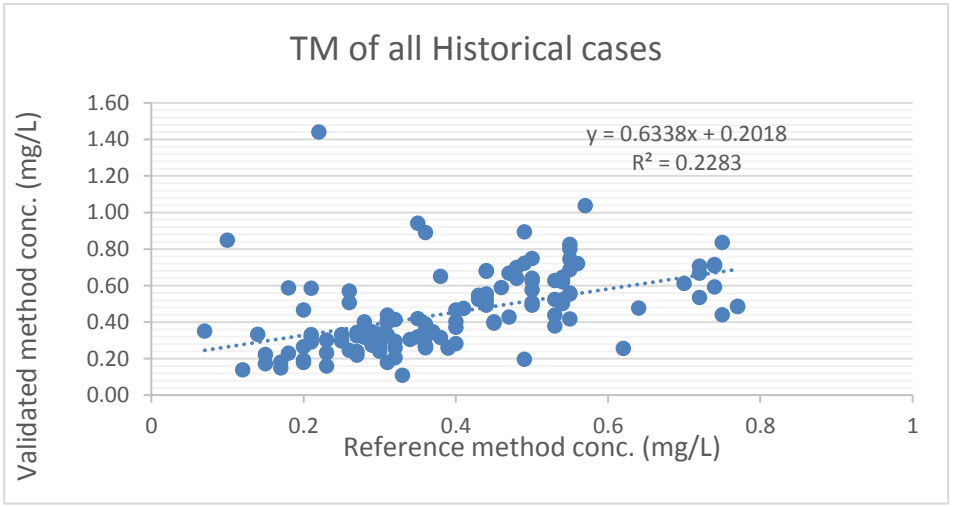
Paired results of historical cases were used to create a regression equation describing the line of best fit between initial analysis and reanalysis. The correlation coefficient comparing these 2 results was R^2 = 0.38 and 0.22 for free and total morphine, respectively in Figure 6-2 below.

The closeness of agreement between the morphine concentrations of the initial test results and the re-test results were examined in each case and Bland-Altman plots were used. For free morphine, the mean difference between the transferred method and the reference laboratory was -0.018 ± 0.153 mg/L indicating that the concentration of free morphine is relatively unstable during long-term storage at -20^{o}C. The lower and upper levels of agreement were -0.318 and 0.282. Out of 226 samples, only 16 samples were considered outliers.

For total morphine, the mean difference between the transferred method and the reference laboratory was -0.057 ± 0.195 mg/L indicating that the concentration of total morphine is relatively unstable during long-term storage at -20^{o}C. The lower and upper levels of agreement were -0.326 and 0.441. Out of 132 samples, only 6 samples were considered outliers. The difference from the initial concentrations given in Bland-Altman plots, are also shown in Figure 6-2.







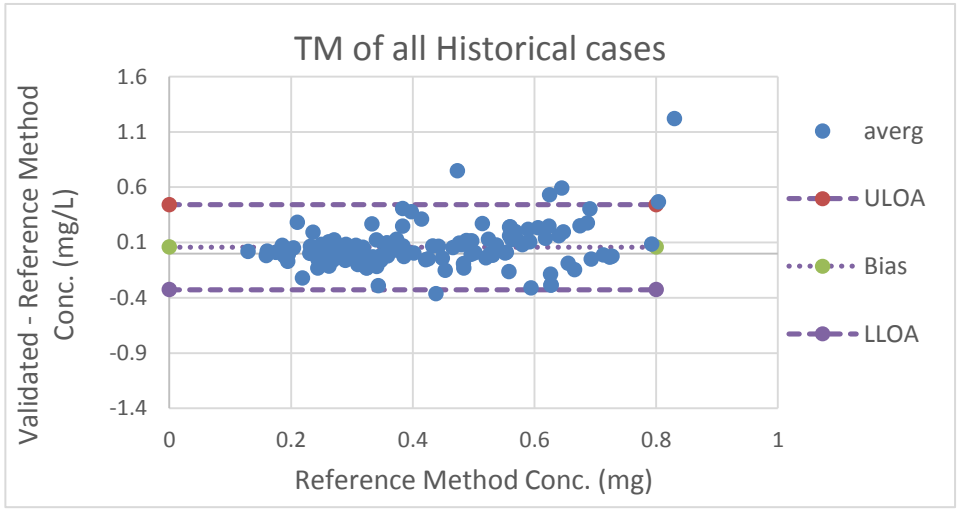


Figure 6-2: Correlation and Bland- Altman plots of the initial analysis versus the reanalysis of FM and TM concentrations of all historical cases.

Figure 6-3 shows the stability of FM compared to the time scale, no trend was observed over time. Moreover, in 94.2% (213 of the 226) of free morphine samples re-analysed, the concentrations were within upper and lower limits of agreement. Decreased concentrations were found in 2.6% (6 of the 226) of the samples and increased in 3.0% (7 of the 226).

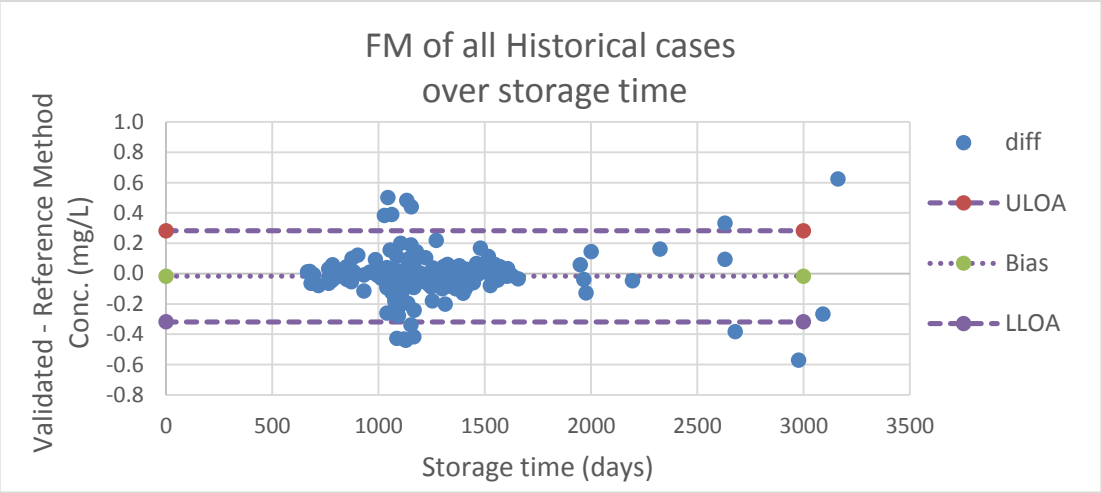


Figure 6-3 Percentage concentration changes in FM of 274 historical cases after storage at -20°C for a period time ranged from 1 – 10 years

Figure 6-4 shows the stability of TM compared to the time scale, no trend was observed over time. Moreover, in 92.4% (122 of the 132) of total morphine samples re-analysed, the concentrations were within upper and lower limits of agreement. Decreased concentrations were found in 3.0% (4 of the 132) of the samples and increased in 4.5% (6 of the 132).

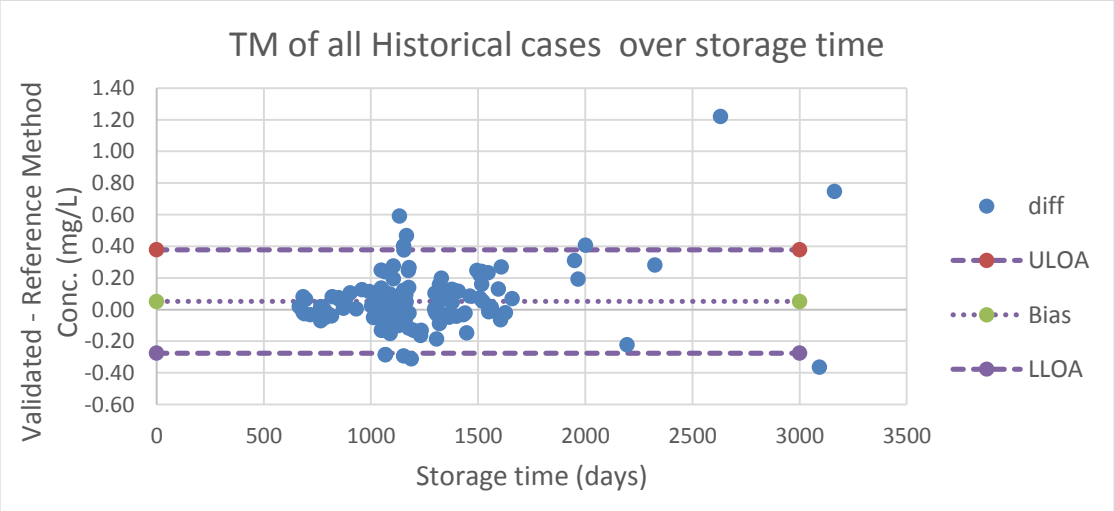


Figure 6-4: Percentage concentration changes in TM of 274 historical cases after storage at -20°C for a period time ranged from 1 – 8.5 years

The results were very similar for both concentration values; there was an agreement between the initial concentrations and the re-test concentrations. No differences in the morphine concentrations were observed between samples with low (0.06 mg/L) and high (0.60 mg/L) morphine concentrations, after storage. Overall the differences were not considered to be marked enough to invalidate any statistical significance derived from, or interpretations based on, the re-test data.

B) Free / Total morphine ratio

The correlation coefficient for free to total morphine ratio between the two groups gave an R^2 of 0.54. The mean difference between the initial test results and the re-test results was -0.072± 0.139. The lower and upper levels of agreement were -0.345 and 0.202. Out of 132 samples, only 10 samples were considered outliers, as shown in Figure 6-5 and Figure 6-6.

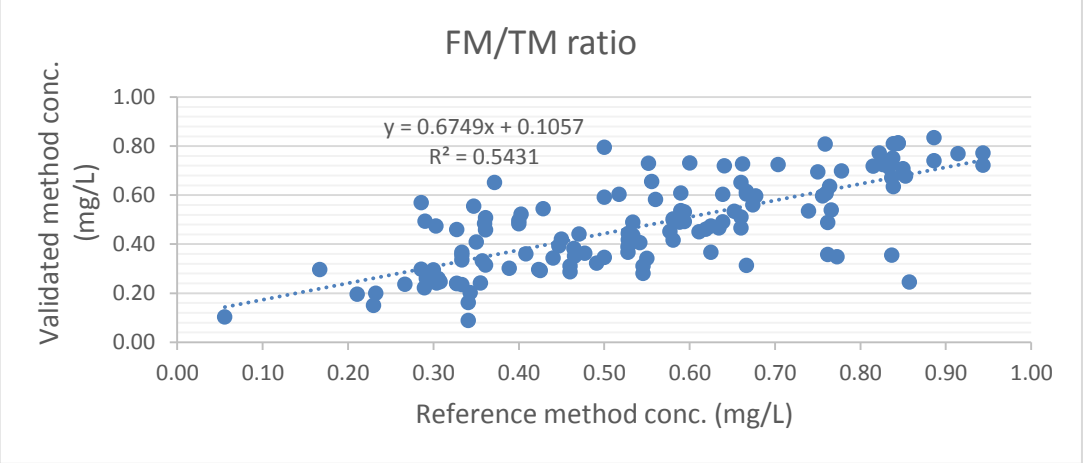


Figure 6-5 Correlation of the initial analysis versus the reanalysis of FM and TM concentrations of all historical cases.

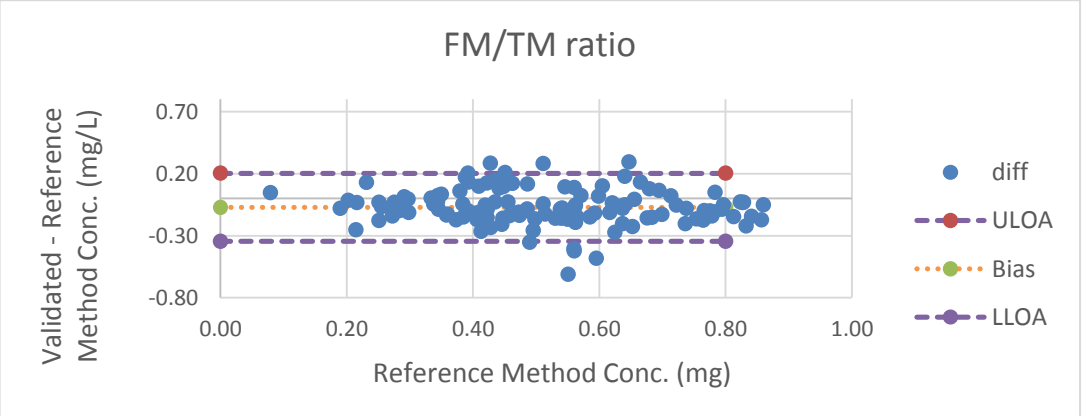


Figure 6-6: Bland- Altman plot of the initial analysis versus the reanalysis of FM and TM concentrations of all historical cases.

Bland-Altman plots in the current study showed that, the results were very similar for both groups, there was an agreement between all analytes and their ratio values in the initial test results and the re-test results, the results reflect the agreement betw een them.

Altogether, 226 authentic, postmortem samples positive for FM and TM were reanalysed after 1–8.5-year storage at less than -8 ºC. It appeared that for most of the drugs no significant change in concentration takes place during storage at -20 ºC. Therefore, in 92 % of the samples reanalysed, the results for all drugs present were within upper and lower limit of agreement of the initial concentration.

These results are in concordance with previous studies in blood [70, 303, 313]. The long-term stability of opioids in authentic postmortem blood samples was previously studied. 73 samples were reanalysed after storage at -20°C for 16-18 years. Samples containing morphine showed the results within acceptance criteria ±30% of the initial concentration [313]. Also, in another separate study, total morphine and free morphine were stored in glass culture tubes (without preservatives) and stored at room, refrigerator, and freezer temperatures and analysed at 30-day intervals for an 11-month period. Total morphine concentration decreases (10 to 40%) were observed for all specimens in all storage conditions, whereas free morphine showed slight but steady increases [70].

In a previous study, the long-time stability of real-life postmortem blood samples (n = 37) and living person blood samples (n = 22) was investigated. All samples contained fluoride and were initially analysed and stored in normal conditions (-20ºC) for 4–9 years. The results showed that, the concentrations of morphine are relatively stable during long-term storage at -20ºC [303]. However, Giorgi and Meeker found that, blood samples from living subjects (not spiked) showed morphine concentrations decreased significantly after two years of storage in tubes containing sodium fluoride and potassium oxalate; the tubes were stored at ambient temperature, but with further storage, increased concentrations were observed [75].

6.4 Stability of Morphine and its glucuronides in Paired Preserved and Unpreserved Postmortem Blood Samples

6.4.1 Introduction

Postmortem examinations carried out in the West of Scotland requiring toxicological investigation are routinely tested for drugs. Femoral blood samples are collected and submitted as preserved and unpreserved samples. The preserved samples stored within blood collection tubes contain chemical additives (screw cap vials containing 0.2% sodium fluoride and potassium oxalate), which in combination with the pre-determined vacuum guarantee the correct mixing ratio for the blood sample.

Following submission to the toxicology laboratory within FMS, the preserved sample is reserved for alcohol testing and may also be tested for known labile drugs e.g. cocaine, the unpreserved blood sample is tested for a wide range of prescription and illicit drugs including opiates. All blood samples are stored in the refrigerator for approximately 3 months while investigations are on-going before being moved to a freezer for long-term storage.

For this reason, the stability of morphine, M3G and M6G in blood was carried out by studying the effect of the preservative (sodium fluoride and potassium oxalate) in sample tubes and the storage temperature.

6.4.2 Ethical Considerations

The same ethical considerations were given as in section 6.2.2 and was approved by the West of Scotland Research Ethics Service (WoSRES), (reference: 17/WS/0102) in Appendix 2. The only difference to this study was the effect of the preservative in sample tubes and the storage temperature, however the ethical considerations concerning testing were the same.

6.4.3 Methodology

Twenty-eight paired preserved and unpreserved blood samples were analysed after being stored in the refrigerator for three months and a further ~2 years following storage at -20ºC. The initial analysis of unpreserved blood samples was carried out by FMS using the accredited method (GC-MS). The reanalysis of the

paired unpreserved and preserved blood was carried out using the validated method described in Chapter 4. FM, M3G and M6G were quantified in these cases using a calibration curve which was linear over the range of 0.02-0.30 mg/L for M6G and 0.05-0.75 mg/L for M3G and FM.

6.4.4 Results and Discussion

Medians, means, standard deviations, Pearson correlations and regression equations were calculated for free and total morphine, these are shown in the Table 6-3.

Table 6-3: Overall comparison of results for both methods.

		Conc. Range (mg/L)	Mean ± SD (mg/L)	Median (mg/L)
FM	Preserved	0.054-0.720	0.231 ± 0.18	0.161
	Unpreserved	0.050-0.555	0.25 ± 0.163	0.215
M3G	Preserved	0.010-0.980	0.243 ± 0.221	0.166
	Unpreserved	0.000-0.917	0.243 ± 0.225	0.162
M6G	Preserved	0.013-0.324	0.061 ± 0.063	0.037
	Unpreserved	0.015-0.223	0.064 ± 0.057	0.038

Paired results of both groups were used to create a regression equation describing the line of best fit between two groups. Pearson correlations and Bland-Altman Plots of (FM, M3G and M6G) concentration were calculated. The closeness of agreement between the preserved and unpreserved in the present study was examined in each case by comparison of all the analyte concentrations measured at each time. The agreement between the two groups was tested by calculating the systemic error (bias), and the 95% limits of agreement as bias ± 2 SD.

A) Free morphine

The correlation coefficient for comparison of the quantitation results between two groups was good, giving an R^2 of >0.73. The mean difference between the preserved and unpreserved was -0.019 ± 0.094mg/L indicating that the concentrations of free morphine is relatively unstable during long-term storage of unpreserved samples at -20^{o}C. The lower and upper levels of agreement were -0.203 and 0.165. Out of 28 samples, only 3 samples were outliers (~11% of the total number of compared tests), as shown in Figure 6-7 and Figure 6-8.

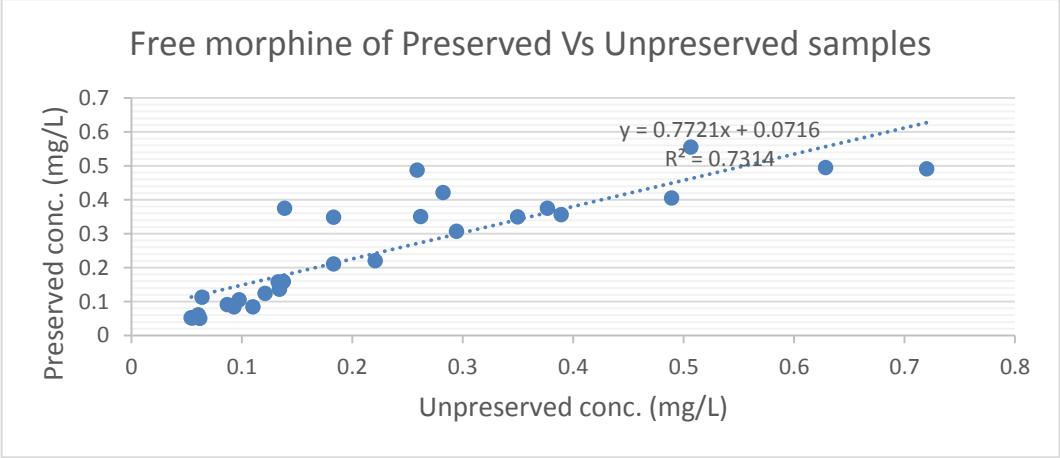


Figure 6-7 Pearson correlations of preserved and paired unpreserved historical samples

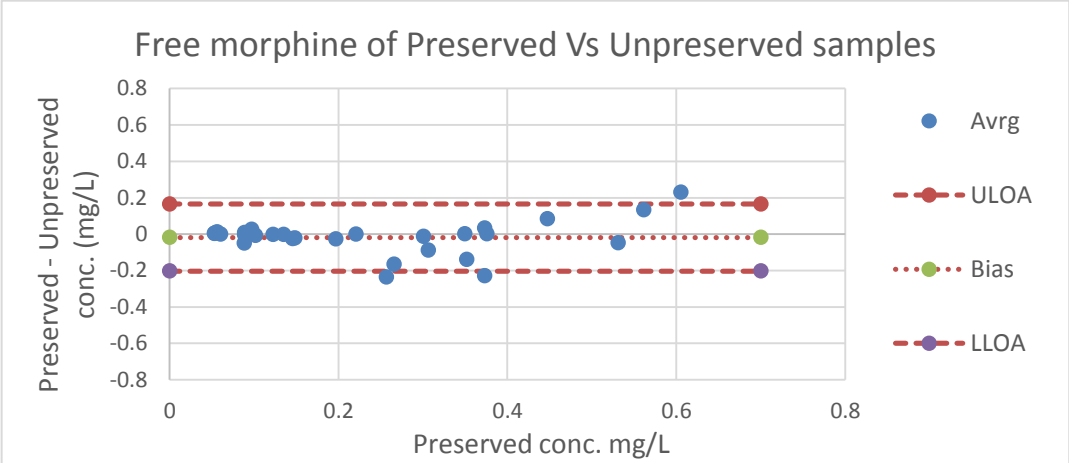


Figure 6-8: Bland and Altman Plot of preserved and paired unpreserved historical samples

B) M3G

The correlation coefficient for comparison of the quantitation results between two groups was good, giving an R^2 of 0.90. The mean difference between the preserved and unpreserved was -0.019 ± 0.066 mg/L indicating that the concentrations of M3G is relatively unstable during long-term storage of unpreserved samples at -20°C. The lower and upper levels of agreement were -0.149 and 0.111. Out of 24 samples, only 1 sample was an outlier (~4% of the total number of compared tests), as shown in Figure 6-9 and Figure 6-10.

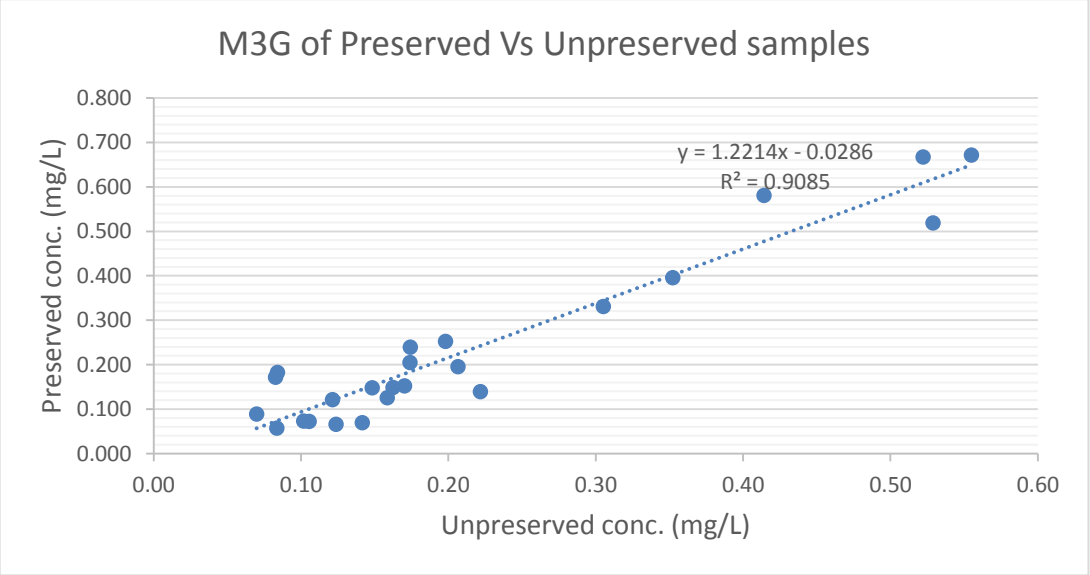


Figure 6-9 Pearson correlations of preserved and paired unpreserved historical samples

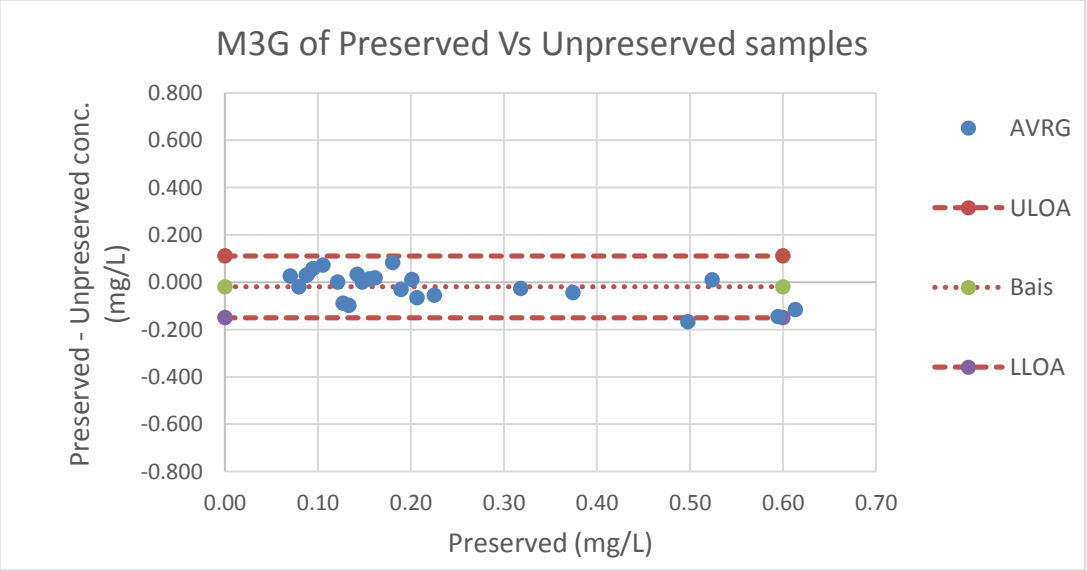


Figure 6-10: Bland and Altman Plot of preserved and paired unpreserved historical samples

C) M6G

The correlation coefficient for comparison of the quantitation results between two groups was good, giving an R^2 of ~0.80. The mean difference between the preserved and unpreserved was -0.006 ± 0.024 mg/L indicating that the concentrations of M6G is relatively unstable during long-term storage of unpreserved samples at -20°C. The lower and upper levels of agreement were -0.053 and 0.040. Out of 26 samples, only 3 samples were outliers (~12% of the total number of compared tests), as shown in Figure 6-11 and Figure 6-12.

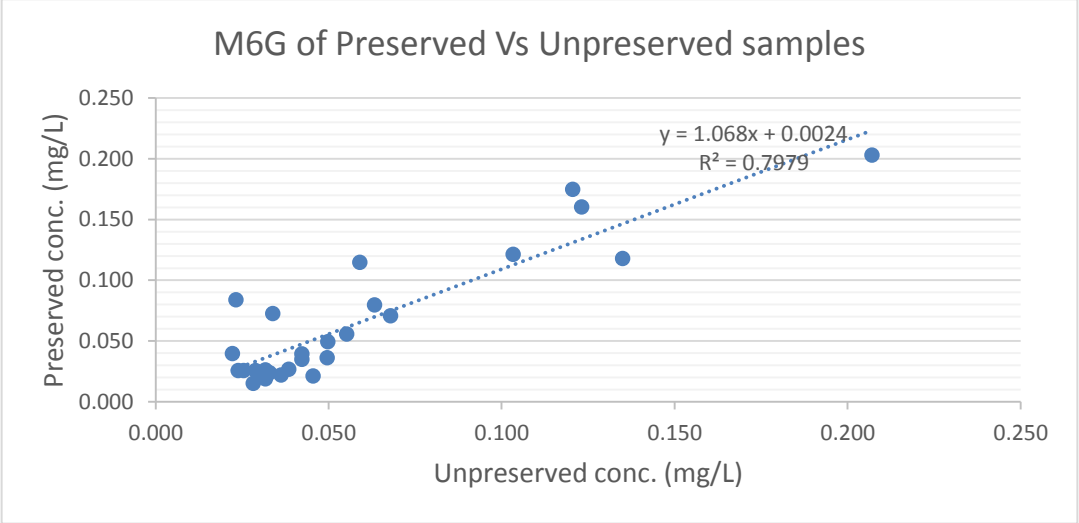


Figure 6-11 Pearson correlations of preserved and paired unpreserved historical samples

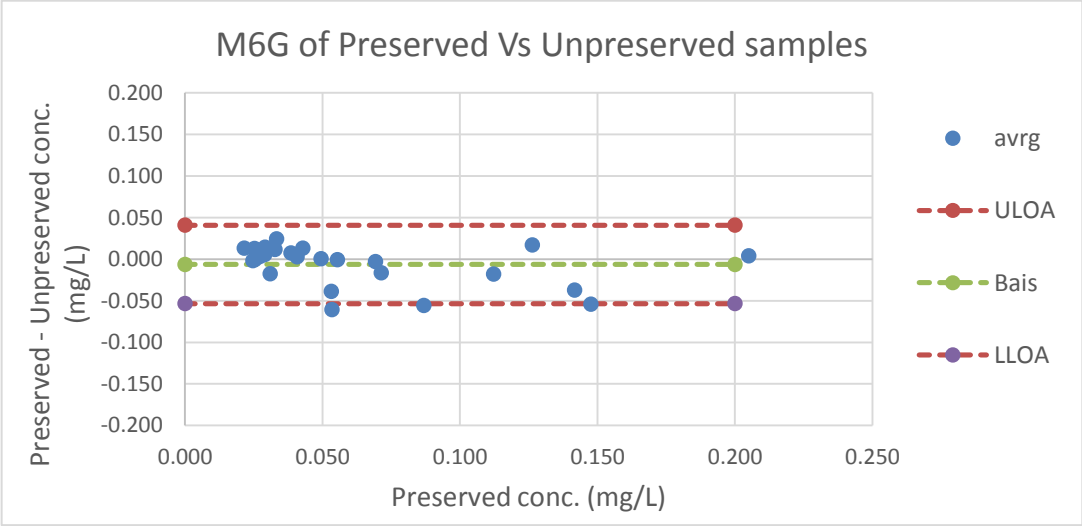


Figure 6-12: Bland and Altman Plot of preserved and paired unpreserved historical samples

Bland-Altman plots in the current study showed that, the results were very similar for both groups, there was an agreement between all analyte concentrations in the preserved and unpreserved blood samples, the results reflect the agreement between them.

The results of our stability study are in agreement with those reported by other respective studies of morphine [51, 69, 75, 274, 311] in blood samples. Some limited data concerning the stability of these three analytes in blood are also published, but during a method validation process [354-356, 398]. To our knowledge, it is the first stability study of FM, M3G and M6G in blood concerning the effect of the addition of preservative and the storage temperature in real case samples.

However, Carroll *et al*. have demonstrated further that the hydrolysis of M3G to free morphine in *vitro* occurs and may persist for months in antemortem and postmortem specimens under various conditions, despite using tubes containing sodium fluoride and potassium oxalate for inhibition of bacterial growth [294]. Rees, K.A., *et al*. (2011) reported that the addition of NaF slowed but did not prevent the breakdown of 6MAM in blood stored at room temperature for 84 days [262]. Other studies have shown a considerable decrease in 6MAM concentrations, even with the addition of NaF, hydrolytic activity may persist for months [294, 303].

However, other studies have highlighted the importance of preserving postmortem blood samples to inhibit bacterial growth. The stability of morphine and 6MAM in blood was studied after different sampling conditions with or without the addition of sodium fluoride (NaF). Spiked blood samples were stored at two different temperatures (4 and -20°C) for 3 months. The addition of NaF as preservative improved the stability of opioids at all conditions studied [302]. Spiehler and Brown (1987) found the ratio of free morphine to total morphine to be stable in postmortem blood preserved with 1% NaF and potassium oxalate after more than a year of storage at room temperature [250].

6.4.5 Conclusions

From the parameters studied, under storage temperatures of -20°C, there were statistically no significant differences ($p < 0.05$), between the same blood samples with and without sodium fluoride and potassium oxalate. When sodium fluoride and potassium oxalate were added to the samples, the addition of preservative did not influence significantly the stability of FM, M3G and M6G over different storage periods.

6.5 Study the effect of femoral blood sampling on drug concentration

6.5.1 Introduction

When investigating drug-related deaths, it is routine practice within the mortuary at the Queen Elizabeth University Hospital, Glasgow to collect two unpreserved blood samples. The second unpreserved blood sample is retained for analysis by any potential defence representation. It is unclear if all the femoral blood is collected in one container initially, and then poured into separate vials, or if they are taken sequentially directly into each vial. The purpose of this study was to assess the homogeneity of these two unpreserved samples.

6.5.2 Methodology

The study was approved by the West of Scotland Research Ethics Service (WoSRES), (reference: 17/WS/0102) in Appendix 2. During the period from Jul 2017 to Jun 2018, one hundred and twenty paired unpreserved femoral blood samples were analysed after being stored at -20ºC up to 10 years using the validated method described in Chapter 4 and 5. Of the one hundred and twenty postmortem femoral blood samples analysed not all had concentrations of the 3 analytes which were within the calibration range. 58, 72 and 73 results were statistically analysed because they fall within the concentration range of the validated method of each analyte FM, M3G and M6G, respectively.

The closeness of agreement between the paired samples in the present study was examined in each case by comparison of the FM, M3G, and M6G concentration measured at each time. The agreement between the two groups was tested by calculating the systemic error (bias), and the 95% limits of agreement as bias ± 2 SD, as described by Bland and Altman. The statistical data of Pearson correlations and regression equations were calculated for all analytes.

6.5.3 Results and Discussion

Paired results of both groups were used to create a regression equation describing the line of best fit between two groups. Moreover, the closeness of

agreement between the paired samples in the present study was examined in each case by comparison of all analyte concentrations measured at each time. The agreement between the two groups was tested by calculating the systemic error (bias), and the 95% limits of agreement as bias ± 2 SD.

A) Free Morphine

The correlation coefficient for comparison of the quantitation results between two groups was 42%. Bland-Altman plots in the current study showed that the mean difference between both groups was 0.012 ± 0.151 mg/L indicating that the first unpreserved group measured slightly higher concentrations than second unpreserved group and that the scatter increases when the concentration is greater than 0.4 mg/L. The lower and upper levels of agreement were -0.284 and 0.308 mg/L. Out of 58 samples, 3 samples were considered outliers ~5% as shown in Figure 6-13 and Figure 6-14. The current study showed that, there was some variation between the FM value in the paired samples from both groups.

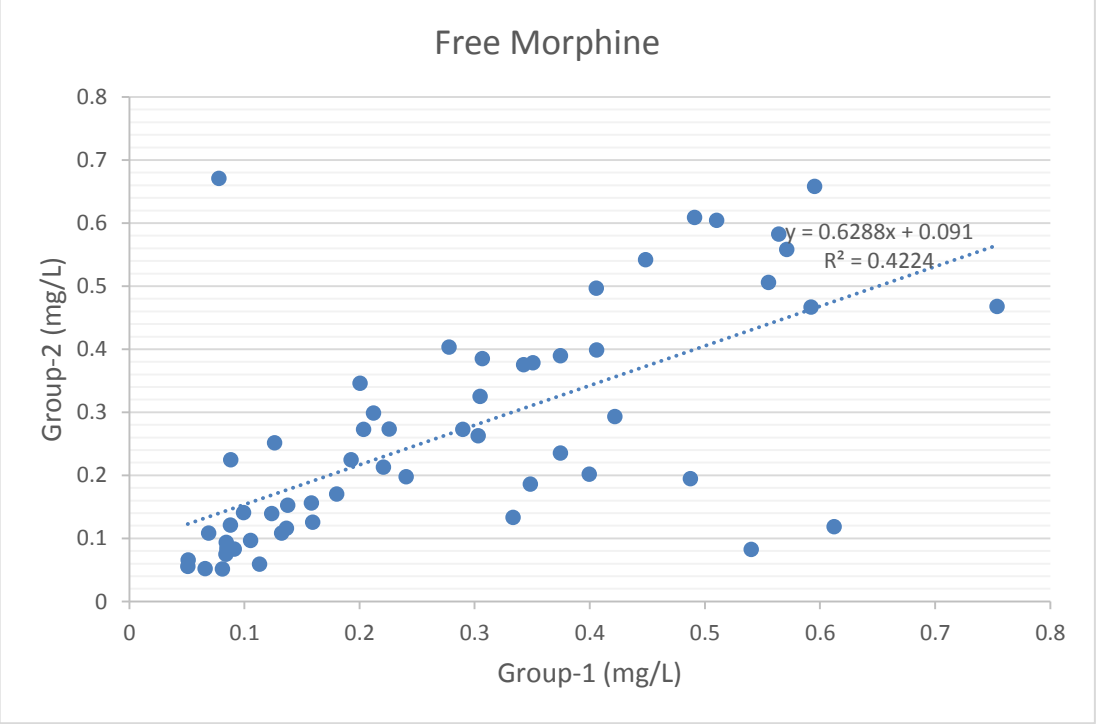


Figure 6-13 Correlation Plot of group-1 Vs group-2

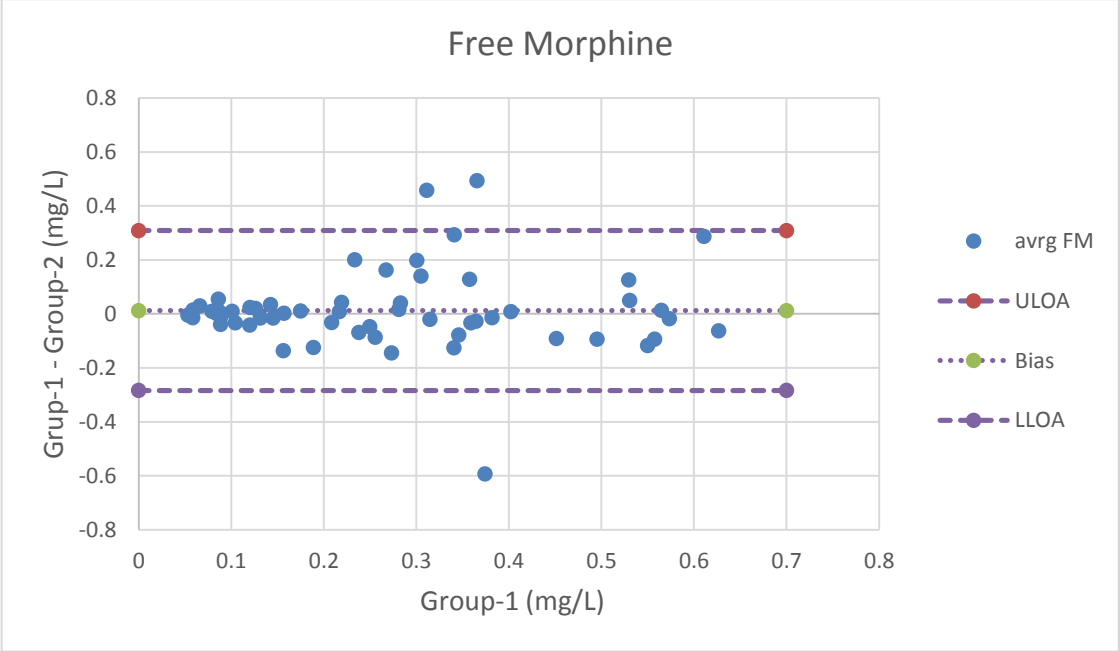


Figure 6-14 Bland-Altman Plot of group-1 Vs group-2

B) M3G

The correlation coefficient for comparison of the quantitation results between two groups was (53%), as shown in Figure 6-15. Bland-Altman plots in the current study showed that the mean difference between both groups was (-0.006 ± 0.155) mg/L indicating that the 1ST unpreserved group measured slightly lower concentrations than 2nd unpreserved group and that the scatter increases when the concentration is greater than 0.3 mg/L. The lower and upper levels of agreement were (-0.309 and 0.297) mg/L. Out of 72 samples, 5 samples were considered outliers (~7%), as shown in and Figure 6-16. The current study showed that, there was some variation between the M3G value in the paired samples from both groups.

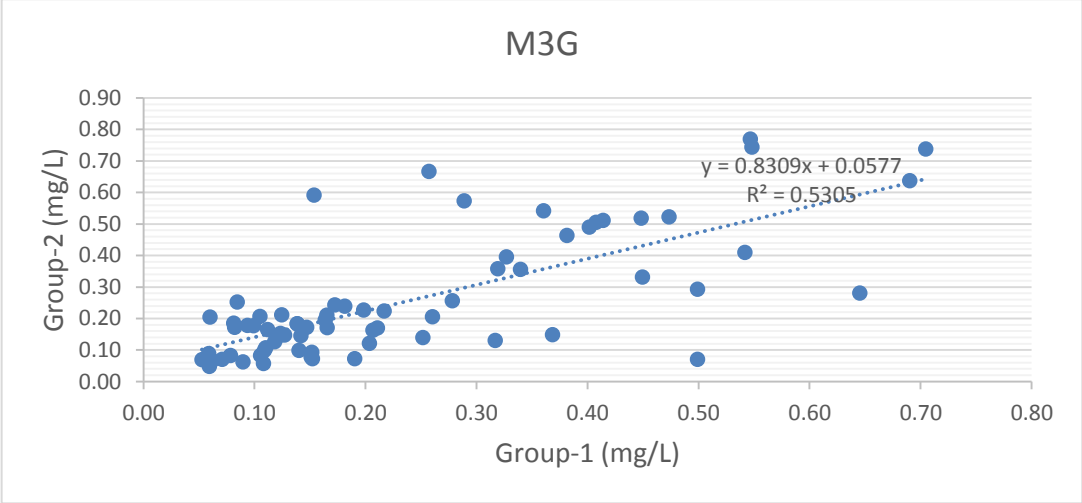


Figure 6-15 Correlation of group-1 Vs group-2

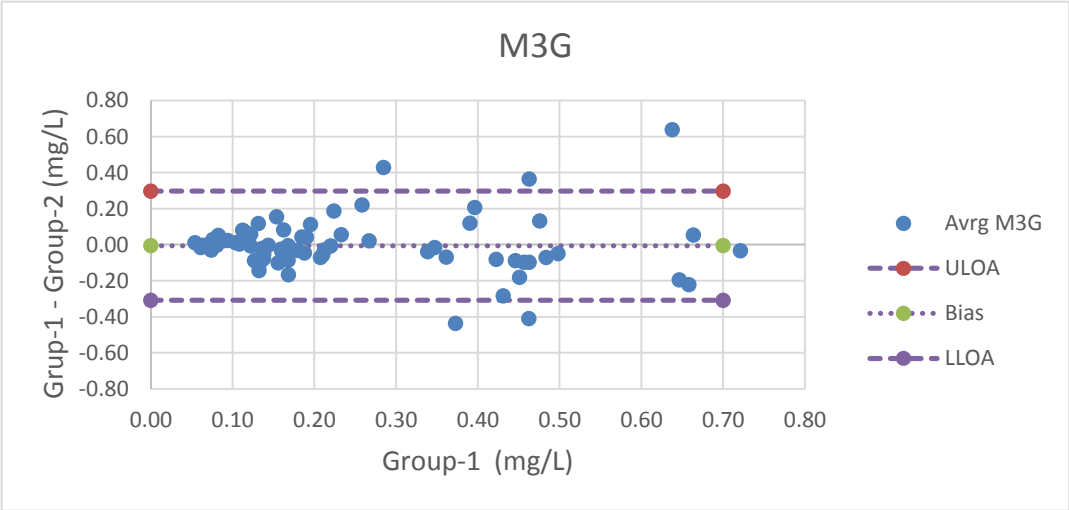


Figure 6-16: Bland-Altman Plot of group-1 Vs group-2

C) M6G

The correlation coefficient for comparison of the quantitation results between two groups was 37%, as shown in Figure 6-17. Even though both groups showed a low correlation for each analyte. Bland-Altman plots in the current study showed that the mean difference between both groups was 0.000 ± 0.031 mg/L. The lower and upper levels of agreement were -0.061 and 0.061 mg/L. Out of 73 samples, 7 samples were considered outliers (~10 %), as shown in Figure 6-18. The current study showed that, there was some variation between the M6G value in the paired samples from both groups.

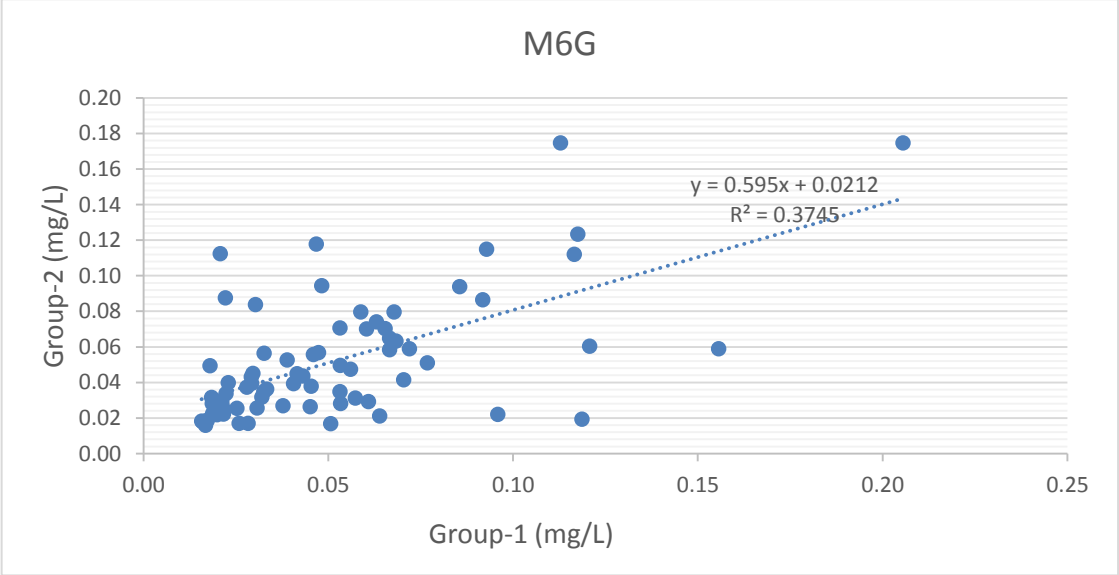


Figure 6-17 Correlation of group-1 Vs group-2

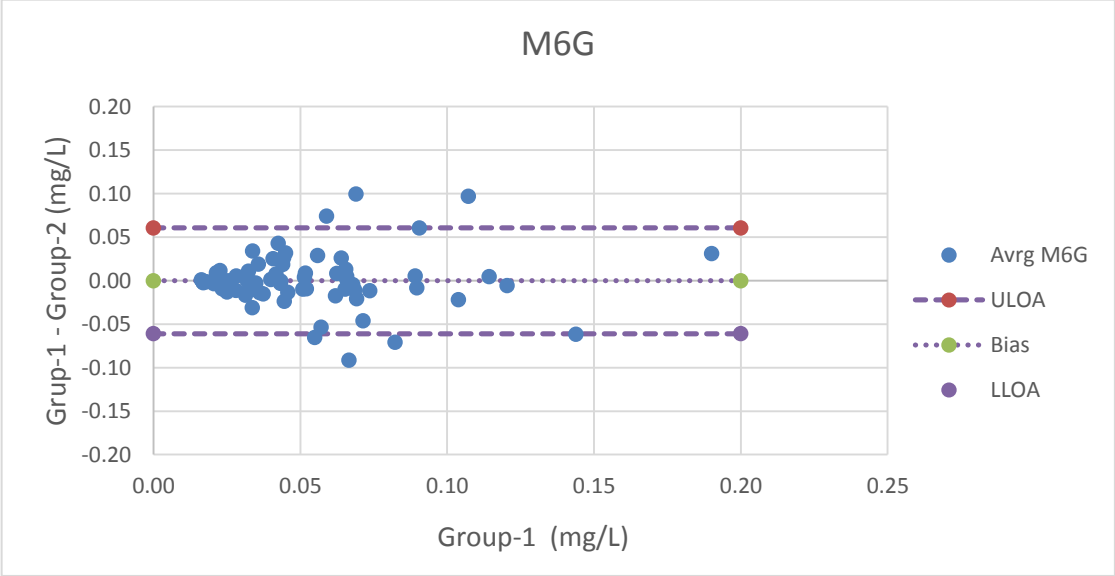


Figure 6-18: Bland-Altman Plot of group-1 Vs group-2

In general, FM, M3G and M6G had only a 42%, 53% and 37% correlation coefficient, respectively. It was clear that, there was significant variation in drug concentration between 2 unpreserved blood samples, even with both samples being collected and analysed at the same time by same method.

6.6 Stability of Opioids in Authentic and Spiked Blood

6.6.1 Introduction

The present study was designed to determine the stability of morphine and its glucuronides in three authentic postmortem blood specimens as well as in spiked fresh blood for a time interval of up to one month. The samples were stored in glass vials at 4^{o}C and 25^{o}C. Samples were analysed using a protein precipitation extraction and liquid chromatography mass spectrometry for isolation and quantitation, providing a sensitive and specific detection method for the parent drug in the presence of its glucuronide metabolites as detailed in sections 5.2.4 and 5.2.5. It is the first stability study of FM, M3G and M6G, comparing spiked blank blood and authentic post mortem blood samples.

6.6.2 Methodology

The validated method was applied on real postmortem femoural blood samples and additional spiked blood samples. These samples had been kept at less than -8^{o}C after they were originally received and analysed. Ethical issues surrounding the use of blood samples specifically for this project has been mentioned in (section 6.2.2) and (Appendix 2). Three groups of spiked blank blood samples were prepared using the method of extraction and analysis mentioned in sections 5.2.5 and 4.5. The first group was spiked with only morphine, the second group spiked with only M3G and the last group was spiked with only M6G. In order to achieve that, the drug working solution was prepared fresh and blood samples were spiked with high concentration (QC3) of each analyte as detailed in section 4.3.2.7.

All prepared samples were divided into two groups and were then stored at 4 ^{o}C and 20 ^{o}C. The times selected for analysis were at day 0, 1, 2, 3, 4 and 5 for room temperature and day 0, 7, 14, 21 and 30 for refrigerator. All blood samples were tested in duplicate and the mean concentration of two replicates of each analyte was considered to be the day zero concentration. They were analysed in two replicates with freshly prepared calibrators and QCs run in the same batch. Each time point samples were analysed, two replicates were taken for analysis from the storage racks for each of the different storage conditions, extracted by

protein precipitation within the day and left to run on the LC-MS/MS instrument overnight as detailed in chapter 4.

6.6.3 Results and Discussion

The stability results from the experiments of FM, M3G and M6G in 3 historical cases and spiked blood sample after storage at 4˚C and 20˚C are presented in percentage concentration changes.

A) Morphine

At room temperature, morphine in both real case samples (1, 2 and 3), and spiked blood was stable for 5 days with insignificant changes (12%, 11%, 3% and 4%) of the original concentrations, respectively.

In the refrigerator (4°C), morphine in both real case samples (1, 2 and 3), and spiked blood was stable for 30 days with insignificant changes (6.1%, 3.2%, 12.9% and 16.1%) of the original concentrations, respectively. All results shown in Figure 6-19 and Figure 6-20.

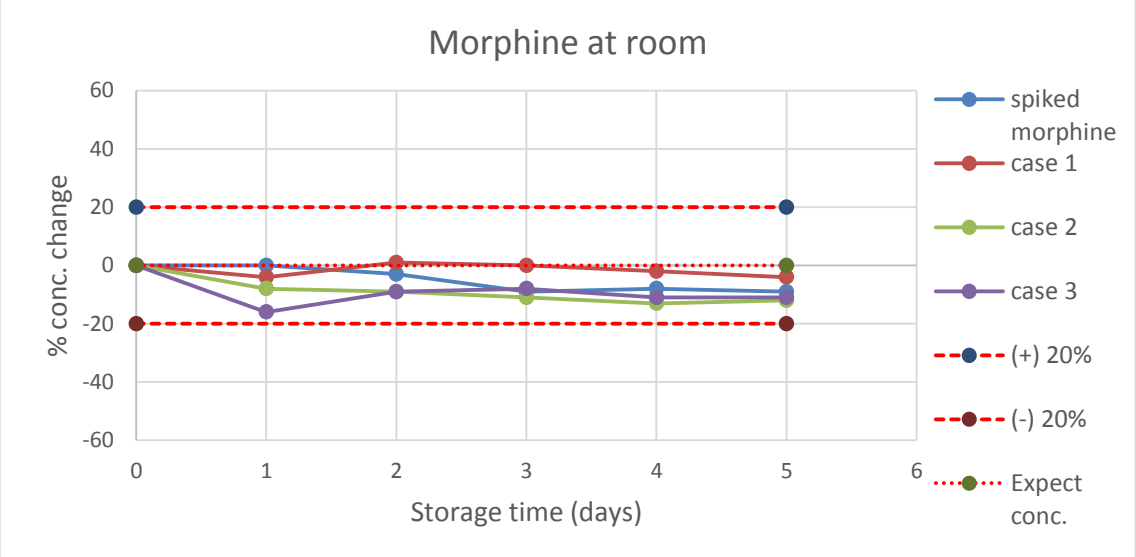


Figure 6-19 Percentage concentration changes in FM of 3 historical cases and spiked blood sample after storage at room temperature.

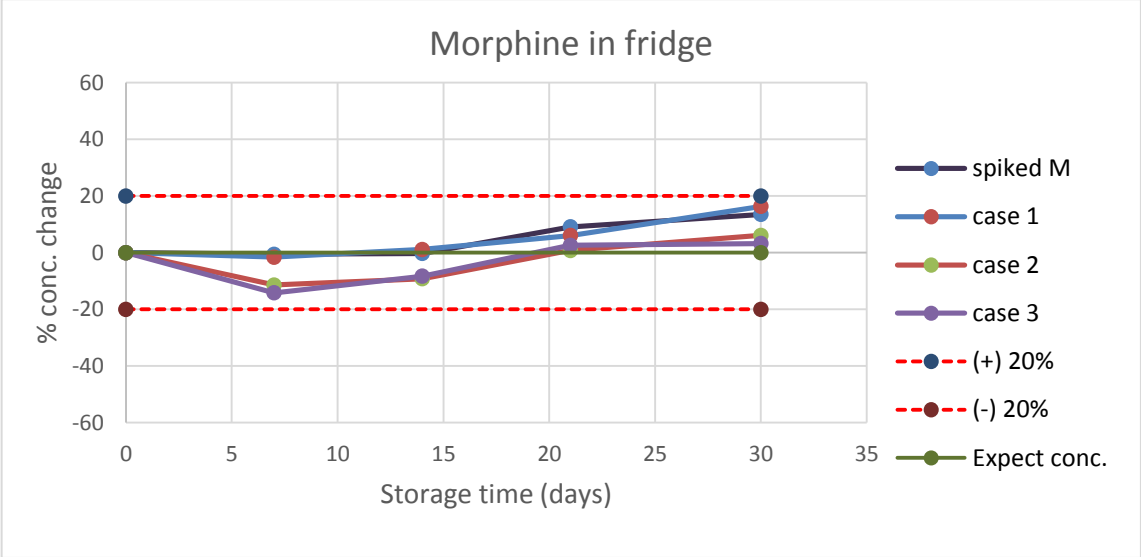


Figure 6-20 Percentage concentration changes in FM of 3 historical cases and spiked blood sample after storage at 4°C.

B) M3G

At room temperature, M3G was stable for 5 days in both real case samples (1, 2 and 3), and spiked blood with insignificant changes (8%, 13%, 18% and 13%) of the original concentrations, respectively.

In the refrigerator (4 ^{o}C), M3G concentration was stable for 30 days in both real case samples (1, 2 and 3), and spiked blood with insignificant changes (7.1%, 4%, 4.7% and 12.7%) of the original concentrations, respectively. All results shown in Figure 6-21 and Figure 6-22.

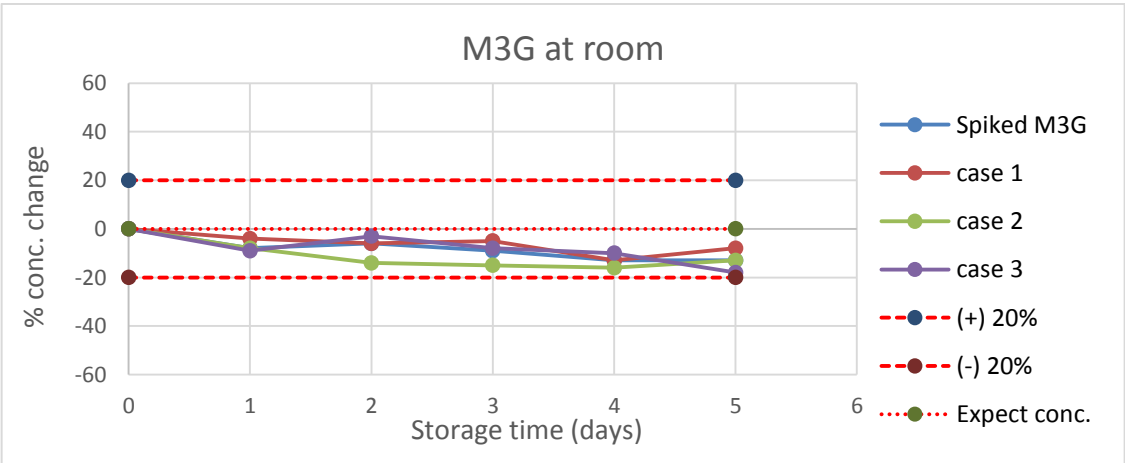


Figure 6-21: Percentage concentration changes in M3G of 3 historical cases and spiked blood sample after storage at room temperature.

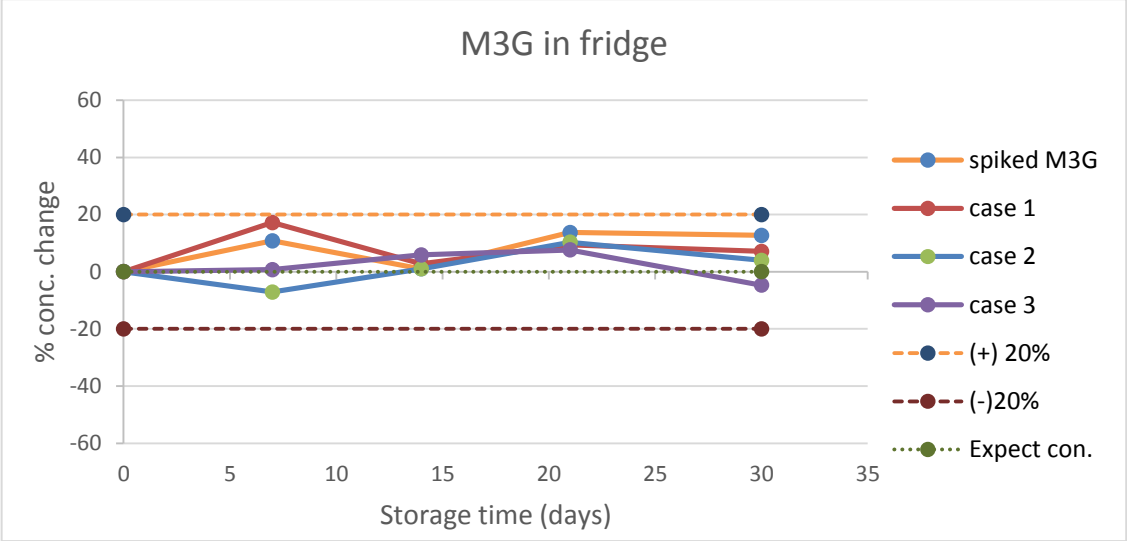


Figure 6-22 Percentage concentration changes in M3G of 3 historical cases and spiked blood sample after storage at 4°C

C) M6G

At room temperature, M6G concentration was stable for 5 days in both real case samples (case 1, 2 and 3), and spiked blood with insignificant changes (4%, 13%, 13% and 1%) of the original concentrations, respectively.

In the fridge, M6G concentration was stable for 30 days in both real case samples (case 1, 2, and 3), and spiked blood with insignificant changes (2, 2.7, 10.7% and 16.7) of the original concentrations, respectively. All results shown in Figure 6-23 and Figure 6-24.

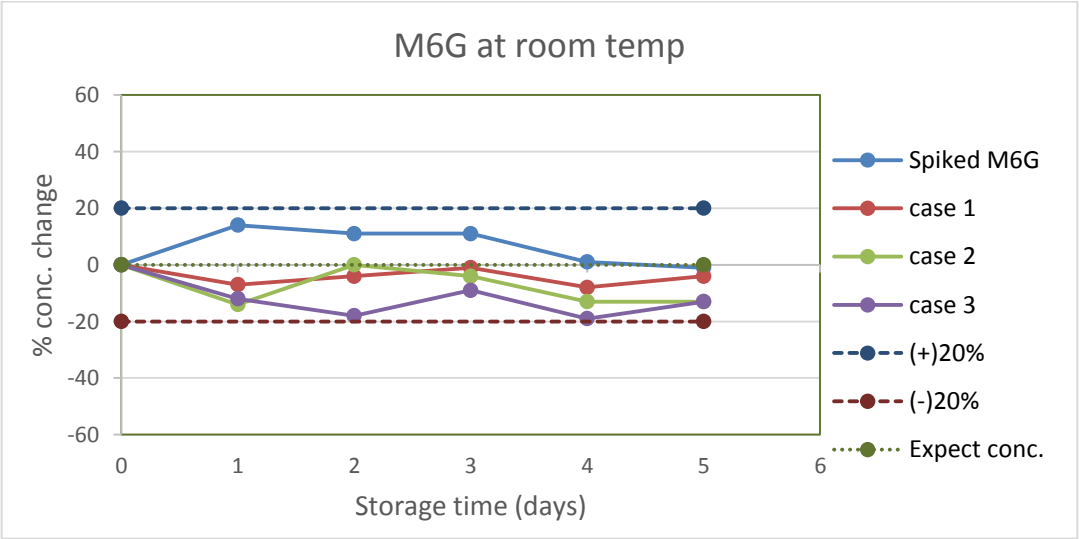


Figure 6-23 Percentage concentration changes in FM, M3G and M6G of 3 historical cases and spiked blood sample after storage at room temperature

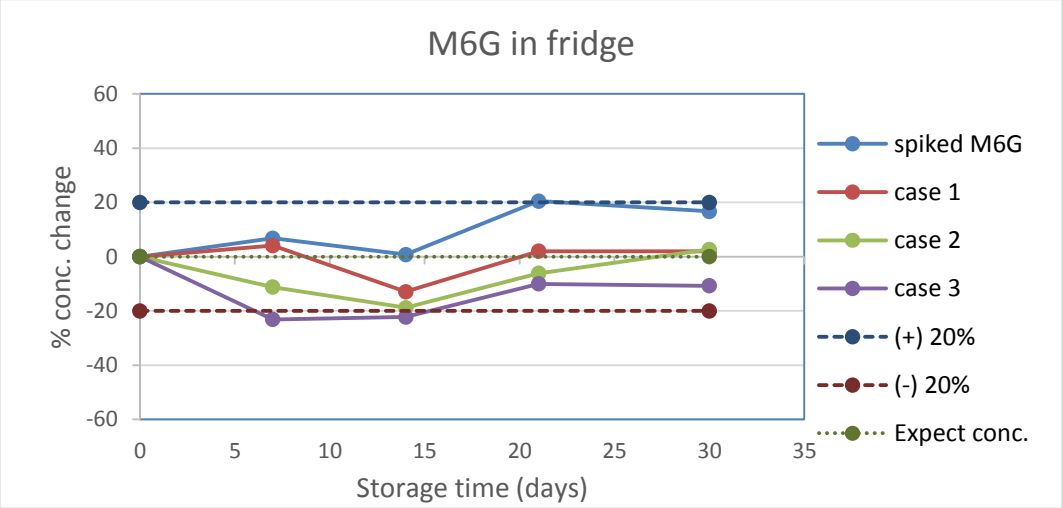


Figure 6-24: Percentage concentration changes in FM, M3G and M6G of 3 historical cases and spiked blood sample after storage at 4°C.

Interestingly, morphine was detected in the M3G-spiked blood samples to some extent after storage in refrigerator and room temperature. However, morphine concentrations detected were below the LLOQ. As expected, morphine cannot be observed in M6G-spiked samples, potentially due to the low concentrations which were spiked. Moreover, M3G and M6G were only detected in their respective spiked samples under the same conditions.

Opioid concentrations in this study seem to provide little support for the concept of bioconversion of morphine to its metabolites. No systematic patterns indicating formation or degradation of any of the investigated analytes could be established in this material. It warrants further investigations of opioid bioconversion with higher concentrations than the one used presently.

Overall in authentic postmortem samples, the degradation pattern was similar to that of spiked blood specimens, where no significant changes were observed throughout the time period. Concentrations of morphine and its metabolites did not change significantly in samples stored at 4 ºC and 20 °C when expressed as a percentage of the initial concentration.

These results are in concordance with previous studies in blood. Holmgren *et al.* (2004) have investigated long-time storage in post mortem blood samples (not spiked). They found excellent stability for morphine in storage at -20°C during 12 months [268] and another two separated studies for three months [69, 311]. In spiked samples from living subjects, Giorgi *et al*, (1195) found excellent

stability for morphine and their metabolites during 6 months of storage at 4°C and -20°C in blood [75]. Several studies have also found that, morphine with their metabolites remained stable at least 6 days at room temperature [72-74]

Inconsistent with other studies, in spiked post mortem samples analytes were stable for 11 months of storage in both 4°C and -20°C, some decrease in the concentration of M3G and M6G and an apparent increase in morphine concentration was observed at room temperature which could be the breakdown of M3G and M6G to morphine over time [312]. Skopp *et al*. (2001) also found that, in spiked post mortem samples, the analytes were stable only when stored at -20°C [69].

6.7 Morphine Ratios and Survival Time since Death

6.7.1 Introduction

Monitoring blood concentrations of morphine and its metabolites is important for the understanding of time between last injection of heroin and death. The ratio between the blood morphine concentration and the respective glucuronide concentrations, in addition to the presence of 6MAM in the blood is suggestive of survival times following heroin administration. In this chapter, we investigate the morphine and morphine metabolites, together with their ratios, in order to achieve a comprehensive interpretation of time since death.

Some toxicology laboratories report the concentration of free-morphine in blood when investigating heroin-related deaths whereas others report total-morphine (free + conjugated), which is the sum of free-morphine along with the amounts released after hydrolysis of the morphine-3-glucuronide and morphine-6-glucuronide metabolites [254, 258]. The use of free morphine (FM) to total morphine (TM) concentration ratios as a means of evaluating the time of survival following heroin or morphine injection has been advocated in several studies [134, 250, 254, 256, 264-267] and may be of use in cases in which 6MAM is not detectable in the blood (a high FM/TM ratio is thought to reflect insufficient time for morphine metabolism to occur and thus be indicative of a relatively rapid death) [135, 268, 269].

6.7.2 Methodology

The study was approved by the West of Scotland Research Ethics Service (WoSRES), (reference: 17/WS/0102) in Appendix 2. During the period from July 2017 to June 2018, two hundred and forty-six postmortem femoral blood samples were analysed for free morphine, M3G and M6G using the validated LC-MS/MS method described in Chapter 4 and 5. All of these samples had previously been analysed for free morphine, total morphine and 6MAM by FMS using an accredited GC-MS method and then stored at -20 ^{o}C over a period of 1-8.5 years until re-analysed.

All cases were selected if they had 6MAM positive in the blood and/or urine to confirm the samples were from heroin users. The samples were classified into

two groups; the first group (n=36) was positive for 6MAM in the blood and the second group (n=210) was positive for 6MAM in the urine only. Both groups were further divided into two subgroups: first subgroup was FM/TM ratio greater than or equal to 0.5 (suggesting quick death) and second subgroup was FM/TM ratio lesser than 0.5 (suggesting delayed death), as illustrated in Figure 6-25.

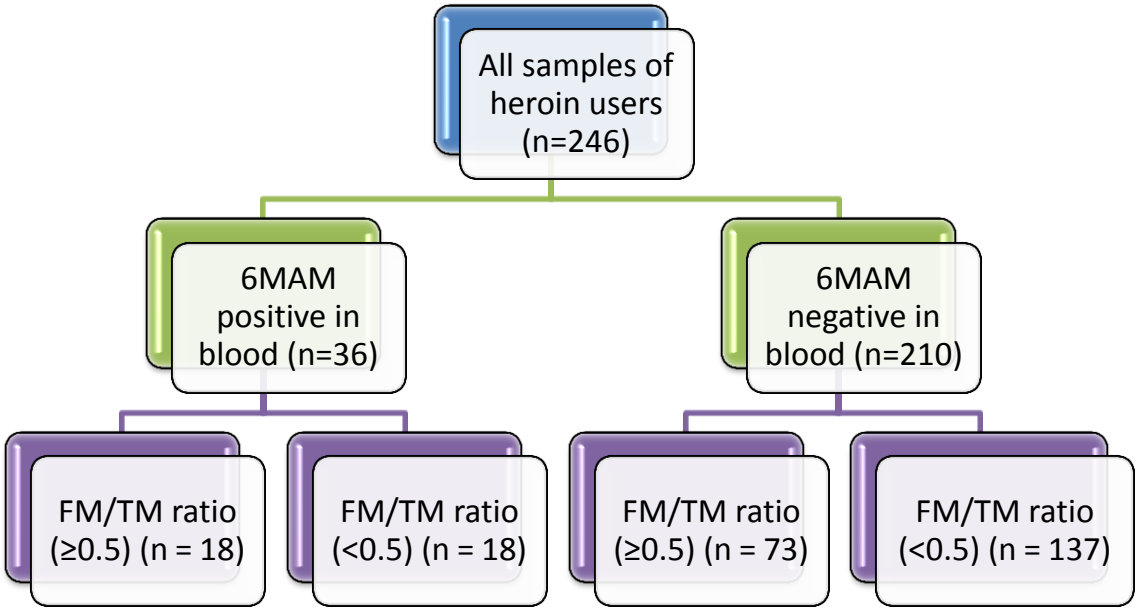


Figure 6-25: Distribution of all postmortem blood samples according to 6MAM and FM/TM ratios

6.7.3 Results and Discussion

Looking at the range for the different parameters that we have investigated in 246 postmortem blood samples, we found that, FM concentration ranged from 0.051 – 0.740 mg/L and mean 0.252 mg/L, M3G ranged from 0.058 – 0.738 mg/L and mean 0.255 mg/L, and M6G ranged from 0.020 – 0.206 mg/L and mean 0.056 mg/L. Distribution of Morphine, M3G and M6G in 6MAM positive and negative cases are detailed in Table 6-4.

Table 6-4: Distribution of Morphine, M3G and M6G in 6MAM blood positive and negative cases

	6MAM Present in blood (n=36)		6MAM Absent in blood (n=210)	
	FM/TM ratio (≥0.5) (n = 18)	FM/TM ratio (<0.5) (n = 18)	FM/TM ratio (≥0.5) (n = 73)	FM/TM ratio (<0.5) (n = 137)
	Median, (Range) mg/L	Median, (Range) mg/L	Median, (Range) mg/L	Median, (Range) mg/L
FM	0.494 (0.239 - 0.671)	0.297 (0.070 - 0.639)	0.624 (0.135 - 0.740)	0.380 (0.051 - 0.467)
M3G	0.178 (0.063 - 0.330)	0.340 (0.105 - 0.575)	0.309 (0.058 - 0.487)	0.602 (0.062 - 0.738)
M6G	0.052 (0.022 - 0.154)	0.069 (0.021 - 0.175)	0.088 (0.020 - 0.121)	0.134 (0.020 - 0.206)
TM	0.724 (0.402 - 1.142)	0.705 (0.197 - 1.362)	0.983 (0.258 - 1.215)	1.044 (0.175 - 1.286)

6.7.3.1 6MAM blood positive heroin cases

6MAM was detected in 14.6% (*n* = 36) of all cases. All 6MAM positive cases were divided into two groups; in first group, 6MAM-positive cases with a higher FM/TM ratio, and in second group, 6MAM-positive cases with a lower FM/TM ratio.

A) First group (6MAM positive with FM/TM ratio ≥0.5)

According to FM/TM ratios, 50% (n=18) of 6MAM positive cases was detected with higher FM/TM ratio (≥0.5). This group was characterised by low M3G/TM and M6G/TM ratios; 100% of this group had lower M3G/TM ratio (≤0.4). Also, this group was characterised by low M6G/TM ratio; 83.3% of this group had a lower M6G/TM ratio (≤0.09). Further details are displayed in Figure 6-27 and Table 6-5.

B) Second group (6MAM positive with FM/TM ratio <0.5)

According to FM/TM ratios, 50% (n=18) of 6MAM positive cases was detected with lower FM/TM ratio (<0.5). This group was characterised by high M3G/TM and M6G/TM ratios; 100% of this group had higher M3G/TM ratio (>0.4). While, 55.6% of this group had higher M6G/TM ratio (>0.09). Further details are displayed in Figure 6-26 and Figure 6-27 and Table 6-5.

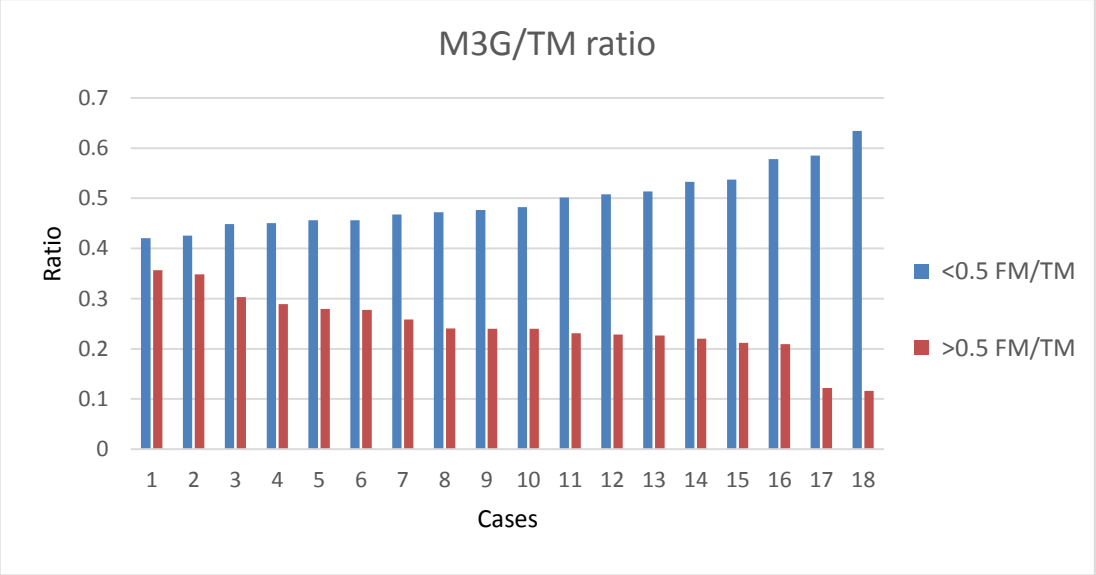


Figure 6-26 M3G/TM ratio in 6MAM blood positive cases

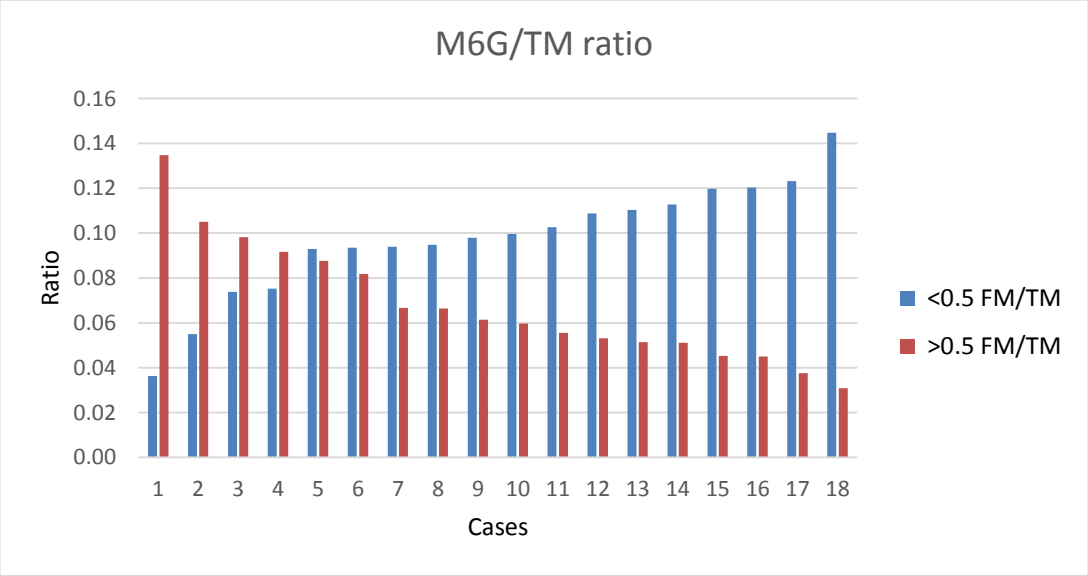


Figure 6-27: M6G/TM ratio in 6MAM blood positive cases

6.7.3.2 6MAM negative in blood of heroin cases

This group was characterised by 6MAM being negative in blood and positive in urine. 85.4% (*n* = 210) of this group was detected from all heroin cases. This group was divided into two subgroups; in first subgroup, 6MAM-negative cases with a higher FM/TM ratio, and in second group, 6MAM-negative cases with a lower FM/TM ratio.

A) First group (6MAM negative with FM/TM ratio ≥0.5)

According to the FM/TM ratio, 34.8% (n=73) of 6MAM negative cases was detected with higher FM/TM ratio (≥0.5). This group characterised by low

M3G/TM and M6G/TM ratios; 93.5% of this group had lower M3G/TM ratio (≤0.4). In addition, 69.8% of this group had lower M6G/TM ratio (≤0.09). As shown in Figure 6-29 and Table 6-5.

B) Second group (6MAM negative with FM/TM ratio <0.5)

According to the FM/TM ratio, 65.2% (n=137) of 6MAM negative cases was detected with lower FM/TM ratio (<0.5). This group characterised by high M3G/TM and M6G/TM ratios; 97.3% of this group had higher M3G/TM ratio (>0.4). In addition, 72.5% of this group had higher M6G/TM ratio (>0.09). As shown in Figure 6-28 and Figure 6-29 and Table 6-5.

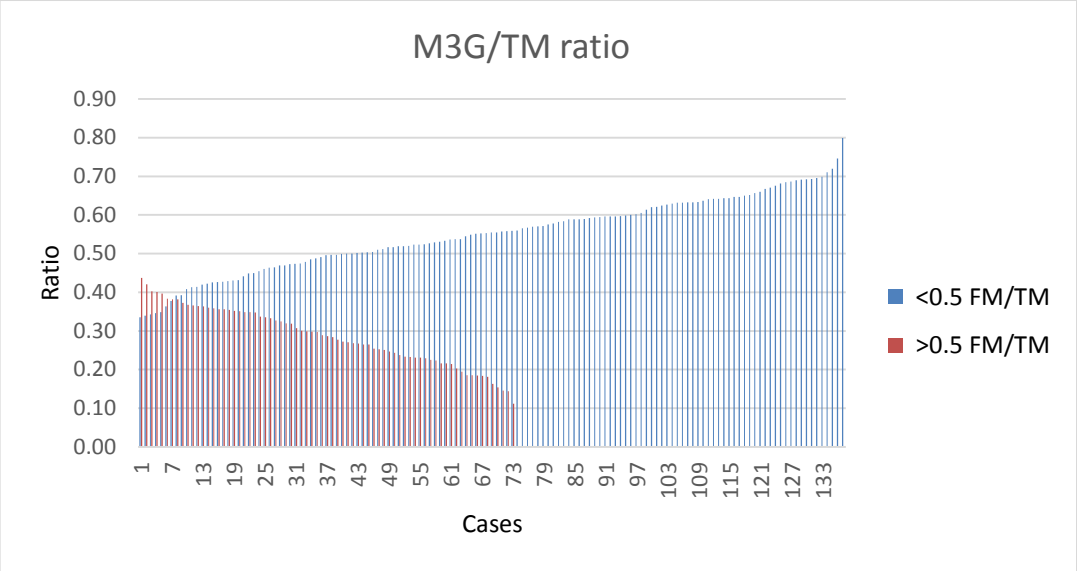


Figure 6-28 M3G/TM ratio in 6MAM blood negative cases

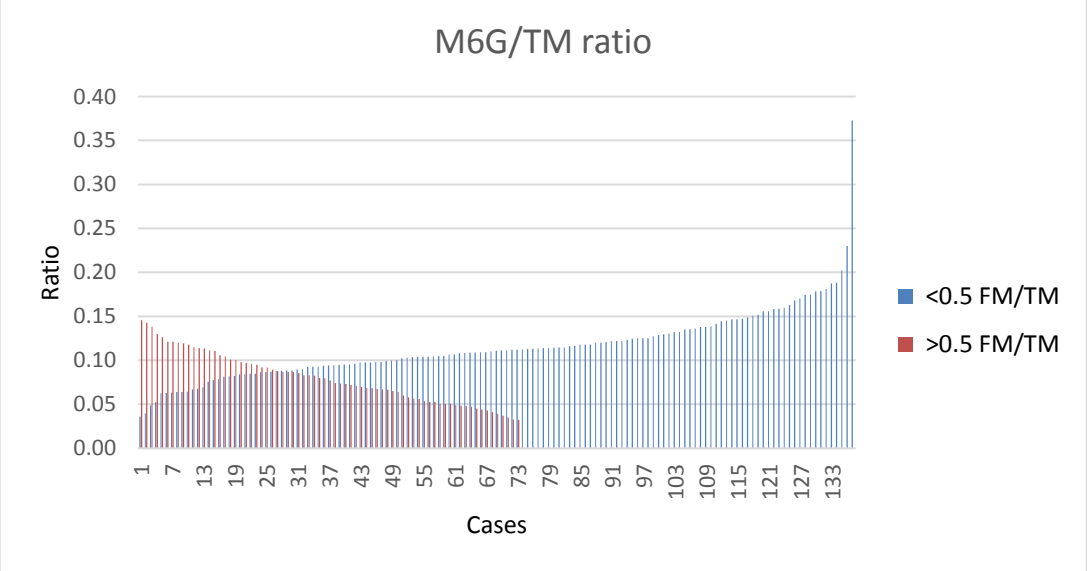


Figure 6-29: M6G/TM ratio in 6MAM blood negative cases

Firstly, in comparing the 6MAM positive-cases (rapid death) with FM/TM ratio in blood, 50% (n=18) of 6MAM-positive cases had a higher FM/TM ratio (≥0.5) and the other 50% (n=18) of 6MAM-positive cases had a lower FM/TM ratio (<0.5), thus a large overlap was observed. While, in comparing the 6MAM negative-cases with FM/TM ratio in blood, more than 65 % (n=137) of 6MAMnegative cases had a lower FM/TM ratio (<0.5) and less than 35% (n=73) of 6MAM-negative cases had a higher FM/TM ratio (≥0.5), some overlap was observed, as illustrated previously in Table 6-5.

Secondly, in comparing the M3G/TM with FM/TM ratio in all cases, cases with higher FM/TM ratio had significantly lower M3G/TM ratio, which is consistent with morphine metabolism. Similarly, cases with lower FM/TM ratio had significantly higher M3G/TM ratio. No significant overlap was observed between this two groups, as illustrated previously in Table 6-5.

Lastly, in comparing the M6G/TM with FM/TM ratio in all cases, cases with higher FM/TM ratio had lower M6G/TM ratio, which is consistent with morphine metabolism. Similarly, cases with lower FM/TM ratio had higher M6G/TM ratio. Some overlap was observed between these two groups, as illustrated previously in Table 6-5.

Table 6-5: Distribution of Morphine, M3G and M6G in 6MAM positive and negative cases

	6MAM Present in blood (n=36)		**6MAM Absent in blood (n=210)**	
	FM/TM ratio (≥0.5) (n = 18)	**FM/TM ratio (<0.5) (n = 18)**	**FM/TM ratio (≥0.5) (n = 73)**	**FM/TM ratio (<0.5) (n = 137)**
M3G/TM Ratio (>0.4)	0 % (n=0)	100 % (n=18)	2.7 % (n=2)	93.5 % (n=128)
M3G/TM (≤0.4)	100 % (n=18)	0 % (n=0)	97.3 % (n=71)	6.5 % (n=9)
M6G/TM (>0.09)	16.7 % (n=3)	55.6 % (n=10)	30.2 % (n=22)	72.5 % (n=98)
M6G/TM (≤0.09)	83.3% (n=15)	44.4 % (n=8)	69.8 % (n=51)	28.5 % (n=39)

In this study, we divided morphine-positive cases into rapid heroin deaths, based on the detection of 6MAM in blood, and delayed heroin deaths, if 6MAM was detected in matrices other than blood. Several studies have also found higher free morphine/total morphine ratios in blood in rapid deaths compared with the

more delayed deaths [67, 250, 254], which also indicates less glucuronidation in the rapid death cases.

In the present study, we found significantly lower M3G/TM ratios in the rapid compared with the delayed death group, which is in concordance with previous studies in blood [61, 399]. Thus, our results indicate that the M3G/TM ratios generally show the same differences between rapid and delayed deaths as those of FM/TM ratio and could be useful when assessing whether death occurred rapidly or more delayed after intake of heroin.

Regarding the M6G/TM ratios, our findings were less clear than the M3G/TM ratios, where considerable overlap in the ratios was observed, but this could perhaps be caused by the generally lower concentrations of M6G compared with M3G. The strength of using ratios between metabolite and parent drug depends on the administered dose of heroin [400]. However, it must be noted that morphine glucuronides can accumulate in blood with repeated use of heroin or morphine [67, 250, 254], particularly in those with renal failure [148, 401], and the concentrations can also change after death [69, 273]. In this work, it has been assumed that morphine glucuronide accumulation was roughly the same between rapid and delayed deaths, but accumulation could perhaps explain the high morphine glucuronide/total morphine ratios found in some of the rapid death cases.

The toxicological data on morphine and its major metabolites supported 6MAM as a measure of survival times. Consistent with the few studies that have compared apparent rapid and delayed overdose deaths cases in which 6MAM was present had higher free morphine concentrations and lower concentrations of M3G and M6G than other cases [60, 254, 399]. Moreover, both M3G and M6G concentrations were independent correlates of the presence of 6MAM. Overall, the morphine ratio results were consistent with the designation as rapid or delayed death.

6.8 Conclusion

The developed and validated LC/MS/MS method for the simultaneous quantification of morphine, M3G and M6G was verified on postmortem blood which made it suitable for routine forensic toxicology. It was successfully verified using 31 authentic case samples with a correlation higher than 92 %. Bland-Altman plots showed good agreement between both methods with a mean difference of 0.043 ± 0.032 mg/L and limits of agreement were -0.019 – 0.107, 95% CI. Moreover, the study of current postmortem case samples reveals that, the concentrations of FM and TM were stable during storage in fridge for six months and no significant losses were noted.

Ideally, analyses should be performed as soon as samples are received, and the samples should be stored at -20 °C or lower to keep the drugs stable for as long as possible. 226 authentic, postmortem samples positive for FM and TM were reanalysed during 8.5-year storage at -20 ºC. In 92 % of the samples reanalysed, the concentrations of FM and TM were within ±2 SD of the initial concentration. The result reveals that, the concentrations of FM and TM were stable during storage in freezer for 10 years and no significant losses were observed.

In comparing current cases with historical cases, the most strongly correlated values were those between paired samples of current cases. Analytes concentrations of historical samples stored for long periods of time were not as strongly correlated as current case samples.

The preservative should be carefully selected according to the analyte. Sodium fluoride and potassium oxalate as a preservative to blood samples does not affect morphine, M3G and M6G stability stored in the freezer. However, if samples are stored in other conditions, the effect of the additives on the stability of these compounds may be greater. From the parameters studied in postmortem samples, under storage temperatures of -20 °C for two years, there were statistically no significant differences ($p < 0.05$), between the same blood samples with and without sodium fluoride and potassium oxalate.

However, many other factors must be considered in the evaluation of drug concentrations in a postmortem sample, such as techniques of blood sampling. It is routine practice to collect two unpreserved blood samples, the second

unpreserved blood sample is retained for analysis by any potential defence representation. This study showed that femoral blood sampling had an effect on the blood concentration when we compared two groups of paired unpreserved blood samples. There was significant variation in drug concentration between 2 unpreserved blood samples, even when both samples were collected and analysed at the same time by same validated method in chapter 4 and 5.

In postmortem and spiked blood samples stored in glass vials at 4^{o}C and 25^{o}C, Morphine, M3G and M6G concentrations did not change significantly in samples stored under different storage conditions when expressed as a percentage of the initial concentration throughout one-month observation period.

The current study provides data on survival times in heroin users. Of primary importance, 6MAM was present in the blood in less than 15% of cases. Using 6MAM as a proxy for rapid death, this suggests that a minority of cases had survival times after administration of less than 20–30 minutes. The metabolism of heroin offers a potential means of estimating the proportions of rapid and delayed deaths in fatal overdoses The presence of 6MAM in the blood of a heroin toxicity death suggests a more rapid death, while its absence suggests a more prolonged survival time. Similarly, lower blood concentrations of M3G and M6G, and lower ratios of these conjugates to total morphine, are suggestive of a more rapid death, as there has been less time for the metabolism of morphine to occur. Conversely, higher concentrations and ratios to total morphine are indicative of longer survival times. The concentration ratios selected in this study represent cases where there have been difficulties with the interpretation of the findings, with respect to heroin ingestion. However, the determination of morphine glucuronide ratios with the ratio of free to total morphine, in parallel with 6MAM, is of relevant value in forensic cases. The knowledge from this study provides important information that can be applied to other cases, where such a conclusion is challenging.

Chapter 7 A comparison of mortuary admission and autopsy blood specimens

7.1 Introduction

Postmortem redistribution (PMR) is a well-known toxicological phenomenon which affects the interpretation of postmortem (PM) blood concentrations. The process of redistribution can affect the concentration of drugs in postmortem blood as a result of a disruption of cellular membranes, causing alterations of drug concentrations within tissue elements and diffusion from one tissue to another [402]. This process is particularly significant for drugs with high lipid solubility or high tissue concentrations relative to blood [16, 19, 281]. The degree of redistribution cannot be accurately assessed for any particular drug. All drugs will undergo some type of redistribution depending upon a number of factors; these include, but are not limited to, the physiochemical properties of the drug (i.e. volume of distribution) and the site of sampling (i.e. central vs peripheral). The time between death and sampling, and the conditions in which the deceased body is found, transported, and stored, also have considerable impact on subsequent toxicological analyses [403].

The time between death and sampling (pre-autopsy interval) should be considered in the evaluation of drug concentrations in the postmortem sample, the storage of bodies for long periods of time may cause greater changes in blood drug concentrations. Therefore, in order to avoid the effect of the pre-autopsy interval on drug concentrations, early collection of samples for analysis is preferable. For this reason, this study was carried out to evaluate the effect of early collection of blood samples by using needle puncture in the upper thigh as opposed to blood samples during autopsy from upper thigh.

There can often be a number of days between discovering the body of the deceased and the autopsy taking place. Usually the body is stored in the refrigerator at the mortuary. This study was designed to evaluate the impact this delay has on the drug concentrations found and to assess whether it is necessary to obtain blood specimens as they are received at the mortuary, before autopsy, for drugs analysis. In addition, we also sought to compare peripheral and central

blood over a period of time after death to further define the extent of PMR of drugs and identify a possible mechanism of PMR.

7.2 Ethical considerations

Ethical approval to obtain postmortem samples in drug-related deaths, with the reference number 17/WS/0026 was obtained from NHS West of Scotland Research Ethics Committee and number 200150153 from the Research Ethics Committee within the College of Medical, Veterinary and Life Sciences (MVLS), University of Glasgow. Copies of ethical approval letters from both authorities, the participant information sheet and the consent form are attached in the Appendix 3, Appendix 4, Appendix 5, Appendix 6.

7.3 Methodology

All cases were selected from adult deaths falling under the Scottish Fatalities Investigation Unit, Crown Office and Procurator Fiscal Service, which are being investigated as drug related deaths. There is likely to be a range of combinations of drugs, drug concentrations, interval between death and arrival at mortuary, and interval between arrival at mortuary and autopsy.

The participant was identified by a pathologist carrying out the autopsy who would decide whether the samples could be available and if they were likely to be positive for the drugs. The next of kin were approached after identifying the body on the same day of identification. They were asked whether they would consider additional samples from the deceased being collected to be part of the research. The researcher would then explain the nature of the research. Only when the next of kin gave their consent would pathologists collect additional blood samples. As detailed in participant information sheet in Appendix 4.

Informed consent from next of kin to obtain additional samples was sought during the period from June 2017 to April 2018. As detailed in participant information sheet in Appendix 5. Sample collection took place within the mortuary at the Queen Elizabeth University Hospital, Glasgow. The samples were taken by experienced pathologists and mortuary technicians. Routine blood samples taken for toxicological examination were taken during the autopsy from the right femoral vein by dissection of the vein in the upper thigh (PB3).

Additional samples for this study were taken by needle puncture in the upper thigh (blind stick) at a time close to when the body was received in the mortuary (PB1) and again at the autopsy (PB2). Lastly, a heart blood sample was taken by dissection of the heart during autopsy (HB). 4 mL blood were collected in plain tubes for each blood sample.

All blood specimens were labelled and identified as PB1, PB2, PB3 and HB as shown in Figure 7-1. The times and dates of the blood draws were noted and sent for toxicological analysis.

On the receipt of the samples at Forensic Medicine and Science, the samples were stored at -20 ^{o}C until analysis. The samples were then analysed using validated methods as described in (sections 5.2.45.2.5) and the data analysis was performed using Excel (Microsoft 2016).

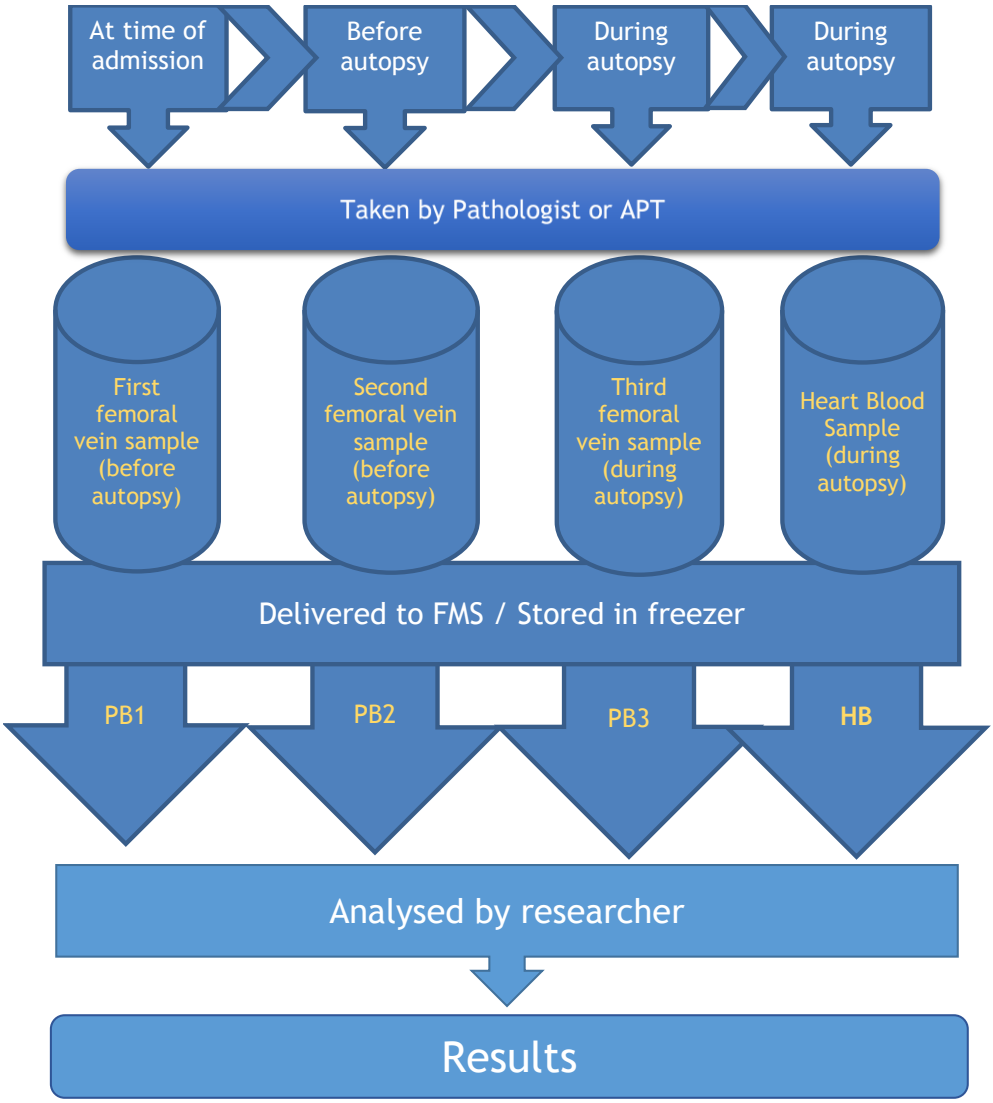


Figure 7-1: Flow chart of blood sampling

7.4 Case History and Background:

A) Case sample (1)

The deceased was a 54 year old male with a history of misuse of drugs including alcohol dependency and was previously prescribed 40 mL of methadone a day. In the month prior to death a urine screening test using the LC/MS/MS screening method showed opiates and cocaine. He was not on any prescribed medication at time of death. In the afternoon, a person was returning to their home and upon entering the close observed the deceased slumped on the stairs with a needle in his hand. Police and ambulance were contacted, and loss of life was confirmed. The postmortem interval between death and sampling was 7 days. The blood sample tested positive for morphine, codeine and alcohol using FMS routine laboratory methods.

B) Case sample (2)

The deceased was a 56-year-old man who had a history of chronic alcoholism, and previous intravenous drug misuse. He had been last seen by his own doctor in the last month in relation to longstanding right-hand pain – his thumb had been amputated some time before, but he had been complaining of pain in the scars – longstanding right knee pain and bleeding from his back passage for which he refused further investigation. At the time of his death he was prescribed thiamine, ibuprofen gel and buprenorphine tablets. The day before he died, the deceased met a male who supplied gabapentin tablets. A witness saw him take ten tablets and consume alcohol before falling asleep on the sofa with his partner. His partner awoke at 0100hrs and found that he was leaning against her and cold to touch with no signs of life. The postmortem interval between death and sampling was 8 days. The blood and urine samples tested positive for morphine, alcohol and buprenorphine, and blood samples were positive for gabapentin, desmethyldiazepam, phenazepam and etizolam using FMS routine laboratory methods.

C) Case sample (3)

The deceased was a 37 year old man who had a medical history of post-traumatic stress disorder, depression and anxiety and alcohol dependency. He had previously overdosed on sertraline, fluoxetine, alcohol and heroin, and was admitted to hospital and referred to mental health services. At the time of his death he was prescribed sertraline, zopiclone, naproxen, and omeprazole. At around 2200hrs, the witness noted he was safe and well and around 1000hrs the following day, he was found lying unresponsive in the kitchen area with a belt around his arm, his trouser leg rolled up, and a used syringe next to his left leg. The postmortem interval between death and sampling was 9 days. The blood samples tested positive for morphine, sertraline, etizolam, phenazepam and alcohol. The urine sample was positive for morphine, codeine, 6MAM and alcohol using FMS routine laboratory methods.

D) Case sample (4)

The deceased was a 27 year old man who had a past medical history of alcohol abuse, controlled drug use including use of injected heroin, and possible cocaine use. On the day of his death around 1900hrs, he was believed to be under the influence of alcohol or possibly drugs, but thereafter watched TV for a period of time before falling asleep on the sofa with witness. The witness thereafter heard a gurgling noise coming from the deceased and became concerned and then called the ambulance. Despite cardiopulmonary resuscitation (CPR) life was pronounced extinct at 2218hrs. The witness confirmed that he was on a methadone programme, but also took heroin. Within the locus, police noticed cannabis (THC) smoking paraphernalia. The postmortem interval between death and sampling was 11 days. The blood samples tested positive for morphine, codeine, citalopram, and alcohol. The urine sample was positive for morphine, codeine, 6MAM and alcohol using FMS routine laboratory method.

7.5 Results and discussion

A total of 5 informed consent forms were signed. All cases were men with the mean age of the decedents 42 years, with a range from 27 to 54 years. However, of the five sets of samples collected, one of the sets was not positive with the drugs of interest. Moreover, morphine and its glucuronides in cases 3 and 4 were below the LLOQ, and gabapentin in case 2 was over the ULOQ of the validated method. The results of analyses are detailed in Table 7-1.

Table 7-1: Distribution of Gabapentin, Morphine, M3G and M6G in the Cases Studied

	Sample no.	Number of days after 1st blood sample taken	FM (mg/L)	M3G (mg/L)	M6G (mg/L)	GBP (mg/L)
Case 1	PB1	0	0.339	0.191	0.021	0.0
	PB2	1	0.597	0.405	0.069	0.0
	PB3	1	0.615	0.568	0.099	0.0
	HB	Not available				
Case 2	PB1	0	<0.05	<0.05	<0.02	91
	PB2	2	<0.05	<0.05	<0.02	139
	PB3	2	0.039	0.090	<0.02	338
	HB	Not available				
Case 3	PB1	0	<0.05	<0.05	0.034	0.0
	PB2	Not available				
	PB3	4	<0.05	0.060	<0.02	0.0
	HB	4	<0.05	<0.05	<0.02	0.0
Case 4	PB1	0	0.137	0.123	0.022	0.0
	PB2	6	0.190	0.152	0.027	0.0
	PB3	6	0.225	0.226	0.046	0.0
	HB	6	0.267	0.224	0.054	0.0

A) Comparing admission (PB1) and pre-autopsy (PB2) drug concentrations in peripheral blood samples

This study focused predominantly on the impact of postmortem time interval (PMI) on a postmortem drug concentration, rather than the influence of the sampling site. The first (PB1) and second (PB2) vein puncture were available for cases 1, 2 and 4 where the postmortem interval was 2 to 11 days. The drug concentration ratios (PB1/PB2) and time interval between PB1 and PB2 for each case are detailed in Table 7-2.

Table 7-2: Comparing pre-autopsy and admission needle puncture (PB2/ PB1) ratio

Cases	Postmortem interval (Days)	PB1/PB2 ratio			
		FM	M3G	M6G	GBP
CASE 1	7	0.57	0.47	0.31	-
CASE 4	11	0.72	0.80	0.81	-
CASE 2	2	-	-	-	0.65

Comparing pre-autopsy and admission needle puncture in both cases found that, FM, M3G and M6G concentrations at time of admission were lower than at pre-autopsy. It was not surprising to see FM, M3G and M6G concentrations increase substantially from mortuary admission to autopsy, presumably due to PMR. In contrast to the previous study, morphine and its metabolite concentrations showed little or no potential for PMR [404]. Similarly, in cases of gabapentin, a significant difference was observed between the two samples.

In Table 7-3, comparing pre-autopsy and admission needle puncture in cases 1 and 4 found that, the changes in (FM/TM), (M3G/TM) and (M6G/TM) ratio were negligible and did not result in any significant changes from mortuary admission to autopsy in both cases.

Table 7-3: Morphine and its glucuronide ratios in all cases

	Sample no.	FM/TM	M3G/TM	M6G/TM
Case 1	PB1	0.61	0.34	0.04
	PB2	0.56	0.38	0.06
Case 4	PB1	0.48	0.43	0.07
	PB2	0.52	0.41	0.07

Overall this study shows that morphine, M3G, M6G and gabapentin exhibit significant increases in drug concentration when femoral blood was collected at autopsy compared to similar blood taken at the time of mortuary admission about 2–11 days later. It may have been expected from previous studies that morphine and its glucuronides would show some redistribution [404], and this was also the case for gabapentin [240]. However, as there has been no assessment in previous studies of the potential for gabapentin postmortem redistribution, a comparison of this was not possible.

In general, this study suggests minimal potential for morphine, M3G, M6G and gabapentin PMR. This interpretation is also consistent with the concept that

compounds with a low volume of distribution show minimum PMR or are not prone to PMR. As this deduction results from a single observation, however, it should be viewed with caution.

In previous studies, Logan and Smirnow found an agreement between morphine concentrations in admission and autopsy blood [249]. While, Saar et al found that storing bodies for long periods of time will cause greater changes in blood drug concentrations [14].

On the other hand, it was expected to see the glucuronide concentrations decrease substantially from mortuary admission to autopsy and subsequently morphine levels increase. It has been known that during life conjugated drugs can revert to their free form from the effects of bacteria and also during the postmortem period [294, 405] although it may be that longer time frames are required to deconjugate significant amounts of morphine glucuronides. However, this study found that the changes in free morphine and glucuronide ratios are negligible and did not result in any significant changes from mortuary admission to autopsy in both cases. The concentrations of morphine glucuronides giving rise to morphine are negligible and did not result in any changes to morphine from mortuary admission to autopsy. Compared to the findings of recent study, the investigation of underlying redistribution mechanisms indicated that concentration change (i.e., increase) of morphine in femoral blood resulted from diffusion processes rather than from release of morphine from its conjugates [404].

Overall, the current study is not without limitations, which include a small case number per analyte and slightly varying sampling time points across cases. By evaluating the data in a time-dependent manner, the latter was attempted to be corrected. Further a limitation of this study is the degree to which postmortem redistribution may have already taken place prior to the mortuary admission specimen being collected. This may affect the magnitude of change during the postmortem interval. Indeed, the study of Hilberg showed that much redistribution occurs in the first 2 h following death, although changes can continue to occur after that time [406]. It is not possible to perform dissections on bodies or vein puncture prior to formal orders by Procurator Fiscal in Scotland to conduct dissections including orders to conduct vein puncture. This may affect the magnitude of change during the postmortem interval. Nevertheless,

the presented results provide valuable information that aid in the understanding of PMR of opioids and are a unique possibility to study time-dependency of redistribution mechanisms with in the human body.

B) Comparing vein puncture (PB2) and dissection (PB3)

The other factor which must be considered in the evaluation of drug concentrations in a postmortem sample, is blood sampling techniques. We compared needle puncture in the upper thigh (blind stick) with dissection of the vein in the upper thigh. The drug concentration ratios between vein dissection and needle puncture were available for three cases.

Firstly, (PB2/PB3) ratios for the FM concentration were 0.97 and 0.84 (averaged 0.90) for case 1 and 4, respectively. Secondly, the M3G concentration ratios were 0.71 and 0.67 for cases 1 and 4, respectively. Thirdly, the M6G concentration ratios were 0.69 and 0.58 for cases 1 and 4, respectively. Lastly, the GBP concentration ratios were 0.41 for case 2. As detailed in Table 7-4.

Table 7-4: PB2/PB3 ratios of gabapentin, morphine and its glucuronides

Cases	Postmortem interval (Days)	PB2/PB3 ratio			
		FM	M3G	M6G	GBP
CASE 1	7	0.97	0.71	0.69	-
CASE 4	11	0.84	0.67	0.58	-
CASE 2	2	-	-	-	0.41

We compared needle puncture in the upper thigh (PB2) and dissection of the vein in the upper thigh (PB3) with central blood (HB). The drug concentration ratios between vein dissection, needle puncture and central blood were available only for case 4. Table 7-5 shows HB/PB2 and HB/PB3 ratios for morphine and its glucuronides, the FM concentrations were 1.40 and 1.18, respectively, the M3G concentration ratios were 1.47 and 0.99, respectively, and the M6G concentration ratios were 2.00 and 1.17, respectively.

Table 7-5: HB/PB2 and HB/PB3 ratios

	Sample no.	FM	M3G	M6G
Case 4	HB/PB2	1.40	1.47	2.00
	HB/PB3	1.18	0.99	1.17

For all analytes at femoral sites (blind stick and dissection sampling), cardiac concentrations are greater than those at the femoral site, whereas femoral concentrations tend to be closer to cardiac concentrations with the dissection (PB3) sampling, even though this is more marked for M6G with the blind stick than with the dissection sampling.

In Table 7-6, comparing needle puncture and vein dissection in both cases found that, the changes in (FM/TM), (M3G/TM) and (M6G/TM) ratios are negligible and did not result in any significant changes from mortuary admission to autopsy in both cases.

Table 7-6: Morphine and their glucuronide ratios in PB2 and PB3

	Sample no.	FM/TM	M3G/TM	M6G/TM
Case-1	PB2	0.56	0.38	0.06
	PB3	0.48	0.44	0.08
Case-4	PB2	0.52	0.41	0.07
	PB3	0.45	0.45	0.09

How blood is sampled may also affect the measurement of drug concentrations. In this study, the effect of the femoral blood sampling protocol (needle puncture) could account for some variation in drug concentrations in blood. Similarly, several reviews have considered the factors that can influence the concentration of drugs measured postmortem including techniques of blood sampling [31-33]. While, a limited study has shown that needle puncture in the upper thigh did not affect the blood concentration as opposed to dissection of the vein in the upper thigh [28, 34], this may not be consistent for all drugs.

It has been suggested that clamping the femoral vessel before sampling may prevent possible contamination from more central sites due to the retrograde flow of blood as can happen with a blind stick sampling. Therefore, femoral sampling done after dissection and clamping of the vein is currently considered the method of choice since it prevents the caudal flow of blood from more central sources such as iliac vessels and the inferior vena cava [19, 281, 407]. However, this procedure results in added time to the external examination as well as additional incisions, and some medico-legal offices simply perform a blind stick femoral sample without tying off the femoral vein.

There are only few references comparing techniques: some authors used dissection and clamping of the vein, others did a blind stick method, and some did not mention which sampling method they used. Hargrove *et al*. concluded that the blind stick method of drawing femoral blood, the easiest, least invasive as well as least time-consuming procedure did not have significant redistribution from central sites and was of equivalent quality to a clamped femoral sample for opiates, for sampling volumes up to 30 mL [408]. The same authors did not observe significant changes in either clamped or unclamped femoral vein morphine concentrations over time either as well as at any period of sampling within the first 24 h after death in bodies kept refrigerated at 4°C.

C) Comparing central (HB) to peripheral (PB3) drug concentration (HB/PB3) ratio

This study focused predominantly on the impact of sampling site on a postmortem drug concentration, rather than the influence of the sampling techniques. In this study, the investigated heart (HB) to femoral (PB3) drug concentration (HB/PB3) ratio was available in one case; firstly, (HB/PB3) ratios for FM concentration was 1.19, M3G concentration was 0.99, and M6G concentration was 1.17.

Earlier studies have proved that for some, but not all drugs, there are significant differences in concentrations between peripheral and corresponding heart blood. The drug concentrations in the heart blood are often higher than in the peripheral blood [284] and do not reflect concentration at the time of death [276]. However, Logan and Smirnow examined 40 heroin-related deaths where the mean postmortem interval was 59 hours and found the cardiac to femoral blood concentration ratios averaged 1.1 for morphine, 1.3 for M6G and 1.1 for M3G. They found no evidence of time-dependent changes in morphine concentrations at either central or peripheral blood sites in 32 cases. They did, however, report consistently higher morphine concentrations in ventricular compared with femoral blood, with the greatest differences observed in cases where the ventricular morphine concentration exceeded 0.3 mg/L [249]. Other authors have also reported obvious differences between central and peripheral blood concentrations of morphine, M3G and M6G in humans [25, 60, 61, 273, 274].

The high Vd of morphine (3 – 5 L/kg) would indicate that it undergoes PMR and it has been shown to do so in animal models [20, 251, 282]. There are, however, conflicting reports on its redistribution in humans and PMR was found not to be a factor in diamorphine fatalities [255]. Considering the low Vd of M3G (0.14 L/kg) and M6G (0.15 L/kg) (Hunt *et al*. 1999), these metabolites would not necessarily be expected to undergo PMR.

In this study, morphine and its metabolites exhibited no significant changes in drug concentration when femoral blood was collected compared to similar blood taken from central samples. It is generally accepted that drugs, such as morphine, with a central to peripheral ratio of less than (or about) 1.0 are not prone to redistribution. It may have been expected from previous studies that morphine and its glucuronides would show no significant redistribution [404].

Although the results of this study are promising, the small sample size for each drug and the limited number of drugs tested limit the utility of this study. Additional peripheral blood versus central blood sample studies with larger sample size and more drugs being compared are needed to fully evaluate potential drug redistribution to peripheral blood vessels.

7.6 Conclusions

This study is the first to concurrently evaluate three aspects of PMR of gabapentin, morphine and its glucuronides. The first aspect, concerning sampling site for all substances, femoral blood concentrations were significantly lower than those found in cardiac blood, a site commonly used for peripheral sampling, indicating that femoral blood is probably less prone to PMR. Although the current study shows that this is true for selected drugs, further research is needed for other drugs, especially those with higher volume of distribution, as refrigeration may not be sufficient to prevent postmortem redistribution to femoral vessels.

Secondly, sampling method also appears to have an effect on femoral drug concentrations depending on the substance considered, since femoral concentrations tend to be closer to cardiac concentrations with the dissection sampling than blind stick sampling technique.

Finally, the analytical results derived from sampling (blind stick) as soon as possible to assess whether it is necessary to obtain blood samples prior to autopsy and prevent contamination of PMR, suggest that PMR is a continuous phenomenon in central as well as in peripheral compartments, but also that the femoral blood appears more resistant to it. Therefore, to avoid the effect of the pre-autopsy interval on drug concentrations it is always preferable for early collection of specimens for analysis, which are collected closer to the time of death, and would enable a better assessment of the likely contribution of drugs to the death.

Chapter 8 General Conclusions, Limitations and Future Work

8.1 General Conclusions

Opioids are one of the most widely abused drug groups in the world. The majority of drug related deaths in the UK relate to opiate use, chiefly heroin/morphine. There is considerable evidence that many instances of opioid overdose are due to the combined effects of opioids with other drugs and this kind of poly-drug use is highly prevalent among illicit drug users in the UK. Almost all DRDs occurred after the consumption of multiple substances. Opioids (methadone, diamorphine/ morphine or buprenorphine) were implicated in over three quarters of DRDs. Diamorphine/morphine, alcohol, anti-depressants, diazepam, etizolam and gabapentin were the most common substances found at post mortem, all have increased in prevalence since 2011, except diazepam.

The use of GC–MS and LC-MSMS for the identification and measurement of drugs of abuse and medication is crucial to the acceptance of evidence in legal proceedings because of its sensitivity and specificity. Analytical methods were developed and validated to measure drug concentrations in the blood of drug related death cases. A GC-MS method with SPE was developed and validated for the simultaneous determination of opioids and antidepressant drugs in whole blood. In addition, a LC-MS/MS with protein precipitation method was developed and validated for simple and accurate analysis of the gabapentin, morphine, M3G and M6G in whole blood samples. The methods were successfully verified using authentic postmortem blood samples. The concurrent method is used to confirm the toxicity of diamorphine and antidepressants drugs simultaneously, especially in the case of multidrug detection, it can be a major advantage in routine procedure to save time and cost .

The stability of drugs in different collection tubes is vital, particularly when analyses cannot be performed promptly. The stability of opioids, gabapentin and antidepressant drugs in spiked blood was studied after different sampling and storage conditions. In both separated clot activator tubes and plain tubes, all analytes were stable under all conditions. While, the separated clot activator tube affected the stability of gabapentin, 6MAM, EDDP, citalopram, sertraline,

amitriptyline and mirtazapine especially when they were stored more than one-month in fridge or freezer. It may be the gel in the tubes absorbed these analytes preferentially compared to other. The results of this study show that, forensic laboratories that are required to store biological samples for long periods should collect these in plain tubes. Furthermore, the stability of other drugs, especially stored in separated clot activator tubes, requires further investigation under different storage conditions and time periods to ensure accurate quantitative analysis that reflect the drug concentrations in actual biological matrices.

In authentic postmortem samples, a stability study of current and historical case samples was carried out. The FM and TM concentrations and their ratio were not affected after incubation of current cases sample stored in the fridge for six months. Moreover, 92% of the historical postmortem blood samples stored long-term at -20°C were within acceptable criteria of the initial concentration. The result reveals that, the concentrations of FM and TM were stable during storage in the freezer for ~10 years and no significant losses were observed

The stability study of authentic case samples with and without preservative, stored in the freezer for three years was carried out. There were no significant differences between paired preserved and unpreserved blood samples. Sodium fluoride and potassium oxalate as a preservative to postmortem blood samples does not affect MOR, M3G and M6G stability under storage temperatures of -20°C for three years.

In authentic postmortem samples, the degradation pattern was similar to that of spiked blood specimens. Morphine and its metabolite concentrations did not change significantly in samples stored under different storage conditions when expressed as a percentage of the initial concentration.

In this study, the investigation revealed that the ratio of morphine to the respective glucuronide concentration predicts survival times after the administration of heroin. The presence of 6MAM in the blood of a heroin toxicity death suggests a more rapid death. Similarly, lower blood concentrations of M3G and M6G, and lower ratios of these conjugates to total morphine, are suggestive of a more rapid death, as there has been less time for the metabolism of morphine to occur. More specifically, when M3G/TM is less than 0.40 could show

a quick death in the absence of 6MAM. Conversely, higher concentrations and ratios to total morphine are indicative of longer survival times. Moreover, both M3G and M6G concentrations were independent correlates of the presence of 6MAM.

This study is the first to evaluate concurrently three aspects of PMR. Concerning sampling site, for all substances, femoral blood concentrations are significantly lower than those found in cardiac blood, a site commonly used for peripheral sampling, indicating that femoral blood is probably less prone to PMR. Sampling method also appears to have an effect on femoral drug concentrations, since femoral concentrations tend to be closer to cardiac concentrations with the dissection sampling than blind stick sampling technique. Finally, the analytical findings derived from techniques for specimen collection (blind stick) as soon as possible to assess whether it is necessary to obtain blood specimens before autopsy and prevent contamination by PMR. This study shows that morphine, M3G, M6G and gabapentin exhibit a significant increase in drug concentration when femoral blood was collected at autopsy compared to similar blood taken at the time of mortuary admission.

Generally, our results conclude that PMR is an ongoing phenomenon in central as well as in peripheral compartments, but also that femoral blood seems more resistant to it. Therefore, to avoid the effect of the pre-autopsy interval on drug concentrations it is always preferable for early collection of specimens for analysis, which are collected closer to the time of death, and would enable a better assessment of the likely contribution of drugs to the death.

8.2 Limitations and Future work

The present study had several limitations. The major one is that the GC-MS and LC-MSMS method were developed and validated to test drugs in blood only. It is recommended to develop a method that can be used to analyse different matrices such as liver and muscle, which would provide greater insight and better understanding of postmortem redistribution.

The LC-MSMS method was only looking for a limited number of drugs (gabapentin, morphine, M3G and M6G). It is necessary in forensic toxicology to develop sensitive and selective methods to identify new drugs and their metabolites for the increasing scope of analyses and increasing numbers of drug of abuse cases submitted to the laboratory.

The evaluation of the stability of morphine, M3G and M6G in blood in paired preserved and unpreserved samples when stored at different temperatures and concentrations of preservative would provide an insight into the presence of these substances at the time of death versus the time of collection.

The limitation of this study was that there were only a few samples analysed. Even though there were many drug-related deaths within this period, it was clearly a very difficult time to ask the next of kin for consent. At the beginning of the study, the next of kin was notified about the research before they came to the mortuary to identify the deceased. However, this strategy was not the best strategy to obtain enough samples.

Although the current study shows an effect of PMR on gabapentin, morphine and its glucuronides, more research needs to be done for other drugs, specifically those with higher volumes of distribution, because refrigeration may not be enough to prevent postmortem redistribution to femoral vessels. Also, the present study of postmortem redistribution was conducted on a small number of samples. Therefore, more samples, as well as testing more drugs to confirm the results of this study is required to achieve a better understanding of this phenomenon.

List of References

1. *Drug-related-deaths-in-scotland-in-2014.* https://www.nrscotland.gov.uk/news/2015/drug-related-deaths-in-scotland-in-2014].
2. Drummer, O.H., *Recent trends in narcotic deaths.* Therapeutic drug monitoring, 2005. **27**(6): p. 738-740.
3. Zador, D., S. Sunjic, and S. Darke, *Heroin-related deaths in New South Wales, 1992: toxicological findings and circumstances.* The Medical journal of Australia, 1996. **164**(4): p. 204.
4. Ochoa, K.C., et al., *Overdosing among young injection drug users in San Francisco.* Addictive Behaviors, 2001. **26**(3): p. 453-460.
5. Monforte, J.R., *Some observations concerning blood morphine concentrations in narcotic addicts.* Journal of forensic sciences, 1977. **22**(4): p. 718.
6. McGregor, C., et al., *Experience of non-fatal overdose among heroin users in Adelaide, Australia: circumstances and risk perceptions.* Addiction, 1998. **93**(5): p. 701-711.
7. Fugelstad, A., et al., *Use of morphine and 6-monoacetylmorphine in blood for the evaluation of possible risk factors for sudden death in 192 heroin users.* Addiction, 2003. **98**(4): p. 463-470.
8. Darke, S., et al., *Hair morphine concentrations of fatal heroin overdose cases and living heroin users.* Addiction, 2002. **97**(8): p. 977-984.
9. Darke, S., J. Ross, and W. Hall, *Overdose among heroin users in Sydney, Australia: I. Prevalence and correlates of non-fatal overdose.* Addiction (Abingdon, England), 1996. **91**(3): p. 405-412.
10. Coffin, P.O., et al., *Opiates, cocaine and alcohol combinations in accidental drug overdose deaths in New York City, 1990-98.* Addiction (Abingdon, England), 2003. **98**(6): p. 739-747.
11. Smith, G.W., et al., *Patterns of polydrug use in Great Britain: Findings from a national household population survey.* Drug and Alcohol Dependence, 2011. **113**(2): p. 222-228.
12. Griffiths, P., et al., *Addiction Research Centres and the Nurturing of Creativity. Monitoring the European drug situation: the ongoing challenge for the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA).* Addiction, 2016. **107**(2): p. 254-258.
13. *Drug-related deaths in Scotland in 2016.* https://www.nrscotland.gov.uk/news/2017/drug-related-deaths-in-scotland-in-2016].
14. Saar, E., et al., *The time-dependant post-mortem redistribution of antipsychotic drugs.* Forensic science international, 2012. **222**(1-3): p. 223.
15. Butzbach, D.M., *The influence of putrefaction and sample storage on post-mortem toxicology results.* Forensic Science, Medicine, and Pathology, 2010. **6**(1): p. 35-45.
16. Ferner, R.E., *Post-mortem clinical pharmacology.* British Journal of Clinical Pharmacology, 2008. **66**(4): p. 430-443.
17. Launiainen, T. and I. Ojanperä, *Drug concentrations in post-mortem femoral blood compared with therapeutic concentrations in plasma.* Drug Testing and Analysis, 2014. **6**(4): p. 308-316.
18. Flanagan, R.J., A. Amin, and W. Seinen, *Effect of post-mortem changes on peripheral and central whole blood and tissue clozapine and*

norclozapine concentrations in the domestic pig (Sus scrofa). Forensic Science International, 2003. **132**(1): p. 9-17.
19. Pélissier-Alicot, A.-L., et al., *Mechanisms Underlying Postmortem Redistribution of Drugs: A Review.* Journal of Analytical Toxicology, 2003. **27**(8): p. 533-544.
20. Crandall, C.S., et al., *The Influence of Collection Site and Methods on Postmortem Morphine Concentrations in a Porcine Model.* Journal of Analytical Toxicology, 2006. **30**(9): p. 651-658.
21. Hilberg, T., J. Mørland, and A. Bjørneboe, *Postmortem release of amitriptyline from the lungs; a mechanism of postmortem drug redistribution.* Forensic Science International, 1994. **64**(1): p. 47-55.
22. Pounder, D.J., A.K. Hartley, and P.J. Watmough, *Postmortem redistribution and degradation of dothiepin. Human case studies and an animal model.* The American journal of forensic medicine and pathology, 1994. **15**(3): p. 231-235.
23. Jaffe, P.D., et al., *A Study Involving Venlafaxine Overdoses: Comparison of Fatal and Therapeutic Concentrations in Postmortem Specimens.* Journal of Forensic Sciences, 1999. **44**(1): p. 193-196.
24. Kunsman, G.W., R. Rodriguez, and P. Rodriguez, *Fluvoxamine distribution in postmortem cases.* The American journal of forensic medicine and pathology, 1999. **20**(1): p. 78-83.
25. Prouty, R.W. and W.H. Anderson, *The forensic science implications of site and temporal influences on postmortem blood-drug concentrations.* Journal of forensic sciences, 1990. **35**(2): p. 243.
26. Levine, B., A.J. Jenkins, and J.E. Smialek, *Distribution of sertraline in postmortem cases.* Journal of analytical toxicology, 1994. **18**(5): p. 272.
27. Rohrig, T.P. and R.W. Prouty, *Fluoxetine overdose: a case report.* Journal of analytical toxicology, 1989. **13**(5): p. 305.
28. Gerostamoulos, D., et al., *The effect of the postmortem interval on the redistribution of drugs: a comparison of mortuary admission and autopsy blood specimens.* Forensic Science, Medicine, and Pathology, 2012. **8**(4): p. 373-379.
29. Dinis-Oliveira, R.J., et al., *Collection of biological samples in forensic toxicology.* Toxicology Letters, 2011. **205**: p. S198-S199.
30. Skopp, G., *Preanalytic aspects in postmortem toxicology.* Forensic Science International, 2004. **142**(2): p. 75-100.
31. Hearn, W.L., et al., *Site-dependent postmortem changes in blood cocaine concentrations.* Journal of forensic sciences, 1991. **36**(3): p. 673.
32. Staeheli, S.N., et al., *Development of CT-guided biopsy sampling for time-dependent postmortem redistribution investigations in blood and alternative matrices—proof of concept and application on two cases.* Analytical and Bioanalytical Chemistry, 2016. **408**(4): p. 1249-1258.
33. James, S.H., *Forensic Science : An Introduction to Scientific and Investigative Techniques, Fourth Edition*. Vol. 4th. 2014: CRC Press.
34. Krinsky, C.S., S.L. Lathrop, and R. Zumwalt, *An Examination of the Postmortem Redistribution of Fentanyl and Interlaboratory Variability.* Journal of Forensic Sciences, 2014. **59**(5): p. 1275-1279.
35. *Fatal versus non-fatal heroin "overdose": blood morphine concentrations with fatal outcome in comparison to those of intoxicated drivers.* 2002. **130**(1): p. 49–54.
36. Burt MJ , e.a., *Postmortem blood free and total morphine concentrations in medical examiner cases. - PubMed - NCBI.* 2001.

37. AW, C.G.a.J., *Concentration ratios of morphine to codeine in blood of impaired drivers as evidence of heroin use and not medication with codeine. - PubMed - NCBI*. 2001.
38. L, R.R.a.L., *Comparison of the hydrolysis rates of morphine-3-glucuronide and morphine-6-glucuronide with acid and beta-glucuronidase. - PubMed - NCBI*. 1995.
39. Jung, B.F., et al., *Interpretation of Opioid Levels: Comparison of Levels During Chronic Pain Therapy to Levels from Forensic Autopsies.* Clinical Pharmacology & Therapeutics, 2005. **77**(4): p. 324-334.
40. Mason, J.L., S.P. Ashmore, and A.R. Aitkenhead, *Simple method for the determination of morphine and its active glucuronide metabolite in human plasma by high-performance liquid chromatography with electrochemical detection.* Journal of Chromatography B: Biomedical Sciences and Applications, 1991. **570**(1): p. 191-197.
41. Wright, A.W., et al., *Quantitation of morphine, morphine-3-glucuronide, and morphine-6-glucuronide in plasma and cerebrospinal fluid using solid-phase extraction and high-performance liquid chromatography with electrochemical detection.* Therapeutic drug monitoring, 1994. **16**(2): p. 200-208.
42. Wright, A.W. and M.T. Smith, *Improved one-step solid-phase extraction method for morphine, morphine-3-glucuronide, and morphine-6-glucuronide from plasma and quantitation using high-performance liquid chromatography with electrochemical detection.* Therapeutic drug monitoring, 1998. **20**(2): p. 215-218.
43. Gerostamoulos, J. and O.H. Drummer, *Solid phase extraction of morphine and its metabolites from postmortem blood.* Forensic Science International, 1996. **77**(1): p. 53-63.
44. Pawula, M., D.A. Barrett, and P.N. Shaw, *An improved extraction method for the HPLC determination of morphine and its metabolites in plasma.* Journal of Pharmaceutical and Biomedical Analysis, 1993. **11**(4): p. 401-406.
45. Ary, K. and K. Róna, *LC determination of morphine and morphine glucuronides in human plasma by coulometric and UV detection.* Journal of Pharmaceutical and Biomedical Analysis, 2001. **26**(2): p. 179-187.
46. Hartley, R., et al., *Pharmacokinetics of morphine infusion in premature neonates.* Archives of disease in childhood, 1993. **69**(1 Spec No): p. 55-58.
47. Huwyler, J., et al., *Rapid and highly automated determination of morphine and morphine glucuronides in plasma by on-line solid-phase extraction and column liquid chromatography.* Journal of chromatography. B, Biomedical applications, 1995. **674**(1): p. 57.
48. Beike, J., et al., *Immunoaffinity extraction of morphine, morphine-3-glucuronide and morphine-6-glucuronide from blood of heroin victims for simultaneous high-performance liquid chromatographic determination.* Journal of Chromatography B: Biomedical Sciences and Applications, 1999. **726**(1): p. 111-119.
49. Glare, P.A., T.D. Walsh, and C.E. Pippenger, *A simple, rapid method for the simultaneous determination of morphine and its principal metabolites in plasma using high-performance liquid chromatography and fluorometric detection.* Therapeutic drug monitoring, 1991. **13**(3): p. 226-232.
50. Svensson, J.O., Q.Y. Yue, and J. Säwe, *Determination of codeine and metabolites in plasma and urine using ion-pair high-performance liquid*

chromatography. Journal of chromatography. B, Biomedical applications, 1995. **674**(1): p. 49.
51. Milne, R.W., et al., *High-performance liquid chromatographic determination of morphine and its 3- and 6-glucuronide metabolites: improvements to the method and application to stability studies.* Journal of Chromatography B: Biomedical Sciences and Applications, 1991. **565**(1): p. 457-464.
52. Chari, G., et al., *High-performance liquid chromatographic determination of morphine, morphine-3-glucuronide, morphine-6-glucuronide and codeine in biological samples using multi-wavelength forward optical detection.* Journal of chromatography 1991. **571**(1-2): p. 263.
53. Brandšteterová, E., E. Blahová, and J. Netriová, *SIMPLE GENERIC SPE ASSAY FOR HPLC ANALYSIS OF MORPHINE AND ITS GLUCURONIDES IN SERUM SAMPLES.* Journal of Liquid Chromatography & Related Technologies, 2002. **25**(16): p. 2521-2534.
54. Wielbo, D., et al., *High-performance liquid chromatographic determination of morphine and its metabolites in plasma using diode-array detection.* Journal of Chromatography B: Biomedical Sciences and Applications, 1993. **615**(1): p. 164-168.
55. Blanchet, M., et al., *Routine determination of morphine, morphine 3-Β-d-glucuronide and morphine 6-Β-d-glucuronide in human serum by liquid chromatography coupled to electrospray mass spectrometry.* Journal of Chromatography A, 1999. **854**(1-2): p. 93-108.
56. Zheng, M., K.M. McErlane, and M.C. Ong, *High-performance liquid chromatography-mass spectrometry-mass spectrometry analysis of morphine and morphine metabolites and its application to a pharmacokinetic study in male Sprague-Dawley rats.* Journal of Pharmaceutical and Biomedical Analysis, 1998. **16**(6): p. 971-980.
57. Naidong, W., et al., *Simultaneous assay of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human plasma using normal-phase liquid chromatography–tandem mass spectrometry with a silica column and an aqueous organic mobile phase.* Journal of Chromatography B: Biomedical Sciences and Applications, 1999. **735**(2): p. 255-269.
58. Shou, W.Z., et al., *An automatic 96-well solid phase extraction and liquid chromatography–tandem mass spectrometry method for the analysis of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human plasma.* Journal of Pharmaceutical and Biomedical Analysis, 2002. **27**(1): p. 143-152.
59. Zuccaro, P., et al., *Simultaneous Determination of Heroin, 6-Monoacetylmorphine, Morphine, and its Glucuronides by Liquid Chromatography-Atmospheric Pressure Ionspray-Mass Spectrometry.* Journal of Analytical Toxicology, 1997. **21**(4): p. 268-277.
60. Bogusz, M.J., R.-D. Maier, and S. Driessen, *Morphine, Morphine-3-Glucuronide, Morphine-6-Glucuronide, and 6-Monoacetylmorphine Determined by Means of Atmospheric Pressure Chemical Ionization-Mass Spectrometry-Liquid Chromatography in Body Fluids of Heroin Victims.* Journal of Analytical Toxicology, 1997. **21**(5): p. 346-355.
61. Bogusz, M.J., et al., *Determination of morphine and its 3- and 6-glucuronides, codeine, codeine-glucuronide and 6-monoacetylmorphine in body fluids by liquid chromatography atmospheric pressure chemical ionization mass spectrometry.* Journal of Chromatography B: Biomedical Sciences and Applications, 1997. **703**(1): p. 115-127.

62. Tatsuno, M., et al., *Simultaneous Determination of Illicit Drugs in Human Urine by Liquid Chromatography-Mass Spectrometry.* Journal of Analytical Toxicology, 1996. **20**(5): p. 281-286.
63. Tatsuno, M., et al., *Determination of Oxazepam Glucuronide in Human Urine by Liquid Chromatography/APCI-Mass Spectrometry.* Eisei kagaku, 1992. **38**(2): p. 162-167.
64. Chari, G., et al., *High-performance liquid chromatographic determination of morphine, morphine-3-glucuronide, morphine-6-glucuronide and codeine in biological samples using multi-wavelength forward optical detection.* Journal of Chromatography, 1991. **571**(1-2): p. 263.
65. Xu, W., et al., *Simultaneous determination of morphine-6-d-glucuronide, morphine-3-d-glucuronide and morphine in human plasma and urine by ultra-performance liquid chromatography-tandem mass spectrometry: Application to M6G injection pharmacokinetic study.* Biomedical Chromatography, 2018. **32**(2): p. n/a-n/a.
66. Franken, L.G., et al., *Pharmacokinetics of Morphine, Morphine-3-Glucuronide and Morphine-6-Glucuronide in Terminally Ill Adult Patients.* Clinical pharmacokinetics, 2016. **55**(6): p. 697-709.
67. Rees, K.A., D.J. Pounder, and M.D. Osselton, *Distribution of opiates in femoral blood and vitreous humour in heroin/morphine-related deaths.* Forensic Science International, 2013. **226**(1): p. 152-159.
68. Mao, J., M.S. Gold, and M.M. Backonja, *Combination Drug Therapy for Chronic Pain: A Call for More Clinical Studies.* Journal of Pain, 2011. **12**(2): p. 157-166.
69. G, S., et al., *Stability of Morphine, Morphine-3-Glucuronide, and Morphine-6-Glucuronide in Fresh Blood and Plasma and Postmortem Blood Samples.* Journal of Analytical Toxicology, 2001. **25**(1): p. 2-7.
70. Lin, D.L., H. Liu, and C.Y. Chen, *Storage temperature effect on the stability of morphine and codeine in urine.* Journal of analytical toxicology, 1995. **19**(5): p. 275.
71. Verplaetse, R. and J. Henion, *Quantitative determination of opioids in whole blood using fully automated dried blood spot desorption coupled to on-line SPE-LC-MS/MS.* Drug Testing and Analysis, 2016. **8**(1): p. 30-38.
72. Clavijo, C.F., et al., *A sensitive assay for the quantification of morphine and its active metabolites in human plasma and dried blood spots using high-performance liquid chromatography-tandem mass spectrometry.* Analytical and Bioanalytical Chemistry, 2011. **400**(3): p. 715-728.
73. Whittington, D. and E.D. Kharasch, *Determination of morphine and morphine glucuronides in human plasma by 96-well plate solid-phase extraction and liquid chromatography-electrospray ionization mass spectrometry.* Journal of Chromatography B, 2003. **796**(1): p. 95-103.
74. Leis, H.J., et al., *Quantitative gas chromatographic/mass spectrometric analysis of morphine glucuronides in human plasma by negative ion chemical ionization mass spectrometry.* Journal of Mass Spectrometry, 2002. **37**(4): p. 395-400.
75. Giorgi, S.N. and J.E. Meeker, *A 5-year stability study of common illicit drugs in blood.* Journal of analytical toxicology, 1995. **19**(6): p. 392.
76. Krogh, M., A.S. Christophersen, and K.E. Rasmussen, *Automated sample preparation by on-line dialysis and trace enrichment: Analysis of morphine, 6-monoacetylmorphine, codeine, ethylmorphine and pholcodine in plasma and whole blood by capillary gas chromatography and capillary gas chromatography—mass spectrometry.* Journal of

Chromatography B: Biomedical Sciences and Applications, 1993. **621**(1): p. 41-48.
77. Geier, A., D. Bergemann, and L. von Meyer, *Evaluation of a solid-phase extraction procedure for the simultaneous determination of morphine, 6-monoacetylmorphine, codeine and dihydrocodeine in plasma and whole blood by GC/MS.* International journal of legal medicine, 1996. **109**(2): p. 80-83.
78. Wang, W.L., W.D. Darwin, and E.J. Cone, *Simultaneous assay of cocaine, heroin and metabolites in hair, plasma, saliva and urine by gas chromatography-mass spectrometry.* Journal of chromatography. B, Biomedical applications, 1994. **660**(2): p. 279.
79. Goldberger, B.A., et al., *Measurement of heroin and its metabolites by isotope-dilution electron- impact mass spectrometry.* Clinical Chemistry, 1993. **39**(4): p. 670.
80. Musshoff, F. and T. Daldrup, *Evaluation of a method for simultaneous quantification of codeine, dihydrocodeine, morphine, and 6-monoacetylmorphine in serum, blood, and postmortem blood.* International journal of legal medicine, 1993. **106**(2): p. 107-109.
81. Guillot, J.G., M. Lefebvre, and J.P. Weber, *Determination of Heroin, 6-Acetylmorphine, and Morphine in Biological Fluids Using their Propionyl Derivatives with Ion Trap GCaMS.* Journal of Analytical Toxicology, 1997. **21**(2): p. 127-133.
82. Gjerde, H., et al., *Evaluation of a method for simultaneous quantification of codeine, ethylmorphine and morphine in blood.* Forensic Science International, 1991. **51**(1): p. 105-110.
83. Fuller, D.C. and W.H. Anderson, *A simplified procedure for the determination of free codeine, free morphine, and 6-acetylmorphine in urine.* Journal of analytical toxicology, 1992. **16**(5): p. 315.
84. Allen, D.L., K.S. Scott, and J.S. Oliver, *Comparison of Solid-Phase Extraction and Supercritical Fluid Extraction for the Analysis of Morphine in Whole Blood.* Journal of Analytical Toxicology, 1999. **23**(3): p. 216-218.
85. Watson, D.G., et al., *Analysis of unconjugated morphine, codeine, normorphine and morphine as glucuronides in small volumes of plasma from children.* Journal of Pharmaceutical and Biomedical Analysis, 1995. **13**(1): p. 27-32.
86. Namera, A., et al., *Simple Analysis of Tetracyclic Antidepressants in Blood using Headspace-Solid-Phase Microextraction and GCMS.* Journal of Analytical Toxicology, 1998. **22**(5): p. 396-400.
87. Truta, L., et al., *Antidepressants detection and quantification in whole blood samples by GC-MS/MS, for forensic purposes.* Journal of Pharmaceutical and Biomedical Analysis, 2016. **128**: p. 496-503.
88. Edinboro, L.E., R.C. Backer, and A. Poklis, *Direct Analysis of Opiates in Urine by Liquid Chromatography-Tandem Mass Spectrometry.* Journal of Analytical Toxicology, 2005. **29**(7): p. 704-710.
89. Langman, L.J., et al., *Therapeutic monitoring of opioids: a sensitive LC-MS/MS method for quantitation of several opioids including hydrocodone and its metabolites.* Therapeutic drug monitoring, 2013. **35**(3): p. 352.
90. French, D., A. Wu, and K. Lynch, *Hydrophilic interaction LC-MS/MS analysis of opioids in urine: significance of glucuronide metabolites.* Bioanalysis., 2011. **3**(23): p. 2603.
91. Venn, R.F. and A. Michalkiewicz, *Fast reliable assay for morphine and its metabolites using high-performance liquid chromatography and native*

fluorescence detection. Journal of Chromatography B: Biomedical Sciences and Applications, 1990. **525**: p. 379-388.
92. Kubiak, E.J. and J.W. Munson, *High-Performance Liquid Chromatographic Analysis of Codeine in Syrups Using Ion-Pair Formation.* Journal of Pharmaceutical Sciences, 1980. **69**(2): p. 152-156.
93. Taylor, R.B., A.S. Low, and R.G. Reid, *Determination of opiates in urine by capillary electrophoresis.* Journal of Chromatography B: Biomedical Sciences and Applications, 1996. **675**(2): p. 213-223.
94. Clarke, E.G.C., *Clarke's isolation and identification of drugs : in pharmaceuticals, body fluids, and post-mortem material*. 2nd / senior consulting: A.C. Moffat, consulting: J.V. Jackson, M.S. Moss, B. Widdop, assist by E.S. Greenfield ; prepar in the Department of Pharmaceutical Sciences of the Pharmaceutical Society of Great Britain ed. 1986, London: Pharmaceutical Press.
95. Rop, P.P., et al., *Determination of 6-monoacetylmorphine and morphine in plasma, whole blood and urine using high-performance liquid chromatography with electrochemical detection.* Journal of chromatography. B, Biomedical applications, 1994. **661**(2): p. 245.
96. Bogusz, M.J., et al., *Applicability of various brands of mixed-phase extraction columns for opiate extraction from blood and serum.* Journal of Chromatography B: Biomedical Sciences and Applications, 1996. **683**(2): p. 177-188.
97. Liaw, W.J., et al., *Determination of morphine by high-performance liquid chromatography with electrochemical detection: application to human and rabbit pharmacokinetic studies.* Journal of chromatography. B, Biomedical sciences and applications, 1998. **714**(2): p. 237.
98. Verwey-Van Wissen, C.P.W.G.M., P.M. Koopman-Kimenai, and T.B. Vree, *Direct determination of codeine, norcodeine, morphine and normorphine with their corresponding O-glucuronide conjugates by high-performance liquid chromatography with electrochemical detection.* Journal of Chromatography B: Biomedical Sciences and Applications, 1991. **570**(2): p. 309-320.
99. Bouquillon, A.I., D. Freeman, and D.E. Moulin, *Simultaneous solid-phase extraction and chromatographic analysis of morphine and hydromorphone in plasma by high-performance liquid chromatography with electrochemical detection.* Journal of Chromatography B: Biomedical Sciences and Applications, 1992. **577**(2): p. 354-357.
100. Besner, J.G., et al., *Determination of opiates and other basic drugs by high-performance liquid chromatography with electrochemical detection.* Journal of Pharmaceutical and Biomedical Analysis, 1989. **7**(12): p. 1811-1817.
101. Heybroek, W.M., et al., *Automatic on-line extraction coupled with electrochemical detection as an improved method for the HPLC co-analysis of codeine and morphine in plasma and gastric juice.* Journal of Pharmaceutical and Biomedical Analysis, 1990. **8**(8): p. 1021-1027.
102. *The National DrugRelated Deaths Database (Scotland) Report Analysis of Deaths occurring in 2015 and 2016*. 2018.
103. Saarto, T. and P.J. Wiffen, *Antidepressants for neuropathic pain.* The Cochrane database of systematic reviews, 2007(4): p. CD005454.
104. Aragona, M., et al., *Randomized double-blind comparison of serotonergic (Citalopram) versus noradrenergic (Reboxetine) reuptake inhibitors in outpatients with somatoform, DSM-IV-TR pain disorder.* European Journal of Pain, 2005. **9**(1): p. 33-38.

105. Kroenke, K.M.D., E.E.M.D.M.P.H. Krebs, and M.J.M.D.M.S. Bair, *Pharmacotherapy of chronic pain: a synthesis of recommendations from systematic reviews.* General Hospital Psychiatry, 2009. **31**(3): p. 206-219.
106. Liu, S.J. and R.I. Wang, *Increased analgesia and alterations in distribution and metabolism of methadone by desipramine in the rat.* Journal of Pharmacology and Experimental Therapeutics, 1975. **195**(1): p. 94.
107. Pick, C.G., et al., *Potentiation of opioid analgesia by the antidepressant nefazodone.* European Journal of Pharmacology, 1992. **211**(3): p. 375-381.
108. Ozdemir, E., S. Gursoy, and I. Bagcivan, *The effects of serotonin/norepinephrine reuptake inhibitors and serotonin receptor agonist on morphine analgesia and tolerance in rats.* The Journal of Physiological Sciences, 2012. **62**(4): p. 317-323.
109. Baldo, B.A., *Opioid analgesic drugs and serotonin toxicity (syndrome): mechanisms, animal models, and links to clinical effects.* Archives of Toxicology, 2018. **92**(8): p. 2457-2473.
110. Mozayani, A., L.P. Raymon, and SpringerLink, *Handbook of drug interactions: a clinical and forensic guide*. 2nd ed. 2012, New York: Humana Press.
111. Pert, C.B. and S.H. Snyder, *Opiate Receptor: Demonstration in Nervous Tissue.* Science, 1973. **179**(4077): p. 1011-1014.
112. Caplan, Y.H., *Disposition of Toxic Drugs and Chemicals in Man.* Journal of Analytical Toxicology, 2015. **39**(5): p. 417-417.
113. Rook, E.J., et al., *The quantitative analysis of heroin, methadone and their metabolites and the simultaneous detection of cocaine, acetylcodeine and their metabolites in human plasma by high-performance liquid chromatography coupled with tandem mass spectrometry.* Journal of Chromatography B, 2005. **824**(1): p. 213-221.
114. von Euler, M., et al., *Interpretation of the presence of 6-monoacetylmorphine in the absence of morphine-3-glucuronide in urine samples: evidence of heroin abuse.* Therapeutic drug monitoring, 2003. **25**(5): p. 645-648.
115. Petrides, A.K., et al., *Monitoring opioid and benzodiazepine use and abuse: Is oral fluid or urine the preferred specimen type?* Clinica Chimica Acta, 2018. **481**: p. 75-82.
116. Vree, T.B. and C.P. Verwey-van Wissen, *Pharmacokinetics and metabolism of codeine in humans.* Biopharmaceutics & drug disposition, 1992. **13**(6): p. 445-460.
117. Barceloux, D.G., *Disposition of Toxic Drugs and Chemicals in Man , 10th Edition edited by Randall C. Baselt , Biomedical Publications , Seal Beach, CA , 2014 , 2,211 pp, $324.50 , ISBN 978-0-9626523-9-4.* Clinical toxicology (Philadelphia, Pa.), 2015. **53**(2): p. 140.
118. Fromm, M.F., et al., *Dihydrocodeine: A new opioid substrate for the polymorphic CYP2D6 in humans.* Clinical Pharmacology & Therapeutics, 1995. **58**(4): p. 374-382.
119. Al-Asmari, A.I. and R.A. Anderson, *The Role of Dihydrocodeine (DHC) Metabolites in Dihydrocodeine-Related Deaths.* Journal of Analytical Toxicology, 2010. **34**(8): p. 476-490.
120. Winek, C.L., *Barry Levine (Ed.), Principles of Forensic Toxicology, second ed., AACC Press, Washington, DC, 2003, 385 pp., US$ 67.00, softcover, ISBN 1-890883-87-5.* 2005, Elsevier Ireland Ltd. p. 113-113.

121. Reichel, A. and P. Lienau, *Pharmacokinetics in Drug Discovery: An Exposure-Centred Approach to Optimising and Predicting Drug Efficacy and Safety*. Handbook of experimental pharmacology, 2016. **232**: p. 235.
122. Pleuvry, B.J., *Factors affecting drug absorption and distribution.* Anaesthesia & Intensive Care Medicine, 2005. **6**(4): p. 135-138.
123. Pruskowski, J. and R.M. Arnold, *Opioid Pharmacokinetics #307*. Journal of Palliative Medicine, 2016. **19**(6): p. 668-670.
124. Velez de Mendizabal, N., et al., *A Compartmental Analysis for Morphine and Its Metabolites in Young Children After a Single Oral Dose*. Clinical Pharmacokinetics, 2015. **54**(10): p. 1083-1090.
125. Ashby, M., et al., *Plasma morphine and glucuronide (M3G and M6G) concentrations in hospice inpatients.* Journal of pain and symptom management, 1997. **14**(3): p. 157-167.
126. Klepstad, P., et al., *Routine drug monitoring of serum concentrations of morphine, morphine-3-glucuronide and morphine-6-glucuronide do not predict clinical observations in cancer patients.* Palliative Medicine, 2003. **17**(8): p. 679-687.
127. Lam, L.H., R.D. Pirrello, and J.D. Ma, *A Case-Based Approach to Integrating Opioid Pharmacokinetic and Pharmacodynamic Concepts in Cancer Pain Management*. The Journal of Clinical Pharmacology, 2016. **56**(7): p. 785-793.
128. Simonsen, K.W., et al., *Fatal poisoning in drug addicts in the Nordic countries in 2012.* Forensic science international, 2015. **248**: p. 172-180.
129. Kriikku, P., et al., *Phenazepam abuse in Finland: Findings from apprehended drivers, post-mortem cases and police confiscations.* Forensic Science International, 2012. **220**(1–3): p. 111-117.
130. Minett, W.J., et al., *Concentrations of Opiates and Psychotropic Agents in Polydrug Overdoses: A Surprising Correlation Between Morphine and Antidepressants.* Journal of Forensic Sciences, 2010. **55**(5): p. 1319-1325.
131. Hull, M.J., et al., *Fatalities associated with fentanyl and co-administered cocaine or opiates.* J Forensic Sci, 2007. **52**(6): p. 1383-8.
132. Smith, M.L., et al., *Detection Times and Analytical Performance of Commercial Urine Opiate Immunoassays Following Heroin Administration.* Journal of Analytical Toxicology, 2000. **24**(7): p. 522-529.
133. Gottas, A., et al., *Pharmacokinetics of heroin and its metabolites in vitreous humor and blood in a living pig model*. Forensic Toxicol, 2016. **34**(2): p. 277-285.
134. Mitchell, J.M., et al., *Forensic drug testing for opiates. II. Metabolism and excretion rate of morphine in humans after morphine administration.* Journal of analytical toxicology, 1991. **15**(2): p. 49.
135. Pragst, F., et al., *Detection of 6-Acetylmorphine in Vitreous Humor and Cerebrospinal Fluid-Comparison with Urinary Analysis for Proving Heroin Administration in Opiate Fatalities.* Journal of Analytical Toxicology, 1999. **23**(3): p. 168-172.
136. Antonilli, L., et al., *High levels of morphine-6-glucuronide in street heroin addicts.* Psychopharmacology, 2003. **170**(2): p. 200-204.
137. Thorn, C.F., T.E. Klein, and R.B. Altman, *Codeine and morphine pathway.* Pharmacogenetics and Genomics, 2009. **19**(7): p. 556-558.
138. Yue, Q.Y. and J. Säwe, *Different effects of inhibitors on the O- and N-demethylation of codeine in human liver microsomes.* European Journal of Clinical Pharmacology, 1997. **52**(1): p. 41-47.

139. He, Y.J., et al., *CYP2D6 Ultrarapid Metabolism and Morphine/Codeine Ratios in Blood: Was it Codeine or Heroin?* Journal of Analytical Toxicology, 2008. **32**(2): p. 178-182.
140. Advokat, C.D., J.E. Comaty, and R.M. Julien, *Julien's Primer of drug action: a comprehensive guide to the actions, uses and side effects of psychoactive drugs*. Thirteenth, 40th anniversary / Claire D. Advokat, Joseph E. Comaty, Robert M. Julien, M.D. ed. 2014, New York: Worth Publishers.
141. Julien, R.M., *A primer of drug action: a comprehensive guide to the actions, uses, and side effects of psychoactive drugs*. 10th ed. 2005, New York, N.Y: Worth Publishers.
142. Kieffer, B.L. and B.L. Kieffer, *Opioids: first lessons from knockout mice*. 1999, Elsevier Ltd: England. p. 19-26.
143. Kapp, R.W., *Clarke's Analysis of Drugs and Poisons, 3rd edition Edited by Anthony C. Moffat, M. David Osselton, and Brian Widdop Publisher: Pharmaceutical Press: London. 2004. ISBN: 0-853-69473-7. Volume I: 480 pages; Volume II: 1176 pages. Price: $545.00.* International Journal of Toxicology, 2006. **25**(1): p. 81-82.
144. *Clarke's Analysis of Drugs and Poisons.* 2018.
145. Inturrisi, C.E., et al., *Evidence from opiate binding studies that heroin acts through its metabolites*. Life Sciences, 1983. **33**: p. 773-776.
146. Selley, D.E., et al., *mu Opioid receptor-mediated G-protein activation by heroin metabolites: evidence for greater efficacy of 6-monoacetylmorphine compared with morphine*. Biochemical pharmacology, 2001. **62**(4): p. 447.
147. Osborne, R., et al., *Morphine and metabolite behavior after different routes of morphine administration: Demonstration of the importance of the active metabolite morphine-6-glucuronide*. Clinical Pharmacology and Therapeutics, 1990. **47**(1): p. 12-19.
148. Osborne, R., et al., *The pharmacokinetics of morphine and morphine glucuronides in kidney failure*. Clinical Pharmacology and Therapeutics, 1993. **54**(2): p. 158-167.
149. Heppell, S.P.E. and G.K. Isbister, *Lack of respiratory depression in paracetamol-codeine combination overdoses: Paracetamol-codeine toxicity*. British Journal of Clinical Pharmacology, 2017. **83**(6): p. 1273-1278.
150. Smith, H.S.M.D., *Opioid Metabolism*. Mayo Clinic Proceedings, 2009. **84**(7): p. 613-624.
151. Rudd, R.A., et al., *Increases in Drug and Opioid Overdose Deaths--United States, 2000-2014*. MMWR. Morbidity and mortality weekly report, 2016. **64**(50-51): p. 1378-1382.
152. Siegel, S., et al., *Pavlovian psychopharmacology: The associative basis of tolerance*. Experimental and Clinical Psychopharmacology, 2000. **8**(3): p. 276-293.
153. Fugelstad, A., et al., *Use of morphine and 6-monoacetylmorphine in blood for the evaluation of possible risk factors for sudden death in 192 heroin users*. Addiction, 2003. **98**(4): p. 463-470.
154. Fugelstad, A., et al., *Use of morphine and 6-monoacetylmorphine in blood for the evaluation of possible risk factors for sudden death in 192 heroin users*. Addiction, 2016. **98**(4): p. 463-470.
155. Darke, S. and D. Zador, *Fatal heroin 'overdose': a review*. Addiction, 1996. **91**(12): p. 1765-1772.

156. Lehmann, K.A., *Opioids: overview on action, interaction and toxicity.* Supportive Care in Cancer, 1997. **5**(6): p. 439-444.
157. Broussard, L., *Disposition of Toxic Drugs and Chemicals in Man, Seventh Edition. Randall C. Baselt. Foster City, CA: Biomedical Publications, 2004, 1250 pp., $139.50, hardcover. ISBN 09626523-6-9.* Clinical Chemistry, 2005. **51**(3): p. 680-680.
158. Middleberg, R.A., *Disposition of Toxic Drugs and Chemicals in Man—11th Edition.* Journal of Analytical Toxicology, 2018. **42**(2): p. 139-139.
159. Kerr, B., et al., *Concentration-related effects of morphine on cognition and motor control in human subjects.* Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 1991. **5**(3): p. 157.
160. Stanski, D.R., et al., *Kinetics of high-dose intravenous morphine in cardiac surgery patients.* Clinical pharmacology and therapeutics, 1976. **19**(6): p. 752.
161. Davis, G.G., *Review of: Karch's Pathology of Drug Abuse, 4th edition.* Journal of Forensic Sciences, 2009. **54**(6): p. 1495.
162. Levine, B., D. Green, and J.E. Smialek, *The role of ethanol in heroin deaths.* Journal of forensic sciences, 1995. **40**(5): p. 808.
163. Darke, S., et al., *A comparison of blood toxicology of heroin-related deaths and current heroin users in Sydney, Australia.* Drug and Alcohol Dependence, 1997. **47**(1): p. 45-53.
164. Hall, D.a., *Heroin overdose: Research and evidence-based intervention | SpringerLink.* 2003.
165. Kim, I., et al., *Plasma and Oral Fluid Pharmacokinetics and Pharmacodynamics after Oral Codeine Administration.* Clinical Chemistry, 2002. **48**(9): p. 1486.
166. Winek, C.L., W.W. Wahba, and T.W. Balzer, *Drug and chemical blood-level data 2001.* Forensic Science International, 2001. **122**(2): p. 107-123.
167. Frost, J., et al., *Post-mortem levels and tissue distribution of codeine, codeine-6-glucuronide, norcodeine, morphine and morphine glucuronides in a series of codeine-related deaths.* Forensic Science International, 2016. **262**: p. 128-137.
168. Tormey, W.P., M. Sabah, and T.M. Moore, *Methadone, codeine and acute haemorrhagic necrotising pancreatitis: Which came first?* Forensic Science International, 2013. **226**(1): p. e52-e53.
169. Baselt, R.C., *Disposition of toxic drugs and chemicals in man*. 2002, United States.
170. Seymour, A., et al., *The role of dihydrocodeine in causing death among drug users in the west of Scotland.* 2001.
171. Kleine-Brueggeney, M., et al., *Pharmacogenetics in palliative care.* Forensic Science International, 2010. **203**(1): p. 63-70.
172. Costa, I., et al., *Postmortem redistribution of tramadol and O-desmethyltramadol.* J Anal Toxicol, 2013. **37**(9): p. 670-5.
173. Pilgrim, J.L., D. Gerostamoulos, and O.H. Drummer, *Deaths involving contraindicated and inappropriate combinations of serotonergic drugs.* International Journal of Legal Medicine, 2011. **125**(6): p. 803-815.
174. *Drug-**related deaths in Scotland in 2016**.*
175. Bastami, S., et al., *Pharmacogenetic aspects of tramadol pharmacokinetics and pharmacodynamics after a single oral dose.* Forensic Science International, 2014. **238**: p. 125-132.
176. Paar, W.D., P. Frankus, and H.J. Dengler, *The metabolism of tramadol by human liver microsomes.* The Clinical investigator, 1992. **70**(8): p. 708.

177. Poulsen, L., et al., *The hypoalgesic effect of tramadol in relation to CYP2D6.* Clinical Pharmacology & Therapeutics, 1996. **60**(6): p. 636-644.
178. Paar, W.D., et al., *Polymorphic CYP2D6 mediates O-demethylation of the opioid analgesic tramadol.* European Journal of Clinical Pharmacology, 1997. **53**(3): p. 235-239.
179. Subrahmanyam, V., et al., *Identification of Cytochrome P-450 Isoforms Responsible for cis-Tramadol Metabolism in Human Liver Microsomes.* Drug Metabolism and Disposition, 2001. **29**(8): p. 1146.
180. Wu, W.N., L.A. McKown, and S. Liao, *Metabolism of the analgesic drug ULTRAM ® (tramadol hydrochloride) in humans: API-MS and MS/MS characterization of metabolites.* Xenobiotica, 2002. **32**(5): p. 411-425.
181. Grond, S. and A. Sablotzki, *Clinical Pharmacology of Tramadol*. 2004, Adis International: Cham. p. 879-923.
182. Klotz, U., *Tramadol--the impact of its pharmacokinetic and pharmacodynamic properties on the clinical management of pain.* Arzneimittel-Forschung, 2003. **53**(10): p. 681.
183. Roughead, E.E., B. McDermott, and A.L. Gilbert, *Antidepressants: prevalence of duplicate therapy and avoidable drug interactions in Australian veterans.* Australasian Psychiatry, 2007. **41**(4): p. 366-370.
184. Tirkkonen, T. and K. Laine, *Drug interactions with the potential to prevent prodrug activation as a common source of irrational prescribing in hospital inpatients.* Clinical Pharmacology & Therapeutics, 2004. **76**(6): p. 639-647.
185. Nikolaou, P.D., et al., *Validated method for the simultaneous determination of methadone and its main metabolites (EDDP and EMDP) in plasma of umbilical cord blood by gas chromatography-mass spectrometry.* J Chromatogr B Analyt Technol Biomed Life Sci, 2008. **867**(2): p. 219-25.
186. Bizzarri, J.V., et al., *Erratum to: Agonist Opioid Treatment as historical comprehensive treatment ('Dole & Nyswander' methodology) is associated with better toxicology outcome than Harm Reduction Treatment.* Annals of general psychiatry, 2017. **16**(1): p. 10.
187. Frame, L., G. McKay, and M. Fisher, *Methadone*. Practical Diabetes, 2017. **34**(1): p. 34-35a.
188. Pimentel, L.M.D. and D.M.D. Mayo, *Chronic Methadone Therapy Complicated by Torsades De Pointes: A Case Report.* Journal of Emergency Medicine, 2008. **34**(3): p. 287-290.
189. Nielsen, M.K.K., S.S. Johansen, and K. Linnet, *Evaluation of metabolite/drug ratios in blood and urine as a tool for confirmation of a reduced tolerance in methadone-related deaths in Denmark.* Drug and Alcohol Dependence, 2013. **133**(2): p. 447-451.
190. Lewanowitsch, T., J.M. White, and R.J. Irvine, *Use of radiotelemetry to evaluate respiratory depression produced by chronic methadone administration.* European Journal of Pharmacology, 2004. **484**(2): p. 303-310.
191. Dole, V.P., *Implications of methadone maintenance for theories of narcotic addiction.* JAMA: The Journal of the American Medical Association, 1988. **260**(20): p. 3025-3029.
192. Seymour, A., et al., *The role of methadone in drug-related deaths in the west of Scotland.* Addiction, 2003. **98**(7): p. 995-1002.
193. Shields, L.B.E., et al., *Methadone Toxicity Fatalities: A Review of Medical Examiner Cases in a Large Metropolitan Area.* Journal of Forensic Sciences, 2007. **52**(6): p. 1389-1395.

194. *COMMENTARIES - Comments on White Irvine's "Mechanisms of fatal opioid overdose".* Addiction, 1999. **94**(7): p. 973-980.
195. Kuhn, R., *THE TREATMENT OF DEPRESSIVE STATES WITH G 22355 (IMIPRAMINE HYDROCHLORIDE).* American Journal of Psychiatry, 1958. **115**(5): p. 459-464.
196. Klerman, G.L. and J.O. Cole, *CLINICAL PHARMACOLOGY OF IMIPRAMINE AND RELATED ANTIDEPRESSANT COMPOUNDS.* Pharmacological Reviews, 1965. **17**(2): p. 101.
197. Ban, T.A., *Pharmacotherapy of depression: a historical analysis.* Journal of Neural Transmission, 2001. **108**(6): p. 707-716.
198. Feighner, J.P., *Mechanism of action of antidepressant medications.* The Journal of clinical psychiatry, 1999. **60 Suppl 4**: p. 4.
199. Al-Majed, A., et al., *Mirtazapine.* Profiles of drug substances, excipients, and related methodology, 2018. **43**: p. 209.
200. Khurshid, F., et al., *Effect of herb-drug interactions of Bacopa monnieri Linn. (Brahmi) formulation on the pharmacokinetics of amitriptyline in rats.* Brazilian Journal of Pharmaceutical Sciences, 2018. **53**(4).
201. Preskorn, S. and S. Werder, *Detrimental Antidepressant Drug-Drug Interactions: Are They Clinically Relevant?* Neuropsychopharmacology, 2006. **31**(8): p. 1605-1612.
202. Sangkuhl, K., T.E. Klein, and R.B. Altman, *PharmGKB summary: citalopram pharmacokinetics pathway.* Pharmacogenetics and Genomics, 2011. **21**(11): p. 769-772.
203. McIntyre, I.M. and P. Mallett, *Sertraline concentrations and postmortem redistribution.* Forensic Science International, 2012. **223**(1): p. 349-352.
204. Salmon, A.Y., et al., *HUMAN ERYTHROCYTE BUT NOT BRAIN ACETYLCHOLINESTERASE HYDROLYSES HEROIN TO MORPHINE.* Clinical and Experimental Pharmacology and Physiology, 1999. **26**(8): p. 596-600.
205. Wenzel, S., et al., *Tissue distribution of mirtazapine and desmethylmirtazapine in a case of mirtazapine poisoning.* Forensic Science International, 2006. **156**(2): p. 229-236.
206. Anderson, D.T., K.L. Fritz, and J.J. Muto, *Distribution of Mirtazapine (Remeron) in Thirteen Postmortem Cases.* Journal of Analytical Toxicology, 1999. **23**(6): p. 544-548.
207. Lundberg, J., et al., *Serotonin transporter occupancy with TCAs and SSRIs: a PET study in patients with major depressive disorder.* The International Journal of Neuropsychopharmacology, 2012. **15**(8): p. 1-6.
208. Goodwin, G.M., *How do antidepressants affect serotonin receptors? The role of serotonin receptors in the therapeutic and side effect profile of the SSRIs.* The Journal of clinical psychiatry, 1996. **57 Suppl 4**: p. 9.
209. Schreiber, S., A. Bleich, and C.G. Pick, *Venlafaxine and mirtazapine: Different mechanisms of antidepressant action, common opioid-mediated antinociceptive effects—A possible opioid involvement in severe depression?* Journal of Molecular Neuroscience, 2002. **18**(1): p. 143-149.
210. Maany, I., et al., *Increase in desipramine serum levels associated with methadone treatment.* American Journal of Psychiatry, 1989. **146**(12): p. 1611-1613.
211. Richelson, E., *Pharmacokinetic drug interactions of new antidepressants: a review of the effects on the metabolism of other drugs.* Mayo Clinic proceedings, 1997. **72**(9): p. 835.
212. Plummer, J.L., et al., *Estimation of methadone clearance: application in the management of cancer pain.* Pain, 1988. **33**(3): p. 313-322.

213. Bleakley, S., *Antidepressant drug interactions: evidence and clinical significance: Antidepressant drug interactions.* Progress in Neurology and Psychiatry, 2016. **20**(3): p. 21-27.
214. Nieuwstraten, C., N. Labiris, and A. Holbrook, *Systematic Overview of Drug Interactions with Antidepressant Medications*. Vol. 51. 2006. 300-16.
215. Taylor, D., *Selective Serotonin Reuptake Inhibitors and Tricyclic Antidepressants in Combination.* British Journal of Psychiatry, 1995. **167**(5): p. 575-580.
216. Kirkton, C. and I.M. McIntyre, *Therapeutic and Toxic Concentrations of Mirtazapine.* Journal of Analytical Toxicology, 2006. **30**(9): p. 687-691.
217. Nedahl, M., S.S. Johansen, and K. Linnet, *Reference Brain/Blood Concentrations of Citalopram, Duloxetine, Mirtazapine and Sertraline.* Journal of Analytical Toxicology, 2018. **42**(3): p. 149-156.
218. Pounder, D.J., V. Owen, and C. Quigley, *Postmortem changes in blood amitriptyline concentration.* The American journal of forensic medicine and pathology, 1994. **15**(3): p. 224-230.
219. Reis, M., et al., *Reference Concentrations of Antidepressants. A Compilation of Postmortem and Therapeutic Levels.* Journal of Analytical Toxicology, 2007. **31**(5): p. 254-264.
220. Blackwell, B., *Adverse effects of antidepressant drugs. Part 1: monoamine oxidase inhibitors and tricyclics.* Drugs, 1981. **21**(3): p. 201.
221. Nelson, J.C., et al., *Major Adverse Reactions During Desipramine Treatment: Relationship to Plasma Drug Concentrations, Concomitant Antipsychotic Treatment, and Patient Characteristics.* Archives of General Psychiatry, 1982. **39**(9): p. 1055-1061.
222. Barbey, J.T. and S.P. Roose, *SSRI safety in overdose.* The Journal of clinical psychiatry, 1998. **59 Suppl 15**: p. 42.
223. Minett, W.J., et al., *Concentrations of Opiates and Psychotropic Agents in Polydrug Overdoses: A Surprising Correlation Between Morphine and Antidepressants.* Journal of Forensic Sciences, 2010. **55**(5): p. 1319-1325.
224. *Mirtazapine: Various toxicities: case report.* Reactions Weekly, 2017. **1652**(1): p. 187-187.
225. Taylor, C.P., et al., *A summary of mechanistic hypotheses of gabapentin pharmacology.* Epilepsy Research, 1998. **29**(3): p. 233-249.
226. Marson, A.G., et al., *Gabapentin add-on for drug-resistant partial epilepsy.* The Cochrane database of systematic reviews, 2000(3): p. CD001415.
227. Rice, A.S.C., S. Maton, and G. Postherpetic Neuralgia Study, *Gabapentin in postherpetic neuralgia: a randomised, double blind, placebo controlled study.* Pain, 2001. **94**(2): p. 215-224.
228. Cabras, P.L., et al., *Clinical experience with gabapentin in patients with bipolar or schizoaffective disorder: results of an open-label study.* The Journal of clinical psychiatry, 1999. **60**(4): p. 245-248.
229. Pande, A.C., et al., *Treatment of social phobia with gabapentin: a placebo-controlled study.* Journal of clinical psychopharmacology, 1999. **19**(4): p. 341-348.
230. Bonnet, U., et al., *Treatment of alcohol withdrawal syndrome with gabapentin.* Pharmacopsychiatry, 1999. **32**(3): p. 107.
231. Bozikas, V., et al., *Treatment of alcohol withdrawal with gabapentin.* Progress in Neuropsychopharmacology & Biological Psychiatry, 2002. **26**(1): p. 197-199.
232. Myrick, H., et al., *Gabapentin in the treatment of cocaine dependence: a case series.* The Journal of clinical psychiatry, 2001. **62**(1): p. 19-23.

233. Bialer, M., et al., *Progress report on new antiepileptic drugs: A summary of the Eigth Eilat Conference (EILAT VIII).* Epilepsy Research, 2006. **73**(1): p. 1-52.
234. Bialer, M., et al., *Progress report on new antiepileptic drugs: a summary of the Sixth Eilat Conference (EILAT VI).* Epilepsy Research, 2002. **51**(1): p. 31-71.
235. Bockbrader, H.N., et al., *A Comparison of the Pharmacokinetics and Pharmacodynamics of Pregabalin and Gabapentin.* Clinical Pharmacokinetics, 2010. **49**(10): p. 661-669.
236. Eckhardt, K., et al., *Gabapentin enhances the analgesic effect of morphine in healthy volunteers.* Anesthesia and analgesia, 2000. **91**(1): p. 185-191.
237. Zand, L.M.D., K.P.M.D. McKian, and Q.M.D. Qian, *Gabapentin Toxicity in Patients with Chronic Kidney Disease: A Preventable Cause of Morbidity.* American Journal of Medicine, The, 2010. **123**(4): p. 367-373.
238. Hiemke, C., et al., *AGNP consensus guidelines for therapeutic drug monitoring in psychiatry: update 2011.* Pharmacopsychiatry., 2011. **44**(6): p. 195.
239. Hamm, C.E., R.D. Gary, and I.M. McIntyre, *Gabapentin concentrations and postmortem distribution.* Forensic Sci Int, 2016. **262**: p. 201-3.
240. Hamm, C.E., R.D. Gary, and I.M. McIntyre, *Gabapentin Concentrations and Postmortem Distribution.* Forensic Science International, 2016. **262**: p. 201-203.
241. Gomes, T., et al., *Gabapentin, opioids, and the risk of opioid-related death: A population-based nested case-control study.* PLoS medicine, 2017. **14**(10): p. e1002396.
242. Cantrell, F.L., et al., *An acute gabapentin fatality: a case report with postmortem concentrations.* Int J Legal Med, 2015. **129**(4): p. 771-5.
243. Slavova, S., et al., *Prevalence of gabapentin in drug overdose postmortem toxicology testing results.* Drug and Alcohol Dependence, 2018. **186**: p. 80-85.
244. Apple, F.S., *A Better Understanding of the Interpretation of Postmortem Blood Drug Concentrations.* Journal of Analytical Toxicology, 2011. **35**(6): p. 381-383.
245. Jung, B.F. and M.M. Reidenberg, *Interpretation of Opioid Levels: Comparison of Levels During Chronic Pain Therapy to Levels from Forensic Autopsies.* Clinical Pharmacology & Therapeutics, 2005. **77**(4): p. 324-334.
246. Felby, S., H. Christensen, and A. Lund, *Morphine concentrations in blood and organs in cases of fatal poisoning.* Forensic Science, 1974. **3**(1): p. 77-81.
247. Richards, R.G., D. Reed, and R.H. Cravey, *Death from intravenously administered narcotics: a study of 114 cases.* Journal of forensic sciences, 1976. **21**(3): p. 467.
248. Logan, B.K., J.S. Oliver, and H. Smith, *The measurement and interpretation of morphine in blood.* Forensic Science International, 1987. **35**(2): p. 189-195.
249. Logan, B. and D. Smirnow, *Postmortem Distribution and Redistribution of Morphine in Man.* 1996.
250. Spiehler, V. and R. Brown, *Unconjugated morphine in blood by radioimmunoassay and gas chromatography/mass spectrometry.* Journal of forensic sciences, 1987. **32**(4): p. 906.

251. Sawyer, W.R. and R.B. Forney, *Postmortem disposition of morphine in rats.* Forensic Science International, 1988. **38**(3): p. 259-273.
252. Steentoft, A., K. Worm, and H. Christensen, *Morphine concentrations in autopsy material from fatal cases after intake of morphine and/or heroin.* Journal of the Forensic Science Society, 1988. **28**(2): p. 87-94.
253. Kintz, P., et al., *Toxicological data after heroin overdose.* Human toxicology, 1989. **8**(6): p. 487.
254. Goldberger, B.A., et al., *Disposition of heroin and its metabolites in heroin-related deaths.* Journal of analytical toxicology, 1994. **18**(1): p. 22.
255. Gerostamoulos, J. and O.H. Drummer, *Postmortem redistribution of morphine and its metabolites.* Journal of forensic sciences, 2000. **45**(4): p. 843.
256. Burt, M.J., J. Kloss, and F.S. Apple, *Postmortem blood free and total morphine concentrations in medical examiner cases.* Journal of forensic sciences, 2001. **46**(5): p. 1138.
257. Cardona, P.S., et al., *Simultaneous analyses of cocaine, cocaethylene, and their possible metabolic and pyrolytic products.* Forensic Science International, 2006. **157**(1): p. 46-56.
258. Jones, A.W., et al., *Driving Under the Influence of Opiates: Concentration Relationships Between Morphine, Codeine, 6-Acetyl Morphine, and Ethyl Morphine in Blood.* Journal of Analytical Toxicology, 2008. **32**(4): p. 265-272.
259. Tiseo, P.J., et al., *Morphine-6-glucuronide concentrations and opioid-related side effects: a survey in cancer patients.* Pain, 1995. **61**(1): p. 47-54.
260. Joynt, B.P. and N.Z. Mikhael, *Sudden death of a heroin body packer.* Journal of analytical toxicology, 1985. **9**(5): p. 238.
261. Rook, E.J., et al., *Population Pharmacokinetics of Heroin and its Major Metabolites.* Clinical Pharmacokinetics, 2006. **45**(4): p. 401-417.
262. Rees, K.A., et al., *The effect of sodium fluoride preservative and storage temperature on the stability of 6-acetylmorphine in horse blood, sheep vitreous and deer muscle.* Forensic Science International, 2011. **217**(1): p. 189-195.
263. Antonides, H.M., E.R. Kiely, and L.J. Marinetti, *Vitreous Fluid Quantification of Opiates, Cocaine, and Benzoylecgonine: Comparison of Calibration Curves in Both Blood and Vitreous Matrices with Corresponding Concentrations in Blood.* Journal of Analytical Toxicology, 2007. **31**(8): p. 469-476.
264. Cone, E.J., et al., *Forensic drug testing for opiates: I. Detection of 6-acetylmorphine in urine as an indicator of recent heroin exposure; drug and assay considerations and detection times.* Journal of analytical toxicology, 1991. **15**(1): p. 1.
265. Garriott, J.C. and W.Q. Sturner, *Morphine concentrations and survival periods in acute heroin fatalities.* The New England journal of medicine, 1973. **289**(24): p. 1276.
266. Reed, D., *Comparison of spectrofluorometric and GC/MS procedures for the quantitation of morphine in blood and brain.* Clinical toxicology, 1979. **14**(2): p. 169.
267. Staub, C., R. Jeanmonod, and O. Fryc, *Morphine in postmortem blood: its importance for the diagnosis of deaths associated with opiate addiction.* International journal of legal medicine, 1990. **104**(1): p. 39-42.

268. Holmgren, P., et al., *Stability of drugs in stored postmortem femoral blood and vitreous humor.* Journal of forensic sciences, 2004. **49**(4): p. 820.
269. Avella, J., M. Katz, and M. Lehrer, *Assessing Free and Total Morphine following Heroin Overdose when Complicated by the Presence of Toxic Amitriptyline Levels.* Journal of Analytical Toxicology, 2007. **31**(8): p. 540-542.
270. Hasselström, J. and J. Säwe, *Morphine pharmacokinetics and metabolism in humans. Enterohepatic cycling and relative contribution of metabolites to active opioid concentrations.* Clinical pharmacokinetics, 1993. **24**(4): p. 344.
271. Romberg, R.W. and L. Lee, *Comparison of the hydrolysis rates of morphine-3-glucuronide and morphine-6-glucuronide with acid and beta-glucuronidase.* J Anal Toxicol, 1995. **19**(3): p. 157-62.
272. Brunk, S.F. and M. Delle, *Morphine metabolism in man.* Clinical pharmacology and therapeutics, 1974. **16**(1): p. 51.
273. Skopp, G., et al., *Postmortem distribution pattern of morphine and morphine glucuronides in heroin overdose.* International journal of legal medicine, 1996. **109**(3): p. 118-124.
274. Moriya, F. and Y. Hashimoto, *Distribution of free and conjugated morphine in body fluids and tissues in a fatal heroin overdose: is conjugated morphine stable in postmortem specimens?* Journal of forensic sciences, 1997. **42**(4): p. 736.
275. Gyr, E., et al., *Pharmacodynamics and pharmacokinetics of intravenously, orally and rectally administered diacetylmorphine in opioid dependents, a two-patient pilot study within a heroin-assisted treatment program.* International journal of clinical pharmacology and therapeutics, 2000. **38**(10): p. 486.
276. Pounder, D.J. and G.R. Jones, *Post-mortem drug redistribution — A toxicological nightmare.* Forensic Science International, 1990. **45**(3): p. 253-263.
277. Jones, A.W., et al., *Post-mortem concentrations of drugs determined in femoral blood in single-drug fatalities compared with multi-drug poisoning deaths.* Forensic Science International, 2016. **267**: p. 96-103.
278. Lemaire, E., et al., *Popliteal Vein Blood Sampling and the Postmortem Redistribution of Diazepam, Methadone, and Morphine.* Journal of Forensic Sciences, 2016. **61**(4): p. 1017-1028.
279. Pounder, D.J., *The nightmare of postmortem drug changes.* Legal medicine, 1993: p. 163.
280. Leikin, J.B. and W.A. Watson, *Post-mortem Toxicology: What The Dead Can And Cannot Tell Us.* Clinical Toxicology, 2003. **41**(1): p. 47-56.
281. Yarema, M.C. and C.E. Becker, *Key Concepts in Postmortem Drug Redistribution.* Clinical Toxicology, 2005. **43**(4): p. 235-241.
282. Maskell, P.D., et al., *Postmortem redistribution of the heroin metabolites morphine and morphine-3-glucuronide in rabbits over 24 h.* International Journal of Legal Medicine, 2016. **130**(2): p. 519-531.
283. Frost, J., et al., *Post-mortem levels and tissue distribution of codeine, codeine-6-glucuronide, norcodeine, morphine and morphine glucuronides in a series of codeine-related deaths.* Forensic Science International, 2016. **262**: p. 128-137.
284. Pos Pok, P.R., et al., *Cardiac and Peripheral Blood Similarities in the Comparison of Nordiazepam and Bromazepam Blood Concentrations.* Journal of Analytical Toxicology, 2008. **32**(9): p. 782-786.

285. Yonemitsu, K. and D.J. Pounder, *Postmortem changes in blood tranylcypromine concentration: Competing redistribution and degradation effects.* Forensic Science International, 1993. **59**(2): p. 177-184.
286. McIntyre, I.M., *Liver and peripheral blood concentration ratio (L/P) as a marker of postmortem drug redistribution: a literature review.* Forensic Science, Medicine, and Pathology, 2014. **10**(1): p. 91-96.
287. Maskell, P.D., et al., *Postmortem tissue distribution of morphine and its metabolites in a series of heroin related deaths.* Drug testing and analysis, 2018.
288. Musshoff, F. and B. Madea, *Fatality due to ingestion of tramadol alone.* Forensic Science International, 2001. **116**(2): p. 197-199.
289. Langford, A.M. and D.J. Pounder, *Possible markers for postmortem drug redistribution.* Journal of forensic sciences, 1997. **42**(1): p. 88.
290. Fu, K., et al., *An Unusual Multiple Drug Intoxication Case Involving Citalopram.* Journal of Analytical Toxicology, 2000. **24**(7): p. 648-650.
291. Rodda, K.E. and O.H. Drummer, *The redistribution of selected psychiatric drugs in post-mortem cases.* Forensic Science International, 2006. **164**(2): p. 235-239.
292. Moore, K.A., et al., *Tissue Distribution of Mirtazapine (Remeron) in Postmortem Cases.* Journal of Analytical Toxicology, 1999. **23**(6): p. 541-543.
293. Zezulak, M., J.J. Snyder, and S.B. Needleman, *Simultaneous analysis of codeine, morphine, and heroin after B-glucuronidase hydrolysis.* Journal of forensic sciences, 1993. **38**(6): p. 1275.
294. Carroll, F.T., et al., *Morphine-3-D glucuronide stability in postmortem specimens exposed to bacterial enzymatic hydrolysis.* The American journal of forensic medicine and pathology, 2000. **21**(4): p. 323-329.
295. Lockridge, O., et al., *Hydrolysis of diacetylmorphine (heroin) by human serum cholinesterase.* Journal of Pharmacology and Experimental Therapeutics, 1980. **215**(1): p. 1.
296. Nakamura, G.R., J.I. Thornton, and T.T. Noguchi, *Kinetics of heroin deacetylation in aqueous alkaline solution and in human serum and whole blood.* Journal of Chromatography A, 1975. **110**(1): p. 81-89.
297. Smith, D.A. and W.J. Cole, *Identification of an arylesterase as the enzyme hydrolysing diacetylmorphine (heroin) in human plasma.* Biochemical pharmacology, 1976. **25**(4): p. 367.
298. Barrett, D.A., P.N. Shaw, and S.S. Davis, *Determination of morphine and 6-acetylmorphine in plasma by high-performance liquid chromatography with fluorescence detection.* Journal of Chromatography B: Biomedical Sciences and Applications, 1991. **566**(1): p. 135-145.
299. Barrett, D.A., A.L.P. Dyssegaard, and P.N. Shaw, *The effect of temperature and pH on the deacetylation of diamorphine in aqueous solution and in human plasma.* Journal of Pharmacy and Pharmacology, 1992. **44**(7): p. 606-608.
300. Pichini, S., et al., *The role of liquid chromatography–mass spectrometry in the determination of heroin and related opioids in biological fluids.* Mass Spectrometry Reviews, 1999. **18**(2): p. 119-130.
301. Garcia Boy, R., et al., *Determination of Morphine and 6-Acetylmorphine in Blood With Use of Dried Blood Spots.* Therapeutic Drug Monitoring, 2008. **30**(6): p. 733-739.

302. Papoutsis, I., et al., *Stability of Morphine, Codeine, and 6-Acetylmorphine in Blood at Different Sampling and Storage Conditions.* Journal of Forensic Sciences, 2014. **59**(2): p. 550-554.
303. Høiseth, G., et al., *Long-term stability of morphine, codeine, and 6-acetylmorphine in real-life whole blood samples, stored at −20 °C.* Forensic Science International, 2014. **239**: p. 6-10.
304. Beaumont, I. and T. Deeks, *Determination of morphine, diamorphine and their degradation products in pharmaceutical preparations by reversed-phase high-performance liquid chromatography.* Journal of Chromatography A, 1982. **238**(2): p. 520-524.
305. Romolo, F.S., et al., *Optimized conditions for simultaneous determination of opiates, cocaine and benzoylecgonine in hair samples by GC–MS.* Forensic Science International, 2003. **138**(1): p. 17-26.
306. Stabler, P.J. and N.C. Bruce, *Oxidation of morphine to 2,2'-bimorphine by Cylindrocarpon didymum.* Applied and Environmental Microbiology, 1998. **64**(10): p. 4106-4108.
307. Vermeire, A. and J.P. Remon, *Stability and compatibility of morphine.* 1999, Elsevier B.V: NETHERLANDS. p. 17-51.
308. Yadlapalli, J.S.K., et al., *Stability studies of potent opioid analgesic, morphine-6-O-sulfate in various buffers and biological matrices by HPLC-DAD analysis.* Biomedical Chromatography, 2017. **31**(9): p. n/a-n/a.
309. Schmid, R., et al., *The stability of a ketamine-morphine solution.* Anesthesia and analgesia, 2002. **94**(4): p. 898-900.
310. Chang, B.-L., M.-K. Huang, and Y.-Y. Tsai, *Total Morphine Stability in Urine Specimens Stored under Various Conditions.* Journal of Analytical Toxicology, 2000. **24**(6): p. 442-447.
311. Hadidi, K.A. and J.S. Oliver, *Stability of morphine and buprenorphine in whole blood.* International Journal of Legal Medicine, 1998. **111**(3): p. 165-167.
312. Taylor, K. and S. Elliott, *A validated hybrid quadrupole linear ion-trap LC–MS method for the analysis of morphine and morphine glucuronides applied to opiate deaths.* Forensic Sci Int, 2009. **187**(1-3): p. 34-41.
313. Karinen, R., et al., *Long-Term Storage of Authentic Postmortem Forensic Blood Samples at −20°C: Measured Concentrations of Benzodiazepines, Central Stimulants, Opioids and Certain Medicinal Drugs Before and After Storage for 16–18 Years.* Journal of Analytical Toxicology, 2014. **38**(9): p. 686-695.
314. Lombardo, A., et al., *Enzymes of lysosomal origin in human plasma and serum: Assay conditions and parameters influencing the assay.* Clinica Chimica Acta, 1980. **108**(3): p. 337-346.
315. Dixon, R.B., F. Mbeunkui, and J.V. Wiegel, *Stability study of opioids and benzodiazepines in urine samples by liquid chromatography tandem mass spectrometry.* Journal of Analytical Science and Technology, 2015. **6**(1): p. 1-10.
316. Jones, J.M., et al., *Stability of heroin, 6-monoacetylmorphine, and morphine in biological samples and validation of an LC-MS assay for delayed analyses of pharmacokinetic samples in rats.* Journal of pharmaceutical and biomedical analysis, 2013. **74**: p. 291-297.
317. Karinen, R., et al., *Determination of Heroin and Its Main Metabolites in Small Sample Volumes of Whole Blood and Brain Tissue by Reversed-Phase Liquid Chromatography–Tandem Mass Spectrometry.* Journal of Analytical Toxicology, 2009. **33**(7): p. 345-350.

318. King, R., et al., *Mechanistic investigation of ionization suppression in electrospray ionization.* Journal of the American Society for Mass Spectrometry, 2000. **11**(11): p. 942-950.
319. Crump, K.L., I.M. McIntyre, and O.H. Drummer, *Simultaneous determination of morphine and codeine in blood and bile using dual ultraviolet and fluorescence high-performance liquid chromatography.* Journal of analytical toxicology, 1994. **18**(4): p. 208.
320. Lerch, O., O. Temme, and T. Daldrup, *Comprehensive automation of the solid phase extraction gas chromatographic mass spectrometric analysis (SPE-GC/MS) of opioids, cocaine, and metabolites from serum and other matrices.* Anal Bioanal Chem, 2014. **406**(18): p. 4443-51.
321. Reagen, W.K., et al., *Comparison of extraction and quantification methods of perfluorinated compounds in human plasma, serum, and whole blood.* Analytica Chimica Acta, 2008. **628**(2): p. 214-221.
322. Poole, C.F., *New trends in solid-phase extraction.* Trends in Analytical Chemistry, 2003. **22**(6): p. 362-373.
323. Freiermuth, M. and J.-C. Plasse, *Determination of morphine and codeine in plasma by HPLC following solid phase extraction.* Journal of Pharmaceutical and Biomedical Analysis, 1997. **15**(6): p. 759-764.
324. Papadoyannis, I., et al., *Comparative Study of Different Solid-Phase Extraction Cartridges in the Simultaneous RP-HPLC Analysis of Morphine and Codeine in Biological Fluids.* Journal of Liquid Chromatography, 1993. **16**(14): p. 3017-3040.
325. Theodoridis, G., et al., *A Comparative Study of Different Solid Phase Extraction Procedures for the Analysis of Alkaloids of Forensic Interest in Biological Fluids by RP-HPLC/Diode Array.* Journal of Liquid Chromatography, 1995. **18**(10): p. 1973-1995.
326. Mortier, K.A., et al., *Simultaneous, quantitative determination of opiates, amphetamines, cocaine and benzoylecgonine in oral fluid by liquid chromatography quadrupole-time-of-flight mass spectrometry.* Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 2002. **779**(2): p. 321-330.
327. Saint-Marcoux, F., G. Lachâtre, and P. Marquet, *Evaluation of an improved general unknown screening procedure using liquid-chromatography-electrospray-mass spectrometry by comparison with gas chromatography and high-performance liquid-chromatography—diode array detection.* Journal of the American Society for Mass Spectrometry, 2003. **14**(1): p. 14-22.
328. Bogusz, M.J., et al., *Poor reproducibility of in-source collisional atmospheric pressure ionization mass spectra of toxicologically relevant drugs.* Journal of Chromatography A, 1999. **844**(1): p. 409-418.
329. Herrin, G.L., H.H. McCurdy, and W.H. Wall, *Investigation of an LC-MS-MS (QTrap®) Method for the Rapid Screening and Identification of Drugs in Postmortem Toxicology Whole Blood Samples.* Journal of Analytical Toxicology, 2005. **29**(7): p. 599-606.
330. Drummer, O.H., *Requirements for bioanalytical procedures in postmortem toxicology.* Analytical and Bioanalytical Chemistry, 2007. **388**(7): p. 1495-1503.
331. Mackey-Bojack, S., J. Kloss, and F. Apple, *Cocaine, Cocaine Metabolite, and Ethanol Concentrations in Postmortem Blood and Vitreous Humor.* Journal of Analytical Toxicology, 2000. **24**(1): p. 59-65.

332. Wyman, J. and S. Bultman, *Postmortem Distribution of Heroin Metabolites in Femoral Blood, Liver, Cerebrospinal Fluid, and Vitreous Humor*. Journal of Analytical Toxicology, 2004. **28**(4): p. 260-263.
333. Horikoshi, R., et al., *Illustrating the Basic Functioning of Mass Analyzers in Mass Spectrometers with Ball-Rolling Mechanisms.* Journal of Chemical Education, 2017. **94**(10): p. 1502-1506.
334. Snyder, D.T. and R.G. Cooks, *Single Analyzer Precursor Ion Scans in a Linear Quadrupole Ion Trap Using Orthogonal Double Resonance Excitation.* Journal of The American Society for Mass Spectrometry, 2017. **28**(9): p. 1929-1938.
335. Thevis, M., et al., *Mass spectrometry of stanozolol and its analogues using electrospray ionization and collision-induced dissociation with quadrupole-linear ion trap and linear ion trap-orbitrap hybrid mass analyzers.* Rapid Communications in Mass Spectrometry, 2005. **19**(22): p. 3369-3378.
336. Kang, J.-S., *Principles and Applications of LC-MS/MS for the Quantitative Bioanalysis of Analytes in Various Biological Samples, Tandem Mass Spectrometry - Applications and Principles.*, Dr Jeevan Prasain (Ed.), Editor. 2012.
337. Kruve, A. and K. Kaupmees, *Adduct Formation in ESI/MS by Mobile Phase Additives.* Journal of The American Society for Mass Spectrometry, 2017. **28**(5): p. 887-894.
338. Rainville, P.D., et al., *Comprehensive investigation of the influence of acidic, basic, and organic mobile phase compositions on bioanalytical assay sensitivity in positive ESI mode LC/MS/MS.* Journal of Pharmaceutical and Biomedical Analysis, 2012. **59**: p. 138-150.
339. Martens-Lobenhoffer, J. and S.M. Bode-Boger, *Fast and Efficient Determination of Arginine, Symmetric Dimethylarginine, and Asymmetric Dimethylarginine in Biological Fluids by Hydrophilic-Interaction Liquid Chromatography-Electrospray Tandem Mass Spectrometry.* Clinical Chemistry, 2006. **52**(3): p. 488-493.
340. Oertel, R., V. Neumeister, and W. Kirch, *Hydrophilic interaction chromatography combined with tandem-mass spectrometry to determine six aminoglycosides in serum.* Journal of Chromatography A, 2004. **1058**(1): p. 197-201.
341. Aronov, P.A., et al., *Metabolic profiling of major vitamin D metabolites using Diels-Alder derivatization and ultra-performance liquid chromatography-tandem mass spectrometry.* Analytical and Bioanalytical Chemistry, 2008. **391**(5): p. 1917-1930.
342. Licea-Perez, H., et al., *Development of a highly sensitive and selective UPLC/MS/MS method for the simultaneous determination of testosterone and 5a-dihydrotestosterone in human serum to support testosterone replacement therapy for hypogonadism.* Steroids, 2008. **73**(6): p. 601-610.
343. Ventura, R., et al., *High-Throughput and Sensitive Screening by Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry of Diuretics and other Doping Agents.* European Journal of Mass Spectrometry, 2008. **14**(3): p. 191-200.
344. Nordström, A., et al., *Nonlinear data alignment for UPLC-MS and HPLC-MS based metabolomics: quantitative analysis of endogenous and exogenous metabolites in human serum.* Analytical chemistry, 2006. **78**(10): p. 3289-3295.

345. Booth, B., et al., *Workshop Report: Crystal City V—Quantitative Bioanalytical Method Validation and Implementation: The 2013 Revised FDA Guidance*. The AAPS Journal, 2015. **17**(2): p. 277-288.
346. *Laboratory guidelines and standards in clinical and forensic toxicology | SpringerLink*. 2006.
347. Elliott, S.P., D.W.S. Stephen, and S. Paterson, *The United Kingdom and Ireland association of forensic toxicologists forensic toxicology laboratory guidelines (2018)*. Science & Justice, 2018. **58**(5): p. 335-345.
348. Scientific Working Group for Forensic, T., *Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology*. Journal of Analytical Toxicology, 2013. **37**(7): p. 452-474.
349. Branum, G.D., et al., *The Feasibility of the Detection and Quantitation of β-Adrenergic Blockers by Solid-Phase Extraction and Subsequent Derivatization with Methaneboronic Acid*. Journal of Analytical Toxicology, 1998. **22**(2): p. 135-141.
350. Boonjob, W., et al., *Retention and selectivity of basic drugs on solid-phase extraction sorbents: Application to direct determination of β-blockers in urine*. Analytical and Bioanalytical Chemistry, 2014. **406**(17): p. 4207-4215.
351. Vessman, J., *Selectivity or specificity? Validation of analytical methods from the perspective of an analytical chemist in the pharmaceutical industry*. Journal of Pharmaceutical and Biomedical Analysis, 1996. **14**(8): p. 867-869.
352. Vessman, J., et al., *Selectivity in analytical chemistry (IUPAC Recommendations 2001)*. Pure and Applied Chemistry, 2001. **73**(8): p. 1381-1386.
353. Peters, F.T., O.H. Drummer, and F. Musshoff, *Validation of new methods*. Forensic Sci Int, 2007. **165**(2-3): p. 216-24.
354. de Castro, A., et al., *Development and validation of a liquid chromatography mass spectrometry assay for the simultaneous quantification of methadone, cocaine, opiates and metabolites in human umbilical cord*. Journal of Chromatography B, 2009. **877**(27): p. 3065-3071.
355. Santos, V., et al., *Determining plasma morphine levels using GC-MS after solid phase extraction to monitor drug levels in the postoperative period*. Clinics (Sao Paulo, Brazil), 2008. **63**(3): p. 307-314.
356. Coles, R., et al., *Simultaneous Determination of Codeine, Morphine, Hydrocodone, Hydromorphone, Oxycodone, and 6-Acetylmorphine in Urine, Serum, Plasma, Whole Blood, and Meconium by LC-MS-MS*. Journal of Analytical Toxicology, 2007. **31**(1): p. 1-14.
357. Polettini, A., et al., *Determination of opiates in hair. Effects of extraction methods on recovery and on stability of analytes*. Forensic Science International, 1997. **84**(1): p. 259-269.
358. Ventura, M., et al., *Stability studies of principal illicit drugs in oral fluid: preparation of reference materials for external quality assessment schemes*. Therapeutic drug monitoring, 2007. **29**(5): p. 662-665.
359. Bosch, M.E., et al., *Morphine and its metabolites: analytical methodologies for its determination*. J Pharm Biomed Anal, 2007. **43**(3): p. 799-815.
360. Barroso, M., et al., *Bioanalytical procedures and recent developments in the determination of opiates/opioids in human biological samples*. Anal Bioanal Chem, 2011. **400**(6): p. 1665-90.

361. Deventer, K., et al., *Direct quantification of morphine glucuronides and free morphine in urine by liquid chromatography–tandem mass spectrometry.* Forensic Toxicology, 2012. **30**(2): p. 106-113.
362. Van Thuyne, W., P. Van Eenoo, and F.T. Delbeke, *Urinary concentrations of morphine after the administration of herbal teas containing Papaveris fructus in relation to doping analysis.* Journal of Chromatography B, 2003. **785**(2): p. 245-251.
363. Meatherall, R., *GCMS Confirmation of Codeine, Morphine, 6-Acetylmorphine, Hydrocodone, Hydromorphone, Oxycodone, and Oxymorphone in Urine.* Journal of Analytical Toxicology, 1999. **23**(3): p. 177-186.
364. Yang, H.S., A.H.B. Wu, and K.L. Lynch, *Development and Validation of a Novel LC-MS/MS Opioid Confirmation Assay: Evaluation of β-glucuronidase Enzymes and Sample Cleanup Methods.* Journal of analytical toxicology, 2016. **40**(5): p. 323-329.
365. Duer, W.C. and S. McFarland, *Comments on "Incomplete recovery of prescription opioids in urine using enzymatic hydrolysis of glucuronide metabolites".* J Anal Toxicol, 2007. **31**(7): p. 419-20; author reply 421.
366. Wang, P., et al., *Incomplete recovery of prescription opioids in urine using enzymatic hydrolysis of glucuronide metabolites.* J Anal Toxicol, 2006. **30**(8): p. 570-5.
367. Kolmonen, M., et al., *Hydrophilic interaction liquid chromatography and accurate mass measurement for quantification and confirmation of morphine, codeine and their glucuronide conjugates in human urine.* Journal of Chromatography B, 2010. **878**(29): p. 2959-2966.
368. Svensson, J.O., et al., *Electrospray LCMS Method with Solid-Phase Extraction for Accurate Determination of Morphine-, Codeine-, and Ethylmorphine-Glucuronides and 6-Acetylmorphine in Urine.* Journal of Analytical Toxicology, 2007. **31**(2): p. 81-86.
369. Netriova, J., et al., *HPLC determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human serum of oncological patients after administration of morphine drugs.* Die Pharmazie, 2006. **61**(6): p. 528.
370. Tyrefors, N., et al., *Determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human serum by solid-phase extraction and liquid chromatography-mass spectrometry with electrospray ionisation.* Journal of Chromatography A, 1996. **729**(1): p. 279-285.
371. Mallet, C.R., Z. Lu, and J.R. Mazzeo, *A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts.* Rapid Communications in Mass Spectrometry, 2004. **18**(1): p. 49-58.
372. Jemal, M., *Negative ion electrospray high-performance liquid chromatography–mass spectrometry method development for determination of a highly polar phosphonic acid/sulfonic acid compound in plasma Optimization of ammonium acetate concentration and in-source collision-induced dissociation.* Journal of Chromatography B: Biomedical Sciences and Applications, 1997. **703**(1-2): p. 167-175.
373. Gergov, M., et al., *Simultaneous screening and quantification of 25 opioid drugs in post-mortem blood and urine by liquid chromatography–tandem mass spectrometry.* Forensic Science International, 2009. **186**(1): p. 36-43.
374. Yan, Z., et al., *Isobaric metabolite interferences and the requirement for close examination of raw data in addition to stringent chromatographic*

separations in liquid chromatography/tandem mass spectrometric analysis of drugs in biological matrix. Rapid Communications in Mass Spectrometry, 2008. **22**(13): p. 2021-2028.
375. Claessens, H.A., *Trends and progress in the characterization of stationary phases for reversed-phase liquid chromatography.* Trends in Analytical Chemistry, 2001. **20**(10): p. 563-583.
376. Acevska, J., et al., *Chemometric approach for development, optimization, and validation of different chromatographic methods for separation of opium alkaloids.* Analytical and Bioanalytical Chemistry, 2012. **403**(4): p. 1117-1129.
377. Kazemipour, M., I. Fakhari, and M. Ansari, *Gabapentin Determination in Human Plasma and Capsule by Coupling of Solid Phase Extraction, Derivatization Reaction, and UV-Vis Spectrophotometry.* Iranian journal of pharmaceutical research : IJPR, 2013. **12**(3): p. 247.
378. Cao, L., et al., *Determination of gabapentin in human plasma by capillary electrophoresis-laser induced fluorescence detection with and without solid-phase extraction.* Microchimica Acta, 2012. **178**(3): p. 285-292.
379. <*Analysis of Gabapentin in Serum and Plasma by Solid-Phase Extraction and Gas Chromatography-Mass Spectrometry for Therapeutic Drug Monitoring (2).pdf*>.
380. Wolf, C.E., et al., *Determination of Gabapentin in Serum Using Solid-Phase Extraction and Gas-Liquid Chromatography.* Journal of Analytical Toxicology, 1996. **20**(6): p. 498-501.
381. <*Review Morphine and its metabolites Analytical methodologies for its determination.pdf*>.
382. <*Quantification of morphine, morphine 6-glucuronide, buprenorphine, and the enantiomers of methadone by enantioselective mass spectrometric chromatography in whole blood.pdf*>.
383. Kim, S.-E., et al., *Simple and accurate quantitative analysis of ten antiepileptic drugs in human plasma by liquid chromatography/tandem mass spectrometry.* Journal of Pharmaceutical and Biomedical Analysis, 2011. **56**(4): p. 771-777.
384. Bu, J., et al., *Distinguishing Heroin Abuse from Codeine Administration in the Urine of Chinese People by UPLC-MS-MS.* Journal of Analytical Toxicology, 2013. **37**(3): p. 166-174.
385. Chen, C.P., et al., *Gabapentin Enacarbil and Morphine Administered in Combination Versus Alone: A Double-blind, Randomized, Pharmacokinetic, and Tolerability Comparison.* Clinical Therapeutics, 2015. **37**(2): p. 349-357.
386. Matuszewski, B.K., M.L. Constanzer, and C.M. Chavez-Eng, *Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS.* Analytical chemistry, 2003. **75**(13): p. 3019-3030.
387. Shibata, M., et al., *Detection of 22 antiepileptic drugs by ultra-performance liquid chromatography coupled with tandem mass spectrometry applicable to routine therapeutic drug monitoring.* Biomedical Chromatography, 2012. **26**(12): p. 1519-1528.
388. Van Eeckhaut, A., et al., *Validation of bioanalytical LC–MS/MS assays: Evaluation of matrix effects.* Journal of Chromatography B, 2009. **877**(23): p. 2198-2207.
389. Penninckx, W., et al., *Validation of the calibration procedure in atomic absorption spectrometric methods.* Journal of Analytical Atomic Spectrometry, 1996. **11**(4): p. 237.

390. López, M.L., M.D. Baño, and J.L. Guillén, *Long-Term Stability of Methadone in Clinical Plasma Samples Stored at −20°C*. Journal of Analytical Toxicology, 2002. **26**(4): p. 236-238.
391. Dugan, S., et al., *Stability of drugs of abuse in urine samples stored at -20 degrees C*. Journal of analytical toxicology, 1994. **18**(7): p. 391.
392. Weatherall, M., et al., *Avoiding pitfalls of correlation coefficients in the assessment of measurement instruments in rehabilitation research*. Clinical Rehabilitation, 2004. **18**(2): p. 186-194.
393. Bland, J.M. and D.G. Altman, *Measuring agreement in method comparison studies*. Statistical methods in medical research, 1999. **8**(2): p. 135-160.
394. Petersen, P.H., et al., *Graphical interpretation of analytical data from comparison of a field method with a Reference Method by use of difference plots*. Clinical Chemistry, 1997. **43**(11): p. 2039.
395. Bland, J.M. and D.G. Altman, *Statistical methods for assessing agreement between two methods of clinical measurement*. International Journal of Nursing Studies, 2010. **47**(8): p. 931-936.
396. Karelitz, J.L., V.C. Michael, and K.A. Perkins, *ANALYSIS OF AGREEMENT BETWEEN EXPIRED-AIR CARBON MONOXIDE MONITORS*. Journal of smoking cessation, 2017. **12**(2): p. 105-112.
397. Dewé, W., *Review of statistical methodologies used to compare (bio)assays*. Journal of Chromatography B, 2009. **877**(23): p. 2208-2213.
398. Al-Asmari, A.I. and R.A. Anderson, *Method for Quantification of Opioids and their Metabolites in Autopsy Blood by Liquid Chromatography-Tandem Mass Spectrometry*. Journal of Analytical Toxicology, 2007. **31**(7): p. 394-408.
399. Darke, S. and J. Duflou, *The toxicology of heroin-related death: estimating survival times*. Addiction, 2016. **111**(9): p. 1607-1613.
400. Aderjan, R., et al., *Morphine and morphine glucuronides in serum of heroin consumers and in heroin-related deaths determined by HPLC with native fluorescence detection*. Journal of analytical toxicology, 1995. **19**(3): p. 163.
401. Bodd, E., et al., *Morphine-6-Glucuronide might Mediate the Prolonged Opioid Effect of Morphine in Acute Renal Failure*. Human and Experimental Toxicology, 1990. **9**(5): p. 317-321.
402. Drummer, O.H. and J. Gerostamoulos, *Postmortem drug analysis: analytical and toxicological aspects*. Therapeutic drug monitoring, 2002. **24**(2): p. 199-209.
403. Skopp, G., *Postmortem toxicology*. Forensic Sci Med Pathol, 2010. **6**(4): p. 314-25.
404. Staeheli, S.N., et al., *Time-dependent postmortem redistribution of morphine and its metabolites in blood and alternative matrices—application of CT-guided biopsy sampling*. International Journal of Legal Medicine, 2017. **131**(2): p. 379-389.
405. Sousa, T., et al., *The gastrointestinal microbiota as a site for the biotransformation of drugs*. International Journal of Pharmaceutics, 2008. **363**(1): p. 1-25.
406. Hilberg, T., et al., *Diffusion as a mechanism of postmortem drug redistribution: an experimental study in rats*. International journal of legal medicine, 1992. **105**(2): p. 87-91.
407. Cook, D.S., R.A. Braithwaite, and K.A. Hale, *Estimating antemortem drug concentrations from postmortem blood samples: the influence of postmortem redistribution*. Journal of clinical pathology, 2000. **53**(4): p. 282-285.

408. Hargrove, V.M. and D.K. Molina, *Peripheral postmortem redistribution of morphine*. Am J Forensic Med Pathol, 2014. **35**(2): p. 106-8.

Appendices

Appendix 1

Drugs included in Selectivity/Specificity/Interference Studies

Drugs used to assess Selectivity and Specificity		
Atenolol	Alprazolam	Halazepam
Verapamil	Estazolam	Prazepam
Lidocaine	Triazolam	Lorazepam
Ethylmorphine	Climazolam	Lormetazepam
Codeine	Loprazolam	Oxazepam
Dihydrocodeine	Midazolam	Temazepam
Methadone	Phenazepam	Clonazepam
EDDP	Etizolam	Flunitrazepam
EMDP	Allobarbital	7-Aminoflunitrazepam
Pethidine	Amobarbital	Nimetazepam
Propoxyphene(Dextro-/Levo-)	Aprobarbital	Nitrazepam
Fentanyl	Alphenal	Adinazolam
Norfentanyl	Barbital	Sertraline
Oxycodone	Brallobarbital	Moclobemide
Hydrocodone	Pentobarbitone	Mirtazapine
Tramadol	Phenobarbitone	Nefazodone
Dipipanone	Primidone	Venlafaxine
Buprenorphine	Secobarbital	Chlorpromazine
Norbuprenorphine	Amitriptyline	Promazine
Cocaine	Nortriptyline	Triflupromazine
Ecgonine methyl ester(EME)	Clomipramine	Levomepromazine
Benzoylecgonine(BZE)	Dosulepin	Methotrimeprazine
Cocaethylene	Doxepin	Mesoridazine
Ecgonine	Imipramine	Thioridazine
Chlordiazepoxide	Desipramine	Perphenazine
Clorazepate	Citalopram	Prochlorperazine
Diazepam	Fluoxetine	Trifluoperazine

Drugs included in the optimised/developed methods were not included in the selectivity studies. All were at a concentration of 1 µg/mL.

Appendix 2

WoSRES
West of Scotland Research Ethics Service

NHS
Greater Glasgow and Clyde

Mr Abbas Kablan
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Date 04 July 2018
Direct line 0141 232 1807
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Dear Mr Kablan

Study title:	**Quantification and interpretation of morphine and its glucuronide metabolites in current and historical postmortem cases.**
REC reference:	**17/WS/0102**
Protocol number:	**N/A**
IRAS project ID;	**227157**

This study was given a favourable ethical opinion by the Committee on 02 June 2017.

Research Ethics Committees are required to keep a favourable opinion under review in the light of progress reports and any developments in the study. You should submit a progress report for the study 12 months after the date on which the favourable opinion was given, and then annually thereafter. Our records indicate that a progress report is overdue for 2018. It would be appreciated if you could complete and submit the report by no later than one month from the date of this letter.

Guidance on progress reports and a copy of the standard NRES progress report form is available from the Health Research Authority website.

The Health Research Authority website also provides guidance on declaring the end of the study.

If you fail to submit regular progress reports – which is a condition of the favourable ethical opinion – the REC may wish to consider suspending or terminating its opinion.

17/WS/0102:	**Please quote this number on all correspondence**

Yours sincerely

Abibat Adewumi-Ogunjobi
REC Manager

Copy to: *Dr Hazel Torrance*
Ms Lyndsay Mcdade, NHS Greater Glasgow and Clyde

Appendix 3

WoSRES
West of Scotland Research Ethics Service

NHS
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Mr Abbas Kablan
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Date 3 May 2017
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E-mail WoSREC4@ggc.scot.nhs.uk

Dear Mr Kablan

Study title: **An Investigation into the Post-Mortem Redistribution of Drugs**
REC reference: **17/WS/0026**
Protocol number: **N/A**
IRAS project ID: **213185**

Thank you for your submission of 3 May 2017. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 28 April 2017

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Participant consent form [Consent_Form]	4	29 April 2017
Participant information sheet (PIS) [Participant_Information]	7	29 April 2017

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Clinical Trial Insurance]		12 August 2015
Other [CV for Berlian Isnia]	1.2	

Document	*Version*	*Date*
Other [CV for Dr Marjorie]	1	26 October 2016
Other [Clarification]	1	15 March 2017
Other [Clarification 2]	1	20 April 2017
Participant consent form [Consent_Form]	4	29 April 2017
Participant information sheet (PIS) [Participant_Information]	7	29 April 2017
REC Application Form [REC_Form_17012017]		17 January 2017
Research protocol or project proposal [Postgraduate PhD Forensic Toxicology student project]	2	31 October 2016
Summary CV for Chief Investigator (CI) [Abbas Kablan cv]	1.2	
Summary CV for supervisor (student research) [Hazel Torrance cv]		
Summary CV for supervisor (student research) [CV Dr John Williams]	1.2	
Summary, synopsis or diagram (flowchart) of protocol in non technical language [Flow chart]	2	31 October 2016

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

17/WS/0026	**Please quote this number on all correspondence**

Yours sincerely

Rozanne Suarez
REC Manager

Copy to: *Dr Hazel Torrance, University of Glasgow*
Lyndsay McDade, NHS Greater Glasgow and Clyde

Appendix 4

26/07/16

MVLS College Ethics Committee

Project Title: An Investigation into the Post-Mortem Redistribution of Drugs
Project No: 200150153

Dear Dr Torrance,

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study but the project requires approval by the NHS Research Ethics Committee.

- Project end date:
- The data should be held securely for a period of ten years after the completion of the research project, or for longer if specified by the research funder or sponsor, in accordance with the University's Code of Good Practice in Research: (http://www.gla.ac.uk/media/media_227599_en.pdf)
- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely,

Appendix 5

University of Glasgow | College of Medical, Veterinary & Life Sciences

PARTICIPANT INFORMATION SHEET

First and foremost, I offer you my deepest condolences for your recent loss. Thank you very much for taking time to read this participant information sheet, even though this must be a very difficult time for you.

An Investigation into the Post-Mortem Redistribution of Drugs

This research project is organized by the Department of Forensic Medicine and Science at the University of Glasgow. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether you wish to give consent or not for the samples from your relative to be taken as part of this project.

What is the purpose of the study?

The purpose of this study is to understand better how the body changes after death and what effect this has on the way we investigate deaths.

Why have I been chosen?

Your relative is having an autopsy (post-mortem examination) for determining the cause of death. Due to the likelihood of these samples being positive for medication we would like to take additional samples for this study.

Do I have to take part?

No. If you decide to give consent, you will be given this information sheet to keep for reference and be asked to sign a consent form.

What will happen if I give consent?

If you decide to give consent, you will be given this information sheet to keep for future reference. Three additional blood samples will be collected by the pathologist as follows:

Sample 1 - Taken after consent is given before the autopsy.

Samples 2 and 3 - Taken during the autopsy.

The amount of blood taken for each sample is approximately 1 teaspoon.

What do I have to do?

Consider giving consent to allow additional blood samples to be taken before and at the autopsy.

College of MVLS 2 Version No.:005 Ethics Committee 17 Mar 2017

What are the possible disadvantages and risks of taking part?

There are no disadvantages. There will be no delays in the autopsy and you will be able to proceed with funeral arrangements as you would do so in any case.

What are the possible benefits of taking part?

Your kin will not directly benefit from this research; however, the results will give Forensic Toxicologists and Pathologists a greater understanding of the movement of drugs after death and this may benefit future cases.

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information will be handled in confidence. Results will be used as part of the research process only and may be published in peer-reviewed papers and presented at international conferences but only presented as a group of data and they would be completely anonymised.

What will happen to the blood samples?

Most of the sample will be destroyed during the testing; however, any remaining will be destroyed within 1 year of the samples being taken.

What will happen to the results of the research study?

The results of the tests will be kept anonymous with only the researchers knowing the original identity. It is only the combined results from all the participants that is of interest and will be published.

Who is organising and funding the research?

The research is funded by PhD student fees.

Who has reviewed the study?

This research has been assessed by the College of Medical, Veterinary and Life Sciences Research Ethics Committee, University of Glasgow, United Kingdom. It has also been reviewed by the West of Scotland Research Ethics Committee 4.

Contact for Further Information

For any further information concerning this project OR if you or any member of your family change your mind, both before or after the autopsy+, please contact Abbas Kablan or Hazel Torrance at 0141 330 4574. If you have any other concerns/questions and you like to speak with someone out-with the research team, please contact Calum Morrison at 0141 330 4574.

Thank you for considering giving consent for this project.

Appendix 6

University of Glasgow | College of Medical, Veterinary & Life Sciences

Subject Identification Number for this research:

CONSENT FORM

Title of Project: An Investigation into the Post-Mortem Redistribution of Drugs

Name of deceased :

Date of birth :

Date of death :

Please initial box

I confirm that I have read and understand the information sheet dated 29 Apr 2017 (Version No.:004) For the above study and have had the opportunity to ask questions and I have had these answered satisfactorily.	☐
I understand that the participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights being affected.	☐
I agree that additional biological samples may be retained and used for ethically approved medical research	☐
I confirm that to the best of my knowledge, neither the deceased nor other members of the family had any objection to this.	☐
I agree that data may be collected and stored on a secure database	☐
I understand that most blood samples will be discarded during testing and any residual samples will be discarded on conclusion of the study.	☐
I authorise that the tissue may be kept for medical research and will be disposed of in accordance with the Human Tissue Authority's Code of Practice when the research is complete or they are no longer required.	☐
I understand that any information collected will be treated as confidential and made available to researchers only in a form which preserves anonymity.	☐
I agree to take part in the above study.	☐

Name of next of kin	Date	Signature

Relationship to deceased:_____

Researcher Name	Date	Signature

Name of Researcher(s):

Abbas Mohamed Kablan, Berlian Isnia Fitrasanti, Dr Hazel Torrance, Dr Edward John Williams, Dr Marjorie Turner.

(1 copy for subject; 1 copy for researcher)

Bibliography

Oral presentation, Posters and Award in Support of this Thesis

Oral presentation

- Abbas Kablan, Edward John Williams, Hazel Torrance. “Quantification and interpretation of morphine with its glucuronide metabolites of current and historical cases”. Presented at the United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT) AGM meeting, Oxford, UK (31st August/1st September 2017).

Posters

- Abbas Kablan, Edward John Williams, Hazel Torrance. “New method development and validation of the most commonly encountered drugs”. Presented at and in 3rd Scottish Student Forensic Research Symposium, University of Glasgow, Glasgow, UK (1st April 2016).

- Abbas Kablan, Edward John Williams, Hazel Torrance. “Quantification and Interpretation of Morphine and its Glucuronide Metabolites in Current and Historical Postmortem Cases”. Presented at Emerging Analytical Professional held in Knutsford on 11-13th May 2018.

Award:

- Royal Society of Chemistry Book Prize Awarded at the United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT) AGM meeting, Oxford, UK (31st August/1st September 2017).

UKIAFT

The United Kingdom and Ireland
Association of Forensic Toxicologists

Present this

CERTIFICATE OF ATTENDANCE

TO

Abbas Kablan

For participation in the UKIAFT 2017 Conference held in Oxford, UK
31st August – 1st September 2017

Dr Sue Paterson, UKIAFT Chair

Dr Susan Grosse, 2017 Meeting Host

The Scottish Student Forensic Research Symposium

University of Glasgow, 1st April 2016

This is to certify that:

Abbas Kablan

Attended the 3rd Scottish Student Forensic Research Symposium 2016.

Sponsored by:

CHIRON CHIRON CHIRON
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Kinesis Scientific Experts

On behalf of the organising committee
1st April 2016

New method development and validation of the most commonly encountered drugs

Scottish Student Forensic Research Symposium

Abbas Kablan[1], Hazel J Torrance[2], Edward John Williams[3]
1,3 Forensic Medicine and Science, University of Glasgow, Glasgow, Scotland, UK, 2 Manager of Forensic Toxicology Service, Forensic Medicine and Science, School of Medicine, University of Glasgow, Scotland, UK

Introduction

Reliable analytical data are a prerequisite for correct interpretation of toxicological finding in the evaluation of scientific studies, as well as in daily routine work. Therefore, this study describes a validated, simple, sensitive and selective method to determine opiate in blood using solid phase extraction and GC–MS detection.

Method

The method validated by determining selectivity, precision and accuracy, linearity, limit of quantification (LOQ) and limit of detection (LOD), robustness and stability study of analytics during specimen processing. 1,2

Results

Linearity

The method provides high chromatographic resolution, good sensitivity and good linearity with correlation coefficients (R^2) higher than 0.995 were obtained for all substances. Such as 6-MAM shown in fig.1

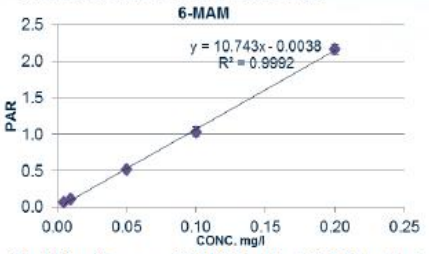


Fig.1 Linearity curve of 6-MAM in blood GC-MS method

Table 1 Precision and accuracy data at lower conc.

	Inter-assay Precision (n = 8)				Intra-assay Precision (n = 8)			
Analyte	Mean Conc. (mg/l)	Std Dev (mg/l)	CV (%)	Bias	Mean Conc. (mg/l)	Std Dev (mg/l)	CV (%)	Bias
MOR	0.05	0.01	13.1	7.5	0.05	0.003	5.8	3.4
COD	0.05	0.01	16.5	-1.9	0.05	0.009	9.0	-0.8
DHC	0.05	0.01	16.9	-4.0	0.05	0.009	8.7	-0.4
MAM	0.01	0.00	8.6	0.4	0.01	0.000	4.0	6.1

Precision and accuracy

Intra and Inter-assay precision and accuracy evaluated by analysing at three different concentration. Here the lower level shown in table.1

Conclusion

The validation data of this method for the analysis of opiates in blood is sufficiently reproducible, robust and sensitive to carry out routine analysis within the laboratory.

Future work

Rapid, and selective analytical procedures will developed for the simultaneous determination of basic drugs in a biological mixture using solid phase extraction, GC–MS and LC–MS techniques.

References:

1. SWGTOX Doc 003 Revision 1 Published May 20, 2013
2. SOFT / AAFS Forensic Laboratory Guidelines – 2006

Quantification and interpretation of morphine and its glucuronide metabolites in current and historical post-mortem cases

Abbas Kablan, Edward John Williams; Forensic Medicine and Science, Glasgow, Scotland

Abstract

The concentration of morphine is often determined by gas chromatography-mass spectrometry (GC-MS), and the content of total morphine can be estimated after hydrolysis of the glucuronides and described as "Total Morphine". However, less attention has been paid to the specific morphine metabolites, (M3G) & (M6G). It is useful to understand the ratio between free and conjugated morphine to assist in interpretation of events in forensic cases, especially after heroin misuse. Moreover, M3G and M6G can undergo degradation after death and post-mortem hydrolysis increases apparent free morphine concentrations. It is also known that hydrolysis of the glucuronides to free morphine is increased in unpreserved tissues, and this is hastened by factors such as storage temperature, bacterial contamination and pH changes (1-5). The purpose of this study is to compare specific morphine-glucuronide concentrations to previously quantitated results by GCMS in real post-mortem case samples. Whole blood post-mortem samples were re-tested in this study from recent (less than 6 months) and historical (around 4 years) cases which were previously investigated by the Procurator Fiscal as a "drug-related" death. Recent (n=30) and historical (n=30) cases were analysed with a range of morphine concentrations from 0.18 to 1.2 mg/L and compared to previous results by accredited GCMS method. The free morphine results gave a correlation coefficient of 0.92 and total morphine results gave a correlation coefficient of 0.91 across all time periods (n=53) with outliers removed due to extensive decomposition leading to extremely heterogeneous samples. This result show, not only the comparison between these 2 methods of analysis, but also suggesting the ratio between free and total morphine remains stable up to 4 years in post-mortem whole blood when stored at freezing temperatures long-term.

References

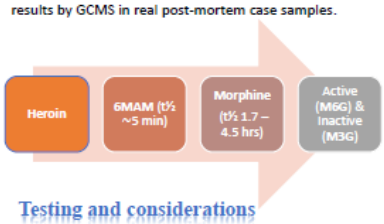
1. Joachim Frost, et.al, Post-mortem levels and tissue distribution of codeine, codeine-6-glucuronide, norcodeine, morphine and morphine glucuronides in a series of codeine-related deaths, Forensic Science International 262 (2016) 128–137
2. Riikka Mari Berg-Pedersen, et, al. Codeine to Morphine Concentration Ratios in Samples from Living Subjects and Autopsy Cases after Incubation. Journal of Analytical Toxicology 2014; 38:99–105
3. Peter D. Maskell, et, al. Postmortem redistribution of the heroin metabolites morphine and morphine-3-glucuronide in rabbits over 24 h. Int J Legal Med (2016) 130:519–531.
4. He S. Yang†, Alan H. B. Wu, and Kara L. Lynch*. Development and Validation of a Novel LC-MS/MS Opioid Confirmation Assay: Evaluation of β-glucuronidase Enzymes and Sample Cleanup Methods. Journal of Analytical Toxicology, 2016;40:323–329 doi: 10.1093/jat/bkw026
5. Susanne Lott, et, al. LC/MS/MS method of 6-MAM, morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) for quantitative analysis in serum. Toxichem Krimtech 2013;80(Special Issue):363

Quantification and interpretation of morphine with its glucuronide metabolites of current and historical cases

Abbas Kablan, Edward John Williams, Hazel J Torrance

Introduction

The concentration of morphine is often determined by gas chromatography-mass spectrometry (GC-MS), and the content of total morphine can be estimated after hydrolysis of the glucuronides and described as "Total Morphine". However, less attention has been paid to the specific morphine metabolites, (M3G) & (M6G). It is useful to understand the ratio between free and conjugated morphine to assist in interpretation of events in forensic cases, especially after heroin misuse. Moreover, M3G and M6G can undergo degradation after death and post-mortem hydrolysis can increase apparent free morphine concentrations. It is also known that hydrolysis of the glucuronides to free morphine is increased in unpreserved tissues, and this is hastened by factors such as storage temperature, bacterial contamination and pH changes (1-5). The purpose of this study is to compare specific morphine-glucuronide concentrations to previously quantitated results by GCMS in real post-mortem case samples.



Testing and considerations

Whole blood post-mortem samples were re-tested in this study after the ethical approval has been sought from the NHS (ref. no. 17/WS/0102). All cases were previously investigated by the Procurator Fiscal as a "drug-related" death. Positive opioid cases were selected in this study.

- Different ways of measuring glucuronides
- Bacterial contamination – PM samples
- Storage conditions
- Time
  - Between death & sample collection
  - Long term storage of samples

Case sample selection

Positive opioid cases were selected in this study, which had previously been investigated by GCMS method and were stored for months / years.

The case samples (n = 108) were divided into two groups:

- First group (n = 31) were current cases 2017 (<6 months stored in Fridge).
- Second group (n = 77) were historical cases 2013/14 (around 4 years stored in freezer).

Current cases result after re-analysis

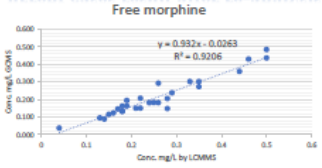


Fig.1 The correlation coefficient between GCMS and LCMSMS

Historical cases result after re-analysis

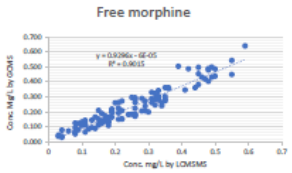


Fig.2 The correlation coefficient between GCMS and LCMSMS

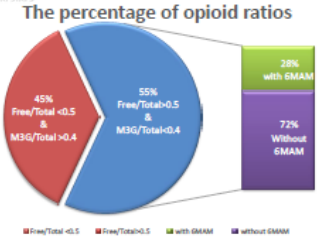


Fig. 3: Free/Total, M3G/Total and M6G/Total ratio with and without 6MAM of all cases by GCMS and LCMSMS

Tab. 1: Free morphine and Free/Total ratio in current and historical cases by GCMS and LCMSMS

	Current cases		Historical cases	
	GCMS	LCMSMS	GCMS	LCMSMS
Mean Free Morphine conc.	0.30	0.27	0.26	0.24
Median Free	0.25	0.22	0.24	0.18
Free Morphine conc. range	0.03-3.2 mg/L	0.03-2.14 mg/L	0.04-0.5 mg/L	0.03-0.64 mg/L
Mean F/T ratio	0.55	0.54	0.49	0.49
Median F/T ratio	0.53	0.55	0.47	0.45
Range F/T ratio	0.2-1.03	0.19-1.00	0.25-0.84	0.18-0.91

Table 1 shows the morphine concentration and ratios of all cases when were analysed. The ratio between free and total morphine remains stable up to 4 years in post-mortem blood when stored at -20°C for long-term.

Interpretation:

All cases were divided into two groups:

1. First group with ratio less than 0.5 **(Delayed death)**
2. Second group with ratio more than 0.5
   - With 6MAM present **(Recent death)**
   - Without 6MAM present

First group with ratio less than 0.5 **(Delayed death)**

- 91% of first group give a M3G/Total ratio more than 0.4
- The same pattern was not observed for M6G/Total ratio

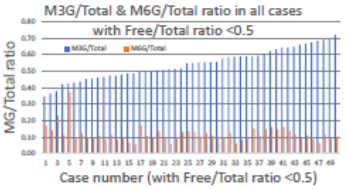


Fig.4 M3G/Total & M6G/Total ratio in all cases with Free/Total ratio <0.5

Second group with ratio more than 0.5

- 100% of this group had M3G/Total ratio of less than 0.4

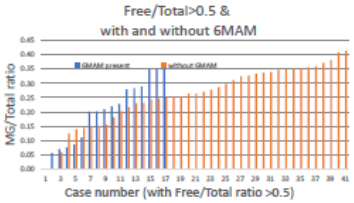


Fig.5 M3G/Total ratio in all cases with Free/Total ratio >0.5 with and without 6MAM present

Conclusion

The LCMSMS method was Comparable with GCMS method for the simultaneous quantification of Free and total morphine. it has been successfully verified through the use of 108 samples of authentic cases with a correlation higher than 90%. In the other hand, the ratio between free and total morphine remained stable in the freezer up to 4 years. Moreover, simultaneous analysis M3G presents additional evidence of recent heroin intake, where M3G/Total less than 0.4 and 6MAM present. While, delayed deaths have Free/Total ratio less than 0.5 and M3G/Total ratio more than 0.4.

References:

1. Joachim Frost, et al, Post-mortem levels and tissue distribution of codeine, codeine-6-glucuronide, norcodeine, morphine and morphine glucuronides in a series of codeine-related deaths, Forensic Science International 262 (2016) 128–137
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4. He S. Yang†, Alan H. B. Wu, and Kara L. Lynch*. Development and Validation of a Novel LC-MS/MS Opioid Confirmation Assay: Evaluation of β-glucuronidase Enzymes and Sample Cleanup Methods. Journal of Analytical Toxicology, 2016;40:323–329 doi: 10.1093/jat/bkw026
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ROYAL SOCIETY OF CHEMISTRY

Royal Society of Chemistry Books

This is to certify that

Abbas Kablan

Has been awarded a Royal Society of Chemistry Books Prize at

the 7th Annual UK and Ireland Association of Forensic Toxicologists AGM Meeting

Oxford, UK

31st of August – 1st of September 2017

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Roheena Anand

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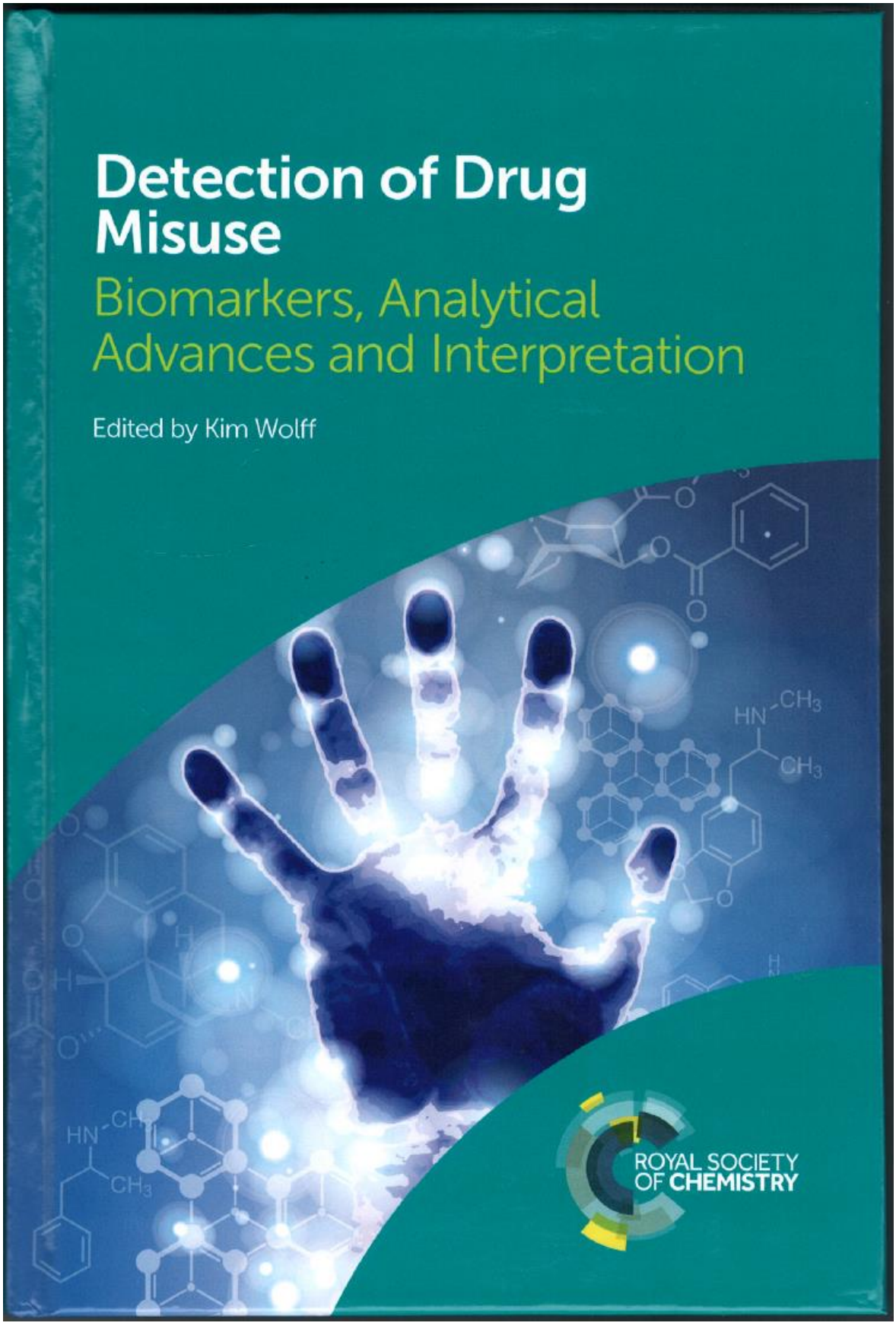
Detection of Drug Misuse
Biomarkers, Analytical Advances and Interpretation
Edited by Kim Wolff
ROYAL SOCIETY OF CHEMISTRY

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