Applications of LC-MS/MS in Forensic Toxicology for the Analysis of Drugs and their Metabolites

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List of Abbreviations

AAFS	American Academy of Forensic Sciences
6-AC	6-Acetylcodeine
ALAT	Alanine Aminotransferase
APCI	Atmospheric Pressure Chemical Ionisation
API	Atmospheric pressure Ionisation
APPI	Atmospheric Pressure Photoionisation
APLI	Atmospheric Pressure Laser Ionisation
ASAT	Aspartate Aminotransferase (ASAT)
AVE	Average
BAC	Blood Alcohol Concentration
BSTFA	*N,O*-Bis (Trimethylsilyl) trifluoroacetamide
BUP	Buprenorphine
BUP3G	Buprenorphine-3-glucuronide
CDT	Carbohydrate-deficient Transferase
CE	Collision energies
CID	Collision-Induced Dissociation
COD	Codeine
C6G	Codeine-6-glucuronide

DAD	Diode Array Detector
DHC	Dihydrocodeine
DHC6G	Dihydrocodeine-6-glucuronide
DHM	Dihydromorphine
DHM3G	Dihydromorphine-3-glucuronide
DHM6G	Dihydromorphine-6-glucuronide
DIM	Diamorphine
DM	Direct determination
DUID	Driving Under the Influence of Drugs
ELISA	Enzyme Linked Immunosorbent Assay
ESI	Electrospray Ionisation
ETG	Ethyl Glucuronide
ETS	Ethyl Sulfate
FAB	Fast Atom Bombardment
FAEE	Fatty Acid Ethyl Ester
FCOD	Free Codeine
FMOR	Free Morphine
GC	Gas Chromatography
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas Chromatography-Mass Spectrometry

GGT	Gamma-glutamylTransfererase
GTOL	5-hydroxytryptophol gluucronide
GUS	General Unknown Screening
Hcl	Hydrochloric acid
5-HIAA	5-Hhydroxyindole Aacetic Aacid
H3G	Hydromorphone-3-glucuronide
HE	High ethanol group (BAC ≥ 100 mg/100mL)
HILIC	Hydrophilic Liquid Chromatography
HMOR	Hydromorphone
HM	Hydrolysis method
HPLC	High performance Liquid Chromatography
5-HTOL	5-Hydroxytryptophol
IH-DIM	inhalation diamorphine
IM-DIM	Intramuscular Diamorphine
INDIM	Intranasal Diamorphine
IPA	Ion Pair Agent
IPs	Identification Points
IT-MS	Ion Trap-Mass spectrometry
IVDIM	Intravenous Diamorphine
LC	Liquid Chromatography

LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LE	Low ethanol group (BAC ≤ 100 mg/100 mL)
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
6-MAM	6-Monoacetylmorphine
MCV	Mean Corpuscular Erythrocyte Volume
METH	Methadone
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
MIP	Molecularly Imprinted Polymer
MOR	Morphine
MRM	Multiple Reaction Monitoring
NAL	Naloxone
NAL3G	Naloxone-3-glucuronide
NBUP	Norbuprenorphine
NBUP3G	Norbuprenorphine-3-glucuronide
NCI	Negative Chemical ionisation
NCOD	Norcodeine

NMOR	Normorphine
NOXY	Noroxycodone
NPLC	Normal Phase Liquid Chromatography
OXY	Oxycodone
OXYM	Oxymorphone
RF	Radiofrequency
RIA	Radioimmunoassay
RPLC	Reverse Phase Liquid Chromatography
RSD	Rrelative Standard Deviation
RT	Retention Time
SAMSHA	Substance Abuse and Mental Health Services
SIM	Selective Ion Monitoring
SOFT	Society of Forensic Toxicologists
SRM	Selective Reaction Monitoring
SPE	Solid Phase Extraction
STA	Systematic Toxicological Analysis
STD	Standard Error
TBUP	Total Buprenorphine
TCOD	Total Codeine
TIC	Total Ion Count

TQ-MS	Triple Quadrupole-Mass spectrometry
TMOR	Total Morphine
TNBUP	Total Norb
TOF	Time of flight
UAC	Urine Alcohol Concentration
UGT	UDP-glucuronosyltransferase
VH	Vitreous humour
WADA	World Anti-Doping Agency

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Summary

This thesis studied opioids and alcohol in forensic toxicology by LC-MS/MS, which avoids time-consuming procedures involving hydrolysis, extraction and derivatisation. Initially, a method was validated for quantification of opioids and unhydrolysed polar metabolites in autopsy specimens and was used to develop procedures for interpretation of forensic toxicology results. The LC-MS/MS method developed has been validated for the simultaneous determination of 24 opioids in human whole blood, including, for the first time in human whole blood, naloxone-3-glucuronide. Although a large number of drugs of interest were included in the method, acceptance criteria for linearity, precision, and recovery for all analytes were achieved. The method was found useful for differentiating between users of heroin and other opioids, such as codeine and morphine, and for determining the survival time in deaths attributed to heroin use.

Subsequently, the efficiencies of hydrolytic and non-hydrolytic methods for opioid analysis were compared for buprenorphine (BUP) analysis. The aims were to develop and validate a method for the direct determination (DM) of buprenorphine (BUP), norbuprenorphine (NBUB), buprenorphine-3-glucuronide (B3G) and norbuprenorphine-3-glucuronide (NBUP3G). This method was compared with an in house enzymatic hydrolysis method (HM) for the determination of total buprenorphine (TBUP) and norbuprenorphine (TNBUP), using real positive BUP urine case samples. A comparison between the drug and metabolite concentrations obtained by direct and hydrolysis methods was reported for the first time in this work.

LC-MS analysis was also applied to paediatric plasma specimens obtained from a clinical pharmacokinetic study of intravenous and intranasal administration of diamorphine. This work was aimed at obtaining pharmacokinetic data for diamorphine and its metabolites in children following intravenous (IVDIM) and intranasal (INDIM) administration in a blind study. It was intended that the concentrations of active metabolites would be used to evaluate whether or not IN-DIM can deliver rapid and efficient analgesia in children comparable to that obtained with IV-DIM. The pharmacokinetics of DIM and its metabolites following INDIM and IVDIM administration in children have been compared for the first

time in this study, which confirmed that INDIM can achieve therapeutic plasma concentrations of active metabolites, although these were lower than those obtained with IVDIM and occur at later times after administration.

In Scotland, the number of prescriptions for oxycodone has risen by 430% since prescribing began in 2002. Blood samples from fatalities in the West of Scotland involving oxycodone were analysed using an LC-ESI-MS/MS method developed for the determination of oxycodone and its metabolites in post-mortem specimens. To the author's knowledge, this is the first report of blood and urine concentrations of noroxycodone and oxymorphone in acute oxycodone overdoses. Also, it is the first LC-MS/MS application to be reported with oxycodone related fatalities cases in forensic toxicology as most of previous reports used GC or HPLC applications. Moreover, this work reported for the first time vitreous humour levels of noroxycodone following oxycodone intoxication. Ten oxycodone-related deaths were identified in the short period of this study in the Strathclyde region of Scotland alone, highlighting the importance of including this drug in routine laboratory screening and confirmation procedures.

Polar alcohol metabolites ethyl glucuronide and ethyl sulfate are biomarkers of ante-mortem alcohol consumption and are used to test for post-mortem artefactual formation of alcohol. An LC-MS method for these metabolites using a novel hydrophilic interaction liquid chromatography column was validated and applied to routine forensic casework. Ninety urine case samples were divided into three groups depending on the ethanol concentration found in blood and analysed by the developed method: group A with post-mortem blood ethanol higher than 200 mg/100 mL; group B with ethanol concentration in the range 80 to 200 mg/100 mL and group C with ethanol concentration less than 80 mg/100 mL.

It was concluded that the risk of false positive ethanol results increased in the low ethanol concentration group as several cases tested negative for both biomarkers. ETG was detected at low concentrations in some cases for which ETS tested negative, suggesting that either ETG may have a longer half-life in urine or else ETS is unstable. The data was compared with previous studies and confirmed that both ethanol biomarkers should be determined in heavily putrefied cases and when the ethanol level in post-mortem blood is low,

suggesting the production of ethanol after death. To the authors' knowledge, this is the first report of the determination of ETS using an LC-ESI-ion trap-MS/MS method, and of a HILIC-ESI-ion trap-MS/MS method for the simultaneous determination of ETG and ETS in post-mortem urine samples.

1 General Introduction

1.1 Forensic Toxicology

Toxicology is the study of chemicals or poisons which could potentially result in harmful effects or death after exposure in living subjects. Forensic toxicology is defined as the method of identification of the presence of these harmful substances for the purposes of law. The toxic effects of such poisons depend on their quantity present or concentration and differ between individuals. Tolerance amongst users varies and is dependent on the individual patient with factors including dose, age, gender, body mass and the length of time the person used or abused. Everything in nature can be a poison, such as water if it is taken in abnormal quantities [1-5]. Illicit and licit drugs have been described by Kintz *et al* [6] and Trujols *et al* [7] as "chemical weapons" whose effect on society is that of a double-edged sword: they may relieve pain, and yet they may also kill. Drug testing is also important for identifying the cause and manner of deaths in post-mortem cases, keeping the workplace drug free and reducing accidents due to driving under the influence of drugs (DUID).

The analysis of biological fluids to obtain evidence linking to drug use which may cause death is one of the main activities in the field of post-mortem forensic toxicology. Forensic toxicology also involves the study of the pharmacology and biochemical properties of drugs or poisons such as route of administration, absorption, metabolism and excretion from the body. Information is also needed concerning the therapeutic and lethal dose of drugs, in order to interpret the cause of death and whether the drug concentrations detected in post-mortem specimens could lead to death or the amount of drug found in a specimen from a driver could lead to impairment.

Samples collected at autopsy for toxicology analysis depend on the case under investigation. In most cases blood, urine and liver specimens are commonly used. However, in putrefied cases these common specimens may not be available for use and instead hair, muscular tissue and bone may be more suitable for analysis. However, in general, a variety of specimens can be processed in forensic laboratories such as blood, urine, vitreous humour, bile,

stomach contents, liver and other organ tissues, hair, nails and oral fluid [8-10]. Traditionally, urine samples are the sample of choice for the screening and identification of unknown drugs or toxic compounds. A urine specimen is preferable for living subjects for drug testing such as driving under the influence of drugs (DUID) and workplace drug testing, as urine is non-invasive compared to withdrawing blood samples. Also, drug concentrations in urine are much higher than those detected in blood, for example, it has been found that the concentration of amphetamine in urine was 200 times greater than in blood [11]. In addition, detection windows of drugs in urine are longer. Moreover, the volume of a urine specimens is usually enough for initial analysis and repeat analysis compared, for example, to hair analysis in which there is often not enough sample available for multiple analysis. Analytes of interest have been found to be stable in urine for long periods if the samples are frozen, which is important for re-analysis [12,13]. However, it has been shown that urine samples can be adulterated using several substances to bring about a false negative results for drugs [12,14,15]. Also, urine samples do not give information on the concentrations of drugs in blood and hence on their effects at the time a urine sample is collected: in driving cases this means that impairment cannot be assessed based on urine drug concentrations.

The analysis of blood samples obtained at autopsy has acquired a considerably greater value in comparison to other specimens, particularly in forensic toxicology, as concentrations of drugs found in venous blood can give a close approximation of what the brain has been exposed to before death [8,9,16,17]. In contrast, other matrices such as urine could be used to confirm that a person has taken drugs before death but the concentration cannot be linked to the cause of death. Hair is the matrix of choice to assess history of drug use, with long widows of detection, but not for the last few hours to weeks before death. It is standard practice in many jurisdictions such as in which hair is used in an investigation to take a hair sample at the time of reporting an alleged offence (as a control sample) and to ask the complainer to come back a month later to provide a second sample (which may indicate the presence of drugs at the time of the reported offence). Another advantage of blood is that repeat analysis should give the same concentration if there is sufficient sample. During the last 20 years of the 20th century, post-mortem toxicology practice has improved due to significant advances in extraction and analytical

procedures which make blood samples suitable as screening specimens. In addition, there has been improvement in the data available from case study samples for use as interpretive aids [8,18].

In forensic post-mortem toxicology, one of the more important factors which might affect the interpretation of post-mortem toxicology results is the phenomenon of post-mortem redistribution, which results in changes in blood concentrations of drugs after death due to diffusion of drugs from surrounding tissues into the blood [5,9]. It is believed to occur widely, with drugs diffusing from high to low concentration and is site-dependent, with the largest increases in concentration occurring in the blood vessels in or near major organs such as the heart and liver rather than in peripheral sites [19]. Redistribution starts within an hour after death, with the major changes occurring in the first 24 hours. Post-mortem redistribution is influenced by the post-mortem interval time [9] and by the volume of distribution (VD) of a drug, as drugs with a high VD, i.e. greater than 3 L/Kg, are more susceptible to post-mortem redistribution [9,20]. The occurrence of post-mortem redistribution can be determined by analysing blood samples from different collection sites including the body core, such as from the heart, and periphery, such as the femoral vein. Differences in drug concentrations in the blood from these sites indicates the probable presence of a post-mortem redistribution problem [18]. Blood from the femoral vein is considered to be the specimen of choice because it is spared from redistribution phenomena as well as bacterial invasion [21].

Post-mortem redistribution has been studied widely for many drugs, including the opioids studied in this thesis. Morphine, for example, exhibited little or no redistribution in man at either central or peripheral sites [22] whereas methadone concentrations changed by -30% [5]. In another study, no significant post-mortem redistribution of morphine and its glucronides was observed in 40 heroin related deaths. In that study, blood was analysed for morphine and its metabolites from subclavian, heart and femoral sites and no significant changes were observed [23]. Skopp *et al* [24] studied the post-mortem redistribution of dihydrocodeine and its metabolites and found no significant changes from site to site due to the low volume of distribution (1-1.3 L/Kg for dihydrocodeine). The presence of oxycodone at high concentrations in blood

has been attributed to its accumulation in tissue following chronic administration and subsequent release back into the blood [25].

1.2 Drug misuse

In this thesis the term "heroin" will be used with reference to the illicit form of diamorphine (also referred to as "street heroin"), which contains impurities derived from opium constituents as well as diluents and adulterants. The pharmacological name "diamorphine" will be used with reference to the pure substance and considered to be synonymous with the chemical name diacetylmorphine.

In the most recent United Nations World Drug report, published in 2009[26], the United Nations Office On Drugs and Crime estimates that between 172 and 250 million persons used illicit drugs at least once in 2007 and 18 to 38 million aged 15-64 had problem drug use. The prevalence of abuse of many drug classes has continued to increase, for example, the number of cannabis users increased from 3.8% to 3.9%, cocaine from 0.34 to 0.37% and opioids from 0.37 to 0.39% of the world population in the periods 2005-2006 to 2006-2007, respectively. Heroin misuse also increased from 0.27 to 0.28% of the word population.

Europe has an estimated 3-4 million opiate users (0.6-0.7% of the population aged 15-64). The level of use observed has been stable, despite some countries reporting an increase in fatalities due to opioids. The largest market for opioids in Western Europe is in the UK, with 404,000-434,000 users.

According to the most recent report of the General Registrar of Scotland [27], published in 2009, the number of drug-related deaths (cases attributed to drug abuse) has increased over the last ten years, rising from an average of 189 deaths per annum in the period 1996-2000 to 246 deaths per annum in the period 2003-2007. Also, unintentional/accidental drug-related deaths have increased from 13 (7%) to 43 deaths (17%) on average in the same periods, while suicide or intentional drug related deaths increased only slightly.

There were 574 drug-related deaths in Scotland in 2008, which represents increases of 26 % and 131% over those reported in 2007 and 1997, respectively. Heroin was involved in 336 (59 %) of the drug related deaths, followed by methadone which was found in 181 (32 %) and diazepam in 362 (63%) of the deaths. Alcohol was involved in 273 cases (48%), while cocaine, ecstasy and amphetamine were involved in 79 (14%), 7 (1%) and 12 (2%) cases, respectively.

The majority of drug related deaths were drug abusers, who increased from an average of 189 per year in the period 1996-2000 to an average of 277 in 2004-2008. Also, accidental deaths showed an increase from an average of 13 to an average of 42, and deaths due to undetermined causes rose from an average of 25 to an average of 74 in the same periods. Ninety six drug related deaths in the period 2004-2008 (21%) were 25-34 years old while 151 (35 %) were 35-44 years old. The number of deaths on average increses from 134 per year from 2004 to 2008 (31%) were 35-44 years old. One hundred and ninety seven (34%) of the deaths occurred in Greater Glasgow and Clyde.

The average numbers of fatalities related to heroin/morphine, cocaine and alcohol increased from 128 to 229, 6 to 38 and 91 to 129 per year in the periods 1996-2000 and 2003-2007, respectively. Methadone (average numbers of deaths 74 and 90), diazepam (average numbers 116 and 103) and ecstasy (average numbers 7 and 13) have shown no significant change in their trend in the same periods of time. In addition, temazepam related deaths declined sharply from 47 to 12 cases, following a decrease in the number of prescriptions for temazepam over the same period.

1.3 Systematic Toxicological Analysis

Systematic toxicological analysis is a term describing the process of how each forensic analysis test should be performed when searching for unknown drug and toxic compounds. This standard approach consists of two steps in the laboratory [28-31]: immunoassay has been employed as a preliminary method for the detection of abused drugs, especially in urine, serum and plasma, due to its ability to detect drugs even in very low concentrations as well as being able to process large numbers of samples without pre-treatment.

Immunoassays suitable for hair and other matrices are now available. However, LC-tandem MS and LC-time of flight (TOF) have also been used for screening of drugs in several matrices. The LC-MS/MS instrument may become the method of choice for screening of drug and toxic compounds in the future [10,32-40].

The confirmation step is usually came out using chromatographic techniques coupled with more specific methods of identification and detection, for example, the most commonly used confirmation procedure is gas chromatography coupled with mass spectrometry (GC-MS) or HPLC coupled with photo diode array (DAD) detection, LC-MS and LC-tandem MS [10,28].

The identification of illicit or licit drug use depends on analytical toxicology findings which, until recently, involved tedious procedures for hydrolysis, extraction and derivatisation which are potentially hazardous because of the toxic chemicals involved, and samples might need to be analysed several times to achieve these requirements. The amount of blood available is inadequate in some cases if multiple target analytes need to be analysed using different confirmatory techniques plus the screening method.

Although GC-MS has greatly enhanced the potential of analytical toxicology, many obstacles have been encountered due to lack of applicability towards polar and thermolabile compounds, and high mass molecules [41-45]. The arrival of LC-MS techniques in analytical toxicology has allowed these expensive, time-consuming procedures to be replaced by methods involving a single extraction and chromatographic analysis. Also, LC-MS/MS has been used as both a screening and confirmation method [32].

2 Method validation

2.1 Introduction

Reliable results are important in medico-legal investigations which in turn require reliable methods of analysis that provide accurate, rapid and reducible data. Good laboratories practices applied by well trained staff are

essential to accomplish those requirements for methods. Forensic laboratory methods should be able to distinguish between a variety of drugs in use which sometimes share the same chemical properties and mechanisms of toxicity and may be present in the analytical specimens at the same time, such as heroin, morphine and codeine. Also, for some drugs good separation from matrix components eluted at the void volume and other interferences are important. Therefore, the sensitivity, and selectivity of a method of analysis should be examined before the use of the method for forensic toxicology investigation in order to ensure that the method is fit for the purpose of use and is suitable for the equipment in daily routine use [10,46-50].

Many useful protocols for method validation have been published in the literature by different organisations to ensure high quality and reliable data, among them, SOFT/AAFS Forensic Toxicology Laboratory Guidelines and United Nation Guidelines for Analytical Methodology. These and many other sources were used in the work carried out for this thesis [46,49,51-57].

The requirements for a method differ depending on whether the method will be used for qualitative or quantitative analysis [5,47,57,58]. In the case of a qualitative method, few parameters need to be examined. The limit of detection, precision (intra and inter-day precision), specificity/selectivity and stability of analyte(s) in given matrices have to be investigated. In biological fluids there are often concentration thresholds for target analytes: the presence of a drug can be interpreted in three ways: concentration within the therapeutic range, lower than the therapeutic range and higher than the therapeutic range [5]. For some drugs such as digoxin the therapeutic index between therapeutic and toxic concentrations is narrow. Therefore, positive results of some analytes are reported if they are higher than certain thresholds. Linearity, accuracy and precision should be examined for qualitative methods with pre-defined threshold at the threshold concentrations; accuracy and precision should be examined within the laboratory under repeatability and/or reproducibility conditions [57-59].

Quantitative methods are more important in forensic toxicological investigations and require more parameters to be optimised [47]. Limit of detection, specificity /selectivity, linearity within the quantitation range,

precision and accuracy, recovery and stability have to be investigated. Ruggedness and robustness have also been suggested for quantitative method but are not essential for the method validation process. In addition, it is often suggested that matrix effects should be determined for quantitative methods using LC-MS/MS [46-48,52,53,57,58,60].

2.2 Specificity/Selectivity

Specificity is defined as the ability of an optimised method to produce a response for only a single analyte, while selectivity is the ability of method to distinguish the response of single target analytes from other responses [1,46,49,52,53,56]. Specificity/selectivity of the method is an important issue for both qualitative and quantitative methods of analysis. The effects of possible interfering substances from unseen endogenous matrix components or compounds with similar structures (isomers, metabolites, etc) that commonly occur in real cases and degradation product from unwanted compounds and metabolites or endogenous material of matrices are found to interfere with the analyte of interest and to influence method accuracy. It has been recommended that selectivity/specificity should be investigated with six independent matrices [46]. It is also recommended 10-20 different sources of blank specimens [47,53,61]. Specificity of the method could also be examined by testing real case samples that are known to be positive for target analytes for the presence of possible interfering components and by testing real case samples that are known to be negative for target analytes to assess the effect of these interferences on the response of target analytes [47,58]. In the case of high resolution LC-tandem mass spectrometry, one blank matrix source would be sufficient [46]. A method can still be specific and selective even with the presence of small interferences which have little effect on the method precision and accuracy if less than ± 20% at the LLOQ [49,51,52]. Specificity is concentration-dependent which should examined of the low end of calibration range [57].

2.3 Limit of detection and lower limit of quantification

The limit of detection is defined as the lowest concentration that can be detected using an optimised method which also can be distinguished from the matrix background noise at a signal to noise ratio of 3:1 [1,49,54]. The lower limit of quantification (LLOQ) is described as the lowest concentration of the analyte of interest that can be quantified with acceptable precision and accuracy; in most reports a limit of ± 20% has been recommended. LLOQ is recommended in most guidelines to be determined as a signal to noise ratio of 10:1 [46-48,50].

LOD and LLOQ can be determined using more than one procedure [47,58]. Traditionally, the method of baseline noise is the most used which relies on signal to noise (S/N) ratios of 3 and 10, respectively [47,54]. This method is time consuming and difficult to obtain using chromatographic techniques [54,62].

Secondly, LLOQ is also 'defined in the term of concentration as the lowest calibrator can be detected with acceptable precision and accuracy; therefore no need to be determined experimentally'. This is the most used procedure with LC-MS/MS method of quantification [46-48,52,54].

Thirdly, LOD and LLOQ are calculated statistically as the intercept (Y_B) of the calibration graph and the standard error (S_B) of regression line using equations 2-1,2-2 and 2-3,2-4 for LOD and LLOQ respectively [3,50,62-64]:

Equation 2-1 $y_{LOD} = yB + 3S_B$

Equation 2-2 $LOD = (y_{LOD}-y_B)/m$

Where y_B is the intercept, S_B is the standard error of the regression line and m is the gradient.

LLOQ values were calculated using the same method but using 10 times the standard error of the regression line (equations 3 and 4).

Equation 2-3 $y_{LLOQ} = y_B + 10S_B$

Equation 2-4 $LLOQ = (y_{LLOQ}-y_B)/m$

The latter procedure has been used in the current project and found sufficient for the intended purpose of use. This was because many target analytes (more than 27 analytes of interest) were detected in the thesis projects. Finally, the LOD and LLOQ can be estimated using especial calibration curve which constructed within expected LOD and LLOQ ranges in order to determined LOD and LLOQ of the optimised method [47].

2.4 Precision and accuracy

Precision is defined as 'the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous samples under the prescribed conditions' [1,3,56,57]. Method precision is measured using two main condition sets which are called repeatability and reproducibility and which are also known as intra-assay and inter-assay precision, respectively. Intra-assay precision is determined by analysing replicate specimens containing analytes of interest in the same day using the same extraction and analytical method [57]. The inter-assay precision is measured in a similar manner to the intra-assay precision on different days. Five different determinations are carried out for each quality control standard which should include low, medium and high levels relative to the calibration curve range. Intra and inter-assay precision were assessed using the percentage relative standard deviation (RSD) [61]. The acceptable limit for method validation is less than ±15 %, except at the LLOQ value where precision should not exceed ± 20% [1,48,49,57].

Accuracy or trueness is 'the degree of closeness of the determined value to the nominal or known true value under method conditions'; it is expressed as a percentage [1,3,56,57]. Both precision and accuracy should be examined using certified references materials. In this thesis, precision and/ or accuracy were usually determined using blank samples spiked at three different concentrations (low, medium and high) as quality control standards (QCs) across the calibration range. A calibration curve should be prepared with each batch of QCs using the optimised method. The acceptable limits for method accuracy are the same as for method precision. In addition, blank samples

which tested negative for target analytes and blank samples spiked with internal standards were usually included with each batch samples which could be to check the absence of such interferences [47,54].

2.5 Linearity

Linearity is described as the ability of the optimised method to produce a straight line response for the range of analyte concentrations in the sample within working range [62]. The linear range is the range of concentrations over which a linear response is obtained. The range of the calibration curve chosen should depend on the concentrations of target analytes found in the body. Precision and accuracy are often measured using the calibration curve for the optimised method which can also be used in turn to demonstrate that the optimised method has suitable levels of precision, accuracy and linearty [46,55]. The equation for a straight line used to describe a linear calibration curve given in Equation 2-5.

Equation 2-5 $y = mx + b$

Where y is the response (in forensic toxicology the peak area ratios between analytes versus their corresponding internal standards) [47,65], x is the concentration, m is the slope and b is the intercept on the y axis of the best fit line for the data. Calibration curve standards should be spiked into blank matrix and then analysed using the optimised method for more matrix compatibility due to the fact that the pure standard response may be not the same as extracted standards, because a percentage of each analyte is usually lost during the extraction procedure. It is recommended that five to eight concentrations across the calibration range are used [46,49,56]. Also, a correlation coefficient (r^2) of 0.99 or better is preferred for quantitative analysis [54,55,57] but less an 0.99 may still fit for purpose [57].

2.6 Stability

Stability is described by Shah *et al* [46] as 'the chemical stability of analyte in a given matrix under specific conditions for a given time interval'. A study of the stability of analytes during the extraction and analysis was recommended

as part of full method validation for more reliable methods of quantitation [16,46,47,49,52,53]. It is believed that most drugs are stable in biological fluids when subject to proper storage condition. However, drugs which have ester bonds are unstable and can be easily hydrolysed in body fluids [49]. The assessment of analyte stability in matrix during the validation process is required for reliable quantification, especially if no information is available from previous work [47]. Dadgar *et al* [53] point out that stability studies measure the differences in analyte concentration that may be encountered between the time of sampling and the time of analysis; they suggested that two concentrations have to be investigated at low and high points of the calibration range. In fact, stability studies can be conducted in many different ways, for example, to cover the storage conditions before and during the analysis in the laboratory. Four storage conditions are most commonly used and these are described below [47,49,53,58,60].

Short-term temperature stability at room temperature is required for sample preparation. Freeze-thaw stability should be also conducted due to the fact that samples are often frozen and thawed in the event of re-analysis: freeze/thaw stability of analytes of interest is recommended to be determined after at least three cycles (thawed, left at room temperature then refrozen) on consecutive days. Analytes could also be lost during sample processing if a large batch of samples is analysed, which sometimes extends overnight and even over one or more days. Therefore, autosampler stability using reconstituted extracted samples is required for the expected period of an analytical run [47,49,53]: it is recommended that stability is assessed at 24 and 48 hours [52].

Long-term stability of analytes in a given matrix should be examined during method validation with the same storage conditions and times that are expected to be used for real samples. The stability often is expressed as percentage (100%) of spiked concentration which survives. Analytes are considered stable if the concentrations of stability samples are within the range of ± 10-20% compared to the spiked standard concentrations [52,53]. Also, it is recommended that the stability of standard solutions is assessed [53].

2.7 Recovery

Recovery is defined as 'the extraction efficiency of an analytical process, reported as percentages of the known amount of an analytes of interest which are extracted and analysed by an optimised method' [46]. This is to be compared with detector response of neat standard solution which is considered to be 100%. Some believe that the recovery experiments are not required as part of method validation as long as the LLOQ is adequate and the precision and accuracy of the method are within the acceptable limit for method validation (± 20%) [46,49,53,60]. The loss of analyte during extraction should be investigated with at least five replicates at three QC levels or at least using two levels at low and high concentration. Internal standard is added after extraction to allow direct comparison with unextracted standards. Low recoveries can be accepted if LLOQ, accuracy and precision of the method are within the acceptable limits. The acceptable limit of reproducibility for recoveries should be higher than 50% of recovery value [47]. Recovery is reported sometimes as being higher than 100%, which indicates the presence of matrix effects, especially when using an LC-MS/MS procedure [51]. Matrix effects should therefore be examined together with recovery during method validation.

3 Liquid Chromatography Tandem Mass Spectrometry

3.1 Introduction

A liquid chromatography column was used successfully for the first time in 1903 for the isolation of plant pigments [2]. Since then many chromatography techniques have been created for isolation and identification of substances in nature, some of which are suitable for qualitative and/ or quantitative analysis. Among them gas chromatography (GC) and high performance liquid chromatography (HPLC) are the techniques most used in forensic laboratories for routine analysis; both are powerful separation techniques [16,66-68].

The basic principles of separation are similar for both techniques: two phases are used for the separation of compounds, the stationary phase and the mobile phase, but the nature of these two phases differs between GC and HPLC. The separation of components occurs due to their distribution between these two phases, determined by the partition coefficient of the analyte between the stationary and mobile phases. The column is the stationary phase in both techniques. In GC an inert gas is used as a mobile phase while in HPLC a liquid mobile phase using organic or non-organic solvents is used. Long columns (meters) are preferred for GC and shorter columns (centimetres) for HPLC applications. Temperature is the most important factor influencing the speed of analyte separation in GC while the percentage of organic solvent is the most important factor in HPLC.

These mobile phases move through GC and HPLC columns towards the instrument detector. Many detectors have been used for both GC and HPLC such as the flame ionisation detector (FID) and electron capture detector (ECD) for GC and ultraviolet (UV), diode array detector (DAD), electrochemical detector (ECD) for HPLC. Detectors used with GC are not suitable for HPLC and vice versa and differ in their application, sensitivity and selectivity with the exception of one detector which used for the analysis of explosives, the so-called thermo energy analysis detector, which can be

coupled with both GC and HPLC [69-71]. GC is applicable for components that are stable and which can be volatilised at temperatures up to 350 ^{0}C.

GC-MS has been considered the gold standard technique in toxicology due to generation of reducible and repeatable mass spectra which then can be matched with spectra available in libraries for identification purposes [16]. However, many limitations have been reported, for example, GC-MS is not suitable for the determination of non-volatile or thermally labile substances [44,45,68,72] and heavy and thermally unstable compounds such as morphine glucuronides should be hydrolysed to the free (unconjugated) form in order to be analysed by GC-MS. New GC columns are available with ionin liquid based phases that can be oberated up to 450 ^{o}C for the analysis of polar compounds and would be available commercially in the future.

Also, analytes of interest often need to be chemically derivatised using toxic reagents to enhance the sensitivity of the analysis [43,73,74]. For example, in most reported methods for determination of diacetylmorphine (heroin) after extraction from biological specimens, the extract is divided into two portions, one injected directly for heroin analysis and other portion subjected to derivatisation steps for the determination of 6-monoacetyl morphine (6-MAM) and free morphine (MOR) [75-77].

In forensic toxicology, drugs and their metabolites are determined for a better understanding of the cause of death, driver impairment and for workplace drug testing. Therefore, toxic components in the specimens should be identified accurately [59]. Some drugs are extensively metabolised to polar metabolites such as glucuronides, and hydrolysis of specimens is necessary to obtain free drug which can then be analysed using GC-MS. The ratio of total drug to free drug can then be used for interpretation of cases. This sometimes requires a large volume of sample to be analysed because of the relatively low sensitivity often obtained in GC-MS analysis.

HPLC is an alternative to GC for analysis of less volatile, thermolabile and polar analytes. More than 70% of drugs commonly encountered in routine forensic toxicology can be quantified using HPLC methods [78]. However, the identification of these drugs, which is important in the toxicology field, is

often problematic due to interferences and overlapping of analyte peaks, which makes simultaneous determination of drugs and metabolites difficult. In some reports [79-82], two different detectors have been used for identification of morphine glucuronides [83]. Also, some analytes cannot be oxidised, such as noroxycodone, and so cannot be detected using an electrochemical detector [84]. Quantitative analysis by HPLC coupled with conventional UV and DAD detectors is limited because not all analytes absorb UV light and identification of substances is limited due to the small databases of spectra available. However, the presence of a chromophore can allow analogous analytes such as metabolites to be detected. Also, the absorbance of analytes can be affected by the mobile phase composition, including solvent strength and pH [44,59,72,85].

An LC-MS/MS procedure usually requires fewer sample preparation steps and analytes of interest can be identified and quantified directly without the need for hydrolysis and derivatisation procedures [16,86-88]. Different LC-MS interfaces are available which are applicable to many type of analysis [89]. The ionisation of analytes can be optimised and the best instrumental settings can be chosen by continuous infusion of a standard solution of the analyte into the mass spectrometer. LC-MS is considered to be a highly selective means of identification, which is achieved in two dimensions: HPLC is used to obtain chromatographic separation of analytes from matrix components on one hand, and the mass spectrometer device is used to obtain mass to charge resolution of analytes of interest from other matrix components, interferences or other drugs [42,90].

In the present work, a Thermo Finnigan LCQ™ Deca XP plus was used for identification and quantification of analytes of interest. The LCQ™ Deca XP plus consists of three main parts: the HPLC system, electrospray interface (ESI)/atmospheric pressure ionisation source and ion trap mass analyser. Figure 3-1 shows the components of LCQ Deca XP plus system.

3.2 Sample preparation

Sample preparation is very important for method optimisation; clean extracts are often required when using HPLC analysis to reduce the interference

problem and concentrate the analytes into a small volume that can be injected into the HPLC system [65]. In the work presented in this thesis, whole blood obtained at autopsy was used for analysis which is known to be a dirty matrix. In addition, whole blood material cannot be injected directly to the LC-MS instrument which could cause damage to the HPLC column and ion source contamination [65,91].

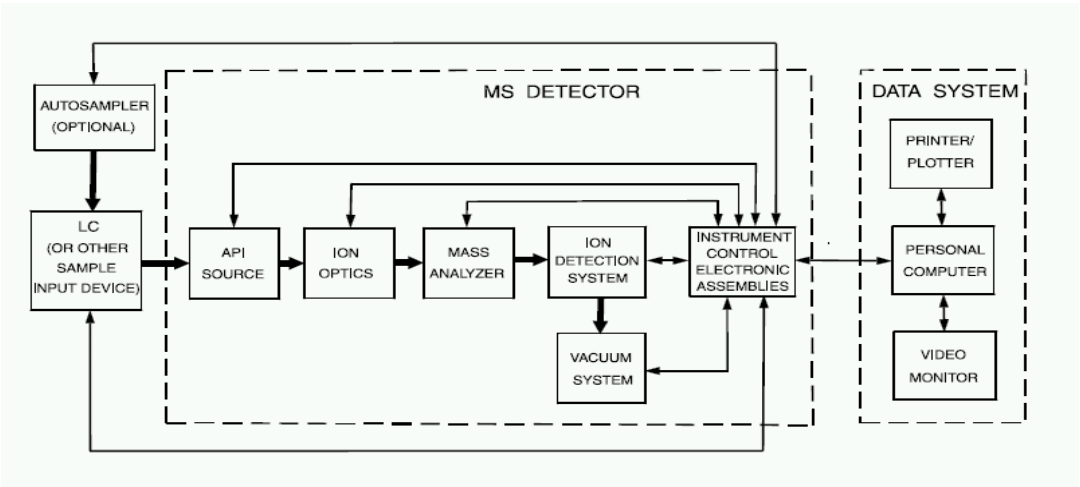


Figure 3-1: Diagram of Thermo Finnigan LCQ Deca system including HPLC, mass spectromer detector and data system [92].

In the work reported in Chapter 10, pretreatment of urine samples using acetonitrile followed by centrifugation was found efficient for the isolation of ethanol polar metabolites but a hydrophilic liquid chromatography (HILIC) method of separation was employed. HILIC can be used for the separation of very polar metabolites but is not suitable for non-polar metabolites. Reverse phase liquid chromatography (RPLC) was applied to simultaneous analysis of opioids and their metabolites and HILIC was used for very polar ethanol conjugates. The selectivity of the HILIC phase is opposite to that of RPLC but similar to that of normal phase liquid chromatography (NPLC). The mobile phase typically contains only a minor percentage of water, usually less than 30%. More information about the HILIC phase is given later in Chapter 10.

3.3 HPLC

An HPLC system includes the mobile phase reservoir, degasser, pump, auto-sampler and injector port, column oven containing the pre-column and

analytical column and connection to the detector. Sample extracts are placed in the auto-sampler and are then injected by syringe into the injection port containing the sample loop held in a six-port valve. The sample in the mobile phase is transferred through a connecting line to the HPLC-column which is connected via the sample inlet line to the detector interface system.

The mobile phase and column are very important in traditional HPLC methods in achieving good peak shapes. Complete separation is required with most HPLC detectors used, with the exception of the mass spectrometric detector, which can distinguish overlapping peaks using the mass to charge ratios of molecular and fragment ions. Many solvents can be used depending on the analytes to be separated and their solubility. The separation of analytes can be achieved using a single solvent or using a mixture of organic solvents and water [55].

In fact, RPLC is the most commonly-used system in forensic and clinical toxicology in which the mobile phase is more polar than the stationary phase; analytes of interest are partitioned between a polar mobile phase and non-polar stationary phase. A silica support modified with C8 or C18 chemically-bonded substituents phase is the most common non-polar stationary phase used for RPLC applications. The separation depends on the polarities of the analytes, with more polar components being eluted early, near to the void volume, while non-polar analytes are strongly retained and often require high percentages of organic modifiers to be eluted. Acetonitrile and methanol are the most common organic modifiers used with RPLC-MS [41,42,55].

In NPLC, the stationary phase is more polar than the mobile phase. Applications have been reported involving an ion pair agent (IPA) which is used with polar compounds to increase their retention times. NPLC and mobile phases containing IPA are not often used in LC-MS applications due to toxic solvents used in the NPLC mobile phase such as chloroform and hexane, which is carcinogenic and because of accumulation and contamination of the ion source by non-volatile IPA[42]. Recently, HILIC has been introduced as an alternative to RPLC for separation of polar compounds but it is not efficient with non-polar compounds [88,93]. In the current work, RPLC and HILIC were used for separation of drugs and metabolites.

Isocratic elution is common in RPLC but generally requires longer run times and is not suitable for separation of drugs and their polar metabolites for which gradient elution is preferred [55]. The percentages of organic modifiers is fixed in isocratic elution but is changed during the run time using gradient elution. The latter is often used to increase retention of polar metabolites on RPLC columns and decrease retention of non-polar analytes to provide a reasonable run time suitable for routine analysis [42]. In isocratic LC-MS or LC-MS/MS, the detector response can be enhanced by increasing the percentage of organic modifier but interferences from the matrix are often encountered [88,94]. Gradient elution also leads to enhanced detector sensitivity while avoiding the interference problem. The peak width also can be narrowed by as much as half compared to isocratic elution which is also desirable [42,55]. It is known that changing other parameters of the chromatographic system, such as buffer concentration, pH and temperature, can lead to retention time changes [55,65,95].

In regular RPLC, the mobile phase contains an aqueous and an organic phase. Phosphate buffer is commonly used in traditional HPLC but is not used with a mass spectrometric detector due to the non-volatile phosphate salts, which would precipitate in the ion source and cause a major contamination problem that results in reduction of the instrument performance [88,96]. In addition, other inorganic salts and IPAs should also be avoided in LC-MS due to the possibility of interference with analyte spectra, for example, trifluoroacetic acid (TFA) is known to decrease ionisation efficiency of analytes. Volatile mobile phase salts such as ammonium acetate and ammonium formate are more suitable for LC-MS applications. These buffers can be adjusted using acetic or formic acid or ammonium hydroxide to obtain the desired range of pH [3,41,42,65]. Although the use of a higher buffer concentration often leads to good chromatographic separation, a low buffer concentration is preferable in LC-MS [97], in which buffer concentrations higher than 0.01 M have been found to affect the sensitivity and linearity of the method [42,88]. This may be explained by an insufficient charge on electrospray droplets due to ion signal saturation by higher buffer concentrations, which decreases the ESI response [98]. It has been found that an increase in buffer concentration leads to suppression of the target ion signal, this conclusion having been made after an investigation of the effect of different buffer concentrations (0.001, 0.005

and 0.01 M) in the ESI responses for some peptides. In that study the lower the buffer concentration used the higher the ESI response obtained [99]. A similar observation was made by Torrance [63] for the ionisation of flunitrazepam during LC-MS, when it was found that the ESI response decreased with higher ammonium acetate buffer concentrations. Before samples are submitted to LC-MS, extracts are reconstituted with initial mobile phase in order to ionise analytes before they are injected on-column.

A step by step method of optimising buffer and organic modifier percentages was used in the present study which takes longer than using other methods of optimisation such as computer software programmes [42,55] or using a published method for analytes of interest. The analyte retention time depends on the flow rate, column capacity and diameter [96]; the injection volume is recommended not to exceed 1% of column volume to preserve maximum separation efficiency [100]. In the present work parent drugs and their polar metabolites were determined which were found to be well separated using gradient elution. Morphine-3-glucuronide, hydromorphone-3-glucuronide and dihydromorphine-3- glucuronide eluted with very low percentages of organic modifier and methadone eluted last with a high organic modifier content. Metabolites which are eluted early are more susceptible to matrix effects and sample preparation based on SPE was efficient in preventing matrix effects.

3.4 Mass spectrometry

3.4.1 Introduction

Mass spectrometry is the detection system of choice in forensic toxicology because it is a reliable, sensitive, specific and selective method of analysis and because of the useful information it provides about the chemical structures of analytes [65,67]. It is aneasy means of identification of substances compared to other types of spectral information obtained such as the ultraviolet spectrum. A wide range of analytes can be analysed with less sample volume and pre-treatment required [3,101]. Although GC-MS methods have been in use since 1970 and became the methods of choice in the 1980s, the combination between HPLC and mass spectrometry was not achieved

easily [16]. The most challenging problem was to find a suitable way to remove the aqueous mobile phase when delivered at a high flow rate of 1 mL/min without affecting the performance of the mass spectrometer. The use of interface techniques means that the combination between LC and MS techniques is possible. These interface systems have two main tasks: elimination of as much of the unwanted mobile phase solvent as possible and introduction of components of interest to the MS detector in the gas phase. These two steps require a high vacuum system [65,98,101-103]. Many interfaces have been used such as thermospray ionisation, particle beam ionisation, fast atom bombardment, desorption electrospray ionisation, matrix-assisted laser desorption ionisation and atmospheric pressure ionisation (API). The most common interface in use with LC-MS is API; this interface has two main types: atmospheric pressure chemical ionisation (APCI) or electrospray ionisation (ESI). New API interfaces have been created and used but with few applications in toxicology such as atmospheric pressure photo-ionisation, atmospheric pressure laser ionisation and sonic spray ionisation [3,42,88,89,94].

All interfaces used on LC-MS apparatus use soft ionisation, which provides relatively little structural information [42,59,96] compared to the hard ionisation mode used in GC-MS which means the library used with GC-MS is not useful with LC-MS. In most cases, data for analytes of interest can be optimised manually in LC-MS using certified standards and real case samples in order to obtain analyte spectra which have been found to be reproducible and accurate.

Components in the matrix or generated in the ion source may interfere with the information obtained in the single stage mass spectrometer. The use of tandem stage mass spectrometry introduces more selectivity into LC-MS/MS techniques [31,94]; analytes can be fragmented to product ions and thus the fragment ion is selected as the target ion in the analysis. This is one of the great advantages of using tandem MS, which not only provides the molecular weight of the parent drug, which is the single most important identification criterion required by the toxicologist, but which can also provide information regarding the structures of the components of interest [42,89,104]. There are two types of tandem MS instruments in use. The first type is a hybrid combination of two mass analysers based on quadrupoles or magnetic sectors. A triple

quadrupole instrument is the most common construction. The second type uses a quadrupole prefilter and ion trap, i.e. only a single mass analyser is used. In hybrid instruments the product ion is created using two separate mass spectrometers by isolation of the ions of a given m/z ratio in the first mass spectrometer which are then activated by collision and allowed to dissociate before the product ions are scanned in the second mass spectrometer. In the ion trap specific masses are selected by ejecting the other masses and then selected ions are fragmented during a selected time event or retention window [42,66,94,102,104]. The product ions are obtained by a sequence of operations in the scan function and are formed by collision-induced dissociation (CID) of the selected ion using helium buffer gas [89]. The latter type of tandem mass spectrometry was used for the current study.

3.4.2 Electrospray ionisation

In the current study, electrospray ionisation (ESI) (Figure 3-2) was used as it is usually recommended for highly polar compounds. The recognition of the value of ESI has increased since its applicability for large bio-molecules was discovered. It is useful for the determination of small, medium and large molecules; ESI is the most commonly used interface in forensic toxicology due to its relative ease of use, low solvent consumption and wide polarity range capability [88,89]. APCI is less susceptible to matrix effect phenomena compared to ESI [105-108]; however, application of APCI is less common due to the limited polarity range of APCI, incompatibility with thermally labile analytes and high background noise compared to ESI [28,42,109]. However, both interfaces are sensitive, reproducible and robust and have replaced all other LC-MS interfaces such as fast atom bombardment, thermospray, and particle beam [88,94,96].

Analytes are required to be ionised in the mobile phase before passing through the ESI probe. The advantage of introducing analytes in solution is to limit the degradation of some thermally labile analytes which are successfully separated by HPLC [42,88]. The stream of liquid mobile phase enters the ESI needle by a narrow capillary sample inlet at high voltage (typically, from 3-5 kV) then a fine mist of highly charged droplets is formed (positive or negative charge, depending on voltage polarity) while they pass through the ESI needle

tip. Once the charged droplets pass out of the ESI needle, mobile phase components start to be evaporated and the charged droplets become smaller and smaller. The size of the droplets is estimated to be 1 μm which decreases upon evaporation until the droplet size becomes about 10% of the original size. A series of coulombic explosions occur when the electrostatic repulsion becomes higher than the surface tension of the charged droplets, called the 'Rayleigh limit', in which the droplet can no longer retain the ions. The solvated ions are ejected into the gas phase and then enter the ion transfer capillary and move into the mass spectrometer to be analysed [3,42,88,98].

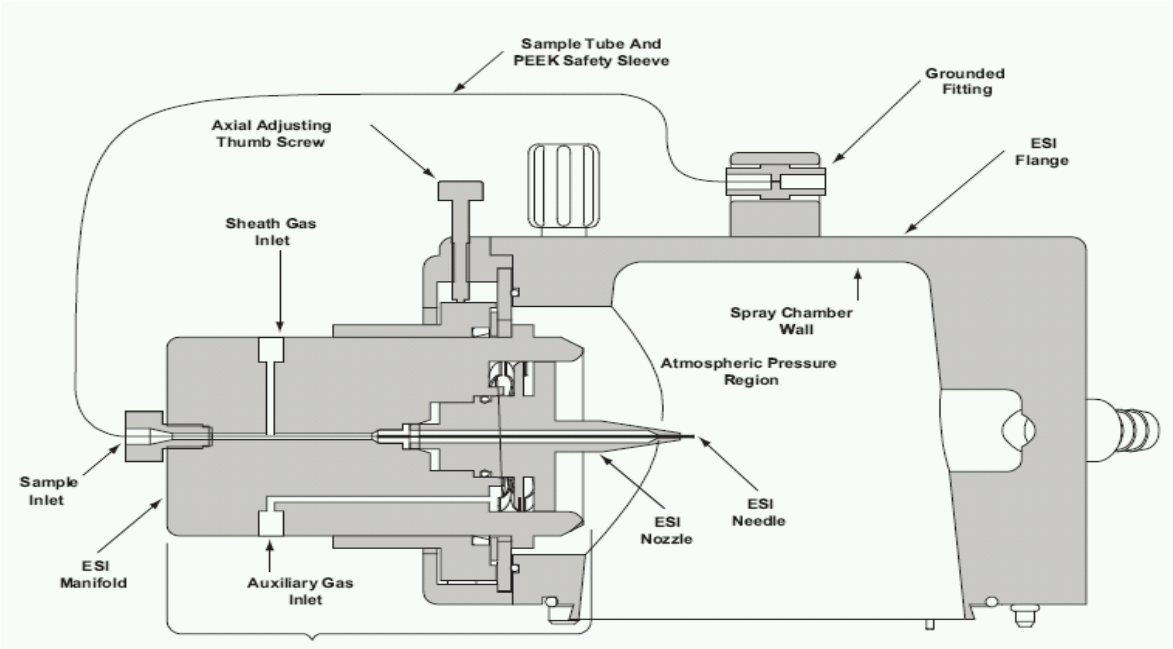


Figure 3-2: Electrospray probe of LCQ Deca system [92].

In the interface system, mobile phase is partly or completely removed during transportation of ionised analytes under high vacuum. The ionisation of analytes can be influenced by many factors such as flow rate, background noise, mobile phase composition including additives and sample interference, all of which have been found to affect the ESI signal [88,97,109]. Flow rate, for example, directly affects the size and distribution of droplets formed at the ESI needle and is preferred to be within the range of 5 to 10 μL/min. However, flow rates used for HPLC analysis are usually higher than 1 mL/min. Therefore, the reduction of flow rate amount is crucial for ESI in order to

obtain good ionisation. Splitting the flow rate has been suggested but this leads to loss of sensitivity. This problem has been solved using an ESI probe with a concentric flow of nebulising gas which allows flow rates up to about 200 μL/min to be used. A heated source inlet aids the evaporation of mobile phase before it reaches the lens, also, a heated stainless-steel block is placed in the capillary for the same purpose [42]. Figure 3-3 shows ESI in the positive ion mode using the LCQ deca system.

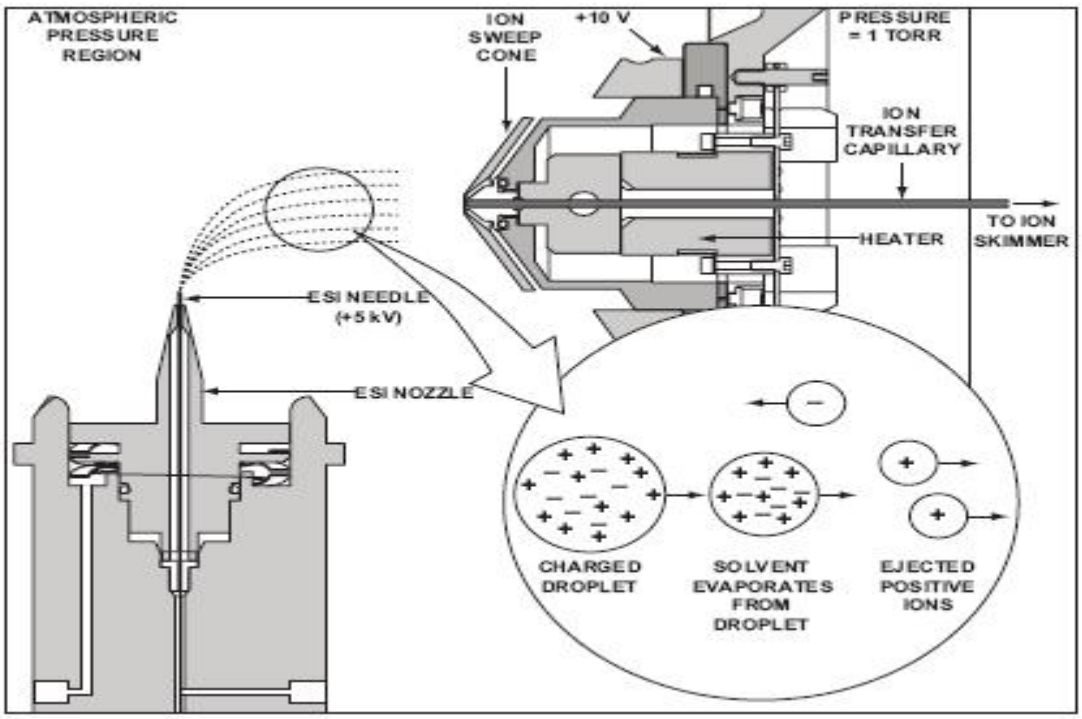


Figure 3-3: ESI positive ion mode process in LCQ Deca system [92].

Thermo Finnigan [92] recommends that the electrolyte in the mobile phase eluents should be at a concentration higher than 0.5 mM and less than 10 mM in order to promote the ESI signal. The target ion can be singly or multiple charged depending on its chemical structure and the mobile phase composition. Mobile phases with high aqueous contents reduce the efficiency of ESI whereas a high organic modifier content in the mobile phase enhances ionisation. Higher water or aqueous content in the mobile phase leads to higher surface tension in the charged droplets, which produces larger droplets that require a longer time to be evaporated during ESI. This is found to affect the breakdown of the initial charged droplets to smaller droplets in order to

facilitate the conversion of these tiny droplets to gas phase ions. However, applications of ESI using highly aqueous phase have been reported and some HPLC columns are capable of operating with 100% aqueous phase, which is required for the determination of very polar compounds, for example, ethyl glucuronide could only be determined using very low organic modifiers of 5% or less in RPLC applications. Many solutions can be used to tackle the loss of sensitivity using highly aqueous phases such as the use of sheath flow or post-column addition of organic solvent which was found to improve ESI-MS sensitivity [88,95]. In RPLC, a combination of polar solvents and volatile additives are required for two main tasks: good chromatographic separation and better ESI response, which makes choosing the mobile phase easy as few phases are recommended [65].

Nitrogen has been used as nebuliser gas which repels charged droplets from the ESI needle towards the ion source [42]. Ion spray is a term often used to describe the use of sheath and auxiliary gas to spray the mobile phase under reduced pressure through the interface. A combination of an organic sheath liquid and aqueous mobile phase solvent reduces the surface tension which accelerates the evaporation of the charged droplets [42,55,66].

3.4.3 Ion optics

After passing the skimmer, ions are filtered and transferred into the mass analyser through ion optics. In the LCQ Deca XP Plus instrument, the ion optics system (Figure 3-4) consists of an interoctapole lens placed between quadrupole and octapole. Both quadrupole and octapole act as ion transmission devices. RF and DC offset voltages are applied to the rods which lead to an electric field that guides the ions through the quadrupol and octapole. During this transmission the offset voltage is negative for positive ions and vice versa. Selected masses are allowed to enter the mass analyser.

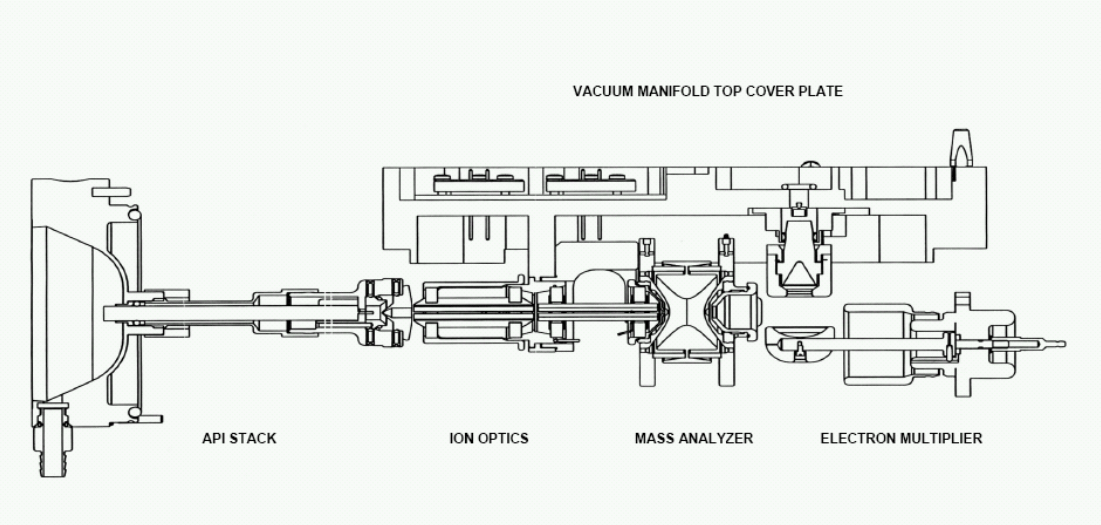


Figure 3-4: Cross section of LCQ Deca ion optics system [92].

3.4.4 Ion trap mass analyser

An ion trap mass analyser (IT) was used in the current study (Figure 3-5). An IT is also known as a quadrupole ion trap mass analyser. It is a three dimensional quadrupole electrodynamic field or 'electric field test-tube' to trap ions which are created in the ESI interface. It is considered as a high-throughput analytical tool especially for providing structural information about substances [110]. It has a wide mass range, up to m/z 70,000, making it applicable to protein analysis [96]. Ionised molecules enter the ion trap through an electrostatic gate that pulses open and closed. Ions are accumulated with each pulse and transferred to the ion detector which makes it highly sensitive in the full scan mode compared to other transmission quadrupole instruments. Ion masses in the IT are accumulated selectively over time in which target ion(s) can be selected to ensure constant signal to noise ratios over a wide range of concentrations [41,42,110,111]. However, the discontinuous measurement is a noticeable drawback of using IT which allows few ions to be quantified during the same chromatographic retention window.

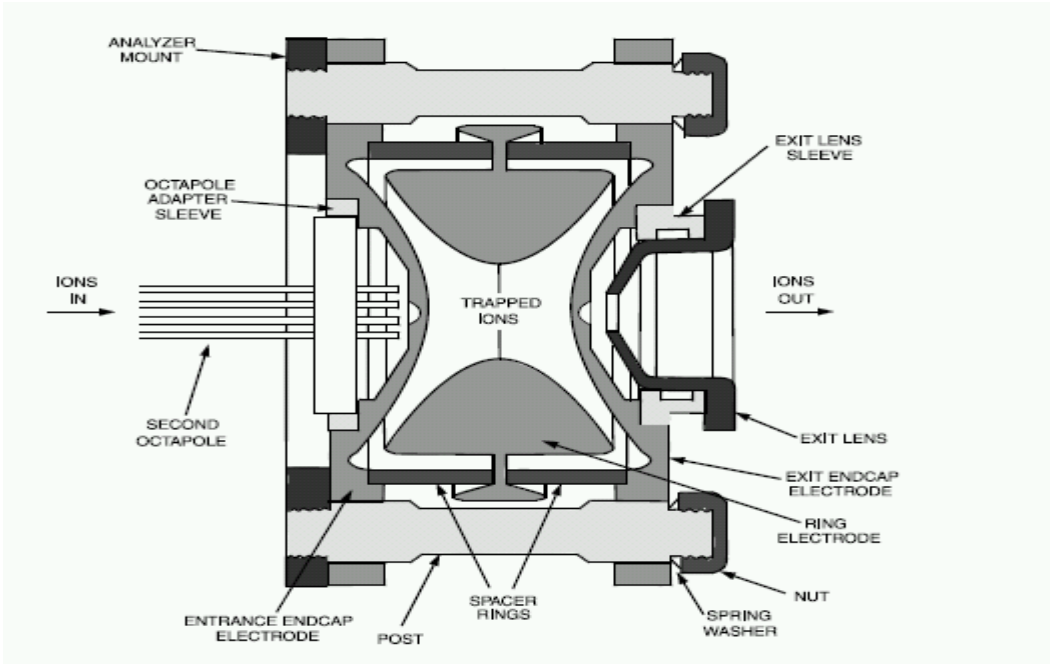


Figure 3-5: Ion trap mass analyser equipped with LCQ Deca plus XP [92].

In the LCQ Deca Plus XP, selection of more than five target ions per retention time segment widow was found to reduce the data point number across chromatographic peaks, which can be overcome using multiple short retention widows for target masses during the chromatography run [90,112,113]. Therefore, good LC separation of analytes is required using the IT in order to use many segment windows and avoid co-elution of analytes, allowing a higher scan speed and increase in the number of data points per chromatographic peaks. In contrast, when a triple quadrupole instrument (TQ) is used, co-elution is of no concern due to the selectivity of the tandem mass spectrometer, which allows a shorter chromatography run and increased sample throughput. However, the cross-talk or isobaric phenomenon occurs when the interval between two or more analytes of interest which share the same chemical structure is very short, resulting in overlapping between them. Cross-talk occurs in both IT and TQ mass spectrometry techniques and affects method accuracy [90,96,102]. Cross talk or isobaric metabolites interference is different than matrix effects. Matrix effects are generated from non-drug related compounds eluting at the same time as an analyte, while cross-talk usually results from co-elution of an analyte with one or more other drug-like substances which fragment to give the same (or similar) product ions used for

identification. Cross talk phenomena are not specifically matrix effects [90,114] but often interfere with the quantification of the analyte. This so-called isobaric metabolic interference can also be the result of in-source contamination or be due to degradation products of phase I and II metabolites [28,90,115].

The operating principle of the ion trap is that ions are generated in an external ion source and injected into the ion trap cavity [102]. Helium gas is introduced into the cavity to move the ions towards the centre of the trap [110]. Ions of a given m/z value are trapped, while all other masses are held back during the admission time in the trap. The ion trap uses segment windows or reaction times which can be extended for some time [104]. Selected ions are fragmented to product ion(s) within the trap space and series of MS^n data can be generated up to $MS^{(n=10)}$ using the LCQ Deca instrument. However, few applications using more than $MS^{(n=3)}$ have been reported [94].

The ion trap consists of three hyperbolic electrodes: a central ring electrode and two virtually identical end-cap electrodes. The end-cap electrodes are placed at the entrance and exit of the ion trap cavity. The ring electrode is symmetrically placed between the end-cap electrodes. Ions are passed into and out of the ion trap cavity using two small holes in the end-cap electrodes. Trapped ion(s) are retained for collision with a damping inert gas with relatively high pressure (usually 10^{-2} to 10^{-3} torr of helium) and a radiofrequency voltage is applied to the ring electrode which makes ions of a certain m/z ratio become unstable and fragment to give product ions, This process is called collision induced dissociated (CID). The trajectories of ions with a given m/z value become unstable and then the ions are ejected through the exit lens to the detector [102,116].

3.4.5 Detector

The ejected ions are converted into measurable electric signals upon reaching the detector which is necessary to have a fast acquisition system, have a wide dynamic range and high sensitivity. In the LCQ Deca Plus XP detector (Figure 3-6) ions are converted by a conversion dynode to electrons which are further converted into photons by means of a phosphorous screen and these are

amplified by an electron multiplier. When an ion strikes the conversion dynode, secondary particles are formed which are focused by a curved surface and accelerated into the electron multiplier by means of a voltage gradient. The secondary particles will strike the inner surface of the cathode to produce electrons. A cascade of electrons is created in which original signals are magnified and converted into a voltage that can be recorded by the data system [67,92].

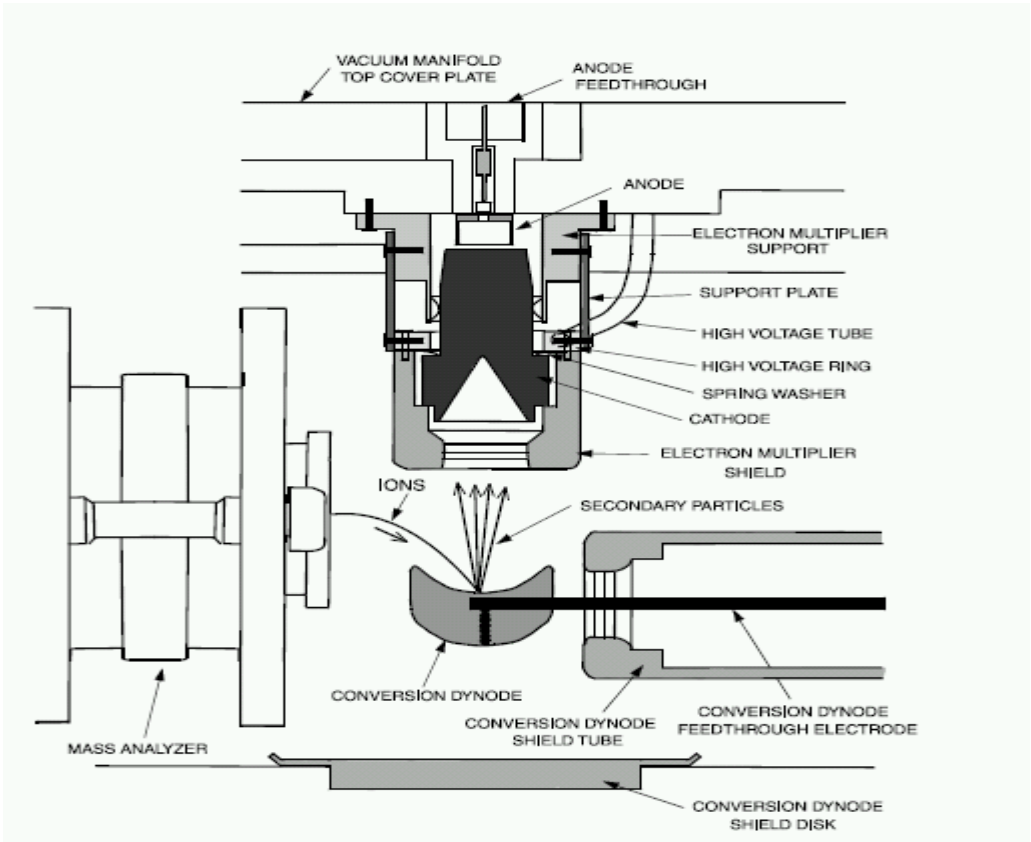


Figure 3-6: Cross section of ion detector system in the LCQ Deca [92].

3.5 Matrix effects and LC-MS/MS

The susceptibility of LC-MS/MS procedures to matrix effects has been reported using different methods of extraction such as protein precipitation, direct injection, liquid-liquid extraction and SPE [16,97,105-108,117-120]. The mechanism of the matrix effect in Atmospheric Pressure Interference (API), especially, electrospray ionisation (ESI) is unknown [51,97]. It has been suggested that the matrix effect originated as a result of the competition between

analytes of interest and endogenous matrix components co-eluting at the same time leading to a decrease or increase of the ionisation efficiency of the analyte of interest in ion source [51,117]. Method validation parameters such as recoveries, reproducibility, accuracy, LOD and LLOQ may be affected by the presence of a significant matrix effect [105,117]. Therefore, the assessment of the matrix effect during the method development process has become an important issue in method validation and is recommended to ensure that the selectivity, sensitivity and precision of the method are not compromised [16,51,97,105-108,117-120].

King *et al* [106] reported that ionisation suppression is not likely to be caused by reactions in the gas phase. They stressed that non-volatile solutes were most likely to cause ion suppression and non-volatile substances can be any type of chemical structure. For example, salts, sulfates and phosphates would compete with analytes of interest in the ionisation process. Therefore, these interferences should be removed using clean up procedure such as SPE before samples are analysed by LC-MS(MS). Although SPE methods are efficient in concentrating the analytes of interest, co-eluting, endogenous, matrix components are also concentrated [51,108,117]. Chambers *et al* [117] also concluded that the cleaning steps would concentrate endogenous matrix components that may shorten the lifetime of the LC column, because of deposited materials, and require more MS maintenance.

Matrix effects due to mobile phase additives were found to be less significant than those from endogenous matrix components [42,105]. In addition, pharmaceutical impurities or degradation products can cause matrix effects [117]. It has been reported that hydrophilic residual components i.e. organic salts present in urine samples are the major cause of matrix effects [108]. Ion suppression can be the result of some ionic substance or substances at the surface transition zone of charged droplets [121]. Ion suppression is magnified by increasing the injection volume [42]. Many researchers believe that matrix effects phenomena in LC-MS^n procedures are a result of inadequate sample preparation and the use of short analytical column with fast gradient LC-methods or short isocratic methods [107,119,121].

Elimination of matrix effects is important in order to gain more sensitivity and selectivity of analytes of interest, especially for early eluting (low capacity factor, k′) [121] analytes such as M3G and DHM3G in the present study. Many methods have been used to eliminate matrix effects. Nissan *et al* [105] recommended two approaches: the best way is to improve the sample preparation and the separation of analytes of interest [108]. However, this approach is hard to achieve, especially in methods aimed at simultaneous detection of a variety of drugs [105]. The second approach is to change the mobile phase composition, use negative mode LC-API-MS/(MS) instead of the positive mode and use internal standards [109]. Matrix effects can decrease if urine samples are diluted and injected directly without sample pre-treatment steps. However, dilution results in loss of method sensitivity [108,122].

The use of internal standards is the best solution for overcoming matrix effects [42,51,105-108,117-120]. However, isotopically labelled analogues are not available commercially for all analytes and the cost of synthesising internal standards is high as well as being time consuming. In this case structurally similar analogues are frequently used as internal standards, with a preference for analogues that elute close to the analytes of interest and show the same matrix effect profiles [118]. The use of unlabeled therapeutic drugs as internal standards is not acceptable as these may be found in case samples even if they are not marketed in the country in which the laboratory is located and that would affect the accuracy of the method [16]. The use of two solid phase extraction columns or LLE followed by SPE were reported although the recoveries obtained were very poor. Finally, matrix effects can be reduced using diluted samples and a small injection volume, but this leads to a reduction in sensitivity. LC-MS(MS) methods are still valid, even in the presence of matrix effects, if the internal standard shows the same matrix effects. If an isotopically-labeled internal standard is available and the precision and accuracy of the method at the LLOQ are within the acceptable limits of the validation procedure then a method is considered valid even in the presence of minor interferences [51].

Two methods are commonly used to determine matrix effects [51,97,107]: the post-column infusion procedure and post-extraction spiked analyte procedure. The latter method was used in this work as it was easier to perform with 26

analytes and 13 internal standards than the first method which requires optimising each drug separately. The post extraction method compares the ESI response of an unextracted standard dissolved in mobile phase with the response of the same standard dissolved in blank matrix extract. Matrix effects are determined as a percentage relative to the response of the unextracted standard: a value of > 100% demonstrates ion enhancement while a value of < 100% points to ion suppression.

Matrix effects may also affect analyte recovery measurements, which are usually determined by comparing the peak area ratios of extracted analytes with those of unextracted standards solution at corresponding amounts. The effect of matrix on these ratios is not examined using the traditional method, which may lead to inaccurate estimates of recovery for analytes without an isotopically-labeled internal standard. In the present study the true extraction recovery was determined using the procedure of Matuszewski *et al* [51]. In this method the effects of matrix interference and ESI ionisation were excluded. Absolute matrix effects as well as relative matrix effects were assessed. The latter refers to variations in response depending on the source of blank urine. Matrix effect (ME) and recovery (RE) are given by the following equations:

Equation 3-1 $ME\ (\%) = B/A \times 100$

Equation 3-2 $RE\ (\%) = C/B \times 100$

Where A is the average peak area ratio of the unextracted standard to internal standard, B is the average peak area ratio of the same standards spiked into blank extract and C is the average peak area ratio of extracted standard to unextracted internal standard.

4 Opioids

4.1 Introduction

Opiates are obtained from the opium poppy (*Papaver somniferum)* and have been used medicinally for more than 2000 years [2,123,124]. The term opioids 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 [2,125]. Opioid drugs have been used for many purposes including management of moderate to severe pain resulting from surgery or illnesses such as cancer, as antitussives and for the treatment of diarrhoea [125,126]. Unfortunately, opioids are globally misused drugs. For example, it is estimated that approximately 0.3% of the world's population (11 million people) misuse the opioid heroin each year [26]. Injection heroin abuse is a major problem in society, contributing to the spread of infectious diseases and carrying adverse economic consequences.

Opioids are defined as compounds which have similar pharmacological effects to those of morphine, and they can be divided into three main groups [125]: naturally occurring (morphine and codeine); semi-synthetic or derived from morphine by chemical modification (oxycodone, dihydrocodeine, hydrocodeine, hydromorphone, etc.); and obtained synthetically (fentanyl, methadone, etc.). Simple modification of the morphine structure, for example methylation or acetylation of morphine, can result in products which have different molecular weights and whose pharmacological properties are altered. For example, codeine, which is a naturally occurring opiate, can be derived from morphine by the methylation process. Synthetic drugs such as methadone offer the same narcotic analgesic action as that of morphine but may differ in their potency. Some pharmacological parameters of opioids included in this thesis are summarised in Table 4-1

Table 4-1: Dose, relative potencies to morphine, half-life and bioavilability for selected opioids

Drug	Usual dose (mg) [a]	Relative Potency [b]	Half-life (h) [c]	Bioavail. [d]	Usual therapeutic blood Concentrations (ng/mL)
Buprenorphine[e]	0.2-24	25-50	3-6	30	0.1-1
Codeine	8-60	0.08	2-4	High	10-250
Dihydrocodeine	30-60	0.1	2-4	High	
Heroin	>5	1.5	<0.1	<1%	10-100 [g]
Hydromorphone	1.3-2.6	5-10	2-5	50%	8-32
Methadone	5-120	1	8-55	50%	100-500
Morphine	>10	1	1-8	20-30%	10-1000
Oxycodone	10-15	0.8	2-8	80%	10-200

[a] Dose range normally prescribed; [b] pharmacological potency relative to morphine; [c] plasma half-life of terminal elimination phase; [d] oral bioavailability, relative to intravenous dose; [e] sublingual dose; [f] usually given parenterally; [g] as morphine [127].

Other than classifying opioids according to chemical structure, another method of classification was introduced which is dependent upon the mode of action and divided into three classes [2,124,125]. The first class includes full agonist drugs that have abilities to specific type of opioid receptors. These compounds are toxic and potent and they are active even in amounts as small as a 'few micrograms'. This group includes dihydrocodeine, hydromorphone, hydrocodone, methadone, pethidine and fentanyl. The second class includes mixed agonist-antagonists; morphine, for example, has both agonist and antagonist actions. These drugs have an agonist effect with certain types of receptors and an antagonist effect with others. Buprenorphine, butrophenol and pentazone belong to this group. The third class contains opioids known as full antagonists. Naloxone and naltrexone are two of these drugs, which have been used as antidotes to morphine and heroin toxicity because of their abilities to inhibit agonist binding; however, these drugs are themselves inactive.

Opiate toxicity has attracted a great deal of interest since its side effects became known. It has been amply demonstrated that opiates are a double-edged sword, they may relieve pain, and yet they may also kill. One reason is that tolerance of the patient or addict increases from using opiates;

therefore, the opiate dosage soars dramatically after the first use in order to attain a similar therapeutic effect. This increase may be more than 35-fold after three weeks of chronic use [2]. As a result, sudden and unexpected deaths have risen sharply due to overdoses of these drugs [125]. Moreover, repeated consumption of these drugs results in physical dependence. Although these side effects are common, they are also complex, and the mechanism of physical dependence remains unclear. One explanation holds that by decreasing the number of μ-receptors, the efficiency of receptor binding along with their biochemical responses would therefore also reduce. Consequently, entry of calcium into cells is affected, and thus activity of the adenylate cyclase enzyme and protein phosphorylation would be inhibited [125]. Although each drug has its own toxic effect, it can be concluded that all opiates are expected to cause depression of respiratory centres and cardiovascular collapse may also occur [2,125,128].

Opiates have been administrated by many routes: oral, intravenous, intramuscular, smoking, nasal insufflation and many others, but where the abuse of drugs is concerned, intravenous administration is optimum, owing to its fast analgesia effect after injection compared to the delayed effect obtained with other routes of administration [129]. Opiates in general are rapidly absorbed, with some variation depending on route of administration. Drugs such as opiates are metabolised in the body by two types of enzymatic reactions: phase 1 and phase 2 metabolism. These reactions change the chemical structure of the drugs to be less toxic, and more water-soluble to facilitate excretion in urine; this process is called detoxification [43,76].

In addition, the metabolism of opiates differs considerably depending on their chemical structures. Opiates are commonly metabolised by reactions, such as oxidation, hydroxylation, O- and N-demethylation, N and O-dealkylation and deacetylation, which result in phase one metabolites. The main function of Phase 1 metabolism is to prepare compounds for phase 2 metabolism [130]. Phase 2 metabolites produced by glucuronidation and sulfatation reactions are acidic compounds with very polar and hydrophilic functional groups. These are usually inactive metabolites but may also be active; for example, morphine is glucuronidated into morphine-3-glucuronide (M3G), which is an inactive metabolite, and into morphine-6-glucuronide (M6G), which is active [5,131].

However, metabolites differ from parent drugs in potency and action [76,132]. The process of glucuronidation is a crucial enzyme reaction in detoxification. Glucuronic acid is derived from glucose, which is readily available in the body and is readily transferred enzymatically to many functional groups such as hydroxyl, amino, carboxyl and sulfhydryl. Uridine diphosphate-glucuronosyl transferase catalyzes this process [14,76,130,133].

It is known that codeine, dihydrocodeine, oxycodone and hydrocodone are prodrugs and exert their analgesia effects via their active metabolites produced by O-demethylation, which have much stronger μ-receptor binding than the parent substances. This reaction is mediated by the polymorphic cytochrome P450 2D6 isozyme (CYP2D6) [126,134]. It is known that approximately 10% of the Caucasian population are deficient in this isozyme and as a result are poor metabolisers, which reduces active metabolite production. The desired analgesia cannot be achieved with these four opioids at the usual dose. On the other hand, there may be clinical advantages in using the active metabolites morphine, dihydromorphine, oxymorphone and hydromorphone instead of the prodrugs as small dose controlled-released tablets, to provide strong analgesia for those patients who are poor metabolisers [134]. By contrast, a higher opioid effect may occur with the usual dose of codeine in rapid opioid metabolisers, which can be explained by the formation of higher concentrations of morphine following codeine administration.

Poor metabolisers may experience reduced codeine efficiency due to the reduction of codeine metabolism to morphine which may require higher concentrations of codeine to reach optimal effects, and this may lead to codeine toxicity [135,136]. A high risk of codeine intoxication with codeine doses in the usual range may be encountered in ultrarapid CYP2D6 metabolisers [126,136,137]. Patients who are poor CYP2D6 metabolisers may experience oxycodone toxicity due to the reduction of oxymorphone production and accumulation of oxycodone and noroxycodone [135] although other studies have shown that there are no differences in the analgesic effects of oxycodone or dihydrocodeine between poor or extensive metabolisers [138,139].

4.2 Heroin

4.2.1 Background

Heroin (Diamorphine; Acetomorphine; Diacetylmorphine) or (5α,6α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol diacetate (ester) is a semi-synthetic morphine derivative [1,132]. 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 [75,128,140,141]. It was intended to be used in medication as an antitussive agent [141], but unfortunately, the misuse of heroin has become a major cause of death in the world today [128,132,140,142,143].

4.2.2 Metabolism and Excretion

In all administration routes, heroin is quickly deacetylated to 6-monoacetylmorphine (6-MAM), which is considered to be a specific marker of heroin use. Heroin has a short plasma half-life, which has been reported to be between 2-8 minutes after intravenous injection [144]. Despite the short half-life of heroin, it can be detected in blood samples under certain experimental conditions. In particular if the blood sample has been taken directly after a heroin injection or in the case of a massive drug overdose [129].

6-MAM is not always found in blood due to its short half-life, which is reported to be between 10-40 minutes [145,146] but can be detected in urine up to eight hours after heroin administration [2,147]. 6-MAM is hydrolysed to morphine (MOR), and then morphine is conjugated to form two glucuronides: morphine-3-glucuronide and morphine-6-glucuronide and 5% of MOR is metabolised by N-demethylation to normorphine (NMOR). The biotransformation of heroin is illustrated in Figure 4-1. 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 heroin use [12,128,140,148]. Eighty percent of heroin metabolites are eliminated within 24 hours after administration [2].

4.2.3 Toxicity

Heroin is known as the drug most likely to kill as a result of an overdose [125] and is two times more potent than its precursor morphine. Deaths attributed to diamorphine may occur with a dosage as low as 200 mg but also depends on the tolerance of the deceased [1]. The lethal dose of heroin may be much more, up to ten fold, for chronic drug abusers. However, deaths attributed to heroin have been reported following doses of 10 mg [1]. As indicated earlier, heroin has a very short half-life and is rarely detected [5]. As a result, levels of its active metabolite MOR have been employed for the interpretation of cause of death and elapsed time after heroin administration. The presence of 6-MAM in blood has been used as evidence of a short elapsed time after administration because 6-MAM has a short half-life of less than 40 minutes after administration [149]. There is an overlap between concentrations encountered in fatal and non-fatal overdoses. Morphine levels determined in heroin fatalities vary between cases and there is an overlap between deaths attributed to heroin and those not attributed to heroin [149-152]. The heroin overdose mechanism is not fully understood and many deaths are attributed to respiratory depression [153].

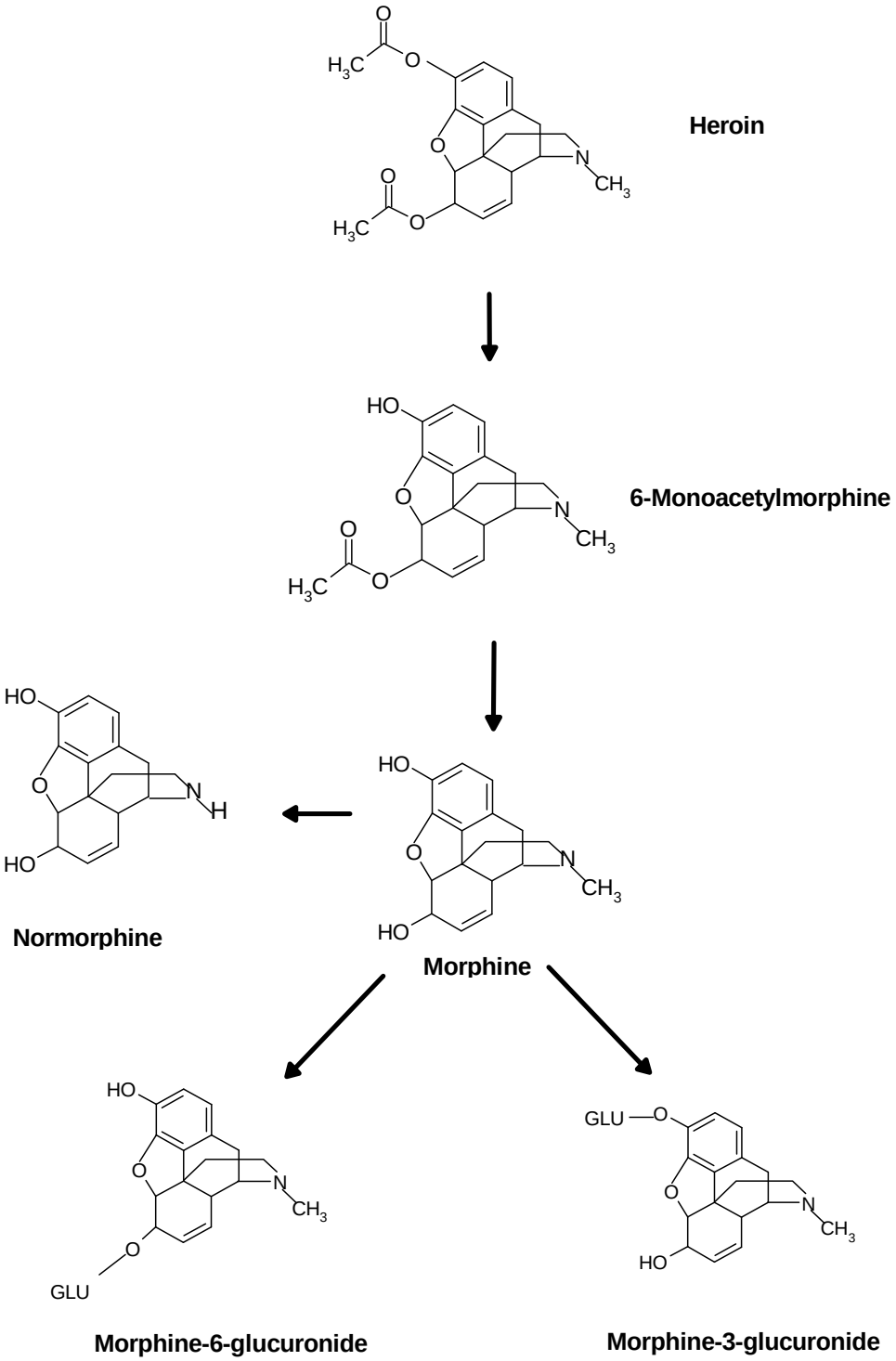


Figure 4-1: Heroin metabolism. (GLU: Glucuronic acid)

Toxic levels of MOR present in autopsy blood have been suggested to be equal to or higher than 300 ng/mL [149,154]. However, a value of 240 ng/mL was the

most common concentration in actual post-mortem cases in one study [155]. Fugelstad *et al* [149] found that 10 ng/mL of 6-MAM with fatal cases could be attributed to fatal cases. Darke and Rose [156] reported levels of MOR in 10 cases attributed to non-injected diamoprhine fatalities; the median was 0.31 µg/mL and ranged from 0.06-0.99 µg/mL. In 54 people who survived acute heroin overdose, the plasma concentrations of free and total morphine were 0.088 and 0.277 µg/mL respectively. However, levels of MOR detected in living subjects overlapped with those found in acute heroin overdoses [151,152]. The median blood level of MOR detected was 0.35 µg/mL (range 0.08-3.2 µg/mL) and 0.09 µg/mL (range 0.05-1.45 µg/mL) in fatal and non-fatal heroin users, respectively.

The majority of heroin-related deaths occurred with the presence of other drugs such as ethanol, benzodiazepines, cocaine and other opioids. It has been found that these deaths may occur at low levels of MOR in blood as a result of poly-drug intoxication, especially with other central nervous drugs. Deaths involving heroin can be classified in three categories [157]: intoxication with heroin alone, in combination with other centrally acting drugs and non-heroin related. Heroin users are known to build their tolerance with chronic use and can administer high doses without leading to overdose. Heroin overdose deaths due to high doses would be expected to result in high concentrations of heroin metabolites such as morphine in post-mortem blood. However, lower levels of morphine are observed in many heroin deaths than are encountered in living heroin users. These can be explained in many ways, for example death may occur due to a lack of or loss of tolerance or a long time may have elapsed between injection and death (delayed death). However, low levels of morphine are considered toxic in the presence of other centrally-acting drugs and may contribute to death [149-151,153,158].

4.2.3.1 Morphine and its glucuronides

As a result of the difficulties encountered in detecting heroin and its specific markers in biological fluids in post-mortem cases, great emphasis has been placed on the analysis of morphine and its metabolites due to their abundance and stability in biological fluids. Therefore, the concentrations of free morphine, total morphine, and 6-MAM in biological fluids have been

determined to be interpretative tools which provide information for the estimation of the survival time after heroin ingestion [159-162]. Carriott and Sturner [162] were the first to show a relationship between the survival period after a heroin overdose and the free morphine level found in the deceased's blood. In addition, the ratio of free to total morphine has been used by Staub *et al* [163] to differentiate between rapid and delayed deaths.

For several reasons, difficulties have been encountered when the ratio of free to total morphine is used to interpret a heroin-related death, for example, the inability to distinguish between users of heroin and other opiates such as morphine and codeine [164]. This is because morphine can be formed from the ingestion of heroin, morphine, codeine, and poppy seeds contaminated with opium. Burt *et al* [165] point out of that an accurate toxic range could not be established for morphine due to the considerable overlapping of therapeutic, toxic and lethal concentrations, and due to the differences in opiate tolerance between these groups. In addition, determining total morphine without distinguishing between morphine and its metabolites will not provide information useful to understanding the mechanism of intoxication [159]. Also, the discovery of the role of morphine-6-glucuronide at the opioid receptor, which has been found to contribute to the toxic effect of heroin in subjects with renal failure or with a history of heroin use over a long period, highlights the importance of measuring morphine glucuronides [165].

Determination of morphine and its metabolites is crucial in interpreting both forensic and clinical cases, for many reasons. One reason is the lack of necessary data in most post-mortem cases such as route of administration, time of intake and dose. Therefore, the concentration of 6-MAM and the ratio of morphine to its glucuronides can be a useful tool to determine survival time between ingestion and death [166-168]. Dienes-Nagy *et al* [159] reported that the molar ratios M6G/morphine and M3G/morphine in blood are associated with elapsed time since the last injection. The ratios can also be used to interpret the mode of death and if death was delayed, if morphine is almost completely metabolised to its glucuronide [2,45,76].

4.2.4 Previous analytical work

Specific immunoassays for morphine detection in human blood and urine have been described as screening methods [169]. Although immunoassay techniques save time and effort, immunoassays can be manipulated by adulteration in order to bring about false negatives. Moreover, immunoassay methods lack the specificity to discriminate between drugs and their metabolites [170,171]. Gas chromatography coupled with mass spectrometry (GC-MS) has been frequently employed to confirm positive results obtained using immunoassays [68]. Only one method has been reported for morphine glucuronide by GC/ negative-ion chemical ionisation MS [172]. Although GC-MS has greatly enhanced the potential of analytical toxicology, many obstacles have been encountered due to lack of applicability towards a polar and thermolabile compounds and high mass molecules. Another problem which has been reported is that sample preparation is time-consuming owing to the sample cleaning step. Moreover, polar compounds have to be hydrolysed and derivatised in order to be analysed, which is hazardous because of the toxic chemicals used in this step [41-43].

Svensson *et al* [83,173] developed an ion-pair HPLC method with UV and electrochemical detectors for the detection of morphine, M3G, M6G and normorphine in biological fluids for the first time. Problems have been encountered in determining morphine glucuronides due to the difference in their detectability. However, these have been solved by using multiple detectors such as an electrochemical detector [80], a fluorescence detector for M3G, a colourmetric detector for other metabolites [82,174] and a fluorescence detector for all metabolites [81] (Table 4-1).

In recent years, in addition to the success of HPLC for the detection of morphine glucuronides, the analysis of low concentrations of opiates and their glucuronides has become achievable due to the development of LC-MS techniques (Table 4-2). LC-MS has become the technique of choice and has been successfully used to achieve excellent separations and accurate identifications. Another advantage of LC-MS is its ability to determine a variety of compounds such as non-volatile samples, ionic, polar, thermally labile and high molecular weight molecules without derivatisation.

Table 4-2: Previous HPLC and GC-MS methods for opioids and metabolites.

Analyte	Sam *	HPLC or GC conditions (Mobile phase)	HD **	SPE ∋	Findings	R &
MOR	B U	TLC & UV spectrometry & GC	-	-	Free MOR for those dying within 3 hours following heroin injection was between 10-93 µg/100mL and in those dying from 3-24 hours after the last injection, it ranged from 3-10 µg/100mL.	162
MOR **M3G** **COD** **HER**	P U	Ion-pair HPLC Column: Reversed Phase (RP) Ultrasphere ODS 150 x 4.6mm (10 mM sodium dihydrogen phosphate buffer pH 2.1 + 1 mM dodecyl sulphate and 26% cetonitrile) Flow rate (FR) 1.5 mL/min	UV	Sep-Pak C18 SPE	M6G was identified tentatively in this method	173
MOR **M6G** **NMOR**	P U	Ion-pair HPLC Column: RP Ultrasphere ODS 150 x .6mm (10 mM sodium dihydrogen phosphate buffer pH 2.1 + 1 mM dodecyl sulphate and 26% acetonitrile), FR: 1.5 mL/min	ECD	Sep-Pak C18 SPE	M3G is not detectable by ECD detector	83
MOR **M3G** **M6G** **NMOR**	P	Ion pair HPLC Column: RP Apex ODS (150 x 4.6 mm) (10 mM sodium dihydrogen phosphate buffer pH 2.1 + 1 mM dodecyl sulphate and 26% acetonitrile)	ECD UV	Sep-Pak C18 SPE	The use of M6G as internal standard gives this method reliability. They used another electrode with ECD to remove an interference peak that eluted close to MOR	82
MOR **M6G**	P	Ion-pair HPLC Column: RP C18 Nucleosil 25cm x 4.6mm I.D. 5 µm particle size (10 mM sodium dihydrogen phosphate buffer pH 2.1 and 26% acetonitrile); FR: 1.5 mL/mi	ECD	Sep-Pak C18 SPE		80
MOR **M3G** **M6G** **NMOR**	P	Ion-pair-HPLC Column: µBondapak C18 RP 300mm x 3.9 mm, 10 µm particle size (10 mM sodium dihydrogen phosphate buffer pH 2.1 and 26% acetonitrile); FR: 1.0 mL/min	FD	Bond-Elut C8 SPE		81
MOR **M3G** **M6G**	P	HPLC Column: Nva-Pak 4-µm phenyl 3.9 x 75 mm, with Nova-Pak guard column 4 µm. (methanol: 0.01 M phosphate monobasic pH 4 (10: 90) FR: 1 mL/min	ECD FD	Bond Elute C2 SPE		174
MOR **6-MAM**	B U	Immunoassay HPLC GC-MS	-	-	Free MOR in blood makes up 50-100% of the total MOR in rapid deaths, and 20-40% in those which survive for a long time	165

* Sam.: Sample types; B: Blood; U: Urine; P: Plasma; ** HP: HPLC detector used; UV: Ultraviolet; ECD: Electrochemical detector; FD: fluorescence detector. SPE: Solid phase extraction; & R: Reference.

Table 4-3: Previous LC-MS methods for morphine glucuronides.

Analyte	Sam. *	HPLC or GC conditions (Mobile phase)	Extraction Method ϶	Ref. π
MOR M3G	U	RPLC & -MS# -APCI ** + ## (SIM &&) Column: L-column ODS; (150 mm x 4.6 mm) M.P ☼: (50 mM ammonium acetate: methanol (86: 14) FR *f*: 1.0 mL/min	Sep-Pak C18 SPE	175
MOR M3G M6G COD	S	PRLC-MS-ESI+ (SIM) Column: ABZ (25cm x 4.6 mm, 5 µm) M.P: (water: ACN § with linear gradient from 96:4 to 30:70 with 3 nmol/L formic acid). FR: 1 mL/min.	Ethyl SPE C2	176
MOR M3G M6G	S	RP-LC-MS ESI #* +(SIM) Column: YMC ODS-AL 100 x 4.6 mm (water: ACN (94/4 to 30/70 with mmol/L formic acid) FR: 1 mL/min	Sep-Pak C18 SPE	171
MOR, M3G M6G, COD C6G 6-MA	B U CSF VH	RPLC-MS-APCI (SIM) Column: Supersgher RP 18 (125 x 3 mm, 4µm) M.P: (50 mM ammonium formate pH3: ACN (95: 5) FR: 0.6- 1.1 mL/min	Bond Elut C18 SPE	177
MOR M3G M6G 6-MA	B U CSF VH	RP LC-MS-APCI+ (SIM) Column: supersgher RP 18, (125 x 3 mm, 4µm) M.P: (50 mM ammonium formate pH3: ACN (90: 10)) FR: 0.6 mL/min	Bond Elut C18 SPE	86
MOR, M3G M6G, COD DIM 6-MA	S	Normal phase (NP) LC-MS-APCI+ (SIM) Column: Suplelcosil LC-Si (25 cm x 2.1 mm, 5 µm) M.P: (methanol: ACN: Water : formic acid (59.8: 5.2: 34.65: 35 respectively). FR: 0.230 mL/min	Ethyl SPE C2	178
M3G,M6G MOR,C6G COD, 6MA DHM, DHC BUP	B U CSF VH	RPLC - MS-APCI+ (SIM) Column: supersgher RP 18 (125 x 3 mm, 4 µm) M.P: (50 mM ammonium formate pH3: ACN (90: 10)) FR: 0.3 mL/min for M3G, DHM M 6G, and MOR and 0.6 mL/min for C6G, DHC, COD, and 6-MAM.	Bond Elut C18 SPE	86
MOR M3G M6G	P	NPLC-MS/MS- turbo ion spray (MRM ζ) Column: Inertsil silica (, 50 x 3 mm, 5µm) M.P: (Formic acid: water: ACN (1: 10: 90)) FR: 1.0 mL/min	Bond Elut C18 SPE	170
MOR,M3G M6G, COD C6G, 6-MA ,DHC6G, DHC, DHM BUP	B U	RPLC-MS- APCI+ (SIM) Column: Supersgher RP 18 (125 x 3 mm I.D., 4 µm) M.P: (50 mM ammonium formate pH3: ACN (90: 10) FR: 0.3 mL/min for M3G, DHM, M 6G, and MOR, 0.6 mL/min for C6G, DHC6G, DHC, COD and 6-MAM, and 0.4 mL/min for BUP.	Bond Elut C18 SPE	179

* Sam.: Sample types; B: Blood; U: Urine; P: Plasma; CSF: Cerebrospinal fluid ; VH: vitreous humour; * Ref: Reference.& RPLC-MS: Reverse phase liquid chromatography, NPLC: Normal Phase liquid chromatography; # MS: Mass spectroscopy; ** APCI: Atmospheric pressure chemical ionisation; #* ESI: Electrospry ionisation;&& SIM: Selected ion monitoring, *# MRM: Multiple reaction monitoring, ##+ : positive ion mode.☼ M.P: Mobile phase; *f* FR: Flow rate. §ACN: Acetonitrile.

Furthermore, LC-MS techniques provide sensitivity, robustness, flexibility, and save time [78,101,180]. Methods for determining morphine and its glucuronides have been developed using LC-MS with both API interfaces (Table 4-2.) Nishikawa *et al* [175] reported the first application for detection of MOR and M3G using LC-APCI-MS. Pacifici *et al* [176] developed the first method using LC-ESI-MS for serum samples. Tyrefors *et al* [171] and others developed several methods using LC-ESI-MS. Bogusz developed a series of reversed phase LC-APCI-MS methods for detecting morphine and its glucuronides [86,177,179,181]. Most of these methods were developed using reversed phase HPLC for determining morphine and its glucuronides [170,178]. Also, methods using normal phase have been developed [170,178].

SPE methods reported for sample preparation have frequently used a single cartridge or two cartridges in sequence as well as automatic 96-well SPE methods [182,183].

4.3 Codeine

4.3.1 Background

Codeine (Codeinum; Methylmorphine; Metilmorfina; Morphine Methyl Ether) or (5α, 6α)-7,8-didehydro-4,5-epoxy-3-methoxy-17-methylmorphinan-6-ol monohydrate) is a natural opioid obtained from opium. This naturally occuring opioid can also be prepared by methylation of morphine. Codeine was isolated for the first time in 1832 [1,132]. It has been prescribed widely for the relief of mild to moderate acute pain as an analgesic and antitussive agent [116,184-186] . Codeine is often combined with non-opioid painkillers such as acetaminophen and aspirin. Although codeine has been extensively used as a medication, illicit use of codeine has been reported [2,125,132,187].

4.3.2 Metabolism and Excretion

Codeine is metabolised in the liver to norcodeine via N-demethylation and to morphine via O-demethylation by the cytochrome P450 isoenzyme CYP2D6. Codeine is also conjugated to form codeine-6-glucuronide. Morphine and norcodeine in turn are metabolised to normorphine, M3G, M6G and

norcodeine glucuronide [1,132,141,186,188]. The metabolites found in urine three days after codeine administration are the same as those obtained when morphine or heroin is consumed [2]. Although the presence of norcodeine in urine is considered to be evidence of codeine use [2], norcodeine has never been detected without the presence of codeine [187] and so will not be present three days after consumption.

Codeine glucuronides, especially codeine-6-glucuronide, have attracted a great deal of interest for several reasons: firstly, codeine glucuronides have been found to constitute 50-80% of codeine excretion [126,186]. Secondly, the concentration of codeine glucuronides is 15 times greater than codeine itself [125,126,189]. It has been found that 10% of codeine is metabolised to morphine and codeine analgesia is attributed to morphine [124,126,136,190]. However, many people are deficient in the cytochrome P-450 enzyme that is necessary for metabolising codeine to morphine [125,136,190] and the analgesia in poor metabolisers following codeine administration is attributed to codeine itself or codeine-6-glucuronide [126,184,191]. Moreover, codeine-6-glucuronide (C6G) is thought to be an active metabolite [126,184,192], which gives C6G clinical advantages [186]. It has been suggested that C6G has the same properties as M6G and immunocompromised patients may use C6G to relieve their pain [193]. This hypothesis has been supported, C6G observed anti-conceptive effects in animal [193] and human experiments [194]; ultra-rapid, extensive, intermediate and poor metaboliser subjects produced CNS effects after codeine administration which strongly indicated that C6G is an active metabolite of codeine [126]. Biotransformation of codeine is illustrated in Figure 4-2.

4.3.3 Toxicity

Deaths attributed to codeine are rare, with estimated minimum lethal doses between 500-1000 mg [1,132]. Overdose of codeine leads to unconsciousness and convulsions, with death likely to happen as a result of respiratory failure within 2-4 hours [132]. Codeine intoxication often involves other toxic substances which enhance its adverse respiratory effects; codeine abusers may increase their dose up to 10 times the normal codeine dose without experiencing toxic effects.

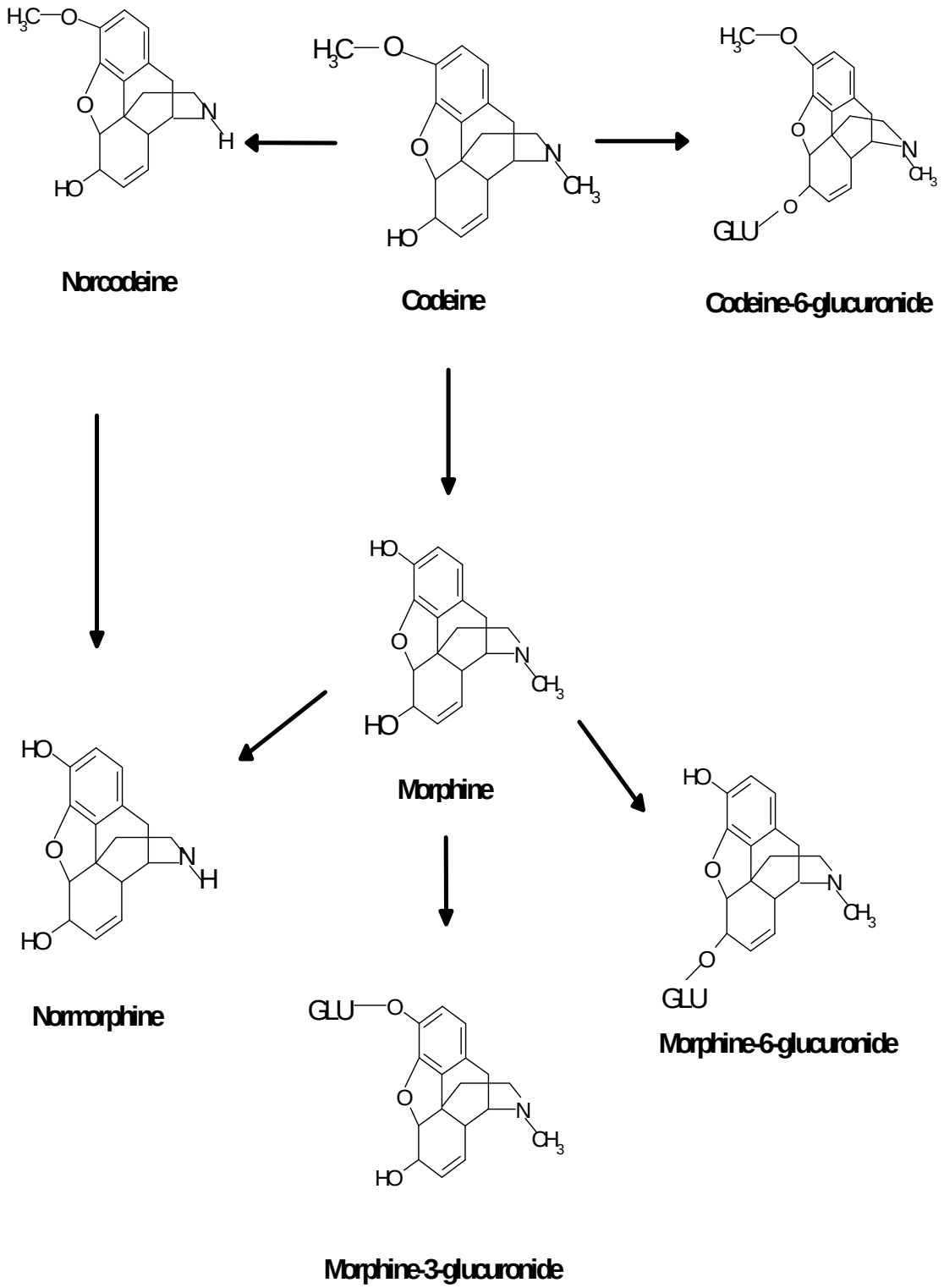


Figure 4-2: Codeine metabolism. (GLU: glucronic acid)

Blood codeine levels in eight overdose deaths ranged between 1.4-5.6 µg/mL [195]. Concentrations of codeine in post-mortem blood from two codeine fatalities was 15 µg/mL 48 hours after the consumption of a large amount of codeine tablets [196]. Also, blood codeine concentrations in 39 fatalities attributed to codeine ranged between 0.1-8.8 µg/mL [197].

Gerostamulos *et al* [198] studied the role of codeine in drug related deaths, finding that the average total codeine concentration in 6 deaths attributed solely to codeine was 4 µg/mL (range 2.1-8 µg/mL), free codeine was 1.3 µg/mL (range 0.4-2.8) in blood samples. The authors also presented an additional 101 deaths involving codeine but combined with other substances, total and free codeine was 1.8 µg/mL (range 0.04-26 µg/mL) and 0.82 µg/mL (range 0.02-9 µg/mL), respectively. However, codeine deaths are dependent upon the tolerance of users in which lethal levels can be detected with living subjects after codeine administration. In one report, blood codeine levels of 21.6 and 7 µg/mL were reported in two drivers under the influence of codeine [132].

Blood levels of active metabolites (morphine) were found to be within toxic levels as detected in morphine-related fatalities after heroin administration; the mean concentrations of morphine found in codeine overdoses deaths were in the range 0.2-0.3 µg/mL[197,199,200]. Blood morphine concentrations in deaths due to codeine ranged between 0.1-0.7 µg/mL [197]. Alcohol, paracetamol, diazepam and salicylate are the most common drugs accompanying codeine intoxication [1,198].

4.3.4 Previous work

Methods for the detection of codeine and its metabolites in biological fluids have been reported and used for forensic toxicology analysis. Radioimmunoassay techniques have been used for morphine and codeine screening, while GC-MS has also been used for confirmation [187,201-203]. HPLC methods have been employed successfully to determine codeine and its metabolites using two detectors, UV and electrochemical (ECD), as it has been found that using an ECD alone did not reach the selectivity obtained by using both UV and ECD detectors [204]. Also, HPLC methods with fluorescence detectors [186,205] and with ECD [184,206] were reported.

Few methods have been developed to detect codeine-6-glucuronide in biological fluids. Svensson *et al* [206] developed an HPLC method with a fluorescence detector to detect C6G in plasma and urine. LC-MS methods for detecting codeine and its glucuronides have been reported [86,159,179]. An LC-ESI-

MS/MS method for the determination of codeine and its glucuronides in human urine samples has been described [116].

4.4 Dihydrocodeine

4.4.1 Background

Dihydrocodeine (Dihydroneopine, Drocode, hydrocodeine, 6- α-hydrocodol, drocol, DHC-plus, Synalgos-DC) or (5α,6α)-4,5-epoxy-3-methoxy-17-methylmorphinan-6-ol is a semisynthetic analgesic opioid [1,132]. DHC was prepared for the first time in 1920 by hydrogenating the double bond between carbon atoms 7 and 8 in codeine [2]. It has been used for the relief of moderate to severe pain, and has also been commonly used as an antitussive and analgesic [207,208]. Since 1961, it has been widely used in some countries for the treatment of opiate addicts as an alternative to methadone [209,210]. In Germany, for instance, DHC has been used for treating heroin users since the late 1980s [129,179,208,209,211-213]. However, abuse of DHC has soared sharply in recent years [24,211,212,214]. Fatal intoxication has been reported due to polydrug use [215].

DHC is prescribed in the UK in two forms; oral and parenteral (50 mg/mL). DHC is less addictive than methadone which has led to an increase in its prescription by general practitioners (GPs) as a safe alternative to methadone and less monitoring is required since DHC is a class B drug [157]. DHC-related fatalities in the West of Scotland increased from 12 cases in 1995 to 42 cases in 1999; another drug was detected in most of these cases and all of the deceased were drug abusers with the exception of two [157]. However, no data is available on more recent DHC-related deaths.

4.4.2 Metabolism and Excretion

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) [132]. DHC is also sulphated at this position [132]. O-demethylation is responsible for metabolising DHC to its activate metabolite dihydromorphone (DHM) which is then conjugated with glucuronic acid to

form dihydromorphine 3- and 6-glucuronide. Nordihydrocodeine (NDHC) is formed by N-demethylation of DHC. Cytochrome P-450 enzyme CYP2D6 has been found responsible for O-demethylation of DHC. However, deficiency of this enzyme in about 7% of the Caucasian population and 50% of Chinese has been reported, and formation of dihydromorphone and its glucuronides is inhibited because of this. It has been stated that only 1-2% of dihydromorphine and its glucuronides are formed in poor metabolisers compared to 9% in extensive metabolisers and no differences have been noted between metabolism of DHC and nordihydrocodeine [207,213,216]. In conclusion, dihydromorphone and its glucuronide are found in small amounts in poor metabolisers, which in some cases cannot be detected. Biotransformation of DHC is shown in Figure 4-3.

4.4.3 Toxicity

DHC is often identified due to the presence in biological specimens of unchanged parent drug and metabolites dihydromorphine and nordihydrocodeine. Toxic concentrations reported were 0.8 µg/mL or higher [24,196,212,213] and therapeutic levels were suggested to be 0.03-0.25 µg/mL [217]. Symptoms of DHC toxicity involve dizziness, drowsiness, light-headedness, nausea and constipation. In severe exposure respiratory depression occurs followed by coma, convulsion, cardiovascular collapse and death [132]. In 54 living subject cases involving DHC the mean concentration was 0.7 µg/mL (range 0.1-3.3 µg/mL) [212]. Levels of free DHC in three fatalities ranged between 1.92-18.45 µg/mL [212]. In four overdose deaths attributed to DHC, the average blood DHC was 9 µg/mL (range 7.2-12 µg/mL) [218].

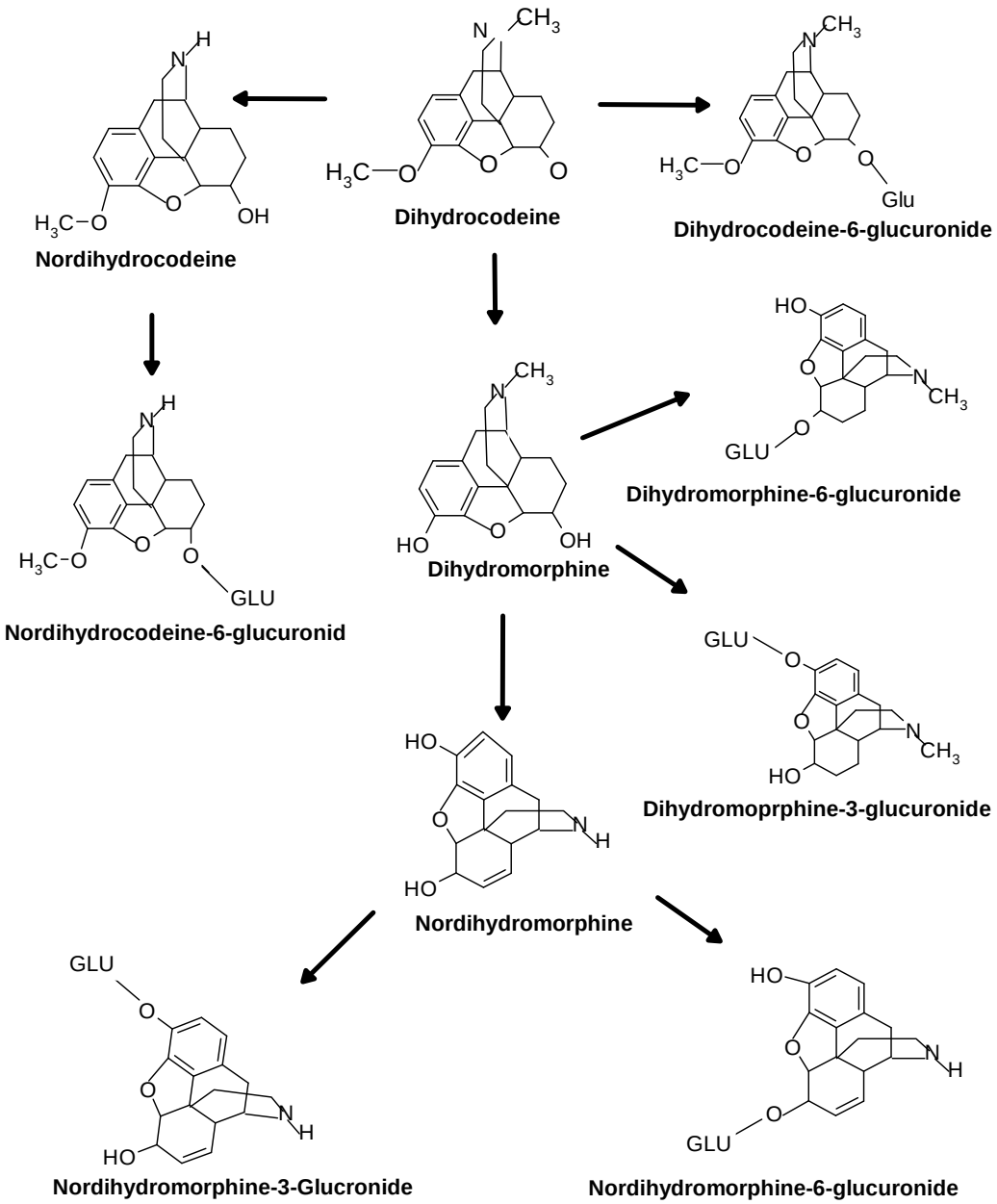


Figure 4-3: DHC metabolism. (Glu: Glucronic acid)

However, levels of DHC that cause death may be lower in polydrug intoxication. Levels found at autopsy overlapped between toxic and therapeutic levels due to the presence of other harmful substances while death can occur with concentrations below lethal levels. Deaths involving DHC can be classified in three categories [157,219]: DHC only intoxication, poly-drug intoxication involving DHC or 'DHC related' and cases in which DHC was found but deaths were not attributed to DHC intoxication.

Seymour *et al* [219] found that DHC levels in cases attributed to DHC alone were higher than in the other two categories. Blood DHC levels ranged between

0.9-19.9 µg/mL, 0.03-17.5 µg/mL and 0.01-1.6 µg/mL for deaths attributed to DHC alone, poly-drug intoxication and non-DHC related deaths, respectively. However, there was obvious overlapping between these three categories with median concentrations of 3.8 and 0.9 µg/mL for DHC alone and DHC-related categories, respectively. Skopp *et al* [24] reported that DHC levels of DHC alone cases (≤ 3 µg/mL) overlapped with those in living subjects. Seymour [157] found over half of cases (57%) positive for both DHC and morphine indicating that both heroin and DHC have been used at the same time which increases the risk of overdose.

4.4.4 Previous work

Methods for the detection of DHC have been reported using various techniques such as GC-FID [196], GC-MS [201,208,211], HPLC-fluorescence detector [24,212] and LC-MS(/MS) . DHC and its active metabolites in serum using negative –ion chemical ionisation GC-MS/MS was reported [210]. Kirkwood *et al* [207] described an HPLC method with UV detector for determining DHC , DHM, and norDHC in human liver microsomal incubations. HPLC equipped with fluorescence detector has been reported in two studies for the detection of DHC, DHM, NDHC, DHC6G, DHM3G and DHM-6-G in blood and serum samples after fatal overdose. C8 Bond Elut SPE was applied for extraction of samples [24,212]. Bogusz [179] described a method for the detection of DHC and its metabolites in biological samples based on SPE (Bond Elut C-18) followed by LC-APCI-MS. Applications using electrophoresis for the determination of DHC, DHC6G and DHM have been reported [220-222].

4.5 Buprenorphine

A thorough review of buprenorphine can be found in Chapter 7 and will not be repeated here.

4.6 Hydromorphone

4.6.1 Background

Hydromorphone (dihydromorphinone; dilaudid and dimorphone) or 4,5-epoxy-3-hydroxy-17-methylmorphinan-6-one is a potent semisynthetic opioid agonist and strong analgesic [1,132]. It has been used to relieve moderate to severe pain such as cancer pain and postoperative pain and has been used as an alternative to morphine [132,223-226]. It has been used to avoid some of the adverse effects of morphine due to its water solubility which is estimated to be five times higher than that of morphine sulphate [227], making hydromorphone the best choice when concentrated doses and small amounts have to be taken, such as in subcutaneous infusion. For this reason, hydromorphone has become an attractive drug for the relief of chronic cancer pain [225,228-230].

Hydromorphone was synthesised in Germany in 1921, and has been used clinically since 1926 [223]. It has become a drug of abuse due to its tendency to induce dependence. Furthermore, although the structure of hydromorphone is very similar to that of morphine, slight differences in its chemical structure have a considerable affect on its metabolism pathway. There are two differences: the presence of a 6-keto group, and the hydrogenation of the double bound at the C7-8 position of the hydromorphine molecule [231].

4.6.2 Metabolism and Excretion

Hydromorphone is metabolised into hydromorphone-3-glucuronide, conjugated dihydromorphine, unconjugated dihydroisomorphine, hydromorphine-3-sulfate, norhydromorphine, and nordihydroisomorphine [132,223]. Chemical structures of hydromorphone and its metabolites are shown in Figure 4-4. The existence of hydromorphone-6-glucurnide (H6G) has continually been brought into question. It has been thought that H6G is formed as a hydromorphine metabolite [232]. However, there was confusion in distinguishing between H6G, DH-6 G and DHI-6-G, and the presence of H6G has been assumed in the study by Cone *et al* [232]. Theoretically, H6G can be

formed by altering the 6-keto group on the hydromorphone molecule to the enol form, and then another glucuronidation process will form H6G. However, H6G is not likely to be formed in hydromorphone detoxification , and instead of forming H6G, hydromorphone is metabolised to H3G and dihydroismorphine [223,231].

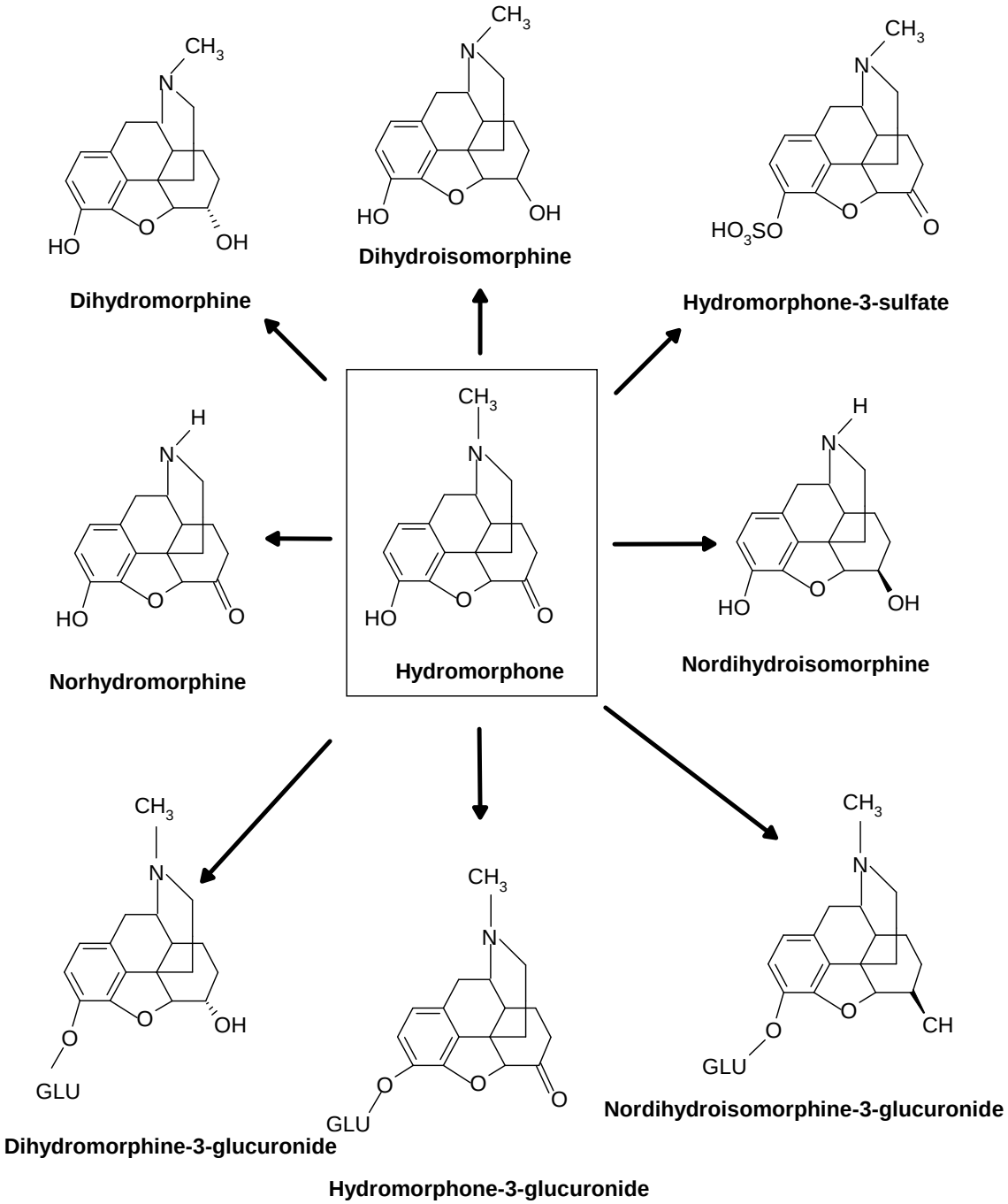


Figure 4-4: Chemical structures of hydromorphone and its metabolites.

4.6.3 Previous work

Many methods have been developed to detect hydromorphone and its metabolites in plasma using GC-MS [232] and HPLC with electrochemical [233] and UV detectors [234]. The use of LC-MS/MS for the determination of hydromorphone and its metabolites in biological fluid has also been reported [224-226,235].

4.7 Oxycodone

A thorough review of oxycodone can be found in Chapter 8 and will not be repeated here.

4.8 Naloxone

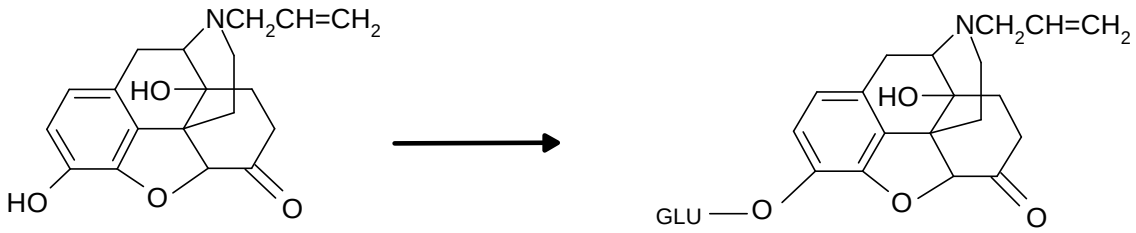
4.8.1 Background

Naloxone (N-allylnoroxymorphone, Narcan) or (5α)-4,5-epoxy-3,14-dihydroxy-17-(2-propenyl) morphinan-6-one is a synthetic opioid antagonist [1,132]. It has been used since 1962 for the treatment of opioid overdoses and cardiovascular depression after surgery [236]. It has also been shown to raise the blood pressure in patients with shock and has a mild diuretic effect in patients with liver cirrhosis [237]. Also, naloxone binds non-selectively to opioid receptors; it has been used in the short term to reverse opioid overdose effects [126]. Naloxone is the drug of choice for the treatment of opioid overdose and appears to be safe. Its use is well established in treating narcotic-induced respiratory depression due to intoxication by opiates such as morphine, codeine, oxymorphone and hydromorphone [238]. In addition, there is an increase in the use of combinations of buprenorphine and naloxone for maintenance therapy. This was found to minimise the abuse and misuse of buprenorphine and this combination was found to be safe and offered an alternative therapy to the use of methadone or buprenorphine in addiction programme maintenance [239]. Also, a combination of oxycodone and naloxone as a prolonged release tablet for the treatment of chronic pain was found to be safe and efficient [240,241].

An increase in cases of heroin overdose has been observed in recent years; however, the danger of heroin overdose can be prevented if naloxone is injected within a certain suitable time and the distribution of naloxone to heroin users and training of heroin users to inject themselves has been piloted both in Glasgow [242] and elsewhere [243,244]. The detection of naloxone in biological fluids has not gained attention until now and, due to the use of naloxone with combinations of other opioids for pain management, it would be found with cases at autopsy. In the present work there were two cases found due to the fact that if naloxone was used following opioid overdose, the user will survive and if not the samples from the deceased will be negative. Also, short half-life of naloxone could be another factor causing it to be missed at analysis. Chemical structures of naloxone and its glucuronide are shown in Figure 4-5.

4.8.2 Metabolism and Excretion

Naloxone is metabolised by N-dealkylation, O-glucuronidation and reduction of the 6-keto group to produce naloxone-3-glucuronide, nornaloxone (noroxymorphine) and naloxol [132]. Naloxone-3-glucuronide is the major metabolite of naloxone which can be formed in the human brain and may contribute to the short duration of action of naloxone when it reaches the brain [126]. Naloxone has a short plasma half life (30 minutes) in which respiratory depression caused by long-acting opioids (methadone, heroin and morphine) may return after the effect of naloxone has ended. Therefore, monitoring of the patient is necessary after recovery when starting naloxone intravenous infusion. High dose or rapid infusion of naloxone may result in catecholamine release and consequently pulmonary oedma and cardiac arrhythmias [238,239].



Naloxone **Naloxone-3-glucuronide**

Figure 4-5: Chemical structures of Naloxone and Naloxone-3-glucuronide, (GLU: Glucuronic acid).

4.8.3 Previous work

A few methods have been reported for the detection of naloxone in biofluids using different techniques such as radioimmunoassay, gas-liquid chromatography [245] and HPLC with electrochemical [236] or UV detectors [246]. Until recently, no method has been described for detecting naloxone glucuronide [241].

5 Method for Quantification of Opioids and their Metabolites in Autopsy Blood by Liquid Chromatography-Tandem Mass Spectrometry.

5.1 Introduction

Until recently, opioids and their metabolites were detected in biological specimens as free (unconjugated) substances or as the total concentration of the drug following cleavage of the conjugates, particularly those formed with glucuronic acid. Acid and enzyme hydrolysis procedures have been reported, although several have been found to give inaccurate results, to be time consuming and to have limitations, including incomplete hydrolysis and introduction of interferences in the analysis [76,247]. Also, this approach does not allow different phase II metabolites to be distinguished and measured individually [159].

Success has been achieved in recent years in the detection of low concentrations of morphine glucuronides in biological specimens using HPLC [80,81,83,173,174,248] and LC/MS/(MS) techniques [75,86,116,170,171,175,176,178,179,183]. The latter has become the technique of choice to provide good selectivity and accurate identification and quantification of analytes of interest. Another advantage of LC-MS/MS lies in its ability to determine ionic, polar, thermally labile, high molecular weight and other non-volatile substances without derivatisation. LC-MS/MS methods are also characterised by excellent sensitivity, robustness, flexibility and efficient sample preparation [41,78,180]. These advantages permit the simultaneous determination of a variety of drugs of interest [112,142,249]. LC-MS/MS has also been considered as an alternative to immunoassay screening which saves time, and reduces the cost of the analysis [32].

5.2 Aims

Although methods for determining morphine and its glucuronides have been described [75,86,116,170,171,175,176,178,179,183], few methods have been developed for other opioid glucuronides. The primary aim of the present study was to develop a reliable, sensitive, and selective method based on LC-MS/MS for the identification and quantification in human whole blood of the opioids and metabolites which are most commonly encountered in post mortem toxicology. The second aim was to employ this method in the routine toxicology laboratory for opioid-related fatalities and to evaluate its usefulness in interpreting the cause of death, in determining the type of death (rapid, sub-acute death, or delayed death) and in distinguishing between heroin, morphine and codeine users.

5.3 Method and Materials

5.3.1 Reagents and Standards

Methanol and acetonitrile (HPLC grade) were obtained from BDH (Poole, UK). Ammonium carbonate, formic acid and ammonium hydroxide were also purchased from BDH. Ammonium formate was obtained from Acros Organics (New Jersey, USA). The method was developed using human blank blood obtained from West infirmary Hospital, Glasgow.

Morphine (MOR), morphine-D3 (MOR-D3), 6-monoacetylmorphine (6-MAM), 6-monoacetylmorphine-D3 (6-MAM-D3), codeine (COD), codeine-D6 (COD-D6), 6-acetylcodeine (6-AC), hydromorphine (HMOR), hydromorphine-D3 (HMOR-D3), dihydrocodeine (DHC), dihydrocodeine-D6 (DHC-D6), buprenorphine (BUP), buprenorphine-D4 (BUP-D4), norbuprenorphine (NBUP), norbuprenorphine-D3 9NBUP-D3), naloxone (NAL), oxymorphone (OXYM) and oxymorphone-D3 (OXYM-D3) were obtained from Promochem (Middlesex, UK). Morphine-3-glucuronide (M3G), morphine-3-glucuronide-D3 (M3G-D3), morphine-6-glucuronide (M6G), morphine-6-glucuronide-D3 (M6G-D3), normorphine (NORM), codeine-6-glucuronide (C6G), codeine-6-glucuronide-D3 (C6G-D3), norcodeine (NCOD), dihydrocodeine-6-glucuronide (DHC6G), dihydromorphine

(DHM), dihydromorphine-3-glucuronide (DHM3G), dihydromorphine-6-glucuronide (DHM6G) and naloxone-3-glucuronide (NAL3G) were purchased from Lipomed (Arlesheim, Switzerland). All standards and internal standards were obtained as solutions in methanol at a concentration of 0.1 mg/mL or 1 mg/mL and each had a purity of more than 99%. Bond Elut LRC-C18 cartridges were purchased from Varian (CA, USA).

Individual working standards were prepared at a concentration of 1 µg/mL by dilution of the stock solutions. Working mixtures of standards and internal standards were similarly prepared.

The method was developed using packed human red blood cells, which had passed their usable date, obtained from the Scottish National Blood Transfusion Service and re-suspended in an equal volume of isotonic saline.

5.3.1.1 Preparation of 0.01M Ammonium carbonate buffer (pH 9.3)

1.571 g of ammonium carbonate was weighed and added to a l L volumetric flask and 800 mL of DI H_2O was added. The pH was then adjusted to 9.3 using concentrated ammonium hydroxide. The volume was made up to 1 L using deionised water. The buffer was stored at 4 ^{0}C until used and unused solution was discarded after 4 weeks from the date of preparation.

5.3.1.2 Preparation of 0.01 M Ammonium Formate (pH 3 or 4.5)

0.630 g of ammonium formate was added to a 1 L volumetric flask and 800 mL of DI H_2O was added. The pH was adjusted to 3 or 4.5 using concentrated formic acid. Then volume was made up to 1 L with deionised water. Ammonium formate buffer was prepared freshly and stored at room temperature until used; unused solution was discarded after 4 weeks from the date of preparation.

5.3.2 Solid Phase Extraction

One millilitre of whole blood was added to 3 mL of 0.01 M ammonium carbonate, pH 9.3 and 100 µl of the internal standard working solution was

added. The mixture was vortex mixed, allowed to equilibrate for 10 minutes and then centrifuged for 10 minutes at 2500 rpm. The supernatant was applied to a Bond Elut C18 SPE cartridge preconditioned with 3 mL methanol, 3 mL of deionised water, and 3 mL of 0.01 M ammonium carbonate (pH 9.3). The SPE cartridge was washed twice with 3 mL 0.01 M ammonium carbonate (pH 9.3), and then dried for 10 minutes. Retained drugs were eluted with 3 mL methanol, after that, the eluate was evaporated to dryness under nitrogen at 50 ^{o}C. The extract was reconstituted with 150 µl of initial mobile phase and the tube was centrifuged for 10 minutes at 4000 rpm before transferring the supernatant into an autosampler vial. 20 µl were injected into the LC-MS/MS instrument.

5.3.3 Chromatography conditions

Chromatographic separation was achieved using a Synergy Polar RP column (150 x 2.0 mm, 4-µm particle size), protected by a guard column with identical packing material (4 x 2.0 mm, Phenomenex, Torrance, CA). Gradient elution was based on a mobile phase consisting of 10 mM ammonium formate adjusted to pH 3 (A) and acetonitrile (B) at a flow rate of 0.3 mL/min in the first 8 min, decreasing to 0.2 mL/min at 13 min for the next 13 min. After that, the initial flow rate was applied until the end of analysis. The gradient conditions were initially 97% of solution A for 3 min; decreasing to 84.5% at 8 min, to 74% at 13 min and to 20% at 26 min, 5% of solution A was maintained for the next 3 min before returning to 97% for 7 min prior to the next injection.

5.3.4 Instrumentation

Analysis of opiates and their metabolites was performed using a Thermo Finnigan LCQ DECA XP Plus ion trap instrument (Thermo Finnigan, San Jose, USA) equipped with a surveyor LC system interface.

Ionisation of analytes of interest was carried out using electrospray positive ion mode. The capillary temperature, sheath gas flow rate, auxiliary gas flow rate and collision energies were optimized for each analyte separately. All internal standards were analysed using selected ion monitoring mode (SIM),

Table 5-1: LC-MS/MS Parameters for Opiates and their Metabolites

Analyte	Internal standard	Precursor ion (m/z)	Sheath Gas (AU)	Auxiliary Gas (AU)	Capillary Temp. (°C)	CID (%)	Product ion (s)	RT [#] (min)	Injection (segment) no.
MOR	**MOR-D6**	286	30	0	225	35	201, 228	7.99	2 (2)
M3G	**MOR-D6**	462	20	10	270	28	286	3.67	1 (1)
M6G	**MOR-D6**	462	20	115	270	30	286	6.83	1 (2)
6-MAM	**MAM-D3**	328	25	0	300	35	211, 268	14.02	1 (3)
NMOR	**MOR-D6**	272	25	10	270	31	254, 229	4.97	2 (1)
COD	**COD-D6**	300	20	0	270	35	215, 243	12.47	2 (3)
C6G	**C6G-D3**	476	25	10	285	30	300	11.17	1 (3)
NCOD	**MOR-D6**	286	30	5	270	35	268, 243	11.19	1 (3)
6-AC	**MOR-D6**	342	20	15	290	35	225, 225	18.44	2 (4)
HMOR	**HMOR-D3**	286	25	0	280	35	185, 229	10.25	1 (3)
H3G	**HMOR-D3**	462	30	10	290	30	286	5.01	1 (1)
DHC	**DHC-D6**	302	25	10	270	35	245, 201	11.90	2 (3)
DHC6G	**DHC-D6**	478	25	5	270	30	302	11.05	2 (3)
DHM	**MOR-D6**	288	25	5	270	35	213, 231	6.99	2 (2)
DHM3G	**MOR-D6**	464	20	15	290	28	288	3.36	2 (1)
DHM6G	**MOR-D6**	464	15	15	290	30	288	6.98	2 (2)
BUP	**BUP-D4**	468	33	10	270	36	414, 396	22.49	1 (5)
BUP3G	**BUP-D4**	644	30	0	295	30	644	18.30	1 (4)
NBUP	**NBUP-D3**	414	30	0	300	31	396, 340	19.40	1 (4)
NBUP3G	**NBUP-D3**	590	30	0	280	30	414	14.23	2 (3)
NAL	**MOR-D6**	328	20	0	300	30	310	12.34	1 (3)
NAL3G	**MOR-D6**	504	20	0	290	30	486, 328	8.04	1 (2)
OXY	**OXY-D6**	316	25	10	250	30	298	13.75	1 (3)
OXYM	**OXYM-D3**	302	5	5	250	25	284	9.26	2 (2)

[#] Retention time (minutes).

whereas analytes were identified and quantified based on their retention times and product ion full scan spectra, except buprenorphine-3-glucuronide, which was analysed in the SIM mode. The spray voltage used was 5 kV. The MS/MS parameters are detailed in Table 5-1.

5.3.5 Method Validation

5.3.5.1 Linearity

Analytes of interest were added to human whole blood to obtain eight concentrations ranging from 5-400 ng/mL. Internal standards were added at 100 ng/mL. The blood samples were extracted by SPE as described earlier and analysed by LC-MS/MS. Peak area ratios of analytes of interest to their internal standards were calculated (see Table 5-1). Calibration curves were obtained by plotting peak area ratios against concentration. The correlation coefficient (r^2) was obtained for each linear regression curve.

5.3.5.2 Recovery

The recoveries of the analytes by SPE were calculated by comparing the peak area ratios of analytes extracted from blood at three concentrations (10, 50 and 200 ng/mL, n=5) with those obtained with unextracted standards at corresponding amounts. Internal standards were added after the blood samples were extracted by SPE.

5.3.5.3 Limit of Detection and Lower Limit of Quantitation

The Limit of detection (LOD) and the Lower Limit of Quantitation (LLOQ) for each analyte were calculated by lowering the concentrations of calibration standards in blood in the range of expected LODs. Calibration curves were obtained for all analytes at five concentrations ranging from 0.1- 10 ng/mL. LODs and LLOQs for all analytes were obtained using equations 2-1, 2-2 and 2-3, 2-4, respectively.

5.3.5.4 Intra-assay precision

Intra-assay precision was determined by analysing replicate human whole blood samples (n=5) containing analytes of interest at three concentrations (10, 50, and 200 ng/mL) in the same day. Concentrations of the analytes were obtained using calibration curves prepared with standards at 5, 10, 20, 25, 50, 100, 200 and 400 ng/mL as well as blanks.

5.3.5.5 Inter-assay precision

The inter-assay precision was measured in a similar manner to the intra-assay precision at three concentrations over the linear dynamic range (10, 50, and 200 ng/mL) on five different days.

5.3.5.6 Matrix effects

The effect of co-extracted human whole blood matrix components on opioid ionisation in LC-MS/MS was assessed using the procedure of Matuszewsk *et al* [51]. In brief, the peak area ratios of unextracted standards (n=5) prepared in mobile phase were compared with those of 200 ng/mL standards (n=3) extracted from five different blank human whole blood sources. Matrix effects for each analyte were calculated using equation 3-1, refer to section 3.5.

5.3.5.7 Stability

Stability was assessed using human whole blood spiked with the analytes of interest at 100 ng/mL (n=3). Short-term temperature stability at room temperature was investigated for human whole blood stored for 4 and 24 hrs. Freeze-thaw stability of analytes of interest was determined after four cycles (thawed, left at room temperature for 3 hrs then refrozen) on consecutive days. Auto-sampler stability using reconstituted extracted sample was determined at 48 hrs and at one week after extraction. Long-term stability for analytes of interest at -20 and 4 ^{o}C for period of 24hrs, 48hrs, 1 week and 1 month were investigated. Calibration curves were prepared for each batch samples using standards in whole blood at 5, 10, 20, 25, 50, 100, 200 and 250 ng/mL plus blanks. The storage temperatures were monitored daily using

permanently mounted digital thermometers with a resolution of 0.1 C°at the start of the study and subsequently at weekly intervals during the period of study. Temperatures were stable within a range of approximately 1 °C (freezer and refrigerator) or 5 °C (room temperature, average temperature 20 °C).

5.3.6 Case Materials

Samples of autopsy blood were analysed using the proposed method as part of the investigation of medico-legal cases submitted to Forensic Medicine and Science, University of Glasgow which were drug-related deaths. Analyses were repeated after dilution of the blood sample when analyte concentrations outside the calibration range were obtained. A comparison of the results obtained by this method with those obtained by the established methods of the Forensic Medicine and Science Section laboratory is given in Table 9-10, 9-11 and 9-13 and these are discussed in Chapter 9.

5.4 Results and Discussion

5.4.1 Solid Phase Extraction

The SPE extraction efficiencies for all analytes were between 80.3% and 101.4% which was acceptable taking into account the large number of analytes in this method. Recoveries of opioid metabolites included in the current work are detailed in Table 5-2. Bond Elut C18 columns were selected after comparing them with Bond Elut Certify (Varian, CA, USA), and Strata X-C (Phenomenex, Torrance, CA) columns. Bond Elut Certify was excluded due to significant losses of glucuronides during extraction. However, parent drugs were retained with good recoveries. A method involving Strata X-C columns, published by Murphy and Huestis [116] for urine as the matrix, was evaluated for use with human whole blood. However, the extracts obtained had many co-eluting interferences, with the potential to cause column contamination and significant matrix effects and so Strata X-C was also excluded. Methods using Bond Elut C18 for the extraction of opioid glucuronides have already been published [170,172,177,179,250] and the method developed in the present study,

giving clean extracts with excellent recoveries, was based on the procedures of Bugusz [179] and Leis *et al* [172].

5.4.2 Chromatography

Good chromatography of all analytes was obtained using a gradient starting with a high percentage of aqueous buffer (97% 10 mM ammonium formate). Different concentrations of ammonium formate between 1-10 mM at pH 3 were not found to have significant effects but a slight shift in retention times was observed at pH 4.

Table 5-2: Extraction recovery from human whole blood

Analytes	Nominal Concentration		
	10 (ng/mL)	**50 (ng/mL)**	**200 (ng/mL)**
	Mean Recovery % [#] (R.S.D of recovery [*])		
MOR	97.9 (3.4)	99.2 (5.5)	100.3 (6.1)
M3G	96.2 (4.5)	99.5 (3.1)	99.7 (3.8)
M6G	86.0 (1.4)	90.6 (4.7)	90.5 (2.8)
6-MAM	91.2 (4.2)	93.6 (7.4)	96.0 (3.6)
NMOR	91.6 (7.7)	96.1 (7.3)	99.6 (2.4)
COD	94.6 (2.8)	101.8 (4.4)	101.4 (3.6)
C6G	98.2 (4.8)	99.8 (4.2)	100.0 (4.9)
NCOD	90.3 (4.9)	94.6 (3.4)	97.4 (2.9)
6-AC	88.0 (7.7)	89.6 (5.9)	93.0 (3.0)
HMOR	90.3 (4.2)	96.2 (4.1)	97.0 (3.2)
H3G	92.8 (3.5)	95.6 (3.5)	100.0 (3.0)
DHC	90.4 (2.6)	93.0 (4.5)	99.3 (3.4)
DHC6G	91.7 (3.7)	94.6 (3.5)	96.8 (7.2)
DHM	80.7 (4.5)	83.3 (7.2)	84.4 (5.4)
DHM3G	80.9 (11.5)	84.0 (2.7)	84.9 (3.7)
DHM6G	80.3 (11.2)	89.5 (0.1)	87.7 (4.8)
BUP	82.0 (8.7)	85.8 (5.8)	94.5 (5.2)
BUP3G	91.0 (3.8)	92.7 (6.1)	98.0 (3.9)
NBUP	84.6 (6.2)	92.7 (5.1)	98.0 (4.3)
NBUP3G	81.8 (2.4)	86.0 (4.7)	98.1 (1.8)
NALOX	90.3 (6.5)	92.4 (6.3)	98.7 (4.2)
NALOX3G	84.3 (3.4)	98.7 (2.6)	100.1 (2.9)
OXY	89.7 (4.5)	92.0 (4.5)	99.5 (3.8)
OXYM	97.9 (9.6)	98.4 (4.0)	99.1 (2.7)

[#] The mean percentages for the replicate analysis (n=5).
[*] R.S.D: Relative standard deviations.

5.4.3 LC-MS/MS

LC-MS/MS has the capability of analysing a variety of drugs with the same molecular weight and polarity, although overlapping peaks can decrease the number of available data points for each analyte across the peaks with an adverse effect on precision [112]. In the present method, several analytes of similar polarity were shown to co-elute, including. M3G/DHM3G and MOR/NAL3G. As a result, two HPLC runs were used with two sets of MS/MS parameters to avoid more than 5 scan events per retention time window, in order to achieve good repeatability between injections. Mass Retention windows and their scan events are shown in Table 5-1.

Deuterated internal standards were used when possible although some are not yet commercially available (NMOR, NCOD, 6-AC, H3G, DHC6G, DHM, DHM3G, DHM6G, B3G, NBUP3G, NAL and NAL3G). However, analyte co-elution with simultaneous mass spectral pattern overlaps occurred between M3G-D3/DHM3G, M6G-D3/DHM6G, MOR-D3/DHM and COD-D3/DHC. The problem was difficult to solve, as the differences in molecular weights of analytes and isotopically-labelled internal standards is just 1 amu and the mass window of the ion trap is also ± 1, resulting in a degree of cross-talk. Analytes were still able to be measured and identified in the presence of partial overlap but it was found preferable to replace these deuterated internal standards with alternative deuterated analogues with similar structure. The problems disappeared when M3G-D3, M6G-D3, and MOR-D3 were replaced by MOR-D6 and COD-D3 was replaced with COD-D6. The use of individual internal standards for each drug is recommended but the use of analogues that give a linear response between analytes of interest and peak area ratio is acceptable. Some of these problems might be solved using a triple quadrupole or Q-TOF instrument.

Mass spectral data for all analytes were obtained in the electrospray positive ion mode. The product ion of all opiate glucuronides resulted from loss of the glucuronic acid moiety (m/z 176) from the protonated molecular ion. It was observed that there was no advantage, with the instrument used in this study, in performing selected reaction monitoring (SRM) rather than full MS/MS scanning and the latter was chosen for identification and quantitation of all

analytes with the exception of B3G, due to the variable peak area ratios obtained for its main product ion at m/z 468. Optimisation of the mobile phase flow rate and composition did not solve this problem. Moody *et al* [251] reported the same observation when using a triple quadrupole instrument. The BUP product ion abundance was relatively constant at low collision energies (CE) up to -20eV then dropped sharply using CE above -30 eV. Product ion intensities were low at low CE values and could not be used for quantitation. Increasing the CE above -40eV formed many low intensity product ions and as a result the protonated molecular ion at m/z 468 was used for quantitation. Reproducible fragmentation was obtained even at the LOD concentration of 0.1 ng/mL. The same approach was used in the present study and B3G was determined in the SIM mode using the protonated molecular ion at m/z 644, which gave acceptable linearity over the concentration range 5-400 ng/mL.

5.4.4 Method Validation

5.4.4.1 Linearity

The concentration range for calibration curves was chosen on the basis of previous work [75,86,112,212,252]. All linear regression lines had r^2 values greater than 0.999 which is very satisfactory.

5.4.4.2 LOD and LLOQ

LODs and LLOQs obtained for the analytes compared well with previously published work, and were in the ranges 0.16 - 1.2 ng/mL and 0.5 - 4.09 ng/mL, respectively (Table 5-3). The calculation of LOD and LLOQ has been carried out by different methods but in this work they were obtained using Equations 2-1, 2-2 and 2-3, 2-4, respectively [62,64,112,253,254]. LODs and LLOQs of opioids and metabolites are listed in Table 5-3 and chromatograms of all analytes at 3 ng/mL are shown in Figure 5-1 and 5-2.

Table 5-3: LODs and LLOQs of opioids extracted from human whole blood.

Analytes	r^2 #	y-intercept	Standard error	Gradient (m)	LOD * (ng/mL)	LLOQ & (ng/mL)
MOR	0.9998	-2.9E-05	9.96E-05	0.001422	0.20	0.70
M3G	0.9995	-2.5E-05	0.000138	0.001487	0.28	0.93
M6G	0.9998	1.89E-06	9.75E-05	0.001461	0.20	0.67
6-MAM	0.9999	-8.8E-06	9.04E-05	0.001743	0.16	0.50
NMOR	0.9997	-0.00012	9.13E-05	0.000402	0.68	2.27
COD	0.9994	-4.5E-05	0.000161	0.001481	0.32	1.08
C6G	0.9977	-0.00102	0.000861	0.00402	0.38	2.10
NCOD	0.9994	-0.00024	0.000302	0.002706	0.33	1.10
6-AC	0.9995	-0.00209	0.002612	0.029658	0.26	0.88
HMOR	0.9997	-2.1E-05	0.000127	0.001654	0.23	0.77
H3G	0.9995	-7.7E-05	0.000218	0.002128	0.30	1.02
DHC	0.9995	3.74E-05	0.000193	0.001839	0.3	1.05
DHC6G	0.9997	-5.4E-05	0.000233	0.000961	0.7	2.42
DHM	0.9992	-0.00023	0.000305	0.002897	0.3	1.05
DHM3G	0.9992	-0.00081	0.000716	0.006751	0.3	1.06
DHM6G	0.9996	-0.00052	0.000683	0.002747	0.7	2.50
BUP	0.9998	-0.00022	0.000199	0.001084	0.55	1.84
BUP3G	0.9992	-4E-05	0.000687	0.005353	0.39	1.28
NBUP	0.9998	-0.00019	0.000339	0.00083	1.2	4.09
NBUP3G	0.9987	-1.4E-05	2.05E-05	0.000124	0.49	1.65
NALOX	0.9999	-0.00093	0.000663	0.005038	0.39	1.32
NALOX3G	0.9996	0.00027	5.73E-05	0.000677	0.25	0.85
OXY	0.9984	-0.01363	0.006931	0.035863	0.58	1.93
OXYM	0.9993	-0.00011	0.000757	0.007702	0.30	0.98

r^2: Correlation coefficients.
* LOD: Limit of detection;
& LLOQ: Lower limit of quantitation;

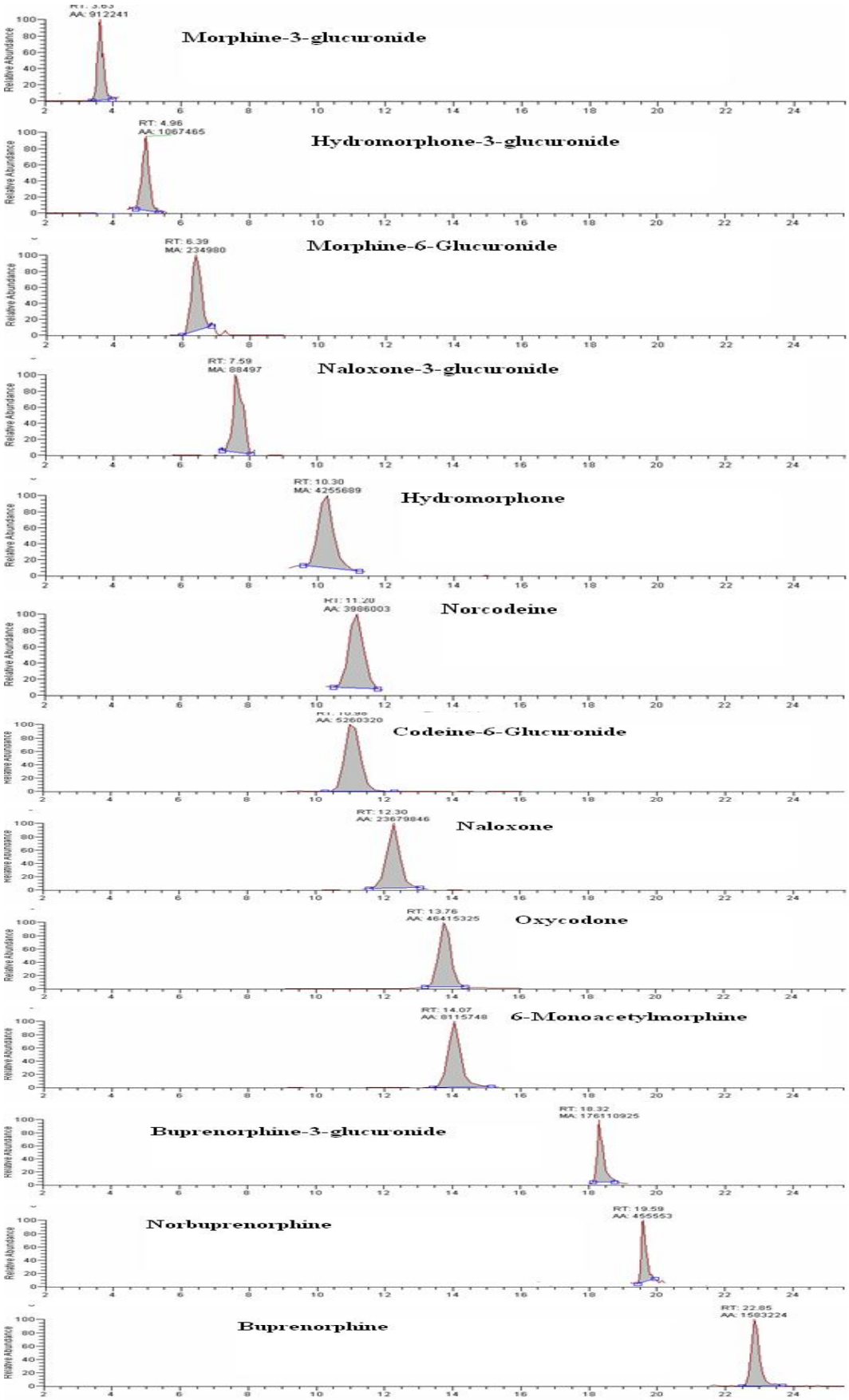


Figure 5-1: LC-MS/MS product ion chromatograms for opioids in blood (concentration 3 ng/ml) detected during the first injection.

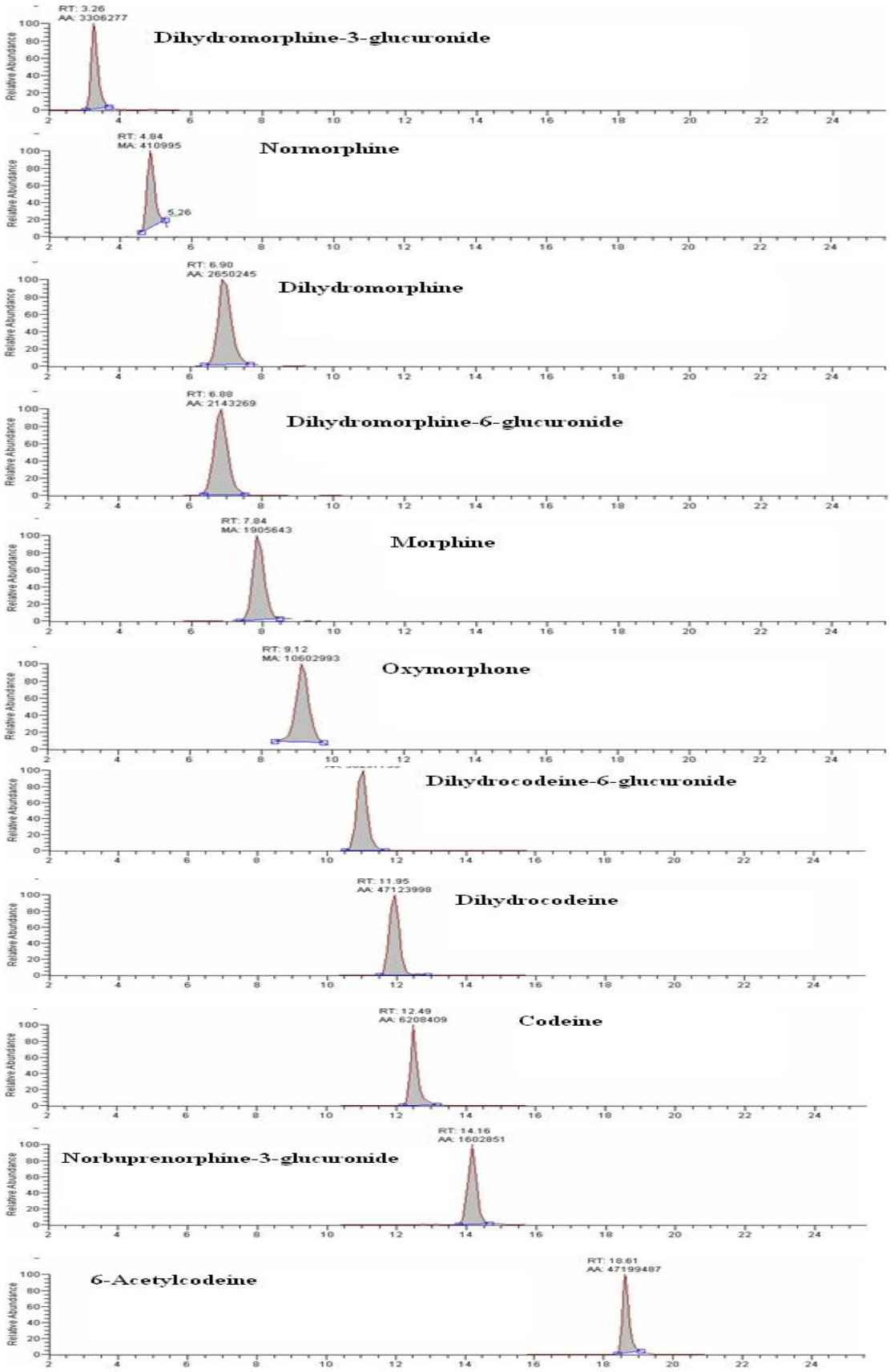


Figure 5-2: LC-MS/MS product ion chromatograms for opioids in blood (concentration 3 ng/ml) detected during the second injection.

5.4.4.3 Method precision

Intra- and inter-assay precision were assessed as the percentage relative standard deviation (RSD) on analyses of standards at three different concentrations (10, 50, and 200 ng/mL). Good precision was obtained for all analytes in the range 0.6 to 13.8 % RSD (Tables 5-4, 5-5).

Table 5-4: Intra-day precision

	Nominal Concentrations (ng/mL)			
	3.0	**10.0**	**50.0**	**200.0**
Analytes	**Measured concentration ng/mL # (R.S.D % *)**			
MOR	2.8 (8.9)	11.3 (7.2)	49.3 (6.0)	197.9 (4.2)
M3G	3.1 (11.5)	11.5 (4.9)	51.2 (9.0)	206.3 (3.9)
M6G	3.5 (14.4)	11.3 (8.8)	52.2 (10.0)	199.6 (4.4)
6-MAM	2.7 (10.7)	11.0 (8)	52.7 (5.9)	199.0 (3.3)
NMOR	3.1 (10.4)	10.3 (6.9)	46.4 (6.2)	197.0 (5)
COD	2.7 (15.6)	9.6 (5.6)	47.8 (4.5)	197.0 (3.1)
C6G	3.1 (8.9)	10.3 (12.1)	50.9 (13.0)	198.5 (4.4)
NCOD	3.4 (9.2)	9.5 (13.4)	53.1 (4.0)	204.7 (5.6)
6-AC	3.5 (7.5)	11.7 (9)	53.1 (1.0)	205.5 (3.3)
HMOR	3.0 (10.0)	9.6 (11.3)	43.6 (3.0)	189.3 (3.6)
H3G	3.4 (6.6)	11.0 (7)	50.0 (4.4)	197.0 (4.7)
DHC	2.9 (13.0)	9.5 (11.6)	51.2 (3.6)	195.5 (3.3)
DHC6G	2.7 (15.3)	11.4 (13.3)	51.8 (4.0)	204.7 (5.6)
DHM	3.4 (12.2)	11.0 (6.2)	54.6 (1.9)	193.4 (1.8)
DHM3G	3.3 (5.0)	8.2 (8.2)	45.3 (7.0)	192.6 (4)
DHM6G	3.6 (10.4)	10.8 (4.6)	47.4 (4.3)	210.9 (1.9)
BUP	3.1 (11.2)	11.1 (6.9)	53.4 (3.3)	199.0 (3.2)
BUP3G	3.1 (13.5)	11.7 (13.8)	53.0 (9.5)	197.4 (2.4)
NBUP	3.2 (15.5)	10.1 (10.5)	44.9 (4.5)	202.9 (4.1)
NBUP3G	3.2 (13.9)	11.5 (10.3)	53.5 (8.8)	205.2 (3.8)
NALOX	3.3 (14.2)	10.2 (13)	49.5 (7.9)	193.4 (5.3)
NALOX3G	2.8 (14.4)	10.8 (13.4)	53.0 (10.9)	205.3 (2.8)
OXY	2.3 (13.5)	9.4 (5.5)	43.9 (3.4)	192.2 (6.0)
OXYM	3.4 (8.3)	9.3 (9.4)	50.9 (3.6)	199.2 (4.7)

* The mean percentages for the replicate analysis (n=5).
# R.S.D: Relative standard deviations.

Table 5-5: Inter-day precision

	Nominal Concentration		
	10.0	**50.0**	**200.0**
	Measured concentration ng/mL [#] (R.S.D *)		
MOR	10.3 (3.2)	52.4 (4.6)	200.0 (0.8)
M3G	11.0 (7.3)	49.6 (4.9)	204.6 (1.0)
M6G	10.1 (8.7)	47.6 (6.2)	201.6 (1.0)
6-MAM	10.8 (10.2)	51.4 (1.8)	202.5 (1.9)
NMOR	11.2 (2.9)	46.6 (6.7)	195.0 (1.6)
COD	11.0 (5.6)	53.6 (3.9)	201.0 (2.5)
C6G	9.3 (13.0)	50.8 (9.2)	198.0 (2.0)
NCOD	10.0 (6.0)	49.5 (3.5)	196.8 (1.3)
6-AC	9.8 (5.6)	53.6 (4.4)	198.0 (1.4)
HMOR	10.8 (3.3)	47.6 (3)	196.7 (0.9)
H3G	10.9 (6.3)	51.8 (5.3)	201.9 (1.0)
DHC	11.1 (3.8)	49.2 (3.9)	196.4 (0.6)
DHC6G	10.0 (8.6)	49.0 (4.2)	197.7 (1.2)
DHM	10.4 (9.2)	50.7 (9.2)	199.8 (4.2)
DHM3G	9.0 (10.0)	45.7 (2.5)	199.6 (1.4)
DHM6G	9.8 (7.2)	45.9 (7.1)	205.0 (2.7)
BUP	10.9 (6.3)	48.8 (5.5)	200.7 (3.1)
BUP3G	11.3 (5.0)	48.1 (2.7)	200.0 (2.2)
NBUP	10.0 (12.0)	47.3 (8.5)	200.0 (3.4)
NBUP3G	9.7 (11.5)	51.3 (4.0)	199.7 (2.4)
NALOX	9.9 (12.5)	47.6 (8.1)	201.0 (2.3)
NALOX3G	10.3 (7.5)	52.8 (8.5)	194.4 (1.4)
OXY	10.2 (6.5)	53.7 (5.7)	197.3 (1.3)
OXYM	9.8 (4.9)	51.1 (3.8)	201.0 (2.3)

[*] The mean percentages for the replicate analysis (n=5).
[#] R.S.D: Relative standard deviations (%).

5.4.4.4 Matrix effects

Assessment of possible matrix effects indicated that ion enhancement and ion suppression occurred with some analytes and the average value ranged from < 1% to 17% and from < 1% to 18%, respectively. In Table 5-6, a value of > 100% demonstrates ion enhancement while a value of < 100% points to ion suppression. Although the average value of the ion suppression and the ion enhancement of analytes of interest were within the accepted limit of the validation procedure (± 20 %), further studies were conducted to evaluate matrix effects due to endogenous blood components by testing samples (n=3) of blank blood, blank blood spiked with internal standards and blank blood spiked with analytes of interest at 3 ng/mL (LLOQ), refer to Figure 5-1 and 5-2.

The specificity of the method was investigated using a mixture of drugs that are routinely detected in forensic toxicology cases (cocaine, benzoylecgonine, cocaethylene, ecgonine methyl ester, amphetamine, methamphetamine, methylenedioxyamphetamine, methylenedioxyethyleamphtamine, methylenedioxymethamphetamine, nitrazepam, 7- aminoflunitrazepam, chlordiazepoxide, diazepam, oxazepam and temazepam) at a concentration of 400 ng/mL in whole blood. No endogenous blood components nor any of the drugs co-eluted with any of the analytes of interest or their internal standards. Matrix affects results are listed in Table 5-6.

5.4.4.5 Stability

The assessment of analyte stability in matrix during the validation process is required for reliable quantitation, especially if no information is available from previous work [47]. The stability of heroin metabolites, including 6-MAM, morphine and its glucuronides have been studied and data is available in the literature [12,75,116,172,182,183,255]. Heroin was stable for 14 months in frozen plasma [75] and 6-MAM was stable in frozen urine over a period of 12 months [12]. Morphine and its glucuronides were found to be relatively stable in frozen specimens for a long period. However, these analytes were found unstable at 4 ºC after a few days [12]. Skopp *et al* [255] concluded that the major factor that affects the stability of morphine and M6G is storage temperature.

Table 5-6: Blood matrix effect during extraction (% relative to drug in buffer).

	Mean matrix effect (%, n=3)**					All five human sources	
Analyte	Source 1[#]	Source 2	Source 3	Source 4	Source 5	Mean %	R.S.D. [&]
MOR	104	101	98	96	111	102	5.8
M3G	101	89	90	97	110	97	8.6
M6G	94	80	82	82	90	86	7
6-MAM	101	101	105	101	87	99	7
NMOR	94	100	82	84	100	92	9
COD	100	99	107	96	118	104	8
C6G	95	105	109	106	107	104	5
NCOD	101	112	109	84	99	101	11
6-AC	85	86	102	96	96	93	8
HMOR	107	108	107	97	112	106	5
H3G	83	98	82	107	106	95	13
DHC	111	106	99	100	103	104	5
DHC6G	103	84	76	93	90	89	11
DHM	110	98	102	93	98	100	6
DHM3G	93	75	84	80	85	83	8
DHM6G	77	90	83	81	83	83	6
BUP	95	95	109	112	108	104	8
BUP3G	123	106	112	116	125	116	7
NBUP	98	128	110	120	129	117	11
NBUP3G	106	93	104	97	113	103	8
NALOX	94	87	84	90	95	90	5
NALOX3G	102	93	82	83	104	93	11
OXY	104	95	82	88	102	94	10
OXYM	101	108	109	113	113	109	4

Human blood was sourced from completed post-mortem blood samples that were scheduled for destruction and contained no analytes of interest.
** Matrix effect is expressed as the response obtained for a standard chromatographed along with matrix extract compared to that obtained with an unextracted standard chromatographed in mobile phase only, expressed as a percentage. Standard was spiked into matrix extract at a concentration of 200 ng/mL.
[&] R.S.D. %: Relative standard deviation expressed as a percentage.

They found that M3G was stable at room temperature for 4 weeks and that analytes were stable in the dark for at least 181 days. BUP and its metabolites were stable in human plasma and urine for 119 and 85 days respectively at -20 °C [250], but were stable for only a short time at 4 °C or at room temperature [256]. Dawson *et al* [252] reported that oxycodone was stable for 24hrs at room temperature in an autosampler. Hydromorphone and its glucuronide were found stable during storage, sample extraction and chromatography [235]. Codeine and its glucuronide were found stable in urine for a short time [159] and codeine alone was stable for more than seven months in frozen plasma [205].

In the present study, from the results listed in Table 5-7, analytes of interest were subjected to different stability experiments as the stability of some analytes has not previously been studied in human whole blood, such as NAL3G, DHC6G, DHM3G, and DHM6G. Analytes of interest were stable after being subjected to four freeze-thaw cycles.

Concentration variations after 48 hours and after a week of storage in the auto-sampler were less than ± 10% which were within the acceptable limits of the validation procedure ±20% [46,53]. 6-MAM was stable after 4 hours storage at room temperature but decreased by 21% after 24 hours at room temperature. Similarly, 6-AC was stable for 4 hours at room temperature but decreased by 63% after 24 hours.

Long-term stability studies were performed at two different storage temperatures used routinely in the forensic toxicology laboratory, 4 °C and -20°C, to assess the stability of analytes of interest. In general, opioids and their metabolites were stable for the whole period of this stability study (Table 5-7). However, the concentration of 6-AC sharply decreased by 56% after 24hrs at 4°C and by more than 77% after 48 hrs. 6-MAM was stable up to 24 hours at 4°C but decreased by 15% after 48 hours and continued decreasing over time by more than 30 % within the week of the stability study. Concentration changes with other analytes were less than ± 10% which was within the acceptable limits of the validation procedure. MOR and COD concentrations increased after storage for 24 hours at 4 °C by 8 and 9%

Table 5-7: Stability studies (% relative to starting concentration).

Storage Conditions	Room Temperature		Auto-sampler		Freeze-thaw	Freeze at -20 0C				Refrigerate at 4 0C			
Analytes	**4 hours**	**24 hours**	**48 hours**	**week**	**4 cycles**	**24 hours**	**48 hours**	**week**	**Month**	**24 hours**	**48 hours**	**week**	**Month**
MOR	104 *	144	100	98	98	99	102	106	103	108	128	140	145
M3G	105	108	104	108	111	94	103	108	104	101	104	106	103
M6G	97	105	101	98	105	109	102	107	102	98	108	103	97
6-MAM	102	79	99	100	102	104	101	102	108	98	85	67	67
NMOR	109	110	101	99	98	109	101	107	101	107	105	103	95
COD	109	142	100	104	98	105	109	105	107	109	130	136	148
C6G	98	106	102	103	96	105	99	103	103	105	104	109	104
NCOD	111	110	102	93	96	106	98	99	105	106	103	105	108
6-AC	100	37	100	106	103	105	110	92	97	72	44	22	22
HMOR	105	104	100	105	96	108	105	105	104	105	95	105	109
H3G	105	150	103	95	104	102	95	104	103	104	95	105	95
DHC	107	100	101	106	99	103	105	99	102	101	104	109	99
DHC6G	92	92	104	93	100	95	99	102	105	99	93	101	98
DHM	102	104	100	107	99	99	104	96	109	98	101	102	98
DHM3G	99	95	102	99	100	105	102	96	97	98	105	92	95
DHM6G	97	110	101	102	102	100	97	94	109	95	104	109	113
BUP	99	103	99	104	100	101	104	100	99	106	109	103	93
BUP3G	94	105	102	106	99	99	98	100	102	94	108	109	100
NBUP	104	102	100	108	104	105	104	105	110	106	105	102	108
NBUP3G	96	109	96	94	91	98	105	97	99	104	106	108	95
NALOX	108	103	95	99	92	99	117	97	104	100	101	107	106
NALOX3G	97	108	104	90	108	107	112	101	103	109	107	96	99
OXY	109	110	103	105	105	103	105	106	111	109	107	107	103
OXYM	105	106	101	106	99	110	103	99	97	108	99	106	97

* Mean % (n=3).

respectively and increased by 45% and 48%, respectively, after one month of storage as a result of the hydrolysis of 6-MAM and 6-AC to MOR and COD respectively. Increases in MOR and codeine concentrations after 24 hours at room temperature were similar to those observed after one month at 4 ^{o}C.

5.4.5 Case samples

The method was employed for the quantitative determination of opioids and their metabolites in thirty-two post-mortem blood samples (Table 5-8). Seventeen cases tested positive for morphine, codeine, M3G, M6G and C6G. 6-MAM was found in seven of these cases and NCOD was detected in six of these cases.

5.4.5.1 Heroin cases

Eleven cases (3, 5, 7, 13, 15, 17, 19, 22, 29, 30 and 31) showed of the presence of free morphine at concentrations ranging from 116 to 799 ng/mL. In addition, M3G and M6G were present at concentrations ranging from 5 to 2477 and 5 to 175 ng/mL, respectively. NMOR was found in all of these cases with the exception of 31, which had a high concentration of 6-MAM and a low concentration of morphine glucuronide, suggesting that death occurred shortly after heroin was administered and before morphine was demethylated to NMOR (Figure 5-3).

Total morphine (TMOR) to total codeine (TCOD) ratios were calculated to determine which opioid had been used (Table 5-9). The ratios were in the range 12 to 70, suggesting that heroin had been used by the deceased and that the codeine detected resulted from acetylcodeine present in the street drug [202,257-260]. Heroin use could be confirmed in seven of these cases because of the presence of 6-MAM. Figure 5-4 shows a chromatogram of 6-MAM in heron related fatalities case detected in the current work.

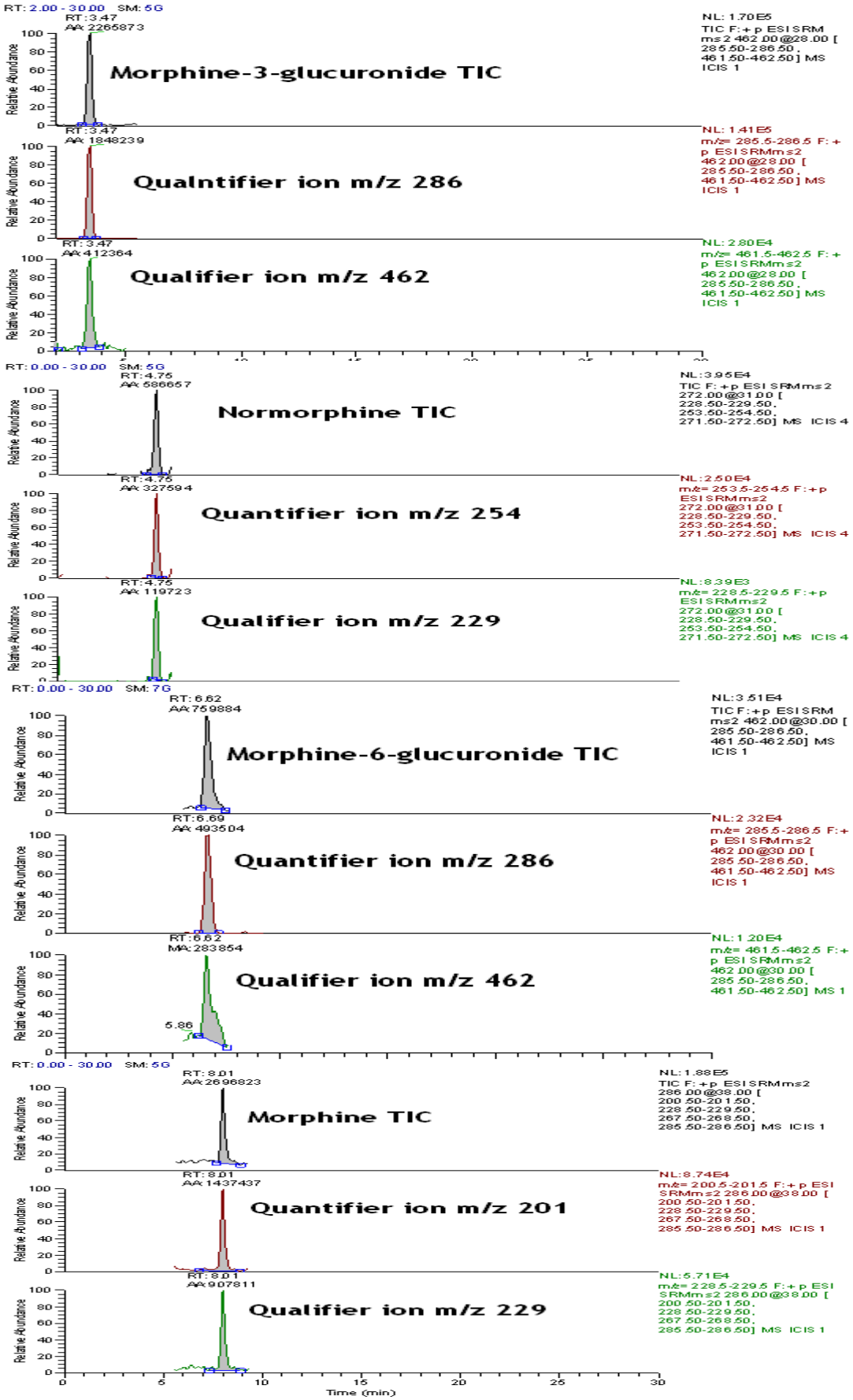


Figure 5-3: Morphine and its metabolites detected in heroin related fatalities.

Table 5-8: Concentrations of opioids and their metabolites (ng/mL) in autopsy blood from 31 cases.

Analytes [*]	MOR	6-MAM	M3G	M6G	NMOR	COD	NCOD	C6G	DHC	DHC6G	DHM	DHM3G	DHM6G
Serial no.	Measured concentration (ng/mL) each samples has been analysed in duplicate												
1	N.D [*]	N.D	N.D	N.D	N.D	N.D	N.D	N.D	225	66	2	5	N.D
2	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	420	1862	21	122	10
3	799	1	208	13	2	68	N.D	13	N.D	N.D	N.D	N.D	N.D
4	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	2132	383	15	14	Trace
5	418	N.D	4.91	6	Trace [#]	33	1.5	Trace	N.D	N.D	N.D	N.D	N.D
6	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	840	147	100	65	9
7	346	1	319	34	Trace	29	N.D	5	N.D	N.D	N.D	N.D	N.D
8	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	1641	5364	1	4	N.D
9	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	42	105	2	8	N.D
10	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	518	19	44	28	trace
11	162	N.D	874	69	84	724	Trace	496	N.D	N.D	N.D	N.D	N.D
12	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	70	18	N.D.	3	N.D
13	559	3	490	56	Trace	36	N.D	11	N.D	N.D	N.D	N.D	N.D
14	26	N.D	427	31	94	520	51	38145	N.D	N.D	N.D	N.D	N.D
15	568	1	175	8	1	33	N.D	4	N.D	N.D	N.D	N.D	N.D
16	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	2882	1997	94	63	15
17	116	N.D	1966	122	3	9	N.D	86	N.D	N.D	N.D	N.D	N.D
18	14	N.D	206	15	Trace	2	N.D	8	N.D	N.D	N.D	N.D	N.D
19	406	Trace	704	19	18	7	N.D	7	N.D	N.D	N.D	N.D	N.D
20	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	157	76	8	7	N.D
21	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	2013	654	5	2	3
22	196	N.D	2476	175	5	20	N.D	118	N.D.	3	N.D.	16	6
23	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	248000	260177	25	1493	482
24	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	3735	821	18	12	4
25	161	N.D	201	21	144	848	145	493	N.D	N.D	N.D	N.D	N.D
26	129	N.D	859	54	127	20000	2596	5306	N.D	N.D	N.D	N.D	N.D
27	25	N.D	33	5	19	296	42	275	N.D	N.D	N.D	N.D	N.D
28	Trace	Trace	2	2	Trace	88	5	21	1	N.D	6	1	trace
29	788	4	1920	161	5	90	N.D	19	N.D	N.D	N.D	N.D	N.D
30	417	3	216	20	Trace	35	N.D	7	N.D	N.D	N.D	N.D	N.D
31	369	87	82	5	0	34	N.D	4	N.D	N.D	N.D	N.D	N.D

[*] N.D: not detected;
[#] Trace: < LLOQ

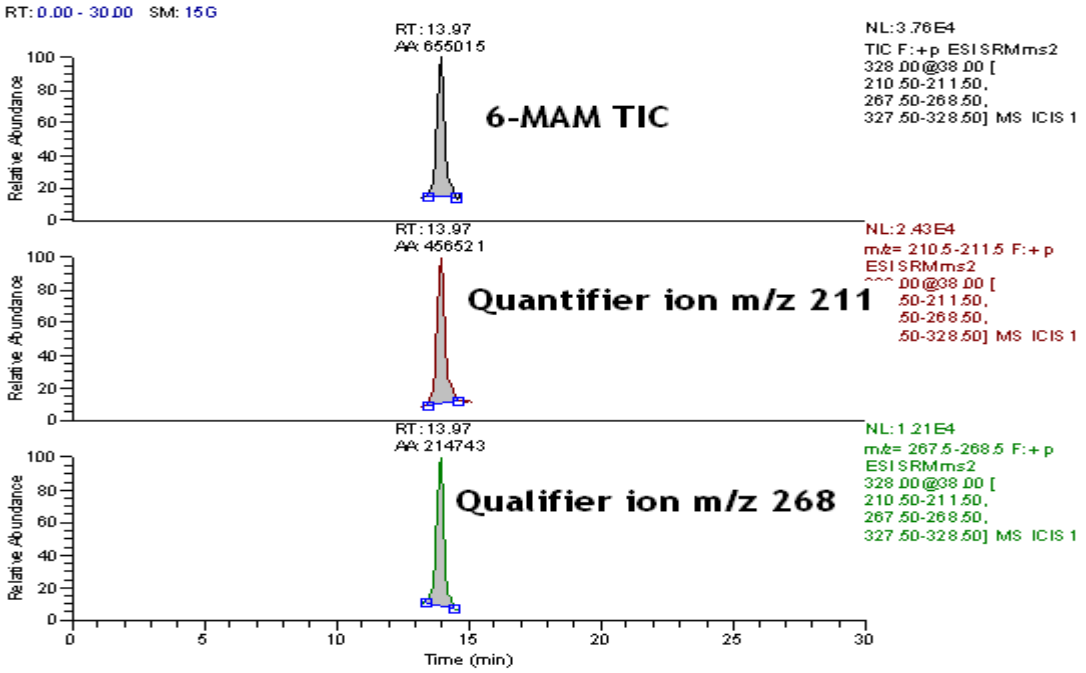


Figure 5-4: 6-MAM detected in heroin related deaths.

Determination of the type of death (immediate or delayed) and survival time after last injection have been investigated in several previous studies [125,162,163,165,166,177,261]. The concentration of free morphine alone has been used to interpret the survival time: free morphine concentrations ranging from 100-930 ng/mL indicate a short survival time, whereas concentrations less than 60 ng/mL was determined three days after the last injection in one case [162]. Staub *et al* [163] used the concentration of free morphine as a percentage of total morphine in blood, and suggested that a percentage higher than 53% indicated rapid death, and less than 35% indicated a delayed death . Spiehler and Brown [166] reported an average percentage ratio of 68% in rapid deaths . However, the use of free morphine to interpret the cause of death has also been found to be unreliable because free morphine was negative in some heroin-related deaths [125,150,152]. In the last twenty years, the concentrations of morphine metabolites in blood have been used in conjunction with free morphine concentration to assess the survival time [125,177,248,261]. Deaths attributed to heroin use were classified into three types using the concentration of free morphine and its glucuronides (in blood: immediate death, sub-acute death (death in less than 3h), and delayed death (death after 3h) [125].

In the present study, the latter method was used for eleven cases attributed to heroin use. Cases 3, 13, 15 and 31 were classed as immediate deaths, i.e. free morphine was higher than 500 ng/mL, 6-MAM was detected and the percentages of free morphine to total morphine were 78, 75 and 80%, respectively. Three cases (5, 7, and 30) were classed as sub-acute deaths. In these cases the free morphine concentration was less than 500 ng/mL, the ratios of free morphine to morphine glucuronides and free morphine plus morphine-6-glucuronide to morphine-3-glucuronide were higher than 1, and the total morphine was less than 1000 ng/mL. The percentages of free morphine to total morphine were 97, 49, 51 and 64%, respectively. Cases 17, 19, 22 and 29 were classified as delayed deaths. These showed concentrations of free morphine of less than 200 ng/mL with the exception of 19 and 29 which showed high concentrations of free morphine, 406 and 788 ng/mL respectively. The ratios of free morphine to morphine glucuronides and free morphine plus morphine-6-glucuronide to morphine-3-glucuronide were less than 1. Total morphine was higher than 1000 ng/mL, and the percentages of free morphine to total morphine were 5, 35, 7, and 27%, respectively.

5.4.5.2 Codeine cases

Codeine and its main metabolite C6G were found in each of these eleven cases with concentrations ranging from 7 to 90 and trace levels to 118 ng/mL, respectively. Six cases (11, 14, 25, 26, 27 and 28) were attributed to the use of codeine because of the presence of NCOD, which is considered a codeine marker and because the TMOR/TCOD ratio was less than 1 in all of these cases (Table 5-10). Also, the concentrations of NMOR found in codeine cases (2 to 144 ng/mL) were generally higher than those in heroin cases (trace level to 18 ng/mL). Figure 5-5, shows norcodeine, codeine glucuronide and codeine in codeine positive case sample.

Codeine was found at concentrations within the therapeutic range in cases 27 and 28 and at concentrations higher than the therapeutic level in cases 11 and 25. Codeine overdose was determined in cases 14 and 26. Free codeine was less than 1000 ng/mL in case 14 but case 26 which had a free codeine concentration estimated at 20,000 ng/mL. Total codeine was found to be higher than 1000 ng/mL in cases 11, 14, 25 and 26; and less than 1000 ng/mL

Table 5-9: Ratios of morphine and its glucuronide total morphine to total codeine, and free morphine to free codeine in post-mortem blood in 11 heroin cases.

Serial no	Mode of deaths [§]	M3G / MOR	M6G /MOR	M3G/ M6G	TMOR [#] (ng/mL)	FMOR [*]/ CONJ MOR [**]	FMOR+ M6G/M3G	MOR/ TMOR	TCOD[##] (ng/mL)	TMOR/ TCOD	FMOR/ FCOD [&]
3	A	0.26	0.016	15.98	1022.61	3.60	3.89	78.11	80.82	12.63	11.78
5	SA	0.01	0.02	0.77	431.47	36.99	86.51	96.96	34.89	12.37	12.57
7	SA	0.92	0.10	9.31	700.86	0.98	1.19	49.41	33.49	20.93	11.98
13	A	0.87	0.10	8.78	1105.63	1.02	1.25	50.52	47.67	23.19	15.36
15	A	0.31	0.01	22.3	753.09	3.09	3.28	75.48	36.72	20.51	17.48
17	P	16.89	1.05	16.11	2208.26	0.06	0.12	5.28	95.3	23.17	12.40
19	P	1.74	0.04	36.21	1148.04	0.56	0.60	35.36	16.38	70.09	59.97
22	P	12.61	0.90	14.12	2853.48	0.07	0.15	6.89	138.58	20.59	9.59
29	P	2.44	0.20	11.9	2874.72	0.38	0.49	27.42	108.59	26.47	8.8
30	SA	0.52	0.05	11.01	654.66	1.77	2.02	63.75	42.57	15.38	11.89
31	A	0.22	0.01	18.01	456.3	4.25	4.54	80.96	37.86	12.05	10.81

[#] TMOR: Total morphine; [*] FMOR: Free morphine, [**] CONJ MOR: morphine conjugates; [##] TCOD: Total codeine; [&] FCOD: Free Codeine.
[§] (A) acute death, (SA) Sub-acute and Prolonged (P).

in cases 27 and 28. In case 14 the concentration of free codeine alone would not give an accurate interpretation as this case showed a C6G concentration

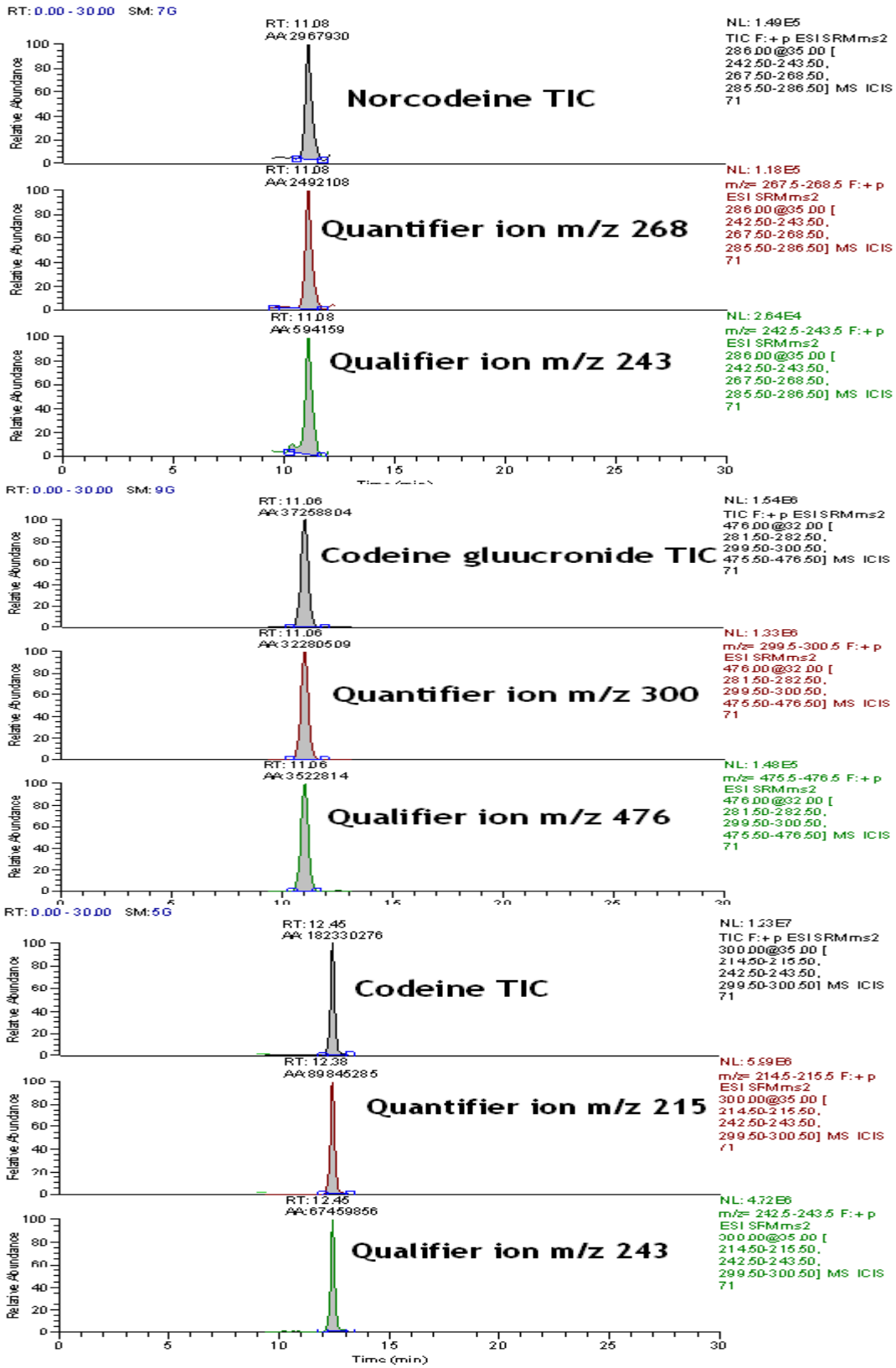


Figure 5-5: Codeine metabolites (norcodeine, codeine glucuronide and codeine) detected in real case samples.

Table 5-10: Ratios of morphine, codeine and their metabolites in post-mortem blood from 6 codeine cases.

Serial no	M3G / MOR	M6G/ MOR	M3G/ M6G	TMOR [#] (µg/mL)	FMOR [*] / CONJ MOR **	FMOR+ M6G/ M3G	MOR/ TMOR	TCOD [##] (µg/mL)	TMOR/ TCOD	TCOD/ TMOR	FCOD [&] /TCOD %	FMOR /FCOD
11	5.38	0.43	12.60	1.19	0.17	0.26	13.63	1.22	0.97	1.03	59.32	22.40
14	16.24	1.17	13.90	0.58	0.06	0.13	4.55	4.39	0.13	7.59	11.86	5.05
25	1.25	0.14	9.48	0.53	0.72	0.90	30.47	1.49	0.35	2.82	57.08	18.94
26	6.67	0.42	16.05	1.17	0.14	0.21	11.03	27.89	0.04	23.88	71.66	0.64
27	1.34	0.23	6.06	0.08	0.64	0.91	30.02	0.61	0.13	7.44	48.30	8.36
28	3.27	3.04	1.08	0.01	0.16	1.23	8.89	0.11	0.06	18.13	76.96	0.64
AVE	5.69	0.90	9.86	0.59	0.32	0.61	16.43	5.95	0.28	10.15	54.2	9.34

[#] TMOR: Total morphine; [*] FMOR: Free morphine, [**] CONJ MOR: Morphine conjugates; [##] TCOD: Total codeine; [&] FCOD: Free Codeine.
[§] A: Acute death; SA: Sub-acute; P: Prolonged.

six times higher than that of free codeine. The total codeine concentration is in the toxic range, suggesting an overdose of codeine with a long survival time, see Table 5-10. The ratios of total morphine to total codeine were less than 1 in all codeine cases. The concentration of free morphine relative to free codeine was less than 10 % in these cases with the exception of cases 11 and 25, in which it was 22 and 19 % respectively. It has been reported that free morphine was found in serum after a single oral codeine dose at a concentration of more than 10% relative to free codeine [262].

5.4.5.3 Dihydrocodeine cases

DHC was detected in fourteen cases (1, 2, 4, 6, 8, 9-10, 12, 16, 20-21, 23, 24 and 28, Tables 5-8 and 5-11). DHM3G, DHM and DHC6G were also found in each case except cases 12 and 28 which were found negative for DHM and DHC6G, respectively. DHM6G was found to be negative in five cases (1, 8, 9, 12 and 20). Total DHC to DHM ratios ranged from 6 to 1319, with an average of 159, which was due to the high concentration of DHC determined compared to DHM and its glucuronide in all cases.

The concentrations of total DHC (parent drug and its 6-glucuronide) in six cases (2, 4, 8, 16, 21, 23 and 24) were higher than 2000 ng/mL (average 5855 ng/mL) which are high concentrations that would have caused death, especially in those who have no tolerance to the drug [212]. Deaths attributed to DHC overdose were reported in the literature with DHC concentrations of 800 ng/mL or higher [196]. In contrast, the concentration of total DHC in four cases (1, 9, 12, and 20) was within the therapeutic range. Case 10 showed a DHC concentration of 518 ng/mL and total DHC of 537 ng/mL. These concentrations were higher than the usual therapeutic level.

DHC6G is the main metabolite of DHC and the ratio of DHC to its glucuronide can be used to estimate the elapsed time after drug ingestion [212]. The concentration of DHC was higher than DHC6G in five cases (4, 6, 16, 21, and 24) which indicates a short survival time after ingestion. In contrast, cases 2 and 8 showed DHC6G concentrations exceeding that of the parent drug, indicating a longer survival time. DHM and its glucuronide were detected in the present study, but at lower concentrations than those reported in

Table 5-11: Ratios of DHC and its metabolites in autopsy blood from 13 DHC cases.

Serial no	DHC	TDHC (µg/mL)	TDHM (ng/mL)	TDHC/ TDHM	DHC/ DHC6G	DHM3 / DHM	DHM6 / DHM	DHM6/ DHM3	FDHM /DHMG
1	222.3	0.29	6.70	44.00	3.40	2.30	n.d. [#]	n.d.	0.40
2	420.1	2.28	143.00	16.00	0.20	5.80	0.47	0.08	0.20
4	2132.4	2.52	30.00	85.00	5.60	0.90	0.04	0.05	1.00
6	840.3	0.99	166.00	6.00	5.70	0.70	0.09	0.14	1.40
8	1640.8	7.00	5.00	1319.00	0.30	4.30	n.d.	n.d.	0.20
9	42.2	0.15	10.00	14.9.00	0.40	5.20	n.d.	n.d	0.20
10	518.3	0.54	72.00	7.5.00	27.00	0.60	0.02	0.03	1.50
12	70.0	0.09	2.90	31.00	3.90	n.d.	n.d.	n.d.	n.d.
16	2881.8	4.88	156.00	31.00	1.40	0.70	0.15	0.23	1.20
20	156.8	0.23	15.00	15.00	2.10	0.90	n.d.	n.d.	1.20
21	2013.2	2.67	6.00	416.00	3.10	0.40	0.72	2.06	0.90
23	248000.0	123.63	694.00	178.00	1.00	2.70	0.74	0.28	0.30
24	3735.4	4.56	30.00	150.00	4.60	0.70	0.20	0.29	1.20

[#] n.d.: Not detected.

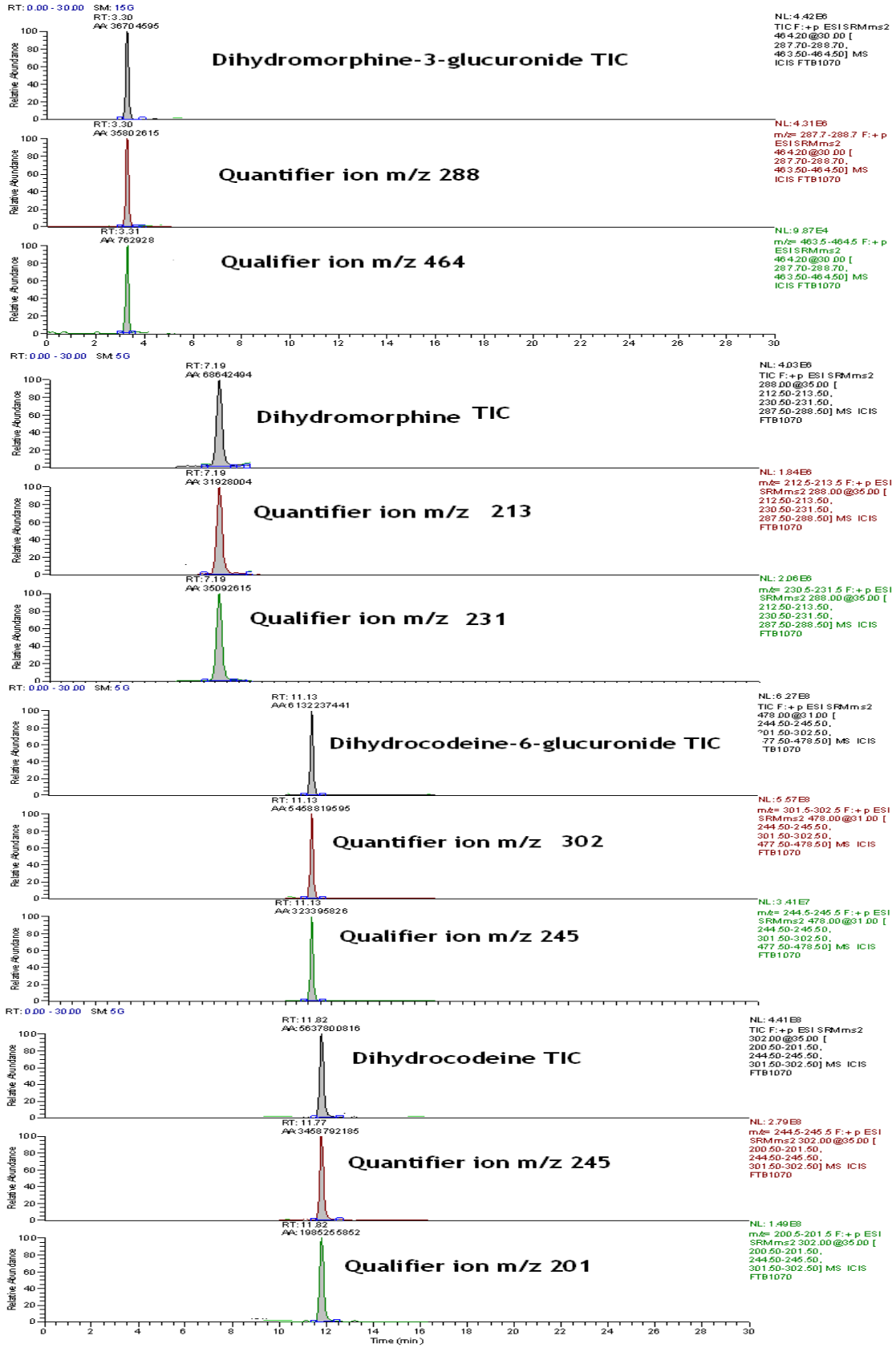


Figure 5-6: Case samples positive for DHC metabolites apart from DHM-6-G which was not detected.

previous studies [24,212], and DHM6G was not detected at all in some cases, see Figure 5-6. This may be due to differences in DHC metabolism between extensive and poor metabolisers. The highest concentrations of DHM glucuronides detected in the present study were 1490 ng/mL and 482 ng/mL for DHM3G and DHM6G respectively, in the presence of high concentrations of DHC and its glucuronide. These results are in agreement with previous studies in concluding that the detection of DHC metabolites is a reliable tool for an accurate interpretation of the role of DHC in causing death, especially when the parent drug is found at a low concentration. Refer to Table 5-11.

5.4.5.4 Poly-drug intoxication

Overdose has been considered the most important cause of death of heroin users. However, polypharmacy has sharply increased the risk of acute respiratory depression due to additive drug interactions [127,151,158,263]. Other drugs that could cause respiratory depression are frequently detected in opioid fatalities, such as ethanol and benzodiazepines. In this study other drugs that could interact with opioids were investigated to provide an accurate interpretation of the cause of death (Table 5-12).

Ethanol was detected in 47% of these cases with a concentration range from 12 to 245 mg/100 mL. Diazepam metabolites were present in 62% of these cases. Cocaine was found with its metabolites benzoylecgonine in case 5, and benzoylecgonine alone was found in case 19. Chlordiazepoxide was detected in cases 4 and 31 at concentrations within the therapeutic range. A low concentration of 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid (0.022 µg/mL) was found in case 16. Paracetamol was detected in 31% of these cases, with concentrations ranging from 3.4 to 278 mg/mL. An overdose level of chlorpromazine (0.73 µg/mL) was observed in case 26, which also found overdose levels of codeine and paracetamol and a high concentration of Mirtazapine (0.81 mg/mL).

Ethanol is the most frequently detected drug in heroin fatalities [150,151,264-268], in up to one third of cases [125]. Ruttenber *et al* [266] determined total morphine (TM) in many cases and reported that a low concentration of TM was detected in cases that had a blood alcohol concentration (BAC) greater than 100 mg/

100 mL. Leven *et al* [150] investigated the role of ethanol in heroin deaths and found that alcohol at a concentration of less than 100 mg/100mL may contribute to heroin deaths. The authors observed that morphine concentration increased significantly when the BAC was between 200 mg/100 mL and 290 mg/100 mL. They stressed that when the BAC exceeds 300 mg/100mL, ethanol is main cause of death and the effect of morphine is a minor factor. Polettini *et al* [264] compared two groups which had morphine and alcohol in their blood: a low-ethanol group (LE) with BAC ≤ 100 mg/100mL, and a high ethanol group (HE) with BAC ≥ 100 mg/100mL. The authors found that the concentrations of heroin metabolites were lower in the HE group. Another study found that the mean morphine concentration remains unchanged if the BAC is less than 200 mg/100 mL [150].

Three mechanisms have been suggested for the role of ethanol in heroin deaths. Many studies concluded that alcohol has an inhibitory effect on the phase II metabolism of morphine, thus increasing the level of free morphine in blood and reaching the brain. Another explanation is that the high BAC combined with morphine would have an additional depressant action on the central nervous system and then more acute deaths would occur due to the combined effects of these two substances. In addition, it has been suggested that alcohol may also inhibit the hydrolysis of 6-MAM to morphine, and indirectly the biotransformation of morphine would also be inhibited. The excretion of free morphine and total morphine would be also reduced [150,264,265].

In the present study, alcohol was detected in eight out of eleven cases that were attributed to heroin use. Cases 3, 7, 13, 15, 30, and 31 were classified as HE as they had a BAC above 100 mg/100 mL and cases 5 and 29 were classified as LE with a BAC of less than 100 mg/ 100 mL. The average free morphine in LE cases was found to be slightly higher than in the HE group. However, the average TMOR was 782 and 1652 ng/mL for HE and LE, respectively. Morphine alone was detected in case five and no glucuronides were observed. In contrast, case 29 had high concentrations of morphine metabolites. In the HE group, free morphine to total morphine percentages ranged from 49 to 81%. In this study, 6-MAM was detected in heroin deaths in

Table 5-12: Other drugs detected in cases investigated in this study (µg/mL).

Serial no.	Methadone	Paracetamol	Cocaine	Benzoyl ecogonine	Alcohol (mg/100mL)	Chlorodiaz-epoxide	Mirtazapne	Amitriptyline	Nortriptyline	Fluoxetine	Norfluoxetine
1	#				14						
2		14.9									
3	0.18				124						
4	0.58					0.39	0.18				
5			0.01	0.42	42						
6											
7					138		0.08				
8		25.4						0.65	0.11		
9		3.4									
10											
11		41.4			58						
12	0.38				182						
13					210						
14		72									
15					180						
16								0.15			
17	0.12										
18	0.89							12.5	0.5		
19	0.32			0.06				0.16			
20	0.21										
21		129			12			0.9	0.12	0.64	0.19
22											
23					71			0.3	0.03		
24					236						
25		220									
26		278					0.81				
27		48									
28	0.52	28			139						
29					33						
30					160						
31					245	1.63				0.09	0.14
32											

Negative.

the HE group, and survival times after last injection were shorter than in the LE group. The ranges of free morphine concentrations in both groups overlapped, indicating that the use of the free morphine concentration alone is insufficient to interpret the cause of death.In case 31, low concentrations of free morphine and its glucuronides along with the presence of a high concentration of 6-MAM perhaps met the criteria of the third mechanism for the role of ethanol in heroin deaths proposed by Polettini *et al* [264]. In addition, the combination of alcohol, heroin, and chlordiazepoxide in this case would have caused enhanced respiratory depression and death probably occurred shortly after heroin injection.

The combination of alcohol and codeine probably increased the risk of toxic side effects due to codeine in case 11. Likewise, the combination of alcohol and DHC in case 24 is likely to have produced enhanced sedation and respiratory depression [132,212].

Methadone was detected in cases 3, 4, 12, 17, 18 19, 20 and 28 with concentrations ranging from 120 to 890 ng/mL; the average methadone concentration in these cases was 400 ng/mL. The overlap of therapeutic and toxic concentrations of methadone, as for morphine, makes the inclusion of methadone as a cause of death dependent on the circumstances, for example, if there is no other identifiable cause of death such as the presence of natural disease [125]. The concentration of methadone in blood for patients on methadone maintenance programmes ranged from 100 to 1000 ng/mL. However, in a series of 10 methadone fatalities, the average methadone concentration in blood was 1000 ng/mL, and the range of concentrations was 400 to 1800 ng/mL [132]. In one study, methadone was detected in only one heroin fatality but was found in 27% of living heroin users [151].

Methadone was within the therapeutic range in cases 3, 17 and 20 and was above the normal range for therapeutic administration, except in high dose maintenance therapy, in cases 4 and 18. The combinations of heroin and methadone in case 19 and methadone and alcohol in cases 12 and 28, are likely to have more potent effects than those for each drug on its own [132].

Cocaine is believed to interact with heroin as both are metabolised by carboxylesterases. TMOR and FMOR were lower in cocaine positive cases than in cocaine negative samples, whereas the FMOR/TMOR ratio was the same in both groups. Analysis of data on the role of ethanol in heroin deaths in the presence of cocaine showed that the high ethanol group had higher FMOR/TMOR ratios lower TMOR concentrations than the low ethanol group [265].

In the present study, case 5 was positive for morphine (418 ng/mL), cocaine (10 ng/mL cocaine and 400 ng/mL benzoylecogonine) and alcohol (42 mg/100mL). The ratio of FMOR/TMOR was the highest found in the study (Table 5-9). Morphine phase 2 metabolite concentrations were less than 13 ng/mL. This may indicate that the formation of morphine metabolites was inhibited by the presence of alcohol and cocaine or that the survival time was short.

The concentrations of diazepam and its metabolites detected in this study were within the therapeutic range in most cases except case 18, which had a concentration higher than the usual therapeutic range. Diazepam or its metabolites were found in all heroin cases except cases 15 and 17. Benzodiazepines were found to increase the CNS depressant action of opioids in general [151].

Paracetamol concentrations detected in this study were within or slightly higher than the therapeutic range, except in cases 21, 25 and 26 which had overdose levels of paracetamol plus DHC or codeine. In case 25, a high concentration of codeine (0.85 µg/mL) was observed and in this case, the cause of death would have been attributed to codeine (respiratory depression) rather than paracetamol (220 µg/mL) (liver damage) [132].

Other opioid drugs were detected in a few cases, including naloxone and its glucuronide (case 14), see Figure 5-7. Buprenorphine and its metabolites were detected in case 29, oxycodone was found in case 23, hydromorphone and its glucuronide (case 32) with concentrations of 5 and 183 ng/mL, respectively, see Figure 5-8. Levels of hydromorphone and its glucuronide were within therapeutic range. The detection of multiple opiates in some cases (14, 29 and 23) illustrated the advantages of the method which has been developed.

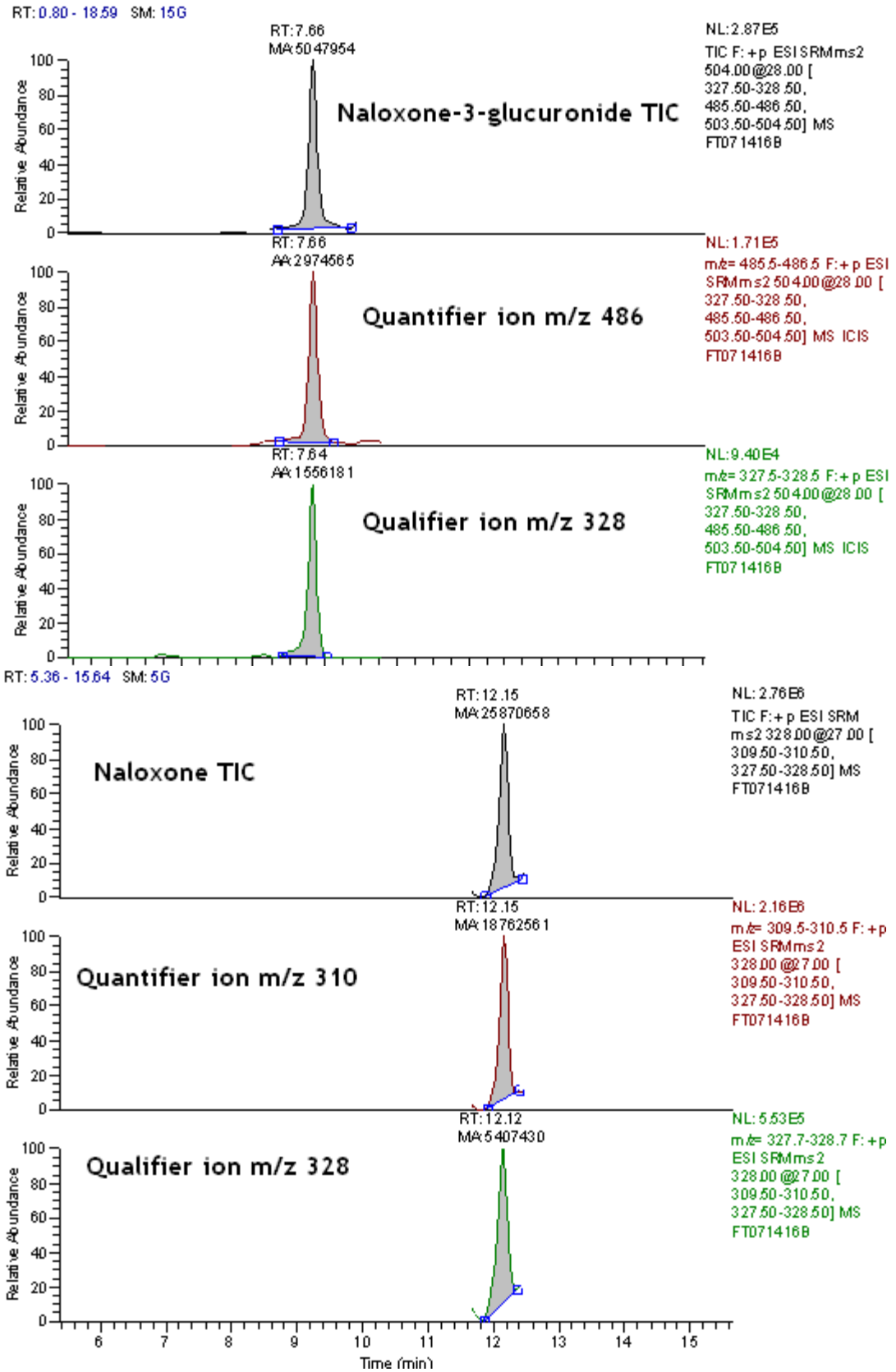


Figure 5-7: Reconstructed mass chromatograms for naloxone and its glucuronide in case 14.

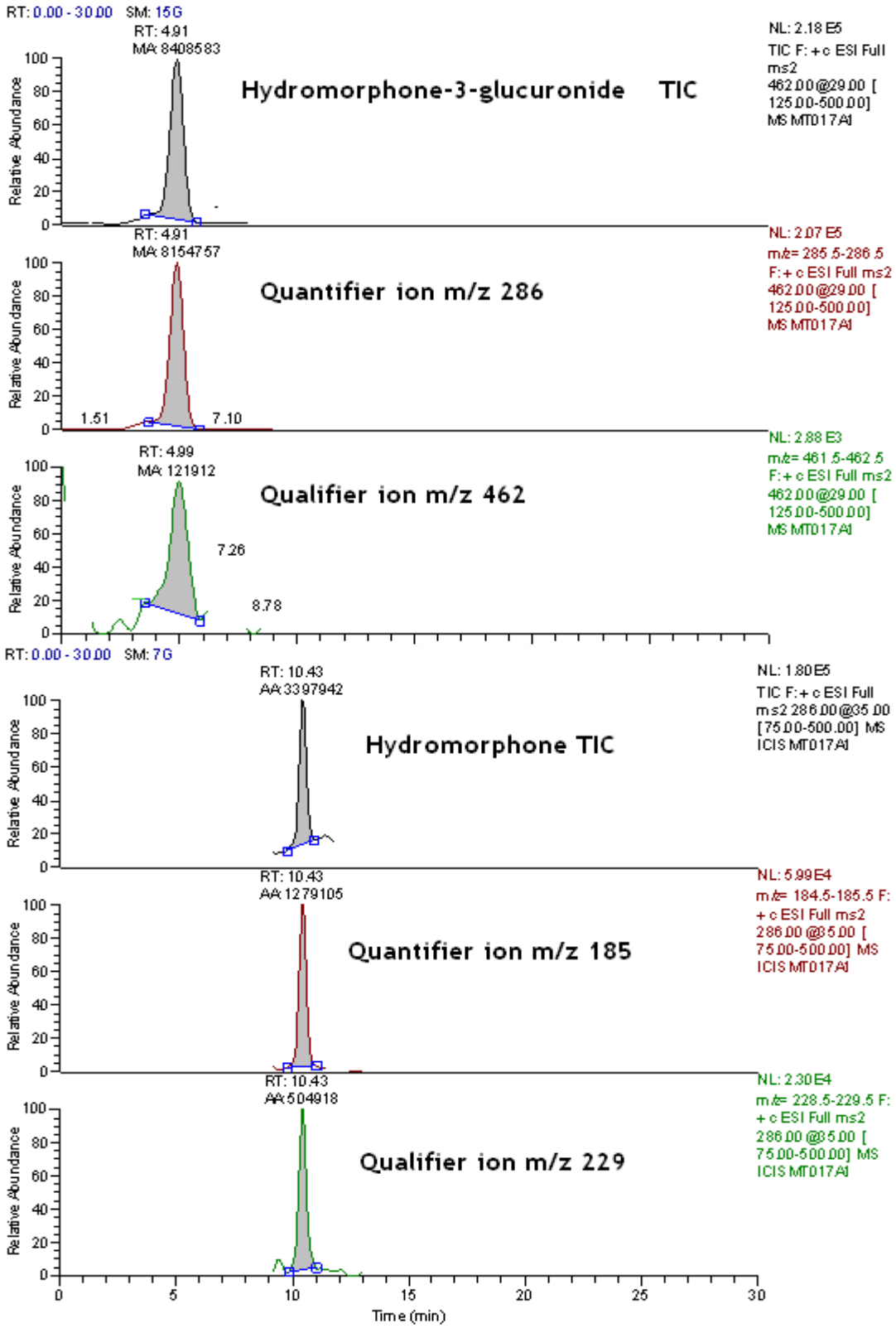


Figure 5-8: Reconstructed mass chromatograms for hydromorophone and its glucuronide in case 32.

5.5 Conclusions

The LC-MS/MS method developed has been validated for the simultaneous determination of 24 opioids in human whole blood, including, for the first time in human whole blood, naloxone-3-glucuronide. Although a large number of drugs of interest were included in the method, acceptance criteria for linearity, precision, and recovery for all analytes were achieved. The method was found useful for differentiating between users of heroin and other opioids, such as codeine and morphine, and for determining the survival time in deaths attributed to heroin use.

6 Analysis of Diamorphine and its Metabolites in Paediatric Plasma Samples

6.1 Introduction

Although diamorphine (DIM) is a widely abused drug around the world, pharmaceutically prepared DIM has been used successfully for the treatment of diamorphine-dependent patients and for the management of moderate to severe pain resulting from surgery or illnesses such as cancer [269,270]. DIM is more potent and faster acting than its precursor morphine (MOR) and can pass the blood-brain barrier easily due to the presence of two acetyl groups in its structure, compared to hydroxyl groups in MOR [123]. It also produces more euphoria and less depression than morphine. The high water solubility of DIM hydrochloride allows it to be injected in small volumes [269,271-274].

DIM metabolite concentrations have been found to be influenced by the route of administration [270]. Intravenous diamorphine (IVDIM) is considered the gold standard route of therapeutic administration due to the rapid delivery of the correct quantities of analgesic needed for pain management. However, alternative ways of administration are often preferred by recreational users. Smoking and intranasal administration (snorting) of diamorphine are less efficient than IVDIM but offer many advantages such as being 'needle free', leaving no external marks on the user's body, being inexpensive and reducing HIV infection resulting from needle sharing amongst users [129,144,270].

Clinically, the intranasal route of DIM administration provides as much effective analgesia as the intramuscular route, with fewer side effects compared to intravenous administration [270,275]. It has been found to be well tolerated with an acceptable safety profile. High concentrations of DIM hydrochloride can be dissolved in small volumes of water (0.1 mL) and then dropped into the nasal cavity. It has been reported that the potency of intranasal DIM is reduced due to swallowing of DIM from the nasal cavity. DIM is more likely to be hydrolysed, its administration half-life is reduced to 5 min and it has lower bioavailability when compared to the intravenous route of administration [270,275].

Children are often exposed to accidents after which admission to hospital is required, including childhood fractures which result in severe pain and which account for 20/1000 of the population/year [276]. There are still problems encountered in providing immediate pain relief due to limitations in the 'therapeutic armoury' and to problems in route of administration and communication [277]. Routes of administration using needles i.e. intravenous and intramuscular have been found to cause distress and pain In children. In addition, oral administration produces less efficient analgesia and rectal administration is not preferable for patients because of difficulty in obtaining consent [270,274].

In recent years intranasal DIM (INDIM) has been recommended as an alternative to intravenous administration for the treatment of severe acute pain in children. This provides a less traumatic means of administering rapid powerful analgesia to children, in whom obtaining intravenous access may be technically difficult and distressing, without decreasing the effectiveness of the analgesic properties. In addition, lower side effects have been reported [270,274,275,278].

Questions must be answered with rewpect to each new medicine for pain relief regarding the potential risks and benefits of its use. However, the degree of efficiency of DIM analgesia for children is rarely addressed by scientific studies and much of the evidence and expertise comes from studies in adults.

The efficiency of INDIM has been compared with intramuscular MOR (IM-MOR) and it was found that 0.1 mg/kg of INDIM is as efficient as 0.2 mg/kg of IM-MOR in children aged 3-16 years with clinically diagnosed limb fractures. In that study the unwanted side effects of MOR were not observed with INDIM [277]. INDIM and IM-MOR were compared in these children using pain scores. Although both administration routes were found effective and to have 'expedient pain reduction', lower pain scores were achieved with INDIM at 5, 10, and 20 minutes. Also, INDIM was found to provide quicker pain relief than IM-MOR. INDIM was considered to be much safer and was preferred by patients and staff as it was needle free. An INDIM spray was recommended as an

alternative to IM-MOR for pain relief in children and teenagers who were diagnosed with clinical fractures [274].

Hallett *et al* [279] reported on the acceptability and effective self-administration of INDIM, which was found to produce sufficient analgesia for postoperative pain management. They recommended further investigation into INDIM analgesia. Pharmacokinetic data was not reported but the results were based on questionnaires completed by both patients and nurses. The efficacies of IVDIM and INDIM were compared during the early postoperative period in 52 subjects (26 patients for each group). Pain scores recorded after IVDIM were significantly higher than in the INDIM patients and no side effects were observed with INDIM compared to IVDIM, which caused a higher incidence of vomiting. The authors indicated that the lack of side effects was due to the estimated 50% lower bioavailability, which was a consequence of higher body weights or else difficulties in operating the INDIM delivery device properly, resulting in a decrease in analgesia and associated side effects [275].

6.2 Diamorphine metabolites and analgesia

DIM is known to act as a pro-drug and to achieve its analgesia via its active metabolites due to its short half-life [5,75,270,280,281]. In fact, DIM and its major metabolite morphine-3-glucuronide (M3G) have been found to lack affinity for opioid receptors. However, M3G has been reported to have CNS stimulatory effects and to contribute to the side effects of morphine [2,76,282]. In contrast, 6-monoacetylmorphine (6-MAM), MOR and morphine-6-glucuronide (M6G) bind to opioid receptors and are considered to be active DIM metabolites [283].

Although the plasma half-life of 6-MAM is also short, it is longer than that of DIM [75]. It is believed that the initial analgesic effects of DIM depend on the efficacy of 6-MAM [270,280,281]. The fast degradation of DIM to 6-MAM produces a metabolite with increased affinity for opioid receptors [280,281]. 6-MAM is metabolised to MOR, which is also an active metabolite of DIM and which reaches its peak concentration shortly after DIM intravenous injection together with DIM and 6-MAM. MOR has been fully studied and it is considered to be one of the most effective drugs used for pain management in illness or disease, especially cancer [76,282]. Respiratory depression, nausea and vomiting

are the most common adverse effects associated with use of MOR for treatment [271,282].

MOR is mostly metabolised via glucuronidation to morphine-3-glucuronide and less than 10% is metabolised to morphine-6-glucuronide (M6G) [141]. The analgesic effect of M6G is equal to or more potent than MOR itself, with fewer side effects due to its high affinity for opioid receptors [2,282,284-286] and M6G has been found to be more efficient than MOR in the treatment of postoperative pain. However, M6G crosses the blood-brain barrier much more slowly than MOR and with difficulty, and so its analgesic effect is delayed compared to DIM or MOR [285-287]. Because of this, it has been suggested that M6G is responsible for the late stages of diamorphine impairment in drivers [288].

On the other hand, the role of M6G in DIM intoxication in a subject with renal failure or with chronic diamorphine abuse is known [125,126,132,148,165,289-294]. Severe side effects of M6G in patients with renal failure refer to the reduction of its metabolism and elimination leading to accumulation in the tissues which is a result of acidosis and changes in plasma protein binding [283,295]. Furthermore, only a short space of time (10 minutes) is required for M6G to reach the brain compared to that of normal patients [282,296].

MOR is also known to metabolise to normorphine (NMOR), accounting for approximately 5% of MOR [132]. However, NMOR was not detected in any of the published DIM pharmacokinetics studies. Also, the concentration of NMOR was found to be much higher in codeine cases than heroin cases and this is explored further in Chapter 5. The detection of NMOR may be dependent on the route of administration as codeine is usually ingested orally and heroin is usually injected and NMOR is likely to form after oral administration [248,283,297]. NMOR is a secondary codeine metabolite produced by N-demethylation of norcodeine after codeine administration [298].

In conclusion, DIM and all of its metabolites should be determined in pharmacokinetic studies owing to their contributions to DIM analgesia and toxicity [75]. Re-evaluation of studies of the role of morphine glucuronides in clinical analgesia and their contribution to side effects after DIM or MOR intake has been suggested [283].

6.3 Aims

IVDIM is the 'gold standard' and the method of choice for pain relief. Many clinicians believe that IVDIM delivers more rapid and effective analgesia than INDIM but no scientific evidence has been reported [276]. Although both IVDIM and INDIM have been used in the UK for many years for pain relief following fracture accidents in children [273], no direct comparison has yet been made of their pharmacokinetic profiles in children. Pharmacokinetic data after IVDIM administration in children is available but DIM concentrations have not been reported and no pharmacokinetic data is available after INDIM administration in children.

A sensitive technique for the detection and quantification of DIM and its metabolites is essential due to the low concentrations of DIM and metabolites in children's plasma, which is a result of the low dose of DIM given and the limited sample volume obtained from children (0.2 mL or less). In addition, DIM can be easily hydrolysed to 6-MAM during sample preparation and extraction, so this must be considered when developing a solid-phase extraction method to prevent the hydrolysis of DIM.

The initial aim of this work was to develop and validate a method for the determination of DIM and its metabolites, namely 6-MAM, MOR, M3G, M6G and NMOR in human plasma. Previous methods for the detection of DIM and its metabolites were reviewed for better understanding of the chromatographic behaviour of the analytes of interest. The second part of this study was aimed at obtaining pharmacokinetic data for DIM and its metabolites in children following intravenous and intranasal administration in a blind study. Since DIM is known to act as a pro-drug and to achieve analgesia via its metabolites, it was intended that the concentrations of active DIM metabolites would be used to evaluate whether or not INDIM can deliver rapid and efficient analgesia in children comparable to that obtained with IVDIM.

6.4 Review of previous analytical methods for the determination of diamorphine

Many analytical methods have been described and used in forensic and clinical toxicology for the quantification of DIM but have not yet been reviewed. As methods for the quantification of morphine and its metabolites have been reviewed elsewhere [76,101,299], only analytical methods for the quantification of DIM are reviewed below. Previous analytical methods for quantification of DIM and its metabolites in biological samples are summarised in Table 6-1.

The high deposition of parent drug in hair compared to metabolites has been reported and many applications were published in the early 1990s, including a GC-MS method for the determination of DIM in hair by Goldberger *et al* [300]. This method was used to differentiate between DIM and other opiate users by the presence of DIM and its marker (6-MAM) in hair samples, which are difficult to determine in blood, plasma and urine due to the short half-life of both metabolites. Liquid-liquid extraction was efficient for isolating DIM, 6-MAM and MOR from hair samples with recoveries ranging from 55-75%. Methanolic hair extracts were diluted with 2 mL of deionised water and saturated sodium bicarbonate (pH 8.4). This mixture was extracted using 7 mL of (toluene: heptane:isoamyl alcohol 70:20:10 v:v:v). The extracts were then derivatised using MBTFA before analysis by GC-MS. The column used was HP-5 (25 m X 0.32 mm, 0.17μm coating) and the quantification limit of all analytes was 5 ng/50 mg hair. This method suffered from interference with the diamorphine peak.

Goldberger *et al* [301] also reported a method involving SPE and GC-MS for detection of the same analytes in blood, plasma, saliva and urine. CleanScreen SPE cartridges were used for extraction of analytes with some modifications. Acetonitrile was used for preparing standard solutions and as an SPE wash solvent instead of methanol to minimise the hydrolysis of DIM to 6-MAM. The SPE elution solvent was methanol, as commonly used elution solvents for CleanScreen (methylene chloride: isopropanol: ammonia) caused promotion of diamorphine hydrolysis. In this method there was no interfering peak observed with diamorphine. The calibration curves were linear over the

Table 6-1: Procedures for quantification of diamorphine and its metabolites in biological samples published between 1991-2009

Analytes	Sample types [*]	Extraction SPE [¥] & LLE [§] Chromatography method	Analytical method	LOD [#] / LOQ [**] (ng/mL)	Ref.
DIM, 6-MAM MOR	H	LLE: GCCL[&*] : HP-5 (25 m X 0.32 mm, 0.17 µm)	GC-MS [##]	5ng/50mg	[300]
DIM, 6-MAM MOR	B, P U, Sa	SPE: Clean Screen (ZSDAU020) GCCL: RTx5 (15 m X 25 mm X 5 µm)	GC-MS	~ 1 ng/mL	[301]
DIM , 6-MAM MOR , NM	P, Sa U, H	SPE: Clean Screen (DAU, 200mg) GCCL: HP-1 (12 m X 0.2 mm X 0.33 µm)	GC-MS	~ 1 ng/mL	[302]
DIM, 6-MAM MOR	B U VH	LLE: GCCL: DB-1 (30 m X 0.32 mm X 0.25 µm)	GC-MS	10 50 100	[303]
DIM 6-MAM MOR M3G M6G	S	SPE: Bond Elut (C8, 50 mg)HPCL*[#]: RP-C18 column (250 X 4 mm; 5 µm)	GC-MS & HPLC-Fluorescence Detector	n.d n.d 10 3 10	[304]
DIM 6-MAM MOR M3G M6G NM	P	SPE: ODS C18 HPCL: Spherisorb C18 ODS-2 column (125 X 2 mm; 3 µm)	HPLC- coupled with photo diode array detector	25 25 25 25 25 25	[305]
DIM 6-MAM MOR M3G M6G	S	SPE: C2-substituted silica HPLC: Supelco LC-SI (250 X 2.1 mm; 5 µm)	LC-APCI-MS	0.5 4 4 1 4	[178]
6-MAM MOR M3G M6G	B U VH CSF	SPE: Bond Elut C18 (200 mg) HPCL: Ecocart column (125 X 3 mm)	LC-APCI-MS	0.5 0.1 1 1	[177]
DIM, 6-MAM MOR, NM	U	Direct injection HPCL: Synergi Polar RP (150 X 2 mm, 4 µm)	LC-APCI-MS/MS	Between 10-100	[142]
DIM, 6-MAM MOR	U	SPE: Online switching SPE SCX HPCL: SCX (150 X 1.5mm)	LC-ESI-MS [&]	0.1-3	[306]
DIM 6-MAM MOR	H	SPE: Chrombond drug (3 mL, 200 mg) GCCL: HP-5MS (30 m X 0.25 mm X 0.25 µm)	GC-MS	0.04 / 0.21 0.02/0.15 0.03/0.11	[307]
DIM, 6-MAM MOR, M3G M6G	P	SPE: Oasis MCX HPCL: RP Zorbax Bouns column (150 X 4.6 mm X 5 µm)	LC-ESI-MS/MS [&&]	Not mentioned	[75]
DIM 6-MAM MOR	S	Direct injection HPCL: Kromasil C18 (250 X 4 mm, 5 µm)	HPLC coupled with ultraviolet detector	23 & 36 15 & 28 11 & 23	[308]

[*] Sample types: B: Blood; CSF: Cerebrospinal fluid ; H: Hair; P: Plasma; S: Serum, Sa: Saliva, U: Urine; VH: vitreous humor; [#]LOD: Limit of detection; [**] LOQ: Limit of quantification; [¥] SPE: solid phase extraction; [§] LLE: liquid-liquid extraction;
[##] GC-MS: Gas Chromatography-Mass Spectrometry; [&] LC-APCI-MS or LC-(ESI)-MS: Liquid chromatography coupled with atmospheric pressure chemical ionisation (electrospray ionisation) mass spectrometry, [&&] LC-ESI-MS/MS: Liquid chromatography coupled with electrospray ionisation tandem mass spectrometry, [&*] GCCL: Gas chromatography column; *[#] HPCL: Liquid chromatography column.

range 1-500 ng/mL for all analytes of interest with correlation coefficients greater than 0.995. The limit of detection for all analytes was 1 ng/mL and intra- and inter-assay precisions were between 1.1-4.2% and 5.4-8.9%, respectively. This method was applied to clinical samples and was found sufficient for the determination of the analytes of interest. Morphine glucuronides were not included in this study and no hydrolysis method was involved as this method determined only free morphine.

Wang *et al* [302] described a method for the determination of DIM, 6-MAM, MOR and NMOR in hair, plasma, saliva and urine. Cocaine metabolites were also included in the study. The SPE method of Goldberger *et al* was used [301] but analytes of interest were eluted with methylene chloride: isopropanol: ammonia (80:20:2 v:v:v). Analytes were analysed by GC-MS as trimethylsilyl derivatives using a fused-silica capillary column (12 m X 0.2 mm X 0.33 µm). The method focused on hair analysis and validation parameters were not provided for the other biological specimens. DIM and 6-MAM tested positive in one urine case sample while other DIM metabolites were detected in all cases. In saliva, DIM and 6-MAM were determined and achieved their peak concentrations at 10 min. However, the authors did not exclude the presence of DIM, 6-MAM and MOR as a result of re-contamination of the oral cavity.

In 1997, Guillot *et al* [303] described a procedure for the identification and quantification of DIM, 6-MAM and MOR based on liquid-liquid extraction at pH 9.5, derivatisation at room temperature using propionyl anhydride and ion trap GC-MS analysis. Recoveries of analytes of interest were 95% for DIM and 80% for 6-MAM and MOR. The limits of quantification were 10, 50 and 100 ng/mL, respectively. Intra and inter-assay precisions ranged from 5.3-8.1% and 5.2-11.6% in blood and urine matrices, respectively. There was a significant loss of DIM during the derivatisation process and at temperatures higher than 60^{o}C. The acetyl groups of DIM were easily substituted with propionyl groups used for the derivatisation procedure. This problem was solved by using 4-dimethylaminopyridine as catalyst at room temperature.

Skopp *et al* [304] developed an HPLC method coupled with fluorescence detection for the quantification of MOR and its glucuronides in serum, adapted from previous work reported by Goldberger *et al* [301]. SPE with C8

columns was used for extraction of MOR glucuronide. The calibration curves were linear over the ranges 5-500 and 15-500 ng/mL for M3G and MOR and M6G, respectively. The lower limits of detection were 10, 3 and 10 ng/mL and recoveries were 71%, 71% and 62% for MOR, M3G and M6G, respectively. Intra and inter-assay precisions were less than 8%. These methods were applied to pharmacokinetic analysis after intranasal and intramuscular DIM administration. However, validation data were not reported for DIM and 6-MAM even though SPE columns were used which were different from those reported in the original GC-MS method [15].

Bourquin *et al* [305] described an HPLC-DAD method for the simultaneous detection of DIM, 6-MAM, MOR, M3G, M6G and NMOR in human plasma. SPE with C18 ODS-2 cartridges was applied for extraction of analytes of interest using 1.5 mL of plasma diluted with 4.5 mL of 0.5 M carbonate buffer (pH 9.3). Columns were then washed using 20 mL 0.005 M of carbonate buffer (pH 9.3) and 0.35 mL of 40% acetonitrile in 0.01 M phosphate buffer (pH 2.1). DIM metabolites were eluted with 2 x 0.6 mL of 40% acetonitrile in 0.01 M phosphate buffer (pH 2.1). HPLC separation was achieved using a Nucleosil C18 ODS column. Recoveries of analytes ranged between 88-98%. Linear calibration curves were reported over the range 25-5000 ng/mL with 25 ng/mL as the LLOQ for DIM and its metabolites. The intra- and inter-assay precisions for low, medium and high concentrations of the analytes were less than 5%. Plasma samples from pharmacokinetic studies and intoxication cases were measured by this method.

Zuccaro *et al* [178] developed a normal phase LC-APCI-MS method for the quantification of DIM, 6-MAM, MOR, MOR, M3G and M6G in serum. Analytes of interest were extracted with C2 SPE cartridges; nalorphine was used as internal standard. One millilitre of serum was submitted to SPE which was pre-conditioned with one column volume of methanol and deionised water and two column volumes of 0.001 M carbonate buffer (pH 9.3). Then, the cartridge was washed with one column volume of 0.001 M carbonate buffer (pH 9.3) and eluted with one column volume of methanol. DIM and metabolites were separated using a Supelcosil LC-Si column (25 cm X 2.1 mm, 5 µm) and mobile phase consisting of methanol: acetonitrile:water:formic acid (59.8: 5.2: 34.65: 0.35 v:v:v:v). Recoveries of DIM, 6-MAM, MOR, M3G

and M6G ranged between 72-74%, 99.5-99.8%, 99-99.2%, 77.2-79.6% and 43-44.6% and LOQs were 0.5, 4, 4, 1 and 4 ng/mL respectively. Pharmacokinetic studies in mice after diamorphine administration were carried out using this method.

Bogusz *et al* [177] described a reverse phase LC-APCI-MS method for the determination of 6-MAM, MOR, M3G and M6G in blood, urine, vitreous humour and cerebrospinal fluid. DIM metabolites were extracted using Bond Elut C18 SPE. One millilitre of plasma was loaded on the SPE column which was washed using 0.01 M of ammonium carbonate buffer (pH 9.3). Analytes of interest were then eluted with 0.5 mL methanol-0.5 M acetic acid (9:1 v:v). This method did not quantify DIM but it has been used for quantification of diamorphine in two different pharmacokinetic studies [309,310]. Although these two studies used LC-ESI-MS for the analysis and a different mobile phase from that used by Bogusz *et al* [177], no validation data was given. In these studies, DIM and its metabolites were separated using a mobile phase consisting of 5 mmol/L ammonium formate buffer (pH 3), methanol, and acetonitrile[309,310], whereas, in the method of Bogusz *et al* [177], separation of DIM metabolites was based on 50 mM ammonium formate and acetonitrile. The recoveries of analytes of interest ranged from 82-89%. Calibration curves were linear over the range 5-500 ng/mL and inter-day precision was less than 10%.

An on-line switching SPE method using SCX cartridges coupled with LC-ESI-MS was developed and validated by Katagi *et al* [306] for the identification of DIM, 6-MAM and morphine in human urine. Separation of analytes of interest was performed using a Capcell Pak SCX column (150 X 1.5mm, 5 µm) and the mobile phase consisted of 10 mM ammonium acetate (pH 6): acetonitrile (30:70 v/v) and 30 mM ammonium acetate. The SPE recoveries were 88%, 89% and 86% and LODs were 0.1, 0.5 and 3 ng/mL for DIM, 6-MAM and MOR respectively. Linear calibration curves were obtained over the concentration ranges 1-100 and 10-1000 ng/mL for DIM and 6-MAM and MOR, respectively. Urine samples from cases of opioid intoxication were analysed using this method.

Dams *et al* [142] reported a direct injection method for the determination of DIM, 6-MAM, MOR and NMOR using an LC-APCI-MS/MS method. Analytes of

interest were separated using a Synergi Polar RP column (150 X 2mm, 4µm) and gradient mobile phase with 10 mM of ammonium formate-0.001% formic acid (pH 4.5) and acetonitrile. Although good precision and linearity were obtained, high LODs and LOQs were reported in the range 10-100 ng/mL, which are very high, and no case samples were tested. Correlation coefficients greater than 0.99 were obtained over the range 10-10,000, 50-10,000 and 50-10,000 ng/mL for DIM and 6-MAM, MOR and NMOR, respectively. Intra-assay and inter-assay precisions were less than 15.8% and 16%, respectively.

Musshoff *et al* [307] described a GC-MS method to quantify DIM, 6-MAM and MOR in hair. Hair extracts were reconstituted with phosphate buffer (pH 6) and loaded on Chromabond SPE cartridges. The columns were then washed using 1 mL of water and 2 mL 0.01 phosphoric acid. DIM metabolites were eluted with 2 mL of dichlormethane/propanol/ammonia (80:20:2 v:v:v). GC-MS was performed using a HP-5MS fused silica capillary column (30 m X 0.25 mm, 0.25 µm). Recoveries ranged between 78.8-95.3%, 90.5-96.5% and 92.3-95.1% for DIM, 6-MAM and MOR, respectively. LODs and LOQs were determined to be 0.04, 0.02 and 0.03 and 0.21, 0.15 and 0.11 ng/mL, respectively. Intra-assay and inter-assay precisions were less than 9%. Hydrolysis of DIM to 6-MAM and to MOR was evaluated and 5% degradation was observed during the extraction.

Rook *et al* [75] reported a reverse phase LC-ESI-MS/MS method for the simultaneous determination of DIM, 6-MAM, MOR, M3G and M6G in human plasma. Acidified plasma was submitted to mixed mode SPE (MCX Oasis), which was then washed using acetic acid (pH 3) and eluted using 0.5% ammonium acetate in methanol. Samples were kept on ice during the pre-treatment to minimise the hydrolysis of DIM; also, extracts were collected in elution tubes containing 50 mM ammonium acetate buffer (pH 3). In addition, samples were evaporated carefully until 250 µl was left before injection into the LC-MS/MS instrument.

Gradient elution was used for the separation of DIM metabolites based on a mobile phase consisting of (A) 5 mM ammonium formate adjusted to pH 4 and (B) acetonitrile, at a flow rate of 1 mL/min. SPE recoveries ranged between 79.8-86.6%. Intra-assay and inter-assay precisions were within the acceptable

limits and were ≤ 20% at the LLOQ and ≤ 15% for other samples. However, LODs and LOQs of analytes of interest were not mentioned.

Direct injection and micellar liquid chromatography (MLC) with UV detection of DIM, 6-MAM and MOR in serum was described by Capella-Peiro *et al* [308]. Analytes were separated using a Kromasil C18 coulmn (Scharlab, 250 x 4 mm, 5 μm) at a flow rate of 1 mL/min. Good recoveries of analytes of interest were observed at low, medium and high concentrations and ranged between 97.7-100.3%. Intra-assay and inter-assay precisions were lower than 2% and 3% respectively. LODs and LOQs ranged from 11-23 and 23-36 ng/mL respectively. Real case samples were tested using this method but only 6-MAM and MOR were detected.

6.5 Review of previous studies of the stability of diamorphine

Stability of analytes during extraction and analysis has been recommended as part of full method validation for a more reliable method of quantification [46,47,53]. Stability of DIM was studied in stock solutions, in the auto-sampler and during extraction. The stability of DIM in solution and in solid powder was studied by Wijesekera *et al* [311]. DIM in both conditions was stored at ambient temperature (26 ^{o}C) and under refrigerated conditions (6-8 ^{0}C). They found a great loss of DIM in methanolic solution by 90.8% and 70% of DIM content at ambient and refrigerated conditions, respectively. Complete degradation of DIM to 6-MAM occurred after 8 weeks of storage at ambient temperature. The average percentage of degradation was found to be less than 21% and 17% of DIM content with DIM powder in ambient and refrigerated conditions, respectively.

DIM detected in human hair was reported to be stable and <10% degradation of DIM to 6-MAM was observed during the extraction [300]. Goldberger *et al* [301] reported less than 5% degradation of DIM during extraction when acetonitrile was used for preparing standard solutions and for SPE wash solvent. Extracts were reconstituted in acetonitrile and analysed. DIM, 6-MAM and MOR were found stable in the auto-sampler for up to 18 hours.

In another study, sodium fluoride was added to samples during collection and to SPE buffer. A deuterated internal standard of DIM was spiked in order to monitor DIM hydrolysis. Degradation of DIM to 6-MAM was limited to 10% [302]. DIM, 6-MAM and MOR were found stable for several weeks at room temperature after reconstitution with chloroform-0.1% pyridine [303].

A procedure using C2 SPE cartridges and LC-APCI-MS reported by Zuccaro *et al* [178] failed to detect DIM in mice serum despite the low limit of quantification obtained in this method. DIM stability was not investigated and could be the reason of complete hydrolysis of DIM to 6-MAM. However, an LC-ESI-MS method and C8 SPE procedure was successful at detecting DIM in human subjects [305].

In another study, heroin was detected in heroin user urine collected immediately after injection [306]. Stability of DIM during extraction was not studied in this study. Dams *et al* [142] found DIM unstable after storage at room temperature for 24 hours, but degradation of DIM was not observed when stored at 4 ^{o}C for 24 hours, -20 ^{o}C for five days and after being subjected to a 5 freeze/thaw stability experiment. A slow hydrolysis rate for DIM may be due to the acid pH of urine.

Musshoff *et al* [307] carried out an auto-sampler stability study on the degradation of DIM to 6-MAM and to MOR. Spiked hair samples were extracted and analysed after 0, 1, 3, 12, 18, and 24 hours. No degradation of DIM was reported during the storage period at room temperature.

Direct injection of serum samples by MLC failed to detect DIM in real case samples, however, 6-MAM and MOR were detected [308]. The period of sample collection and sampling was not controlled in this study; therefore, it was not surprising that DIM was completely converted to 6-MAM and MOR. Rook *et al* [75] reported a full stability study of DIM, 6-MAM, MOR, M3G, and M6G. They found analytes of interest stable in a short-time stability test in a water/ice bath for 60 min. Stability of stock solutions of analytes of interest was investigated after storage at 4 ^{0}C for 17 days and at -20 ^{0}C for 25 months. In addition, long-term stability was performed using 10 patient plasma samples which were stored at – 20 ^{0}C for 14 months. Analytes of interest were also found stable

when subjected to three freeze-thaw cycles. In that study, degradation of DIM was prevented using an ice/water bath and acidified plasma after thawing plus an acidic mobile phase at pH 4 for analyte separation and ionisation.

6.6 Review of previous studies on the pharmacokinetics of diamorphine and its metabolites

Table 6-2 defines some pharmacokinetics terms that will be used in this Chapter. Pharmacokinetics of DIM have been reported using many routes of administration. Previous pharmacokinetic studies are listed in Table 6-2. In one study, the peak concentrations of DIM, 6-MAM and MOR in subjects receiving 12 mg of DIM intravenously were achieved at 2 min after injection and concentrations were 141, 151, and 44 ng/mL, respectively [312]. In another study, DIM and 6-MAM reached their peak concentrations rapidly with concentrations of 1490 and 3410 ng/mL after injection of 200 mg of DIM, and levels of both analytes rapidly decreased within two minutes to 814 and 1887 ng/mL, respectively. In that study MOR reached its peak concentration after 6 minutes with 166 ng/mL while peak concentrations of M3G and M6G were detected after 15 minutes of injection and were 3850 and 399 ng/mL, respectively [305].

Cone *et al* [144] published pharmacokinetic data after intranasal and intramuscular administration of 6 and 12 mg of DIM hydrochloride in six healthy adult subjects. Samples were collected from 8 minutes to 24 hours following administration. Peak concentrations of DIM were achieved at 5 min. Levels of DIM metabolites in blood were found dose-dependent in both routes of administration (INDIM and IM-DIM). In that study, MOR following INDIM had a longer half-life (2-4 hours) compared to DIM and 6-MAM. Blood levels of DIM metabolites were higher after 6 mg IM-DIM compared to 6 and 12 mg INDIM which suggests less absorption of DIM due to the hydrolysis of DIM to 6-MAM and MOR in nasal mucosa. The terminal half-life and AUC of DIM following INDIM was 4.2 minutes and 24.5 μg.h/L [144], respectively. DIM was detected in plasma samples up to 25 minutes in most cases following INDIM regardless of

dose. C_{max} was lower with 6 mg INDIM than 12 mg INDIM and ranged between 4.3-13.6 and 14-24.5 ng/mL, respectively.

Table 6-2: Pharmacokinetics parameters used in this study.

Term	Definition
PK	Pharmacokinetics: the study of the quantitative relationship between administration dose of a drug and the observed plasma/blood or tissue concentration or the study and characterisation of the time course of drug absorption, distribution, metabolism and execretion.
T ½	Half-life: a pharmacokinetic term to describe the approximate time taken to halve blood concentration of the drug during the terminal phase of drug elimination.
T_{max}:	Time of maximum observed concentration. For non-steady state data, the entire curve is considered. For steady state data, Tmax corresponds to points collected during a dosing interval.
C_{max}:	Maximum observed concentration, occurring at Tmax.
AUC_{all}:	Area under the curve from the time of dosing (dosing time) to the time of the last observation. If the last concentration is non-zero AUClast=AUCall. Otherwise, AUCall will be greater than AUClast as it includes the additional area from the last measurable concentration
AUC_{last}	Area under the curve from the time of dosing (dosing_time) to the last measurable concentration.
Dosing time	Time of last administered dose. Assumed to be zero unless otherwise specified. Used mainly with steady-state data, which may code time as the time elapsed since the first dose, or the elapsed time since the time of the first dose.
λz	First order rate constant associated with the terminal (log-linear) portion of the curve. Estimated by linear regression of time vs. log concentration.

Table 6-3: Pharmacokinetics data for Diamorphine and its metabolites from literature.

Dose of DIM (mg)	No. of Cases	R *	Analytes	T ½ (min)	C_{max} (ng/mL)	T_{max} (min)	AUC (µg.hr/L)	Ref.
20-60	3	IV	DIM	3	n.d. &	n.d.	57-114	313
6-12	6	IN	DIM	4.2	n.d.	< 5	24.5	144
3-20	2	IV	DIM	3.6	n.d.	n.d.	56.5	312
			6-MAM	9.3	n.d.	n.d.	109	
2.6-10.5	2	IH	DIM	3.3	n.d.	n.d.	n.d.	312
			6-MAM	5.4	n.d.	n.d.	n.d.	
			MOR	18.8	n.d.	n.d.	n.d.	
6	6	IN	DIM	0-6	n.d. -23.3	4.8-15	1.5-3.6	304
			6-MAM	0-26.4	3.8-10.6	4.8-10.2	0.9-7.1	
			MOR	0-102	6.1-6.6	10.2-30.0	6.1-11.7	
			M3G	0-312	32.4-82.3	30.0-180.0	112.6-412.0	
			M6G	n.d.	n.d.	n.d.		
12	6	IN	DIM	n.d. -6	n.d. -44.3	4.8-15	3.7-6.5	304
			6-MAM	0-28.2	4.4-17.4	4.8-10.2	1.7-10.0	
			MOR	0-180.0	5.3-15.0	43.8-90.0	14.1-34.8	
			M3G	0-186	88.2-137.4	60-120	381.5-906.3.	
			M6G	n.d.	n.d. -23.9.0	n.d. -120		
6	2	IM	DIM	n.d. -5.4	45.7	4.8	n.d. -6	304
			6-MAM	n.d. -19.2	22.6	n.d. -4.8	n.d. -10.1	
			MOR	90.0-114.0	7.9	n.d. -10.2	n.d. -16.5	
			M3G	102.0-180.0	93.0	10.2-30.0	240.4-337.1	
200	2	IV	DIM	1.3	1530-2270	n.d.	5.2-8.8	314
			6-MAM	46-52	4620-3400	0.7-1.5	26.3-27.2	
			MOR	182	340-810	3.6-3.9	64.3-48.7	
40-210	8	IV	DIM	3.3	n.d.	n.d.	n.d.	309
			6-MAM	n.d.	n.d.	2.7	n.d.	
			MOR	n.d.	n.d.	6.4	n.d.	
146	8	IV	DIM	3	3960	n.d.	185	310
			6-MAM	3	5740	0.3	257	
			MOR	n.d.	1569	7	128	
			M3G	n.d.	387	10	1469	
			M6G	n.d.	55	58	230	
50	5	CH-D	DIM	n.d.	225.3	1	19.8	315
			6-MAM	n.d.	171.5	1	34.4	
			MOR	143.0	59.9	2.0	179.3	
			M3G	280.1	341.5	120.0	2672.7	
			M6G	269.9	50.8	120.0	390.5	
	5	HE-d	DIM	n.d.	44.3	1	3.4	315
			6-MAM	n.d.	52.4	1	10.7	
			MOR	127.0	37.1	2.0	108.5	
			M3G	252.0	230.8	120.0	1646.7	
			M6G	216.2	36.9	120.0	224.2	
133-450	10	IV	DIM	3.8	3119	n.d.	329	316
			6-MAM	22	1731	n.d.	482	
			MOR	177	829	7.8	2594	
			M3G	276	4287	n.d.	32192	
			M6G	268	678	n.d.	4291	
133-450	12	IH	DIM	3.2	685	2	174	316
			6-MAM	26	289	n.d.	177	
			MOR	184	271	8	1043	
			M3G	283	2284	n.d.	15865	
			M6G	240	461	n.d.	2677	

* R: Route of administration or method of administration (IV: Intravenous; IN: Intranasal; IM: Intramuscular; IH: Inhalation; CH-D: Chasing the dragon; HE-d: Heating device).
& n.d.: Not detected.

In Cone *et al* [144], the plasma peak concentrations of 6-MAM were detected after 0.08 hour in all cases with the exception of one case in which 6-MAM peaked after 0.17 hour (no samples were collected before 8 minutes). C_{max} was lower with 6 mg compared to 12 mg INDIM, in the range of 7.9-13.7 and 8-17 ng/mL, respectively. 6-MAM was determined in two cases up to 0.5 hour after INDIM at concentrations of 2.2 and 4 ng/mL but no DIM was detected after 0.25 hours. Peak concentrations of MOR were in the range of 6.2-26 ng/mL and were little affected by the dose, except for two subjects in which the 12 mg dose produced peak concentrations double that of a 6 mg dose;

Skopp *et al* [304] studied the pharmacokinetics of DIM and its metabolites including morphine glucuronides after injection of 6 mg IM-DIM and 6 and 12 mg IVDIM in four volunteers. Findings were similar to those obtained by Cone at al [144]. DIM reached its peak concentration within 5 min in both routes of administration and blood levels of DIM and 6-MAM were higher in IM-DIM compared to INDIM (no samples were collected before 5 minutes). $T_{½}$ of DIM and 6-MAM for both routes of administrations were 5.4 ± 0.6 and 22.8 ± 4.2 min, respectively. $C_{max,}$ T_{max} and AUC of DIM were 0-44.3 ng/mL, 4.8 minutes and 3.7-6.5 µg/L*hr, respectively. Interestingly, T_{max} for MOR was dose dependent and ranged between 10.2-30.0 and 43.8-90.0 min after 6.0 and 12.0 mg, respectively. $T_{½}$ of MOR after INDIM was estimated to be in the range of 90.0-180.0 minutes and was likely to be higher with 12.0 mg of INDIM. The mean value of T_{max} for M3G was higher after INDIM compared to IM-DIM, and also likely to be dose related with higher blood levels observed with INDIM than IMDIM. M3G plasma levels were 120.0 ± 42.0, 70.8 ± 63.0 and 19.8 ± 9.6 after 6.0 mg or 12.0 mg of INDIM and 6.0 mg of IM-DIM, respectively. The method used could not quantify M6G. $T_{½}$ of M3G was more than 2 hours and ranged between 132.0-312.0 minutes, C_{max} ranged between 32.4-82.0 and 88.0-137.0 ng/mL after 6.0 mg and 12.0 mg of INDIM, respectively.

Jenkins *et al* [317] reported a pharmacokinetic study of IVDIM and DIM inhalation (IH-DIM). $T_{½}$ for DIM was the same for both routes of administration. The AUC reported for IVDIM was 56.5 µg/L*hr and DIM and 6-MAM were detected for up to 60.0 minutes. DIM peak concentrations occurred 2 minutes after IVDIM apart from one case in which the peak concentration was at 10 minutes following injection. T_{max} of 6-MAM was later than for DIM and was at 2 minutes

for most cases. Plasma peak concentrations ranged between 1.0-320.0 ng/mL but 6-MAM could still be detected at levels of 1.3-2.8 ng/mL in some cases up to 60.0 minutes. The T_{max} of MOR was similar to that of 6-MAM and the C_{max} of MOR ranged between 8.4-105.0 ng/mL. MOR level after 60.0 minutes was in the range not detected - 20.6 ng/mL.

Girardin *et al* [310] found a $T_{½}$ of 3 minutes for DIM and 6-MAM after IVDIM. C_{max} was 3960 ± 1369 and 5742 ± 1837 ng/mL, respectively. The AUC was higher with 6-MAM than DIM and was 257 and 185 μg/L*hr, respectively.

MOR is considered to be the active metabolite of DIM; the analgesic effects of DIM are believed to be from its active metabolites MOR [313,318,319]. Two pharmacokinetic studies have been reported in newborn infants and premature neonates after intravenous DIM infusion [273,320]. In these two studies only morphine [273] or morphine and its glucronides [320] were determined. DIM was found safe and efficient and to have a similar metabolic pathway to morphine and its glucuronides as in adult subjects.

Barrett *et al* [273] studied the pharmacokinetics of morphine in 26 newborn premature neonates after a dose of 50 μg/kg. The mean steady state concentration of MOR was 62 ± 22.8 ng/mL with an elimination $T_{½}$ of 8.9 ± 3.3 hours and volume of distribution of 2.7 ± 1.01 L/kg. 6-MAM was detected in only two cases at low concentrations. This concentration was efficient for achieving analgesia and was similar to that in adult subjects, reported to be 20 - 65 ng/mL [321-323].

The pharmacokinetic profiles of morphine and its glucuronides were studied in 19 ventilated newborn infants after an intravenous infusion of DIM at 15 μg. $kg^{-1}.h^{-1}$ after loading doses of 50 and 200 μg/kg. MOR, M3G and M6G were measured at the steady state period and ratios of M3G/MOR, M6G/MOR and M3G/M6G were calculated. The mean steady state concentrations for MOR, M3G and M6G were 86 ± 52, 703±400 and 48±28 ng/mL, respectively. The ratios of M3G/MOR and M6G /MOR at steady state plasma levels were 11 ± 10.8 and 0.8 ± 0.8, respectively. There was no significant difference observed between the two loading doses in reaching MOR steady state, making the low dose preferable and much safer [320].

Rook *et al* [316] reported the pharmacokinetics of IVDIM and IH-DIM. They found no significant changes in $T_{½}$ of DIM metabolites but a noticeable increase in C_{max} and AUC after IVDIM compared to IH-DIM (3119 and 985 ng/mL, and 329 and 52 μg/L*hr, respectively). Bioavailability of IH-DIM was estimated as 52% of IVDIM.

Giradin *et al* [310] found higher a AUC for 6-MAM than DIM, 185 and 257 μg/L*hr, respectively. Gyr *et al* [314] reported AUCs of 5.2, 26-27 and 64.3-84.7 μg/L*hr for DIM, 6-MAM and MOR, respectively. Elsewhere, AUC of DIM was found to be 57-114 μg/L*hr [313] after 3 hours of DIM infusion. Rook *et al* [316] found that the AUC was 174 and 177 μg/L*hr for DIM and 6-MAM, respectively.

6.7 Methods and Materials

6.7.1 Reagents and Standards

Methanol and acetonitrile (HPLC grade) were obtained from BDH (Poole, UK). Ammonium carbonate, formic acid and ammonium hydroxide were also purchased from BDH. Ammonium formate was obtained from Acros Organics (New Jersey, USA). The method was developed using human plasma, which had passed its usable date, obtained from the Scottish National Blood Transfusion Service. Ethical approval for supply of time-expired blood products was given by the given by the Ethics Committee of the Scottish Blood Transfusion Service, Edinburgh.

DIM, DIM-D9 (DIM-D9), morphine (MOR), morphine-D3 (MOR-D3), 6-monoacetylmorphine (6-MAM), 6-monoacetylmorphine-D3 (6-MAM-D3) was purchased from from Promochem (Middlesex, UK). Morphine-3-glucuronide (M3G), morphine-3-glucuronide-D3 (M3G-D3), morphine-6-glucuronide (M6G), morphine-6-glucuronide-D3 (M6G-D3), normorphine (NORM) were obtained from Lipomed (Arlesheim, Switzerland).

All standards and internal standards were obtained as solutions in methanol at a concentration of 0.1 mg/mL or 1 mg/mL and each had a purity of more than 99%. Bond Elut LRC-C18 cartridges were purchased from Varian (CA, USA).

Individual working standards were prepared at a concentration of 1 µg/mL by dilution of the stock solutions. Working mixtures of standards and internal standards were similarly prepared.

6.7.2 Solid Phase Extraction

250 µl of plasma was added to 300 µl of 0.01 M ammonium carbonate (pH 9.3) and 25 µl of the internal standard working solution (1 µg/mL) was added. The mixture was vortexed. The supernatant was applied to a Bond Elut C18 SPE cartridge preconditioned with 2 mL methanol, 1 mL of deionised water, and 2 mL of 0.01 M ammonium carbonate (pH 9.3). The SPE cartridge was washed twice with 1 mL 0.01 M ammonium carbonate (pH 9.3) and then dried for 10 minutes. Retained drugs were eluted with 2 mL methanol. After that, the extracts were evaporated to dryness under nitrogen at 50 ^{o}C. The residues were reconstituted with 80 µl of initial mobile phase and then 20 µl were injected into the LC-MS/MS instrument.

6.7.3 Chromatography conditions

In the current procedure, HPLC methods was as described in a previously method for the analysis of opioids and their metabolites in autopsy blood samples, refer to 5.3.3.

6.7.4 Instrumentation

Analysis of opiates and their metabolites was performed using a Thermo Finnigan LCQ DECA XP Plus ion trap instrument (Thermo Finnigan, San Jose, USA) equipped with a surveyor LC system interface. During the analysis auto-sampler and column oven temperatures were maintained at 4 ^{o}C and 30 ^{o}C, respectively. Ionisation of analytes of interest was carried out using electrospray positive ion mode. The capillary temperature, sheath gas flow rate, auxiliary gas flow rate and collision energies were optimized for each analyte separately. The spray voltage used was 5 kV.

Analytes and their internal standards were identified and quantified based on their retention times and the presence of parent ion and two product ions in

selected reaction monitoring mode (SRM) with the exception of M3G and M6G which were fragmented to a single product ion as follows: m/z310, 328, and 370 for DIM, m/z 211, 229 and 328 for 6-MAM, 201, 229 and 268 for MOR, 254, 229 and 272 for NMOR and m/z 286 and 462 for M3G and M6G. The LC-MS/MS chromatogram was divided into six different segments to enhance analyte responses. The MS/MS parameters are detailed in Table 6-2.

6.7.5 Method Validation

6.7.5.1 Linearity

Two calibration curves were prepared in plasma and extracted by the described method as the study was blind with respect to patient groups: one calibration curve covered low concentrations (0.1, 0.2, 0.3, 0.5, 0.75, 1, 5 and 10 ng/mL) and the other covered high concentrations (5, 10, 20, 25, 50, 100, and 200 and 250 ng/mL). Blanks with no internal standards and blank plasma samples with internal standard at 25 ng/mL were included with each run. Calibration curves were plotted of peak area ratios obtained versus concentration. The linear correlation coefficient (r^2) was obtained for each regression curve.

6.7.5.2 Matrix Effects and Recoveries

Matrix effects and extraction recoveries were measured using the approach of Matuszewski *et al* [51]. For matrix effects, Plasma (obtained from blood bank of West Infirmary Hospital Glasgow University) were obtained from six different human sources and spiked after solid phase extraction with analytes of interest at 5 and 100 ng/mL. Neat standards were diluted in initial mobile phase and injected directly into LC-MS/MS. Matrix effects of endogenous components were calculated by comparing peak areas of these two sets of standards.

Recoveries were investigated for DIM and its metabolites using six different concentrations in plasma across the two calibrations curve ranges (0.5, 1, 5 and 25, 50 and 200 ng/mL). Internal standards were added after extraction

and recoveries were calculated by comparison of the peak area ratios of the extracted and unextracted standards analysed under identical conditions.

6.7.5.3 Limits of Detection and Lower Limits of Quantitation

Sensitivity of the method was evaluated by determining the Limit of Detection (LOD) and the Lower Limit of Quantitation (LLOQ) for each analyte of interest. The lower calibration curve was used for this purpose (n=5) comprising spiked plasma at eight different concentrations 0.1, 0.2, 0.3, 0.5, 0.75, 1, 5 and 10 ng/mL plus blank and blank spiked with internal standards. LODs and LLOQs values were then determined from the calibration curve as described in Chapter 5 (section 5.3.5.3).

6.7.5.4 Intra-assay and inter-assay precision

Three concentrations were used with each calibration curve as QCs to examine the Intra-assay and Inter-assay precision. For the lower concentration calibration curve 0.5, 1 and 5 ng/mL were used as QCs and 25, 50 and 200 ng/mL were used as QCs with the higher concentration calibration curve.

Intra-assay precision of DIM metabolites was determined using QCs (n=5) in one day. The calibration curve of each analyte of interest was prepared using the two calibration concentration ranges mentioned above. The inter-assay precision was measured in a similar manner to the intra-assay precision on five different days.

6.7.5.5 Stability

Stability was assessed using human plasma spiked with the analytes of interest at 5 and 100 ng/mL (n=3). Short-term temperature stability at room temperature was investigated for human plasma stored for 1, 2 and 4 hrs. Freeze-thaw stability of analytes of interest was determined after four cycles on consecutive days. Autosampler stability using reconstituted extracted sample was determined at 24 and 48 hrs after extraction. Long-term stability was determined for analytes of interest at -20°C for periods of 24hrs, 48hrs, 1 week and 1 month. Calibration curves were prepared for each batch of

samples using standards spiked over two calibration curves plus blanks and blanks spiked with internal standards.

6.7.5.6 Specificity

The specificity of the described method was investigated using a mixture of drugs that are routinely detected in forensic toxicology cases (cocaine, benzoylecgonine, cocaethylene, ecgonine methyl ester, amphetamine, methamphetamine, methylenedioxyamphetamine, methylenedioxyethylamphtamine, methylenedioxymethamphetamine, nitrazepam, 7-aminoflunitrazepam, chlordiazepoxide, diazepam, oxazepam and temazepam) at a concentration of 400 ng/mL in whole blood.

In addition, 21 commonly used opioids (codeine, codeine-6-glucuronide, norcodeine, acetylcodeine, hydromorphine, hydromorphone-3-glucuronide, dihydrocodeine, dihydrocodeine-6-glucuronide, naloxone, naloxone-3-glucuronide, Buprenorphine, norbuprenorphine, norbuprenorphine-3-glucuronide, buprenorphine-3-glucuronide, oxycodone, noroxycodone, oxymorphone, dihydromorphine, dihydromorphine-3-glucuronide, dihydromorphine-6-glucuronide and methadone) were extracted and injected at the same concentration to test the effect of the presence of opioids of similar structure on the specificity and selectivity of the described method.

6.7.6 Case samples

Plasma samples were obtained from twenty three children receiving DIM at the A & E department of a city-centre paediatric teaching hospital in Edinburgh. Ethical approval was obtained from the regional ethics committee by the participating paediatricians. Written informed consent was obtained from the parents of each child, and verbal consent from each child.

Twelve children received IVDIM (dose 0.1mg/kg) and eleven subsequent children had INDIM at the same dose in 0.2 mL normal saline dripped into both nostrils. The children were aged 3-13 years, with clinical diagnosis of isolated deforming limb fractures. Sequential blood samples were taken at 2, 5, 10, 20, 30 and 60 minutes post DIM administration. The blood tube was then

centrifuged at 4000 rpm for 2 minutes and plasma was transferred to plain 'Ependorph' tubes that were immediately placed in a -70^{o}C freezer until analysed.

The INDM dose was administered in 0.2 mL of sterile water dropped into both nostrils over a period of 1 minute. At the mid point of the dose, i.e. after 30 seconds, the timing was commenced for the subsequent samples. IVDM was also administered over 1 minute.

Plasma samples were subsequently analysed for DIM, 6-monoacetyl morphine (6-MAM), morphine (MOR), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and normorphine (NMOR) using the method described earler.

6.7.7 Data analysis

Pharmacokinetic data was measured by Dr Alison Thomson from Glasgow Western Infirmary using the pharmacokinetic software package WinNonlin Professional Version 3·3 (Pharsight Corporation, Mountain View, California, USA). The elimination rate constants (k) for IV and IN plasma data were determined by nonlinear regression analysis of the log-linear decline with a 1/weighting (1/concentration) and the elimination half-lives were obtained from 0·693/k. Area under the concentration–time curve from 0 to the last sample (AUC0–t) was determined by the trapezoidal rule and AUC from 0 to infinity (AUC 0–∞) from AUC $_{0-t}$+ C_z /k −C_0/ k, where C_z is the final concentration measurement, k is the elimination rate constant and C_0 is the predose concentration measurement.

6.8 Results

6.8.1 SPE Optimisation

SPE cartridges involved in this study were chosen from a previous method. Sample pre-treatment, washing and elution steps were then optimised. As described in Chapter 5, three SPE cartridges were compared for the detection of opioids and their metabolites including morphine glucuronides, 6-MAM, MOR

and NMOR. Bond Elut Certify® SPE was found sufficient to isolate MOR, 6-MAM and NMOR but loss of morphine glucuronides was observed. Another SPE procedure with the same mixed mode sorbent reported by Rook *et al* [75] and used other studies [269,315,324,325] was not found to provide good recovery of morphine glucuronides. Variations in work environment and equipment could cause this difference; as a result, this method of extraction was excluded. An SPE method using CleanScreen, reported for quantification of DIM, 6-MAM and MOR [301,302,317] was also excluded due to large losses of morphine glucuronides. Methdod optimisation steps are detailed in Table 6-4.

Table 6-4: Optimisation of SPE procedure.

	Recovery %					
	DIM	**6-MAM**	**MOR**	**M3G**	**M6G**	**NMOR**
Sample pre-treatment before SPE application						
0. 0.15 M Hydrochloric acid [75]	93	87	40	7	31	43
0.01 M Ammonium formate pH 9.3 [177]	67	80	76	81	80	88
Citrate buffer pH 3 [301]	30	45	57	5	17	35
SPE wash step optimisation						
Acidified water pH 3 [75]	70	65	83	10	55	58
0.01 Ammonium formate pH 9.3 [177]	103	100	101	72	103	83
0.001 M Ammonium formate pH 9.3 [178]	100	106	105	59	103	82
SPE elution step optimisation						
(DCM: ISO: AM) [302,307]	91	84	92	0	0	98
0.5 % Ammonium acetate in methanol [75]	92	87	92	102	98	104
Methanol [178,301]	91	87	94	107	99	95

6.8.1.1 Sample Pre-treatment

The Bond Elut C18 SPE method used for opioid glucuronides and described in Chapter 5 was applied here with some modifications. Instead of 1:3 sample dilution with ammonium formate buffer, 0.01 M, 1:1 dilution was used to facilitate application of samples through the SPE cartridges and prevent hydrolysis of DIM. Plasma samples were spiked at (50 ng/mL) with analytes of interest before SPE and 25 ng/mL of their corresponding internal standards were added after SPE application. Samples were kept on ice as recommended by Rook *et al* [75] to prevent of hydrolysis of DIM to 6-MAM and to MOR.

Samples were subjected to three different pre-treatment reagents. 0.3 mL pre-treatment reagent (0. 0.15 M hydrochloric acid [75], 0.01 M ammonium formate pH 9.3 [177], or citrate buffer pH 3) [301] were added to 0.25 mL of spiked plasma samples. A good recovery for analytes of interest was observed using 0.01 M ammonium formate buffer: recoveries of DIM, 6-MAM, MOR, M3G, M6G and NMOR were 67, 80, 76,81, 80 and 88% respectively.

Morphine glucuronides were not retained well on SPE cartridges using a lower pH buffer. Recoveries of analytes of interest were 30, 45, 57, 5, 17 and 35% using citrate buffer (pH 3) for DIM, 6-MAM, MOR, M3G, M6G and NMOR, respectively. Hhydrochloric acid was efficient for providing good recoveries of DIM and 6-MAM, which were 93 and 87%; however, lower recoveries were obtained for MOR, M3G, M6G and NMOR which were 40, 7, 31 and 43 respectively. Therefore, 0.01 M of ammonium formate was chosen for sample pre-treatment.

6.8.1.2 SPE Wash Step Optimisation

Wash steps were also investigated involving different wash volumes and different wash reagents: acidified water at pH 3 [75], 0.01 ammonium formate at pH 9.3 [177] and 0.001 M ammonium formate at pH 9.3 [178]. Recoveries of DIM, 6-MAM, MOR, M3G, M6G and NMOR after use of acidified water were 70, 65, 83, 10, 55, 58 %, and after using 0.001 M ammonium buffer were 100, 106, 105, 59, 103 and 82 %, respectively. The recoveries of M3G and other DIM metabolites were improved with use of 0.01 M ammonium formate at pH 9.3

and were 103, 100, 101, 72, 103 and 83 %, respectively. Therefore, the latter wash buffer was chosen and 2 mL of buffer were found more efficient compared with 1 and 3 mL.

6.8.1.3 Elution Step Optimisation

Three different elution solvents were compared: (DCM: ISO: AM) [302,307], 0.5 % ammonium acetate in methanol [75] and methanol [178,301]. High recoveries were obtained for unconjugated metabolites using the first elution solvent which were 91, 84, 92, and 98% for DIM, 6-MAM, MOR and NMOR, but recoveries were zero for morphine glucuronides. However, high recoveries for all analytes of interest were obtained using the last two elution solvents and they were 92, 87, 92, 102, 98, and 104% using the second solvent, and 91, 87, 94, 107, 99 and 95% using methanol for DIM , 6-MAM, MOR, M6G, NMOR and M3G respectively. Therefore, methanol was chosen as the elution solvent. 2 mL of eluant were found more efficient when compared with 0.5, 1, 1.5, 2 and 3 mL of methanol.

6.8.2 Method Validation

6.8.2.1 LC-MS/MS Optimisation

In Chapter 5, 6-MAM, MOR, M3G, M6G and NMOR were determined for the purpose of evaluating of the role of these metabolites in heroin fatalities. The same LC-MS/MS method was used for the analysis of DIM and its metabolites in the current project. DIM was not included with target analytes in the previous method and in addition a different matrix was used; therefore SPE and LC-MS/MS methods were re-evaluated. In addition, more deuterated internal standards were used for increased accuracy and to compensate for matrix effects. As described in Chapter 5, morphine-3-glucuronide-D3 and morphine-6-glucuronide-D3 were found to have cross talk with dihydromorphine-3-glucuronide and dihydromorphine-6-glucuronide, respectively. However, these internal standards could be used with the current study along with deuterated internal standard DIM-D9 because the co-eluting drugs were absent from the clinical samples obtained from children.

Each analyte was dissolved in organic solvent at 10 μg/mL and infused through a T-connection to the MS/MS instrument to obtain the best conditions and responses for LC-ESI-MS/MS. Ionisation of DIM metabolites was carried out using electrospray positive ion mode. The capillary temperature, sheath gas flow rate, auxiliary gas flow rate and collision energies were optimised for each analyte separately. The spray voltage used was 5 kV. Data was then stored as a tune page.

The LC-MS/MS chromatogram was divided into six different time segments or retention widows, one for each analyte and its internal standard, in order to enhance analyte responses. The MS/MS parameters are detailed in Table 6-5. Analytes were identified and quantified based on their retention times and the presence of the parent ions and two product ions in selective reaction monitoring mode (SRM) with the exception of M3G and M6G which were fragmented to a single product ion as given in Table 6-5.

Deuterated internal standards were analysed by selective ion monitoring in Chapter 5. In the present study, deuterated standards were analysed and quantified using SRM as follows; M3G-D3 and M6G-D3 were fragmented to m/z 289, MOR-D3, 6-MAM-D3 and DIM-D9 were fragmented to m/z 201, 211 and 316 respectively. Good chromatographic peak shapes were obtained for DIM metabolites at very low concentrations in the present study as shown in Figures 6-1 and 6-2. The major product ion was used for quantification and the others were used as qualifiers. In the case of M3G and M6G for which no other product ions were formed, 10% of survival product ion was used to qualify the SRM transition. In the case of DIM, three product ions can be obtained and two of them were found at relatively the same amount (m/z 310 and 328). For quantification, m/z 310 was used and m/z 328 and 268 were used as qualifier ions. The major product ions of 6-MAM and MOR are at the same m/z values as their corresponding internal standards which were 201 and 211, respectively. The SRM transition ratios were calculated on five different days using five different calibration curves for both high and low concentrations. The ion intensity ratios of DIM metabolites were 1.0, 1.3, 1.6, 1.4, 2.3, 5.3 and 5.6 for DIM (m/z 310/328), DIM (m/z 310/268), 6-MAM (m/z 211/268), MOR (m/z 201/229), NMOR (m/z 254/229), M3G (m/z 286/462) and M6G (m/z 286/462), respectively (Table 6-5).

Table 6-5: LC-MS/MS parameters for Diamorphine metabolites.

	Parameters	DIM	6-MAM	MOR	M3G	M6G	NMOR
Analytes	Precursor Ion (m/z)	370	328	286	462	462	272
	Product Ion(s) (m/z)	310, 328, 268	211, 268, 226	201, 229, 268	286, 462	286, 462	254, 229
	Quantifier Ion (m/z)	370 →310 370 → 328	328→ 211	286→201	462→286	462→286	272→254
	Qualifier Ion(m/z)	370→268	328→268	286→229	462→462	462→462	272→229
	SRM * transition Quan/Qual	310/328	211/268	201/229	286/462	286/462	254/229
	SRM Transition Ions ratio (R.S.D #)	1 (5)	1.6 (3)	1.4 (4)	5.3 (7)	5.6 (10)	2.4 (2)
	RT & (min)	18.3	14.1	8.2	3.4	6.9	5.0
Internal standards	Internal Standard (IS)	DIM-D9	6-MA-D3	MOR-D3	M3G-D3	M6G-D3	MOR-D3
	IS Precursor Ion(s) (m/z)	379	331	289	465	465	289
	Product Ion (m/z)	379→316	331→211	289→201	465→289	465→289	289→201
	RT & (min)	18.3	14.0	8.1	3.3	6.5	8.1
Analytes and internal standards	Sheath Gas (AU)	15	25	30	20	20	25
	Auxiliary Gas (AU)	5	0	0	10	15	10
	Capillary Temperature (°C)	285	300	300	270	270	270
	Collision energy (%)	38	38	38	28	30	35
	Retention Widow No.	6	5	4	1	3	2

R.S.D: Relative standard deviations
* SRM: Selective reaction monitoring
& Retention time of drugs.

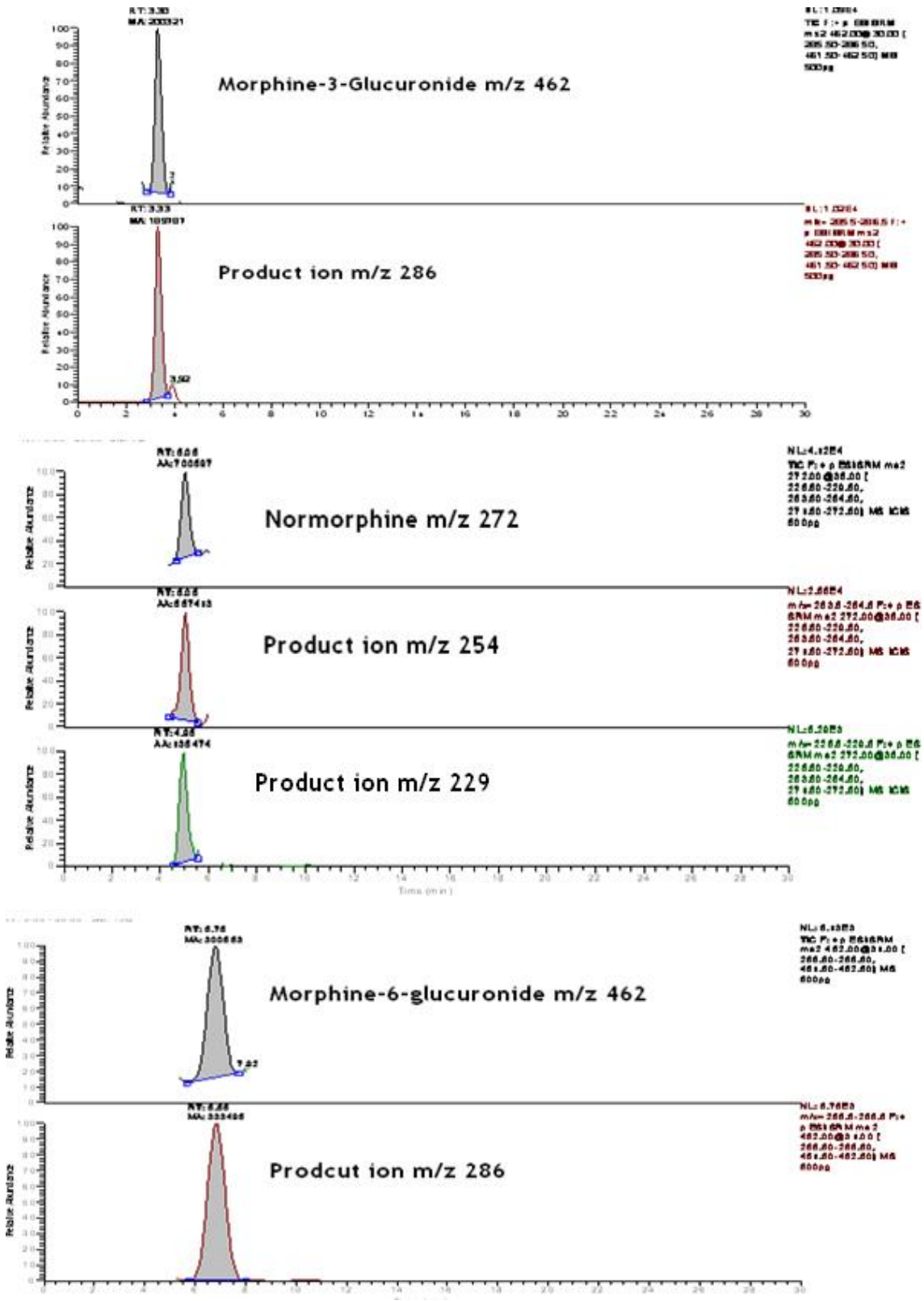


Figure 6-1: SRM chromatograms of morphine-3-glucuronide, normorphine and morphine-6-glucuronide at their LLOQ concentrations.

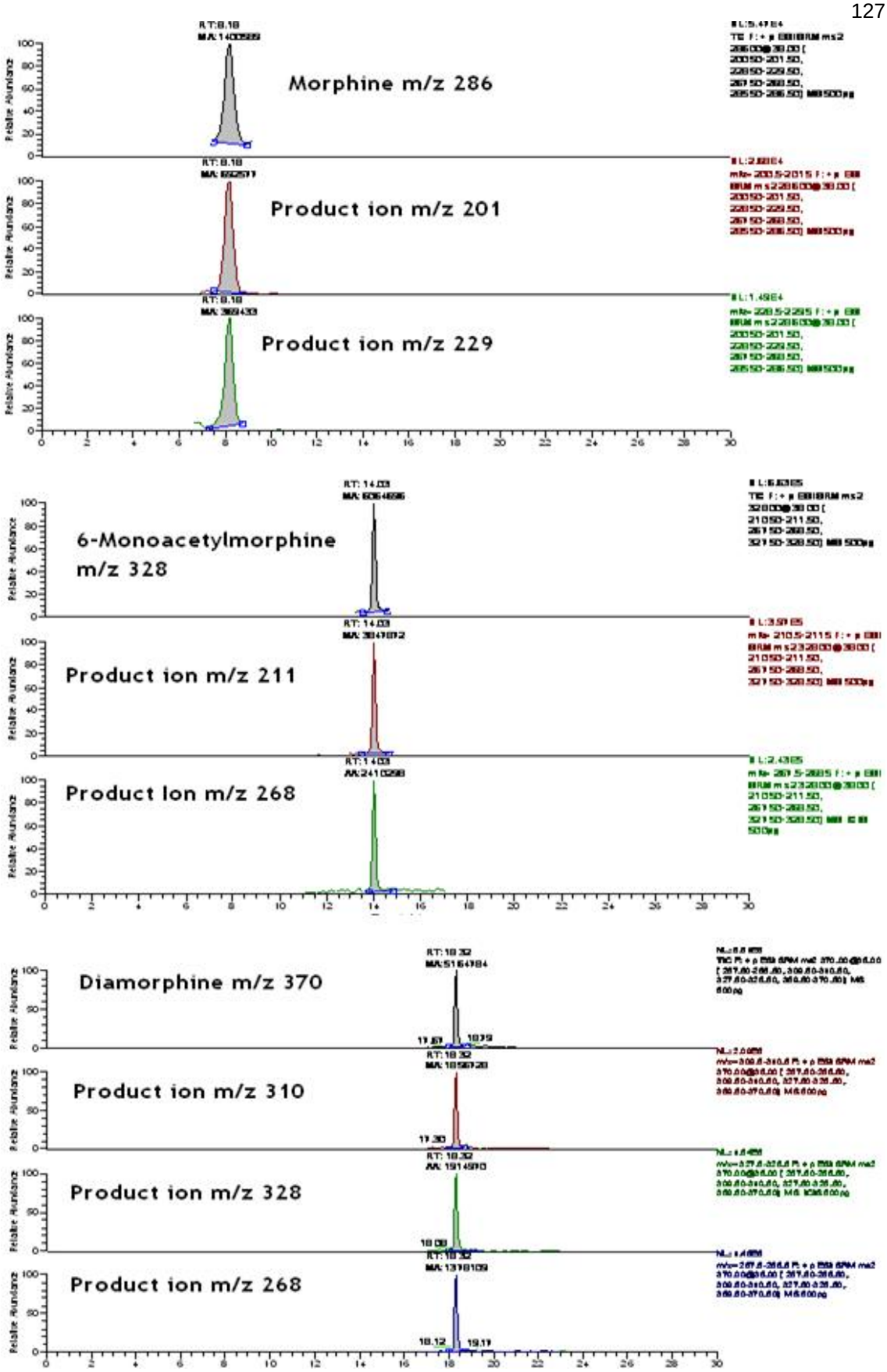


Figure 6-2: SRM chromatograms of morphine, 6-monoacetylmorphine and diamorphine at their LLOQ concentrations.

6.8.2.2 Stability

In the present study, stability was carefully controlled during SPE and LC-ESI-MS/MS. Working standard solutions were prepared weekly and kept at -20 ^{o}C until used. Stability of DIM and metabolites during the extraction and analysis of blank plasma samples spiked with analytes of interest were studied. Stability results are listed in Table 6-6. DIM and its metabolites were subjected to short-term stability tests after 1, 2, and 4 hours using an ice/water bath. Also, quick dilution of plasma samples after thawing without using an ice/water bath and transfer to SPE directly at room temperature at two concentrations (5 and 100 ng/mL) was investigated (Table 6-6). Degradation of DIM was observed after 1, 2 and 4 hours at room temperature. Most of the DIM was converted to 6-MAM after storage for 4 hours, but degradation was less than 45% after 2 hours and less than 10 % of DIM was hydrolysed after 1 hour using an ice/water bath. However, degradation was also investigated at room temperature without using an ice/water bath, when samples were thawed and diluted quickly with ammonium carbonate buffer (pH 9.3) and then submitted to SPE. Degradation of DIM was limited to less than 4%. The later extraction procedure was used for the method validation process and sample analysis.

DIM and its metabolites were found to be stable after four freeze-thaw cycles on consecutive days of plasma spiked at concentrations of 5 and 100 ng/mL and frozen at -20 °C. This method was designed to quantify DIM and metabolites in frozen plasma samples and the stability study was performed under the same conditions. Analytes of interest were spiked into blank plasma samples at low and high concentrations (5 and 100 ng/mL). The use of the lower concentration was aimed at monitoring the degradation of DIM at low levels, expected to be encountered in children's plasma samples due to the low dosage. Plasma samples were then frozen at -20 °C. Samples were analysed after 24 and 48 hours for short-term stability and after 1 week, 1 month and 6 months for long period stability. DIM and its metabolites were stable in both storage conditions.

Storage temperatures were monitored daily using permanently mounted digital thermometers with a resolution of 0.1 °C at the start of the study and

subsequently at weekly intervals during the period of study. Temperatures were stable within a range of approximately 1 °C (freezer) or 5 °C (room temperature, average temperature 20 °C).

Table 6-6: Stability of diamorphine and its metabolites in spiked plasma samples

Storage conditions	Time of storage	Nominal conc. (ng/mL)	Mean Stability (analyte recovery) % * (R.S.D % #)					
			DIM	6-MAM	MOR	M3G	M6G	NMOR
Room temperature	4 hrs	5	52 (6)	134 (4)	113 (4)	102 (6)	114 (10)	115 (10)
		100	39 (10)	150 (7)	115 (7)	102 (6)	117 (15)	94 (7)
	2 hrs	5	83 (6)	113 (7)	104 (8)	101 (6)	99 (10)	108 (4)
		100	70 (11)	123 (3)	108 (3)	102 (9)	96 (8)	108 (13)
	1hrs	5	91 (5)	104 (7)	108 (5)	100 (1)	102 (11)	105 (9)
		100	93 (6)	109 (2)	99 (7)	102 (2)	96 (6)	97 (7)
Directly	0.5 hrs	5	96 (11)	94 (1)	97 (4)	101 (3)	103 (3)	92 (11)
		100	98 (2)	102 (3)	97 (9)	96 (6)	101 (11)	98 (5)
Freeze & thaw	4 cycles	5	95 (7)	109 (5)	95 (8)	94 (5)	101 (2)	90 (2)
		100	92 (5)	108 (4)	98 (7)	95 (10)	103 (7)	112 (11)
Auto-sampler	48 hrs	5	97 (6)	100 (3)	100 (8)	99 (1)	102 (9)	99 (5)
		100	94 (2)	97 (11)	94 (9)	104 (4)	98 (3)	97 (7)
Freezer -20 oC	24 hrs	5	102 (3)	95 (9)	106 (6)	101 (7)	99 (3)	102 (9)
		100	98 (2)	104 (7)	101 (8)	101 (4)	95 (5)	99 (10)
	48 hrs	5	98 (7)	99 (12)	98 (11)	99 (11)	101 (8)	102 (7)
		100	95 (2)	97 (3)	96 (6)	101 (6)	95 (2)	101 (8)
	Week	5	95 (8)	109 (5)	101 (4)	95 (9)	105 (3)	102 (11)
		100	103 (8)	101 (2)	100 (8)	93 (8)	108 (3)	96 (6)
	Month	5	98 (11)	96 (2)	102 (11)	105 (2)	90 (7)	101 (2)
		100	95 (4)	106 (7)	94 (8)	91 (12)	103 (8)	95 (3)
	6 month	5	94 (13)	99 (4)	100 (11)	92 (1)	94 (3)	96 (5)
		100	97 (4)	94 (4)	93 (5)	92 (2)	93 (6)	95 (12)

* The mean percentages for the replicate analysis (n=3).
# R.S.D: Relative standard deviations.

Plasma sample extracts were reconstituted using the initial mobile phase and subjected to an auto-sampler stability study. No degradation of analytes of interest was observed after periods of 6, 12, 24 and 48 hours in the LC-MS/MS auto-sampler, which was set at 4 ºC.

6.8.2.3 Linearity

Two calibration curves were applied together for this study. Linear calibration curves were obtained with correlation coefficients (r^2) greater than 0.999 for each analyte of interest.

6.8.2.4 LODs and LLOQs

LODs and LLOQs of analytes of interest were determined on five different days in duplicate. Relative standard deviations (RSD) of DIM and its metabolites were within the acceptable limit of method validation (less than 20% of LODs and less than 10% of LLOQs). LODs and LLOQs for analytes of interest ranged between 0.08-0.1 and 0.24-0.3 ng/mL respectively. LODs and LLOQs of the present method are detailed in Table 6-7.

Table 6-7: LODs and LLOQs of Diamorphine and its metabolites

	Linearity range (ng/mL)	$r^{2\,\#}$	y-intercept	Standard Error	Gradient (m)	LOD * (ng/mL)	LLOQ ^ (ng/mL)
DIM	0.1-10.0	0.999	-0.00095	0.000917	0.0330	0.08	0.20
6-MA M	0.1-10.0	0.999	-0.00094	0.00102	0.0413	0.07	0.20
MOR	0.1-10.0	0.999	0.000862	0.00147	0.0485	0.10	0.30
M3G	0.1-10.0	0.999	-0.00105	0.00262	0.0783	0.10	0.30
M6G	0.1-10.0	0.999	0.000491	0.000398	0.0134	0.09	0.30
NMOR	0.1-10.0	0.999	0.00006	0.000616	0.0233	0.08	0.26

r^2: Correlation coefficients;
* LOD: Limit of detection; ^ LLOQ: Lower limit of quantitation;

6.8.2.5 Matrix Effects and Recoveries

Matrix effects were within the acceptable limits of method validation (± 20 %) using six different plasma sources. Percentages above 100% indicate the presence of ion enhancement, while those below 100% indicate the presence of ion suppression. The highest ion suppression detected was -14 % and the highest ion enhancement detected was +18%. Next, recoveries of analytes of interest were determined at six different concentrations (0.5, 1, 2.5, 25, 50 and 200 ng/mL) and ranged between 93-102, 90-102, 90-101, 85-102, 83-97 and 92-103% for DIM, 6-MAM, MOR, M3G, M6G and NMOR respectively. Matrix effect results and recoveries of analytes of interest are detailed in Tables 6-8 and 6-9, respectively.

Table 6-8: Matrix effects from six different plasma sources.

Drug	Nominal concentration (ng/mL)	Mean Matrix effect % [#] (R.S.D. %)[&]					
		Source 1	Source 2	Source 3	Source 4	Source 5	Source 6
DIM	5	103 (2)	101 (2)	98 (5)	95 (5)	95 (3)	96 (5)
	100	104 (5)	100 (7)	97 (3)	99 (8)	106 (4)	103 (8)
6-MAM	5	95 (5)	90 (3)	111 (3)	92 (2)	100 (3)	108 (2)
	100	89 (11)	87 (9)	92 (4)	86 (3)	92 (6)	86 (2)
MOR	5	98 (3)	88 (5)	86 (11)	82 (4)	86 (6)	90 (7)
	100	96 (4)	99 (5)	94 (4)	97 (3)	118 (4)	91 (9)
M3G	5	100 (4)	91 (1)	95 (27)	91 (2)	97 (7)	91 (4)
	100	104 (5)	95 (1)	98 (3)	112 (4)	107 (3)	108 (1)
M6G	5	108 (5)	95 (2)	96 (1)	95 (3)	92 (4)	93 (2)
	100	102 (9)	101 (2)	100 (7)	97 (6)	102 (5)	106 (7)
NMOR	5	107 (7)	117 (4)	112 (4)	108 (2)	114 (3)	111 (10)
	100	104 (11)	109 (10)	94 (4)	115 (13)	111 (4)	105 (5)

[#] Value calculated from the average matrix effects for the replicate analyses (n=3).
R.S.D: Relative standard deviations.

Table 6-9: Matrix effects and recoveries of diamorphine and its metabolites (n=5).

Analytes	Nominal concentration (ng/mL)	Matrix effects % (R.S.D. %)	Mean Recovery % [#] (R.S.D. %)
Diamorphine	0.5	94 (8)	93 (13)
	1	89 (2)	92 (3)
	5	98 (1)	97 (6)
	25	102 (4)	94 (13)
	50	95 (8)	102 (9)
	200	98 (3)	97 (9)
6-Monoacetyl-morphine	0.5	93 (6)	95 (8)
	1	98 (7)	90 (4)
	5	94 (1)	95 (10)
	25	107 (4)	102 (2)
	50	92 (1)	101 (9)
	200	101 (7)	99 (10)
Morphine	0.5	84 (4)	90 (14)
	1	92 (2)	96 (2)
	5	94 (12)	98 (8)
	25	98 (5)	101 (6)
	50	93 (10)	99 (11)
	200	107 (11)	98 (10)
Morphine-3-glucuronide	0.5	99 (13)	92 (8)
	1	93 (2)	85 (10)
	5	90 (4)	96 (9)
	25	97 (9)	103 (7)
	50	107 (2)	95 (9)
	200	94 (6)	102 (11)
Morphine-6-glucuronide	0.5	106 (12)	90 (13)
	1	96 (2)	83 (13)
	5	92 (6)	93 (10)
	25	105 (3)	96 (7)
	50	100 (3)	96 (4)
	200	93 (8)	97 (7)
Normorphine	0.5	91 (12)	93 (6)
	1	101 (2)	92 (10)
	5	111 (14)	103 (9)
	25	108 (10)	101 (8)
	50	96 (7)	93 (9)
	200	93 (8)	97 (8)

[#] Value calculated from the average recovery for the replicate analyses (n=5)

6.8.2.6 Precision

Intra-assay and inter-assay precisions were measured using six different concentrations (0.5, 1, 5, 25, 50 and 200 ng/mL) and were based on relative standard deviation (RSD). Precision was found to be less than 15% for both intra-assay and inter-assay conditions (Table 6-10).

6.8.2.7 Specificity and Selectivity

Specificity and selectivity of the optimised method were examined using a mixture of the most common drugs detected in forensic cases. Blank plasma and blank plasma spiked with internal standards were also included with each batch to investigate the effects of unseen endogenous components on the ESI-MS/MS responses. In addition, 23 plasma blank samples provided for the pharmacokinetics of DIM in children for this study were analysed with the optimised method (Figure 6-3). Clean base lines with little or negligible matrix components was found with no interferences detected from the other common drugs.

6.8.3 Case samples

Twenty three sets of children's plasma samples were analysed by the optimised method for DIM and metabolites. The median doses of DIM received by children were 3.3 mg in the IVDIM group and were 2.8 mg in the INDIM children group. Plasma sample volumes used for analysis ranged between 0.1-0.25 mL. DIM and metabolites were detected in all case samples with the exception of NMOR which tested negative in both study groups. Children's body weights were in the range 16-59 kg. The ages of the children ranged from 4 to 13 years. The mean and median body weights were 31 and 33 kg in the IVDIM group and were 33 and 28 kg in the INDIM children group. Plasma samples taken before DIM administration were all negative for DIM and its metabolites in both the IVDIM and INDIM groups.

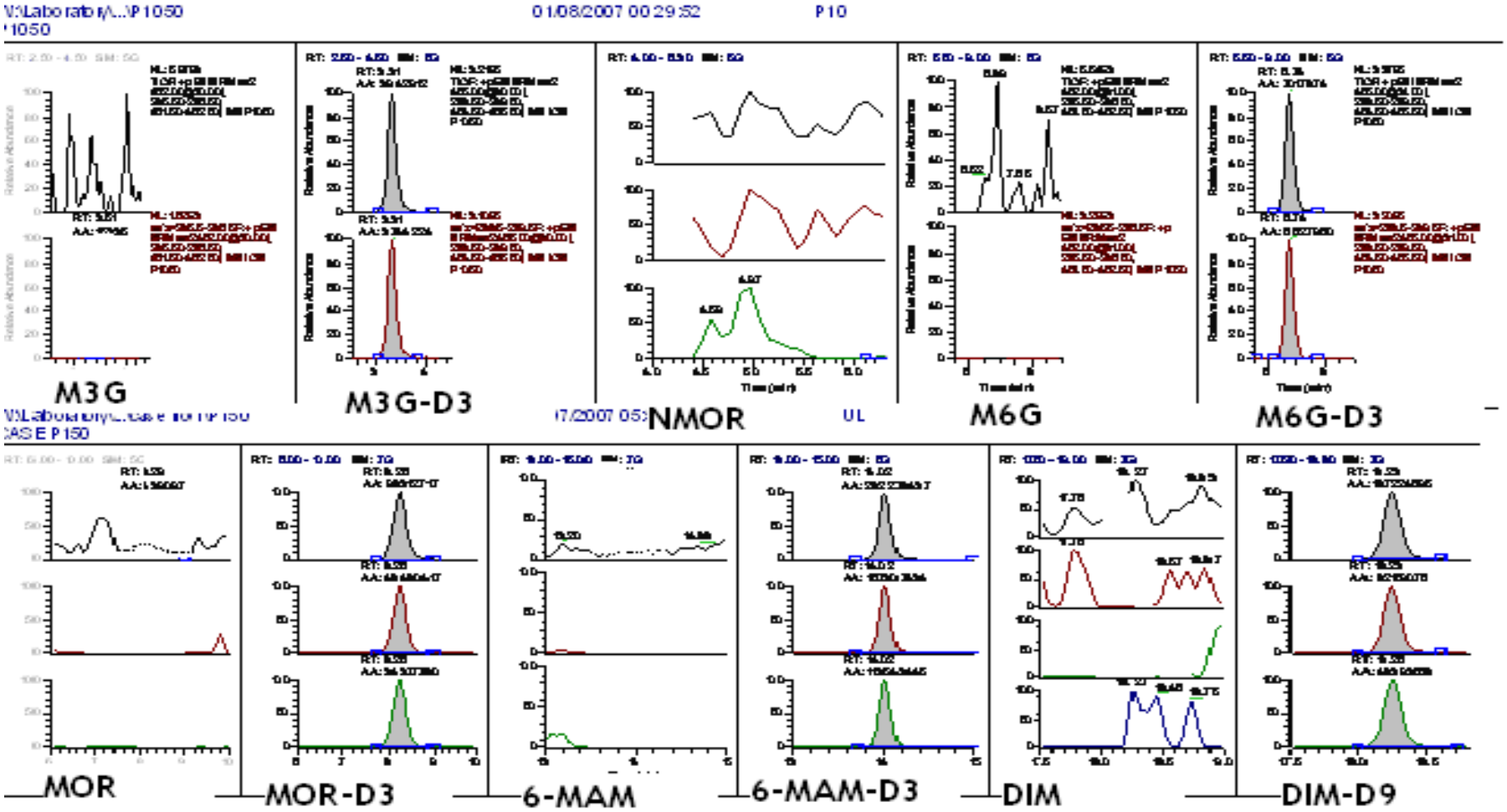


Figure 6-3: SRM chromatograms for a blank plasma sample obtained at time zero (case 1).

Table 6-10: Intra-assay and inter-assay precision.

Analytes	Nominal concentration (ng/mL)	Intra-assay ng/mL [#] (R.S.D. %) [#]	Inter-assay ng/mL [*] (R.S.D. %) [*]
Diamorphine	0.5	0.5 (5.0)	0.5 (5.0)
	1.0	1.2 (10.0)	1.0 (11.0)
	5.0	4.7 (7.0)	4.9 (8.0)
	25.0	24.0 (6.0)	25.0 (2.0)
	50.0	48.0 (5.0)	50.0 (6.0)
	200.0	197.0 (4.0)	200.0 (2.0)
6-Monoacetyl-morphine	0.5	0.49.0 (7.0)	0.5 (9.0)
	1.0	1.1 (10.0)	1.0 (9.0)
	5.0	4.9 (5.0)	4.8 (7.0)
	25.0	24.0 (5.0)	25.0 (5.0)
	50.0	51.0 (4.0)	48.0 (6.0)
	200.0	203.0 (5.0)	200.0 (1.0)
Morphine	0.5	0.5 (10.0)	0.5 (11.0)
	1.0	1.1 (6.0)	1.0 (10.0)
	5.0	4.7 (10.0)	5.0 (5.0)
	25.0	25.0 (5.0)	25.0 (4.0)
	50.0	48.0 (7.0)	48.0 (8.0)
	200.0	198.0 (5.0)	199.0 (1.0)
Morphine-3-glucuronide	0.5	0.5 (7.0)	0.5 (12.0)
	1.0	1.1 (10.0)	1.0 (3.0)
	5.0	4.9 (9.0)	5.0 (3.0)
	25.0	24.0 (3.0)	25.0 (6.0)
	50.0	47.0 (7.0)	49.0 (4.0)
	200.0	203.0 (3.0)	201.0 (1.0)
Morphine-6-glucuronide	0.5	0.5 (9.0)	0.5 (6.0)
	1.0	1.1 (6.0)	1.0 (11.0)
	5.0	4.9 (10.0)	4.9 (5.0)
	25.0	25.0 (6.0)	25.0 (5.0)
	50.0	47.0 (6.0)	48.0 (7.0)
	200.0	196.0 (2.0)	201.0 (1.0)
Normorphine	0.5	0.5 (6.0)	0.5 (8.0)
	1.0	1.0 (9.0)	1.0 (11.0)
	5.0	4.8 (5.0)	4.6 (10.0)
	25.0	25.0 (4.0)	25.0 (6.0)
	50.0	53.0 (10.0)	50.0 (7.0)
	200.0	205.0 (2.0)	199.0 (2.0)

[#] Value calculated from the average measured concentration for the replicate analyses (n=5) in the same day.

[*] Value calculated from the average measured concentration for the replicate analyses (n=5) on five different days.

6.8.3.1 Intravenous diamorphine

Twelve subjects received intravenous DIM (6 male and 6 female, Table 6-9, Figures 6-4 to 6-10). In most IVDIM subjects, The maximum measured concentrations of DIM, 6-MAM and MOR were in the first samples taken after dosing, at 2 minutes, apart from cases 2, 4 and 5 (at 5 minutes) and case 3 (at 10 minutes). In case 2 no 2 minute sample was provided and the highest concentrations were measured in the 5-minute sample. M3G and M6G reached their peak concentrations 20 and 30 min after DIM administration respectively.

Both DIM and 6-MAM were detected in all IVDIM samples collected after 2-20 and 2-30 minutes, respectively. In some cases trace concentrations of DIM and 6-MAM, less than 5 ng/mL, were detected 30-60 minutes after the DIM dose. Plasma concentrations of DIM were measurable in many cases after 20 and 30 minutes with concentrations in the range 0.8-12 and 0.2-0.4 ng/mL, respectively. In addition, DIM was detected in four cases (4, 9, 10 and 19) after 60 minutes with concentrations in the range 0.5-1 ng/mL (Table 6-11). The highest concentration of DIM was found in case 1 (2062 ng/mL, 2 minutes) and the lowest concentration in case 10, at a trace level (estimated 0.1 ng/mL, 60 minutes) which was below the LLOQ but above the LOD of the method. The mean and median peak concentrations of DIM in IVDIM were 706 and 364 ng/mL, respectively, which were detected 2 min after DIM administration (Figure 6-4).

6-MAM tested positive in many IVDIM cases after 30 min with concentrations in the range 1-5 ng/mL, and was detected in six cases after 60 minutes with concentrations in the range 0.3-3 ng/mL (Table 6-11). The highest concentration of 6-MAM was found in case 1 (1876 ng/mL, 2 minutes) and the lowest concentration in case 1 (0.3 ng/mL, 60 minutes). The mean and median peak concentrations of 6-MAM were 522 and 205 ng/mL, respectively, 2 min after DIM administration (Figure 6-5).

MOR was detected in all cases, with the exception of the last sample collected from case 7 at 60 minutes (Figure 6-6). The highest concentration was found in case 9 (227 ng/mL, 2 minutes) and the lowest concentration in

case 6 (0.5 ng/mL, 60 minutes). The mean and median peak concentrations of MOR were 61 and 33 ng/mL, 2 min after DIM administration. The peak plasma concentration in cases 3, 4 and 5 was delayed to 5 minutes. As no 2-minute sample was provided for case 2, the highest MOR plasma level was found in the 5-minute sample. Most cases showed a faster rate of MOR decrease between 2-5 minutes than after 5 minutes, which is likely to reflect the initial distribution phase . In most cases, the plasma MOR concentration in the last sample (at 60 minutes) was higher than 4 ng/mL, exceptions being cases 5 and 6, which had MOR levels of 0.5 and 1 ng/mL, respectively.

M3G was detectable in all IVDIM samples taken after DIM administration, with concentrations ranging from 3-757 ng/mL, but reached its maximum concentration after 20 minutes (Table 6-11 and Figure 6-7). The mean and median peak concentrations of M3G in IVDIM cases were 372 and 331 ng/mL respectively. No change in M3G concentration occurred between 20 and 30 minutes after administration.

The average plasma concentration versus time profile for M6G followed the same pattern as M3G but with lower concentrations. M6G was only detectable in three of the first samples taken after IVDIM, with concentrations of 0.4, 9, and 2 ng/mL in cases 1, 4 and 6, respectively. The concentration of M6G then increased in all cases to reach the peak concentration at 20 - 30 minutes but little or no change was observed at 60 Minutes. The highest plasma concentration for M6G was found in case 1 (56 ng/mL, 30 min). M6G was detectable in the rest of the cases, apart from case 7, with concentrations ranging from 0.5-8 ng/mL after 5 minutes (Figure 6-8).

Table 6-11: Intravenous diamorphine cases.

Patient No.	Time	DIM	6-MAM	MOR	M3G	M6G
	(min)	Measurement concentration (ng/mL)				
1	0	0	0	0	0	0
	2	2060	1880	223	50	0.4
	5	137	121	64	330	4
	10	19	30	54	680	17
	20	2	6	18	706	35
	30	0.6	3	17	757	56
	60	0	0.3	8	415	34
2	0	0	0	0	0	0
	2	0	0	0	0	0
	5	971	529	192	127	4
	10	61	25	47	342	16
	20	12	9	31	318	19
	30	4	3.5	17	312	31
	60	0	0	7	241	19
3	0	0	0	0	0	0
	2	228	80	14	74	0
	5	37	23	18	167	0.8
	10	9	11	19	307	5
	20	0	3	15	343	14
	30	0	2	15	373	12
	60	0	0	9	359	19
4	0	0	0	0	0	0
	2	430	99	20	98	9
	5	283	116	44	403	5
	10	27	38	38	582	14
	20	12	21	15	574	23
	30	2	5	10	480	20
	60	1	1	7	502	19
5	0	0	0	0	0	0
	2	364	57	14	44	0
	5	61	11	22	284	3
	10	10	7	16	453	8
	20	1	1	8	452	20
	30	0	0	4	383	20
	60	0	0	1	386	27
6	0	0	0	0	0	0
	2	1110	487	42	54	2
	5	125	150	24	161	3
	10	9	6	11	246	7
	20	0	1	9	245	12
	30	0	0	4	203	13
	60	0	0	0.5	171	11

Table 6-11: Continue.

Patient No.	Time	DIM	6-MAM	MOR	M3G	M6G
	(min)	Measurement Concentration (ng/mL)				
7	0	0	0	0	0	0
	2	937	768	32	3	0
	5	24	26	8	61	0
	10	5	10	5	83	1.2
	20	4	11	3	191	12
	30	0	0	1.5	101	14
	60	0	0	0	106	13
8	0	0	0	0	0	0
	2	260	237	22	10	0
	5	27	34	7	118	1.4
	10	4	12	9	203	4
	20	4	9	7	310	10
	30	0.2	2	5	177	8
	60	0	1	4	117	12
9	0	0	0	0	0	0
	2	1600	1710	227	82	0
	5	198	237	51	374	0.5
	10	55	144	37	595	9
	20	6	14	15	588	23
	30	0.5	6	9	583	28
	60	0.8	3	7	519	22
10	0	0	0	0	0	0
	2	324	106	15	51	0
	5	85	45	8	258	8
	10	29	18	8	454	13
	20	4	3	7	459	18
	30	0.4	1	7	407	17
	60	0.1	0	4	311	12
19	0	0	0	0	0	0
	2	190	205	34	6	0
	5	36	64	22	41	1
	10	0.6	21	15	80	3
	20	0.8	6	9	107	12
	30	0.7	5	8	113	16
	60	0.5	2.5	5	98	16
20	0	0	0	0	0	0
	2	255	118	31	8	0
	5	58	40	20	91	4
	10	6	18	15	154	6
	20	0	5	14	172	22
	30	0	2	11	142	23
	60	0	2	12	114	21

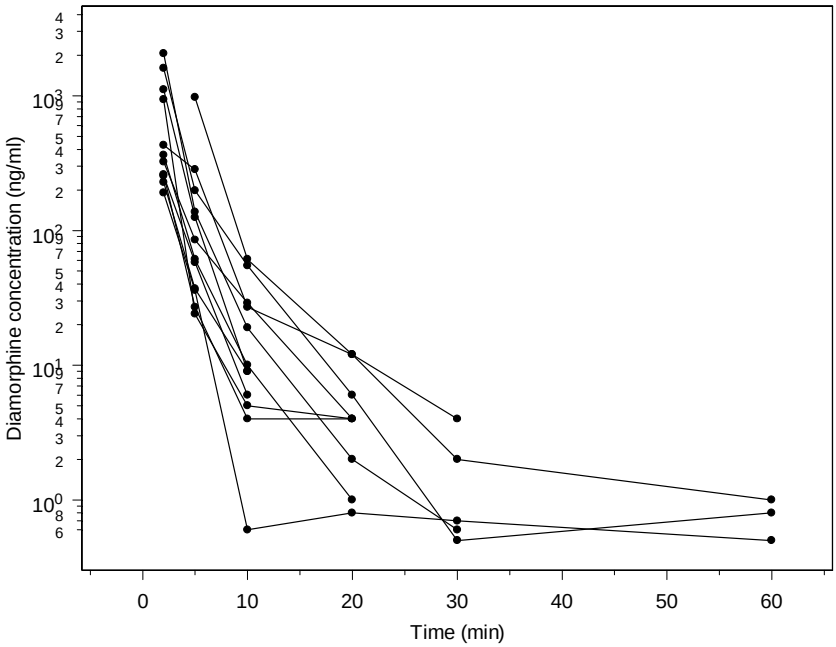


Figure 6-4: Semi-log plots of Diamorphine concentrations versus time after administration in 12 IVDIM cases.

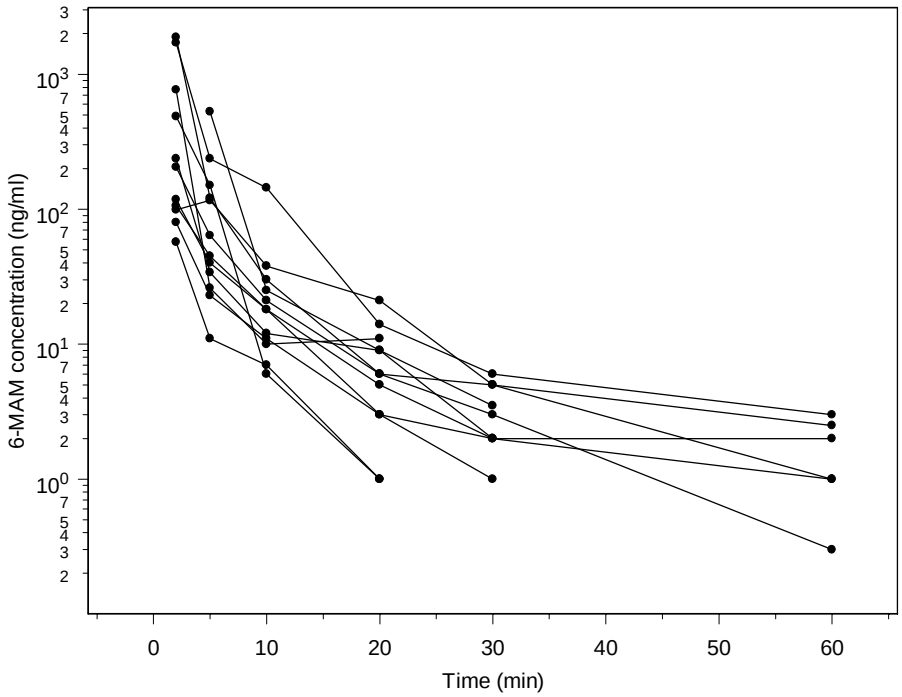


Figure 6-5: Semi-log plots of 6-monoacetylmorphine concentrations versus time after administration in 12 IVDIM cases.

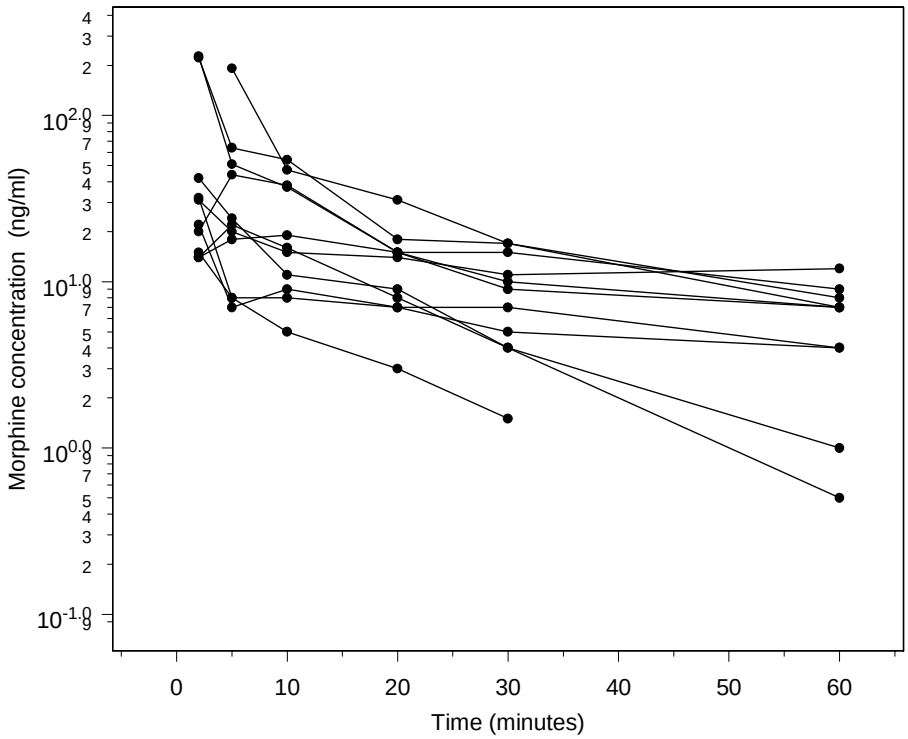


Figure 6-6: Semi-log plots of morphine concentrations versus time after administration in 12 IVDIM cases.

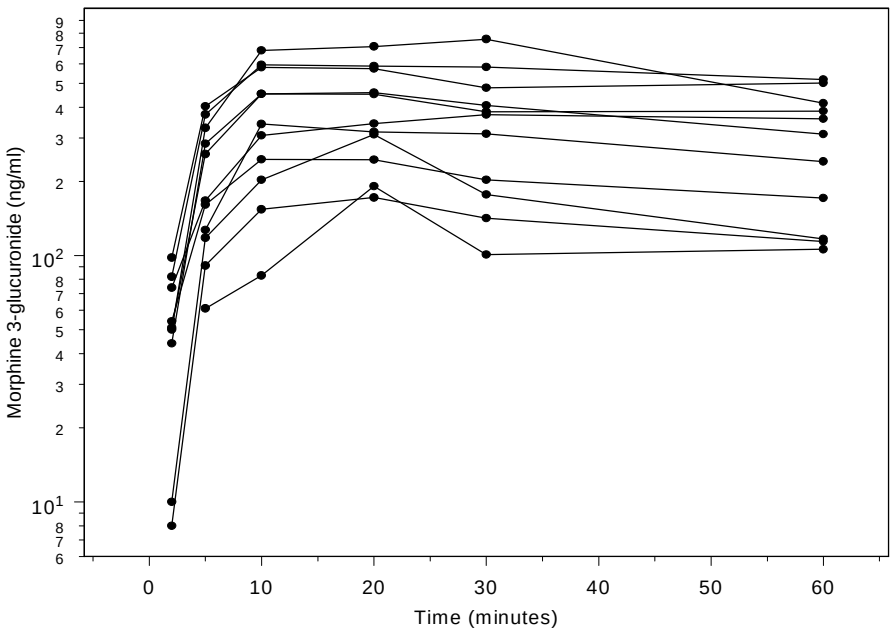


Figure 6-7: Semi-log plots of morphine-3-glucuronide concentrations versus time after administration in 12 IVDIM cases.

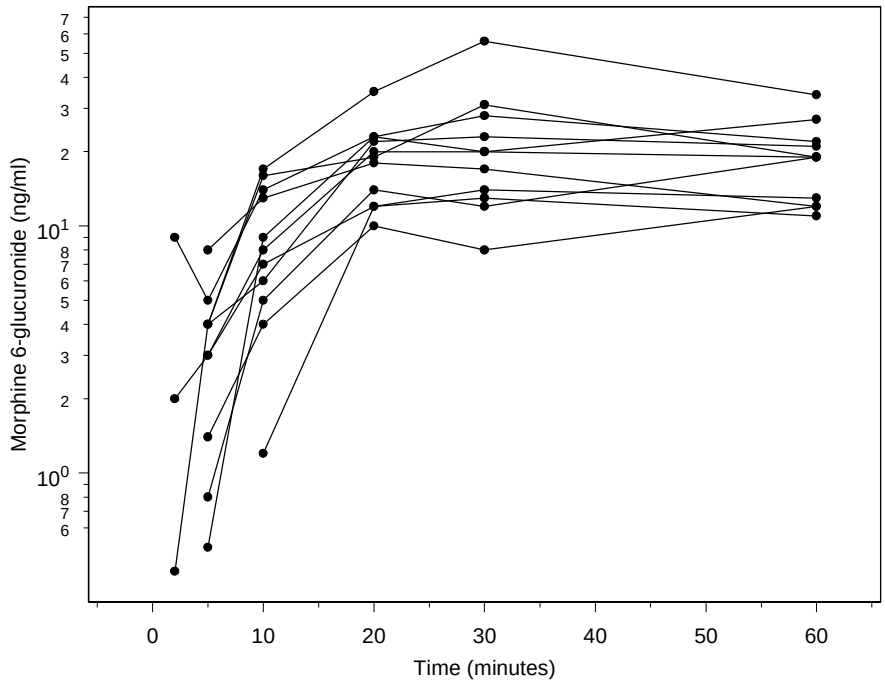


Figure 6-8: Semi-log plots of morphine-6-glucuronide concentrations versus time after administration in 12 IVDIM children.

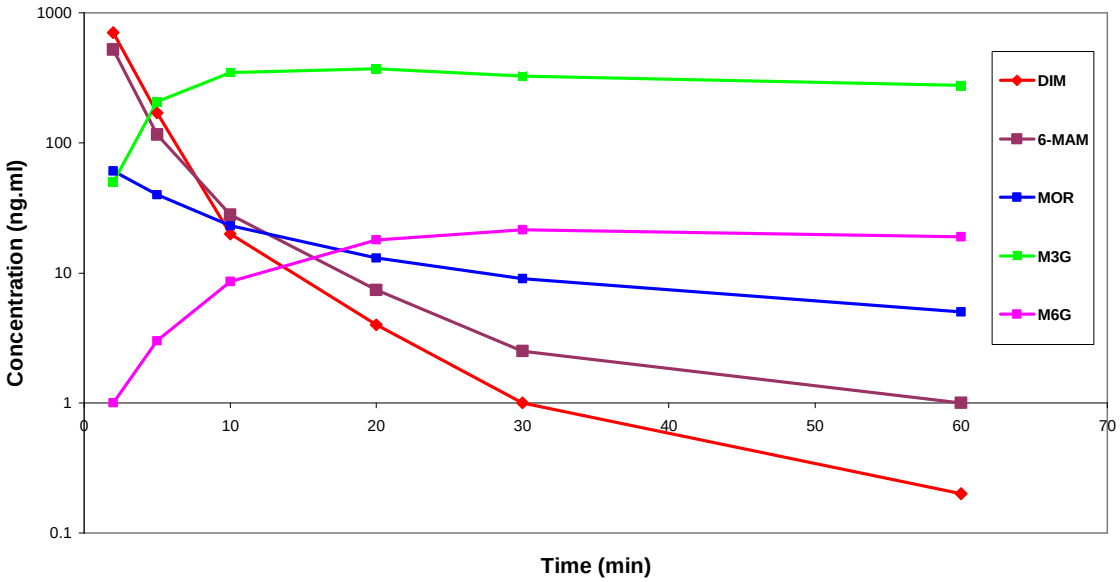


Figure 6-9: Semi-log plots of of mean plasma concentrations of DIM metabolites versus time after administration in 12 children following intravenous DIM.

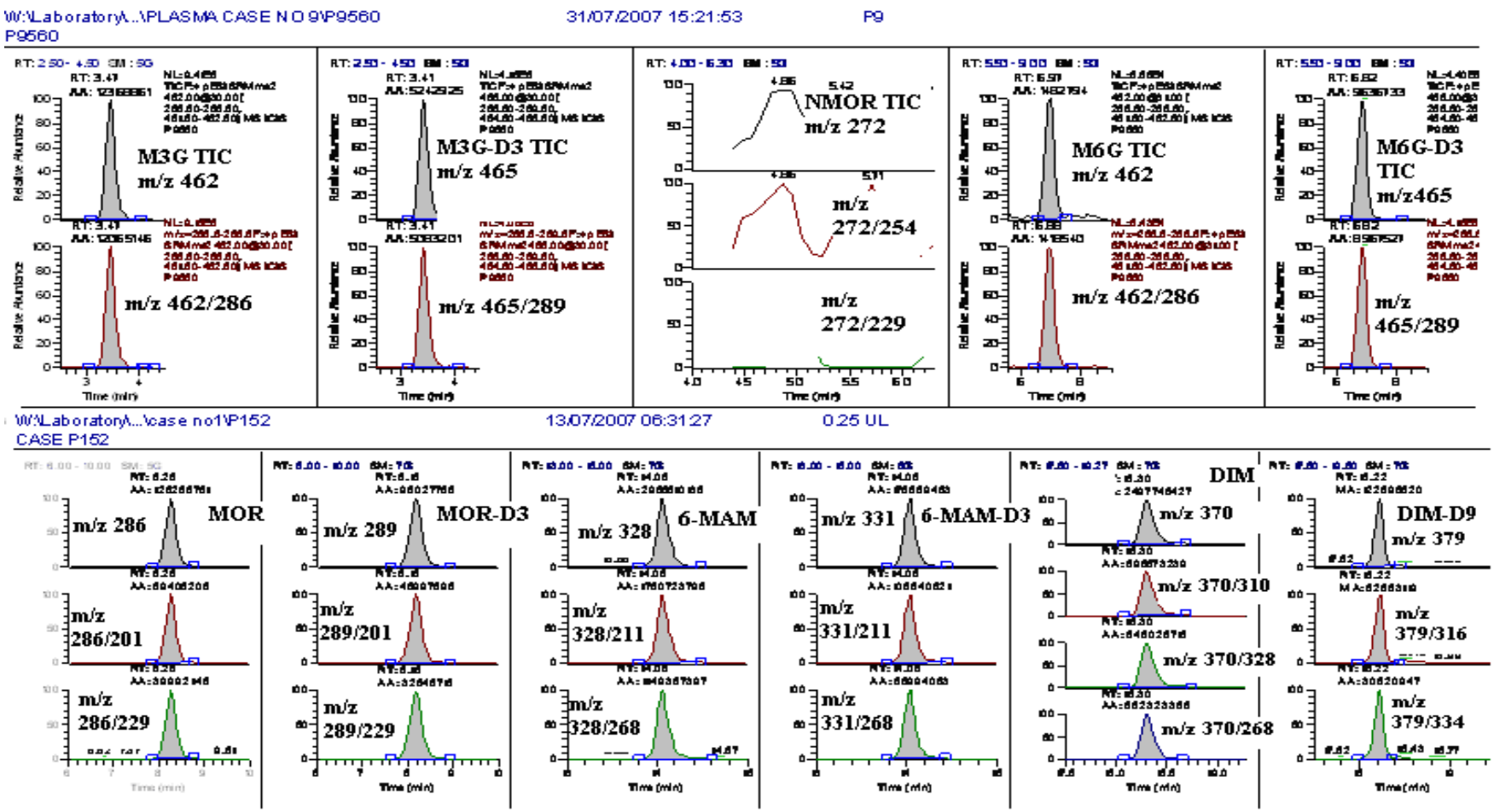


Figure 6-10: SRM chromatograms for DIM metabolies after IVDIM, M3G and M6G (Case9, 60 minutes) and MOR, 6-MAM and DIM (Case1, 2 minutes).

6.8.3.2 Intranasal diamorphine

Eleven children received intranasal diacetylmorphine (5 male and 6 female) for their treatment. Table 6-12, Figures 6-11 to 6-17 summarise the analytical data obtained for these case. DIM and 6-MAM were detected in all INDIM cases from the first sample until 10 and 20 min after DIM administration, respectively. In six cases, the plasma concentrations of DIM ranged between 0.2- 4 ng/mL (20 minutes after DIM administration); three cases were found positive for DIM with plasma levels lower than 1 ng/mL at 30 minutes and only one case had a concentration of DIM at the LLOQ of 0.2 ng/mL at 60 minutes. Maximum DIM concentrations were observed in the first samples, collected 2 minutes after administration in most subjects although in four cases, maximum onbserved DIM concentrations were in the 5–minute samples. However, levels of DIM in plasma were very similar at 2 and 5 minutes after administration. The highest DIM concentration detected was 43 ng/mL (case 22, after 2 minutes). The mean and median peak concentrations of DIM were 17 and 15.5 ng/mL, 2 minutes after administration (Table 6-12).

6-MAM concentrations in the INDIM group were lower than those of DIM in all cases. Maximum observed plasma concentrations of 6-MAM were in the 5-minute samples, with a slow rate of decline after the first sample until 6-MAM disappeared by 60 minutes. 6-MAM was still detectable in all 20-minute plasma samples, with the exception of cases 16 and 22, but was only detectable in three cases at 30 minutes at concentrations ranging between 1.5-2 ng/mL (Figure 6-12).

The highest concentration of 6-MAM was found in case 12 (16 ng/mL, 5 minutes) and the lowest concentration in case 16 (0.3 ng/mL, 2 minutes). The mean and median peak concentrations of 6-MAM were 7.4 and 6 ng/mL after 5 min, respectively.

MOR was detected in all samples collected at 5-60 minutes after DIM administration (Figure 6-13). Only four cases had detectable levels of MOR in the first sample collected at 2 minutes with a concentration range below the LLOQ to 3 ng/mL in case 11, with a mean concentration of 0.6 ng/mL. The maximum observed plasma concentration of MOR in this group was detected

after 20 minutes and little change was observed until the curve started to decline in samples collected after 60 minutes. The highest concentration was found in case 12 (14 ng/mL, 10 minutes) and the lowest concentration in case 18 (0.2 ng/mL, 2 minutes). The mean and median peak concentrations of MOR were 5.5 and 4 ng/mL, respectively, at 10 min after DIM administration. Little change in the MOR level occurred between 10 to 20 minutes with mean and median concentrations in the range of 5.4-5.5 and 4-5 ng/mL, respectively. The plasma MOR concentrations in the last samples, collected at 60 minutes, ranged between 0.8-8 ng/mL, with mean and median concentrations of 3 and 2 ng/mL, respectively.

M3G levels were detectable in all cases (concentrations ranged between 0.2 to 250 ng/mL) from the second sample taken after 5 minutes until the last sample at 60 minutes, with the exception of cases 16 and 18 which tested negative after 5 minutes of the DIM dose. Plasma M3G levels rose after 5 minutes of the DIM dose until the end of sampling at 60 minutes in a straight line which it is supposed would continue rising (Table 6-14). After 5 minutes, all detectable M3G levels were above the LLOQ with concentrations equal to or higher than 1 ng/mL, with the exception of case 17 (0.2 ng/mL). In case 15 (female subject) high levels of M3G were observed from the second sample collected (129 ng/mL) and the maximum measured concentration was in the 20 minute-sample (250 ng/mL), which was the highest plasma level of M3G determined in this group. The mean and median concentrations of M3G at 60 minutes after DIM administration were 80 and 86 ng/mL, respectively (Table 6-12, Figures 6-14).

The mean plasma concentration versus time profile of M6G had the same rising pattern as M3G (Figure 6-15), but with lower concentrations, and M6G was detectable after 20 minutes in all cases. M6G was detectable in four samples at 10 minutes with concentrations of 0.5, 1, 0.8, and 0.7 ng/mL in cases 11, 12, 15 and 22, respectively. Cases 11 and 21 had levels of M6G lower than the LLOQ at 2 minutes but both had a concentration of 3 ng/mL at 5 minutes. Also, the peak plasma concentration of M6G was not reached before the end of sampling at 60 minutes. The highest plasma concentration for M6G was found in case 12 (14 ng/mL, 60 min). The mean and median

concentrations of M6G at 60 minutes after DIM administration were 7 and 8 ng/mL, respectively (Table 6-15).

Table 6-12: Intranasal diamorphine cases.

Patient No.	Time	DIM	6-MAM	MOR	M3G	M6G)
	(min)	Measured Concentration (ng/mL)				
11	0	0	0	0	0	0
	2	15.5	13	3	3	0
	5	14	15	6	5	0.2
	10	4	8	6	14	0.5
	20	0.6	4.5	8	35	2
	30	0	2	8	47	3
	60	0	0	8	87	8
12	0	0	0	0	0	0
	2	30	10	0	0	0
	5	23	16	5	1	0
	10	7	8	14	34	1
	20	0	2	5	79	5
	30	0	0	5	101	7
	60	0	0	3	160	14
13	0	0	0	0	0	0
	2	17	3.5	0	0	0
	5	10	9	6	2.4	0
	10	6	7	10	12	0
	20	4	3	7	21	2.2
	30	0	0	6	29	3
	60	0	0	4	49	8
14	0	0	0	0	0	0
	2	2.5	0	0	0	0
	5	15	9	3.5	1	0
	10	10	11	5	21	0
	20	0	2	5	47	0.4
	30	0	1.5	3	73	0.6
	60	0	0	2	86	1.4
15	0	0	0	0	0	0
	2	3.4	26	0	2	0
	5	3.4	8	4	129	0
	10	2.5	3	4	147	0.8
	20	0.6	3	5	250	2
	30	0	0.7	3.7	121	5.0
	60	0	0	3.4	91	3.5
16	0	0	0	0	0	0.0
	2	7.4	0.3	0.7	0	0.0
	5	8	1.7	0.8	0	0.0
	10	3.8	0	2.2	2	0.0
	20	1	0	2.1	17	2
	30	0.5	0	1.5	45	3
	60	0.2	0	0.8	53	8

Table 6-12: Continue.

Patient No.	Time	DIM	6-MAM	MOR	M3G	M6G)
	(min)	Measured Concentration (ng/mL)				
17	0	0	0	0	0	0
	2	5	7	0	0	0
	5	6	7	2	0.2	0
	10	1	5	6	11	0
	20	0	2	12	32	1
	30	0	1	10	37	2.2
	60	0	0	1.3	46	4
18	0	0	0	0	0	0
	2	12	3.2	0.2	0	0
	5	12	5	1.3	0	0
	10	5	10	3.4	2	0
	20	0.2	4	8	50	0.3
	30	0	0	5	69	2
	60	0	0	4	87	3
21	0	0	0	0	0	0
	2	17	10	1	15	1
	5	5	6	3	32	3
	10	0	2.4	4	40	4
	20	0	0.5	2.3	54	4
	30	0	0	2	54	7
	60	0	0	2	81	13
22	0	0	0	0	0	0
	2	43	8	0	0	0
	5	12	5	5	6	0
	10	0.7	2	3	19	0.7
	20	0	0	1.3	35	3
	30	0	0	1.2	50	4
	60	0	0	0.8	91	13
23	0	0	0	0	0	0
	2	12	4	2	1	0
	5	6	4	3.4	2	0
	10	3	2	3.4	7	0
	20	1.2	1.3	3.2	21	1
	30	1	0	3	33	2
	60	0	0	1.7	44	5

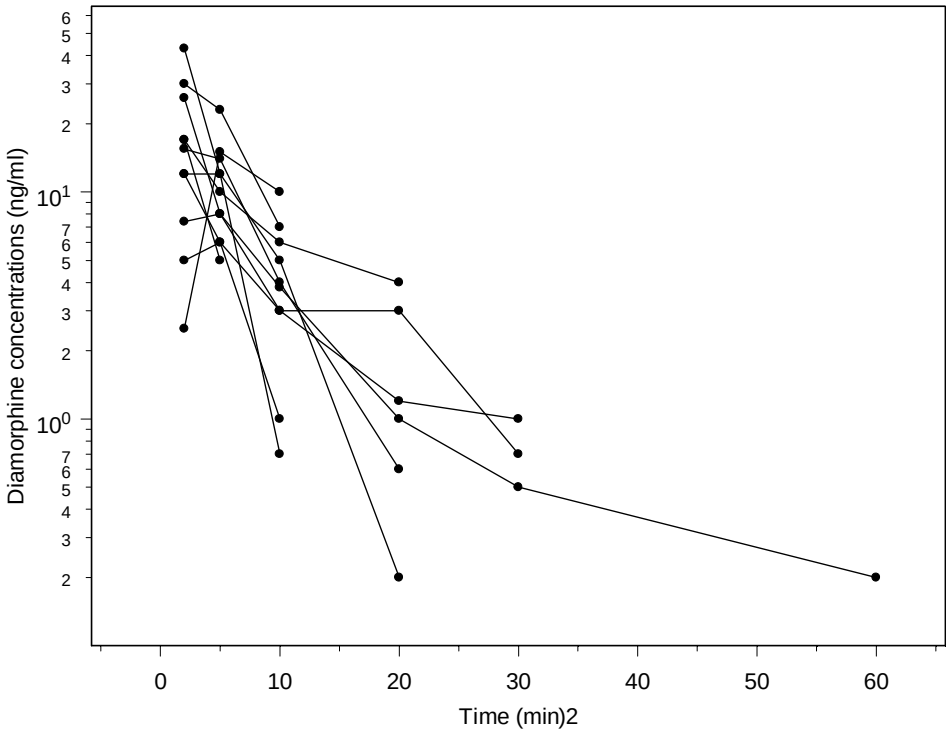


Figure 6-11: Semi-log plots of diamorphine concentrations versus time after administration in 11 INDIM cases.

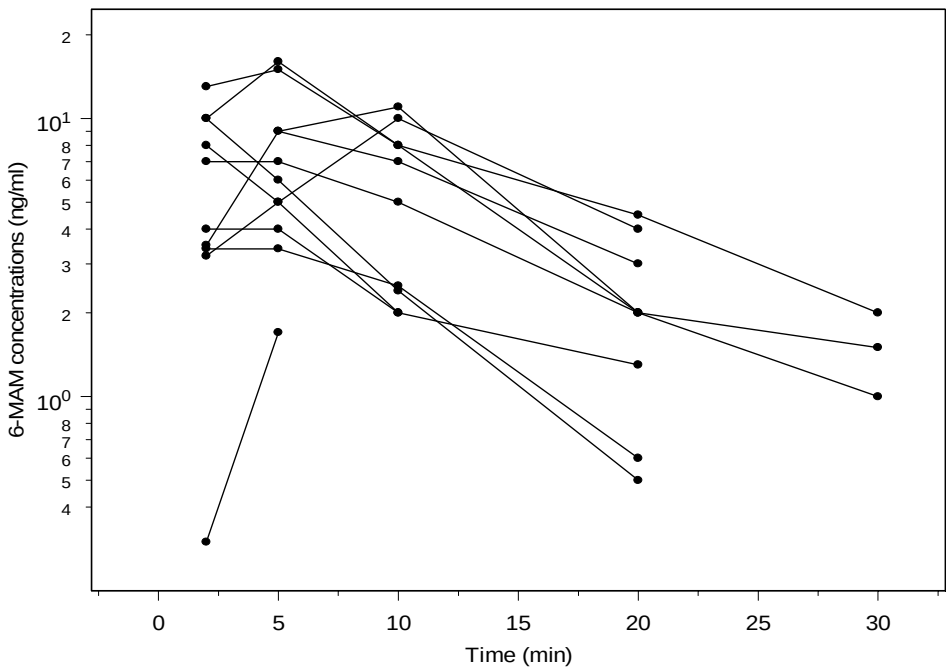


Figure 6-12: Semi-log plots of 6-monoacetylmorphine concentrations versus time after administration in 11 INDIM cases.

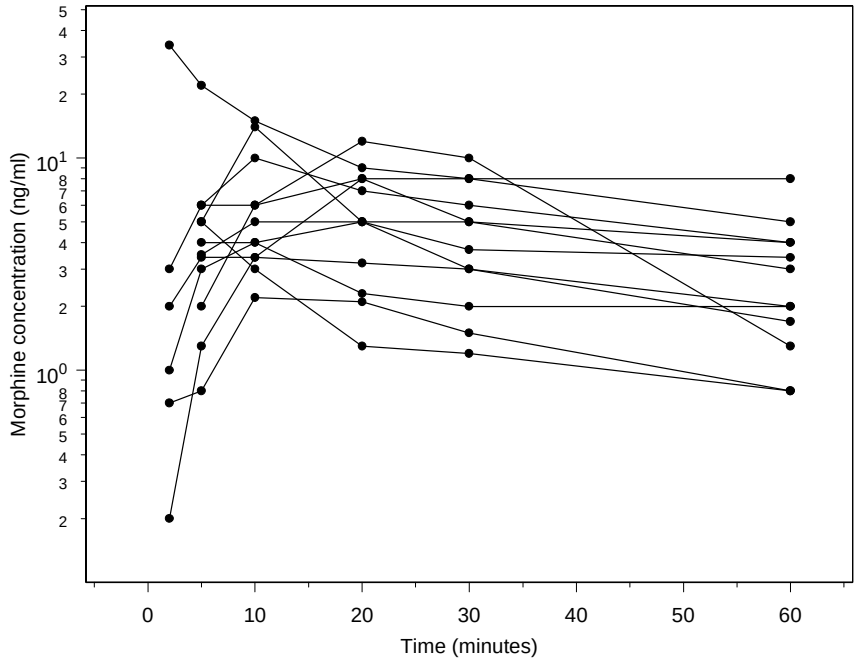


Figure 6-13: Semi-log plots of morphine concentrations versus time after administration in 11 INDIM cases.

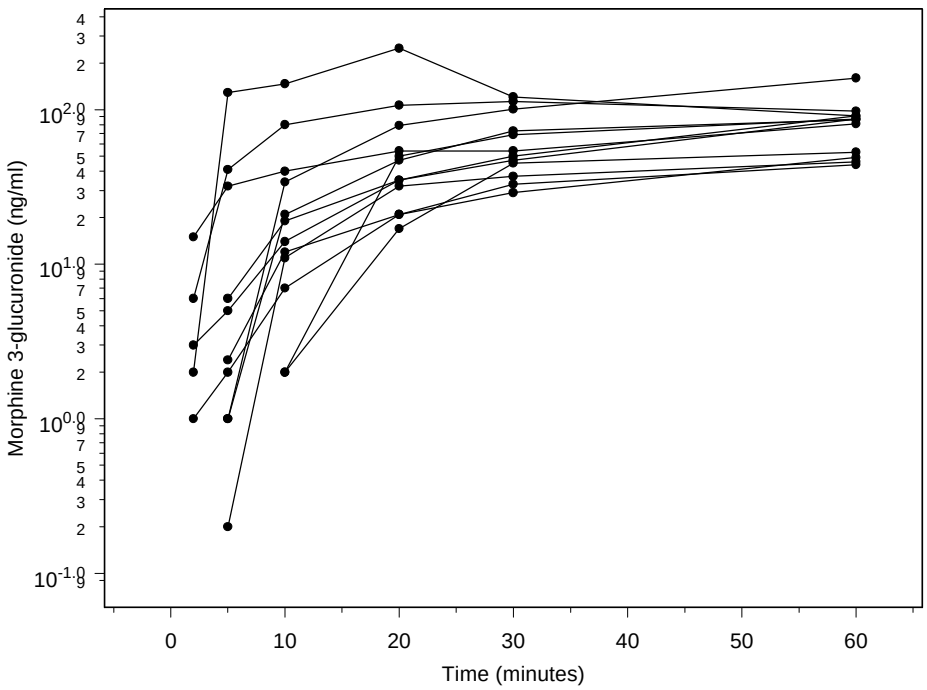


Figure 6-14: Semi-log plots of morphine-3-glucuronide concentrations versus time after administration in 11 INDIM cases.

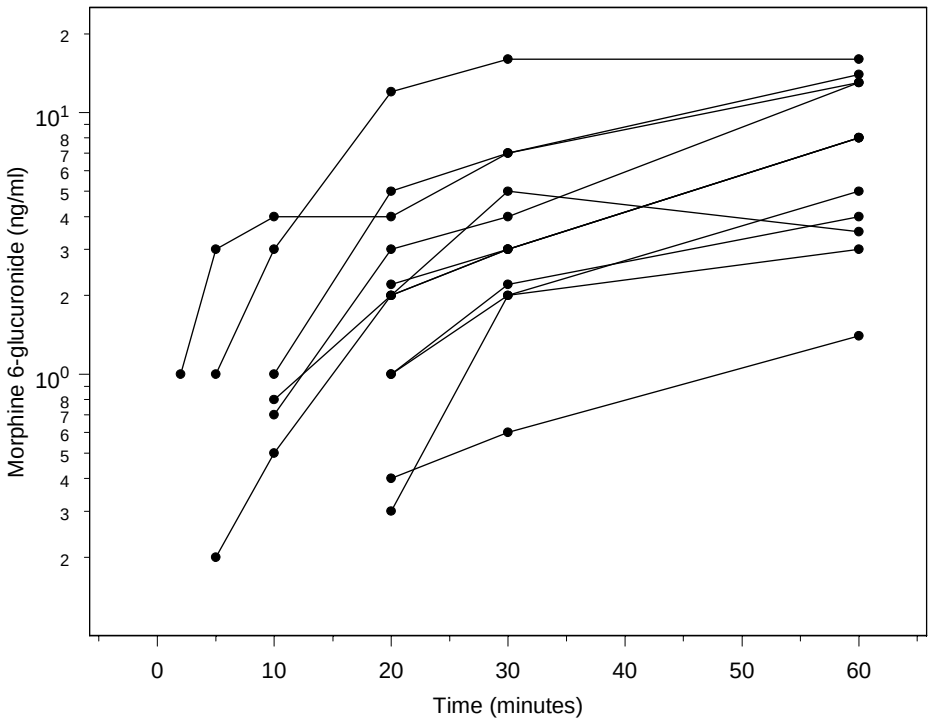


Figure 6-15: Semi-log plots of morphine-6-glucuronide concentrations versus time after administration in 11 INDIM cases.

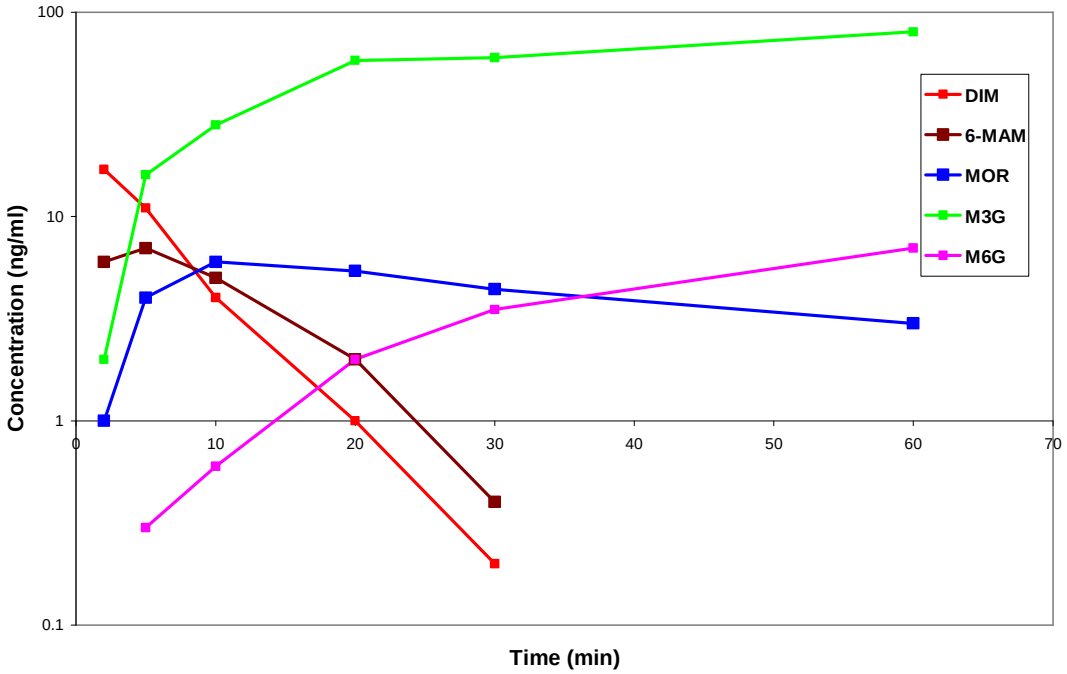


Figure 6-16: Semi-log plots of of mean plasma concentrations of DIM metabolites versus time after administration in children following intranasal DIM.

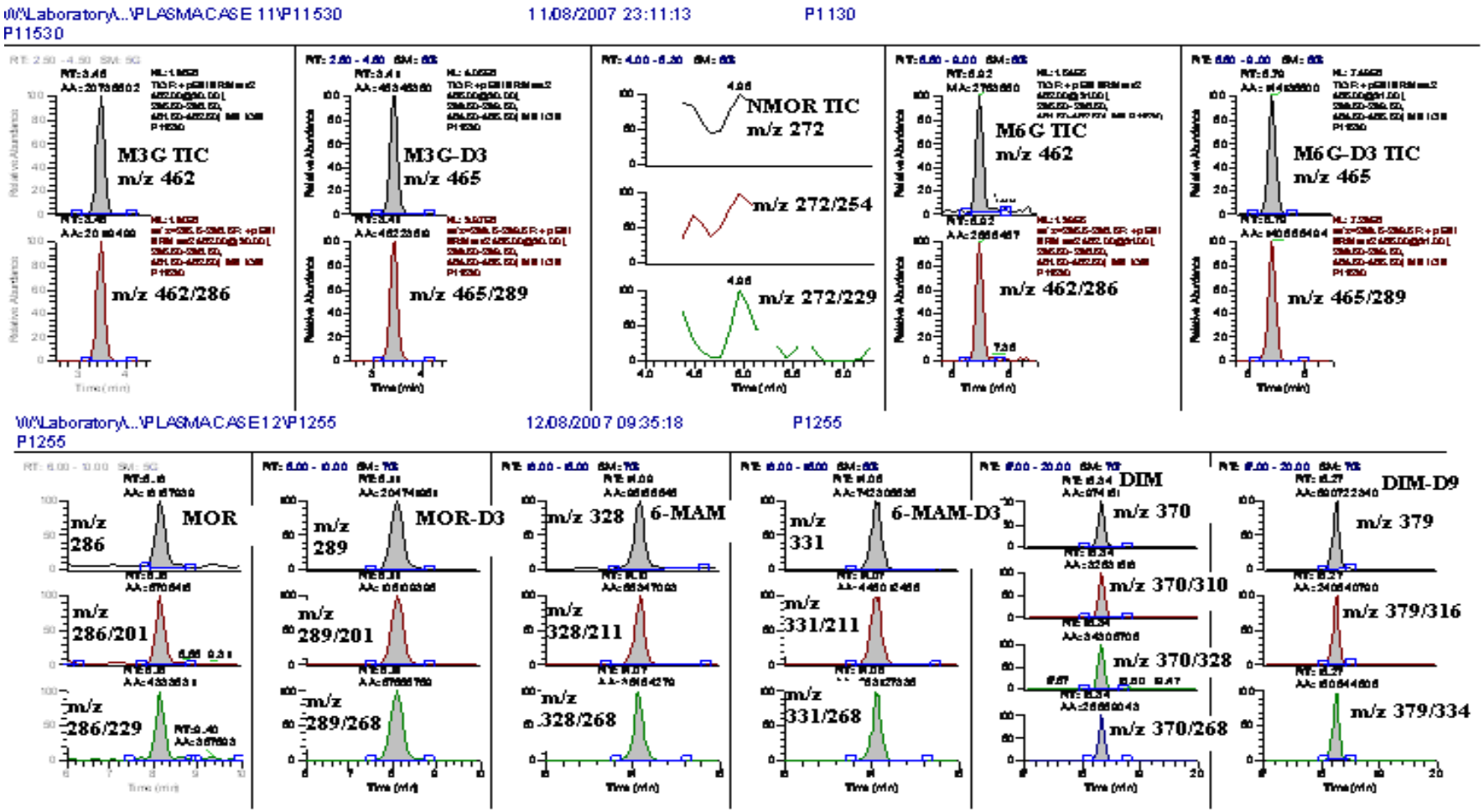


Figure 6-17: SRM chromatograms for DIM metabolies after INDIM, M3G and M6G (Case 11, 30 minutes) and MOR, 6-MAM and DIM (Case 12, 5 minutes).

6.8.3.3 Comparison between IVDIM and INDIM

Concentrations of DIM metabolites were found to depend on the route of administration. The concentrations of DIM metabolites after INDIM were lower than after IVDIM at the same dose and body weight, and were in the ranges 190 to 2062 ng/mL and 2 to 43 ng/mL for IVDIM and INDIM, respectively. The median peak concentrations of analytes of interest after IVDIM were achieved at 2 min for DIM, 6-MAM and MOR, and at 20 and 30 min for M3G and M6G respectively. After INDIM, median peak concentrations were achieved for DIM at 2 minutes but metabolites achieved their peak concentrations later: 6-MAM at 5 minutes, MOR at 10 minutes and the glucuronides beyond the study time window.

Ratios of concentrations (IVDIM/INDIM) were calculated using the median peak concentrations or highest concentration obtained and were 34, 26.4, 6.2, 3.8 and 2.9 for DIM, 6-MAM, MOR, M3G and M6G respectively. Following IVDIM, the concentrations of DIM, 6-MAM and MOR decreased sharply after 2 min whereas the rate of decrease was slower after INDIM.

Plasma levels of DIM and its initial metabolite 6-MAM were found to share some similarities; DIM was higher than 6-MAM until 5 minutes in IVDIM and INDIM but 6-MAM exceeded DIM after 10 minutes until both analytes were completely eliminated from the plasma. By contrast, some differences were found in relative analyte concentrations between the two groups. The plasma concentration of 6-MAM was 74% of the DIM concentration after 2 minutes of IVDIM compared to 30% of the DIM level after INDIM. The percentages at 5 minutes were 68% and 73%, respectively and at 10 minutes were 140% and 150%, respectively.

Interestingly, INDIM achieved similar DIM metabolite concentrations to IVDIM but at later times due to a delay in absorption and differences in metabolism in the nasal or gastric mucosa compared to IVDIM. For example, a mean DIM plasma level of 4 ng/mL was achieved following both IVDIM and INDIM administration, after 10 or 20 minutes, respectively. Also, the mean peak concentration of 6-MAM after INDIM occurred at 5 minutes post-dose and was the same as the mean plasma concentration after IVDIM at 20 minutes post-dose and the plasma

concentrations of M6G at 30 and 60 minutes after INDIM (3.5 and 7.4 ng/mL, respectively) were close to those at 5 and 10 minutes after IVDIM (3 and 8.6 ng/mL, respectively).

DIM, 6-MAM and MOR were detected simultaneously for the first 10 minutes in the IVDIM group. 6-MAM exceeded DIM after 10 minutes and MOR exceeded DIM and 6-MAM after 20 minutes, when MOR levels were 2 and 3 fold higher than DIM and 6-MAM concentrations, respectively (Figure 6-18.) In the INDIM group, the plasma concentration of MOR was higher than DIM and equal to 6-MAM after 10 minutes. The same results were obtained after 20 minutes of INDIM but the level of MOR was 5 and 2.5 times higher than DIM and 6-MAM (Figure 6-19). The mean plasma concentration 30 minutes after INDIM (5.3 ng/mL) was similar to that obtained 60 minutes after IVDIM (5.4 ng/mL).

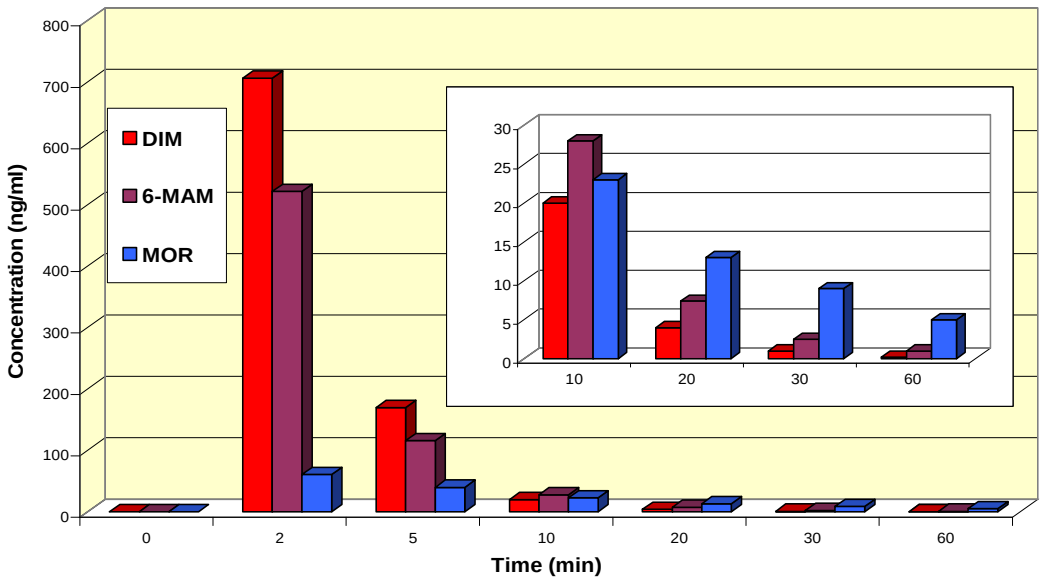


Figure 6-18: DIM, 6-MAM and MOR levels following IVDIM.

In the IVDIM group, 2 minutes after DIM injection, the total MOR (TMOR) concentration was made up by free morphine (FMOR, 55%), M3G (44%) and M6G (1%). After 5 minutes, FMOR decreased to only 15% of TMOR and at 60 minutes accounted for less than 2% of TMOR (Figure 6-20). M3G accounted for 83% of total MOR after 5 minutes of DIM dose and 92% at 60 minutes. FMOR and M3G accounted for 33% and 67% of TMOR at 2 min after INDIM, respectively. The M3G

plasma level increased sharply after 5 minutes to become a major portion of TMOR, which was the same as in the intravenous group. FMOR and M3G accounted for 3.3% and 89% of TMOR, respectively (Figure 6-21.)

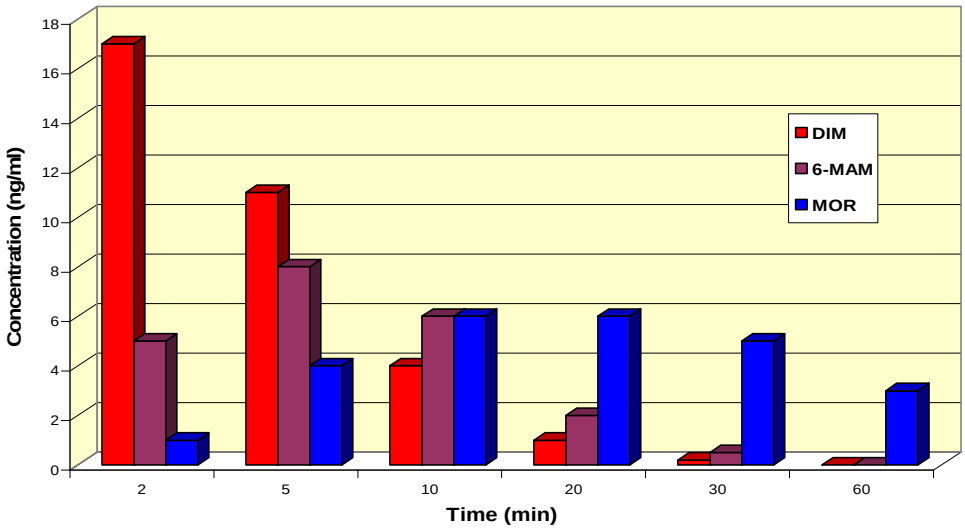


Figure 6-19: DIM, 6-MAM and MOR levels following INDIM.

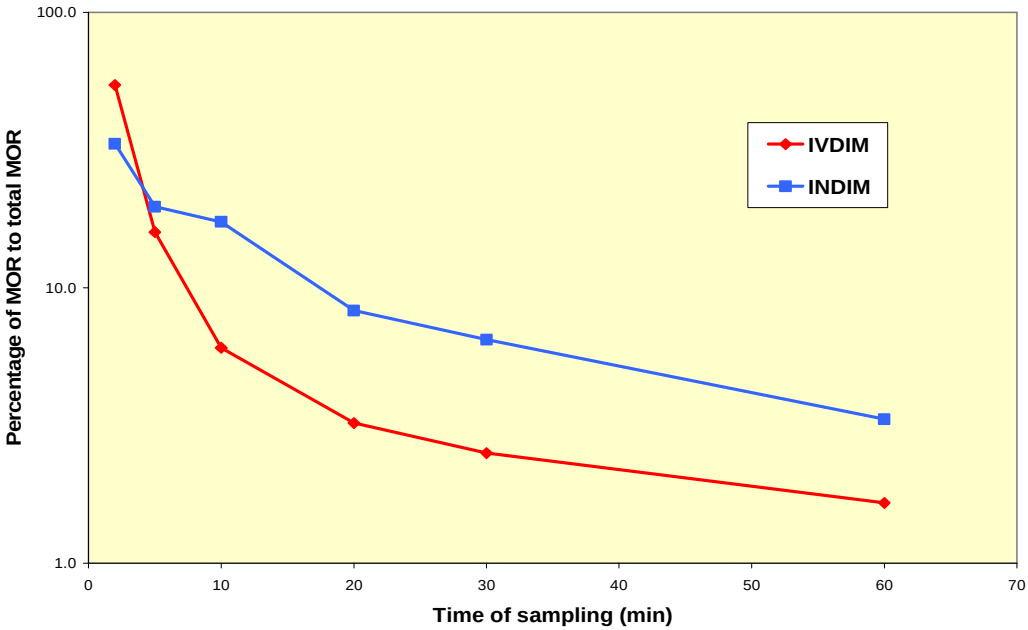


Figure 6-20: Free morphine as a percentage of total morphine after IVDIM and INDIM.

M6G was the minor DIM metabolite in both groups. M6G was formed early, after 2 minutes of IVDIM and accounted for 1% of TMOR, and after that the percentage of M6G increased until it exceeded that of FMOR. M6G accounted for 6.3% of TMOR and was three times higher than FMOR at 60 minutes.

The same pattern was observed after INDIM but M6G accounted for 1% of TMOR after 10 minutes and M6G accounted for approximately 8% of TMOR, and was more than double FMOR at 60 minutes (Figure 6-22).

The ratios of M6G/MOR and M3G/MOR and M3G/M6G were calculated for both groups. Although the peak concentrations of M6G and M3G were not determined in the intranasal group, the ratios of M3G/M6G were close to each other (Figure 6-23). They were about 50 at 2 or 5 minutes after IVDIM and INDIM, respectively, and then declined to 14 and 11 at 60 minutes, respectively. Also, the same ratio of M6G/MOR was obtained at 30 and 60 minutes after IVDIM and INDIM, respectively. It is expected that the M6G/MOR ratio would be the same in both INDIM and IVDIM groups after 60 minutes (Figure 6-24). The ratio of M3G/MOR increased with time in both study groups. However, the ratio of M3G/MOR at 60 minutes after IVDIM was twice that obtained with INDIM due to the delayed peak concentration with INDIM (Figure 6-25).

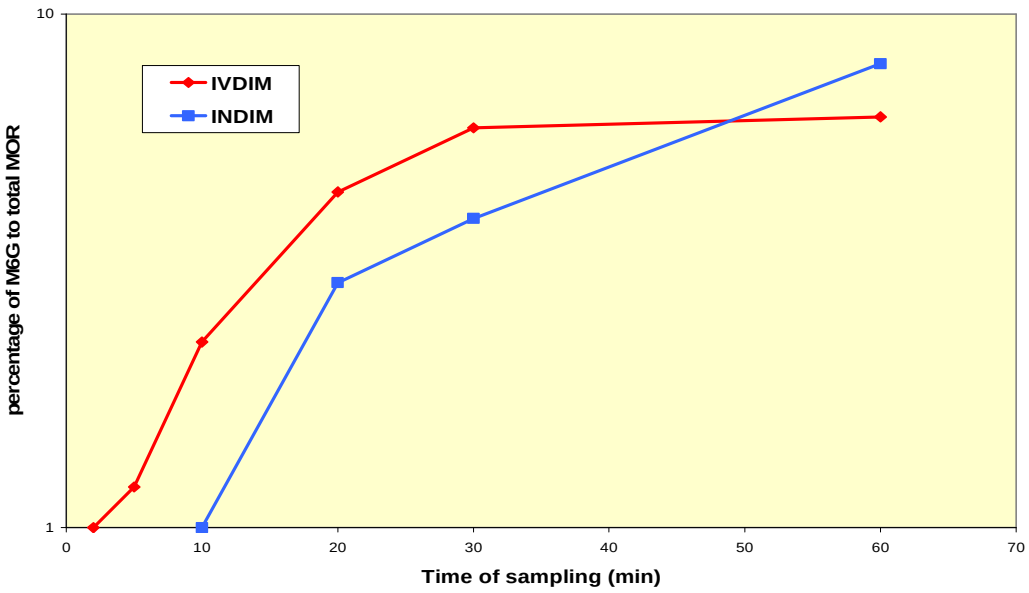


Figure 6-21: M3G as a percentage of total morphine after IVDIM and INDIM.

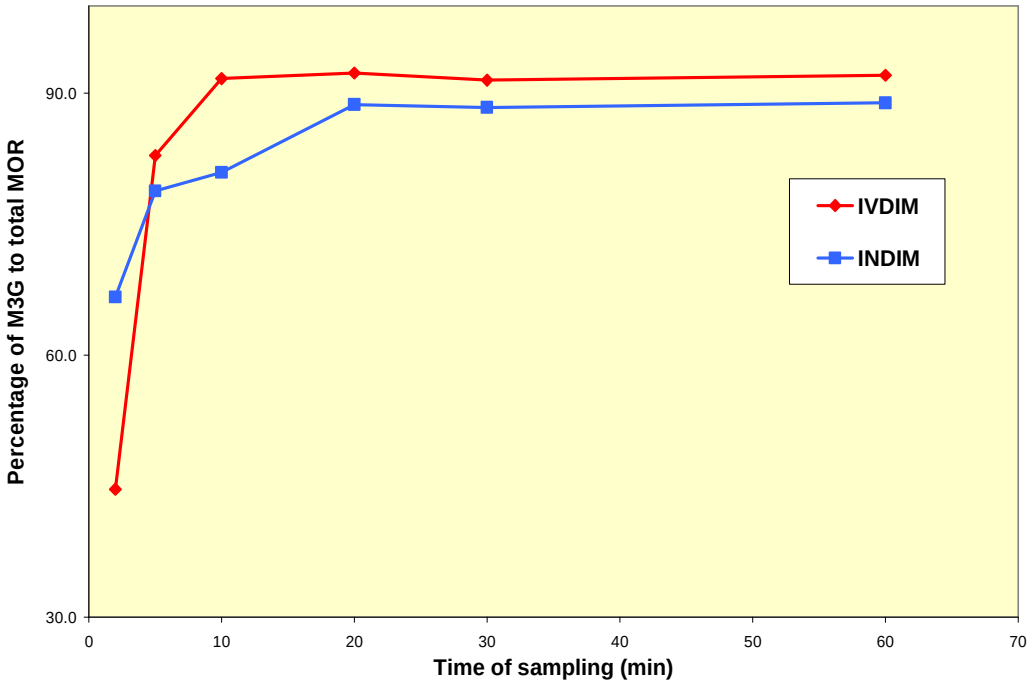


Figure 6-22: M6G as a percentage of total morphine after IVDIM and INDIM.

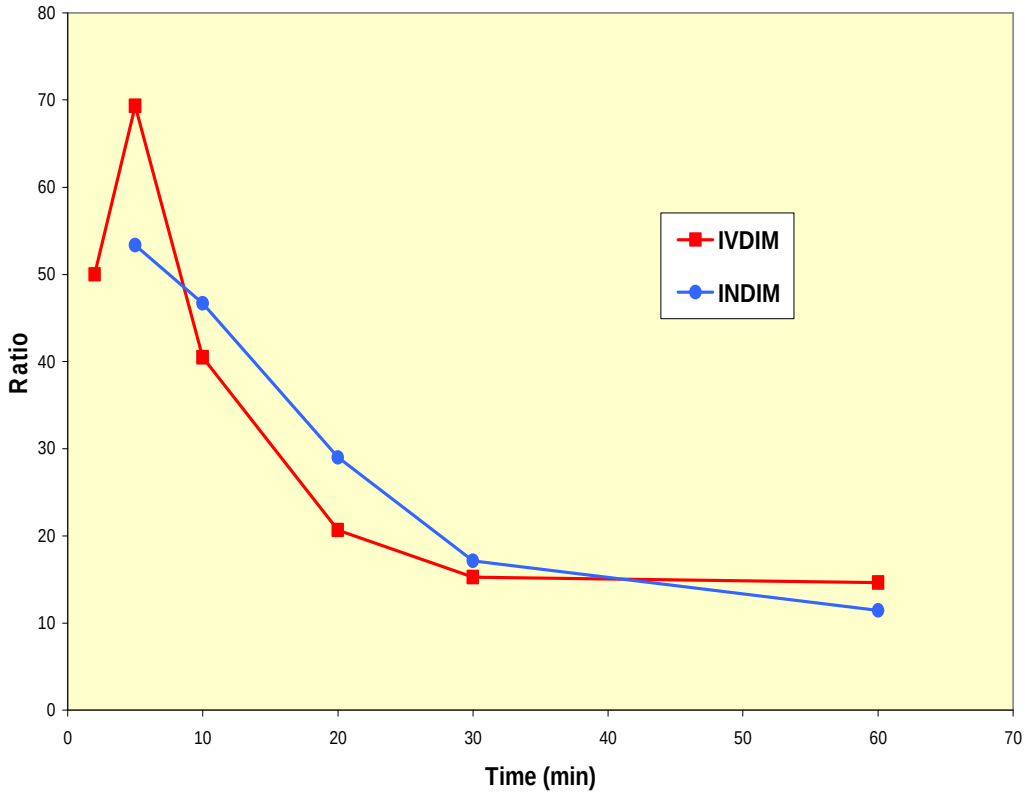


Figure 6-23: Ratio of M3G/M6G after IVDIM and INDIM.

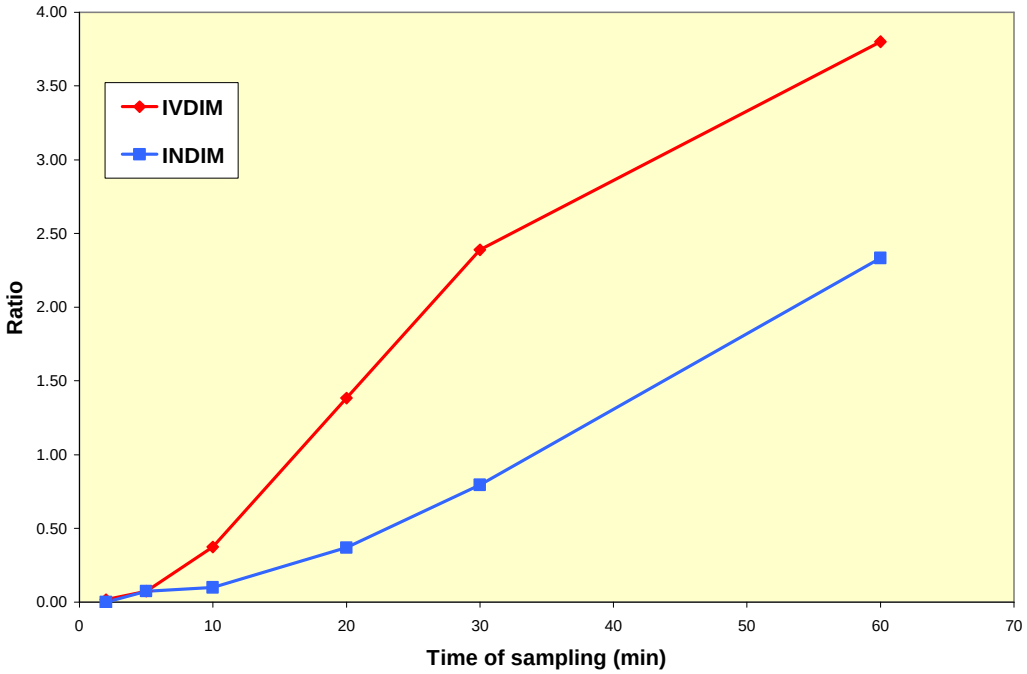


Figure 6-24: Ratio of M6G/MOR after IVDIM and INDIM.

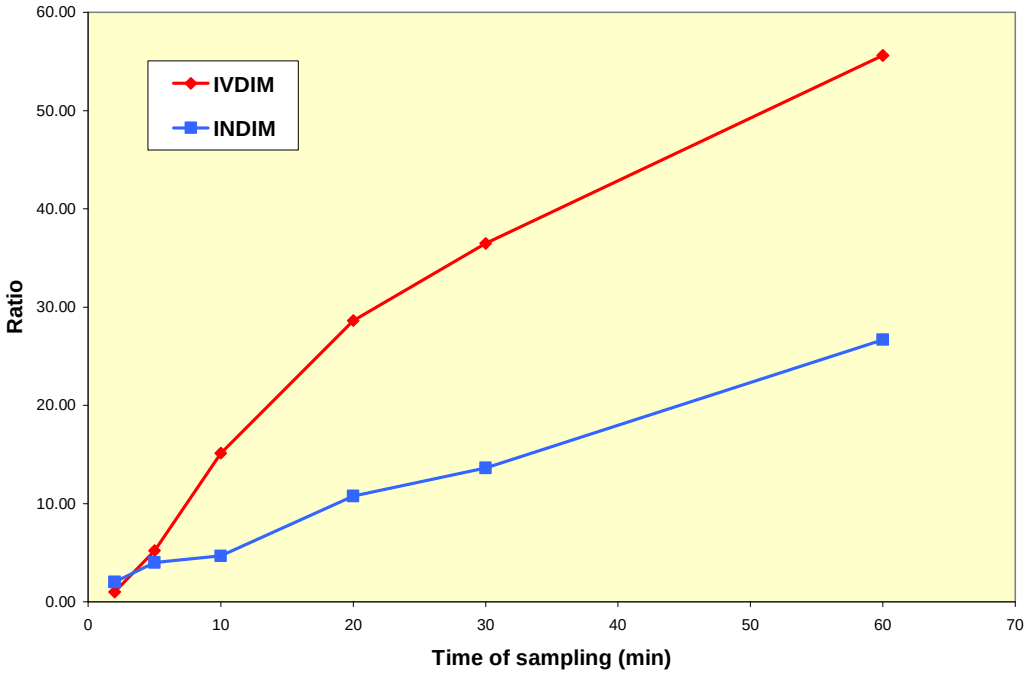


Figure 6-25: Ratio of M3G/MOR after IVDIM and INDIM.

Female subjects in both study groups were found to produce slightly higher metabolite concentrations compared to male subjects but were found to have

the similar plasma profiles in both study groups. These relationships are illustrated in Figures 6-26 and 6-27.

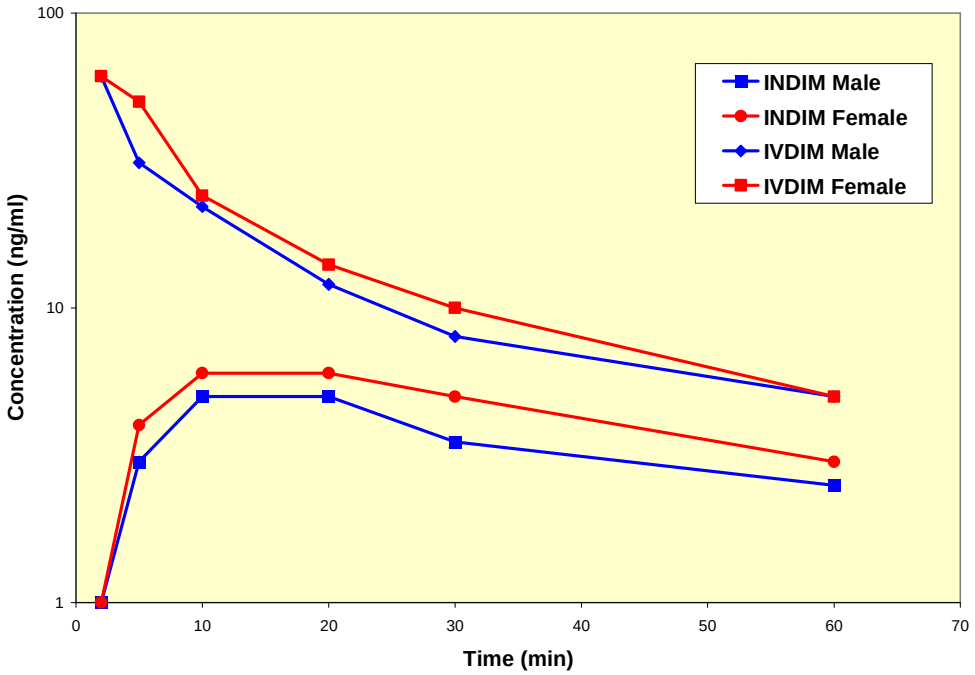


Figure 6-26: Comparison of MOR profiles in males and females after IVDIM and INDIM.

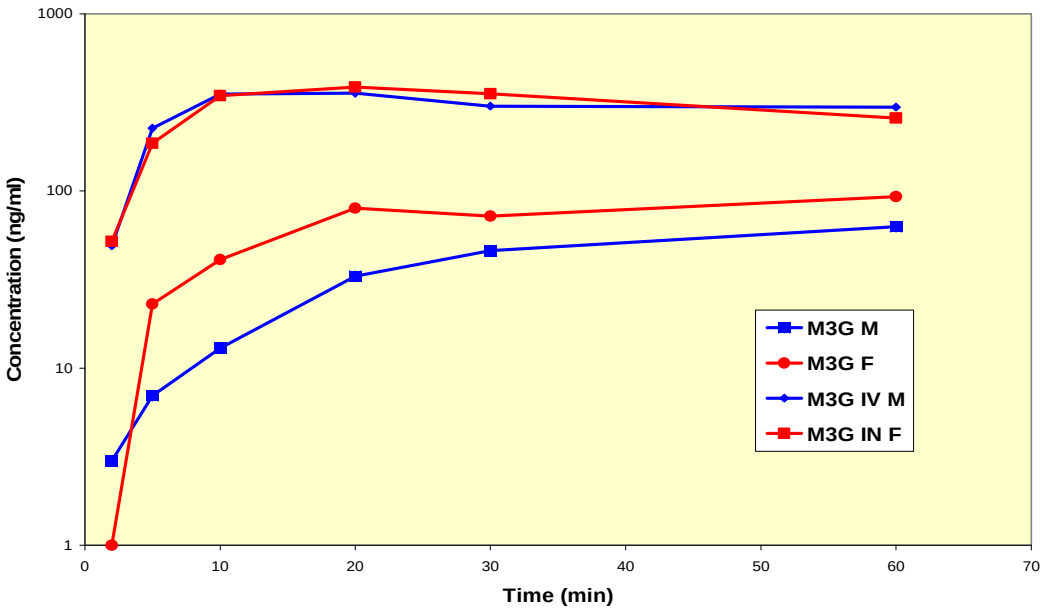


Figure 6-27: Comparison of M3G profiles in males and females after IVDIM and INDIM.

6.8.3.4 Pharmacokinetic data

There was difficulty in obtaining the half-life and other pharmacokinetic data from the plasma time course of DIM after intravenous and intranasal mean of administration. For DIM and 6-MAM only two points were used to define elimination rate (lambda z) or there was a very poor fit in some cases. The experimental data were therefore used to calculate AUCs to the last time point (AUCall). A summary table of the mean values of the main pharmacokinetic parameters is given in Table 6-13.

6.8.3.4.1 Intravenous DIM

The mean terminal half-lives of DIM and 6-MAM after IVDIM were calculated to be 4 minutes and 7 minutes, respectively. The terminal half-life of MOR was found to be 41 ± 11 minutes with a median of 43 minutes. In cases 5-7, however, the half life was very short, ranging between 10-12 minutes.

T_{max} and C_{max} for DIM and 6-MAM following IVDIM are listed in Table 6-14. The median T_{max} was 2 minutes and median C_{max} values were 728, 524 and 75 ng/mL for DIM, 6-MAM and MOR, respectively, although there was significant variability between individuals in C_{max}. The median C_{max} and T_{max} of M£G and M6G were 358 ng/mL and 20 minutes and 21 ng/mL and 30 minutes, respectively (Table 6-14).

AUC calculated for all DIM metabolites are detailed in Table 6-14. DIM exhibited a very high inter-individual variability between subjects and ranged between 1230 to 44150 µg.min/L with a median of 2790 µg.min/L. The gender of the subject affected the AUC, as females had higher AUC values than males with median AUCs of 13610 and 4710 µg.min/L, respectively.

6-MAM had a lower AUC than DIM, in the range 249-6603 µg.min/L with the median at 1220 µg.min/L. Male subjects had a slightly higher mean AUC for 6-MAM compared to female subjects, with mean values of 2130 and 1980 µg.min/L, respectively.

Table 6-13: Summary of pharmacokinetic data

Substance	IVDIM average values in 12 cases				INDIM average values in 11 cases				
	$T½$ min	T_{max} min	C_{max} ng/mL	AUCall ng.min/mL	$T½$ min	T_{max} min	C_{max} ng/mL	AUCall ng.min/mL	Bioavailability
DIM	4	2.0	728	9162	4	2.0	18.0	129	1.4
6-MAM	7	2.0	524	2054	14	5.0	9.0	109	5.3
MOR	41	3.0	75.0	873	46	13.0	7.0	242	27.7
MOR-3-GLUC	n.d	18.0	383	18140	n.d	56.0	94.0	3260	17.9
MOR-6-GLUC	n.d	36.0	23.0	973	n.d	57.0	8.0	208	21.4

Table 6-14: Pharmacokinetic data for diamorphine and 6-MAM in IVDIM group.

	Diamorphine			6-Monoacetyl-morphine		
Case no.	T_{max}	C_{max}	AUCall	T_{max}	C_{max}	AUCall
	min	ng/mL	µg.min/L	min	ng/mL	µg.min/L
1	2	2062	18450	2	1880	5520
2	5	971	44150	5	529	2990
3	2	228	1550	2	80	445
4	2	430	3150	5	116	1320
5 *	2	364	2440	2	57	249
6	2	1112	8120	2	487	1870
7	2	937	13300	2	768	2210
8	2	260	2010	2	237	964
9	2	1601	11740	2	1710	6600
10	2	324	2210	2	106	630
19	2	190	1230	2	205	1120
20	2	255	1600	2	118	710

* No 2 minutes-sample provided.

Table 6-15: Pharmacokinetic data for morphine and its glucuronides in IVDIM group.

	Morphine			Morphine-3-glucuronide			Morphine-6-glucuronide		
Case no.	T_{max}	C_{max}	AUCall	T_{max}	C_{max}	AUCall	T_{max}	C_{max}	AUCall
	min	ng/mL	µg.min/L	min	ng/mL	µg.min/L	min	ng/mL	µg.min/L
1	2	223	1860	30	757	34970	30	56	2130
2	5	192	2070	10	342	16240	30	31	1240
3	10	19	835	30	373	19430	60	19	706
4	5	44	966	10	582	29090	20	23	1060
5 *	5	22	418	10	453	22610	60	27	1080
6	2	42	461	10	246	11700	30	13	615
7	2	32	187	20	191	6390	30	14	604
8	2	22	381	20	310	10420	60	12	476
9	2	227	1480	10	595	31490	30	28	1190
10	2	15	400	20	459	21960	20	18	830
19	2	34	611	30	113	5580	30	16	707
20	2	31	810	20	172	7810	30	23	1060

AUC $_{0\text{-}60\ minutes}$ of MOR after IVDIM was in the range of 187-2070 µg.min/L (median, 710 µg.min/L). Cases 1 and 2 had the highest MOR AUCs (1860 and 2070, respectively) while the other cases had a lower MOR AUC (median, 461 µg.min/L). A higher MOR AUC was observed in females compared to males (means of 955 and 792 µg.min/L, respectively, Table 6-15).

The AUC of M3G and M6G ranged between 5579-35000 µg.min/L (mean, 18140 µg.min/L) and between 476-2130 µg.min/L(mean, 973), respectively. There were no differences between male and female subjects for either glucuronide.

6.8.3.4.2 Intranasal DIM

The mean terminal half-lives of DIM and 6-MAM after INDIM were calculated to 4 minutes 14 minutes for 6-MAM, respectively. The mean terminal half-life of MOR was 46 ± 20 minutes. The MOR half life was very long in Case 21, estimated at 257 minutes, and could not be calculated for case 11 because the MOR concentration was still rising at the end of the study period. Similarly, the terminal half-lives of morphine glucuronides could not be calculated for either group because the study period was too short.

In INDIM cases, AUCs for DIM ranged between 44-220 µg.min/L (mean, 129 µg.min/L). The mean AUC value was higher with female than male subjects (means 143 ± 58 and 112 ± 38 µg.min/L, respectively). After INDIM, the AUC for 6-MAM was found to be slightly lower than for DIM and ranged between 8-238 µg.min/L (mean, 109 µg.min/L); the mean C_{max} and T_{max} were 3 minutes and 5 ng/mL, respectively. Female subjects also had higher AUCs than males with means of 133 and 81 µg.min/L, respectively (refer to Table 6-16).

In INDIM cases, MOR AUCs ranged between 85-437 µg.min/L (mean, 241 µg.min/L. The mean T_{max} and C max was 13 minutes and 7 ng/mL, respectively. Female subjects had higher AUCs than males: mean MOR AUCs were 276 and 201 µg.min/L, respectively. A comparison of pharmacokinetics data of morphine and its glucuronide are detailed between IVDIM and INDIM study groups are listed in Table in Table 6-17.

Table 6-16: Pharmacokinetic data for diamorphine in INDIM group

	Diamorphine			6-Monoacetyl-morphine		
Case no.	**T_{max}**	**C_{max}**	**AUCall**	**T_{max}**	**C_{max}**	**AUCall**
	min	**ng/mL**	**µg.min/L**	**min**	**ng/mL**	**µg.min/L**
11	2	16	131	5	15	238
12	2	30	220	5	16	169
13	2	17	168	5	9	127
14	5	15	141	10	11	169
15	2	26	164	2	3.4	47
16	5	8	102	5	1.7	8
17	5	6	44	2	7	123
18	2	12	118	10	10	143
21	2	17	63	2	10	72
22	2	43	161	2	8	55
23	2	12	109	2	4	54

Table 6-17: Pharmacokinetic data of morphine and its glucuronide in INDIM group

	Morphine			Morphine-3-glucuronide			Morphine-6-glucuronide		
Case no.	**T_{max}**	**C_{max}**	**AUCall**	**T_{max}**	**C_{max}**	**AUCall**	**T_{max}**	**C_{max}**	**AUCall**
	min	**ng/mL**	**µg.min/L**	**min**	**ng/mL**	**µg.min/L**	**min**	**ng/mL**	**µg.min/L**
11	20	8	4387	60	87	2728	60	8	205
12	10	14	320	60	160	5469	60	14	408
13	10	10	349	60	49	1625	60	8	202
14	10	5	192	60	86	3382	60	1.4	37
15	20	5	221	20	250	7909	30	5	179
16	10	2.2	85	60	53	1880	60	8	200
17	20	12	393	60	46	1833	60	4	114
18	20	8	271	60	87	3200	60	3	88
21	10	4	138	60	81	3301	60	13	420
22	5	5	92	60	91	2882	60	13	310
23	5	3.4	162	60	44	1593	60	5	125

In INDIM cases, M3G AUCs ranged between 1593-7909 (mean, 3255 µg.min/L). The mean AUCs of M3G were 4034 and 2320 for female and male subjects, respectively. The mean T_{max} and C_{max} of M3G in this group were 56 minutes and 94 ng/mL, respectively. M6G AUCs were in the range of 37-420 (mean, 208 µg.min/L). There was no difference in M6G AUCs between female and male subjects and were 209 and 207 µg.min/L, respectively. The mean T_{max} and C_{max} were 57 minutes and 8 ng/mL, respectively.

6.9 Discussion

6.9.1 Method validation

An LC-MS/MS method has been developed and found to be sufficient for the analysis of DIM and its metabolites at lower LLOQs than in previously published methods reviewed in Table 6-1. In most of these, the LLOQ of DIM was equal to or above 1 ng/mL [108,302,303,305,308,317,326] and an LOD of 0.5 ng/mL was reported in two studies using LC-APCI-MS [178] and GC-MS [327] which was found to be sufficient with adult subjects. The situation is different with children since very low levels in plasma from INDIM cases are expected in the range 1 - 10 ng/mL.

In the current application, two calibration curves were required since the method was to be applied to the determination of DIM and its metabolites in plasma from children, in a study that was carried out as a blind study. The concentrations of DIM or 6-MAM to be expected in children after DIM intake were not available in the literature, but were predicted to be very low. A calibration curve extending to a low level was crucial for obtaining accurate results and also to avoid false negative results. In the present study, LODs and LLOQs of DIM and its metabolites were included within the calibration range, which helped in examining the method sensitivity at the LLOQ with each sample batch. This is one of the advantages of this method over previously reported methods.

IVDIM subjects had high levels of DIM and its metabolites which were within the range of the high calibration curve; however, low concentrations were obtained when the subject had INDIM but some samples were also found within both the

low and high calibration ranges. If the method was not validated using both calibration curves, it is possible that high concentrations of DIM and its metabolites would be not quantified accurately as they would be out of the calibration range and might lead to false negative results for low DIM concentrations.

Dams *et al* [142] studied the matrix effect by using a post-column infusion of morphine with blank urine samples in duplicate at 10 μg/mL. No matrix effects were observed after 1 min of chromatography. Rook *et al* [75] studied the effects of plasma matrix on the ionisation of DIM and its metabolites; they found that ion suppression was within the acceptable criteria for method validation. However, no information was presented in their work on how they examined the matrix effects and what concentration they used.

In the current study, five human plasma samples were used for the investigation of matrix effects present and to calculate recovery of analytes of interest. Both ion suppression and enhancement were detected but no effects on DIM ionisation of metabolites was observed. This was accounted for by the use of SPE which provided clean extracts, the use of internal standards for each DIM metabolite with the exception of NMOR which was not available commercially and finally a good separation of analytes of interest which made it easy to established separate retention window for each analyte. Matrix effects were examined at low levels, 0.5 ng/mL, which is also the concentration used to measure the intra and inter-assay precision of the optimised method. Recoveries were calculated after excluding matrix effects. Good recoveries have been obtained in the current study which compared well with previous methods.

6.9.2 Stability of diamorphine

In the current method, the samples analysed were stored at -70°C in Edinburgh, as mentioned in Section 6.7.6 and available data indicate that they were stable while frozen, for example a 6-month stability study was carried out (Table 6-6) and a similar median concentration of morphine was obtained in IVDIM cases (31 and 33 ng/mL in Kidd *et al* [276] and current study, respectively). RIA results for

MOR obtained for the current study samples by Kidd *et al* [276] are listed in Table 6-18.

From the review of previous methods above (Section 6-7), degradation of DIM during sample preparation was up to 10% but in the current method only 4% degradation was observed.

Table 6-18: Morphine concentration versus time profile using (RIA) by Kidd *et al* [276].

		IN group n=12 Median (range)	**IV group n=13 Median (range)**
C_{max}	(ng/mL)	10.3 (7.8-18.1)	31.0 (27-56.6)
T_{max}	(min)	10 (5-60)	2(2-5)
AUC	(nmol/L-h)	512 (252-650)	1070 (957-1150)
60 min value	(ng/mL)	9.0 (3.8-10.3)	13.4 (9.5-17.1)

6.9.3 Case Studies

The plasma samples from children used in this work were sent to the Forensic Medicine and Science Section in Glasgow University for the purpose of obtaining DIM levels, and at that time no method was available in the Section for DIM analysis. The A & E department of a city-centre paediatric teaching hospital in Edinburgh tested these cases prior to their delivery to the Section but the method used was found insufficient for the determination of DIM and only MOR could be determined, using a radioimmunoassay [276]. In addition, HPLC coupled with electrochemical detection (HPLC-ECD) was trued in Edinburgh but failed to determine DIM, which suggested DIM may have been completely hydrolysed during extraction or an unsuitable SPE was used resulting in no DIM recovery. However, 6-MAM, MOR, M3G and M6G were detected, although this data is unpublished and unavailable at the time of writing.

The samples were collected during the clinical study in 2003, stored at -70 °C and analysed by the current optimised method in 2007. There were many challenges due to the lack of an existing method and long period of storage of samples as DIM is known to be unstable and could be hydrolysed either *in-vivo* or

in-vitro. Also, doses and routes of administration were unknown and no data from previous RIA or HPLC-ECD results were available when the samples were analysed

Little case information data was available from Dr Kidd. Some information came from an abstract of a paper presented by the group, but this did not include detailed results such as concentrations for individual cases, only the median values for T_{max}, V_{max} and AUC and the concentration of morphine at 60 minutes have noted.

Kidd *et al* [276] used a radioimmunoassay method for their analysis which could not distinguish between morphine and other opioids. Also, Kidd *et al* [276] analysed samples from more INDIM cases than were available in current study. However, as mentioned above, similar median concentrations of morphine were obtained in IVDIM cases. By contrast, different median levels of morphine were obtained for INDIM cases (10.3 and 5 ng/mL in Kidd *et al* and current study, respectively). This can be attributed to cross reactivity of the RIA method to both morphine and its glucuronides. Also, more samples were analysed by Kidd *et al* (25 cases compared to 23 cases in the current study).

In addition, the short period of sampling time (60 minutes) has limited the calculation of pharmacokinetic data for MOR and its glucuronides as a result of a poorly-defined declining curve, since the curve for the elimination phase is normally used for calculating clearance and volume of distribution. In previous reports [144,312], samples were collected from time zero to 24 hours following DIM administration which provides more data points for the elimination phase of MOR and its glucuronides. However, it was difficult for the clinicians to obtain additional plasma samples from children due to their progress through hospital sections to complete their treatment and due to the ethical restriction on the invasive procedure of obtaining blood samples for medical staff.

However, the numbers of patients included in the current study were reasonable compared with published pharmacokinetic studies following DIM administration. The first samples were obtained at 2 minutes and these usually had the highest measured concentrations of DIM, indicating that peak concentrations of DIM

occurred no later than 2 minutes after administration. In many pharmacokinetic studies, sampling started after 5 min which was found insufficient to providing a full description of pharmacokinetics profile of DIM [144,317].

In the present study, pharmacokinetic parameters such as C_{max}, T_{max} and AUC_{all} were calculated for the IVDIM and INDIM study groups. INDIM AUC profiles were lower than those obtained with IVDIM subjects, for example, AUC of DIM after INDIM (Bioavilability, Table 6-13) was only 1.6% of the AUC obtained with IVDIM. This may suggest rapid hydrolysis of DIM following INDIM. DIM is known to convert rapidly to 6-MAM and MOR after IVDIM. 6-MAM and MOR were included as target analytes but were found to have lower AUCs after INDIM, only 5% and 28% during the first hour after administration compared to that of IVDIM, respectively.

Significant losses of DIM following intranasal administration, due to absorption in nasal mucosa or swallowing of DIM, have been reported [304] and may lead to a delayed appearance in plasma and to most of the DIM being hydrolysed to 6-MAM and MOR before reaching the blood circulation. Also, phase I metabolites of DIM (6-MAM and to MOR) may have a longer half-life after INDIM than IVDIM, since this metabolism may take place in the stomach and possibly the presence of food or an empty stomach may influence the bioavailability of DIM after dosing.

In the current study, DIM and 6-MAM similar concentrations at 2 and 5 minutes after INDIM, indicating that the T_{max} was between 2 and 5 minutes. This may be explained by the delay of reaching the blood circulation after INDIM between DIM absorbed by nasal mucosa and that of swallowing. 6-MAM was also found to have the same trends of formation and elimination as its parent drug and both had very high hydrolysis rates shortly after DIM administration. Traces of both DIM and 6-MAM could be detected up to 60 minutes after IVDIM, although most had hydrolysed by 20 minutes following INDIM.

MOR is generated from 6-MAM and seemed to have a steady state period between 20 and 30 minutes following INDIM. Levels of M3G after IVDIM were lower than those of MOR at its T_{max} (2 minutes) but were higher than MOR levels at their own peak times with INDIM cases. Both had similar mean levels at 5 minutes after INDIM administration in most cases. This indicates that some MOR

was conjugated in the liver to form M3G and M6G. M6G may achieve only very low concentrations, lower than the LLOQ of the optimised method, since it was only detected in one case. This may be due to the longer half-life and duration of morphine glucuronides in blood which need at least 30 minutes after the MOR peak concentration to reach their own C_{max} plateau period. That can be seen clearly from two routes of DIM administration. The percentages of AUC within first hours of M3G and M6G following INDIM were 18% and 21% of that observed with IVDIM cases, respectively, which meant that less than 22% of morphine and its metabolites were metabolised in the first hour after INDIM. Comparisons between MOR plasma results obtained in the present study and previous studies are listed in Table 6-19.

In the current study, the plasma concentration versus time curves of the two morphine glucuronides were parallel curves following both routes of administration while the curves for MOR and M6G crossed over. This cross-over point was found after 20 and 40 minutes of IVDIM and INDIM route of administration (Figure 6-10 and 6-17), respectively. In IVDIM, the MOR level decreased after the MOR peak concentration at 2 minutes. By contrast, the MOR level increased to reach its peak concentration and steady state period in INDIM groups.

The mean AUC values following IVDIM and INDIM were comparable with previous pharmacokinetics reported in Table 6-3; in the present study the AUC of DIM was 150 and 2.1 μg/L*hr, and was 34 and 1.8 μg/L*hr for 6-MAM after IVDIM and INDIM, respectively. AUC is dose dependent and also depends on time of sample analysis. The latter one is a limitation of the current study in which samples were collected for only an hour following administration.

Table 6-19: Comparison of morphine pharmacokinetic data from previous work and current study (adapted from Kidd *et al* [276]).

Dose of Diamorphine	Route of administration	Cmax (ng/mL)	Tmax (min)	60 min of morphine (ng/mL)	Ref.
6mg	**Snorted powder**	10	<5	1.8	144
12mg	**Snorted powder**	15	<5	10	
6mg	**IM**	15	<5	5	
10.5mg	**IH**	56	2	20	312
10	**IV**	16	5	10	
12	**IV**	44	2	20	
10.5mg	**IH**	11.9	1	4-7	
4mg	**IM**	15	15	8	313
56mg	**Oral**	140	30	60	
112 µg/hr	**IV infusion**	Not reported	Not obtained	10	
112mg	**Snorted powder**		60	11-12	304
15µg/kg/hr	**IV load+infuse**		120	86 plateau	318
40mg	**Nasal spray**	200	2	180	328
40mg	**IV**	330	15	200	
0.1mg/kg	**IV**	31	2	13.4	276
0.1mg/kg	**IN drops**	10.3	10	9.0	
0.1mg/kg	**IV**	33	2	6	Present study
0.1mg/kg	**IN drops**	5	10	3	

The AUC of 6-MAM obtained here was lower than the AUC of DIM in both routes of administration. This can be explained by the improved LLOQ for DIM in the current method, which enabled DIM to be measured at lower concentrations, and over a longer time period. Also, it has been found that the concentration of DIM was higher than that of 6-MAM following both IVDIM and INDIM in the first two samples, which also had the highest concentrations of each analyte.

Analgesia produced by DIM is usually attributed to its active metabolite, MOR. The median MOR level followed IVDIM was 33 ng/mL in the present study, which was considered enough to produce analgesia (Table 6-19). Also, the level of MOR reported in previous work after INDIM and found to produce analgesia was also found with INDIM cases in the present study. Peak concentrations of MOR were in the range 6.2-26 ng/mL. In the study by Cone *et al* [144], levels of MOR ranged between 2.6-13.4 ng/mL at one hour after administration and were in agreement with Skopp *et al* [304] who found that the C_{max} of MOR ranged between 6.1-13.2 ng/mL after INDIM.

The presence of M6G at later stages was reported to be responsible for DIM analgesia and found to be more effective than MOR. The levels of DIM at 1 hour after administration were reported following oral and DIM infusion at high DIM doses (56 mg and 40 mg), which resulted in MOR concentrations of 60 [313] and 180 -200 ng/mL [328], respectively. In addition, plasma MOR concentrations were within the range obtained in the present study following INDIM and IVDIM, which were 1.8-10 ng/mL [144,313,317] (Table 6-19). Therefore, it can be confirmed that therapeutic levels of MOR can be achieved following INDIM in children based on the pharmacokinetic data presented in the current study.

The median plasma peak concentrations of DIM, 6-MAM, MOR and M3G following INDM in this work were similar to those reported by Cone *et al* [144] and Skopp *et al* [304]. Data obtained after IVDIM were similar to those reported by Jenkins *et al* [317]. $T_{½}$ was similar to most previous studies and ranged between 3-5 minutes for DIM (see Table 6-3). In the current study, the median $T_{½}$ for DIM after IVDIM and INDIM was 4 min and 3 min , respectively.

In previous work, it has been found that peak plasma concentrations of DIM metabolites were lower following smoked or intranasal DIM compared to IVDIM which was the same as in the present work. DIM and 6-MAM were detected with both routes of administrations in the present work which may indicate that DIM and 6-MAM provided the analgesic effects at the early stage when MOR levels were low or not detected, especially in INDIM cases.

In the present study, the two routes of administration can be distinguished on the basis of the plasma metabolite concentrations. IVDIM produces faster and higher pharmacokinetic profiles for DIM and its metabolites compared to INDIM. There is no data available in the literature regarding DIM levels in children treated with DIM to assist in the interpretation of DIM metabolites levels obtained in this study, and so these were compared with the results of studies in adults.

6.10 Conclusions

Methods of DIM analysis published between 1991 and 2009 were reviewed for the first time for this thesis.

Many methods have been reported for extraction of DIM metabolites and among these SPE is the method of choice, producing clean extracts, good sensitivity and minimal matrix effects. In the present study, DIM metabolites were extracted and analysed using SPE and LC-MS/MS procedures. LC separation was performed using the Synergi Polar RP column (150 X 2mm, 4µm) and a gradient mobile phase. DIM and its metabolites were well separated from each other. SPE was sufficient to provide clean extracts and minimize the effect of matrix on the ionisation of analytes. Results of analysis of case samples obtained following intravenous and intranasal DIM were reported and DIM metabolites were determined with the exception of NMOR which was found to be negative in both case groups.

Following IVDIM, the concentrations of DIM, 6-MAM and MOR decreased sharply whereas the rate of decrease was slower after INDIM. Pharmacokinetic parameters, C_{max}, T_{max}, $T_{½}$ and AUC, were calculated for the two patient groups.

These were compared with each other and with previous studies in adult patients. The pharmacokinetics of DIM and its metabolites following IN and IV administration in children have been compared for the first time in this study, which confirmed that INDIM can achieve therapeutic plasma concentrations of DIM and its active metabolites, although these are lower than those obtained with IVDIM and occur at later times after administration.

7 Comparison of Nonhydrolysis and Hydrolysis Methods for the Determination of Buprenorphine Metabolites in Urine by LC-MS/MS.

7.1 Introduction

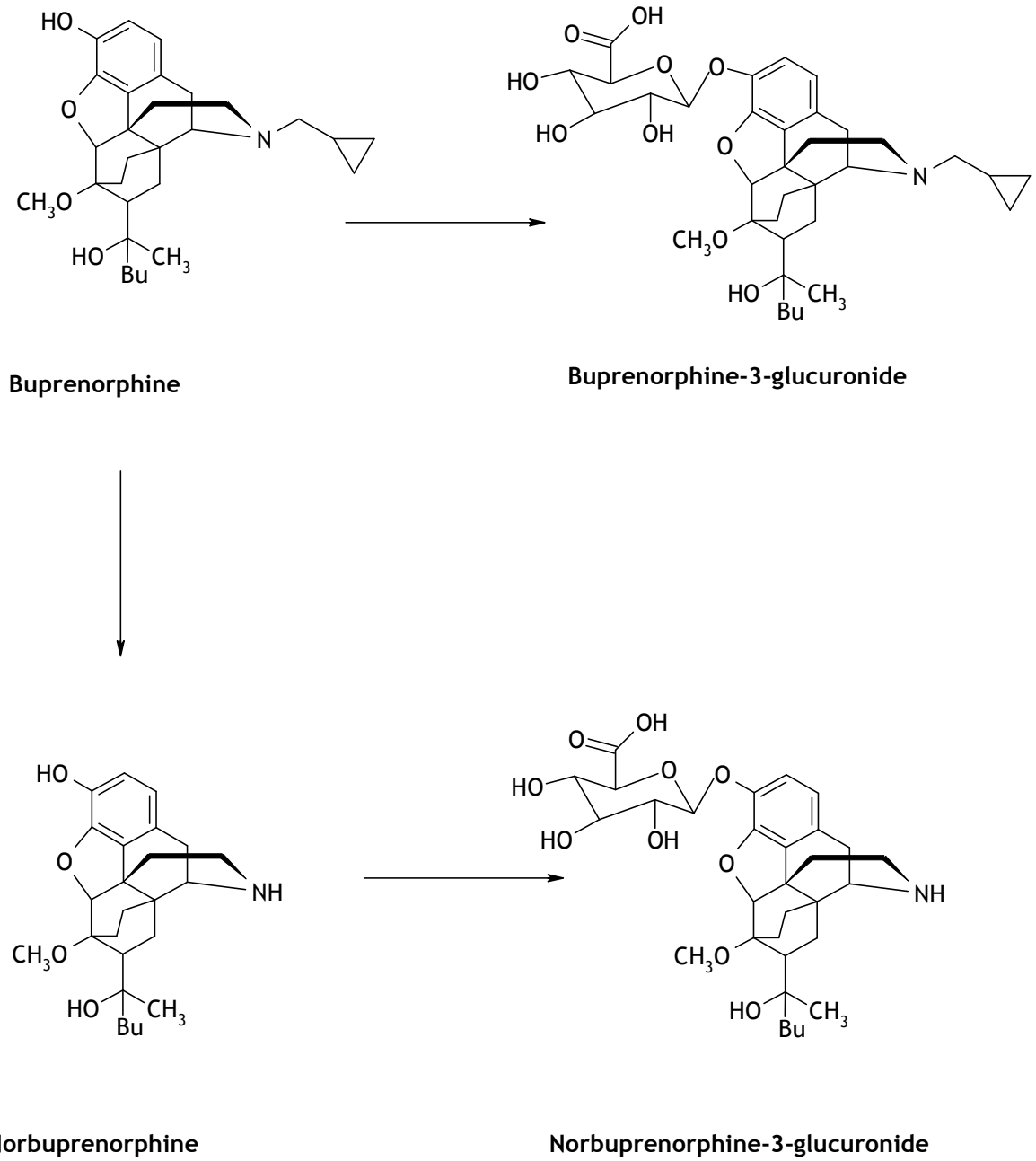
Buprenorphine (BUP) is a semisynthetic opioid, prepared from thebaine [1,125,132] in a seven-step sequence [329], which is 25 to 40 times more potent than morphine and is therefore a powerful analgesic. It is a partial κ-opioid receptor antagonist and μ-opioid receptor agonist [124,126,330,331]. Buprenorphine is used for the treatment of moderate to severe pain, for example in surgical and neoplastic cases, and in the treatment of opiate dependence [124,331-333].

7.1.1 Metabolism and excretion

BUP has a low bioavailability when taken orally due to extensive first pass metabolism. In addition, it retains its potency as an opioid agonist even at high dosage, from 2-32 mg, without leading to overdose. It appears to act as a limited respiratory depressant [124,126,331,332]. BUP has been studied widely. It is metabolised by N-dealkylation to the active metabolite norbuprenorphine (NBUP) and both BUP and NBUP are conjugated with glucuronic acid to form buprenorphine-3-glucuronide (BUP3G) and norbuprenorphine-3- glucuronide (NBUP3G), respectively [132,250,331] (Figure 7-1).

The first application was reported by Polettini and Huestis [332] seven years ago and the first method for the direct determination of polar metabolites in urine was reported in 2003. The determination of conjugated and free metabolites using a hydrolysis method was reported in 1984. Cone *et al* [334] analysed BUP metabolites following subcutaneous, sublingual and oral BUP administration in urine and faeces. The percentage of BUP metabolites in urine was the opposite of the faeces profile as NBUP metabolites exceeded BUP metabolites in urine and vice versa. In addition, pharmacokinetic profiles of BUP and its metabolites,

including glucuronides, reported by Huang *et al* [250] give different concentrations for free BUP and NBUP than those reported by Ceccato *et al* [335] and Polettini and Huestis [332]. The BUP concentration exceeded that of NBUP in Ceccato *et al* [335] and Polettini and Huestis [332] while the NBUP concentration exceed that of BUP in Huang *et al* [250].



Buprenorphine

Buprenorphine-3-glucuronide

Norbuprenorphine

Norbuprenorphine-3-glucuronide

Figure 7-1: Buprenorphine metabolism.

7.1.2 Toxicity

Symptoms associated with buprenorphine overdose include confusion, dizziness, pinpoint pupils, hallucinations, hypotension, respiratory difficulty, seizures and coma [132]. The mechanism of buprenorphine intoxication is not clear due to the low concentrations found in cases. It has been suggested that norbuprenorphine is responsible for burenophine deaths due to its action as a very potent respiratory depressant and can be detected in plasma following buprenorphine intoxication. However, it is not known yet if norbuprenorphine alone could potentially induce respiratory depression. In animal experiments, it has been found that administration of buprenorphine and norbuprenorphine blocks the norbuprenorphine-induced respiratory effect. In addition, the parent drug has been found to be mostly unchanged in buprenorphine fatalities [336-339].

Buprenorphine was considered to be safe until it became an abused drug due to its availability in the black market, especially, if it is taken by intravenous injection, and the use of buprenorphine concomitant with benzodiazepines has been found to increase its toxicity and subsequently the incidence of fatalities sharply [125,340-342]. 150 deaths have been reported [340,342]. Blood concentrations of buprenorphine were reported for buprenorphine related fatalities and ranged between 1-76 and 2-65 ng/mL for buprenorphine and norbuprenorphine, respectively [343]. Kintz *et al* [341] reported buprenorphine levels of 1.1 and 0.2 ng/mL for buprenorphine and norburenorphine, respectively, in fatal drug-facilitated sexual abuse cases. Unhydrolysed and hydrolysed urine were tested and 9.1, 9.6 and 575 and >1000 ng/mL were detected for buprenorphine, norbuprenorphine, respectively [341]. Also, low concentrations of BUP and NBUP were encountered in 20 BUP fatalities which ranged between 1.1-29 and 0.2-13 ng/mL and averaged 8.4 and 2.6 ng/mL; urine levels were in the range of 4-1033 and 6.6-230 ng/mL and averaged 172 and 67 ng/mL for BUP and NBUP, respectively [340].

7.1.3 Previous work

Many methods have been developed for screening and quantification of BUP and its metabolites in biological fluids using several analysis techniques.

Immunoassay techniques were used in early studies for quantification of BUP [344] and many reports describe the use of immunoassay for BUP screening [253,345,346]. Although immunoassay methods for BUP screening are widely used and considered to be rapid, sensitive and capable of processing large numbers of samples compared to chromatographic methods, they are not sufficiently specific to differentiate between BUP and its metabolites due to cross reactivity with metabolites and structurally similar compounds. Gas chromatography with electron capture or mass spectrometric detectors [251,347-349] and HPLC methods with UV [350], fluorescence [351] or electrochemical detectors [352] were reported. These methods were found to lack sensitivity, being unable to detect BUP and NBUP with concentrations of less than 1 ng/mL. In addition, most of the methods were not employed for routine analysis. The methods showed some critical limitations such as being time-consuming due to the use of hydrolysis procedures and derivatisation steps.

Methods using liquid chromatography coupled with mass spectrometry [342,353] and tandem mass spectrometry [250,256,332,335,354-359] have been reported. Most methods validated for BUP analysis used enzymatic hydrolysis although for opioids acid hydrolysis is often favoured for opioids [247,360,361]. Feng *et al* [362] investigated the efficiency of using acid, alkaline and enzymatic procedures for hydrolysis of BUP3G to BUP and found neither acid nor alkaline hydrolysis was suitable for BUP analysis. They reported slow acid hydrolysis rates with only 2% conversion after 2 hours at 50^{o}C. Degradation of analyte was observed with increased temperatures. In the same study, no recovery of BUP was obtained after hydrolysis with 10 M KOH or concentrated hydrochloric acid at room temperature for 16 hours. Complete hydrolysis of BUP3G was achieved enzymatically in 2 hours at 37 ^{o}C using *E.coli,* 4 hours at 60 ^{o}C using *H. pomatia*. and in 1 hour at 60 ^{o}C with *Glusulase*. Elsohly *et al* [348] investigated the enzymatic hydrolysis of NBUP3G and found that it was completely hydrolysed by ß-glucuronidase from *E.coli* or *H. pomatia* following incubation at 60 ^{o}C for 4 hours.

The rate of hydrolysis is different for the two glucuronide metabolites and this could potentially lead to false negative results for NBUP3G as it requires a longer hydrolysis time than BUP3G [348,354]. Different rates of hydrolysis could arise due to differences in the laboratory environment, analysis procedure, amount of

enzyme used and incubation time and temperature. The sensitivity of enzymatic hydrolysis methods was always questionable due to limitations caused by, for example, incomplete hydrolysis, contamination of the enzyme preparation and their inability to cleave some glucuronide metabolites to their free form [76,247].

LC-MS techniques now available should replace traditional hydrolysis methods since they can provide unique methods of identification and quantification of analytes and can detect intact glucuronides without hydrolysis or derivatisation procedures.

In fact, the hydrolysis step is crucial for quantification of BUP as the free drug is found at very low concentration. A method for determination of the polar metabolites was reported which did not include BUP and NBUP [355]. By contrast, a study by Fox *et al* [356] compared the amount of BUP and NBUP in urine samples before and after enzymatic hydrolysis. Interestingly, the contributions made by the free drugs to the total BUP and total NBUP were not detectable-67% (mean 6.4%) and not detectable-100% (mean 34.7%), respectively. The authors noted that the ratios between free and conjugated metabolites are variable and advocated that hydrolysis methods should be replaced by direct determination of glucuronides.

7.2 Aims

In practice, enzymatic hydrolysis methods continue to be widely used and there have been few reports of simultaneous direct analysis of glucuronide metabolites and free drugs [250,256,355]. Also, until now there has not been a direct comparison of these two methods of analysis using real case samples.

In this work, a method for the simultaneous determination of BUP, NBUP, BUP3G and NBU3G was validated and then compared with an in-house method for quantification and identification of BUP and NBUP which uses enzymatic hydrolysis with ß-glucuronidase (*Helix pomatia*). This comparison was carried out using real case urine sample testing positive for BUP. A full description of the in-house method was reported previously by Miller *et al* [253].

7.3 Methods and Materials

7.3.1 Reagents and Standards

Methanol, acetonitrile and acetic acid (HPLC grade) were obtained from BDH (Poole, UK). Ammonium carbonate, formic acid and ammonium hydroxide were also purchased from BDH. Ammonium formate was obtained from Across Organics (New Jersey, USA). ß-glucuronidase was purchased from Sigma-Aldrich (Dorset, UK).

Buprenorphine (BUP), buprenorphine-D4 (BUP-D4), norbuprenorphine (NBUP), norbuprenorphine-D3 (NBUP-D3) and Norbuprenorphine-3-glucuronide (NBUP3G) were purchased from Promochem (Middlesex, UK). Buprenorphine-3-glucuronide (BUP3G) was purchased from ElSohly Laboratories (Oxford, MS, USA). All standards and internal standards were obtained as solutions in methanol at a concentration of 0.1 mg/mL and each had a purity of more than 99%. Bond Elut LRC-C18 cartridges were purchased from Varian (CA, USA).

Individual working standards were prepared at a concentration of 1 µg/mL by dilution of the stock solutions. Working mixtures of standards and internal standards were similarly prepared.

7.3.1.1 Preparation of 0.01M Ammonium carbonate buffer (pH 9.3)

1.571 g of ammonium carbonate was weighed and added to a I L volumetric flask and 800 mL of DI H_2O was added. The pH was then adjusted to 9.3 using concentrated ammonium hydroxide. The volume was made up to 1 L using deionised water. The buffer was stored at 4 ^{0}C until used and unused solution was discarded after 4 weeks from the date of preparation.

7.3.1.2 Preparation of 1 M, pH 5 Sodium Acetate Buffer

42.9 g of sodium acetate trihydrate and 10.4 mL of concentrated (17 M) glacial acetic acid were dissolved in 400 mL of deionised water. The pH was adjusted to

5.0 with 1 M acetic acid and the solution made up to 500 mL with deionised water.

7.3.1.3 Preparation of 0.01 M, pH 3.0 Acetic Acid

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

7.3.1.4 Preparation of Phosphate Buffer (0.1 M, pH 6.0)

1.70 g of potassium hydrogen phosphate and 12.14 g of potassium dihydrogen phosphate were weighed into a 1 L beaker. 800 mL deionised water was added and the solid was dissolved by stirring. After the solid had dissolved, the pH of the solution was adjusted to 6.0 using 1 M potassium hydroxide solution. The solution was then transferred into a 1 L volumetric flask and was made up to the 1 L mark with deionised water.

7.3.1.5 Preparation of 0.01 M Ammonium Formate (pH 3)

0.630 g of ammonium formate was added to a 1 L volumetric flask and 800 mL of DI H_2O was added. The pH was adjusted to 3 or 4.5 using concentrated formic acid. Then volume was made up to 1 L with deionised water. Ammonium formate buffer was prepared freshly and stored at room temperature until used; unused solution was discarded after 4 weeks from the date of preparation.

7.3.1.6 Preparation 0.001 M of ammonium formate pH 3

100 mL of 0.01 M ammonium formate solution were added to 1 L volumetric flask and made up to 1L with deionised water.

7.3.1.7 Preparation of 0.003 M Ammonium Formate + 0.001% formic acid

0.189 g of ammonium formate and 10 µL of concentrated formic acid were added to a 1 L volumetric flask and made up to the mark with deionised water.

7.3.1.8 Preparation of 0.005 M Ammonium Acetate

0.385 g of ammonium formate were added to I L volumetric flask and made up to volume with deionised water. The pH was adjusted to 4.5 using concentrated acetic acid.

7.3.2 Direct determination procedure

7.3.2.1 Solid Phase Extraction (SPE)

One millilitre of urine was added to 3 mL of 0.01 M ammonium carbonate, pH 9.3 and 50 µl of the internal standard working solution was added. The mixture was vortex mixed, allowed to equilibrate for 10 minutes and then centrifuged for 10 minutes at 3000 rpm. The supernatant was applied to a Bond Elut C18 SPE cartridge preconditioned with 3 mL methanol, 3 mL of deionised water, and 3 mL of 0.01 M ammonium carbonate (pH 9.3). The SPE cartridge was washed twice with 3 mL 0.01 M ammonium carbonate (pH 9.3), and then dried for 10 minutes. Retained drugs were eluted with 3 mL methanol, after which the eluate was evaporated to dryness under nitrogen at 50^0C. The extract was reconstituted with 150 µl of initial mobile phase and centrifuged at 4000 rpm and then the supernatant was transferred to an auto-sampler vial and 20 µl was injected into the LC-MS/MS instrument.

7.3.2.2 Chromatography conditions

Chromatographic separation was achieved using a Synergy Polar RP column (150 x 2.0 mm, 4-µm particle size), protected by a guard column with identical packing material (4 x 2.0 mm, Phenomenex, Torrance, CA). Gradient elution was based on a mobile phase consisting of 10 mM ammonium formate adjusted to pH 3 (A) and acetonitrile (B) at a flow rate of 0.3 mL/min for the first 8 min, decreasing to 0.2 mL/min at 13 min for the next 13 min. After that, the initial flow rate was applied until the end of analysis.

The gradient conditions were initially 97% of solution A for 3 min; decreasing to 84.5% at 8 min, to 74% at 13 min, and to 20% at 26 min; 5% of solution A was maintained for the next 3 min before returning to 97% for 7 min prior to the next

injection. Three time segments were used to maximise the sensitivity of analysis: the first segment from 0-16 min was for NBUP3G, the second segment from 16-21 min was for B3G, NBUP and NBUP-D3, and the last segment from 21-25 min was for BUP and BUP-D4.

7.3.3 In-house Hydrolysis Procedure

7.3.3.1 Solid Phase Extraction

1 mL of the urine sample spiked with 50 ng/ml of internal standard (BUP-d4 and NBUP-D3), 40 µl of ß-glucuronidase crude solution (Helixa pomatia) and I ml of acetate buffer (pH 5, 1M) were added to a 20 mL glass tube and incubated in an oven for 3 hrs at 60 ^{0}C. Three ml of phosphate buffer (pH 6, 0.1 M) was added to the mixture after cooling. Samples were centrifuged and submitted to Bond Elut Certify LCR SPE cartridge that was preconditioned with 2 mL of methanol and 2 mL phosphate buffer (pH 6, 0.1 M). Then each column was subject to two washing steps using 1 mL of deionized water following by 0.5 mL acetic acid (pH 3, 0.01 M), and full vacuum was then applied for 10 min. 50 µL of methanol were added, then the columns were dried with full vacuum for 2 min. SPE cartridges were washed with 4 mL Acetone/Dichloromethane (1:1, V/V). BUP and NBUP were eluted with 2 x 1.5 mL ethyl acetate containing 2 % v/v ammonium hydroxide. The eluents were evaporated to dryness under nitrogen at 30 ^{0}C, reconstituted with 80 µL of initial mobile phase and 20 µL were injected to LC-MS/MS.

7.3.3.2 Chromatography conditions

BUP and NBUP were separated using a Gemini C18 Column (150 mm X 2.0 mm, 5 µm particle size), protected with a guard column (4 mm x 2.0 mm, Phenomenex, Torrance, CA) containing identical packing material. Gradient elution was based on a mobile phase consisting of 3 mM ammonium formate + 0.001% formic acid in water (1) and acetonitrile (2) at a flow rate 0.3 ml/min. LC separation was started with 75% (1), decreasing to 20% at 15 min; at 17 min 90% (2) was maintained for 2 min. Then the initial mobile phase was applied for 5 min. The column temperature was maintained at 35 ^{0}C.

7.3.4 Instrumentation

Analysis of BUP and its metabolites was performed using a Thermo Finnigan LCQ DECA XP Plus ion trap instrument (Thermo Finnigan, San Jose, USA) equipped with a Surveyor LC system interface. The column oven and auto-sampler tray were maintained at 30 ^{0}C at 4 ^{0}C respectively. Ionisation of analytes was carried out using electrospray positive ion mode. The capillary temperature, sheath gas flow rate, auxiliary gas flow rate and collision energies were optimized for each analyte separately.

Analytes of interest and internal standards were identified and quantified based on their retention times, precursor ions and two product ions using Selection Reaction Monitoring (SRM) with the exception of BUP3G and NBUP3G which are fragmented by losing the glucuronic acid moiety (m/z 176) from the protonated molecular ions to form single product ions at m/z 468.3 and 414.4, respectively. In this case it was not possible to obtain two product ions from BUP3G and NBUP3G. Therefore, 90% of product ions and 10% of precursor ions were used for the quantification and identification of BUP3G and NBUP3G, respectively. The spray voltage used was 5 kV. The MS/MS parameters are detailed in Table 7-1.

7.3.5 Method Validation

7.3.5.1 Linearity

Eight different calibration concentrations in the range 5-250 ng/mL spiked in urine were extracted and analysed using the developed method. Calibration curves were plotted by dividing the peak area ratios obtained at each concentration to internal standards. The correlation coefficient (r^2) was obtained for each linear regression curve.

7.3.5.2 Recovery and matrix effects

The method described by Matuzewski *et al* [51] was used for the assessment of the SPE recoveries (RE) and matrix effect (ME) of opioids and their metabolites using urine as matrix. REs and MEs of analytes of interest were calculated by dividing

the peak area ratios of analytes of interest over their internal standards using equations 3-1 and 3-2.

Table 7-1: LC-MS/MS parameters, LODs and LLOQs for BUP metabolites.

	Parameters	BUP	BUP3G	NBUP	NBUP3G
Analytes	**Precursor Ion (m/z)**	468.4	644.3	414.3	590.5
	Product Ion(s) (m/z)	414.4, 369.3	468.3, 644.3	396, 340	414.4, 590.5
	Quantifier Ion (m/z)	468.4→ 414.4	644.3→ 468.3	414.3→396	590.5→ 414.4
	Qualifier Ion(m/z)	468.4 →396..3	644.3→644.3	414.3→340	590.5 →590.5
	SRM transition (Quan/Qual)	414.4/396.3	468.3/644.3	396/340	414.4/590.5
	SRM * Transition Ions (R.S.D. #)	2.6 (8.0)	7.0 (17.0)	1.6 (4.0)	5.8 (5.0)
	RT & (minutes)	22.49	18.3	19.4	14.2
Internal Standards	**Internal Standard (IS)**	BUP-D3	BUP-D3	NBUP-D3	NBUP-D3
	IS Precursor Ion(s) (m/z)	472	472	417	417
	Product Ion (m/z)	415, 400	415, 400	399, 343	399, 343
Analytes and their internal standards	**Sheath Gas (AU)**	33	30	30	30
	Auxiliary Gas (AU)	10	0	0	0
	Capillary Temperature (°C)	270	295	300	280
	Collision energy (%)	36	30	31	30
	Retention Widow No.	3	2	2	1
	LOD ## (ng/mL)	0.2	0.2	0.4	0.2
	LLOQ ** (ng/mL)	0.7	0.7	1.2	0.7

* SRM: Selective reaction monitoring.
# R.S.D: Relative standard deviations (%).
& Retention time of drugs.
LOD: Limit of detection.
** LLOQ: Lower limit of quantitation;

The effect of endogenous urine matrix components on analyte ionisation during LC-MS/MS was assessed by analysing blank urine obtained from five different sources. Urine samples were extracted using the developed method and spiked after SPE at two concentrations (5 and 100 ng/mL) of BUP and metabolites and 50 ng/mL of internal standards.

7.3.5.3 Limit of Detection and Lower Limit of Quantitation

Limits of detection (LODs) and lower limits of quantitation (LLOQs) were obtained by extending the calibration curves to the concentration of the expected LODs and LLOQs of analytes of interest. A linear calibration model was established for each analyte of interest at eight concentrations (0.1, 0.2, 0.3, 0.5, 0.75, 1, 2.5 and 5 ng/mL) plus blank, and 50 ng/mL of internal standard working solution (1 µg/mL) was added. Spiked urine samples were then extracted using the developed method. LODs and LLOQs values were obtained using equations 2-1, 2-2 and 2-3, 2-4, respectively.

7.3.5.4 Intra-assay and inter-assay precision

Five replicate human urine samples (n=5) were spiked with BUP and metabolites at three concentrations (5, 25 and 100 ng/mL). The extracts were analysed using the developed method on the same day to determine Intra-assay precision. The inter-assay precision was measured in a similar manner to the intra-assay precision on five different days.

7.3.5.5 Stability

Stability was assessed using human urine spiked with the BUP and its metabolites at 100 ng/mL (n=3). Short-term temperature stability at room temperature was investigated using spiked urine stored at 4 and 24 hours. Freeze-thaw stability of analytes of interest was determined after four cycles (thawed, left at room temperature for 3 hours then refrozen) on consecutive days. Auto-sampler stability using reconstituted extracted sample was determined at 48 hours and at one week after extraction. Long-term stability for analytes of interest at -20 and 4 ^{o}C for period of 24 hours, 48 hours, 1 week and 1 month were investigated.

Calibration curves were prepared for each batch of samples using standards spiked in human urine at 5, 10, 20, 25, 50, 100, 200 and 250 ng/mL plus blanks.

7.3.5.6 Specificity

These were similar to those described in Chapters 6 (section 6.7.5.6).

7.4 Results

7.4.1 Method Validation

Linear regression lines were obtained for each analyte over the calibration range of 5-250 ng/mL and r^2 values were greater than 0.999 for all analytes of interest. Low LOD and LOQ values were required as there are big differences in the concentrations of analytes detected in real case urine samples. The main portion of a BUP dose is excreted as glucuronide at high concentration levels compared to BUP and NBUP. Diluted urine samples are often used to bring glucuronide metabolites within the range of the calibration curve, as concentrations of more than 500 ng/mL for total BUP (TBUP) and total NBUP (TNBUP) have been reported [250,251,253,256,335,354]. If a high LLOQ is obtained, the BUP and NBUP result could result in false negatives. In the present study, lower LODs and LLOQs were aimed at filling the gaps between analytes of interest, and these were in the range of 0.2-0.4 and 0.7-1.2 respectively. LODs and LLOQs for analytes of interest are given in Table 7-1, Figure 7-2.

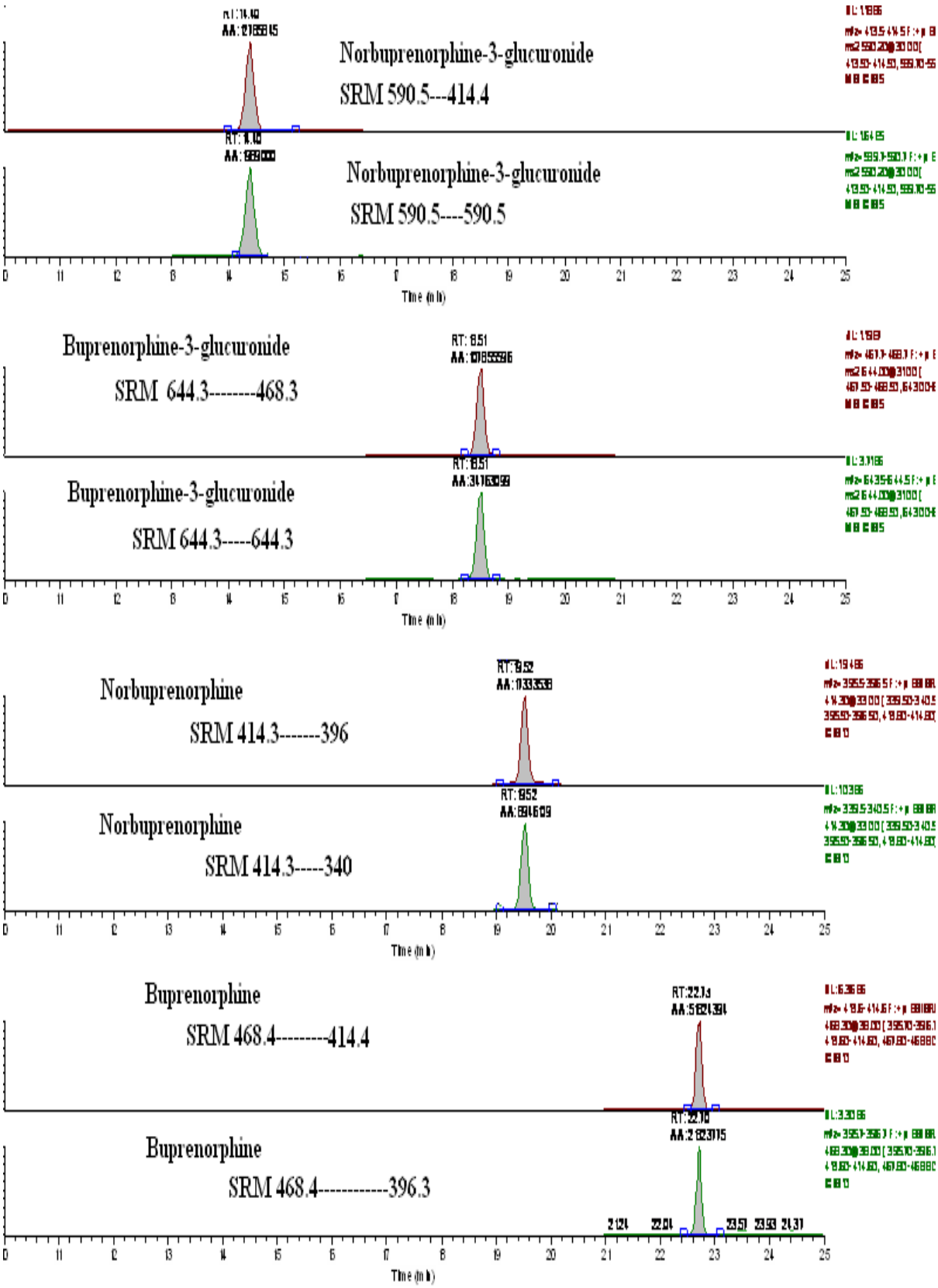


Figure 7-2: Mass chromatograms for buprenorphine and metabolites at 1 ng/ml.

The recoveries of BUP metabolites were calculated using equation 3-2. Good recoveries were obtained for BUP3G, NBUP, BUP and NBUP3G at all three concentrations investigated and were in the ranges 76-93%, 78-95% and 87-96% at 5, 25 and 100 ng/mL, respectively. Recoveries are listed in Table 7-2.

Matrix effects were measured at two concentrations (5 and 100 ng/mL) using equation equation 3-1. From the results listed in Table 7-3, both ion suppression and ion enhancement were observed using urine obtained from five different sources. Percentages above 100% indicate the presence of ion enhancement, while those below 100% indicate the presence of ion suppression. Ion suppression was observed with BUP and NBUP3G at the higher concentration (100 ng/mL) amounting to -4% and -8%, respectively. Ion enhancement was detected with all analytes of interest at the lower concentration (5 ng/mL) and with BUP3G and NBUP at the higher concentration (100 ng/mL).

Table 7-2: Recoveries of buprenorphine metabolites

Drug	Nominal Conc.	Matrix effects [#]	Recovery [##]
	ng/mL	Mean % [*] (R.S.D [&])	
BUP	5	112 (6)	76 (3)
	25	N/A §	78 (6)
	100	96 (7)	87 (10)
BUP3G	5	110 (3)	85 (12)
	25	N/A	91 (12)
	100	105 (5)	96 (3)
NBUP	5	110 (6)	77 (9)
	25	N/A	88 (13)
	100	103 (6)	89 (11)
NBUP3G	5	103 (12)	93 (10)
	25	N/A	95 (3)
	100	92 (8)	92 (4)

[#] Human urine was sourced from completed urine samples that were scheduled for destruction and contained no analytes of interest.
* Matrix effect is expressed as the response obtained for a standard chromatographed along with matrix extract compared to that obtained with an unextracted standard chromatographed in mobile phase only, expressed as a percentage. Standard was spiked into matrix extract at a concentration of 5, 25 and 100 ng/mL.
[##] Value calculated from the average recovery for the replicate analyses (n=5)
[&] R.S.D. %: Relative standard deviation expressed as a percentage.

Percentage relative standard deviations (RSD) were used to assess the intra- and inter-assay precision of the method for BUP and its metabolites at three concentrations. RSD values better than 12% and 14% were for intra- and inter-assay precision, respectively (Table 7-2).

BUP and metabolites were stable after being subjected to different storage conditions, namely: short-term stability at room temperature for 4 hours and 24 hours, for four freeze/thaw cycles, in the auto-sampler for up to one week and long-term at -20 ^{0}C and 4 ^{0}C for up to 1 month (Table 7-3). The stability of BUP and its metabolites has previously been studied and the results agreed with those obtained in the present study [250,256,332,335,363].

Table 7-3: Matrix effects

Analytes	Nominal Conc.	Source 1 #	Source 2	Source 3	Source 4	Source 5
	ng/mL	Mean % ** (R.S.D &)				
BUP	5	115 (12)	118 (1)	105 (11)	117 (3)	103 (3)
	100	103 (1)	103 (4)	91 (8)	88 (1)	95 (5)
BUP3G	5	113 (13)	109 (10)	108 (13)	114 (2)	105 (13)
	100	112 (10)	104 (3)	107 (1)	99 (15)	104 (13)
NBUP	5	111 (1)	112 (5)	103 (6)	119 (12)	106 (9)
	100	96 (5)	97 (6)	107 (6)	105 (4)	109 (1)
NBUP3G	5	89 (8)	103 (1)	122 (5)	103 (4)	98 (4)
	100	98 (11)	87 (5)	101 (1)	92 (15)	82 (4)

Human urine was sourced from completed urine samples that were scheduled for destruction and contained no analytes of interest.
** Matrix effect is expressed as the response obtained for a standard chromatographed along with matrix extract compared to that obtained with an unextracted standard chromatographed in mobile phase only, expressed as a percentage. Standard was spiked into matrix extract at a concentration of 5, 25 and 100 ng/mL.
& R.S.D.: Relative standard deviation expressed as a percentage.

Table 7-4: Method precision

Analytes	Nominal Concentration	Intra-day precision	Inter-day precision
	Measured concentrations (ng/mL) * (R.S.D %#)		
BUP	5	4.7 (12)	5.2 (12)
	25	27 (3)	25 (14)
	100	102 (6)	98 (6)
BUP3G	5	4.6 (7)	5.3 (6)
	25	26 (7)	25 (5)
	100	100 (9)	98 (3)
NBUP	5	4.4 (10)	5.1 (10)
	25	24 (7)	22 (6)
	100	101 (7)	99 (8)
NBUP3G	5	5.4 (9)	4.9 (11)
	25	24 (8)	23 (6)
	100	99 (6)	96 (4)

* Value calculated from the average recovery for the replicate analyses (n=5)
# R.S.D. %: Relative standard deviation expressed as a percentage.

Table 7-5: Stability studies (% relative to starting concentration)

Storage Conditions #		BUP	BUP3G	NBUP	NBUP3G
		Mean % * (R.S.D % &)			
Room Temperature	4 hours	115 (14)	105 (6)	103 (10)	99 (7)
	24 hours	94 (5)	102 (10)	105 (6)	109 (1)
Freeze-thaw	4 cycles	101 (2)	119 (6)	100 (8)	93 (12)
Auto-sampler	48 hours	98 (11)	95 (3)	104 (5)	93 (3)
	Week	108 (1)	100 (6)	102 (10)	99 (6)
Freezer at -20 0C	24 hours	101 (5)	104 (2)	94 (4)	96 (9)
	48 hours	106 (4)	98 (6)	101 (4)	96 (10)
	Week	105 (12)	97 (13	104 (8)	103 (6)
	Month	109 (3)	93 (9)	94 (7)	102 (6)
Refrigerated 4 0C	24 hours	96 (8)	107 (4)	94 (11)	94 (10)
	48 hours	104 (5)	104 (12)	108 (3)	93 (2)
	Week	102 (14)	110 (7)	103 (4)	97 (2)
	Month	107 (5)	100 (12)	109 (10)	99 (10)

Starting concentration is 100 ng/mL;
* Value calculated from the average recovery for the replicate analyses (n=3).
& R.S.D. %: Relative standard deviations expressed as a percentage.

7.4.2 Case Samples

The direct method developed was intended to be employed for routine analysis of urine samples for BUP and to be compared with the in-house hydrolysis method used at present for this analysis. All positive BUP cases received over the course of one year were analysed using the two methods in parallel. From the results listed in Table 7-6 and 7-7, twenty cases tested positive using the hydrolysis method, and twenty-one cases tested positive using the direct method. TBUP and TNBUP concentrations in positive cases obtained after hydrolysis ranged from not detected to 300 ng/mL and not detected to 294 ng/mL with average concentrations of 52 and 69 ng/mL, respectively. However, the concentrations obtained by direct analysis ranged from 6 to 401 (average 61 ng/mL) and 9 to 318 (average 85 ng/mL) for TBUP and TNBUP, respectively (Figure 7-3).

Using the direct method, analyte concentrations averaged 53 ng/mL (range 2-358 ng/mL) for BUP3G, 9 ng/mL (range not detected to 41 ng/mL) for NBUP, 8 ng/mL (range not detected to 43 ng/mL) for BUP and 76 ng/mL (range 9-288 ng/mL) for NBUP3G (Table 7-7). BUP3G and NBUP3G were detected in all cases. However, BUP and NBUP were negative in several cases. Using the hydrolysis method, case 3 tested negative for NBUP and neither BUP nor NBUP were detected in case 13.

Results from the two methods correlated well, with correlation coefficients (r^2) of 0.994 and 0986 for TBUP and TNBUP respectively. Higher concentrations were obtained by the direct method compared to the hydrolysis procedure (Table 7-6 and 7-7, Figures 7-4 and 7-5). The data given in Table 7-8 indicate that the differences between the two methods reach statistical significance ($p<0.05$) based on the confidence intervals for the intercepts and gradients, indicating a systematic error in one of the methods [62]. Possible explanations are incomplete hydrolysis in the conventional method or interference in the direct method. The latter was examined during the validation procedure but no interferences were detected. However, for both methods the median ratio for TNBUP/TBUP was 2.

The median BUP3G/BUP and NBUP3G/NBUP ratios obtained by direct analysis when both were detected were 5 and 6, respectively.

Table 7-6: Results obtained by in-house LC-MS/MS hydrolysis method (ng/mL)

Case no.	TBUP [#]	TNBUB [*]	TBUP+TNBUP	TNBUP/TBUP
	Measured concentration (ng/mL)			
1	63.0	78.0	141.0	1.2
2	15.0	45.0	60.0	3.0
3	29.0	n.d. [&]	29.0	n.d.
4	32.0	67.0	99.0	2.1
5	68.0	13.0	81.0	0.2
6	19.0	66.0	85.0	3.5
7	14.0	33.0	47.0	2.4
8	19.0	11.0	30.0	0.6
9	29.0	62.0	91.0	2.1
10	300.0	29.0	329.0	0.1
11	120.0	240.0	360.0	2.0
12	198.0	277.0	475.0	1.4
13	n.d.	n.d.	n.d.	n.d.
14	6.0	22.0	28.0	3.7
15	52.0	294.0	346.0	5.7
16	43.0	15.0	58.0	0.3
17	17.0	46.0	63.0	2.7
18	10.0	12.0	22.0	1.2
19	24.0	25.0	49.0	1.0
20	19.0	76.0	95.0	4.0
21	15.0	45.0	60.0	3.0

[#] TBUP: Total buprenorphine; [*] TNBUP: Total norbuprenorphine;
[&] n.d: Not detected.

Table 7-7: Concentrations of BUP and metabolites obtained by the direct method (ng/mL).

Case no.	BUP3G	NBUP	BUP	NBUP3G	BUP3G/ BUP	NBUP3G/ NBUP	TBUP #	TNB UP*	TNBUP/ TBUP	TBUP+ TNBUP
	Measured concentration (ng/mL)									
1	46.0	6.0	5.0	95.0	9.2	15.8	51.0	101.0	2.0	152.0
2	20.0	n.d &	n.d	52.0	n.d	n.d	20.0	52.0	2.6	72.0
3	34.0	5.0	3.0	15.0	11.3	3.0	37.0	20.0	0.5	57.0
4	20.0	6.0	4.0	76.0	5.0	12.7	24.0	82.0	3.4	106.0
5	60.0	5.0	13.0	13.0	4.6	2.6	73.0	18.0	0.2	91.0
6	14.0	6.0	n.d	93.0	n.d	15.5	14.0	99.0	7.1	113.0
7	10.0	6.0	6.0	14.0	1.7	2.3	16.0	20.0	1.3	36.0
8	16.0	n.d	n.d	9.0	n.d	n.d	16.0	9.0	0.6	25.0
9	28.0	7.0	6.0	44.0	4.7	6.3	34.0	51.0	1.5	85.0
10	358.0	8.0	43.0	42.0	8.3	5.3	401.0	50.0	0.1	451.0
11	130.0	15.0	14.0	260.0	9.3	17.3	144.0	275.0	1.9	419.0
12	210.0	41.0	35.0	265.0	6.0	6.5	245.0	306.0	1.2	551.0
13	5.0	n.d	4.0	16.0	1.3	n.d	9.0	16.0	1.8	25.0
14	2.0	5.0	4.0	9.0	0.5	1.8	6.0	14.0	2.3	20.0
15	52.0	30.0	23.0	288.0	2.3	9.6	75.0	318.0	4.2	393.0
16	39.0	5.0	n.d	14.0	0.0	2.8	39.0	19.0	0.5	58.0
17	15.0	14.0	n.d	62.0	n.d	4.4	15.0	76.0	5.1	91.0
18	8.0	n.d	n.d	17.0	n.d	n.d	8.0	17.0	2.1	25.0
19	20.0	12.0	1.2	35.0	16.7	2.9	21.2	47.0	2.2	68.2
20	14.0	17.0	n.d	117.0	n.d	6.9	14.0	134.0	9.6	148.0
21	20.0	n.d	n.d	52.0	n.d	n.d	20.0	52.0	2.6	72.0

TBUP: Total buprenorphine; * TNBUP: Total norbuprenorphine; & n.d: Not detected.

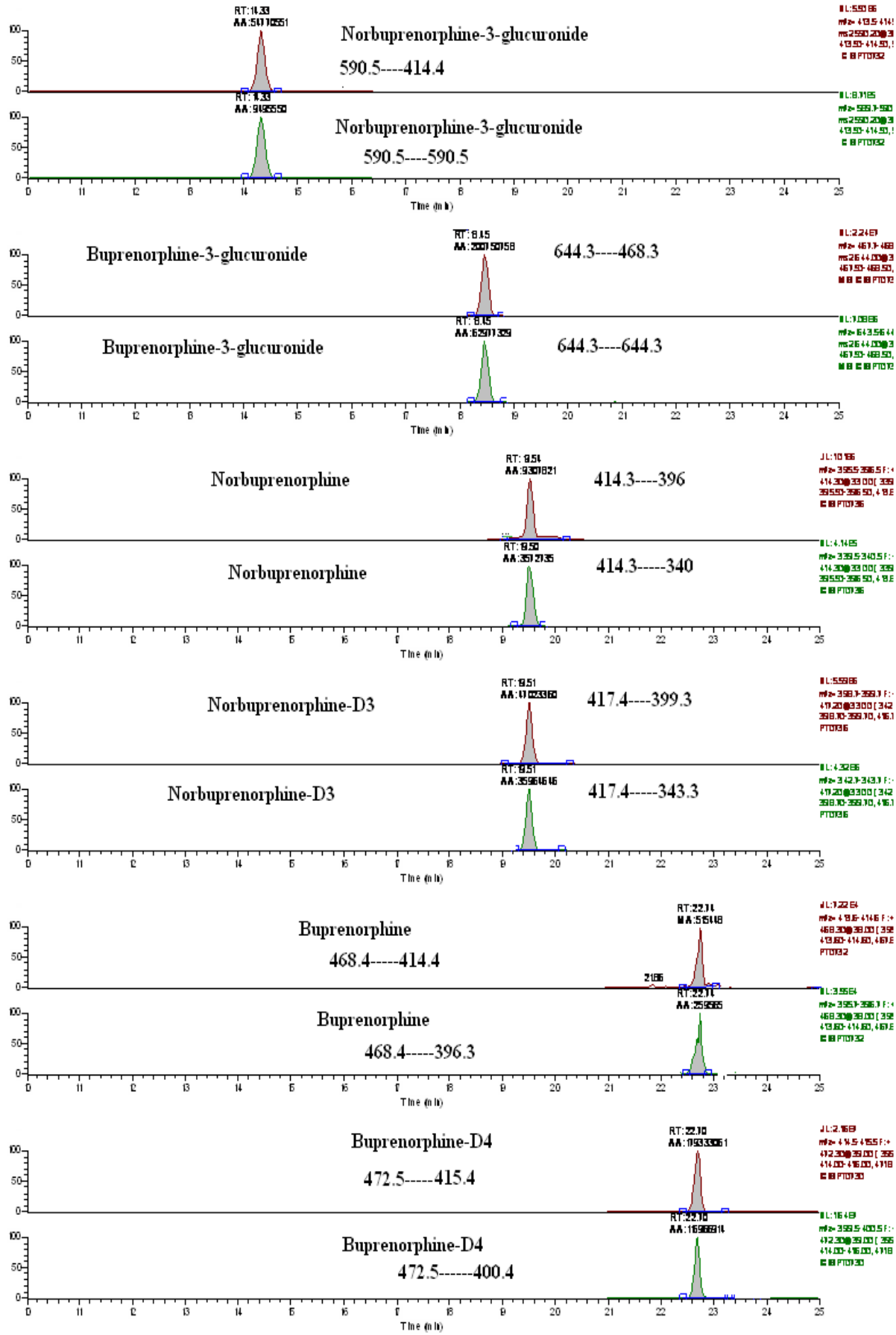


Figure 7-3: Mass chromatograms for buprenorphine and metabolites in Case 19.

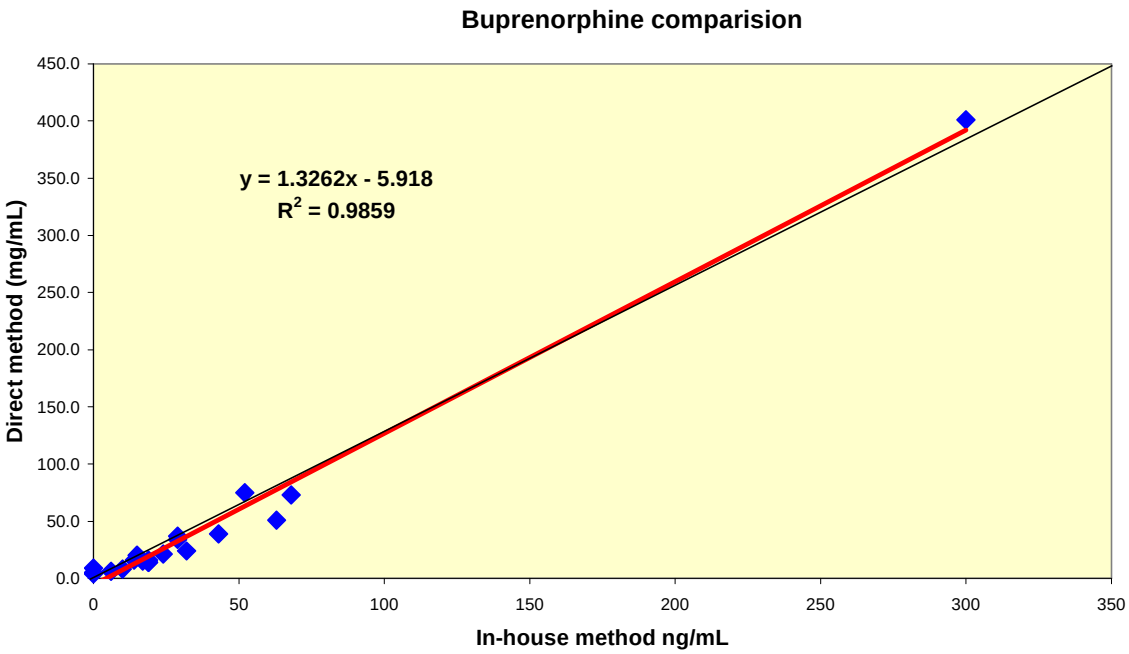


Figure 7-4: Comparison of results from hydrolysis and direct methods for total buprenorphine obtained for 21 case samples.

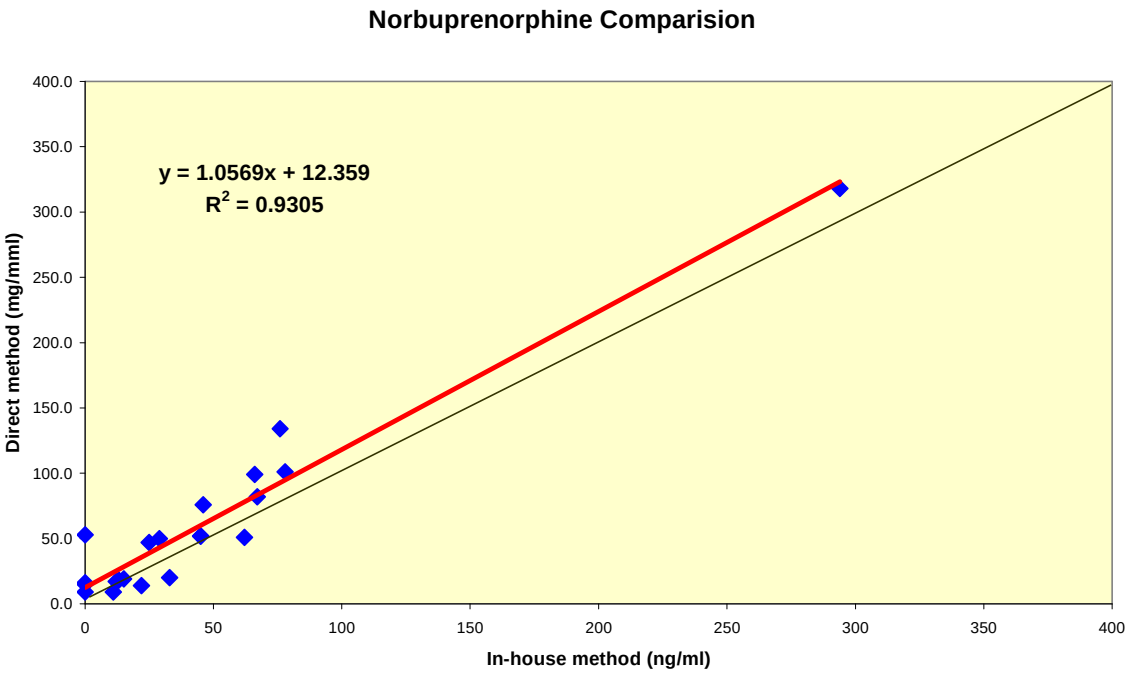


Figure 7-5: Comparison of results from hydrolysis and direct methods for total norbuprenorphine obtained for 21 case samples.

Table 7-8: Regression data for comparison of hydrolysis and direct methods for total buprenorphine andtotal norbuprenorphine in 21 cases

		Total Buprenorphine	Total Norbuprenorphine
	Correlation coefficient (r^2)	0.994	0.986
Intercept data	Intercept	-7.29	9.51
	Intercept 95% confident interval	-12.99 to -1.59	0.056 to 18.97
	p-value	0.015	0.05
Slope data	X-variable 1	1.31	1.08
	95% confident interval	1.25 to 1.38	0.997 to 1.168
	p-value	2.8 E-20	1.91E-16

7.5 Discussion

7.5.1 Method validation

This work was aimed at validating and developing a sensitive method with low LODs and LLOQs to be applied to routine analysis of BUP in urine samples. Both the SPE and LC methods were adapted from previously published method for the determination of 24 opioids and their metabolites in blood specimens (chapter 5). There was no interference from drugs with similar structures such as opioids or from drugs routinely detected in forensic cases.

A comparison of enzymatic and non-enzymatic hydrolysis methods for determining BUP metabolites was report by Kronstrand *et al* [354] using a direct injection method. They found the peaks of glucuronide metabolites were very small. The authors concluded that the used of a direct injection procedure may lead to inaccurate determination of analytes of interest at low concentrations due to the ion suppression in the ES interface which affects ionisation of the polar glucuronides. The authors also recommended using an SPE method for removing endogenous compounds from the matrix.

Direct injection was excluded in the present method due to the higher LODs and LLOQs obtained and small peaks of analytes of interest, which indicate large matrix effects on the ionisation of glucuronide metabolites. The present method was intended to quantify BUP and NBUP with their glucuronides simultaneously. Therefore, a method of extraction which would concentrate the analystes was required due to the low concentrations of BUB and NBUP reported in real case samples, which led to them being excluded in the method by Hegstad *et al* [355].

The assessment of the effects of matrix endogenous components on ionisation of analytes is a critical aspect of LC-MS method validation due to the susceptibility of the technique to ion suppression or enhancement [16,47,51,89]. The mechanisms for these effects are unknown but are influenced by ionisation method (ESI or APCI), type of biological specimens and the adequacy of sample preparation [51,119,120].

Elimination of matrix effects is an objective of method optimisation [117,120]. It has been suggested that improved sample preparation procedures could resolve and minimise the matrix effects. Also, improved LC separation was found to eliminate matrix effects [51,118,364]. However, concentrating analytes of interest using SPE could also result in increased concentrations of matrix endogenous components.

Murphy and Huestis [256] reported ion suppression after using an SPE method for BUP, BUP3G and NBUP. They concluded that SPE reduced the matrix effects to -25 to -30% for plasma samples, plasma being considered a dirtier matrix than urine. In the same study, ion enhancement of +10% was observed for NBUP3G. Hegstad *et al* [355] reported ion enhancement of +153% and ion suppression of -14% with BUP3G and NBUP3G, respectively. The high ion enhancement for BUP3G indicated that SPE is a crucial step before analysis by LC-MS/MS and the authors recommended the production of internal standards for glucuronides which could overcome the matrix effects. Also, the short gradient of 7 min used in that study may have exacerbated the matrix effects.

In the present study, there were no commercially available internal standards for the glucuronide metabolites and so BUP-D4 was used as internal standard for

BUP and B3G and NBUP-D3 was used as internal standard for NBUP and NBUP3G. A similar approach was used by Murphy and Huestis [256] whereas Huang *et al* used M3G-D3 as internal standard to quantify BUP3G and NBUP3G and Hegstad *et al* [355] used NBUP-D3 as internal standard for both glucuronides. In the present study, matrix effects were minimised (-4% and -8% suppression for BUP and NBUP3G, respectively) using SPE and a long gradient elution in which NBUP3G was the first analyte to be eluted at 14 min.

7.5.2 Case samples

Although BUP3G and NBUP3G standards have been available for many years, hydrolysis is still often used as a pre-treatment in the determination of TBUP and TNBUP [250,355] and only two studies have reported concentrations of BUP3G and NBUP3G in case urine samples [250]. Huang *et al* reported concentrations of BUP metabolites in five subjects who were treated daily with 16 mg BUP for 21 days [355]. After 24 hours of administration, the average concentrations of analytes were 1, 112, 95 and 662 ng/mL for BUP, NBUP, BUP3G and NBUP3G, respectively.

Hegstad *et al* [355] developed a method for detecting BUP3G and NBUP3G alone, without including BUP and NBUP. In that study, 928 urine cases were tested and concentrations of both glucuronides were less than 300 ng/mL. Tracqui *et al* [340] reported concentration ranges of 4 to 1033 ng/mL and 7 to 230 ng/mL for BUP and NBUP respectively, with median and average ratios for NBUP/BUP of 0.7 and 0.8. Kronstrand *et al* [354] reported concentrations of TBUP and TNBUP ranging from 3 to 796 ng/mL and 5 to 2580 ng/mL, respectively with a median ratio of TNBUP to TBUP of 2. The median and average ratios obtained in the present study were also in agreement with Kronstrand *et al* [354]. The median and average ratios of TNBUP/TBUP were 2; the concentrations obtained in the current study ranged from 6 to 401 ng/mL (average 61 ng/mL) and 9 to 318 ng/mL (average 85 ng/mL) for TBUP and TNBUP, respectively.

George *et al* [365] determined the mean concentration ratio of NBUP to BUP using positive BUP urine samples which found to be 2.8. BUP and NBUP concentrations were range of 28 to 1458 ng/mL and 28 to 1843 ng/mL.

Miller *et al* [253] reported the concentrations of TBUP and TNBUP in clinical and drug abuse cases obtained using a hydrolysis method. The concentrations of TBUP and TNBUP were much higher in clinical cases, ranging from 123 to 1931 ng/mL and 1001 to 7550 ng/mL respectively, compared to the abuse cases, which ranged from trace concentrations to 179 ng/mL and not detected to 97 ng/mL for TBUP and TNBUP, respectively. In that study, NBUP was not detectable when BUP was at a low concentration.

Fox *et al* [356] measured BUP and NBUP using LC-MS/MS before and after treatment with ß-glucuronidase in real case urine samples. Interestingly, the percentages of free metabolites were in the range not detected to 67% and not detected to 100% with means of 6.4% and 34.7% for BUP and NBUP respectively.

The median TBUP/TNBUP ratios reported by Miller *et al* [253] and Huang *et al* [250] for clinical cases were 12 and 8, respectively. However, the ratio in Miller *et al* [253] for abuse cases was very low, the average being 0.3 and the highest value being 1.8. The median ratios of TNBUP/TBUP in the present study were 2 for both the hydrolysis and direct methods. Differences in the ratio of TNBUP to TBUP could result from methodological parameters such as different ß-glucuronidase sources, incubation times and temperatures, from differences in the study groups, for example, as a result of population pharmacogenomic variations, or from differences in the urine collection protocol, affecting, for example, pH, frequency of collection and elapsed time after drug administration [14].

Enzymatic hydrolysis methods are usually optimised using BUP3G standard then applied to real case samples containing both BUP3G and NBUP3G. The rate of hydrolysis would be different for each glucuronide metabolite and could potentially lead to false negative results for NBUP3G. The hydrolysis of NBUP3G was much slower than BUP3G, with 85% being hydrolysed after incubation at 37°C for 20 hours using ß-glucuronidase from *E.coli* [354]. Increased amounts of enzyme might have interfered in the analysis and for that reason the minimum amount of enzyme was used even though NBUP3G was not completely hydrolysed. George *et al* [365] used 30 minutes incubation at 60 °C with ß-glucuronidase from *Helix pomatia* to hydrolyse glucuronide metabolites to their

free form. The use of the same enzyme sources in another study found that BUP3G needed 4 hours of incubation at 60 ^{o}C and 16 hours at 37 ^{o}C to obtain complete hydrolysis for BUP3G [362].

Elsohly *et al* [348] studied NBUP3G hydrolysis using ß-glucuronidase from *Helix pomatia* and found NBUP3G completely hydrolysed after 4 hours at 60 ^{o}C. The authors reported poor hydrolysis of NBUP3G using *P. vulgata*. However, Fox *et al* [356] used the latter enzyme source for the hydrolysis of glucuronide metabolites after incubation for one hour at 56 ^{o}C.

The in-house method used in this study was similar to the method of Elsohly *et al* [348] but with a 3 hour incubation period. Also, hydrolysis followed by SPE and LC-MS/MS was compared to liquid-liquid extraction followed by GC-MS by Elsohly *et al* [348]. The difference in incubation time could also cause some differences in analyte concentrations obtained by these two different studies and lead to incomplete hydrolysis of NBUP3G.

7.6 Conclusions

A sensitive and selective method for the direct determination of BUP and its metabolites in urine was developed and validated and compared with a conventional in-house hydrolysis method using 21 real case samples. Results from the two methods correlated well, although the direct method gave slightly higher concentrations for BUP metabolites compared to the hydrolysis method. However, it has confirmed that the in-house hydrolysis procedure was effective in cleaving glucuronide conjugates at the range of concentrations encountered in cases in this study, which indicate buprenorphine abuse. The concentrations of BUP metabolites are much higher in clinical cases and this should be taken into consideration when enzymatic hydrolysis procedures are used since the elevated concentrations may result in incomplete hydrolysis. Also, the hydrolysis rates of BUP3G and NBUP3G are different and it is important that hydrolysis methods are optimised using both glucuronide metabolites. The two methods used in this study gave the same ratios for TNBUP/TBUP.

The ratios between conjugated metabolites and their free forms are of interest and can be helpful in the examination of enzymatic hydrolysis methods. In addition, ratios may be of value in interpretation of cases but additional work is required involving controlled administration studies to allow this to be evaluated. Urine could be used as an indicator of recent use of diamorphine, for example if morphine is absent in urine but present in blood it would indicate recent diamorphine use. Lack of glucuronide metabolites in urine would indicate a single dose or intermittent user as opioid addicts administer once a day at least [9,125]. The case is different with BUP as it is mostly excreted as glucuronide metabolites. Huang *et al* [250] reported that NBUP3G is the primary metabolite in both plasma and urine. A more research is needed to investigate the usefulness of using BUP metabolites in determining the time which has elapsed after the last administration. Finally, the sensitive and selective LC-MS procedures now available should replace traditional hydrolysis methods since these permit analysis of BUP and its individual metabolites as well as reducing the analysis time.

8 Oxycodone Related Fatalities in the West of Scotland

8.1 Introduction

Oxycodone (Oxycontin, Roxicodone, Tylox) is a semisynthetic opioid derived from thebaine. Oxycodone can be used alone or in combination with other drugs such as acetaminophen and aspirin and has been successfully used clinically since 1917 for pain relief. A controlled-release form of oxycodone (OxyContin) has been available since 1995 for the treatment of acute to moderate pain extending over long periods of time (>12 hours) and it is completely absorbed in the stomach without being affected by the presence of food [132,366].

Although the oxycodone controlled release preparation has been found to be safe and effective, it has gained wide popularity as a drug of abuse and deaths attributed to oxycodone intoxication have increased [367-369]. OxyContin is an expensive and powerful analgesic with similar potency to morphine. A method for abusing controlled-release oxycodone tablets was developed by drug abusers, which makes oxycodone more immediately available by administration after crushing the tablets. Lethal overdose due to the consumption of numerous tablets has been reported. In many deaths intact tablets were found in stomach but the drug content had been completely absorbed and little or no oxycodone remained in the tablet, which was considered an indicator that suicide was the manner of death [368,369]. Anderson *et al* [369] reported the presence of intact tablets in the stomach of 15 out of 36 oxycodone related deaths.

Owing to the shortage of diamorphine in the United State of Americ a (USA), oxycodone misuse and related fatalities have been widely reported but it is hoped that stricter regulations will prevent a similar trend in the United Kingdom (UK). However, the number of prescriptions for oxycodone in Scotland has risen by 430% since prescribing began in 2002 [370]. This large increase in oxycodone prescriptions increases the possibility of the drug presenting in post-mortem cases and being implicated in the cause of death of accidental or intentional poisonings.

8.1.1 Metabolism and excretion

Oxycodone is metabolised in the liver by cytochrome P450 3A4 and 2D6, primarily to noroxycodone through N-demethylation and to oxymorphone through O-demethylation [132,371,372]. Oxymorphone is a minor metabolite but is 100 times more potent than oxycodone. Noroxycodone is the most abundant metabolite but it is relatively inactive and is reported to be 3-4 times less potent than oxycodone [124,126,366]. Oxycodone and its two primary metabolites also undergo reductive metabolism to form α- and ß-oxycodol, α- and ß-noroxycodol and α- and ß- oxymorphol.

Oxycodone and noroxycodone are excreted unchanged in urine while oxymorphone is excreted only as the glucuronide metabolite [366,371]. Lalovic *et al* [371] have reported on the pharmacokinetics of both oxidative and reduction metabolic pathways. The concentrations of metabolites were found to be dose dependent and the N-demethylation pathway gave the major metabolite. Oxidative metabolites comprised nearly 50% of the oxycodone dose excreted in urine, with only 10% as the unchanged drug and 18% as the reduced metabolites (Figure 8-1).

8.1.2 Review of oxycodone related fatalities

Symptoms reported for oxycodone overdose include severe respiratory depression, skeletal muscle flaccidity, cold, clammy skin, reduction of blood pressure and heart rate, coma, cardiac arrest and death [132,368]. Although many studies focused on oxycodone related fatalities in the last decades, the actual role of oxycodone in fatalities is difficult to determine due to the combination with other central nervous drugs and overlap of concentration of drugs between single drug exposures and poly drug intoxications. Fatalities associated to oxycodone alone are rarely reported despite the high use and abuse pattern which raises a question of whether deaths happened intentionally or accidentally [263,373].

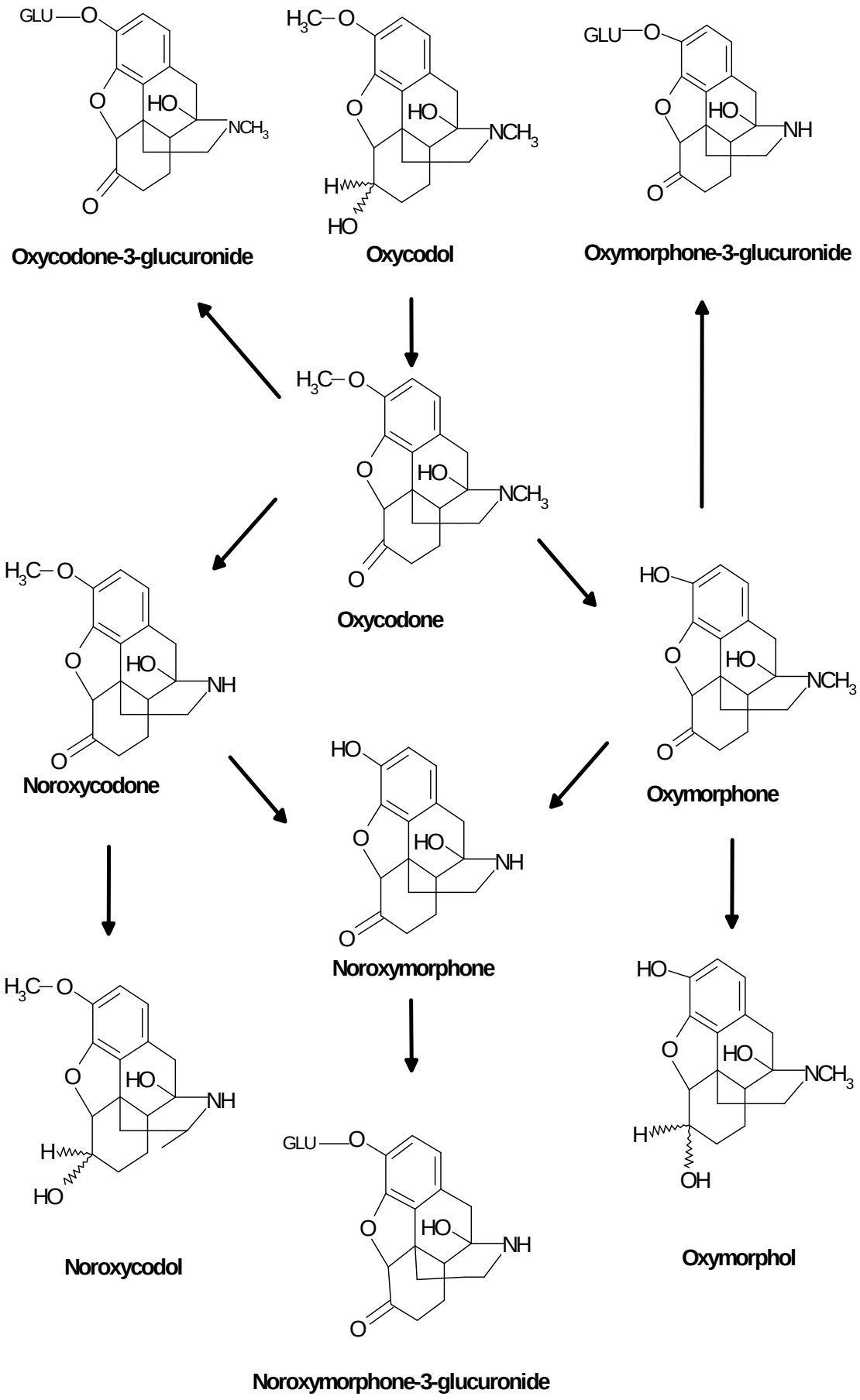


Figure 8-1: Oxycodone metabolism, (GLU: Glucuronic acid).

Oxycodone deaths have mostly been reported in the United States of America due to the high rate of addiction to oxycodone as an alternative to heroin, especially in rural areas [368,370]. However, the first report to discuss oxycodone fatalities was from Australia [25]. Nine deaths involving oxycodone were investigated and it was found that oxycodone can cause death when the blood concentration is higher than 0.6 µg/mL. The concentrations of oxycodone in these deaths ranged from 0.6-1.4 µg/mL with a mean concentration of 0.9 µg/mL. Only one case was attributed to suicidal ingestion of oxycodone. Oxycodone was detected in another 3 cases at concentrations which were in or above the therapeutic range (0.1-0.3 µg/mL) but was not involved in the cause of death. This study may be triggered research on oxycodone related fatalities because of concerns about co-administration of the drug with other central nervous system depressants and whether chronic use of oxycodone could increase user tolerance, resulting in overlap of clinical and toxic drug concentration ranges.

Drugs with a low therapeutic index should be monitored to ensure their concentrations are within the therapeutic range. While this can be achieved under clinical conditions, it is difficult to achieve with drug abusers. Published pharmacokinetic studies on oxycodone involved administration of low doses of the drug and plasma concentrations below 0.1 µg/mL, which are considered low compared to the toxic concentrations reported for overdoses cases and this indicates a higher safety margin between lethal and therapeutic levels. However, the availability of OxyContin tablets containing large doses (40 -80 mg) was believed to reduce the therapeutic window and increase the risk of serious consequences in adult patients receiving chronic treatment with oxycodone. Also, the risk of fatal poisoning is much higher in children who ingest OxyContin accidentally or as a result of homicide [374,375].

The blood concentrations of oxycodone after acute intoxication or suicidal ingestion were investigated in 24 post-mortem deaths attributed to the use of oxycodone alone, in which no other drugs were detected [376]. The average and median blood concentrations were 1.23 and 0.43 µg/mL, respectively. Blood concentrations in suicides were 1 µg/mL and higher, with average and median concentrations of 3.9 and 2.5 µg/mL, in agreement with Cone *et al* [263], who

demonstrated that blood oxycodone concentrations were higher in suicides (1.55 to 1.7 µg/mL) than in other types of death (mixed intoxication, natural).

In addition, Anderson *et al* [369] reported post-mortem oxycodone concentrations in heart and femoral blood from 10 suicides cases which ranged from 0.56-46 µg/mL and 0.59-13 µg/mL respectively. Two of these deaths attributed to oxycodone intoxication had oxycodone levels in heart and femoral blood of 0.58-1.8 and 1.1-3 µg/mL, respectively [369]. Elsewhere, eighteen fatalities related to oxycodone ingestion had post-mortem blood oxycodone concentrations ranging from 0.21-4.71 µg/mL with a mean concentration of 0.69 µg/mL and another 8 cases in which oxycodone was the only drug detected oxycodone concentrations ranged from 0.21-1.22 with a mean concentration of 0.55 µg/mL. One survivor had a blood oxycodone concentration of 2.4 µg/mL 10 hours after ingestion of a massive dose of OxyContin tablets [377].

High concentrations of oxycodone detected at autopsy may not always indicate suicide as the manner of death. Many deaths attributed to natural, traumatic and undetermined causes had post-mortem blood concentrations of oxycodone which overlapped those in the suicide cases mentioned earlier. Wolf *et al* [373] reported 23 cases of death attributed to trauma in which the mean post-mortem blood oxycodone concentration was 0.62 µg/mL, range 0.025-1.99 µg/mL. In another study, the post-mortem blood oxycodone concentrations ranged from 0.14-1 µg/mL and 0.27 -1.3 µg/mL in deaths attributed to natural and undermined causes, respectively [369]. These results were in agreement with Spiller and Abat [376] who reported a mean oxycodone concentration of 0.7 µg/mL and range of 0.17-1.6 µg/mL.

Suggestions have been made concerning why blood concentrations of oxycodone in these groups of cases overlap or why high concentrations are found in non oxycodone related fatalities. One explanation is that oxycodone could accumulate in tissue following chronic administration which is subsequently released back into the blood [25]. The other explanation is oxycodone leaches out of controlled-release tablets after death and diffuses from the stomach to the blood [369]. By contrast, lethal concentrations of oxycodone could be lower than 0.6 µg/mL in deaths attributed to intravenous or nasal administration. Langman

et al [378] reported a case in which oxycodone was injected and another in which it was inhaled nasally. The oxycodone concentrations in blood and urine were 0.5, 21.7 µg/mL and 0.05, 6.858 for intravenous and inhalational administration, respectively.

In most publications concerning oxycodone fatalities, the cause of death has been attributed to concomitant use of other drugs although the mechanism is still not clear [263]. Cone *et al* [263] reported 919 deaths involving oxycodone, of which 889 (96.7 %) were attributed to multiple drug intoxication and only 30 (3.3 %) to intoxication by oxycodone alone. Similarly, Wolf *et al* [373] reported 172 deaths involving oxycodone of which only 18 deaths were attributed to oxycodone alone and the rest to mixed drug intoxication. Potentially lethal concentrations of oxycodone were detected in natural and traumatic deaths as well as in suicidal overdose cases. The authors concluded that it may not be possible to establish a toxic oxycodone blood concentration due to the overlap in concentrations between groups; also, identification of the cause of death in cases involving multiple drugs becomes more difficult. Backer and Poklis [379] reported 70 oxycodone related fatalities of which 38 cases were attributed to mixed drug intoxication and only 4 cases to oxycodone alone. There was no significant difference between the mean oxycodone concentrations in these groups (0.42 and 0.5 µg/mL) or between the ranges of concentrations (0.06-1.6 µg/mL and 0.23-0.76) for mixed drug intoxication and oxycodone only, respectively. Natural deaths had a low mean oxycodone concentration of 0.19 µg/mL.

The most commonly co-administered drugs detected with oxycodone are benzodiazepines, ethanol, cocaine, other opioids, marijuana and tricyclic antidepressants [25,263,373] . In one study of 172 oxycodone related deaths, benzodiazepines were detected in 96 cases, of which 37 involved diazepam and/or nordiazepam [373]. Cone *et al* [263] found that diazepam was present in 286 cases in their study. However, the role of diazepam was not examined further and the concentrations of diazepam were not provided. Diazepam concentrations were within the therapeutic range in cases reported by Drummer *et al* [25] which suggested that the deaths may be attributed to oxycodone with only minor contributions from benzodiazepines. The role of ethanol in heroin

deaths is well known and the same role could be expected in oxycodone related fatalities [150,151,264,267]. Other opioids could have additive effects on the respiratory centre or higher centres [263].

8.1.3 Aims

Methods for identification and quantification of oxycodone using Gas chromatography with nitrogen-phosphorus [368,380] or mass spectrometry detectors (GC-MS) [25,247,381-387] and High performance Liquid chromatography with electrochemical [388,389], ultraviolet [84,390], or tandem mass spectrometry detectors (LC-MS/MS) [249,252,391-394] have been reported. In current study, an LC-ESI-MS/MS method for the identification and quantification of oxycodone and N- and O-demethylation metabolites in post-mortem specimens (blood, urine and vitreous humour) has been developed and validated. The optimised method was applied to the routine analysis of autopsy blood and urine samples from fatalities in the West of Scotland. While the role of oxycodone in fatalities has been studied [25,263,367-369,373-376,378,379,395-397], the role of its metabolites has not yet been investigated and in the present study oxycodone and its N- and O-demethylated metabolites were measured in deaths attributed to oxycodone intoxication for the first time. In addition, the results for free oxycodone are compared with those from other studies.

8.2 Methods and Materials

8.2.1 Reagents and Standards

Methanol and acetonitrile were obtained from BDH (Poole, UK). Ammonium carbonate, formic acid and ammonium hydroxide were also purchased from BDH. Ammonium formate was obtained from Acros Organics (New Jersey, USA). Oxycodone, oxycodone-d_6, noroxycodone, noroxycodone-d_3, oxymorphone and oxymorphone-d_3 were purchased from LGC Standards (Middlesex, UK).

All standards and internal standards were obtained as solutions in methanol at a concentration of 0.1 mg/mL or 1 mg/mL and each had a purity of more than 99%. Bond Elut LRC-C18 cartridges were purchased from Varian Inc (CA, USA).

Individual working standards were prepared at concentrations of 20, 10 and 1 µg/mL by dilution of the stock solutions. Working mixtures of standards and internal standards (1 µg/mL) were similarly prepared.

8.2.2 Solid Phase Extraction and Chromatography conditions

In the current procedure, SPE and HPLC methods were as described in a previously method for the analysis of opioids and their metabolites in autopsy blood samples, refer to chapter 5 (section 5.3.2 and 5.3.3).

8.2.3 Instrumentation

Analysis of opioids was carried out using a Thermo Finnigan LCQ DECA XP Plus ion trap instrument (Thermo Finnigan, San Jose, USA) equipped with a surveyor LC system interface. During the analysis the auto-sampler and column oven temperatures were maintained at 4 °C and 25 °C, respectively. Ionisation of analytes of interest was carried out using electrospray positive ion mode. The capillary temperature, sheath gas flow rate, auxiliary gas flow rate and collision energies were optimized for each analyte separately. The spray voltage used was 5 kV.

Analytes and their internal standards were identified and quantified based on their retention times and the presence of two product ions or one product ion in addition to the parent ion and adjusted to 10% in comparison to the major product ion in selected reaction monitoring (SRM) mode as following: m/z, 298 and 316 for oxycodone, m/z 284, 229 and 302 for noroxycodone and 284 and 302 for oxycmorphone, m/z 287 and 305 for both noroxycodone-D_3 and oxymorphone- D_3 and m/z 304 and 322 for oxycodone- D_6. The MS/MS parameters are detailed in Table 8-1.

Table 8-1: LC-MS/MS data

Parameters		Oxycodone	Noroxycodone	Oxymorphone
Analytes	Precursor Ion (m/z)	316	302	302
	Product Ion(s) (m/z)	298, 316	284, 229	284, 302
	Quantifier Ion (m/z)	316→298	302→284	302→284
	Qualifier Ion(m/z)	316→316	302→302	302→302
	SRM * Transition Ions (R.S.D % #)	18 ± 2 (11)	10 ± 1 (7)	5.4 ± 0.3 (5)
	RT & (min)	14	12.5	9.3
Internal standards	Internal Standard (IS)	Oxycodone-d6	Noroxycodone-d3	Oxymorphone-d3
	IS Precursor Ion(s) (m/z)	322	305	305
	Product Ion (m/z)	322→304	305→287	305→287
Analytes and their internal standards	Sheath Gas (AU)	25	15	5
	Auxiliary Gas (AU)	10	20	5
	Capillary Temperature (ºC)	250	300	250
	Collision energy (%)	30	35	26
	Retention Widow No. (min)	3 (13-17)	2 (11-13)	1 (6-11)
	LOD## Blood (ng/mL)	0.2	0.4	0.3
	LLOQ ** Blood (ng/mL)	1	1	1
	LOD Urine (ng/mL)	0.2	0.2	0.3
	LLOQ Urine (ng/mL)	1	1.2	1

* SRM: Selective reaction monitoring.
# R.S.D: Relative standard deviations (%).
& Retention time of drugs.
LOD: Limit of detection.
** LLOQ: Lower limit of quantitation;

8.2.4 Method Validation

8.2.4.1 Linearity

Two sets of calibration standards were prepared in both blood and urine and extracted in accordance with method reported in Chapter 5 (section 5.3.5.1): one calibration curve of lower concentrations (5, 10, 20, 25, 50, 100 and 250 ng/mL) and the other with higher concentrations (50, 100, 250, 500, 750, 1000, 2500 and 5000 ng/mL).

Blank and spiked controls with internal standard added at 100 ng/mL were included with each run. Calibration curves were plotted of the ratios of analyte peak areas to internal standards against concentration. The correlation coefficient (r^2) was obtained for each linear regression curve.

8.2.4.2 Matrix Effects and Extraction Recoveries

Matrix effects and extraction recoveries were measured using the approach of Matuszewski [51]. For matrix effects, blood and urine were obtained from five different human sources (n=3) and spiked after solid phase extraction with analytes of interest at 100 ng/mL. Neat standards were diluted in initial mobile phase and injected directly onto the LC-MS/MS. Matrix effects of endogenous components were calculated by comparing peak areas of these two sets of standards.

Recoveries were investigated for oxycodone and its metabolites using five different concentrations in blood and urine across the two calibration curve ranges (5, 25, 100, 750 and 2500 ng/mL). Internal standards were added after extraction and recoveries were calculated by comparison of the peak area ratios of the extracted and unextracted standards analysed under identical conditions.

8.2.4.3 Limits of Detection and Lower Limits of Quantification

The sensitivity of the method was evaluated by determining the limit of detection (LOD) and the lower limit of quantification (LLOQ) for each analyte of interest. The lower range calibration curves were used for this purpose (n=5) and

were prepared using blood spiked at eight different concentrations (0.1, 0.2, 0.3, 0.5, 0.75, 1, 2.5, and 5 ng/mL) plus blank without internal standard and blank containing internal standards. The LOD's and LLOQ's were calculated as reported in Chapter 5, see section 5.3.5.3.

8.2.4.4 Intra-assay and Inter-assay Precision

Five different concentrations (5, 25, 100 750 and 2500 ng/mL) of analytes were spiked into five replicate blood and urine samples to assess the intra-assay and inter-assay precision for the optimised method. Intra-assay precisions of oxycodone, noroxycodone and oxymorphone were determined by analysing these samples (n=5) in one day while inter-assay precision was measured by analysing them on five different days.

8.2.4.5 Stability

Stability was assessed using human whole blood and urine spiked with the analytes of interest at 100 ng/mL (n=3). Short-term temperature stability at room temperature was investigated for human whole blood and urine stored for 4 and 24 hrs. Freeze-thaw stability of analytes of interest was determined after four cycles (thawed, left at room temperature for 3 hrs then refrozen) on consecutive days. Auto-sampler stability using reconstituted extracted sample was determined at 48 hrs and at one week after extraction. Long-term stability of analytes of interest was evaluated at 4 ^{o}C for a period of 1 month, and at -20 ^{o}C for periods of 6 months. Calibration curves were prepared for each batch of samples using standards in whole blood at 5, 10, 20, 25, 50, 100, 200 and 250 ng/mL plus negative controls.

8.2.4.6 Specificity

These were similar to those described in Chapters 6 (section 6.7.5.6).

8.2.5 Case samples

Samples of autopsy blood were analysed using the proposed method as part of the investigation of medico-legal cases involving drug-related deaths submitted

to Forensic Medicine and Science, University of Glasgow. Analyses were repeated after dilution of the blood sample when analyte concentrations outside the upper calibration range were obtained.

8.2.5.1 Case 1

66 year old female, heavy smoker and suffered from high blood pressure as well as anxiety and depression, with history of previous suicide attempts. She was last seen alive the night before she died. She stated that she was going to bed and took two diazepam tablets, which were prescribed to her. However the following morning she was found dead in bed with four empty blister packs of OxyContin next to the bed. There were no suspicious circumstances.

8.2.5.2 Case 2

42 year old female had a long history of alcohol, and possibly drug abuse, and also had chronic obstructive airways disease. Medication and painkillers had been prescribed to her for lung disease. She was seen lying on a sofa in the living room the evening before death. The following morning she was still in the same position, had no response and was apparently dead. A packet of Oxycontin tablets and a bottle of vodka were beside the deceased.

8.2.5.3 Case 3

45 year old woman with a history of depression and known to have taken an overdose many years ago. She did not drink alcohol but was on a number of prescribed medications including Oramorph, amitriptyline, Oxynorm (oxycodone), temazepam and acetaminophen. She was last seen the day before death, she did not complain of anything more than the usual stomach pain due to a longstanding illness. The following day she was found lying dead on the kitchen floor.

8.2.5.4 Case 4

45 old lady who suffered from depression, hypertension and hypothyroidism, and had previously undergone a hip replacement operation. She was prescribed

numerous medications including diazepam, zopiclone and propranolol. She was last contacted on the day before death at which point she appeared depressed and was witnessed to consume a number of diazepam tablets. The following day, following concerns as to her whereabouts, her body was discovered lying on top of her bed within her bedroom. There were no suspicious circumstances.

8.2.5.5 Case 5

33 old male who had told his flatmate not to disturb him when he returned home as he wished to sleep late. When his flatmate returned home in the early hours of the evening before death he heard this man snoring in his bedroom and left him to sleep. The following morning the flatmate went to the decedent's bedroom and found the deceased fully dressed on the floor with a suicide note to his girlfriend by his feet. The deceased was depressed about the break-up of this relationship. The flatmate had a prescription for OxyContin 80mg and two blister packs of this medication were missing.

8.2.5.6 Case 6

32 year old man had a melanoma removed from his back ten years prior to his death. His medication included oxycodone, Nozinan (levomepromazine), Oxynorm, omeprazole, dexamethasone and acetaminophen. He was under treatment in hospital for palliative chemotherapy on the day before his death. He asked medical staff if he could go outside for fresh air. Then he was reported missing to police by medical staff when he did not return to the hospital. The following day he was found in undergrowth, unconscious but breathing, and was taken to Accident & Emergency at the local hospital. On admission to hospital his GCS was 3/15 and he was hypothermic. Resuscitation was attempted but this was unsuccessful. Three ampoules of oxycodone hydrochloride had been found next to the deceased as well as a broken syringe driver.

8.2.5.7 Case 7

28 year old man had a history of drug abuse which included diamorphine and cocaine. In 2005 he attempted suicide by hanging and was subsequently on anti-depressant medication. He was involved in a fight and was arrested by the

police. He was taken to hospital for treatment of minor facial injuries and was subsequently released from custody. The following afternoon, he was drinking with friends, and at about midnight they went to the home of one of them where he later fell asleep on a settee in the living room. He remained there for much of the rest of that day and, to the others, appeared to be sleeping. In the early evening however, they became concerned and found him to be unresponsive. An ambulance was called but the crew found him to be dead.

8.2.5.8 Case 8

79 year old female who suffered from osteoporosis and chronic back/shoulder pain for which she was prescribed several medications. She suffered post-operative atrial fibrillation and possible myocardial infarct and was transferred to another hospital for further rehabilitative care. On admission she was found to be frail and lethargic, and was suffering from an apparent infection for which she was prescribed intravenous antibiotics. Over the next couple of days, her condition failed to improve significantly and continued to suffer from a chest infection. She was prescribed analgesic control, and over the next 24 hours or so her condition continued to deteriorate until her death. There were, however, no suspicious circumstances.

8.2.5.9 Case 9

26 year old man with a history of alcohol abuse and depression. He was prescribed Cipramil (citalopram) in July 2008 for the latter. He had previously attempted to take his own life by overdosing on an unknown non-prescribed medication. On the last evening before his death, the deceased was at a party. According to a witness, at some point during the evening the deceased took approximately 5g of cocaine, two morphine tablets and alcohol. A witness said that the deceased informed him that he had jumped from the window and was presently within the garage and was returning to the flat. On arriving a short time later he was observed to be breathing heavily and taking extremely deep breaths which was causing him some difficulty. The witness suggested that he should go to hospital for treatment but he refused. The party continued throughout the weekend with witnesses speaking of seeing the deceased having

a drink but having difficulty walking and breathing. A witness observed the deceased retire to bed; another witness went into the small bedroom of the house where she saw the deceased lying on his back fully clothed, not breathing. He was cold to the touch. Paramedics were contacted but attempts at resuscitation were unsuccessful.

8.2.5.10 Case 10

40 year old man suffering from chronic alcohol and illicit drug abuse (reportedly using only ecstasy and diazepam), pancreatitis and depression. As such he was prescribed numerous medications including oxycodone. He spent most of the weekend in bed, although at one point he reportedly left the flat to buy diazepam and on his return claimed to have overdosed on this drug whilst out. Soon after he complained of feeling unwell and retired to bed which was the last time he was seen alive. That afternoon he was found unresponsive, paramedics in attendance found no signs of life and he was formally declared dead.

8.3 Results

8.3.1 Method Validation

8.3.1.1 LC-MS/MS

A method for quantification of multiple opioids in fatalities, including oxycodone and oxymorphone, was established in previous work (Chapter 5). In the current study another oxycodone metabolite, noroxycodone and its internal standard, were included and the method was full validated for whole blood and urine.

The SRM transition ratios were calculated for both calibration standards and samples analysed. Oxycodone and its O-demethylated metabolite are fragmented to only one major product ion at m/z 298 and 284, respectively. Noroxycodone is fragmented to two product ions at m/z 284 and 229. In the SOFT guidelines [54], the use of two product ions is recommended but it is mentioned that two product ions are not always obtainable using LC-MS techniques. Although LC-MS/MS techniques are more sensitive and specific and

one product ion is acceptable for quantification purposes, a survival precursor ion after fragmentation could be used as qualifier ion by adjusting the cone voltage. This was examined in the current study and Chapter 7. Ratios of 90% of major product ion to 10% of parent ion were adjusted by tuning with standards of oxycodone and oxymorphone and the ratios between precursor and product ions were used to confirm SRM transition (see Table 8-1). Complete separation was necessarily to avoid co-elution between oxymorphone and noroxycodone which have the same precursor ion (mz= 302) and the same major product ion (mz= 284). In addition, their deuterated internal standards also have the same ions. In the current method full separation between all oxycodone metabolites was achieved and each analyte had its own segment window which minimised the issues associated with co-elution. Oxymorphone was eluted first at 9.25 min followed by noroxycodone and oxycodone at 12.25 and 13.75 min, respectively. A low collision energy was required for fragmentation of oxymorphone compared to noroxycodone (Table 8-1).

8.3.1.2 Specificity and selectivity

Opioids may share similar chemical structure characteristics and for that reason specificity should be investigated to ensure that there are no co-elution problems. Common drugs and opioids encountered regularly in routine post-mortem cases were spiked into blood and urine specimens that tested free from oxycodone and its N- and O-demethylated metabolites. Also, blank specimens were spiked with low concentrations of oxycodone and its two metabolites and with the deuterated internal standards of oxycodone, noroxycodone and oxymorphone, to investigate the effects of endogenous components on the ionisation of analytes of interest and their internal standards.

Co-elution of interfering substances during LC-MS is a critical problem and could occur due to similar masses of parent ions or due to undetected endogenous components from the matrix. In the current study, dihydrocodeine had the same molecular ion as noroxycodone and noroxymorphone. The major product ion obtained from these substances was at the same m/z value as the product ion of dihydrocodeine, which required complete separation from both metabolites.

Using the current method of separation, dihydrocodeine eluted close to noroxycodone with about 1 min difference in retention time. The use of deuterium internal standards of noroxycodone and dihydrocodeine has increased method reliability and selectivity even with high concentrations of dihydrocodeine. In one case sample, a high concentration of dihydrocodeine was found not to cause interference with noroxycodone. Similarly, no interference was detected after injection of a mixture of drugs commonly encountered in routine post-mortem cases.

8.3.1.3 Linearity

Linear regression lines were obtained for each analyte over the calibration ranges 5-250 ng/mL and 50-5000 ng/mL. Correlation coefficients were greater than 0.999 for oxycodone and metabolites in both ranges.

8.3.1.4 LOD and LLOQ

Method sensitivity was investigated using the low range calibration curves which encompassed the expected LOD values for each analyte. Analytes could be identified and quantified at concentrations of 1-1.2 ng/mL. LODs and LLOQs are given in Table 8-1 and Figure 8-2.

8.3.1.5 Matrix effects

The effects of endogenous matrix components on ionisation of analytes of interest during LC-MS/MS were assessed by analysing extracts of blank blood and urine samples to which were added analytes of interest at a concentration of 100 ng/mL. Little or no matrix effects were observed with both blood and urine from five different human sources, with an average of less than ± 20 % of spiked standard vlue, indicating that there were no undetected endogenous matrix components co-eluting with the analytes of interest and that the solid-phase extraction (SPE) steps resulted in clean extracts (Table 8-2).

Table 8-2: Matrix effects in LC-MS/MS analysis of blood and urine samples.

Source of blank matrix [#]	Blood Specimens [*] % (RSD %) (n=3)			Urine Specimens [*] % (RSD %) (n=3)		
	Oxycodone	Noroxycodone	Oxymorphone	Oxycodone	Noroxycodone	Oxymorphone
1	102 (1)	105 (7)	93 (6)	100 (13)	87 (13)	102 (4)
2	102 (12)	107 (11)	96 (4)	100 (5)	108 (4)	113 (14)
3	107 (4)	98 (2)	96 (6)	98 (6)	87 (7)	103 (10)
4	98 (3)	115 (10)	103 (1)	116 (7)	109 (8)	124 (4)
5	105 (3)	106 (4)	101 (2)	97 (5)	113 (6)	105 (2)

[#] Human blood and urine were sourced from completed urine samples that were scheduled for destruction and contained no analytes of interest.
[*] Matrix effect is expressed as the response obtained for a standard chromatographed along with matrix extract compared to that obtained with an unextracted standard chromatographed in mobile phase only, expressed as a percentage. Standard was spiked into matrix extract at a concentration of 100 ng/mL.
[&] R.S.D. %: Relative standard deviation expressed as a percentage.

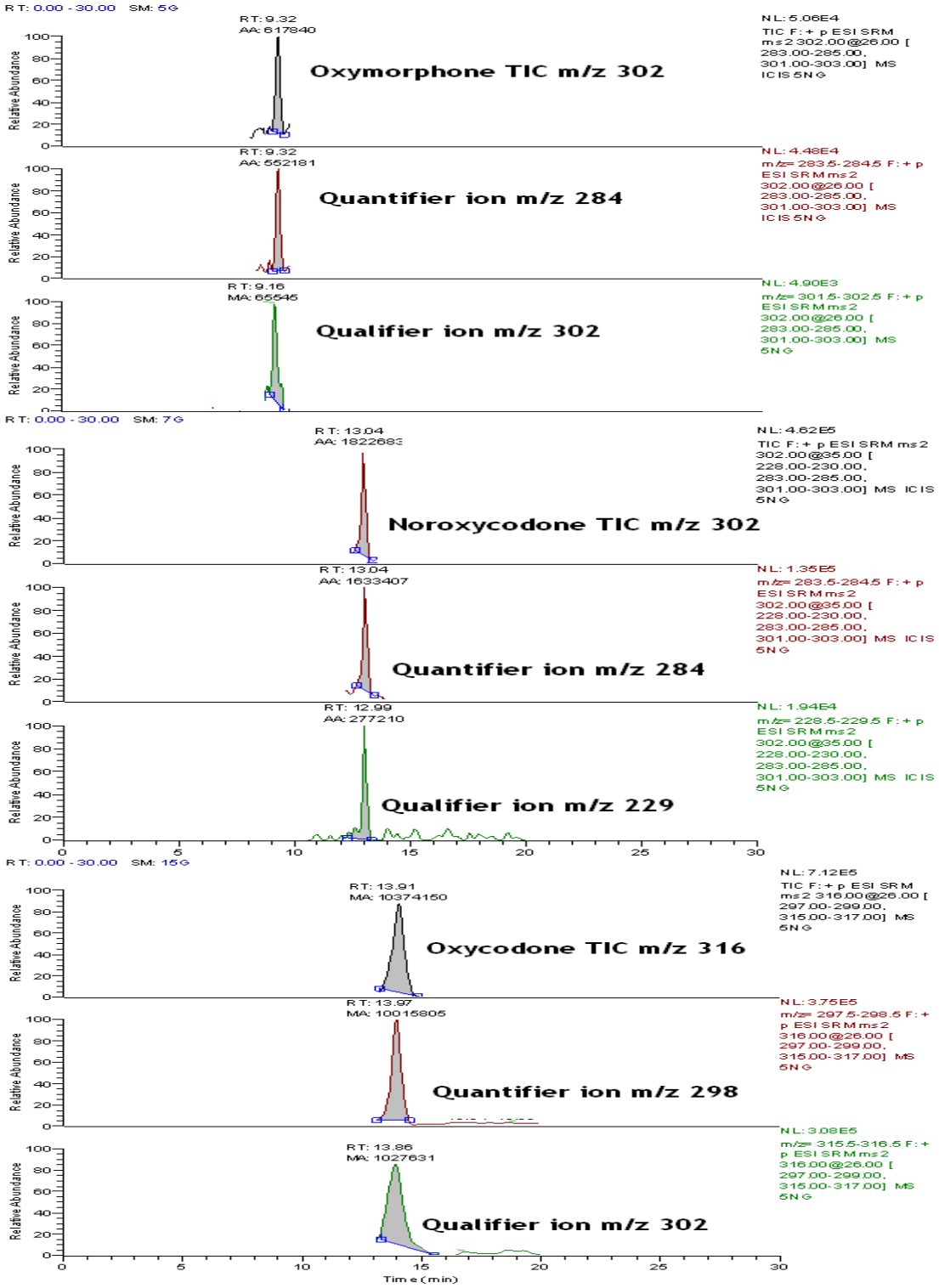


Figure 8-2: Oxycodone metabolites in blood (concentration 1 ng/ml).

8.3.1.6 Recoveries

Recoveries were investigated using five different concentrations across the two calibration curve ranges. The recoveries for oxycodone, noroxycodone and oxymorphone were in the range of 84-101%. Recoveries are listed in Tables 8-3 and 8-4.

Table 8-3: Recoveries from blood.

Analyte	Nominal Concentration (ng/mL)	Matrix effects [#] % [*] (RSD%) [&]	SPE Recovery [**] % (RSD%)
Oxycodone	5	98 (11)	94 (5)
	25	100 (6)	96 (4)
	100	102 (0.2)	99 (10)
	750	102 (7)	100 (6)
	2500	100 (6)	101 (5)
Noroxycodone	5	98 (3)	95 (9)
	25	100 (7)	100 (9)
	100	99 (5)	101 (5)
	750	104 (4)	101 (11)
	2500	105 (7)	100 (10)
Oxymorphone	5	111 (2)	87 (13)
	25	96 (3)	99 (8)
	100	102 (6)	99 (9)
	750	103 (7)	100 (6)
	2500	99 (4)	100 (2)

[#] Human blood was sourced from completed urine samples that were scheduled for destruction and contained no analytes of interest.
[*] Matrix effect is expressed as the response obtained for a standard chromatographed along with matrix extract compared to that obtained with an unextracted standard chromatographed in mobile phase only, expressed as a percentage. Standard was spiked into matrix extract at a concentration of 5, 25, 100, 750 and 2500 ng/mL.
[**] Value calculated from the average recovery for the replicate analyses (n=5)
[&] R.S.D. %: Relative standard deviation expressed as a percentage.

Table 8-4: Recoveries from urine (n=5).

Analyte	Nominal Concentration (ng/mL)	Matrix effects % (RSD %)	SPE Recovery % (RSD %)
Oxycodone	5	100 (9)	88 (10)
	25	96 (6)	91 (10)
	100	102 (1)	94 (9)
	750	106 (11)	102 (6)
	2500	105 (6)	101 (4)
Noroxycodone	5	87 (13)	85 (1)
	25	105 (7)	92 (10)
	100	105 (8)	99 (4)
	750	109 (4)	101 (11)
	2500	104 (8)	101 (5)
Oxymorphone	5	102 (4)	84 (4)
	25	111 (3)	93 (5))
	100	109 (6)	95 (3)
	750	108 (3)	96 (6)
	2500	110 (4)	102 (8)

[#] Human urine was sourced from completed urine samples that were scheduled for destruction and contained no analytes of interest.
[*] Matrix effect is expressed as the response obtained for a standard chromatographed along with matrix extract compared to that obtained with an unextracted standard chromatographed in mobile phase only, expressed as a percentage. Standard was spiked into matrix extract at a concentration of 5, 25, 100, 750 and 2500 ng/mL.
[**] Value calculated from the average recovery for the replicate analyses (n=5)
[&] R.S.D. %: Relative standard deviation expressed as a percentage.

8.3.1.7 Precision

Percentage relative standard deviations (RSD) were used to assess the intra- and inter-assay precision of the method for oxycodone and its metabolites at the same five concentrations used for the recovery study. RSD values better than 12% and 14% were obtained for intra- and inter-assay precision, respectively. The intra and inter-day precisions are listed in Table 8-5.

8.3.1.8 Stability

Oxycodone and metabolites were stable after being subjected to different storage conditions, including (a) at 4 °C for up to one month (b) in the auto-sampler at 5°C for up to week (c) at –20°C for long storage periods up to 6 month (d) at room temperature up to 24 hours and (e) during four freeze/thaw cycles, which are the most frequently encountered conditions in a forensic toxicology laboratory. Stability results for oxycodone, noroxycodone and oxymorphone under different storage conditions are detailed in Table 8-6.

8.3.2 Case samples

Autopsy specimens collected during post-mortem examination were stored in the refrigerator until analysed. The interval between death and post-mortem examination differed between cases, within the range of 24 hours to several days. Full toxicological analysis was carried out for alcohol by GC-FID, drugs of abuse, using ELISA as the screening method and GC-MS or LC-MS/MS for confirmation, prescribed drugs, using GC-MS or LC-MS/MS for screening and confirmation and volatile substances, by GC-FID.

Oxycodone is not recognised as an abused drug in Scotland and is not included in routine post-mortem analysis. If one of the criteria below was met in any case, oxycodone analysis was requested: background information on the deceased's drug abuse, the use of oxycodone for medication, oxycodone tablets found near to the deceased or intact tablets recovered from the stomach. During the period of this study, oxycodone was detected in ten post-mortem cases. Nine of these have been categorised as drug overdose deaths involving one or more drugs, and

in one case as death due to disease, in which oxycodone was used for palliative care.

Table 8-5: Intra- and inter-assay precision and accuracy (n=5).

Analytes	Nominal concentration (ng/mL)	Blood *			Urine *		
		Intra-day precision	Inter-day precision	Accuracy	Intra-day precision	Inter-day precision	Accuracy
		ng/mL (R.S.D.%)#	ng/mL (R.S.D.%)	% (R.S.D.%)	ng/mL (R.S.D.%)	ng/mL (R.S.D.%)	% (R.S.D.%)
Oxycodone	5	5 (12)	5 (10)	98 (6)	5.2 (5)	4.7 (12)	103 (11)
	25	26 (6)	25 (7)	99 (1)	24 (4)	23 (11)	102 (10)
	100	103 (8)	97 (3)	105 (3)	107 (5)	100 (3)	98 (4)
	750	752 (2)	748 (3)	99.5 (3)	746 (4)	743 (2)	99 (2)
	2500	2505 (1)	2492 (1)	100 (0.7)	2498 (1)	2520 (1)	100 (2)
Noroxycodone	5	5 (3)	4.8 (5)	104 (10)	4.7 (7)	5.1 (7)	99 (3)
	25	25 (4)	25 (9)	98 (4)	23 (10)	23 (9)	99 (5)
	100	100 (2)	100 (3)	102 (2)	104 (2)	102 (6)	101 (2)
	750	755 (3)	750 (3)	99.7 (3)	748 (3)	750 (3)	101 (2)
	2500	2513 (2)	2506 (3)	99.8 (2)	2496 (3)	2509 (3)	99 (4)
Oxymorphone	5	5 (8)	5 (8)	98 (7)	4.8 (11)	5.2 (12)	100 (7)
	25	25 (3)	25 (3)	99 (4)	25 (8)	25 (8)	100 (3)
	100	99 (2)	100 (7)	100 (3)	99 (4)	99 (8)	102 (5)
	750	754 (1)	750 (1)	100.3 (1)	752 (1)	750 (1.5)	100 (0.5)
	2500	2520 (1)	2510 (2)	99 (3)	2470 (2)	2512 (1)	101 (3)

* Value calculated from the average recovery for the replicate analyses (n=5)
# R.S.D. %: Relative standard deviation expressed as a percentage.

Table 8-6: Stability (n=3).

Storage Condition [#]		Blood specimens [*] % (R.S.D.%) [&]			Urine Specimens [*] % (R.S.D.%)		
		Oxycodone	Noroxycodone	Oxymorphone	Oxycodone	Noroxycodone	Oxymorphone
Room Temperature	4 Hours	109 (11)	107 (2)	105 (4)	104 (9)	112 (4)	110 (4)
	24 hours	110 (8)	108 (1)	106 (3)	100 (9)	103 (10)	99 (4)
Freeze-thaw 4 cycles		105 (2)	103 (0.5)	99 (6)	100 (9)	103 (10)	99 (4)
Auto-sampler 4 0C	48 hours	103 (3)	94 (1)	101 (1.5)	93 (4)	96 (15)	102 (9)
	Week	99 (4)	105 (6)	106 (8)	108 (5)	97 (5)	104 (1)
Freeze at -20 0C	24 hours	105 (3)	103 (12)	110 (9)	97 (1)	103 (2)	106 (6)
	48 hours	108 (6)	105 (9)	103 (5)	104 (6)	109 (8)	113 (1)
	Week	101 (12)	106 (3)	99 (9)	95 (5)	97 (13)	96 (8)
	Month	103 (7)	111 (5)	97 (1)	107 (6)	100 (8)	110 (2)
	6 month	98 (7)	99 (11)	95 (2)	95 (3)	91 (12)	95 (6)
Refrigerate at 4 0C	24 hours	109 (3)	105 (4)	108 (10)	93 (1)	104 (6)	94 (11)
	48 hours	107 (6)	106 (8)	99 (11)	102 (5)	106 (14)	117 (2)
	Week	96 (8)	108 (5)	106 (7)	103 (2)	106 (6)	97 (5)
	Month	99 (3)	103 (9)	97 (13)	92 (7)	96 (12)	91 (3)

[#] Starting concentration is 100 ng/mL;
[*] Value calculated from the average recovery for the replicate analyses (n=3).
[&] R.S.D. %: Relative standard deviations expressed as a percentage.

Intact tablets were recovered from the stomach in four oxycodone overdose deaths. Oxycodone was prescribed as a pain killer either as OxyContin (2 cases) or OxyNorm (one case) and an undetermined type of oxycodone preparation in one case. In all cases at least one or more than one centrally-acting drug was detected. A summary of the toxicology results is given in Table 8-7.

Blood samples were available in all cases while urine specimens were not available in two cases. Oxycodone and its N-demethylated metabolite were determined in all cases. Oxymorphone was not detected in cases 4, 7, 8 and 10 which may indicate that a short time had elapsed after ingestion of oxycodone before death occurred or else that oxymorphone had been conjugated prior to elimination from the body. In cases 5, 6 and 9, oxymorphone was negative in blood but positive in urine. In case 4, a low level of noroxycodone was detected in blood at a concentration of 0.1 µg/mL along with a high concentration of oxycodone (4.9 µg/mL) which indicated acute oxycodone intoxication. However, propranolol was also present at an overdose level and the cause of death was attributed to intoxication by both oxycodone and propranolol.

In all cases the parent drug was the most abundant analyte detected compared to the major metabolites, with the exceptions of cases 3 and 7. In case 3, oxycodone and noroxycodone had the same concentration and death was attributed to mixed drug intoxication. Oxymorphone was present at low levels in the current study although these were higher than reported in oxycodone pharmacokinetics studies, in which most samples had levels of 0.001-0.003 µg/mL or less [371,391,398,399]. An understanding of oxycodone pharmacokinetics in human subjects could provide information to assist in the interpretation of the cause of death and whether or not oxycodone was taken with suicidal intent. In fact, most cases involved intoxication after oral ingestion of oxycodone controlled-released tablets (OxyContin), and it is not known if the deceased damaged the controlled release system of the tablets, for example by crushing them before administration, which resulted in high concentrations of oxycodone in the blood immediately after ingestion or if the deceased just ingested numerous tablets which resulted in an overdose of oxycodone.

Table 8-7: Concentrations of oxycodone and metabolites in blood and urine samples from 10 forensic autopsy cases

Case no.	Oxycodone (µg/mL)		Noroxycodone (µg/mL)		Oxymorphone (µg/mL)		Other drugs detected (µg/mL)	INS [ϕ]	Cause of death
	Blood	Urine	Blood	Urine	Blood	Urine			
1	5.5	7.6	0.86	2.3	0.04	1.3	Ethanol 28 mg%; Diazepam 0.14; Nordiazepam 0.13	No	Oxycodone overdose
2	0.95	6.8	0.39	2.5	0.06	0.22	Ethanol 111 mg%	3	Combination of oxycodone and ethanol intoxication
3	0.08	0.4	0.08	0.6	0.01	0.1	Codeine 0.12; Morphine 0.06; Temazepam 0.15; Amitriptyline 1.1; Tramadol 1.7; Paracetamol 24	No	Mixed drug intoxication
4	4.9	N/A[a]	0.1	N/A	-[‡]	N/A	Propranolol 3.3; Diazepam 0.68; Nordiazepam 0.56; Valproic acid < 40	> 44	Oxycodone and propanol overdose
5*	3.6	53	1.5	82	-	0.2	Diazepam 0.98; Nordiazepam 0.76; Temazepam 0.05; Morphine 0.025; THC 0.025;	2	Oxycodone intoxication
6	3.6	40	0.5	7.6	-	0.36	Levomepromazine <0.10, Ethanol 17 mg%; Acetaminophen < 20	No	Oxycodone intoxication
7	1.5	N/A	2.3	N/A	-	N/A	Citalopram 0.4	No	Oxycodone intoxication
8	0.09	1.6	0.03	0.5	-	-	Fentanyl 0.005; Diazepam 0.16; Desmethyldiazepam 0.16	No	Broncho-pneumonia due to non-small cell carcinoma of the lung
9[†]	0.76	45	0.5	43	-	0.1	Cocaine 0.20; Benzoylecgonine 3.1; Ecgonine methyl ester 0.72; Diazepam <0.05; Desmethyldiazepam 0.16µg/mL; Acetaminophen 24;	4	Oxycodone intoxication
10	0.82	30	0.55	24	-	-	Total morphine 0.46; Total codeine; 0.03; Diazepam 0.11; Desmethyldiazepam 0.23; Temazepam <0.05; Citalopram 0.36; Amitriptyline 0.35;	No	Heroin and oxycodone intoxication

[ϕ] INS: noumber of the intact tapletes recovered on stomch.
*Vitreous Humour (Oxycodone 2.1 and Noroxycodone 0.76µg/mL); [†] Vitreous Humour (Oxycodone 0.63 and Noroxycodone 0.19µg/mL); [a] (N/A) samples not analysed; [‡] (-) negative result; (case 4 and 5) Gastric contents were analysed qualitatively and tested positive for oxycodone and noroxycodone.

The latter is more likely for those cases in which intact tablets were recovered from the stomach (cases 2, 4, 5 and 9). In three cases the tablets were not dissolved and in case 5 many tablets were present but it was difficult to distinguish these from the gastric contents and only two OxyContin 80 mg tablets were identified. Gastric contents in case 4 and tablets in cases 2, 5 and 9 tested positive for oxycodone and noroxycodone.

In case 1, no intact tablets were recovered from the stomach, which may indicate that oxycodone was injected or crushed and dissolved before administration, but the route of administration is not known. The LC-MS/MS analysis of oxycodone and its metabolites is shown in Figure 8-3. In all cases blood oxycodone concentrations ranged from 0.08 to 5.5 µg/mL with average and median concentrations of 2.18 and 1.2 µg/mL, respectively. The concentrations in overdose deaths ranged from 0.76 to 5.5 µg/mL with mean and median concentrations of 2.7 and 2.6 µg/mL, respectively. Oxycodone concentrations in overdoses attributed to oxycodone alone ranged from 0.76-5.5 µg/mL with average and median concentrations of 3 and 3.6 µg/mL, respectively, while concentrations of oxycodone in cases of mixed drug intoxication were 0.08-4.9 µg/mL, with average and median concentrations of 1.6 and 0.9 µg/mL, respectively.

Noroxycodone was detected in all cases with blood concentrations ranging from 0.03 to 2.3 µg/mL and average and median concentrations of 0.8 and 0.5 µg/mL, respectively. In oxycodone overdose deaths the blood noroxycodone level ranged from 0.5 to 2.3 µg/mL with average and median concentrations of 1.1 and 0.9 µg/mL, respectively. Noroxycodone levels in multiple drug intoxication cases were lower and ranged from 0.08-0.5 µg/mL; mean and median concentrations were 0.28 and 0.25µg/mL, respectively. Oxymorphone was present in blood (3 cases) and urine (6 cases); blood levels ranged from 0.01 to 0.06 µg/mL and the average and median blood concentrations were 0.04 and 0.04 µg/mL, respectively.

Urine samples were analysed for oxycodone in order to determine chronic or single use [9,125]. in the current investigation, urine oxycodone concentrations were higher than 6 µg/mL (6.8-53 µg/mL) in overdose cases attributed to the use of oxycodone. However, in Cases 3 and 8 low levels of oxycodone were

detected at 0.4 and 1.6 µg/mL, respectively, indicative of a single dose of oxycodone. In case 3, noroxyxcodone and oxymorphone were present at concentrations of 0.6 and 0.1 µg/mL , respectively.

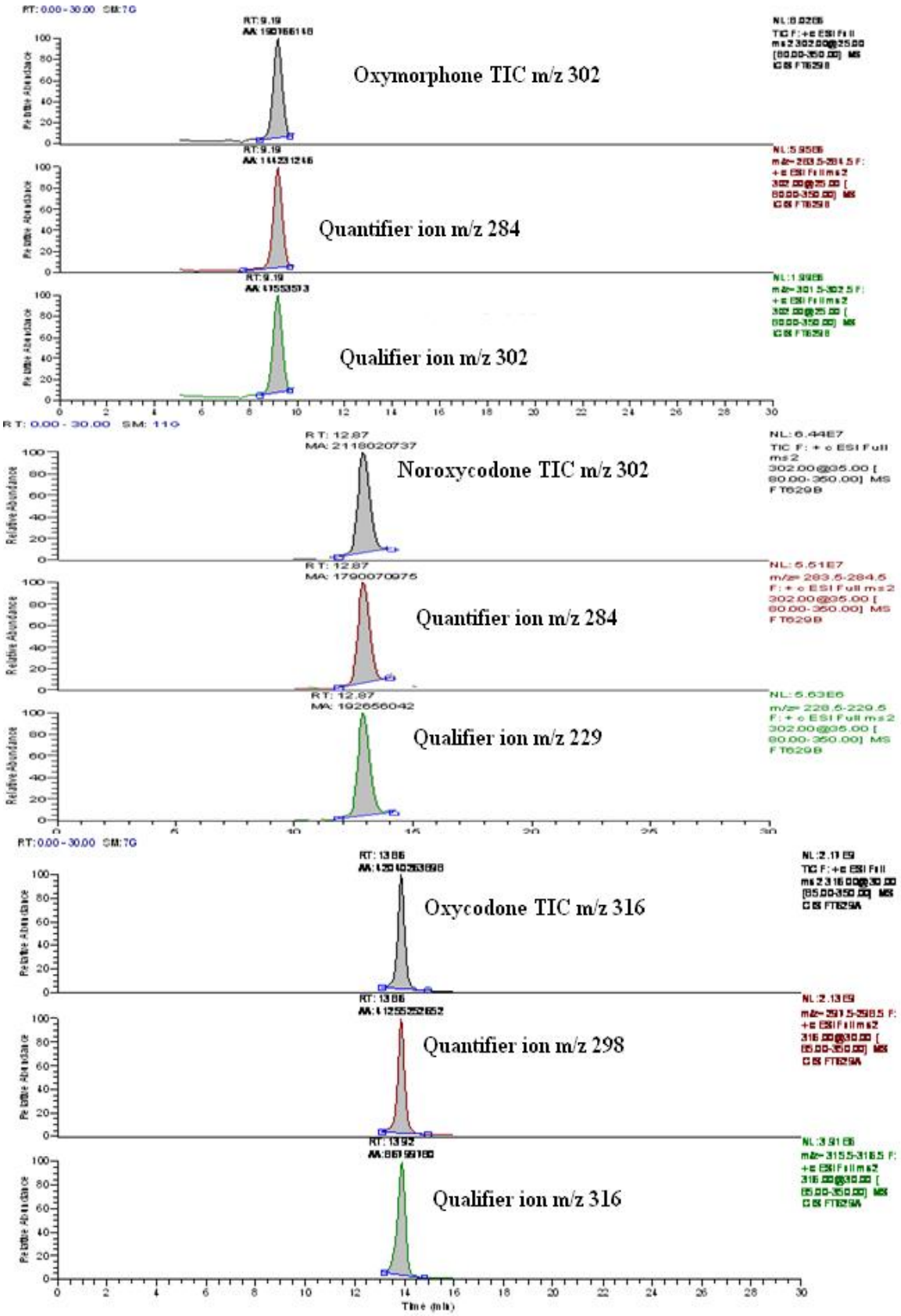


Figure 8-3: Oxycodone metabolites detected in a case of oxycodone intoxication.

In cases 1 and 2, the concentrations of oxycodone detected were 5.5 and 0.95 µg/mL, respectively and noroxycodone was lower in both cases at 0.86 and 0.39 µg/mL, respectively. Urine levels in both cases were quite similar with 7.6, 2.3 and 6.8, 2.5 µg/mL for oxycodone and noroxycodone, respectively. The lower concentration in case 2 compared to case 1 can be explained by the additional presence of a moderate level of alcohol found in case 2, which would increase the risk of fatal oxycodone poisoning even though oxycodone at this concentration could potentialy be a cause of death in the absence of alcohol.

A fatal level of oxycodone was detected in case 6; noroxycodone was also found at a concentration lower than that of the parent drug but oxymorphone was absent from the blood, which indicated a short time had elapsed between administration and death. In this case the cause of death was attributed solely to oxycodone intoxication despite the deceased being in the late stages of cancer. Three ampoules of oxycodone hydrochloride were found next to the deceased as well as a broken syringe driver, which may be an indication that oxycodone was injected. A high dose of oxycodone was administered, as indicated by blood and urine analysis which showed the presence of a high concentration of oxycodone compared to noroxycodone and oxymorphone. The level detected was high and indicative of having taken an excessive dose, whether intentionally or not.

The level of noroxycodone was higher than oxycodone in only one case (7). The cause of death was attributed to oxycodone as no other toxicants were detected apart from citalopram which was found at a therapeutic concentration. The ratio of oxycodone/noroxycodone was 0.7, which is the lowest encountered in the current study, indicating that a longer time had elapsed after administration. Witnesses stated that the deceased had consumed alcohol but none was detected in this case. However, a lack of tolerance for opioids could lead to fatal intoxication: oxycodone was not prescribed for the deceased and the high concentration of oxycodone is indicative of a fatal overdose.

In another two cases, 9 and 10, the concentrations of oxycodone were lower than 1 µg/mL while noroxycodone was above 0.5 µg/mL. The urine

concentrations of oxycodone were 45 and 30 µg/mL and of noroxycodone were 43 and 24 µg/mL, respectively. Death via a centrally-acting respiratory depressant in those with no tolerance to its sedating effects is more likely to cause death despite the presence of cocaine and cannabis in case 9. However, the presence of heroin was more likely to contribute to the cause death in case 10, since the level of morphine detected was consistent with a fatal heroin overdose. This case is in agreement with case 2 and those reported in previous work in which deaths occurred with lower drug concentrations in cases of poly-drug intoxication.

Diazepam and its metabolites were found in cases 1, 4, 5, 8, 9 and 10 and temazepam tested positive in cases 3, 5, and 10. It is known that the combination of other centrally-acting depressant drugs along with opioids may increase the risk of overdose and death compared to a single opioid alone.

8.4 Discussion

There were two main goals for the current study - to validate a method for identification and quantification of oxycodone and its N- and O-demethylated metabolites in blood and urine and to apply the method to routine casework. A previously published method (Chapter 5) based on SPE and LC-MS/MS was adapted and used for analysis of blood, urine, vitreous humour and gastric contents for the current study but was fully validated for blood and urine only since these are available for most cases. Apart from the introduction of a new matrix (urine) validation was necessary to include a new metabolite of oxycodone and its internal standard (noroxycodone and noroxycodone-d_3). The original method for determination of 26 opioids in a single run is expensive to operate due to the cost of standards but can be used conveniently for small groups of opioids when specific analytes need to be determined.

This project provided new information about the concentrations of oxycodone and its metabolites after overdose administration, in some of the cases examined in this study, which involved bypassing the controlled-released system of OxyContin tablets by crushing them, such that the drug was rapidly absorbed after intake. Usually, oxycodone from these slow-release tablets requires 24 hours to be completely absorbed in the stomach. The dose

required for pain relief is limited to a few tablets a day, but in the present work up to 44 tablets were recovered from the stomach of the deceased also indicating that oxycodone overdose can also occur without crushing the tablets (Figure 8-4). LC-MS/MS has been used widely in forensic toxicology. However, no reports have appeared concerning its application to the quantification of oxycodone and its metabolites in autopsy blood from oxycodone-related fatalities, although many pharmacokinetic studies have reported plasma and urine concentrations of oxycodone and its metabolites including noroxycodone, oxymorphone and other oxidative and reductive metabolites. Previous studies of oxycodone-related fatalities have looked only at the concentration of unchanged oxycodone. High concentrations of oxycodone were encountered in most cases of death attributed to oxycodone alone but noroxycodone and oxymorphone were not measured despite the fact that noroxycodone was known to be the major metabolites of oxycodone.

Figure 8-4: More than forty four OxyContin tablets recovered in the stomach of case 4.

8.4.1 Method validation

Most validated methods for oxycodone and its metabolites have been based on GC-MS or HPLC coupled with electrochemical or UV detection. LLOQ values varied between procedures and oxycodone metabolites. Previous GC-MS

procedures generally reported LLOQs higher than 20 and 40 ng/mL for oxycodone and oxymorphone, respectively [381], although some methods achieved LLOQs as low as 1.2 and 3.7 ng/mL for these analytes [382]. In one study using both GC-MS and LC-MS/MS for the determination of oxycodone, LC-MS/MS was 100 times more sensitive than GC-MS [393]. LLOQs of 0.1 to 0.25 ng/mL were reported for oxycodone, oxymorphone and noroxycodone in plasma samples [391]. An LLOQ lower than or equal to 1 ng/mL is required in cases involving oxycodone although it is often found at concentrations higher than 600 ng/mL [25,373]. However, oxycodone has been reported at concentrations as low as 25 ng/mL [391]. Concentrations of analytes in this group vary widely, between oxycodone and noroxycodone on one hand and O-desmethyl metabolites on the other. LLOQs in the present study were in the range I-1.2 ng/mL for both blood and urine.

The calibration intervals used in several previous studies have been in the range 1-100 ng/mL, which is lower than the concentrations of oxycodone and noroxycodone concentrations in overdose cases but is consistent with therapeutic use of oxycodone [400-390]. Wider ranges from 0.1-100 ng/mL and from 25-10000 ng/mL have also been used for a pharmacokinetic study [391] and for overdose cases respectively [381,382]. The selection of the calibration interval depends on the intended application of the method, to clinical or overdose cases or both and is also influenced by the need to avoid re-analysis of samples with high analyte concentrations. Also, the presence of oxycodone and its metabolites at therapeutic concentrations does not mean they are not implicated in the cause of death.

During this study, ten deaths involving oxycodone were investigated and two calibration curves were necessary to accommodate the high concentrations of the parent drug and its major metabolites and the low concentrations of the minor oxycodone metabolite, oxymorphone. Also, low concentrations of analytes could be encountered in non-suspicious deaths or in cases of multiple drug intoxication, as in case 3, in which all analytes were within the lower concentration calibration range. Analyte concentrations in autopsy urine samples were higher than the upper calibration interval, and most cases required dilution and re-analysis. No differences were observed between

calibration curves prepared using blood and urine matrices and one set of calibration curves could be used for both types of sample.

The method developed was capable of simultaneously detecting oxycodone and its metabolites within the recommended ratios for SRM transition recommended. Small sample volumes were used for analysis, 0.1-0.5 mL in most cases, which was possible because of the low LLOQs of the optimised method. Liquid-liquid extraction and SPE have been found to produced good recoveries of oxycodone and its metabolites [391,33-35]. In the present study, recoveries of analytes and matrix effects were assessed at five different concentrations across the calibration range using the approach of Matuszewski *et al* [51]. Matrix effects are now a routine requirement of method validation. But, although ion suppression and enhancement are reported, there are no guidelines concerning the acceptable limits of matrix effects. No matrix effects were found in the current method, for both blood and urine samples, and did not affect the accuracy of results.

Neuvonen *et al* [391] studied the effect of plasma matrix on ionisation in MRM quantification of oxycodone and its metabolites at the LLOQ using a post-column infusion method. In their work, mixtures of other opioids (50 ng/mL) and oxycodone metabolites (2.5 ng/mL) were spiked into plasma and analysed by LC-MS/MS in order to assess interference from structurally similar compounds. Ion suppression ranged from 2-13% in a zone extending from the start of the run to 5.7 minutes. No interference was observed from structurally similar compounds.

Similar findings were obtained in the present study although a different method of examining matrix effects was used and five different blood and urine specimens from different human sources were examined. Recoveries obtained in previous studies were often higher than 100%, indicating ion enhancement had occurred while in the present method recoveries from blood and urine were from 87-101% and 84-102% respectively after excluding matrix effects.

Oxycodone, noroxycodone and oxymorphone were reported to be stable in plasma for up to 6 months under different storage conditions [388,400] and for 12

weeks in another study [392]. Dawson *et al* [252] found oxycodone stable at room temperature for at least 24 hours after three freeze/thaw cycles and for up to 24 hours in the auto-sampler tray. Recently, Neuvonen *et al* [391] examined the stability of oxycodone, noroxycodone, and oxymorphone at two different concentrations in plasma (1 and 100 ng/mL) under three storage conditions and found that less than 10% degradation of oxycodone and its metabolites occurred.

In our previous report (Chapter 5), oxycodone and oxymorphone were found to be stable in blood under different storage conditions and in the present study noroxycodone was also found to be stable (Table 8-6). During the study period, repeat analyses were performed which gave results identical to those from the original analysis, indicating that oxycodone and its metabolites are stable in the refrigerator for up to month. No degradation of analytes was observed during SPE. Also, dilution of specimens was examined when urine samples with high drug concentrations were encountered, in one case by 50 to 100 times. Results obtained after different dilutions were identical.

8.4.2 Case studies

Cone *et al* [263] noticed that lower blood oxycodone concentrations were found in cases involving combined drug toxicity compared to those involving single drug exposure. Blood oxycodone ranged from 0.7-0.93 µg/mL in multiple drug deaths compared to 1.55-1.7 µg/mL in single drug deaths.

In the present study, oxycodone concentrations in mixed drug intoxication cases were in the range 0.08-4.9 µg/mL with average and median concentrations of 1.6 and 0.9 µg/mL respectively. In overdose cases attributed to oxycodone alone, blood concentrations ranged from 0.76-5.5 µg/mL and the average and median concentration were 3 and 3.6 µg/mL, respectively. Despite the overlap between the two groups, it is clear that deaths due to multiple drug intoxication can occur at oxycodone concentrations below 1 µg/mL, whereas most cases involving oxycodone alone have oxycodone concentrations higher than 1 µg/mL, with the exception of naïve users who had no tolerance to oxycodone.

Oxycodone case attributed to multiple drug intoxication by Cone *et al* [263] involved 3.5 drugs on average. In the current investigation an average of 4 drugs was found in oxycodone related cases. Benzodiazepines were found with concentrations either within or slightly higher than the therapeutic range and were considered to have only a minor contribution to the cause of death. A similar conclusion could be reached with respect to the low morphine, cannabis and valproic acid concentrations in cases 4 and 5. Clear effects of multiple drug intoxication were found in case 3, in which morphine, codeine, tramadol, acetaminophen, temazepam and amitriptyline were detected. Although codeine, morphine, acetaminophen and temazepam were within the therapeutic ranges, higher than therapeutic concentrations of amitriptyline and tramadol were detected and could have contributed with other drugs to the cause of death, since oxycodone is known to potentiate the effects of both other opioids and antidepressants [401]. Alcohol was detected in three cases but it was only considered to have contributed to the cause of death in case 2.

In the present study, oxycodone was used only as a painkiller by patients suffering from an acute illness or disease apart from one case in which oxycodone was prescribed to the flatmate of the deceased who administered the drug with clear suicidal intent (case 5). In most cases the deceased was depressed and some had previously attempted suicide, which may indicate that these were suicides, but this is not certain. The median ratio of oxycodone/noroxycodone was 2.4 and ranged from 0.7-49. This ratio may be helpful for estimating the time interval after ingestion before death occurred. A high ratio may indicate a short survival time after ingestion, not excluding other factors surrounding the death such as the presence of other centrally acting drugs which can add to the risk of intoxication with oxycodone.

Oxymorphone is now classified as a drug of abuse in the United State of America as increasing numbers of young people dying due to oxymorphone intoxication have been reported [402-404]. In one study, the median level encountered in post-mortem blood from oxymorphone related fatalities was 0.14 µg/mL with a range of 0.05-0.31 µg/mL [402]. Two other deaths attributed to oxymorphone had blood levels of 0.08 and 0.09 µg/mL. Interestingly, these two cases were known OxyContin users [403]. Recently, Garside *et al* [404]

reported 33 cases of oxymorphone related fatalities. The level of oxymorphone in post-mortem central and peripheral blood were in the range of 0.011-0.59 µg/mL and 0.017-0.82 µg/mL, respectively. The mean and median level of oxymorphone in both matrices were 0.15 and 0.1 µg/mL, respectively [404].

Oxymorphone levels in the current study were within the range for cases attributed to oxymorphone intoxication as mentioned above. Thus, the current study recommended that both oxycodone and oxymorphone be analysed in order to differentiate between the presence of oxymorphone as a consequence of oxymorphone administration or as metabolite of oxycodone.

Very high concentrations of oxycodone and its N-demethylated metabolite were detected in urine in cases 5, 6, 9 and 10. However, oxymorphone concentrations in urine were low compared to the other metabolites and oxymorphone was not detected in the corresponding blood samples. The question arises whether oxycodone was used for first time (single dose) or if it was chronically abused. It is known that oxymorphone is eliminated from the body as the glucuronide [366,371,372] and in the current procedure no hydrolysis method was applied and only free drug was detected in these cases. It is, therefore, expected that the level of total oxymorphone in urine and blood would be higher than reported here if a hydrolysis method was used. In contrast, oxycodone and its major metabolites (noroxycodone) are mostly excreted in the free form which explains the high concentrations encountered here. In one study, the concentrations of oxycodone and noroxycodone in urine samples after a hydrolysis procedure (HM) were not significantly different from those of the free analytes using a non-hydrolysis method [405]. Oxycodone and noroxycodone constituted 8.9% ± 2.6% for the HM versus 8% ± 2.6% for the non-hydrolysis method and 22.1% ± 9% for HM versus 23.1% ± 7.6%, respectively [371].

In the current study, the concentrations are in agreement with previously published pharmacokinetic studies which indicated that oxymorphone in urine accounted for 10.7% ± 5.5% of an oxycodone dose with only 33% ± 0.4% of oxymorphone excreted as the free drug; total oxymorphone excreted in urine accounted for 14.2% ± 7.5% of an oxycodone dose. Oxymorphone was the

lowest concentration metabolite of oxycodone present in urine, with a maximum concentration of 1 ng/mL [366,371]. The absence of O-demethylated metabolite of oxycodone in blood may result from a low concentration below the method detection limits or because death happened rapidly, with no time to convert oxycodone to oxymorphone. The high concentrations of drug and its major metabolites in urine were as expected in acute overdose cases.

By contrast, the urine oxymorphone concentration in case 1 was 1.3 µg/mL, the highest detected in the current study and the blood level was 0.04 µg/mL. In this case, the deceased may have been a chronic user of oxycodone due to her illness. The oxycodone and noroxycodone urine concentrations were not high at 7.6 and 2.3 µg/mL, respectively. Similar concentrations have been found in another study involving two subjects who had received 10 mg of oxycodone. The total oxymorphone did not exceed 2.5 µg/mL and urine oxycodone and noroxycodone concentrations after daily use were 13 and 18 µg/mL respectively [132].

A recently published pharmacokinetic study of oxycodone revealed that noroxycodone concentrations always exceeded those of the parent drug a few hours after administration [371,391]. In the current study, it was observed that the parent drug was the major substance detected in overdose cases rather than its N-demethylated metabolite, which suggested deaths occurred within a few minutes up to three hours after administration, the point at which both analytes should be found at similar concentrations until noroxycodone exceeds the parent drug. Another explanation was suggested by Anderson *et al* [369]: the oxycodone level at autopsy does not reflect its concentration at the time of death because oxycodone continues to be released from controlled-release tablets after death while the intact tablets remain in the stomach. The authors also point out that tablet could be found free of oxycodone as all of the drug was leached out and the tablets remain as 'ghost pills'. This may elevate the parent drug level in gastric compared to its metabolites as the drug released after death was not metabolised. In the current study, the stomach contents of case 4 were tested for oxycodone and found positive with huge concentrations which may be in contrast with the findings of Anderson *et al* [369], taking into consideration that the interval time between death and post-mortem examination was 6 days for case 4.

One of the limitations of the current study was the restricted availability of sample types for analysis, which limited the investigation of correlations between the levels of oxycodone and its metabolities in blood and urine with those in other biological matrices such as vitreous humour (VH). VH is clean and potentially comparable to blood results; unfortunately it is not always collected at autopsy. VH was collected in two cases, 5 and 9. In case 5, VH levels were positive for oxycodone and noroxycodone at levels of 2.1 and 0.76 µg/mL, respectively, giving an oxycodone/ noroxycodone ratio of 2.7. In case 9, VH levels were 0.63 and 0.19 µg/mL for oxycodone and noroxycodone, respectively, with a ratio of 3.3 between oxycodone/noroxycodone. The average ratios between blood/vitreous humour (B/VH) oxycodone and noroxycodone levels were 1.5 and 2.4, respectively. In both cases, oxymorphone was not detected in VH. Few reports are available concerning the B/VH ratios in oxycodone related fatalities: Drummer *et al* [25] reported one case in which the levels of oxycodone in blood and VH were 1.5 and 1.8 µg/mL, respectively, giving a B/VH ratio of 0.8. Anderson *et al* [369] determined oxycodone in blood (heart or femoral blood) and VH in seven fatalities. The B/VH ratios were 1.5 and 1.8 for heart blood/VH and femoral blood/VH, respectively. VH oxycodone levels in that study ranged from 0.18 to 0.82 µg/mL.

8.5 Conclusions

A sensitive and selective method has been developed and validated for the simultaneous determination of oxycodone, noroxycodone and oxymorphone in autopsy blood and urine samples and has been successfully applied to routine cases involving oxycodone intoxication. To the authors' knowledge, this is the first application of LC-MS/MS to oxycodone related fatalities and the first report of blood and urine noroxycodone and oxymorphone levels following acute oxycodone overdose. Oxycodone prescriptions have risen sharply in Scotland in recent years and the identification of ten oxycodone-related deaths in the study period (18 months) in the Strathclyde region of Scotland alone has highlighted the importance of including this drug in routine laboratory screening and confirmation procedures. High levels of oxycodone in combination with low levels of noroxycodone and oxymorphone are likely to

be diagnostic for suicides involving the deliberate ingestion of multiple tablets of OxyContin. The current study presents a novel approach to investigating oxycodone-related fatalities and further research is required to better understand the role of oxycodone metabolites in oxycodone-related deaths. The presence of noroxycodone in vitreous humour following oxycodone intoxication and the stability of noroxycodone in blood and urine was reported for the first time.

9 Identification Criteria for Opioids and Metabolites Using LC-MS/MS

9.1 Introduction

Confirmation of the identity of illicit and licit drugs in a given matrix is a paramount task in forensic and clinical toxicology [406]. Although many reviews have been published on the potential advantages of LC-MS (/MS) in toxicology, only one review has focused on identification issues [59]. After LC-MS was successfully introduced to analytical toxicology in the last decade, the identification of analytes is the most challenging topic needing addressed in order for LC-MS/MS to be used more confidently in the field. Special criteria are needed that fit with most LC-MS/MS techniques and, at the same time, are comparable with GC-MS. At first glance, identification of targetted analytes is not always problematic if standards are available, a suitable method of extraction is used and there is an adequate chromatographic separation. However, problems arise when trying to follow identification criteria using guidelines based on GC-MS analysis [407]. These requirements are easy to meet in theory but few of them can be achieved practically and the final results will depend on the toxicologist's knowledge and assessment of the quality of the results [31,59,407].

The recommendation given in SOFT/AAFS guidelines is to use two different techniques based on different chemical principles for drug identification and confirmation [54,408,409]. The suspected substances are usually identified in two steps. First, using preliminary techniques as a screening method and second, the tentatively identified drug should be confirmed using another technique, mostly a chromatographic method. Screening methods are limited to small drug classes [39] and guide toxicologists to confirm target analytes. Immunoassay techniques are still the methods of choice for drug screening as a first line in systematic toxicological analysis (STA) and could be used as identification power but they are usually unable to discriminate individual drugs as they respond to a group or class of drugs [28]. Monoclonal antibodies, for example to morphine as opposed to opiates, are specific but are not

accepted in the field as being sufficiently reliable for legal purposes. Similarly, thin layer chromatography (TLC) could provide identification power for analytes by a comparison of retention factor (R_f) between standards and samples but could not stand on its own in court [408].

The highest goal in order to avoid misinterpretation of medico-legal cases is the correct identification by laboratories of drugs or poisons; drugs should be discriminated accurately from all other possible interferences, which are more likely to be encountered using LC-MS methods, taking into consideration that other compounds of similar chemically structure may be major source of potential interference [16,59,408].

In many cases, the drugs or poisons that have been consumed or involved in toxicity are unknown. Also, many substances can be detected in each case which share similar properties or else possess different actions. Some of them are considered toxic, therapeutic or sub-therapeutic and some are endogenous components from the matrices themselves. These substances are metabolised by different mechanisms and rates, i.e. heroin can be easily hydrolysed either in *in-vivo* or *in-vitro* within 2-5 min, while some drugs exist for long periods of time in the free form or as phase I and phase II metabolites such as morphine glucuronide and ethanol conjugates. As indicated earlier in Chapter 8, a drug like oxymorphone is a product of oxycodone metabolism; drugs such as hydromorophone or hydrocodone may be found in urine samples as minor metabolites in cases involving high concentrations of morphine and codeine, respectively [410,411]. Cone *et al* [412] found that hydromorphone was excreted in urine with concentrations in the range 120-1400 ng/mL in ten out of thirteen patients treated with morphine using a high method cut-off of 100 ng/mL, which may mean that hydromorphone could be found in “negative” cases if a lower cut-off than 100 ng/mL was applied.

General unknown screening (GUS) is intended to identify or exclude as many harmful substances as possible. This objective is far from achieved as there are millions of substances present in the human environment and only a small portion of them are known to toxicologists [59]. As indicated earlier, known compounds could be subject to interference by unknown compounds. GUS is often based on quick methods of extraction and GC-MS analysis which may

lead to an incompletely resolved peak in which some of the minor metabolites of a drug may be completely overlooked. In addition, the occurrence of non-volatile or thermally labile compounds and the unsuitability of some analytes for derivatisation or which require multiple derivatisation steps could enhance the risk of a false negative result [410,413,414]. The chemical properties of drugs and metabolites to be identified and quantified differ widely, ranging from highly hydrophilic to moderate polarity and basic, acidic or neutral properties. The extraction procedure is still the most important part of identification of drugs even with the availability of highly accurate LC-MS/MS techniques despite the drawbacks of being time consuming, tedious and expensive. However, an extraction procedure using SPE is not only a concentration procedure for trace analytes, but provides more confidence and improved quantification by ruling out the risk of unwanted interferences [17,65,415].

In the field of forensic toxicology, analysis of many target analytes in a method is the preferred approach which can be fulfilled with high-throughput LC-MS/MS procedures in which multiple toxicants and metabolites can be determined in one single procedure, which reduces the cost, labour and time of analysis [16,31,109,416]. However, in most circumstances, only a few metabolites or single drugs need to be determined with mostly known target metabolites [31,54]. In fact, methods for analysis of these known analytes should already be validated and used routinely. Therefore, non-targeted analytes will not be determined leading to the potential risk of false positive results due to non-targeted compounds having similar mass spectra to included analytes [17,59,180,417-419].

Many tissue and matrices are available for analysis in post-mortem cases such as blood, urine, liver, bile, stomach contents, vitreous humour, hair, bone marrow and many others. In contrast, few specimens are available from living subjects, for example, in work place drug testing and driving under the influence of drugs (DUID). Urine samples are usually obtained at both post-mortem and from living subjects but are less important in interpretation of abused drugs, especially in post-mortem cases. Urine is more likely to be used in screening methods using immunoassay or chromatographic methods. However, urine samples are potentially open to manipulation by adulteration

and the interpretation of drug concentrations detected in urine is usually complex [14,202].

LC-MS/MS is new a tool providing simultaneous determination of various drug groups with smaller sample volumes and solvent usage [86]; lower drug cut-offs can be obtained which were found to be 100-fold less than the corresponding GC-MS cut-offs in one study in which the hydrolysis step was removed because free analytes like MOR can be determined with LLOQs of 2 ng/mL [393].

9.2 Aims

The aim of this work was to develop and validate a method for the determination of twenty six opioids and their metabolites which are commonly encountered in post-mortem toxicology in unhydrolysed urine. The method was intended to be complementary to the method for opioids in autopsy blood described in Chapter 5, with addition of new metabolites and their corresponding internal standards (a complete list is given in Table 9.1). The method was then applied to routine analyses of autopsy urine samples in parallel with blood analysis to investigate the value of using both matrices in the interpretation of the cause of death and source of the opioids detected. In addition, it was intended to evaluate identification criterion for drug confirmation recommended by many guidelines using the results obtained and the problems encountered with these identification criteria proposals.

9.3 Methods and Materials

These were similar to those described in Chapters 7 and 8.

9.4 Results and Discussion

9.4.1 Method validation

A method for general routine opioid screening using urine samples was validated as complementary method to that previously developed for blood. Mass spectral data for all analytes were obtained in the electrospray positive ion mode. Difficulties in obtaining product ions for B3G were reported in Chapter 5 has been solved by extanding the mass range of BUP3G to 2 amount mass unit instead of 1 used in Chapter 5 .

Partial cross talk between the internal standards of morphine glucuronide was present even with the use of MS^3 and after decreasing the mass range to 0.5 amu. The morphine glucuronide internal standards improved the method selectivity which can be used in the cases that are positive for morphine or codeine and negative for DHC as few cases were found positive for both MOR/COD and DHC. Morphine glucuronide internal standards are very important in overcoming the matrix effects, especially since both metabolites are eluted early at low percentages of organic modifier. Therefore, these internal standards could be used in cases that were negative for DHC, but for general screening MOR-D6 was employed as internal standard which resulted in good linearity and precision. It is better to have penta- or hexadeuterated morphine glucuronides to avoid cross talk phenomenon with DHM glucuronides. Although optimising LC mobile phase and/or the use of different columns would solve this phenomenon, this would be consuming time. In the first method (Chapter 5) data for all analytes were collected using full scan mode. In the current method, two of the major product ions were chosen for selected reaction monitoring (SRM) for each analyte to obtain two SRM transitions. Only one transition was used with the corresponding internal standards. More internal standards were included in the current method and tested using the SRM mode instead of the SIM mode used in the previous blood autopsy method (Table 9-1). Fourteen internal standards were used in the current method compared to 9 internal standards in the previous method.

Table 9-1: Selected reaction monitoring (SRM) transitions

Analytes	SRM [#] Transition A Quantifier ion m/z	SRM Transition B Qualifier ion m/z	Confirmation ratio A/B (R.S.D. %) [*]	RT [&] (min) n=35.0
Morphine	286 → 201	286 → 286	1.5 (4.0)	8.0
Morphine-D6	292 → 201	n.d.[&]	n.d.	7.9
Morphine-3-glucuronide	462 → 286	462 → 462	5.3 (7.0)	3.4
Morphine-3-glucuronide-D3	465 → 289	n.d.	n.d.	3.3
Morphine-6-glucuronide	462 → 286	462→ 462	5.6 (10.0)	6.6
Morphine-6-glucuronide-D3	465 → 289	n.d.	n.d.	6.5
6-Monoacetylmorphine	328 → 211	328 → 268	1.6 (3.0)	13.9
6-Monoacetylmorphine-D3	331 → 211	n.d.	n.d.	13.9
Normorphine	272 → 254	272 → 229	2.5 (2.0)	4.8
Codeine	300 → 215	300 → 243	1.4 (5.0)	12.4
Codeine-D3	306 → 218	n.d.	n.d.	12.4
Codeine-6-gluucronide	476 → 300	476 → 467	21.0 (12.0)	11.0
Codeine-6-glucuronide-D3	479 → 303	n.d.	n.d.	10.9
Norcodeine	286 → 268	286 → 243	5.0 (2.0)	11.1
6-Acetylcodeine	342 → 225	342 → 225	2.4 (2.0)	18.4
Hydromorphone	286 → 185	286 → 229	2.0 (10.0)	10.3
Hydromorphone-D3	289 → 185	n.d.	n.d.	10.3
Hydromorphone-3-glucuronide	462 → 268	462 → 462	5.6 (7.0)	4.8
Dihydrocodeine	302 → 245	302 → 201	1.6 (6.0)	11.8
Dihydrocodeine-D6	308 → 248	n.d.	n.d.	11.7
Dihydrocodeine-6-glucuronide	478 → 302	478 → 245	14.8 (6.0)	11.0
Dihydromorphine	288 → 213	288 → 231	1.1 (4.0)	6.9
Dinudromorphine-3-gluucronide	464 → 288	464 → 464	4.0 (10.0)	3.1
Dihydromorphine-6-glucuronide	464 → 288	464 → 464	5.0 (2.0)	7.1
Buprenorphine	468 → 414	468 → 396	2.6 (8.0)	23.0
Buprenorphine-D4	472 → 415	n.d.	n.d.	22.9
Buprenorphine-3-glucuronide	644.5 → 468	644.5→ 644.5	7.0 (17.0)	18.4
Norbuprenorphine	414.4 → 396	414.4 → 340	1.6 (4.0)	19.7
Norbuprenorphine-D3	417 → 399	n.d.	n.d.	19.6
Norbuprenorphine-3-glucuronide	590.5 → 414	590.5→ 590.5	5.8 (5.0)	14.3
Naloxone	328 → 310	328 → 328	15.8 (8.0)	12.3
Naloxone-3-glucuronide	504 → 486	504 → 328	1.6 (4.0)	7.9
Oxycodone	316 → 298	316 → 316	18.0 (11.0)	13.7
Oxycodone-D6	322 → 304	n.d.	n.d.	13.7
Noroxycodone	302 →284	302 → 229	10.0 (7.0)	12.9
Noroxycodone-D3	305 → 287	n.d.	n.d.	12.8
Oxymorphone	302 → 284	302 → 302	5.4 (5.0)	9.3
Oxymorphone-D3	305 → 287	n.d.	n.d.	9.2
Methadone	310 → 265	310 → 310	11.0 (10.0)	23.5
Methadone-D3	313 → 268	n.d.	n.d.	23.5

[#] SRM: Selected reaction monitoring
[*] RT: retention Time.
[&] n.d.: only one SRM transition was determined.

9.4.1.1 Linearity

Two calibration curves were prepared for most analytes included in this work with the exception of BUP metabolites, HMOR and HMOR glucuronide due to their expensive standards and only low level calibration curves were used. Two calibration ranges, 5-250 and 50-5000 ng/mL, were spiked into samples. The product ions of standards and internal standards were used to obtain peak area ratios of opioids and their metabolites. Linear regression lines were obtained with correlation coefficients (r^2) greater than 0.999 for all analytes.

High level calibration curves were found sufficient for urine cases but samples were diluted to bring them within the calibration range. SRM transitions are easily monitored for all analytes at high concentrations but the identification criteria may be affected by analyte concentration, for example, retention times can be shifted when a high concentration of target analyte is present. SRM transition ratios and retention times of analytes were similar to those of reference standards within the two calibration ranges used in the current study. Low sample volumes were used for analysis (0.1-0.25 mL) which reduced the matrix effects from endogenous components and avoided repeat analyses due to analyte concentrations being outside the calibration range.

9.4.1.2 LOD and LLOQ

LODs and LLOQs obtained for the analytes compared well with previous work (Chapter 5) and were 0.2 - 0.4 ng/mL and 0.5 - 1.5 ng/mL, respectively. LLOQs were calculated using equations 1-1, 1-2, 1-3 and 1-4. LODs and LLOQs for all analytes of interest are listed in Table 9-2.

9.4.1.3 Intra- and inter-assay precision

Intra- and inter-assay precision were assessed as the percentage relative standard deviation (RSD) on analyses of standards at three different concentrations (10, 50, and 200 ng/mL). Good precision was obtained for all analytes in the range from 1-14 and 1-15 % RSD for intra-and inter-assay precision, respectively (Tables 9-3 and 9-4).

Table 9-2: Linear correlation coefficients, LODs and LLOQs of opioids extracted from human urine.

Analytes	$r^{2\,\#}$	y-intercept	Standard error	Gradient (m)	LOD * (ng/mL)	LLOQ & (ng/mL)
MOR	0.998	0.000105	0.000128	0.001601	0.2	0.8
M3G	0.998	-0.00047	0.000934	0.013056	0.2	0.7
M6G	0.999	-0.000042530	0.000135512	0.002574273	0.2	0.5
6-MAM	0.999	-0.00179	0.001055	0.01998	0.2	0.5
NMOR	0.997	-0.000072	0.0000733	0.000743	0.3	1
COD	0.994	-0.00015	0.000262	0.001912	0.4	1.4
C6G	0.994	0.000863	0.000886	0.00632	0.4	1.4
NCOD	0.992	0.002959	0.003244	0.020331	0.5	1.6
6-AC	0.999	-0.00337	0.002166	0.030524	0.2	0.7
HMOR	0.995	-0.000047	0.000295	0.002304	0.4	1.3
H3G	0.999	0.0000442	0.000133	0.001202	0.3	1.1
DHC	0.997	-0.000081	0.000111	0.001182	0.3	0.9
DHC6G	0.995	0.00002	0.000352	0.002526	0.4	1.4
DHM	0.999	-0.0005	0.000471	0.00347	0.4	1.4
DHM3G	0.999	0.000173	0.000532	0.0045	0.4	1.2
DHM6G	0.998	0.000178	0.000237	0.002739	0.3	0.9
BUP	0.998	0.0000342	0.0000305	0.000413	0.2	0.7
BUP3G	0.998	0.002654	0.019688	0.272071	0.2	0.7
NBUP	0.996	-0.00196	0.001347	0.010989	0.4	1.2
NBUP3G	0.998	0.0000624	0.000254	0.003479	0.2	0.7
NALOX	0.995	-0.03249	0.043185	0.325067	0.4	1.3
NALOX3G	0.993	-0.00028	0.0006	0.004101	0.4	1.5
OXY	0.997	0.00027	0.000431	0.008459	0.2	0.5
NOXY	0.994	-0.0002	0.000856	0.00687	0.4	1.2
OXYM	0.997	0.000123	0.000974	0.009486	0.3	1.0
METH	0.995	0.0000278	0.00012	0.00097	0.4	1.2

r^2: Correlation coefficients.
* LOD: Limit of detection.
& LLOQ: Lower limit of quantitation.

9.4.1.4 Recovery and matrix effects

Matrix effect and recovery experiments were investigated using five different concentrations across the two calibration ranges using five different sources of human urine at two concentration 5 and 100 ng/mL. Both ion suppression and ion enhancement were observed with average values less than 15% and 8% respectively, which were within the accepted limits of the validation procedure (± 20 %). Matrix effect results are detailed in Tables 9-5 and 9-6. Analytes recoveries were calculated at five different concentrations, 5, 25 and 100, 750 and 2500 ng/mL, in the same manner as matrix effects but a third set was included in which analytes were spiked before extraction. The recoveries for analytes were found to be 84-103%. Recoveries of opioids and their metabolites are listed in Table 9-7.

9.4.1.5 Application to case samples

Forty seven post-mortem blood and urine samples were analysed using the validated method. Total morphine (TMOR) to total codeine (TCOD) ratios were calculated to determine which opioid had been administered. The validated method was compared with the existing method in the routine laboratory, which was used only for blood analysis and no urine analysis results were available. Therefore, only blood results were compared. Buprenorphine and oxycodone cases have been discussed in detail in Chapters 7 and 8, respectively. Methadone, DHC, and heroin cases are discussed here.

9.4.1.6 Stability

The stability of many opioid metabolites has not been studied before in urine, such as DHC and its metabolites, naloxone and its glucuronide, hydromorphone glucuronide and acetylcodeine. In the present study, most opioids and metabolites were found to be stable under different conditions: storage at room temperature, long and short term at -20 ^{0}C and 4 ^{0}C, and freeze/thaw cycles, with the exception of 6-AC and 6-MAM which were found to be unstable after 24 hours at room temperature; 6-MAM decreased more than 30 % after a month at 4 ^{0}C. However, 6-AC decreased approximately 80% at the same time.

Table 9-3: Intra-day precision between extractions.

	Nominal Concentrations *				
Analytes	**5.0 (ng/mL)**	**25.0 (ng/mL)**	**200.0 (ng/mL)**	**750.0 (ng/mL)**	**2500.0 (ng/mL)**
	Mean Measured Concentration ng/mL (R.S.D. %) #				
Morphine	5.6 (3)	23.0 (2.0)	99.0 (6.0)	742.0 (4.0)	2492.0 (5.0)
Morphine-3-glucuronide	4.9 (13)	26.0 (11.0)	104.0 (6.0)	753.0 (2.0)	2501.0 (3.0)
Morphine-6-glucuronide	4.8 (7)	26.0 (7.0)	103.0 (2.0)	734.0 (3.0)	2526.0 (3.0)
6-monoacetylmorphine	4.7 (8)	23.0 (2.0)	96.0 (1.0)	746.0 (2.0)	2518.0 (3.0)
Normorphine	5.1 (11)	25.0 (8.0)	96.0 (5.0)	731.0 (2.0)	2514.0 (1.0)
Codeine	4.6 (9)	25.0 (12.0)	100.0 (4.0)	745.0 (2.0)	2510.0 (2.0)
Codeine-6-glucuronide	4.5 (11)	29.0 (4.0)	95.0 (5.0)	767.0 (3.0)	2533.0 (2.0)
Norcodeine	5.7 (1.0)	23.0 (9.0)	105.0 (4.0)	741.0 (4.0)	2505.0 (3.0)
6-Acetylcodeine	5 (10.0)	25.0 (10.0)	98.0 (6.0)	740.0 (2.0)	2472.0 (1.0)
Hydromorphone	5.2 (6.0)	21.0 (7.0)	95.0 (7.0)	N/A &	N/A
Hydromorphone-3-glucuronide	4.9 (14.0)	26.0 (11.0)	101.0 (4.0)	N/A	N/A
Dihydrocodeine	4.5 (7.0)	25.0 (9.0)	98.0 (4.0)	742.0 (4.0)	2498.0 (6.0)
Dihydrocodeine-6-glucuronide	5.5 (8.0)	25.0 (10.0)	100.0 (6.0)	743.0 (3.0)	2507.0 (3.0)
Dihydromorphine	4.7 (10.0)	25.0 (10.0)	98.0 (7.0)	755.0 (7.0)	2502.0 (4.0)
Dihydromorphine-3-glucuronide	5.5 (7.0)	25.0 (14.0)	96.0 (5.0)	743.0 (5.0)	2476.0 (2.0)
Dihydromorphine-6-glucuronide	5.5 (13.0)	25.0 (7.0)	97.0 (6.0)	751.0 (4.0)	2485.0 (3.0)
Buprenorphine	4.7 (12.0)	27.0 (3.0)	102.0 (6.0)	N/A	N/A
Buprenorphine-3-glucuronide	4.3 (7.0)	26.0 (7.0)	100.0 (9.0)	N/A	N/A
Norbuprenorphine	4.4 (10.0)	24.0 (7.0)	100.0 (7.0)	N/A	N/A
Norbuprenorphine-3-glucuronide	5.4 (9.0)	24.0 (8.0)	101.0 (6.0)	N/A	N/A
Naloxone	5.5 (14.0)	24.4 (12.0)	106.0 (5.0)	751.0 (4.0)	2489.0 (6.0)
Naloxone-3-glucuronide	5.3 (5.0)	26.0 (9.0)	99.0 (6.0)	744.0 (2.0)	2474.0 (3.0)
Oxycodone	5.2 (5.0)	24.0 (4.0)	107.0 (5.0)	740.0 (3.0)	2488.0 (1.0)
Noroxycodone	4.7 (7.0)	23.0 (10.0)	104.0 (2.0)	751.0 (3.0)	2482.0 (2.0)
Oxymorphone	4.7 (11.0)	25.0 (8.0)	99.0 (4.0)	750.0 (1.0)	2492.0 (2.0)
Methadone	4.8 (4.0)	25.0 (5.0)	96.0 (3.0)	754.0 (3.0)	2475.0 (5.0)

* Value calculated from the average recovery for the replicate analyses (n=5)
# R.S.D. %: Relative standard deviation expressed as a percentage.
& N/A: Not analysed.

Table 9-4: Inter-day precision between extractions.

	Nominal Concentrations *				
Analytes	**5.0 (ng/mL)**	**25.0 (ng/mL)**	**200.0 (ng/mL)**	**750.0 (ng/mL)**	**2500.0 (ng/mL)**
	Mean Measured Concentration ng/mL (R.S.D. %) #				
Morphine	4.9 (9.0)	24.0 (6.0)	100.0 (5.0)	748.0 (3.0)	2506.0 (1.0)
Morphine-3-glucuronide	5.2 (12.0)	25 (7.0)	95.0 (4.0)	741.0 (5.0)	2534.0 (2.0)
Morphine-6-glucuronide	5.6 (11.0)	24.0 (5.0)	99.0 (5.0)	743.0 (2.0)	2481.0 (1.0)
6-monoacetylmorphine	4.8 (14.0)	25.0 (4.0)	100.0 (3.0)	746.0 (3.0)	2496.0 (5.0)
Normorphine	5.0 (13.0)	23.0 (10.0)	100.0 (4.0)	752.0 (5.0)	2495.0 (7.0)
Codeine	5.0 (15.0)	27.0 (8.0)	99.0 (6.0)	743.0 (3.0)	2487.0 (8.0)
Codeine-6-glucuronide	4.9 (13.0)	26.0 (10.0)	99.0 (4.0)	733.0 (6.0)	2480.0 (3.0)
Norcodeine	4.8 (15.0)	24.0 (11.0)	102.0 (3.0)	743.0 (5.0)	2514.0 (3.0)
6-Acetylcodeine	4.8 (14.0)	27.0 (6.0)	101.0 (3.0)	741.0 (2.0)	2496.0 (5.0)
Hydromorphone	5.0 (13.0)	23.0 (12.0)	100.0 (2.0)	N/A &	N/A
Hydromorphone-3-glucuronide	5.5 (11.0)	25 (12.0)	100.0 (6.0)	N/A	N/A
Dihydrocodeine	4.7 (14.0)	26.0 (6.0)	96.0 (5.0)	753 (6)	2487 (2)
Dihydrocodeine-6-glucuronide	5.6 (9.0)	24.0 (7.0)	101.0 (4.0)	745.0 (4.0)	2523.0 (3.0)
Dihydromorphine	4.8 (7.0)	25.0 (4.0)	103.0 (7.0)	756.0 (2.0)	2515.0 (1.0)
Dihydromorphine-3-glucuronide	5.7 (5.0)	24.0 (1.0)	100.0 (7.0)	742.0 (2.0)	2488.0 (3.0)
Dihydromorphine-6-glucuronide	5.5 (15.0)	24.0 (6.0)	102.0 (4.0)	747.0 (5.0)	2483.0 (2.0)
Buprenorphine	5.2 (12.0)	25.0 (14.0)	98.0 (6.0)	N/A	N/A
Buprenorphine-3-glucuronide	5.6 (6.0)	25.0 (5.0)	98.0 (3.0)	N/A	N/A
Norbuprenorphine	5.8 (10.0)	22.0 (6.0)	98.0 (8.0)	N/A	N/A
Norbuprenorphine-3-glucuronide	4.9 (12.0)	23.0 (11.0)	96.0 (4.0)	N/A	N/A
Naloxone	4.8 (11.0)	23.0 (5.0)	103.0 (2.0)	743.0 (2.0)	2484.0 (1.0)
Naloxone-3-glucuronide	5.5 (14.0)	26.0 (11.0)	100.0 (3.0)	760.0 (6.0)	2480.0 (2.0)
Oxycodone	4.7 (12.0)	23.0 (11.0)	100.0 (3.0)	743.0 (2.0)	2506.0 (3.0)
Noroxycodone	5.1 (7.0)	23.0 (9.0)	102.0 (6.0)	754.0 (3.0)	2507.0 (2.0)
Oxymorphone	5.2 (12.0)	25.0 (8.0)	99.0 (8.0)	746.0 (3.0)	2507.0 (2.0)
Methadone	5.3 (13.0)	25.0 (8.0)	102.0 (2.0)	752.0 (4.0)	2531.0 (3.0)

* Value calculated from the average recovery for the replicate analyses (n=5)
# R.S.D. %: Relative standard deviation expressed as a percentage.
& N/A: Not analysed

Table 9-5: Matrix effects at 5 ng/mL (% relative to drug in buffer).

	Urine Sources [#] spiked at 5 ng/mL after SPE extraction % [*] (R.S.D. %) [&]				
Analytes	**1**	**2**	**3**	**4**	**5**
Morphine	109 (8)	105 (3)	92 (4)	107 (10)	108 (7)
Morphine-3-glucuronide	104 (3)	90 (7)	99 (9)	119 (6)	107 (11)
Morphine-6-glucuronide	91 (10)	108 (4)	103 (6)	116 (2)	99 (14)
6-monoacetylmorphine	113 (5)	108 (11)	102 (12)	116 (10)	93 (3)
Normorphine	95 (5)	110 (8)	84 (2)	117 (10)	114 (9)
Codeine	111 (9)	117 (3)	98 (14)	103 (7)	112 (13)
Codeine-6-glucuronide	126 (7)	107 (10)	106 (9)	118 (3)	115 (6)
Norcodeine	110 (7)	94 (12)	102 (11)	115 (11)	109 (8)
6-Acetylcodeine	95 (2)	94 (11)	97 (6)	109 (3)	101 (3)
Hydromorphone	116 (9)	105 (14)	107 (7)	113 (5)	96 (9)
Hydromorphone-3-glucuronide	106 (9)	96 (15)	116 (15)	118 (8)	103 (14)
Dihydrocodeine	104 (8)	91 (5)	87 (13)	115 (2)	98 (11)
Dihydrocodeine-6-glucuronide	121 (7)	106 (14)	93 (4)	104 (1)	106 (10)
Dihydromorphine	103 (2)	103 (13)	95 (7)	105 (3)	94 (11)
Dihydromorphine-3-glucuronide	99 (3)	89 (10)	87 (8)	98 (10)	105 (6)
Dihydromorphine-6-glucuronide	101 (10)	108 (4)	91 (6)	116 (1)	100 (5)
Buprenorphine	115 (12)	118 (1)	105 (11)	117 (3)	103 (3)
Buprenorphine-3-glucuronide	113 (13)	109 (10)	108 (13)	114 (2)	105 (13)
Norbuprenorphine	111 (1)	112 (5)	103 (6)	119 (12)	105 (13)
Norbuprenorphine-3-glucuronide	89 (8)	103 (1)	122 (5)	103 (4)	98 (4)
Naloxone	108 (6)	102 (9)	96 (7)	94 (6)	86 (4)
Naloxone-3-glucuronide	112 (9)	103 (10)	92 (2)	108 (14)	113 (13)
Oxycodone	100 (13)	100 (5)	98 (6)	116 (7)	97 (5)
Noroxycodone	87 (13)	108 (4)	87 (7)	109 (8)	113 (6)
Oxymorphone	102 (4)	113 (14)	103 (15)	124 (4)	105 (2)
Methadone	101 (12)	98 (12)	96 (10)	91 (8)	104 (4)

[#] Human urine was sourced from completed urine samples that were scheduled for destruction and contained no analytes of interest.
[*] Matrix effect is expressed as the response obtained for a standard chromatographed along with matrix extract compared to that obtained with an unextracted standard chromatographed in mobile phase only, expressed as a percentage. Standard was spiked into matrix extract at a concentration of 5 ng/mL.
[&] R.S.D. %: Relative standard deviation expressed as a percentage.

Table 9-6: Urine matrix effects at 100 ng/mL (% relative to drug in buffer).

	Urine Sources * Spiked at 100 ng/mL after SPE Extraction % ¥ (R.S.D. % Я)				
Analytes	**1**	**2**	**3**	**4**	**5**
Morphine	118 (6)	95 (14)	99 (7)	95 (6)	100 (10)
Morphine-3-glucuronide	100 (1)	91 (3)	87 (1)	105 (5)	102 (2)
Morphine-6-glucuronide	98 (3)	101 (1)	98 (7)	114 (1)	116 (2)
6-monoacetylmorphine	100 (7)	91 (11)	97 (3)	100 (2)	100 (1)
Normorphine	95 (3)	93 (9)	80 (14)	98 (6)	90 (5)
Codeine	93 (3)	98 (3)	90 (1)	103 (1)	99 (5)
Codeine-6-glucuronide	100 (3)	101 (7)	98 (4)	101 (6)	112 (2)
Norcodeine	106 (9)	93 (4)	98 (5)	106 (3)	87 (2)
6-Acetylcodeine	104 (2)	84 (7)	93 (3)	116 (13)	118 (8)
Hydromorphone	97 (3)	93 (5)	102 (9)	97 (3)	99 (8)
Hydromorphone-3-glucuronide	100 (1)	95 (11)	96 (1)	108 (2)	100 (10)
Dihydrocodeine	98 (3)	102 (8)	93 (6)	111 (1)	112 (3)
Dihydrocodeine-6-glucuronide	95 (1)	98 (7)	103 (8)	113 (8)	110 (5)
Dihydromorphine	117 (13)	106 (5)	99 (10)	102 (2)	96 (7)
Dihydromorphine-3-glucuronide	116 (15)	103 (10)	103 (11)	113 (2)	107 (9)
Dihydromorphine-6-glucuronide	118 (7)	103 (7)	100 (8)	105 (2)	100 (5)
Buprenorphine	103 (1)	103 (4)	91 (8)	88 (1)	95 (5)
Buprenorphine-3-glucuronide	112 (10)	104 (3)	107 (1)	99 (15)	104 (13)
Norbuprenorphine	96 (5)	97 (6)	107 (6)	105 (4)	109 (1)
Norbuprenorphine-3-glucuronide	98 (11)	87 (5)	101 (1)	92 (15)	82 (4)
Naloxone	100 (5)	99 (2)	111 (6)	98 (1)	89 (1)
Naloxone-3-glucuronide	93 (1)	100 (4)	99 (7)	108 (4)	97 (8)
Oxycodone	102 (1)	102 (12)	107 (4)	98 (3)	105 (1)
Noroxycodone	105 (7)	107 (11)	98 (2)	115 (10)	106 (1)
Oxymorphone	111 (3)	103 (2)	100 (4)	100 (4)	101 (7)
Methadone	93 (6)	96 (4)	96 (6)	103 (1)	101 (2)

[#] Human urine was sourced from completed urine samples that were scheduled for destruction and contained no analytes of interest.

[*] Matrix effect is expressed as the response obtained for a standard chromatographed along with matrix extract compared to that obtained with an unextracted standard chromatographed in mobile phase only, expressed as a percentage. Standard was spiked into matrix extract at a concentration of 5 ng/mL.

[&] R.S.D. %: Relative standard deviation expressed as a percentage.

Table 9-7: Recoveries in extracted human urine

	Nominal concentration									
	5 ng/mL		25 ng/mL		200 ng/mL		750 ng/mL		2500 ng/mL	
	ME [*]	RE [#]	ME	RE	ME	RE	ME	RE	ME	RE
Analytes	Mean concentration measured % (R.S.D. %) [&]									
MOR	109 (8)	80 (8)	117 (12)	91 (14)	118 (6)	96 (7)	104 (7)	97 (6)	97 (6)	99 (9)
M3G	104 (3)	89 (9)	101 (6)	86 (10)	100 (1)	91 (4)	103 (1)	99 (2)	104 (3)	101 (2)
M6G	91 (10)	84 (11)	88 (12)	91 (11)	98 (3)	93 (2)	106 (3)	98 (1)	109 (3)	97 (2)
6-MAM	113 (5)	91 (9)	100 (5)	90 (12)	100 (7)	94 (11)	107 (4)	95 (2)	92 (5)	100 (3)
NMOR	95 (5)	72 (5)	97 (6)	88 (8)	95 (3)	93 (5)	111 (2)	100 (5)	102 (9)	100 (3)
COD	111 (9)	78 (8)	100 (10)	91 (2)	93 (3)	97 (9)	115 (7)	99 (3)	100 (8)	100 (2)
C6G	126 (7)	79 (3)	102 (9)	87 (14)	100 (3)	94 (11)	117 (5)	93 (7)	103 (2)	101 (5)
NCOD	110 (7)	78 (11)	97 (9)	92 (6)	106 (9)	95 (9)	113 (6)	96 (8)	105 (13)	97 (7)
6-AC	95 (2)	81 (11)	99 (4)	94 (9)	104 (2)	91 (8)	108 (5)	92 (4)	105 (5)	94 (1)
HMOR	116 (9)	80 (2)	100 (7)	84 (12)	97 (3)	90 (11)	N/A [**]	N/A	N/A	N/A
H3G	106 (6)	79 (15)	96 (12)	98 (6)	100	94 (1)	N/A	N/A	N/A	N/A
DHC	104 (8)	78 (13)	98 (8)	89 (7)	98 (3)	97 (13)	108 (5)	93 (5)	108 (7)	98 (5)
DHC6G	121 (7)	75 (8)	103 (10)	85 (5)	95 (1)	92 (5)	102 (2)	99 (6)	103 (10)	98 (4)
DHM	103 (2)	76 (2)	106 (10)	95 (6)	117 (13)	94 (10)	109 (4)	91 (7)	107 (8)	97 (5)
DHM3G	99 (3)	75 (7)	111 (4)	90 (5)	116 (5)	99 (6)	99 (11)	100 (4)	110 (10)	101 (3)
DHM6G	101 (10)	78 (7)	108 (11)	87 (7)	118 (7)	100 (5)	107 (8)	100 (7)	107 (9)	99 (11)
BUP	115 (12)	76 (3)	113 (8)	78 (6)	103 (1)	87 (10)	N/A	N/A	N/A	N/A
BUP3G	113 (13)	85 (12)	116 (7)	91 (12)	112 (10)	96 (3)	N/A	N/A	N/A	N/A
NBUP	111 (1)	77 (9)	106 (5)	88 (13)	96 (5)	89 (11)	N/A	N/A	N/A	N/A
NBUP3G	89 (8)	93 (10)	108 (6)	95 (3)	98 (11)	92 (4)	N/A	N/A	N/A	N/A
NALOX	108 (6)	84 (11)	112 (2)	87 (7)	100 (5)	89 (4)	109 (3)	92 (7)	112 (9)	90 (12)
NALOX3G	112 (9)	91 (13)	103 (10)	89 (6)	93 (1)	90 (6)	103 (10)	98 (6)	107 (11)	96 (4)
OXY	100 (9)	88 (10)	96 (6)	91 (10)	102 (1)	94 (9)	106 (8)	102 (6)	105 (6)	101 (5)
NOXY	87 (13)	85 (1)	92 (10)	105 (7)	105 (7)	99(4)	106 (4)	100 (11)	104 (10)	101 (11)
OXYM	102 (4)	84 (4)	93 (3)	101 (3)	101 (3)	95 (3)	103 (3)	96 (6)	110 (4)	102 (8)
METH	101 (12)	76 (9)	103 (4)	93 (6)	93 (6)	87 (6)	106 (4)	85 (5)	98 (10)	90 (2)

* Matrix effect is expressed as the response obtained for a standard chromatographed along with matrix extract compared to that obtained with an unextracted standard chromatographed in mobile phase only, expressed as a percentage. Standard was spiked into matrix extract at a concentration of 5, 25 and 100 ng/mL.
[#] Value calculated from the average recovery for the replicate analyses (n=5)
[&] R.S.D. %: Relative standard deviation expressed as a percentage. [**] N/A: Not analysed.

Stability of opioids and metabolites during sample processing was investigated using four freeze/thaw cycles and room temperature for four hours. The stability of all other opioids and their metabolites were in ranges of 89-119% and 94-115% respectively. Opioid metabolites in urine samples were stable for a week on the auto-sampler (4 ^{0}C). Long-term stability studies were performed at two different storage temperatures used routinely in the forensic toxicology laboratory, 4^{o}C and -20^{o}C, to assess the stability of analytes of interest. Stability results are listed in Table 9-5.

Opioids and their metabolites were stable for the whole period of this stability study (Table 9.5). However, the concentration of 6-AC decreased sharply by 30% after a month at 4^{o}C. 10% of 6-MAM was lost after a week and 25% after a month at 4^{o}C. Concentration changes with other analytes were less than ± 20% which was within the acceptable limits of the validation procedure.

The stability of naloxone-3-glucuronide, dihydrocodeine-6-glucuronide, dihydromorphone-3-glucuronide and dihydromorphone-6-glucuronide in urine samples was reported for the first time in this work. In Chapter 5, most opioids and metabolites in blood were found stable with the exception of 6-AC and 6-MAM in which large losses were observed previously during blood storage at room temperature and at 4 ^{o}C temperature (Figure 9-1 and 9-2).

The same finding was observed with urine in the current study and a decline in their concentrations was observed but with a lower rate than in blood, possibly because of the lower pH of urine (Figures 9-2 and 9-3). 6-MAM was detected in urine from most heroin-related deaths but in blood in only a few cases. The low concentration of 6-AC plus instability in both matrices has resulted in 6-AC being undetected in all cases except one, in which 6-AC was detected in urine at a very low concentration but in the presence of a high concentration of 6-MAM. O'Neal and Poklis [420] studied the stability of 6-AC in urine at pH 4.7 and pH 8 and found it to be more stable at the lower pH. Also, the authors found 6-AC in 6 out of 69 urine samples testing positive for morphine and codeine. They found 6-MAM in all cases testing positive for 6-AC. In that study, levels of 6-AC and 6-MAM ranged between 1-48 ng/mL and 100-1470 ng/mL, respectively. In the current study, the concentrations of 6-AC and 6-MAM in one living DUID subject were 11 and 329 ng/mL.

Table 9-8: Stability studies (% relative to starting concentration).

Storage condition [#]	Room Temperature		Freeze-thaw	Auto-sampler at 4 ^{0}C		Freezer at -20 ^{0}C				Refrigerator at 4 0C			
	4 hours	24 hours	4 cycles	48 hours	week	24 hours	48 hours	week	month	24 hours	48 hours	week	month
Analytes	**Mean concentration measured % [*] (R.S.D. %)[&]**												
MOR	95 (9)	97 (7)	97 (7)	99 (14)	98 (9)	94 (10)	93 (7)	100 (5)	107 (6)	93 (10)	93 (14)	110 (2)	115 (1)
M3G	97 (7)	104 (9)	103 (9)	96 (5)	90 (8)	95 (5)	96 (10)	93 (2)	102 (5)	98 (6)	91 (9)	91 (12)	89 (8)
M6G	98 (8)	103 (6)	103 (6)	91 (11)	94 (12)	105 (3)	101 (12)	96 (3)	99 (8)	102 (7)	94 (7)	93 (7)	95 (7)
6-MAM	101 (3)	95 (9)	89 (5)	93 (6)	95 (7)	107 (3)	100 (4)	95 (6)	94 (2)	93 (5)	101 (15)	90 (8)	75 (4)
NMOR	98 (1)	100 (4)	100 (4)	100 (11)	101 (5)	109 (11)	101 (1)	91 (5)	96 (8)	100 (2)	104 (4)	104 (7)	108 (5)
COD	115 (5)	108 (3)	109 (2)	94 (5)	103 (2)	108 (14)	114 (5)	97 (10)	102 (5)	95 (7)	114 (8)	100 (8)	110 (6)
C6G	100 (7)	105(11)	105 (11)	99 (4)	102 (5)	104 (2)	102 (3)	87 (3)	115 (7)	91 (7)	101 (8)	91 (6)	103 (6)
NCOD	100 (6)	104 (5)	104 (5)	96 (9)	101 (3)	96 (2)	100 (5)	101 (1)	93 (4)	89 (3)	109 (4)	91 (2)	93 (5)
6-AC	114 (4)	94 (8)	95 (8)	97 (5)	96 (9)	116 (5)	101 (2)	97 (8)	91 (3)	107 (6)	105 (10)	93 (10)	72 (1)
HMOR	104 (6)	102 (2)	102 (2)	97 (5)	109 (1)	104 (7)	102 (7)	94 (6)	95 (6)	93 (13)	104 (7)	94 (2)	101 (10)
H3G	94 (3)	110 (1)	112 (3)	93 (8)	90 (7)	92 (2)	94 (12)	104 (2)	97 (10)	96 (14)	113 (9)	101 (14)	89 (7)
DHC	110 (7)	96 (6)	96 (6)	98 (6)	97 (6)	109 (8)	107 (1)	96 (5)	108 (4)	98 (2)	106 (4)	98 (8)	103 (5)
DHC6G	100 (4)	98 (9)	98 (9)	94 (5)	100 (1)	106 (6)	94 (5)	98 (9)	110 (5)	95 (1)	92 (4)	97 (2)	118 (7)
DHM	96 (8)	95 (6)	95 (6)	108 (4)	104 (3)	93 (5)	105 (8)	101 (11)	105 (7)	93 (3)	97 (6)	111 (1)	106 (9)
DHM3G	97 (2)	102 (2)	102 (2)	103 (8)	96 (12)	94 (2)	93 (3)	103 (3)	105 (11)	92 (4)	88 (6)	95 (2)	108 (6)
DHM6G	94 (8)	98 (6)	98 (6)	98 (5)	90 (4)	95 (8)	97 (7)	95 (7)	100 (2)	93 (11)	93 (6)	99 (8)	117 (5)
BUP	115 (14)	94 (6)	94 (5)	98 (11)	108 (1)	101 (5)	106 (4)	105 (12)	109 (3)	96 (8)	104 (5)	102 (14)	107 (5)
BUP3G	105 (6)	102 (10)	102 (10)	95 (3)	100 (6)	104 (2)	98 (6)	97 (13)	93 (9)	107 (4)	104 (12)	110 (7)	100 (12)
NBUP	103 (10)	105 (6)	105 (6)	104 (5)	102 (10)	94 (4)	101 (4)	104 (8)	94 (7)	94 (11)	108 (3)	103 (4)	109 (10)
NBUP3G	99 (7)	108 (1)	109 (1)	93 (3)	99 (6)	96 (9)	96 (10)	103 (6)	102 (6)	94 (10)	93 (2)	97 (2)	99 (10)
NALOX	114 (5)	98 (2)	98 (2)	105 (5)	106 (8)	112 (12)	111 (12)	106 (3)	98 (11)	94 (8)	109 (8)	91 (5)	101 (8)
NALOX3G	94 (11)	106 (7)	105 (7)	89 (5)	96 (4)	96 (7)	106 (9)	92 (2)	112 (4)	93 (9)	90 (5)	86 (8)	114 (2)
OXY	104 (9)	100 (9)	100 (9)	93 (4)	108 (5)	97 (1)	104 (6)	95 (5)	107 (6)	93 (1)	102 (5)	92 (7)	103 (2)
NOXY	112 (4)	103 (10)	103 (10)	96 (15)	97 (5)	103 (2)	109 (8)	97 (13)	100 (8)	104 (6)	106 (14)	96 (12)	106 (6)
OXYM	110 (4)	99 (4)	99 (4)	102 (9)	104 (1)	106 (6)	113 (1)	96 (8)	110 (2)	94 (11)	117 (1)	91 (3)	97 (5)
METH	100 (5)	99 (11)	99 (10)	95 (10)	100 (2)	96 (2)	106 (5)	95 (6)	112 (1)	95 (11)	107 (2)	95 (4)	109 (8)

[#] Starting concentration is 100 ng/mL;
[*] Value calculated from the average recovery for the replicate analyses (n=3).
[&] R.S.D. %: Relative standard deviations expressed as a percentage.

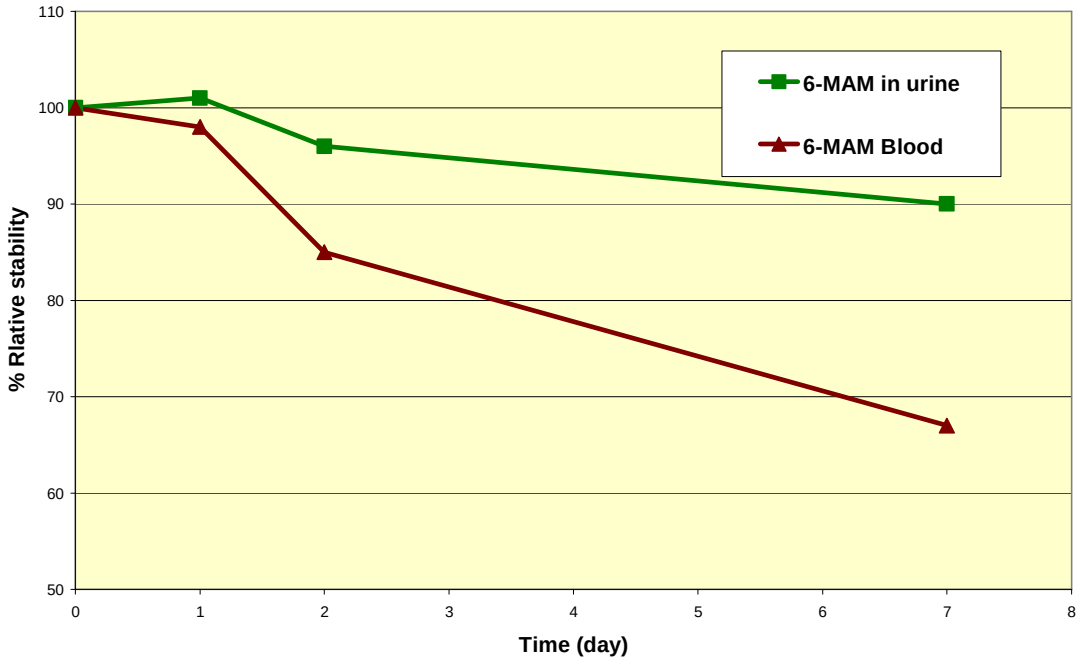


Figure 9-1: Comparison between 6-acetylmorphine stability in blood and urine.

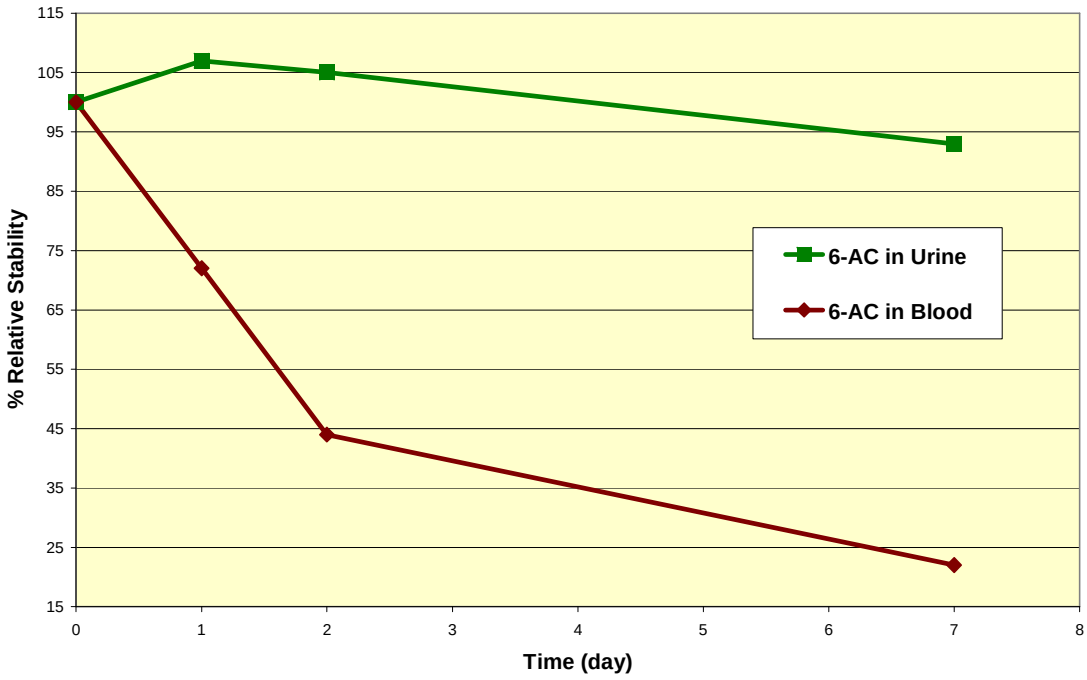


Figure 9-2: Comparison between 6-acetylcodeine stability in blood and urine.

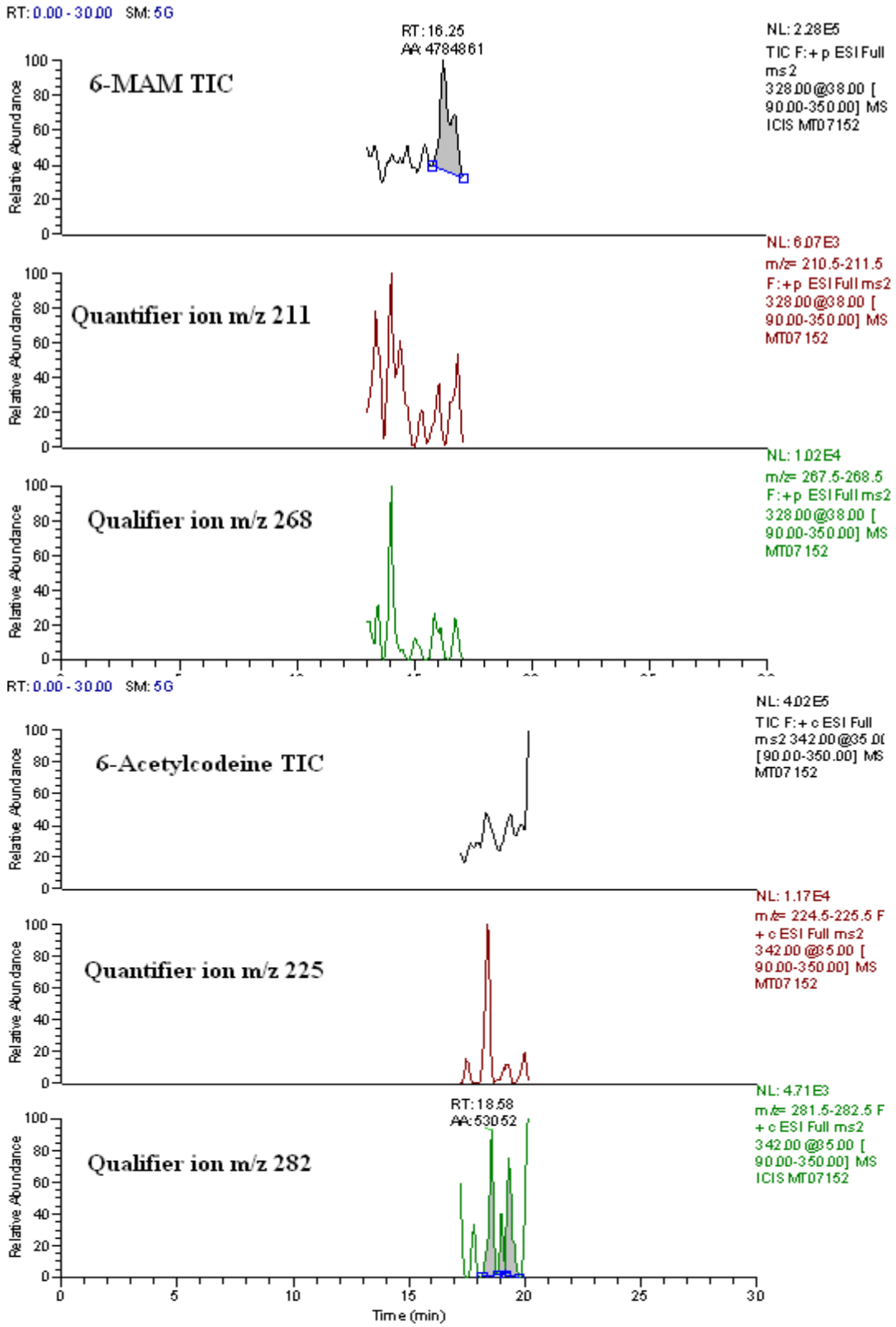


Figure 9-3: Negative results for 6-MAM and 6-AC in blood from a living subject having positive results in urine.

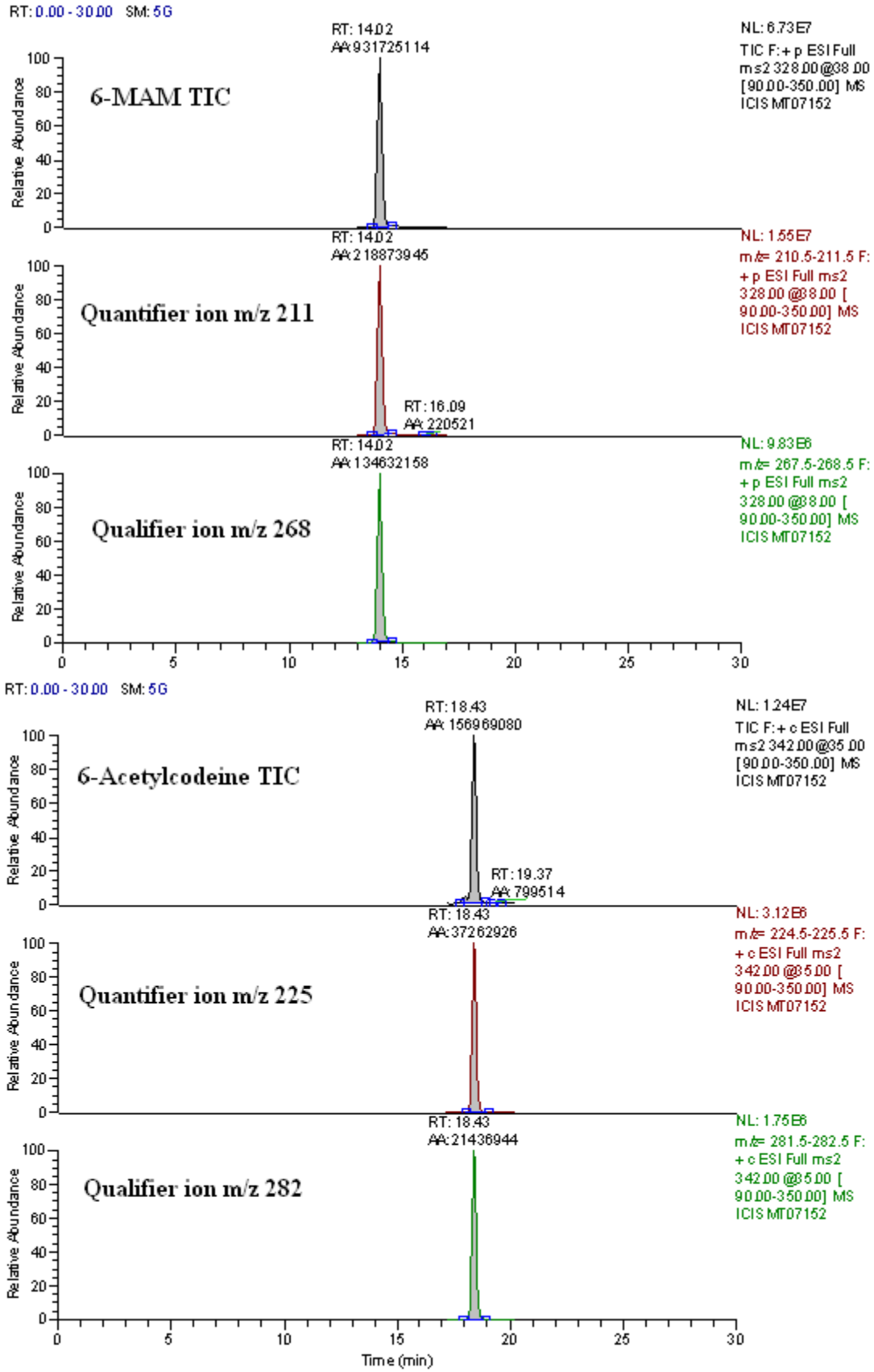


Figure 9-4: LC-MS chromatogram for a case testing positive for both 6-MAM and 6-AC.

6-Acetylcodeine has been detected in heroin users due to impurities which arise from the process of manufacturing heroin. It has been mentioned that heroin contains between 2-20% of 6-AC depending on the source of heroin and could be higher than 80%. O'Neal and Pokils [258] investigated the use of 6-AC as a specific marker of heroin use as an alternative to 6-MAM in urine sample and discovered that small amounts of 6-AC were detected compared to 6-MAM. They concluded that determining 6-AC could be used as a complementary marker of heroin use together with 6-MAM. However, it was not detected when the concentration of 6-MAM was low. Therefore, 6-AC might be a useful tool to distinguish between street heroin and pure heroin given in those programmes where patients are treated with pure heroin and samples were freeze directly to prevent of 6-AC hydrolysis to codeine [258-260].

The present study agrees with previous studies mentioned above that no 6-AC can be present alone without the presence of 6-MAM, dismissing the hypothesis of using 6-AC as heroin biomarker when 6-MAM is absent. Only one case was quantified for 6-AC in the current investigation. A few other cases in which 6-AC was found in trace levels could not be quantified due to the low concentrations present.

9.4.2 Cases samples

9.4.2.1 Methadone

Methadone was included in this method due to the increased incidence of methadone in opioid related deaths. Methadone was detected using autopsy blood in eight cases in the previous study using its pseudomolecular ion at m/z 310 which produced only a single product ion (mz 265). The cone voltage was adjusted to keep 10% of the parent drug in order to calculate the SRM transition ratio between mz 265 and 310. A good recovery and no matrix effects were observed for methadone in both blood and urine. Methadone was eluted at a very high organic modifier content, up to 80 %, which helped to enhance its MS/MS response. LLOQs were 1 and 1.2 ng/mL in blood and urine, respectively.

The stability of methadone was examined in blood at 100 ng/mL, when it was found stable at room temperature for up to 24 hours. Also, methadone was stable after subjection to four freeze/thaw cycles (98%), in the autosampler for

up to 1 week (104%) and in the refrigerator or freezer for up to one month (106% and 103% respectively).

Table 9-9: Comparison between the routine and optimised LC-MS/MS methods for analysis of methadone in autopsy blood samples

	Optimised LC-MS/MS methods [#] results		SOP result [*]	
No cases	**Blood (µg/mL)**	**Urine (µg/mL)**	**Blood (µg/mL)**	**Cause of death**
1	0.21	5.10	0.23	Inhalation of gastric contents; diazepam and methadone intoxication
2	0.41	1.40	0.44	Heroin and methadone intoxication
3	0.25	5.80	0.23	Head injury
4	0.70	5.30	0.70	methadone and alcohol intoxication
5	0.35	1.10	0.34	Heroin, alcohol and methadone intoxication
6	0.90	3.50	0.80	Methadone and cocaine intoxication
7	1.60	25.90	2.20	Methadone intoxication
8	1.20	3.10	1.30	Data not available
9	0.92	6.20	0.80	Methadone and alcohol intoxication
10	5.40	12.00	5.50	Stab wound of heart
11	0.18	0.80	0.14	DHC and methadone intoxication
12	1.40	0.90	1.60	Methadone, olazepine and diazepam intoxication Liver cirrhosis
13	1.80	8.20	1.90	Methadone intoxication
14	0.50	3.70	0.43	Heroin and methadone intoxication

[#] LC-MS/MS method for the analysis of opioids and their metabolites.
[*] SOP: Standard operation procedure or in-house GC-MS method used for routine general opioids analysis in Forensic Medicine and Science; University of Glasgow.

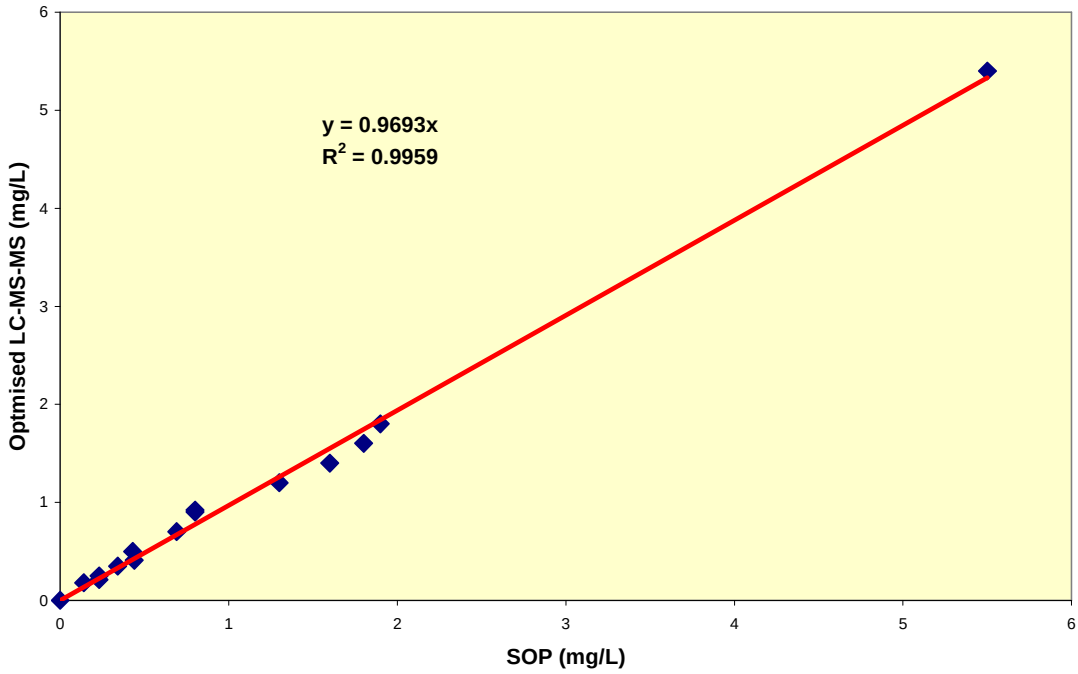


Figure 9-5: Correlation between the routine and optimised methods for the analysis of methadone samples in autopsy blood samples.

Methadone is usually identified in biological fluids by the presence of methadone itself or its metabolite EDDP. The latter eluted near to methadone and good method validation parameters were obtained for it. However, the calibration curve for EDDP was not linear during analysis of real case samples. The problem occured suddenly and may have been the result of standard contamination. As there was limited time left for this study, EDDP was excluded in the final validation process. The results obtained for 14 cases positive for methadone are listed in Table 9-9. Benzodiazepines were detected in all methadone cases, most of which were poly-drug intoxications in which heroin, DHC and alcohol were implicated in the deaths. The results obtained for these cases using the routine laboratory GC-MS (standard operation procedure (SOP)) method were compared with those from the new method. A good correlation was obtained with r^2 of 0.996 (Figure 9-6 and Table 9-9).

9.4.2.2 Heroin and codeine

In Chapter 5, ratios between drugs and metabolites were used for interpretation of the cause of death and the type of opioid administered and these will not be discussed again here.

Total MOR and total COD were not measured in all cases using SOP so only free MOR and COD results were available for direct comparison between optimised autopsy blood method and the SOP. A good correlation was obtained for free MOR and COD with r^2 of 0.99 and 0.996 for FMOR and FCOD, respectively, (Figures 9-7 and 9-8 and Table 9-10).

It can be seen from the results for 29 urine samples from heroin and codeine cases (Table 9-11) that M3G is the most abundant metabolite with average and median concentrations of 45 and 70 µg/mL. The TMOR/TCOD ratios were higher than one in all cases with the exception of five cases (16, 23-25, and 27) suggesting that the opioid ingested was codeine. Levels of COD in urine may be higher than 1 µg/mL following heroin administration. The ratio of free MOR/free COD ranged between 0.05-56 with an average and median of 12 and 10, respectively, suggesting that heroin was used in most cases where the ratio was higher than 1. 6-MAM tested positive in most heroin related fatalities, Norcodeine, considered a marker of codeine use, was detected in most of the heroin cases, probably due to the hydrolysis of 6-AC to codeine and oxidation to norcodeine.

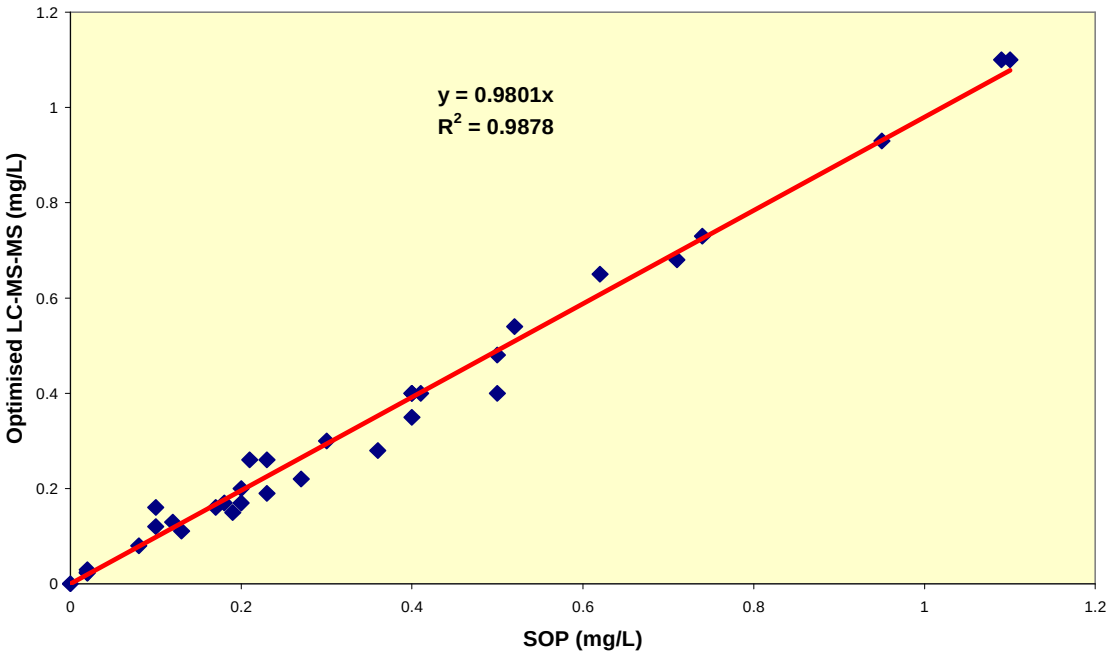


Figure 9-6: Comparison between free morphine in blood obtained using optimised LC-MS/MS and routine methods.

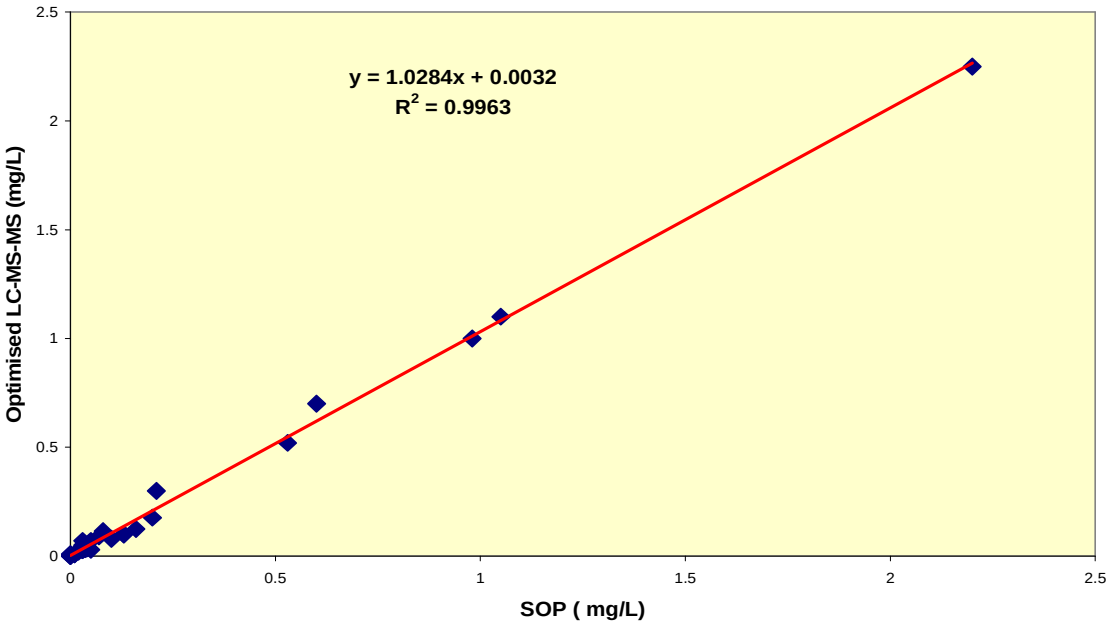


Figure 9-7: Comparison between free codeine in blood obtained using optimised LC-MS/MS and routine methods.

From the results listed in Tables 9-10 and 9-11 for 32 cases, the causes of death was identified in 28 cases in the pathology reports. In cases where heroin was implicated in the death, the causes of death can be classified into three types: sole heroin intoxication, poly-drug intoxication (in which at least another drug contributed to toxicity) and not related to death (in which the cause of death was attributed to another drug or disease) [157]. Eight cases were sole heroin intoxication according to pathology reports: blood MOR levels were in the range 0.2-1.1 µg/mL with average and median concentrations of 0.46 and 0.38 µg/mL, respectively; MOR in urine was in the range of 0.02-8.5 µg/mL with average and median concentrations of 2 and 0.6 µg/mL, respectively. COD concentrations in blood were in the range 0.003-0.1 µg/mL with average and median concentrations of 0.05 and 0.03 µg/mL, respectively. However, in urine, COD ranged between not detected to 3.6 µg/mL.

Heroin contributed with another drug in deaths of nine of these cases; four cases were attributed to a combination of heroin and methadone, three cases to heroin and cocaine and two cases consisted to heroin and alcohol. Blood MOR

was in the range of 0.08-0.7 µg/mL with average and median concentrations of 0.38 and 0.33 µg/mL, respectively. Urine MOR was in the range of 0.04-14.4 µg/mL with average and median concentrations of 3.57 and 0.7 µg/mL, respectively. COD in blood ranged between 0.01-0.1 µg/mL with average and median concentrations of 0.05 and 0.04 µg/mL, respectively; COD in urine was in the range 0.03-1 µg/mL with average and median concentrations of 0.31 and 0.17 µg/mL, respectively.

In the third category, in which the presence of MOR was not related to the cause of death, blood MOR was 0.02-1.1 µg/mL with average and median concentrations 0.4 and 0.2 µg/mL; MOR in urine was 0.04-5.1 µg/mL with average and median concentrations of 2.2 and 1.5 µg/mL, respectively. Three of these cases were attributed to co-codamol intoxication in which high blood levels of COD were encountered (0.001-2.3 µg/mL) with average and median concentrations of 0.6 and 0.1 µg/mL; urine COD levels were in the range of 0.2-4 µg/mL with average and median concentrations of 1.2 and 0.4 µg/mL. There was overlap between cases using free MOR levels in blood and urine even in non-drug related fatalities.

The present work strongly supports the use of the ratio of free MOR/ free codeine in urine to distinguish between heroin and codeine users in which the detectable amount of free MOR was higher than 10 µg/mL and if 6-MAM was not present; these ratios should not be less than one for both free and total drug [257]. Codeine has often been included in the analysis of morphine in biological samples to interpret the opiate administered [201-203,257]. However, interpretation of the ratio of morphine/codeine in urine samples has often been brought into question. Many factors have been found to affect this ratio, such as the pH variation between cases, unusual frequency of urination and amount of urine in the bladder, which influence the reliability of the ratio in forensic analysis. Therefore, the ratio of morphine/codeine in blood is considered strong proof of the source of opiates even if a urine sample is not analysed [202,421].

The absence of codeine and the presence of morphine does not indicate that heroin was used but may suggest codeine intake. A high ratio of COD/MOR in urine could be an indicator for recent codeine ingestion but low ratios cannot be used to distinguish the source of opioid intake [422].

Table 9-10: Comparison between free morphine and codeine in blood obtained using optimised LC-MS/MS and routine methods

Sam no.	MOR µg/mL			Codeine µg/mL			Cause of death
	U[#] (LC) [##]	B [*] (LC)	B (SOP) [**]	U (LC)	B (LC)	B (SOP)	
1	0.60	0.4	0.4	0.02	0.033	0.03	Heroin intoxication
2	0.20	0.26	0.23	0.00	0.028	0.029	Heroin intoxication
3	2.80	0.28	0.36	0.30	0.04	0.035	Methadone intoxication
4	0.60	0.48	0.5	0.20	0.032	0.036	Heroin intoxication
5	3.70	0.4	0.41	0.70	0.01	0	Methadone and heroin intoxication
6	0.30	0.4	0.4	0.02	0.035	0.04	Not-drug related
7	0.04	0.4	0.5		0.03	0.05	Heroin and alcohol intoxication
8	4.30	0.17	0.18	0.40	0.001	0	Morphine and cocaine intoxication
9	4.00	0.35	0.4	3.90	0.028	0.03	Heroin intoxication
10	8.50	0.19	0.23	0.70	0.003	0	Heroin intoxication
11	5.10	1.1	1.09	0.09	0.04	0.03	Hanging
12	1.10	0.08	0.08	0.30	0.05	0.03	Heroin and methadone intoxication
13	0.02	0.17	0.2		0.07	0.03	Heroin intoxication
14	8.00	0.22	0.27	0.50	0.03	0.03	Combined heroin and cocaine toxicity
15	14.40	0.54	0.52	1.00	0.115	0.08	Heroin and cocaine intoxication
16	0.70	0.68	0.71	0.03	0.08	0.1	Heroin and alcohol intoxication
17	0.30	0.023	0.02	1.40	0.124	0.16	not drug related
18	141	0.3	0.3	21.00	0.177	0.2	No information
19	1.50	0.15	0.19	4.00	1	0.98	Co-codamol toxicity
20	0.04	0.93	0.95	0.02	0.1	0.13	Head injury
21	0.25	0.111	0.13	0.03	0.01	0.01	Cocaine, heroin, ecstasy and dihydrocodeine intoxication
22	1.00	1.1	1.1	0.08	0.1	0.13	Heroin intoxication
23	0.50	0.26	0.21	0.04	0.038	0.03	Heroin and methadone intoxication
24		0.65	0.62		0.07	0.05	Heroin and methadone intoxication
25		0.16	0.1		0.7	0.6	No information
26		0.03	0.02		0.52	0.53	No information
27		0.12	0.1		0.01	0.01	No information
28		0.2	0.2		0.02	0.02	Not drug related
29		0.16	0.17		1.1	1.05	Co-codamol overdose
30		0.13	0.12		2.25	2.2	Co-codamol overdose
31		0.024	0.02		0.3	0.21	No information
32		0.73	0.74		0.09	0.07	Heroin intoxication

[#] U: Urine samples result; [*] B: Blood samples result

[##] LC-MS/MS method for the analysis of opioids and their metabolites.

[**]SOP: Standard operation procedure or in-house GC-MS method used for routine general opioids analysis in Forensic Medicine and Science; University of Glasgow.

Table 9-11: Concentrations of heroin, morphine and codeine metabolites (µg/mL) in urine samples

Analytes Sam. no.	MOR	6-MAM	M3G	M6G	NMOR	COD	NCOD	C6G	TMOR #	TCOD *	TMOR/ TCOD	M3G/ MOR	M6G/ MOR	M3G/ M6G	FMOR/ MORG &	FMOR ##+ M6G /M3G	FMOR/ TMOR	FMOR / FCOD **
1	0.6	0.02	30.5	2	0.08	0.02	0.04	0.7	33.18	0.76	43.66	0.02	3.33	15.25	0.02	0.09	0.02	30.00
2	0.2		0.1	0.04		0			0.34	0.00		2.00	0.20	2.50	1.43	2.40	0.59	
3	2.8	0.18	40	2.3	0.04	0.3	0.04	0.4	45.14	0.74	61.00	0.07	0.82	17.39	0.07	0.13	0.06	9.33
4	0.6	0.02	23.5	1.9	0.05	0.2	0.07	1.5	26.05	1.77	14.72	0.03	3.17	12.37	0.02	0.11	0.02	3.00
5	3.7	0.3	118	7.8	0.4	0.7	0.2	3.5	129.90	4.40	29.52	0.03	2.11	15.13	0.03	0.10	0.03	5.29
6	0.3	0.02	12	0.7	0.1	0.02	0.08	0.3	13.10	0.40	32.75	0.03	2.33	17.14	0.02	0.08	0.02	15.00
7	0.04	0.04	0.1	0.04	0.06		0.1		0.24	0.10	2.40	0.40	1.00	2.50	0.29	0.80	0.17	
8	4.3	0.7	26.2	8.8	0.04	0.4		6.3	39.34	6.70	5.87	0.16	2.05	2.98	0.12	0.50	0.11	10.75
9	4	0.1	20.4	7.9	0.2	3.9	0.08	3.9	32.50	7.88	4.12	0.20	1.98	2.58	0.14	0.58	0.12	1.03
10	8.5	0.5	47.5	20.8	0.3	0.7		14.8	77.10	15.50	4.97	0.18	2.45	2.28	0.12	0.62	0.11	12.14
11	5.1		5.5	1.2	1.3	0.09	0.09	0.5	13.10	0.68	19.26	0.93	0.24	4.58	0.76	1.15	0.39	56.67
12	1.1	0.02	0.7	1.3	0.15	0.3	0.08	1.3	3.25	1.68	1.93	1.57	1.18	0.54	0.55	3.43	0.34	3.67
13	0.02	0.03	0.2	0.05					0.27	0.00		0.10	2.50	4.00	0.08	0.35	0.07	
14	5.9	0.14	253	18	1	0.5	0.1	1	277.90	1.60	173.69	0.02	3.05	14.06	0.02	0.09	0.02	11.80
15	14.4	2	428	31.2	0.6	1	0.1	7.8	474.20	8.90	53.28	0.03	2.17	13.72	0.03	0.11	0.03	14.40
16	0.4		0.7			0.7	0.2	9	1.10	9.90	0.11	0.57	0.00		0.57	0.57	0.36	0.57
17	0.7		6.8	1.6	0.4		0.03		9.50	0.03	316.67	0.10	2.29	4.25	0.08	0.34	0.07	
18	0.8		3.9	0.9	0.15	0.05	0.08	0.01	5.75	0.14	41.07	0.21	1.13	4.33	0.17	0.44	0.14	16.00
19	0.05		1	0.3	0.05	0	0.01		1.40	0.01	140.00	0.05	6.00	3.33	0.04	0.35	0.04	
20	0.2	0.01	0.8	0.2	0.02	0.01	0.02	0.2	1.22	0.23	5.30	0.25	1.00	4.00	0.20	0.50	0.16	20.00
21	0.7	0.2	3.4	0.9	0.06	0.08	0.01	1.4	5.06	1.49	3.40	0.21	1.29	3.78	0.16	0.47	0.14	8.75
22	0.05		0.4	0.1	0.02	0.01	0.01	0	0.57	0.02	28.50	0.13	2.00	4.00	0.10	0.38	0.09	5.00
23	0.14		3.2	0.6	0.6	12	0.9	67	4.54	79.90	0.06	0.04	4.29	5.33	0.04	0.23	0.03	0.01
24	0.17		3.5	0.7	2.1	22	7.6	67	6.47	96.60	0.07	0.05	4.12	5.00	0.04	0.25	0.03	0.01
25	0.4		3.5	0.9	0.3	1.4	0.14	20	5.10	21.54	0.24	0.11	2.25	3.89	0.09	0.37	0.08	0.29
26	0.7	0.02	4.2	1.2	0.03	0.03		0.25	6.13	0.28	21.89	0.17	1.71	3.50	0.13	0.45	0.11	23.33
27	1.5		0.55	0.5	0.5	3.8	0.13	28	3.05	31.93	0.10	2.73	0.33	1.10	1.43	3.64	0.49	0.39
28	0.9	0.5	2.8	0.2	0.01	0.08			3.91	0.08	48.88	0.32	0.22	14.00	0.30	0.39	0.23	11.25
29	0.5		6.8	1.1	0.2	11	3.4	12.5	8.60	26.90	0.32	0.07	2.20	6.18	0.06	0.24	0.06	0.05
Ave	2.39	0.28	44.76	4.74	0.23	0.87	0.23	3.62	42.35	11.04	39.03	0.37	1.98	6.78	0.25	0.66	0.14	10.78
Med	3.95		69.7	7.75	0.4	0.8	0.15	4.54	6.13	1.49	14.72	0.13	2.05	4.13	0.10	0.38	0.09	9.04

TMOR: Total morphine; * TCOD: Total codeine; ## FMOR: Free morphine; ** FCOD: Free Codeine; & MORG: Morphine conjugates.

Although the ratio of COD/MOR (0-6 hours) after codeine intake was found to be different between the CYP2D6 genotypes poor, ultra and extensive metabolisers, ratios were higher than one in all three genotypes [136]. He *et al* [188] also reported that the ratio of COD/MOR did not change and remained higher than one in all subjects after codeine administration in the ultra-rapid and extensive metaboliser subjects. Codeine concentrations in urine following codeine intoxication ranged from 0.78 to 108.8 µg/mL, averaging 38 µg/mL [423]. In the present study, total codeine concentrations ranged from not detected to 96 µg/mL with an average concentration of 11 µg/mL. However, in codeine intoxication case, codeine levels ranged from 21.5 to 96 µg/mL with an average concentration of 51.4 µg/mL. Total MOR ranged from 3.1 to 8.6 µg/mL with an average of 5.6 µg/mL in these cases. The ratio of total COD/total MOR ranged between 3.1 and 17 with an average of 10.

9.4.2.3 Dihydrocdeine

DHM and its glucuronides were determined in blood in DHC related deaths and levels of DHM glucuronides were lower than reported previously [24,212]. In the current study, urine and blood samples were analysed in cases attributed to DHC overdose. DHC is metabolised mainly to DHC6G and nordihydrocodeine (nor-DHM); unfortunately, standards of nor-DHM and its glucuronide were not available at the time of this study which would aid in the investigation of their contribution to DHC toxicity. It is estimated that 28 %, 16 % and 32 %, 20% of a DHC dose are metabolised to DHC6G and nor-DHM in extensive and poor metabolisers, respectively. A low percentage is metabolised to DHM, DHM3G and DHM-6-G, estimated to be 9% and 1.3% in the extensive and poor metaboliser populations, respectively [126,208,216].

Previous reports indicate that DHM is responsible for DHC analgesia. DHM has a high affinity to opioid receptors (µ-, δ- and κ) similar to that of morphine and 100 times higher than DHC while nor-DHM and its glucuronide have a lower affinity to opioid receptors, similar to DHC [216]. Reports also suggest that DHM and DHM-6-glucuronide may contribute to DHC toxicity [24,207,209]. Concentrations greater than or equal to 100 and 74 ng/mL, respectively, are believed to be toxic and to contribute to the respiratory depression effect of DHC [24]. Therefore, inclusion of DHM metabolites may add some information to the interpretation of

the cause of death due to DHC intoxication [212]. Levels of DHM and its glucuronide in DHC intoxication were reported in two studies. DHM was found lower or equal to 0.2 µg/mL; DHM3G was always higher than DHM (range 0.3-1.08 µg/mL) and DHM6G was lower or equal to 0.1 µg/mL (range 0.07-0.111 µg/mL) in both studies [24,212].

DHC levels in autopsy blood obtained with the current method were compared with results of routine analysis of free DHC. There were no routine results for urine; twenty eight cases were involved in this comparison and both methods compared well for the analysis of free DHC; good correlation was obtained with an r^2 of 0.996 (Figure 9-9 and Table 9-12). Unfortunately, urine samples were not collected at autopsy in many of these cases and only blood samples were analysed using SOP method.

DHC and its glucuronide were found in all cases investigated in blood and urine matrices. However, DHM and its glucuronide were present at lower levels than DHC and its glucuronide. Unchanged parent drug is the most abundant DHC metabolite in blood samples while DHC glucuronide is the most abundant DHC metabolite in urine. In blood, DHM was found in three cases ≤ 0.1 µg/mL while DHM6G ranged from not detected to 0.01 µg/mL.

Deaths involving DHC can also be classified into three categories: DHC only, DHC related deaths and not DHC related [157,219]. Three cases out of twenty eight were attributed to sole DHC intoxication (blood DHC level, range 0.5-1.7 µg/mL; average and median 1 and 0.8 µg/mL), thirteen deaths were due to polydrug intoxication involving DHC (range 0.6-24.5 µg/mL; average and median 6 and 2 µg/mL) and seven cases where DHC was detected but not related to the cause of death (range 0.04-0.6 µg/mL; average and median 0.3 and 0.2 µg/mL) . The role of DHC was not reported in the other cases.

There was an overlap between DHC only and DHC-related groups and levels in non-DHC related were lower than 0.6 µg/mL. Methadone plus DHC intoxication was encountered in five out of thirteen DHC-related cases, heroin plus DHC in three cases and DHC and alcohol in two cases.

No urine samples tested positive for hydromorphone. One case sample was positive for naloxone-3-glucuronide and trace levels of naloxone.

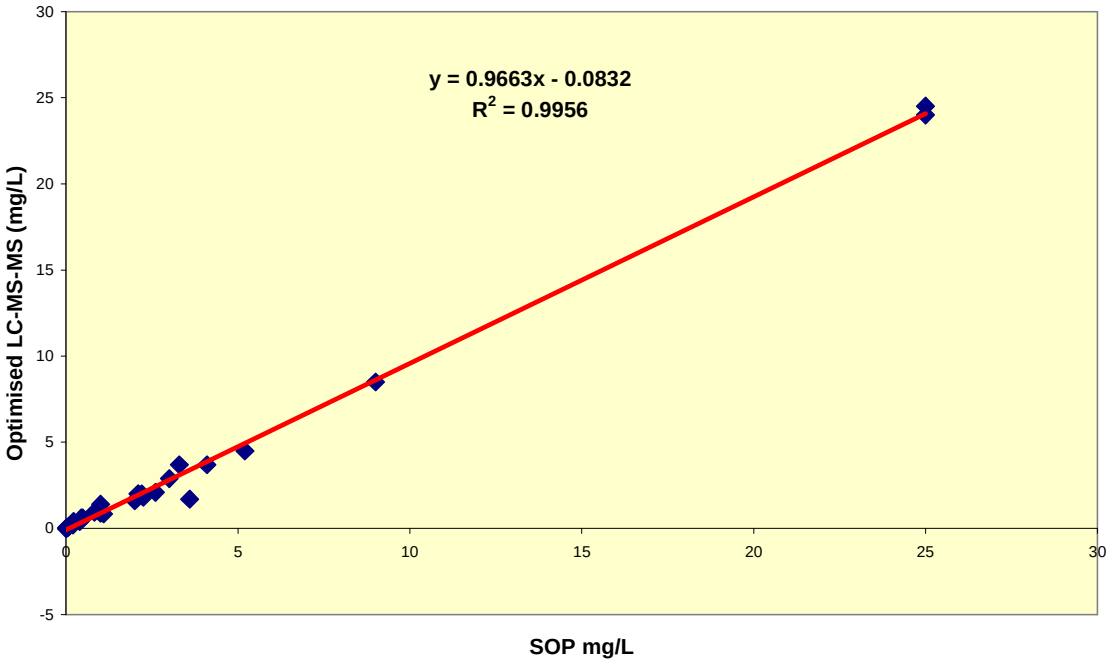


Figure 9-8: Comparison between free DHC results in real autopsy cases using the optimised LC-MS/MS and routine methods for the analysis.

9.4.3 Identification Criteria

Identification criteria proposed by the EU [61] have been employed in the current and previous studies [357-359,419,424,425] and problems encountered with identification using current LC-ion trap mass analysers are discussed below. Many identification parameters are available which may give toxicologists added confidence in making decisions as to the presence of toxicants in biological fluids [408].

The most undesired problem is interference occurring from another drug or from endogenous components that share the same molecular mass. Although the presence of interference is rare in LC-MS/MS [90], high throughput LC-MS/MS methods are needed which aim to shorten the HPLC run time while method sensitivity and selectivity are not affected [31,417]. That leads to an increase in the possibility of matrix effects from co-eluting endogenous components as discussed earlier; however, a greater risk may be caused by cross-talk from drug-related metabolites.

Table 9-12: DHC metabolites in positive case samples (µg/mL) using routine and optimised methods.

Case no	DHC			DHM3G	DHM6G	DHM	DHC6G	DHM3G	DHM6G	DHM	DHC6G	Cause of death
	Blood	BSOP *	Urine	Blood (optimised LC-MS/MS method) #				Urine (optimised LC-MS/MS method)				
1	2.2	2	29	0.1	n.d.	0.01	2.1	6.3	0.93	0.8	122	No information
2	9	8.5	40	0.3	n.d.		7.2	42	6	4	77	Methadone, DHC
3	0.2	0.2	7.2	0.08	n.d.	0.3	0.3	9.3	1.1	0.8	24	Hanging
4	25	24	1.6	0.04	n.d.	0.2	0.2	0.01	0.01	0.01	0.2	DHC, alcohol
5	0.83	0.95	9.5	0.2	0.006	0.06	0.7	6	0.4	0.4	54	Heroin, DHC, diazepam
6	1	0.9	42	0.1	n.d.	0.02	0.3	25	2.4	477	41	Methadone, DHC
7	0.03	0.04	1.2	0.03	n.d.	0.01	0.04	4.5	0.15	0.26	21	Not drug related death
8	2.1	2	31	n.d.	n.d.	n.d.	0.3	5.5	0.34	0.1	68	Heroin, DHC
9	4.1	3.7	20	0.006	n.d.	n.d.	5.6	14	1.1	1.1	163	No information
11	0.22	0.4	0.1	0.002	n.d.	n.d.	0.07	0.02	n.d.	n.d.	0.7	Not drug related death
12	5.2	4.5	159	0.02	n.d.	n.d.	3.3	2	0.2	n.d.	500	DHC, methadone
13	25	24.5	0.2	0.004	n.d.	0.2	4.6	n.d.	1.8	n.d.	0.4	DHC, temazepam
14	2.25	1.8	62	0.007	n.d.	n.d.	0.8	0.7	0.08	0.2	73	Clozapine, DHC, Paracetamol
15	0.45	0.6	9	n.d.	n.d.	n.d.	0.8	0.5	0.14	0.15	45	Cocaine, heroin, ecstasy, DHC
16	0.48	0.6	0.9	n.d.	n.d.	0.004	0.3	n.d.	n.d.	n.d.	0.2	Not drug related death
17	3.6	1.7	109	n.d.	n.d.	n.d.	0.8	6	0.55	0.26	267	DHC
18	0.4	0.4	N/A	0.1	0.01	0.02	1.8	N/A Ω	N/A	N/A	N/A	Not drug related death
19	2.6	2.1	N/A	0.01	n.d.	0.02	0.4	N/A	N/A	N/A	N/A	Methadone and DHC
20	1.1	0.84	N/A	0.07	0.01	0.1	0.15	N/A	N/A	N/A	N/A	DHC intoxication,
21	2	1.6	N/A	0.004	n.d.	0.001	5.4	N/A	N/A	N/A	N/A	DHC and amitriptyline
22	0.04	0.04	N/A	0.01	n.d.	0.002	0.1	N/A	N/A	N/A	N/A	No information
23	0.44	0.5	N/A	0.03	n.d.	0.04	0.02	N/A	N/A	N/A	N/A	DHC intoxication
24	0.06	0.07	N/A	0.003	n.d.	n.d.	0.02	N/A	N/A	N/A	N/A	Stab wound of heart
25	3	2.9	N/A	0.06	0.01	0.09	2	N/A	N/A	N/A	N/A	No information
26	0.1	0.15	N/A	0.01		0.01	0.08	N/A	N/A	N/A	N/A	Methadone intoxication
27	2.1	2	N/A	0.001	0.003	0.005	0.7	N/A	N/A	N/A	N/A	DHC and amitriptyline
28	3.3	3.7	N/A	0.01	0.004	0.02	0.8	N/A	N/A	N/A	N/A	DHC and alcohol intoxication

LC-MS/MS method for the analysis of opioids and their metabolites.
* SOP: Standard operation procedure or in-house GC-MS method used for routine general opioids analysis in Forensic Medicine and Science; University of Glasgow.

This phenomenon results from elution of the two analytes at the same retention time and fragmentation to give similar product ions used for identification; this also so-called isobaric metabolic interference could be the result of in-source contamination or due to degradation products of phase I and II metabolites [28,90,115].

In the current work, many of the drugs analysed share the same precursor ion such as morphine, hydromorphone and norcodeine, which have a pseudo-molecular ion at m/z 286. Despite the use of a selective mass analyser, these analytes also share some of their product ion(s), affecting identification and quantification of these drugs, especially if these shared product ions are used as quantifier or qualifier ions, which results in different SRM transition ratios. Moreover, problems of co-elution are more critical when analytes are not only sharing the same precursor ion but also have the same single product ion. This was encountered with both morphine glucuronide and hydromorphone-3-glucuronide in the present work; they have the same precursor ion at m/z 462 and the same single product ion at m/z 286. Further fragmentation to MS/MS/MS ($MS^{n=3}$) can be used to produce a new product ion [426] but this was at the expense of sensitivity and would not solve the problem of co-elution of the drugs. Retention time can play a crucial role in identification in these cases and analytes should be fully separated from each other. In addition to chromatographic separation, sample preparation also is an important issue to eliminate unwanted drugs and interference [90,419]. It has been suggested that derivatising agents can be used to shift analytes from the co-elution zone to be fully separated but these would lose the advantage of LC-MS in being able to analyse metabolites directly [74,95].

The retention time is a crucial identification power in all chromatography systems which is required to be identical to reference standards or positive controls that should be included in the same run; reliable and repeatable retention time intra and inter-laboratory should be examined [408]. The acceptable limit of difference in the retention time of analytes detected in real cases and that of reference or control materials are recommended to be in the range of 2% (± 0.4 minutes) [409] and 2.5 % [61] using HPLC and 5% for LC-MS. When using a second chromatography system or when using the same system but with a different derivative, the retention times of the analyte should be different in

the two systems, with preference being given to using a second aliquot of the sample and a new extract to rule out the possibility of faulty sample preparation and contamination [54]. The peak of an analyte diagnostic ion should be higher than background with signal-to-noise ratio of at least 3:1 [409]. In the current study, good chromatography separation was obtained as often as possible with a wide range of analytes that were eluted from 3 - 24 minutes; retention times were recorded with each run and compared with calibration standards which were analysed at the same time.

Opioids and their metabolites were included as often as possible in the method which can be a helpful tool for drug identification, with two advantages: firstly, to exclude the potential interference caused by metabolites on drug ionisation and secondly, to increase the method specificity and selectivity for identification [113,418,419]. 6-monoacetylmorphine, for instance, is commonly used as a heroin marker in forensic investigations; 6-acetylcodeine is also considered a heroin marker. However, these two markers are metabolised quickly in vivo to morphine and codeine, respectively. However, morphine and codeine are the most commonly detected drugs after heroin, codeine intoxication which could be a metabolite of heroin, codeine, morphine or after ingestion of poppy seeds. Therefore, the presence of 6-MAM are strongly evidence of idenftification of heroin used.

Drugs such as opioids have relatively similar chemical structures which are metabolised in the same way and many metabolites may be formed from many drugs. For example, oxymorphone is a drug in use and a metabolite of oxycodone. In addition, oxymorphone and noroxycodone share the same molecular weight and product ion. Noroxycodone shares some of its major product ion with DHC: both have the same molecular weight with a slight difference between their mass of 0.2 atomic mass units [425]. This is another reason for the importance of adequate chromatographic separation for correct identification and quantification. Contamination from the ion source can be tackled by the use of high or 100% organic modifier percentages and allowing the LC column to be equilibrated before the next injection in order to eliminate potential in-source dissociation of metabolites [90,113].

The use of internal standardisation has been recommended but is not essential for drug identification according to SOFT/AAFS and WADA recommendations [54,409]. However, in practice internal standard should be included whenever available to provide an accurate identification and quantification procedure [115,427,428]. The recommendation is that an internal standard should be eluted near to the analytes of interest. Three different types could be used in LC-MS applications: analytes labelled with stable isotopes - hydrogen (deuterium) is the most common isotope used in forensic toxicology, structurally similar compounds such as a homologue of the analyte of interest, and any other compounds such as a drug [97,428]. However, the latter are not recommended since they could be present in biological samples due to drug ingestion, leading to a misinterpretation of results and affecting method reproducibility and linearity [16,97,115].

Stable isotopically labelled analogues are the internal standards of choice with masses different from the analytes by at least three mass units in order to avoid interference between analytes and their internal standards, which probably increase if the differences in their masses were less than three amu [427]. In the current work, interferences between isotopically labelled internal standards have been observed with other analytes that differ by 1 amu such as DHM3G (464) and M3GD3 (m/z 465) which interfered with each other, which led to these internal standards not being used in cases positive for both MOR and DHC. This is another example of when full separation of analytes is required to identify metabolites of similar structure, but separation of analytes is not always easily achievable, especially if the analytes are very polar and a reverse phase liquid chromatography method is used.

In addition, internal standards are expensive and not available commercially for all compounds of interest. As indicated earlier, internal standards are the best way to tackle the matrix effect phenomenon and should be treated and extracted together with standard calibration curves and samples. This is also the best way to examine the efficiency of extraction and ionisation of analytes [97,427]. Isotopically labelled internal standards should be free from interference and their fragmentation should be checked to investigate the ratio between analytes and internal standards; this could be checked by injection of similar amounts e.g. 100 ng/mL of both and calculating the intensity ratio of their ions. A ratio of

one is expected in most cases but if the ratio is higher than one the increase could be from interfering ions from the internal standard [428].

LC-MS/MS libraries in most laboratories are created in-house with a collection of the identification parameters from known certified standards and real cases results [30,59]. Manual optimisation of single analytes is facilitated using a direct infusion T connection in the LC-MS/MS instrument. Certified standards are important for both identification and quantification; however, some analytes are not available commercially. In this case analytes were identified tentatively, for example, norbuprenorphine-3-glucuronide was tentatively identified for the first time by Polettini and Huestis using LC-MS/MS [332] and some time later a certified standard was made available. In contrast, some believe that a correct identification cannot be achieved without a certified standard; published mass spectra reported for some analytes could be used for preliminary identification with extreme caution due to the possibility of incorrect data or differences caused by the environment of assays and instrumentation [59,408].

The possibility of creating a universal LC-MS/MS library of analytes is low compared to that of GC-MS for many reasons [66]. Many factors can influence the performance of instruments, ionisation and retention of analytes due to the use of different mobile phases and columns and the many interface and mass analyser available, leading to differences in mass fragmentation patterns, such as ESI, APCI and IT-MS, TQ-MS, Q-TOF, respectively. For example, the differences between CID data collection using IT and TQ lead to different fragment ions for the same drugs [30,44,66,72,429]. Nevertheless, some scientists have established their own library using in-house LC-MS/MS methods; the largest libraries were reported by Dresen *et al* [430] (800 compounds) and Mueller *et al* [39] (301 drugs).

There is an agreement to using a minimum of three identification points (IPs) for identification criteria as proposed by the EU for drug of abuse confirmation using LC-MS/(MS) [61]. IPs are accumulation points depending on the mass spectrometer attached to HPLC whether it is high or low resolution, single or tandem mass spectrometry. For example, in the case of LC tandem MS, four IPs can be obtained by determining one parent drug with two of its product ions (Table 9-13). Also, it is recommended to measure at least one ion intensity ratio in order

to ensure the quality of identification points and the ratio should not exceed EU criteria listed in Table 9-14 [61,418].

The same criteria have been recommended by the World Anti-Doping Agency (WADA) [409]. If these IPs cannot be met, the recommendation is that a second ionisation or fragmentation technique should be used which should result in different diagnostic ions by applying a second derivative. However, SOFT and WADA accepted that the identification of anaytes can be appropriate by monitoring single product ions but it depends on the uniqueness and resolution of the ion [54,409]. Also, another identification system should be used whenever possible. SOFT/AAFS guidelines state that in some cases, drugs or toxicants can only be determined using single identification techniques. Furthermore, single non-selective identification techniques such as validated radioimmunoassay of digoxin are only required to confirm the identity of drug if evidence behind the case strongly indicates no suspicion of suicide or crime.

Table 9-13: Example of Number of IPs earned for confirmation of identity by a range of LC-MS instruments.

Techniques	No. of Ions	IP
LC-MS	N [&]	N
LC-MS/MS	One precursor ion, two products	4
LC-MS/MS	Two precursor ions, each with one product	5
LC-MS/MS/MS	One precursor ion, one product and two grand-products	5.5
HR [*]-MS	N	2N
LC-MS and GC-MS	2+2	4
LC-MS and HR-MS	2+1	4
[&] N: an integer, [*]HR: High resolution		

Table 9-14: Maximum tolerance widows for relative ion intensities in LC-MS(MS) according to European Union criteria for drug identification (Adapted from WADA)

Relative Abundance (% base ion)	Maximum Permitted Tolerance (relative %)
> 50	± 20
> 20-50	± 25
> 10-20	± 30
≤ 10	± 50

Maralikova and Weinmann [407] monitored at least two MRM transitions for each analyte in order to collect IPs as recommended by EU guidelines [61]. The major product ions were chosen for quantification in all drugs with the exception of opioids in which precursor ions were used for quantification ions. Three Collision Energies (CE) were applied with all drugs at 20, 35 and 50 eV; then suspected analytes were compared with reference standards stored in the MS/MS library at the same CEs for proper identification. Also, the ratio of relative intensity of at least one MRM transition was calculated. The ratios obtained from the standard calibration curve (5-50 ng/mL) and from samples positive for the analytes of interest were compared using the percent deviations recommended by EU guidelines.

Fox *et al* [425] reported work using LC-TQ-MS/MS recently using the same EU criteria [61] of identification with opiates: four IPs per analyte have been obtained by monitoring two MRM transitions; the relative retention time and co-eluted internal standards counted as another IP at tolerance of ± 2.5% according to EU criteria [61]. They used two mobile phases A and B; in mobile phase A at pH 6.8, the relative ion intensity did not meet EU criteria when signal-to-noise was less than 10 of one or more MRM transitions of morphine, 6-MAM, codeine and DHC. Also, retention times could not fulfil the criteria when opiate concentrations were above the level of calibration curve (> 10 µg/mL). Isobaric phenomenon between DHC and noroxycodone was detected in one case. However, the authors reported an improvement of morphine and codeine identification when using mobile phase B at pH 8; they could identify 97% of morphine cases using mobile phase B compared to 59% using mobile phase A. They attributed that to

the improvement of analytes separation. However, no improvement was achieved with 6-MAM and DHC. The levels of DHC and oxycodone metabolites are known to be high in urine due to use or abuse of both analytes (Chapter 8 for oxycodone cases). They believed that potential risks of interference between DHC and oxymorphine were impossible due to the excretion of the latter in urine only in glucuronide form, which was untrue. In Chapter 8, oxymorphone was detected in free form in both blood and urine and was more likely to be present in clinical cases than in overdoses in non-regular users. Many analytes can cause isobaric interference with opioids. Naloxone, for example, was eluted in the current study near to 6-MAM which could interfere with 6-MAM as they share the same molecular weight of parent ion but they are fragmented to different product ions. In addition, there is a risk that hydromorphone could also potentially interfere with norcodeine and morphine.

Fox *et al* [425] concluded that the problem of isobaric phenomena due to metabolite interference is a major risk that leads to false positive results which can be tackled by employing more than one MRM transition to confirm the identity of analytes accurately. They also stressed that better chromatographic separation and sample clean up could improve the efficiency of MRM transition of analytes and avoid isobaric interference problems. Parent drugs or metabolites could interfere with each other which should be taken into consideration when detecting MRM transition. They also recommended the use of LC-MS/MS with ion trap mass analyser which can acquire mass spectra of selected metabolites in full scan mode and so enhance method identification as comparable to GC-MS.

In addition to the monitoring of two MRM transitions for increased method selectivity, the calculation of relative ion intensity between the abundances of the two diagnostic fragment ions provides confidence for analyte identity confirmation in which the ratio should be within the acceptable limits proposed by EU guidelines [37,425,431]. The ion ratios are measured as the peak area of the quantifier ion / the peak area of qualifier ion which is also described as 'the peak area of the quantifier transition (100/ion ratio) in such a way that, for instance, the peak area of qualifier ion is half of that of quantifier ion when relative ion intensity is 50%' [424,432]. Relative ion intensity has been monitored in many LC-TQ-MS/MS methods reported for forensic toxicology.

LC-IT-MS/MS has been used successfully in clinical and forensic toxicology but few reports have concerned the identifications criteria. Although these techniques are able to produce two or more selected reaction monitoring (SRM) transitions, relative ion intensities were not calculated according to EU guidelines in most of these methods. One of the earliest methods described by Fitzgerald *et al* [44] using LC-IT-MS/MS showed that some analytes such as benzylecogonine only fragmented to single product ions but morphine was fragmented to five fragments. They mentioned that isobaric interference was expected due to chromatographic overlap between morphine and hydromorphone as both analytes have the same parent and some product ion; good chromatographic separation is recommended for accurate quantification. Although they calculated the relative ion intensity, it was not used to confirm analyte identity as EU guidelines were only published many years later. In fact, the isobaric interference could still be problematic especially as norcocaine has the same parent mass spectrum as benzoylecogonine and they are eluted near to each other. Norcocaine can be detected in urine samples which could co-elute with benzyolecogonine. Therefore, retention time and co-eluted internal standard play a great role to proving an accurate identification when chosen ions were not well separated [419,433]. Cocaine could be interfered by norcocaethylene as they have the same mass and fragment to similar product ions.

In the present work, SRM ratios were calculated with a minimum single to noise of 10 for each transition. It has been noticed that the higher single to noise are used the more accurate the SRM ratio obtained. In the work with diamorphine with clinical plasma samples, three SRM transitions could be determined with ratios of 1-1.3 between them which was also found with 6-MAM in blood, plasma and urine. Ion trap mass spectrometry is known to have a limited product ion mass range in which less energy is needed for fragmentation compared to triple quadrupole mass spectrometry. Ion trap tends to lose water from pesudomolecular ion [42,426]. For opioids in general, two SRM transitions can be obtained such as with heroin, morphine, codeine, DHC, hydromorphone but in some opioids and metabolites only one SRM transition can be obtained. Therefore, in order to obtain two SRM transitions for identification, adjusting the cone voltage has been used in current study. Maralikova and Weinmann [407] applied three different collision energies setting in order to calculate MRM ratios

at 20, 35 and 50 eV. Dresen *et al* [430] also applied this procedure for the same purpose.

A method using the same LC-MS/MS instrument as in the current study was reported by Murphy and Huestis [116]; they used a single product ion for identification and quantification of opioids glucuronide while two product ions were used for morphine and codeine but SRM ratio was not calculated. Rook *et al* [75] identified opioids and metabolites using a single MRM transition. Therefore, it is acceptable to use one single product ion for identification if it is a unique indicator of the parent drug but further SRM ratios calculated could help in providing sufficient IPs for toxicant identification. The adjustment of collision energies for opioids and metabolites that fragment to only one product ion was reliable and repeatable in this study. However, it is not always guaranteed that the criteria proposed by EU guidelines could provide an accurate discrimination between analytes of interest, especially analytes that have the same mass spectra data and are eluted at the same time, even when using relative ion intensities, which could be similar [37,433]. Therefore, good chromatographic separation could be an additional identification point according to the analyte retention time and the presence of internal standards whenever possible [36,90,113,419].

9.5 Conclusions

A sensitive, selective and validated method was described for simultaneous determination of 26 opioids and metabolites by LC-ion trap-MS/MS. The influence of matrix effects was investigated and eliminated by using SPE for sample preparation. It was applied to routine case work as a complementary test to autopsy blood analysis using the same procedure in order to confirm blood analysis, especially in the case of heroin intoxication with no 6-MAM levels detected in the autopsy blood. Stability of heroin markers in urine was compared with blood and the markers were found to be more stable in urine. The results obtained using the optimised LC-MS/MS method in blood samples were compared with those obtained using the routinely laboratory method for analysis of opioids; good correlations were observed for free methadone, MOR, COD and DHC.

The application of identification criteria proposed by EU guidelines, FDA and SOFT/AAFS to the current method were evaluated along with problems encountered with the current LC-MS/MS instrument in providing sufficient identification points. It was found that two SRM transitions can be obtained in most opioids with the exception of some polar metabolites, especially glucuronide metabolites which fragment to only one product ion. Therefore, the adjustment of collision energy voltages was used to obtain two SRM transitions which were found reliable and reducible using both standard and case samples.

10 Direct Determination of Ethyl Glucuronide and Ethyl sulfate in Post-mortem Urine Specimens using Hydrophilic Interaction Liquid Chromatography–ESI-MS

10.1 Introduction

Ethanol (ethyl alcohol, alcohol) is the most common drug used and abused across the world [132,434,435] with the exception of some Islamic countries. For example, in Saudi Arabia, alcohol is not allowed due to religious reasons. There is no doubt that alcohol is a serious problem, for health, workplace and social consequences, if it is misused [436-438]. The risk of sudden death is higher among alcoholics compared to the non-alcoholic person [434,436].

The interpretation of alcohol results for post-mortem specimens can be difficult due to the possibility of post-mortem production of alcohol, which may take place in the body or in the autopsy samples [439-442]. Ethyl glucuronide (ETG) and ethyl sulfate (ETS) are primary ethanol metabolites and have been shown to be useful markers of alcohol consumption for several hours after death or when ethanol itself has been completely eliminated from the body. ETG and ETS are very polar metabolites requiring very low percentages of organic modifiers (less than 5%) for elution from a conventional reversed phase column, which results in poor retention, large matrix effects and low sensitivity in reverse phase LC-MS methods. Post-column addition of organic solvents can enhance ESI-MS/MS response while preserving good chromatographic peak shapes. Recently, hydrophilic interaction chromatography (HILIC) has been introduced as an alternative to reverse phase LC separation of polar compounds. HILIC is suitable for ESI-MS as a high percentage of organic modifiers can be used, up to 95%, without reducing analyte retention.

10.2 Metabolism and Excretion

Ethanol is metabolised to acetic acid by two enzymatic steps in the liver. The formation of acetaldehyde by alcohol dehydrogenase occurs first which in turn is

converted to acetic acid by another liver enzyme, aldehyde dehydrogenase. Then, acetic acid is involved in the Krebs cycle and is rapidly converted to water and carbon dioxide. The oxidised metabolites of ethanol (acetaldehyde and acetic acid) are estimated to account for 90-95 % of ethanol elimination. The rest of the ethanol is excreted unchanged by the kidney (0.5-2%), the lungs (1.6-6%) and skin (0.5%) [132,443]. A small fraction of ethanol undergoes phase two conjugation reactions which is less than approximately 1% of the ethanol dose. These non-oxidative direct ethanol metabolites include ethyl glucuronide (ETG), ethyl sulfate (ETS), phosphatidyl ethanol and fatty acid ethyl esters (FAEE). These metabolites are excreted in the urine with longer elimination times than ethanol, which is attributed to the low molecular weight of ethanol (mz 46) compared to these polar metabolites for example ethyl glucuronide (m/z 222) [444].

Although many researchers have studied alcohol metabolism, few have addressed the pharmacokinetics of polar metabolites of ethanol which are now the most reliable alcohol biomarkers in use. Most of the data available has been obtained with living subjects in whom putrefaction and contamination with bacteria are of no concern. Ethyl glucuronide and ethyl sulfate are formed by two different pathways. The estimated percentage of ETG and ETS are 0.1 % of ethanol consumption using UDP-glucuronosyltransferase (UGT) and sulfotransferase enzyme (SULT) for ETG and ETS, respectively. Although many types of UGT and SULT are found to be involved in the formation of ETG and ETS, the greatest portions are attributed to the UGT1A1, 2B7 and SULT1A3 for ETG and ETS, respectively [445,446].

However, there is a time gap between detection of ethanol and their polar metabolites in blood or urine which was found to be about 2 hours in blood and 1 hour in urine excretion. Subsequent to starting to drink, ETG in serum and blood was found to reach its peak after 2.3 to 5 and 1.3 to 2.1 hours while ETS reached its peak after 2.1 to 3.9 and 0.5 to 2 hours, respectively. The urinary peaks of both analytes were much longer as ETG reaches its peak between 5 to 7.5 hours and ETS reaches its peak between 3.1 and 7.4 hours [447]. ETG could be detected in serum up to 8 hours after complete elimination of ethanol [448-451]. ETG had longer detection times in urine than in blood and could be detected between 15-25 hours after complete elimination of ethanol [452] and up to 80 hours in urine

after intake of alcohol [451,453]. ETS is found to have the same detection window as ETG; ETS was detected in urine after one hour of starting to drink; a longer detection widow for ETS was reported to be between 16-27 hours [454,455].

10.2.1 Production of alcohol after death

The presence of alcohol after death can be interpreted in three ways: ante-mortem alcohol ingestion; post-mortem alcohol synthesis; ante-mortem and post-mortem alcohol formation by microorganisms [441,456,457]. Therefore, it is crucial to distinguish between ante-mortem ingestion and post-mortem alcohol synthesis in medico-legal cases and this phenomenon has to be taken into consideration when investigating accidents on the road, sea or the air [458].

Gas chromatography with a flame ionisation detector (GC-FID) is the method of choice for alcohol analysis in living or autopsy specimens. Although these methods are simple, robust and sensitive and can distinguish between alcohol and other volatile substances [459,460], the interpretation of alcohol results at autopsy is a challenging decision for toxicologists due to many factors associated with post-mortem fermentation which often differ between subjects i.e. diabetes and non-diabetes [461]. In most reports, the cause of alcohol synthesis has been referred to the quality of specimens collected, for example, contamination with microorganisms during sampling or before autopsy. Also, environmental factors such as temperature and humidity with longer periods of time between death and post-mortem examination has been found to enhance the risk of post-mortem alcohol production [440,441,462-465].

O'Neal and Poklis [440] found that ethanol is produced both *in-vivo* and *in-vitro* and 12-57% of alcohol encountered in post-mortem cases was attributed to post-mortem production. In most cases production of ethanol after death was found to have no contribution to the cause of death and less than 12% of ethanol was reported as post-mortem ethanol synthesis. An exception to this is in certain situations where alcohol present reached significant levels such as decomposed and heavy putrefied bodies. The percentage of post-mortem ethanol synthesis in decomposed bodies was reported to be 20% and as high as 40-50% with less common cases such as aviation or naval accidents. 100% of ethanol found at autopsy after the USS Iowa disaster was attributed to post-mortem fermentation

[440,466]. Therefore, the condition of a body at post-mortem examination could indicate whether post-mortem ethanol production should be expected.

Determining blood alcohol concentration (BAC) and/or urine alcohol concentration (UAC) is a part of the routine post-mortem toxicology analysis [441,456,467]. Reports state that a BAC of 40 mg/100mL or higher is indicative of ante-mortem alcohol ingestion [439]. The authors reached their conclusion as a result of positive urine and vitreous humour for alcohol was decreased with a lower BAC range of 10-40 mg/100mL. In other work, it was mentioned that BAC of 10 mg/100mL or lower should be reported negative for two main reasons. The level of BAC is considered lower than the limit of quantification of GC-FID procedure which is commonly used for alcohol analysis. Also, it is most likely to be formed due to alcohol fermentation after death. Urine and vitreous humour have been suggested as complementary specimens for alcohol analysis. These specimens are less susceptible to post-mortem ethanol production due to low or zero glucose found in urine of non-diabetic subjects and microorganism contamination of vitreous humour is limited at the early stages of the putrefaction process [439,468].

However, obtaining a false positive result for alcohol can occur at any concentration (high or low) and production of alcohol after death may be elevated with the passage of time to a significant level such as is detected in alcohol related fatalities [440,457,466,469]. This formation of alcohol occurs by microorganisms and is believed to be part of the post-mortem degeneration or after severe trauma using glucose as a substrate [439]. The ethanol fermentation process is a combination between internal and external factors which lead to body autolysis or self digestion of body tissues. Tissue softening and liquefaction occurs due to aerobic enzymatic digestion which disintegrates the cell membrane and tissues compartments dramatically. As indicated earlier, blood glucose has been found to be elevated after death and then used as a substrate for the alcohol fermentation process. The body starts to decompose using bacteria from the bowel. The skin of the deceased attains a greenish discoloration which is considered the first sign of bacterial activity before spreading to other parts of body. Bacteria reach the blood after death and produce ethanol from glucose using the ‘Embden-Meyerhoff pathway’ [470].

The production of ethanol depends on the degree of putrefaction of the corpse and was found to be higher or more rapid depending on the type of microorganisms present, the substrate available (glucose), air circulation, in-door or out door conditions, immersion in water, the temperature of storage before and after autopsy, and severe damage to the corpse after an accident. Ethanol synthesis is maximised in the presence of proper environmental conditions for the putrefaction process and specimens are more likely to be exposed to microorganisms [439,440,457,468,469,471].

Post-mortem ethanol production can be avoided or minimised by subjecting the body to proper refrigeration within 4 hours of death [470]. This is considered a very short time and unattainable in most cases, especially with drug related deaths in which bodies have been left for a long period of time. In this type of case, levels of ethanol may be elevated due to the post-mortem production up to 50 mg/100mL in most cases [440,471]. However, levels of ethanol higher than 150 mg/100 mL have been reported in severely decomposed cases [441,457,466].

10.2.2 Why are ethanol biomarkers needed?

The blood alcohol concentration is known to decrease over the time due to its oxidation by the liver: ethanol is rapidly cleared from the body at 0.15 g/kg/hr in non-alcoholics and this rate is more rapid in alcoholics, who could reach zero concentration of BAC within hours of last ingestion [438,472,473]. In addition, the presence of alcohol in biological fluids may be the result of recent alcohol consumption or due to ethanol production after death. All of these factors make the interpretation of alcohol complicated and caution should be applied to avoid misinterpretation of either positive or negative results at autopsy. Information available from the scene of accident or death would help but with some cases it is not available. Also, reports suggest that multiple analyses of different matrices, especially, blood, urine and vitreous humour may help in the interpretation of ethanol results. It is believed that positive results for blood with negative urine and vitreous humour are strong evidence of post-mortem ethanol production [434,460,462,474]. However, in the case of putrefaction the specimen available in most cases is blood or urine.

The idea of alcohol biomarkers was established for clinical purposes and much attention was paid to overcome problems of impaired liver function for patients who have been alcoholic for a long term (at least 12 months). This period of excessive consumption causes severe damage to liver cells [434,475]. The risks of alcohol-induced organ and tissue damage are considered higher with alcoholics compared to non-alcoholics [434]. Diagnosis at the early stage of alcohol abuse is important to their treatment and prevention of reaching latter stages of which there may be no cure [438,476,477]. Alcoholism is difficult to observe without medical complication or self reporting of use, the latter has been found unreliable and most alcoholics deny their alcohol abuse problem either because they believe it is part of their culture or to avoid the negative attitude toward them as being alcoholic [446,475,476,478,479]. The level of BAC at post-mortem is only an indicator of recent alcohol ingestion and cannot be used to identify alcoholic abuse problems [434,480]. In one study, negative BAC was detected with half of alcoholics at death [480].

Pathological diagnostics of alcoholics are not unique and are relatively non-specific with similarities between alcoholics and non-alcoholics in liver disease often encountered at post-mortem examination. Poor hygiene and multiple burises of different age has been used to distinguish alcoholics at post-mortem examination [434,480].

In fact, searching for alcohol biomarkers has gained a great deal of interest due to the high alcohol abuse rate among forensic autopsy population which was estimated to be 10% or more [434,475]. The early diagnosis of alcoholics using low cost biochemical makers are without a doubt crucial in order to lower and shorten treatment cost and time, respectively [434,438].

The development of sensitive and specific alcohol biomarkers was found to be challenging due to errors encountered with most clinical ethanol biomarkers which were not direct metabolites of ethanol. Although ethanol blood, urine and breath tests are helpful tools that are complementary with self-reporting and other clinical diagnostics [481], accurate direct alcohol biomarkers are more objective and needed in order to maximise the sensitivity and specificity of the use of these alcohol biomarkers which in turn would reduce the error often encountered with non-alcoholics [434,436,472,476,482].

The availability of high resolution and accuracy mean analyses such as GC-MS and LC-MS(MS) have facilitated these objectives [448]. Phase two metabolites of ethanol have been used as ethanol biomarkers only recently despite them being known since 1953 [483]. During this long journey many alcohol biomarkers have been tested until direct alcohol biomarkers became the method of choice for distinguishing between the sources of ethanol present at autopsy. This does not mean that the other alcohol biomarkers were found not useful but each one of them has its own advantages and disadvantages [482].

Acetaldehyde is primary product of alcohol metabolism and can be as marker for alcohol consumption [484,485] but it has been found not useful as an alcohol biomarker due to its low concentration and lack of proper analytical procedure [459,486]. Methanol has been used to reflect recent alcohol use, as the methanol concentration in the body increases with presence of alcohol above 20 mg/dL due to the ethanol blocking the methanol metabolism pathway [434,479,487,488]. In fact, a method for determining methanol is available and used routinely in many laboratories. It has demonstrated that a level of methanol above 1 mg/dl is an indicator of long-term overconsumption of alcohol. Therefore, it has been suggested that an increase in methanol level may be an indicator of chronic or continuous drinking rather than recent alcohol consumption [434,479,489,490]. However, many alcoholic beverages contain methanol which should be taken into consideration when methanol is used for interpretation as the high methanol result could be a result of the presence of methanol in alcoholic beverages [434,491,492].

Many other alcohol biochemical markers have been developed for more objective ways to identify the overconsumption of alcohol in clinical and forensic subjects. These biomarkers can be classed into short term and long term alcohol biomarkers or direct and indirect ethanol metabolites [472]. The ethanol itself or its primary metabolite such as acetaldehyde has been used as alcohol direct biomarkers [434,484,493]. Also, fatty acid ethyl ester or conjugates metabolites such as ethyl glucuronide, ethyl sulfate , and phosphatidylethanol are minor nonoxidative direct metabolites of ethanol which indicate short term of alcohol ingestion [434,436,448,476,482,494]. The idea of using these biomarkers was to provide 100% sensitivity and specificity of alcohol consumption ante-mortem, however, this was never obtained with ethanol biomarkers [434].

Determining chronic use of alcohol is out of the scope of this Chapter but for completion these biomarkers are discussed briefly. The change of liver function was noticed after long term alcohol consumption and liver enzymes such as gamma-glutamyl transfererase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and mean corpuscular erythrocyte volume (MCV). In addition, carbohydrate-deficient transferase (CDT) is a useful biomarker associated with alcoholics. These biomarkers are indirect alcohol abuse biomarkers which are detected after long overconsumption of alcohol (50-80 g of ethanol daily) for at least one to two weeks [434,477,481,482]. However, there are some limitations of their use which reduces their specificity and sensitivity as alcohol biomarkers, for example, the GGT value has risen with liver damage of non-alcoholics. Although these biomarkers require simple analytical methods for their application and they are available with low cost in each laboratory, these biomarkers are detected only in blood or its derivatives and some of them (GGT, ALT, AST and MCV) are unsuitable in autopsy specimens [436].

Long term biomarkers have their applications with alcohol misuse problems but often suffer from low sensitivity and specificity and two or more biomarkers are used. The use of these biomarkers was found unreliable for diagnosis of alcoholism; only 20-50% of alcoholics can be determined using these biomarkers [478,481,495]. Although the combination of these biomarkers could increase the diagnostic accuracy, the interpretation of them is complex [434,438,477]. False positive or negative results may be obtained for some cases of non-alcoholics who suffer from liver disease apart from those caused by misuse of alcohol as well as alcoholics who show normal levels of these markers [478,496].

There is a gap between short term and long term alcohol biomarkers and single use cannot be detected using long term biomarkers [472,497]. In post-mortem toxicology and Driving Under the Influence of Drugs (DUID) cases, the concentration of drug at the time of death or accident are more important for interpretation [434,447].

In the last decades, many direct metabolites of alcohol have been studied and found useful to determine the recent use of alcohol. These short term markers are able to monitor the intake of alcohol after a single use which can not be identified using the traditional alcohol long term use. These metabolites were

formed from ethanol and are not influenced by liver disease and are detectable in variety of biological specimens and tissues [448,451,482].

Fatty acid ethyl esters (FAEEs), which are esterification products of ethanol and endogenous fatty acids [473,498], have been used as alcohol biomarkers and found to be detectable up 24 hours after ethanol intake [499] and up 44 hours [500] and 99 hours in heavy drinkers. Therefore, they seem to be good markers for the detection of short and long ethanol intake [486,499,501]. Organs damaged by heavy alcohol drinking were found to have high concentrations of FAEEs which may be contributors to alcohol toxicity [486,502-504]. However, their use is limited due to their faster elimination in blood compared to that of other acute alcohol biomarkers which reduces their sensitivity as recent alcohol consumption markers and they have not been found useful for forensic purposes [473]. Levels of FAEEs were found to rapidly decrease after ethanol consumption within 29.4 hours but were found higher than LOQ concentrations and remained stable with low concentrations. The same FAEEs levels were still detected after 4 days of abstinence which limits their usefulness as long term alcohol intake markers [473].

The urinary ratio of the serotonin metabolites 5-hydroxytryptophol (5-HTOL) to 5-hydroxyindole acetic acid (5-HIAA) has been found useful for detection of recent alcohol use. These analytes are not direct alcohol metabolites and are used to detect short term alcohol consumption up to 12 hours in urine; the level of 5-HTOL is increased after alcohol intake [434]. 5-HTOL is excreted mostly in conjugated form as glucuronide or sulfate conjugates and free 5-HTOL accounts for less than 5% of total elimination while about 80% is excreted as 5-hydroxytryptophol glucuronide (GTOL) making the later metabolite promising as an alcohol biomarker for recent intake. Therefore, the urinary ratio of GTOL/5-HIAA has been used for detection of recent alcohol consumption [505-508]. However, these metabolites require sophisticated analytical methods based on GC-MS and LC-MS. In addition to a short detection window of this ratio, which was found to be less than 7 hours using free 5-HTOL, the false positive results after consuming a rich serotonin meal could be obtained [473,505].

Ethyl glucuronide (ETG) and ethyl sulfate (ETS) are direct non-oxidative ethanol metabolites and have been shown to be useful markers of alcohol consumption

for several hours after death or when ethanol itself has been completely eliminated from the body.

10.2.3 Ethyl Glucuronide and Sulfate

10.2.3.1 Introduction

As indicated earlier, two different alcohol biomarkers have been used in routine analysis with alcoholic patients. Blood alcohol concentration (BAC) or urine alcohol concentration (UAC) indicate recent alcohol consumption whereas alcohol biomarkers such as carbohydrate deficient transferrin (CDT), gamma glutamyl transferase (GGT) and mean corpuscular volume (MCV) indicate long term alcohol misuse. Ethyl glucuronide and ethyl sulfate have been found to fill the gap between short and long alcohol biomarkers and show longer detection times. They can be detected while ethanol is still present and even after alcohol has been eliminated from the body, for up to 8 hours in serum and for several days in urine [447,472].

The usefulness of ETG as a recent alcohol consumption biomarker has been studied widely [76,448-450,453,509,510]. It has been found that ETG can only be formed after alcohol ingestion and has been found not to be formed endogenously but in one study ETG has been formed in infected urine samples by Escherchia Coli with present of ethanol [511]. However, the presence of ETG is not definitive evidence of anti-mortem alcohol intake [451,495,512-514]. However, negative results of ETG should be interpreted with caution as false negative results may be obtained. There is a time lag between alcohol present in blood and ETG which may lead to false negative results if death happened shortly after alcohol consumption [512].

Although post-mortem alcohol fermentation has been acknowledged since the 1930's, looking for specific alcohol biomarkers for recent alcohol consumption has gained great deal of interest recently [482]. The reason may be due to the availability of sensitive and selective methods of quantification [474]. In the past, the detection of polar metabolites was tedious and involved extensive labour requiring many steps of isolation and hydrolysis which was often time consuming, expensive and unsuitable for routine analysis. Glucuronide metabolites contain a sugar which is a heavy compound to be detected using GC-MS which needs to be derivatised. At the early stage of discovering the importance of ETG as an

alcohol biomarker in 1990's, LC-MS instrumentation was considered expensive and unavailable in most forensic laboratories; therefore, the first application for the analysis of ETG in serum was conducted using the GC-MS method [449,450]. A few years later, most methods reported were LC-MS or LC-MS/MS as they required less pre-treatment of samples and had high sensitivity and selectivity.

Recently, the stability of ETG has been brought into question which was found to be degraded by bacteria [513,515-517]. Therefore, ETS has been introduced as a complementary marker with ETG due to its stable pattern and resistance to bacterial infection [511]; the presence of ETG and ETS provides strong evidence of recent alcohol consumption even with negative BAC or UAC [454,494,518-523].

ETG and ETS are promising biomarkers because they are phase two ethanol metabolites and their excretion profiles have been studied and documented. Also, their standards and internal standards are available commercially and can be detected using LC-MS/MS method. Both were found specific and sensitive compared to long alcohol biomarkers with longer detection time from 1 to 5 days and are not influenced by disease [447,448,524,525].

10.2.3.2 Previous Work

As indicate earlier, the first application developed to determine ETG in serum was in the mid 1990's using GC-MS [449,450]. A simple protein precipitation (PP) procedure followed by acetic anhydride derivatisation were found sufficient to determine ETG; an LLOQ of 0.1 µg/mL was achieved and a linear calibration line was obtained in the range of 0.1-150 µg/mL. A few years later, ETG was determined using LC-ESI-MS techniques using PP extraction and RPLC-MS-ESI which resulted in lowering the LOD to 0.03 µg/mL compared to 0.1 µg/mL using GC-MS procedure [526]. Both GC-MS [449,450,527-531] and LC-MS/(MS) [444,509,526,532-537] were successfully applied for routine analysis.

The use of LC-MS/MS for identification and quantification of polar glucuronide metabolites has been established and led to establishing a new LC-MS method for direct determination of ETG. Wurst *et al* [453,536] reported LC-ESI-MS/MS and GC-MS methods for the determination of ETG, and both methods were employed for routine analysis in parallel. Good agreement was obtained between both method results from real case samples with correlation coefficient of 0.98.

In the last decade, many methods have been published for the direct determination of ETG using either gas chromatography coupled with mass spectrometry [449,450,527-531] or using LC coupled with electrochemical detection [76,538] or LC-ESI-MS/(MS) [444,526,533,534,537,539] procedures.

The first application for the simultaneous detection of ETG and ETS was reported a decade [447,454,521-523] after the Schmitt *et al* report [449]. The same protein precipitation procedures used for ETG were applied for the direct determination of ETS [447,454,521-523] as well as direct injection of urine samples after dilution [494,520,524]. Three reports aimed at validating methods for detection of ETS in authentic samples [518,540,541]. Protein precipitation of blood and urine samples or dilution of urine samples are the most common pre-treatment procedures for direct determination of both ETG and ETS using LC-ESI-MS/MS [447,454,494,516,520-524,542].

10.3 HILIC and RPLC

ETG and ETS are very polar metabolites requiring very low percentages of organic modifiers (less than 5%) for elution from a conventional reversed phase column, which results in poor retention, large matrix effects and low sensitivity in LC-MS. Post-column addition of organic solvent can enhance ESI-MS/MS response while preserving good chromatographic peak shapes [447,454,516,520,522-524].

Recently, hydrophilic interaction chromatography (HILIC) has been introduced as an alternative to reverse phase LC separation of polar compounds. HILIC is suitable for ESI-MS as a high percentage of organic modifiers can be used, from 40 to 95%, without reducing analyte retention [93,543,544].

Although the name HILIC was suggested by Alpert in 1990 [545], HILIC was in use since the 1950's but with other names such as aqueous normal phase or reverse phase and pseudo normal phase [543,546]. Early 1952 saw an application for the separation of monosaccharides using a gradient elution consisting of 99.5% of ethanol and water was reported. In HILIC phase water is the strong eluent and has been in use since 1975 for the analysis of sugar and oligosaccharides using HPLC [543,547]. Recently, HILIC separations were used to determine cocaine and its metabolites [548] and opioid glucuronides [93,549]. HILIC applications have increased

recently due to the need for analysis of polar compounds and biomarker analysis since in most cases they are very polar metabolites and lack retention using conventional RPLC [416,543,544,546,550-553].

HILIC application could fill the gap between RPLC, ion chromatography liquid chromatography and NPLC. It is completely opposite to RPLC where early eluted compounds using RPLC are eluted later using HILIC systems. This could solve the problem with very polar compounds using HILIC with the possibility of using RPLC solvents. In addition, SPE extracts used with RPLC could be injected directly to HILIC phase saving time and preventing analyte loss during evaporation. HILIC is similar to normal phase liquid chromatography but with a very distinctive difference: HILIC could apply semi-aqueous mobile phase resulting in good solubility and matrix compatibility. However, low solubility of extracts in eluents, such as hexane, was encountered using NPLC mobile phase for the analysis of non-polar and polar metabolites [547,552]. Also, high buffer percentages using RPLC and non-polar eluents from NPLC are not favoured for MS analysis due to susceptibility to matrix effects with RPLC and poor ionisation achieved in totally nonpolar organic mobile phase using NPLC. HILIC could also enhance the sensitivity of ESI-MS/MS techniques. HILIC was found to enhance MS sensitivity from 10-1000 times compared to RPLC for some polar compounds, and lowers the cost of analysis by direct injection which also saves the time of sample preparation [416,543,546,550-554].

The analytes eluting near to the column void volume in RPLC are the most retained analytes using HILIC procedures. The water enriched layer is established within the stationary phase under HILIC phase, and the separation of analytes using HILIC is accomplished by partitioning of mobile phase contents into the HILIC phase. The retention of analytes is based on binding to water which is the primary function of the HILIC stationary phase [544,545,551,552]. The primary HILIC retention is not important; a second HILIC retention has been added by charging the stationary phase which increases selectivity because it increases the interaction with polar metabolites. However, a high buffer concentration is required to disrupt these interactions to allow polar metabolites to be eluted, which is not good for MS detection and sensitivity. The use of columns having a combination of zwitterionic stationary phase and HILIC (ZIC-HILIC) was found to make the overall effect of a weaker electrostatic interaction

leading to lower buffer concentrations, because the electrostatic forces of each charge are partly counterbalanced by the proximity of an ion with opposite charge [552,554].

Another advantage of using ZIC-HILIC is that it is pH-independent which makes the optimisation of mobile phase pH solely determined by the polar metabolites. Other HILIC stationary phases such as silica and amino phases are pH dependent which in turn means the optimisation of mobile phase pH depends on the analytes and stationary phase ionisation [554].

Until now, few studies have been concerned with optimisation of methods using ZIC-HILIC phases for forensic purposes. Only two studies have been reported for the use of ZIC-HILIC for the quantification of opioid polar metabolites [93,549], and two HILIC method was reported for ETG during this work but using urine [555] and hair matrix [556]. The use of HILIC has enhanced MS sensitivity for these polar metabolites which are known to be eluted near to the void volume in RPLC. However, RP is still the method of choice for the simultaneous determination of many drugs and their metabolites while HILIC is suitable for very polar metabolites only.

10.4 Aims

Until now, only one method to determine ETG using an ion trap mass analyser was published at the same time as the present work [539]. However, ETS has not previously been determined using this technique. In addition, a method for combination of LC-ion trap-MS/MS with HILIC for direct determination of both ethanol metabolites has not yet been reported.

This work was aimed at developing and validating an LC-ESI-ion trap-MS/MS method for identification and quantification of ETG and ETS as ethanol biomarkers. The second aim was to employ the optimised method for the separation of these polar metabolites for routine analysis of urine samples obtained at autopsy.

10.5 Methods and Materials

10.5.1 Reagents and Standards

Ethyl glucuronide, ethyl glucronide-D5, ethyl sulfate and ethyl sulfate -D5 were obtained from Lipomed (Arlesheim, Switzerland). Individual working standards were prepared at concentrations of 20, 10 and 1 µg/mL by dilution of the stock solutions. Working mixtures of standards and internal standards were similarly prepared.

10.5.2 Extraction

Following addition of pentadeuterated, internal standards for ETG and ETS, 200 µl of acetonitrile were added to 0.1 mL of urine and centrifuged at 10,000 rpm. The supernatant was then evaporated before reconstituting with 100 µl of initial mobile phase and 5 µl was injected to LC-MS/MS.

10.5.3 Chromatographic conditions and instrumentation

Analytes of interest were separated on a ZIC-HILIC column (150 x 2.1 mm, 3.5 µm), protected by a guard column with identical packing material (4 x 2.0 mm, Merck SeQuant, Umea, Sweden). Gradient elution used a mobile phase with (A) 5 mM ammonium acetate and (B) acetonitrile at a flow rate of 0.2 mL/min for the first 3 min, increasing to 0.4 mL/min at 4 min and maintained for the next 3 min. After that, initial flow rate was applied until the end of analysis.

The gradient conditions were initially 10 % of solution A for 3 min; increasing to 30 % at 4 min, to 50 % at 12 min before returning to 10 % for 8 min prior to the next injection. Two retention segments were used to maximise the sensitivity of analysis: the first segment from 0-4 min was for ethyl sulfate (ETS) and ETS-D5, the second segment from 4-8 min was for ethyl glucuronide (ETG) and ETG-D5.

10.5.4 Instrumentation

A Thermo-Finnigan LCQ Deca Plus LC-MS/MS instrument (Thermo Finnigan, San Jose, CA) equipped with a Surveyor LC system interface was used for

determination of both ethanol biomarkers. The column oven and autosampler tray were maintained at 25 ^{0}C at 4 ^{0}C, respectively. Ionisation of analytes was carried out using electrospray negative ion mode. Both ETG and ETS were optimised separately by infusing 10 μg/mL of each analyte using a syringe pump at fixed flow rate. Then, the capillary temperature, sheath gas flow rate, auxiliary gas flow rate and collision energies were optimized for each analyte separately.

ETG and ETS and their internal standards were identified and quantified based on their retention times, precursor ions using Selection Reaction Monitoring mode (SRM) and negative ESI mode. In the case of ETG and its deuterium internal standard two product ions can be obtained, the MS/MS transition with m/z 221→ 203 (precursor ion → product ion) was used as quantifier, m/z 221 → 113 was used as qualifier; m/z 226 → 208 and 226 → 118 were used for ETG-D5 as quantifier and qualifier ion, respectively . However, a single product ion was obtained with ETS and ETS-D5. Therefore, 90% of product ion and 10% of precursor ion were used for the calculation of SRM transition of ETS in which m/z 125 → 97 was used as quantifier and 125 → 125 was used as qualifier; m/z 130 → 98 and 130 → 130 were used for ETS-D5 as quantifier and qualifier ion, respectively. The spray voltage used was 4 and 5 kV for ETG and ETS, respectively. The MS/MS parameters are detailed in Table 10-1.

10.5.5 Method Validation

10.5.5.1 Linearity

Calibration standards were spiked in urine over the range 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5 and 10 mg/mL and extracted using the protein precipitation (PP) method described earlier. Calibration curves were plotted by dividing the peak area ratios obtained at each concentration to internal standards. The correlation coefficient (r^2) was obtained for each linear regression curve.

Table 10-1: LC-MS/MS data

	Parameters	Ethyl glucuronide	Ethyl sulfate
Analytes	**Precursor Ion (m/z)**	221.5	125.5
	Product Ion(s) (m/z)	103, 113	97,5
	Quantifier Ion (m/z)	221.5 → 103.0	125.5 →97.5
	Qualifier Ion(m/z)	221.5→113.0	125.5 →125.5
	RT * (min)	6.0	1.9
Internal standards	**Internal Standard (IS)**	Ethyl glucuronide-D5	Ethyl sulfate -D5
	IS Precursor Ion(s) (m/z)	226.5	130.5
	Product Ion (m/z)	208.0	98.5
Analytes and their internal standards	**Sheath Gas (AU)**	15.0	10.0
	Auxiliary Gas (AU)	10.0	10.0
	Capillary Temperature (ºC)	275.0	275.0
	Collision energy (%)	32.0	30.0
	Retention Widow No.	2.0 (4.0-10.0)	1.0 (1.0-4.0)
	LOD # (ng/mL)	0.13	0.2
	LLOQ ** (ng/L)	1.0	1.0

* Retention time;
# LOD: Limit of detection;
** LLOQ: Lower limit of quantitation.

10.5.5.2 Recovery and Matrix effect

The method described by Matuzewski *et al* [51] was used for the assessment of the recoveries (RE) of and matrix effects (ME) on ethanol metabolites at three different concentrations 0.05, 0.5 and 2.5 µg/mL with five replicates at each concentration point. REs and MEs of analytes of interest were calculated by dividing the mean peak area ratios of product ion of analytes of interest/their internal standards using equations 3-1 and 3-2, respectively.

The effect of endogenous urine matrix components on analyte ionisation during LC-MS/MS was assessed by analysing blank urine obtained from five different human sources. Analytes of interest and their internal standards were spiked at three concentrations (0.05, 0.5 and 2.5 µg/mL) and analysed by the optimised method.

10.5.5.3 Limits of Detection and Lower Limits of Quantitation

Limits of detection (LODs) and lower limits of quantification (LLOQs) were obtained by extending the calibration curves to the concentration of the expected LODs and LLOQs of analytes of interest. A linear calibration model was established for each analyte of interest at eight concentrations (0.1, 0.2, 0.3, 0.5, 0.75, 1, 2.5 and 5 ng/mL) plus blank. Spiked urine samples were then extracted and analysed using the developed method. LODs and LLOQs values were determined at signal-to-noise ratios of 3 and 10, respectively, using equations 2-1, 2-2 and 2-3, 2-4, respectively.

10.5.5.4 Intra-assay and inter-assay precision

Five replicate human urine samples (n=5) were spiked with ETG and ETS at three concentrations (0.1, 0.5 and 2.5 µg/mL). The extracts were analysed using the optimised method on the same day to determine intra-assay precision. The inter-assay precision was measured in a similar manner to the intra-assay precision on five different days. Linear calibration curve for ETG and ETS were performed with each run batch.

10.5.5.5 Stability

Stability was assessed using human urine spiked with ETG and ETS at 1 µg/mL (n=5). Short-term temperature stability at room temperature was investigated using spiked urine stored for 4 and 24 hrs. Freeze-thaw stability of analytes of interest was determined after four cycles (thawed, left at room temperature for 3 hours then refrozen) on consecutive days. Auto-sampler stability using reconstituted extracted sample was determined for 48 hours after extraction. Long-term stability for analytes of interest at -20 and 4 ^{o}C for period of 24hrs, 48hrs, 1 week and 1 month were investigated. Calibration curves were prepared for each batch of samples using standards spiked in human urine at 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5 and 10 µg/mL plus blanks.

10.5.5.6 Specificity

These were similar to those described in Chapters 6 (section 6.7.5.6).

10.5.5.7 Case samples

Samples of autopsy urine were analysed using the proposed method as part of the investigation of medico-legal cases submitted to Forensic Medicine and Science, University of Glasgow which were drug-related deaths. Measurements were repeated after dilution of the urine sample when analyte concentrations outside the calibration range were obtained.

Ninety urine case samples were divided into three groups depending on the blood alcohol concentration (BAC) and analysed by the developed method: group A with BAC higher than 200 mg/100 mL; group B with BAC in the range 80 to 200 mg/100 mL and group C with BAC less than 80 mg/100 mL.

10.6 Results

10.6.1 Method Validation

The ZIC-HILIC column was first used for the separation of ETG and ETS which therefore required optimising of mobile phase strength, injection volume, and flow rate before applying this method for analysis. The HILIC guideline book

recommended some starting points for method validation such as using 5 mM of ammonium acetate plus high organic solvent from 40- 95% and flow rate at 0.1 mL/min [554,557,558]. These settings gave good separation of two ethanol biomarkers from the first run; however, ETG was eluted at 14 minutes while ETS eluted at 3 minutes plus 15 min for column to be equilibrated before the second injection which resulted in a run time of 30 min. Different injection volumes were investigated at 10, 15 and 20 μl. The sensitivity was good with an injection volume of 5 μl and the peak started to broaden with more than 15 μl, therefore, 5 μl was chosen for the remainder of this study.

For acidic and polar metabolites, a pH of 6 to 7 plus low buffer concentration at room temperature is recommended [554]. Mobile phase strength was also optimised using different organic percentages and different ammonium acetate buffer concentrations (5 and 10 mM). In addition, the pH of mobile phase was examined at 4.5 and 6.4. No differences in separation or peak shape of ETG and ETS were observed with these two pH values and buffer concentrations. However, changes in the percentage of organic modifier and flow rate were sufficient to change analyte retention. Therefore, 90% of acetonitrile (B) and 10 % of 5 mM ammonium acetate (A) were used as a starting point at 0.2 mL/min as flow rate for the first 3 min. A higher flow rate of 0.4 mL/min was necessary to achieve baseline separation in less than 7 minutes and then 3 minunts for column cleaning were used by increasing the buffer (B) percentage to 70% followed by 8 minutes for column conditioning before the next injection (total run time 21 minutes). The LC-chromatogram was divided to two segments for each analyte in order to enhance method selectivity and sensitivity. ETS was eluted first at 1.9 minutes followed by ETG at 6 minutes; internal standards for each analyte were used to correct any matrix effect if present.

10.6.1.1 Linearity

Linear regression lines were obtained for ETG and ETS over the calibration range of 0.05-10 μg/mL with R^2 values greater than 0.999 for ETG and ETS (n=10). Although a higher range of calibration between 0.05 to 50 μg/mL was also achieved with good correlation coefficients, the range of calibration was chosen to be between 0.05-10 μg/mL in order to lower the test cost (Figure 10-1).

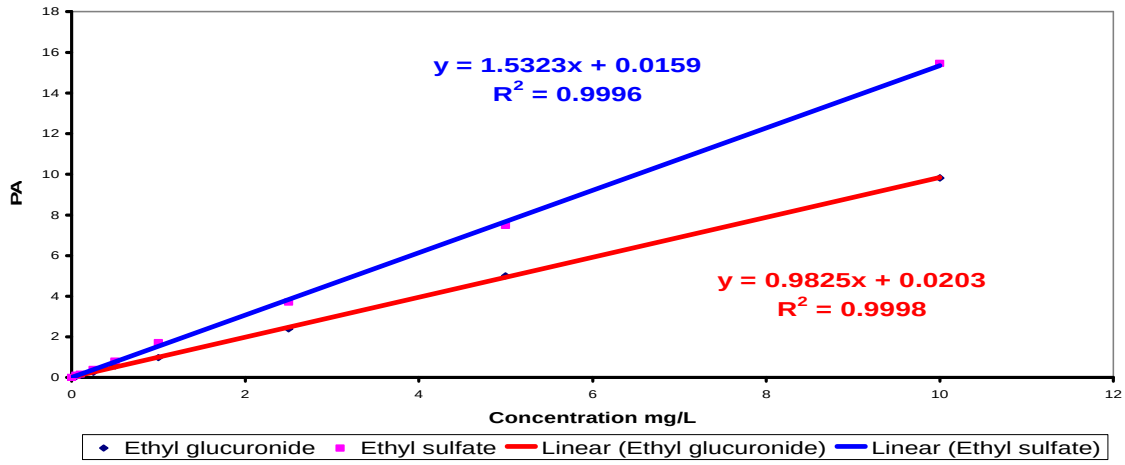


Figure 10-1: Linear calibration curves for ETG and ETS.

10.6.1.2 LOD and LLOQ

Low LOD and LLOQ values were obtained using the ZIC-HILIC procedure. LLOQs of 0.001 µg/mL were obtained which in fact lowered the method cut off of ETG and ETS by 1000 and 100 fold, respectively, see Table 10-1. This was the advantage of using high organic modifiers compared to an RPLC method (Table 10-2 and Figure 10-2).

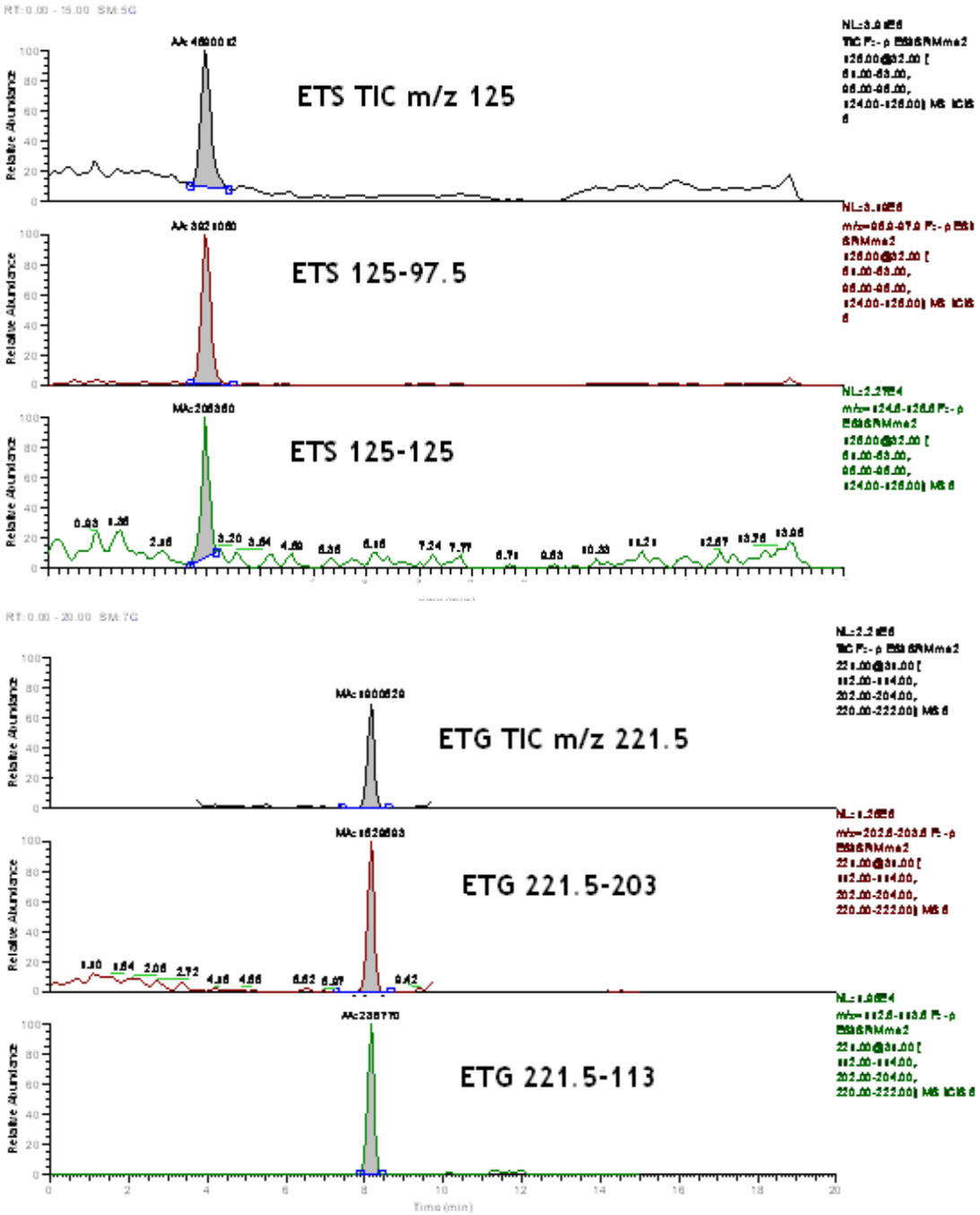


Figure 10-2: ETG and ETS at the LLOQ.

10.6.1.3 Recovery and Matrix Effects

ETG and ETS had high recoveries of 98-99 % using three quality control standards at 0.05, 0.5 and 2.5 µg/mL. The matrix effects were investigated together with recovery experiments in order to determine the true recoveries.

Matrix effects were investigated using five different human urine samples which tested negative for both ETG and ETS. There were no matrix effects observed using three quality control standards at low, medium and high concentration at 0.05, 0.5 and 2.5 µg/mL (Table 10-2).

10.6.1.4 Specificity and Selectivity

In addition, method specificity was examined by injecting high concentrations of common drugs and metabolites encountered with real forensic cases: no interference was observed.

Table 10-2: Matrix effects and recoveries

Analytes	Nominal Concentration µg/mL	Mean of matrix effects [#] % [*] (R.S.D. %) [&]	Mean Recovery % [**] (R.S.D. %)
Ethy glucuronide	0.05	106.0 (13.0)	101.0 (9.0)
	0.50	103.0 (7.0)	96.0 (6.0)
	2.50	99.0 (3.0)	100.0 (2.0)
Ethyl sulfate	0.05	99.0 (7.0)	98.0 (5.0)
	0.50	105.0 (2.0)	101.0 (2.0)
	2.50	100.0 (2.0)	99.0 (5.0)

[#] Human urine was sourced from completed urine samples that were scheduled for destruction and contained no analytes of interest.
[*] Matrix effect is expressed as the response obtained for a standard chromatographed along with matrix extract compared to that obtained with an unextracted standard chromatographed in mobile phase only, expressed as a percentage. Standard was spiked into matrix extract at a concentration of 0.05, 0.5 and 2.5 µg/mL.
[**] Value calculated from the average recovery for the replicate analyses (n=5)
[&] R.S.D. %: Relative standard deviation expressed as a percentage.

10.6.1.5 Method precision

Percentage relative standard deviations (RSD) were used to assess the intra- and inter-day precision of the method for ETG and ETS at three concentrations (0.1, 0.5 and 2.5 µg/mL). RSD values less than 8 % and 11% were observed for intra-

and inter-assay precision, respectively. Accuracy, intra-day and inter-day precision are detailed in Table 10-3.

Table 10-3: Accuracy, Intra-day and inter-day precision of the optimised method

Drugs	Nominal Conc.	Intra-day Precision *	Inter-day Precision *	Accuracy *
	µg/mL	µg/mL (R.S.D %) #	µg/mL (R.S.D %)	% (RSD %)
Ethyl glucuronide	0.1	0.1 (8.0)	0.1 (10.0)	97.0 (6.0)
	0.5	0.5 (2.0)	0.5 (7.0)	99.0 (4.0)
	2.5	2.5 (1.0)	2.5 (2.0)	100.0 (5.0)
Ethyl sulfate	0.1	0.1 (2.0)	0.1 (9.0)	103.0 (10.0)
	0.5	0.5 (6.0)	0.5 (5.0)	101.0 (2.0)
	2.5	2.5 (5.0)	2.5 (2.0)	101.0 (4.0)

* Value calculated from the average recovery for the replicate analyses (n=5)
# R.S.D. %: Relative standard deviation expressed as a percentage.

10.6.1.6 Stability

Stability is the major issue for ETG and ETS analysis. In the current study, stability of ETG and ETS were investigated over the short term, 4 and 24 hours at room temperature, long term stability at two storage conditions 4 °C and -20 °C up to month, four freeze/thaw cycles and auto-sampler for up to 48 hours. There was no degradation of ethanol metabolites observed in all storage conditions (Table 10-4).

Table 10-4: Stability study

Storage Condition [#]	Time	Ethyl glucuronide	Ethyl sulfate
		Mean % [*] (R.S.D %) [&]	
Room Temperature	**4 Hours**	103 (7)	109 (11)
	24 hours	110 (8)	108 (1)
Freeze/thaw	**4 cycles**	105 (6)	105 (3)
Auto-sampler 4 0C	**48 hours**	103 (12	105 (2)
	Week	103 (0.5)	99 (4)
Freezer at -20 0C	**24 hours**	111 (5)	109 (3)
	48 hours	105 (9)	99 (6)
	Week	101 (12)	103 (3)
	Month	103 (9)	99 (3)
Refrigerator at 4 0C	**24 hours**	106 (8)	99 (11)
	48 hours	98 (7	107 (2)
	Week	96 (8)	108 (5)
	Month	105 (4)	106

Starting concentration is 1 μg/mL.
[*] Value calculated from the average recovery for the replicate analyses (n=3).
[&] R.S.D. %: Relative standard deviations expressed as a percentage.

10.6.2 Case Samples

The method was used for analysis of ETG and ETS in case urine samples and has been shown to be a useful tool to indicate recent ethanol consumption. Ninety urine samples obtained at autopsy were tested for BAC and UAC using an in-house GC-FID method. Urine samples were then divided into three groups depending on BAC because in some cases UAC was not determined.

Group A, consisting of 31 cases, had BAC higher than 200 mg/100 mL, the median (mean) BAC and UAC were 261 (318) and 327 (335) mg/100 mL,

respectively. All of these samples were positive for ETG and ETS with the exception of one case which had a negative result for ETS and positive result for ETG. This case had BAC and UAC of 290 and 78 mg/100 mL, respectively. Urine ETG (UETG) concentration was detected at low concentration (4 µg/mL) which was above the ETG cut-off used in this study. The ratio of UAC/BAC was very low at 0.27 which may indicate rapid death within one hour and may indicate that some of the ethanol was still in the stomach [474]. These results also indicate ante-mortem alcohol ingestion due to the presence of UETG, (Figure 10-3 and 10-4, Table 10-5).

In group A, the median and mean UAC/BAC ratios for all cases included were 1.2 and 1.1, respectively. The median UETG and UETS concentrations detected were 181 and 43 µg/mL, with a range of 2 - 1480 and not detected - 340 µg/mL for UETG and UETS, respectively. Mean UETG and UETS levels were 333 and 73 µg/mL, respectively.

Results from 30 cases testing positive for both UETG and UETS correlated well with a correlation coefficient of 0.9. Also, the mean ETG/ETS ratio in group A was 5.3 i.e. UETS was approximately 20% of the UETG in post-mortem urine. Poor correlations were observed with UETG and both BAC and UAC with correlation coefficients of 0.3 and 0.2, respectively. Similarly, correlation coefficients for UETS were 0.137 and 0.2, respectively. In most cases high concentrations of UETG and UETS were detected. Only one case had UETG levels lower than 30 µg/mL and most UETS levels were higher than the cut-off by a factor of at least 17.

There were 22 cases in group B (BAC in the range 80 to 200 mg/100 mL) with median (mean) BAC and UAC of 146 (144) and 197 (209) mg/100 mL, respectively.

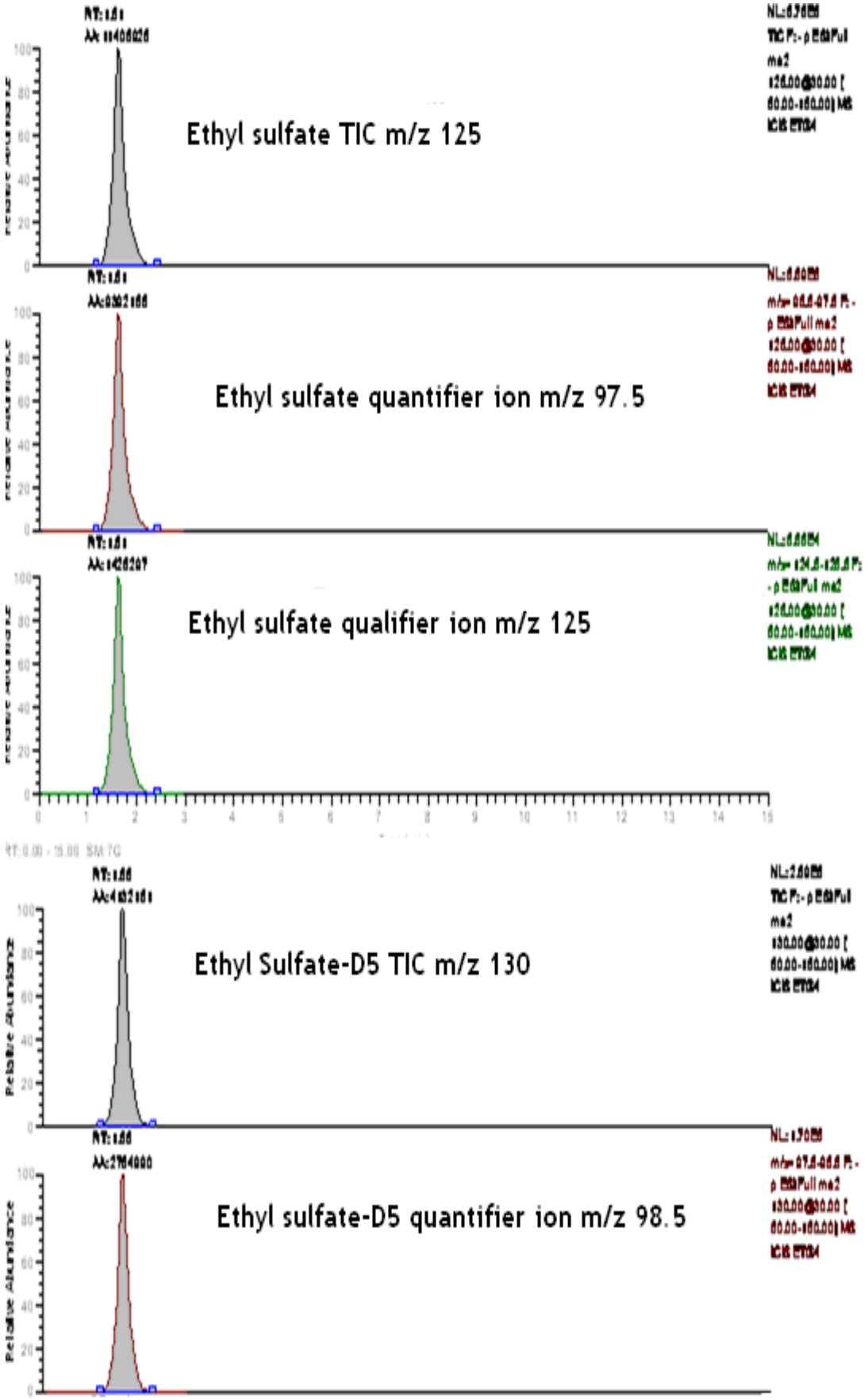


Figure 10-3: Cases sample positive for ETS.

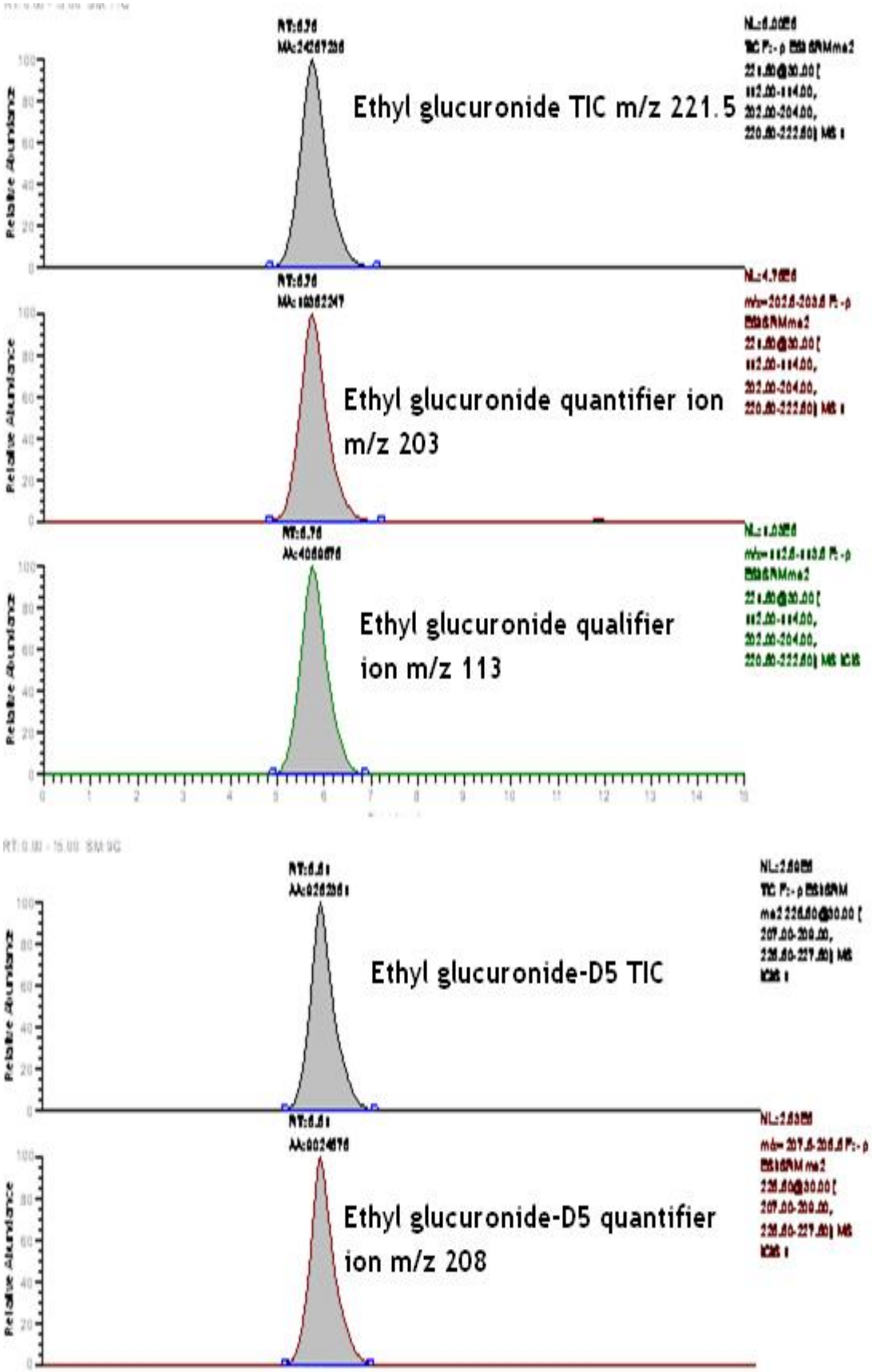


Figure 10-4: Case sample positive for ETG.

Table 10-5: Concentration of ethanol conjugates in post-mortem cases in group A

Case no.	ETS	ETG	BAC	UAC	ETG/ETS	UAC/BAC
	µg/mL	µg/mL	mg/100 mL	mg/100 mL		
1	31.8	220.3	460.0	520.0	6.9	1.1
2	339.8	1047.2	229.0	236.0	3.1	1.0
3	13.9	112.4	219.0	328.0	8.1	1.5
4	5.9	31.6	238.0	365.0	5.4	1.5
5	59.0	1141.8	388.0	390.0	19.4	1.1
6	149.7	679.6	212.0	298.0	4.5	1.4
8	72.0	156.2	243.0	327.0	2.2	1.4
34	22.8	138.7	367.0	390.0	6.1	1.1
40	5.8	34.5	203.0	276.0	6.0	1.4
44	45.6	247.8	470.0	510.0	5.4	1.1
46	12.3	84.5	228.0	256.0	6.9	1.1
48	145.9	552.1	389.0	426.0	3.8	1.1
49	322.3	1479.8	286.0	382.0	4.6	1.3
52	43.0	222.6	252.0	325.0	5.2	1.3
53	6.5	51.3	241.0	297.0	7.9	1.2
59	1.8	36.8	307.0	254.0	20.4	0.8
62	23.8	121.3	316.0	388.0	5.1	1.2
63	76.4	265.6	217.0	273.0	3.5	1.3
66	23.3	2.0	249.0	313.0	0.1	1.3
70	32.8	154.5	234.0	319.0	4.7	1.4
71	158.0	900.4	386.0	487.0	5.7	1.3
75	111.5	373.0	314.0	404.0	3.4	1.3
77	131.0	584.6	1050.0	370.0	4.5	0.4
80	126.6	762.8	251.0	360.0	6.0	1.4
81	28.0	204.1	253.0	332.0	7.3	1.3
82	4.0	29.3	307.0	250.0	7.3	0.8
83	n.d [&]	3.9	261.0	78.0	n.d	0.3
84	62.8	181	383.0	261.0	2.9	0.7
86	12.8	68.7	200.0	248.0	5.4	1.2
87	61.2	163.4	293.0	214.0	2.7	0.8
88	116.0	266.0	430.0	520.0	2.3	1.2
Mean	72.5	332.9	318.6	335.4	5.9	1.1
Median	43.0	181.0	261.0	327.0	5.3	1.2

[&] n.d: Not detected.

Twenty cases had both BAC and UAC: the median and mean UAC/BAC ratio was 1.5 in all these cases which also tested positive for UETG and UETS with higher concentrations than the method cut-off for both biomarkers. The median (mean) UETG concentrations detected in group B were 102 (246) µg/mL, respectively, which were lower than that found with group A. The UETG level was higher than 10 µg/mL in these twenty cases and ranged from 10 to 750 µg/mL. Furthermore, the median of UETS in group B was slightly higher than that of group A, while mean UETS concentrations in group B was lower than group A; the median (mean) UETS level was 44 (60) µg/mL. The UETS levels detected in these cases were higher than 1.7 µg/mL and ranged from 1.7 to 175 µg/mL (Table 10-6).

In group B, two cases had BAC of 133 and 180 mg/100 mL but tested negative for UETG and UETS. One of these cases had been left for many hours after death in an outdoor environment and was heavily putrefied, which suggested post-mortem alcohol production. No information was available for the other case.

In group B, UETG and UETG correlated well with a correlation coefficient of 0.844. The median UETG/UETS ratio was 4.8 which was lower than that of group A and the UETS level was less than 21% of UETG in post-mortem urine specimens. In addition, poor correlation was obtained between UETG and both BAC and UAC with correlation coefficients of 0.13 and 0.4, respectively. The same was observed for UETS which had correlation coefficients of 0.3 and 0.26, respectively. In group B, case samples positive for UETG were also positive for UETS with concentrations higher than 1 µg/mL for both alcohol biomarkers.

Twenty four cases in group C (BAC in the range 0 to 80 mg/100 mL) were positive for both alcohol biomarkers. The median (mean) UAC/BAC ratio was 1.4 for these 24 cases with median (mean) BAC and UAC of 40 (68) and 35 (67) mg/100 mL, respectively. Median (mean) UETG concentrations were found at 106 (197) µg/mL. Also, a wide range of UETG levels were found with concentrations ranging from 5.6 to 996 µg/mL, with approximately the same median as group B. However, a lower median concentration of UETS was observed at 23 µg/mL compared to 43 and 44 in groups A and B, respectively. In addition, UETS concentrations were in the range of 0.3 to 219 µg/mL, which were close to the UETS concentrations in group B.

Table 10-6: Concentrations of ethanol conjugates in post-mortem cases in group B

Case no.	ETS	ETG	BAC	UAC	ETG/ETS	UAC/BAC
	µg/mL	µg/mL	mg/ 100mL	mg/ 100mL		
9	2.7	19.4	172.0	239.0	7.2	1.4
14	18.2	79.8	183.0	123.0	4.4	0.7
15	175.0	330.8	116.0	171.0	1.9	1.5
16	120.7	565.9	90.0	173.0	4.7	1.9
30	132.0	636.3	100.0	234.0	4.8	2.3
33	87.5	357.2	139.0	230.0	4.1	1.7
39	1.7	10.1	113.0	167.0	5.9	1.5
42	110.1	487.6	135.0	196	4.4	1.5
43	32.2	63.1	172.0	242.0	2.0	1.4
45	17.0	87.5	87.0	188.0	5.2	2.2
47	43.4	230.6	127.0	197.0	5.3	1.6
50	15.0	57.5	153.0	181.0	3.8	1.2
56	n.d.[&]	n.d.	133.0	n.a. [*]	n.d.	n.a.
57	78.0	661.4	157.0	243.0	8.5	1.6
60	44.0	14.6	162.0	256.0	0.3	1.6
65	61.4	117.6	129.0	190.0	1.9	1.5
68	8.4	51.4	168.0	200.0	6.1	1.2
73	47.8	350.2	174.0	270.0	7.3	1.6
74	144.6	750.4	184.0	285.0	5.2	1.6
79	7.0	67.5	146.0	193.0	9.6	1.3
90	n.d.	n.d.	180.0	n.a.	n.d.	n.d.
Mean	54.6	235.2	143.8	209.4	4.4	1.4
Median	43.8	102.5	146.0	197.0	4.7	1.5

[&] n.d: Not detected.
[*] n.a: Not analysed.

UETG and UETS correlated well with a correlation coefficient of 0.9. The median ratio of UETG/UETS was 4.5 which means UETS was 22 % of UETG in these urine samples. As before, a very poor correlation was observed between BAC and both UETG and UETS, but a slightly better correlation was obtained between UAC and both UETG and UETS with r^2 values of 0.5 and 0.4, respectively. Positive cases were higher than the method cut-off but UETS concentrations below 1 µg/mL were noted in three cases wherreas the remainder of the cases had UETS higher than 4 µg/mL. Results of group C are listed in Table 10-7.

In group C, six cases tested positive for UETG but not for UETS and in these cases BAC was lower than 10 mg/100 mL, which is considered to be lower than the LLOQ of the GC-FID method used and no UAC results were available for most cases. The median and mean UETG level in these samples was 9 µg/mL and the range was 2.5 to 17 µg/mL. These cases were attributed to ante-mortem alcohol ingestion. In another seven cases in group C, both UETG and UETS tested negative, and in these cases BAC was above 10 mg/100 mL and in the range 11 to 47 mg/100 mL. UAC levels were not available for three of these cases and ranged between 21 to 81 µg/mL in the rest of these cases. The median and mean UAC/BAC ratios were 0.3 and 0.8, respectively. The presence of alcohol in these case may attributed to post-mortem synthesis.

In group C, thirty seven cases had BAC lower than 80 mg/100 mL. The median (mean) BAC and UAC were 24 (34) and 27 (51) mg/100 mL, respectively. The median UAC/BAC was 1. Lower median UETG and UETS were obtained at 23 and 6 µg/mL. Correlations between BAC and both UETG and UETS were 0.2 which were poor. In contrast, better correlations were found between UAC and both UETG and UETS at 0.5 which were better than observed with group A and B.

Both ethanol markers were detected in all groups with overall median concentrations of 114 µg/mL and 23 µg/mL for ETG and ETS, respectively. Nine negative cases were attributed to post-mortem ethanol synthesis out of 90 cases in which neither UETG or UETS was detected. Eighty one cases tested positive for UETG while only 74 cases tested positive for UETS.

Table 10-7: Concentrations of ethanol conjugates in post-mortem cases in group C

Case	ETS	ETG	BAC	UAC	ETG/ETS	UAC/BAC
	µg/mL	mg/mL	mg/ 100mL	mg/ 100mL		
7	n.d [&]	n.d	16.0	n.a. [*]	n.d	n.a.
10	4.9	6.3	77.0	133.0	1.3	1.7
11	0.4	6.4	18.0	n.d	16.0	n.a
12	4.1	6.0	64.0	117.0	1.5	1.8
13	15.0	99.5	78.0	77.0	6.6	1.0
17	0.9	5.6	28.0	n.a	6.2	n.a
18	22.8	91.1	26.0	66.0	4.0	2.5
19	n.d	n.d	26.0	n.d	n.d	n.a
20	n.d	10.6	11.0	n.d	n.d	n.a
21	n.d	n.d	47.0	76.0	n.d	1.6
22	n.d	n.d	n.a	81.0	n.d	n.a
23	35.5	132.3	n.a	17.0	3.7	n.a
24	85.0	220.0	n.a	14.0	2.6	n.a
25	42.0	213.9	n.a	14.0	5.1	n.a
26	62.9	172.2	19.0	71.0	2.7	3.7
27	n.d	n.d	15.0	n.d	n.d	n.a
28	28.0	118.6	15.0	37.0	4.2	2.5
29	n.d	n.d	35.0	21.0	n.d	0.6
31	23.7	111.9	54.0	81.0	4.7	1.5
32	123.0	595.4	55.0	120.0	4.8	2.2
35	12.3	53.7	38.0	35.0	4.4	0.9
36	3.5	14.5	43.0	34.0	4.1	0.8
37	74.9	561.3	n.a	184.0	7.5	n.a
38	n.d	6.8	n.a	12.0	n.d	n.a
41	33.3	166.3	n.a	12.0	5.0	n.a
51	n.d	7.5	n.a	11.0	n.d	n.a
54	219.1	548.6	62.0	149.0	2.5	2.4
55	n.d	2.5	n.a	10.0	n.d	n.a
58	64.4	390.2	70.0	145.0	6.1	2.1
61	208.0	996.0	44.0	73.0	4.8	1.7
64	6.3	68.9	53.0	64.0	10.9	1.2
67	21.0	59.4	n.a	16.0	2.8	n.a
69	n.d	n.d	11.0	32.0	n.d	2.9
72	n.d	17.2	24.0	14.0	n.d	0.6
76	7.7	88.6	61.0	83.0	11.5	1.4
78	n.d	9.3	15.0	n.d	n.d	n.a
85	6.7	22.7	54.0	72.0	3.4	1.3
Mean	29.9	130.0	28.6	50.6	5.3	1.3
Median	6.3	22.7	24.0	34.0	4.5	1.3

[&] n.d: Not detected.
[*] n.a: Not analysed.

Using the median concentration for UETG, there was a gradual decrease from high BAC to the low BAC groups. The case was different for UETS as the median was the same in groups A and B but noticeably lower in the low BAC group (Figures 10-5 and 10-6). This was due to many negative results for UETG and UETS in the low BAC group. Using the positive cases only in group C, the median UETG and UETS increased from 23 to 78 and 6 to 23 µg/mL, respectively, but were still lower than group B.

In addition, the BAC and UAC were plotted for the whole group and a good correlation was obtained, with a linear correlation coefficient of 0.96. Moreover, UETG and UETS correlated well, with a correlation coefficient of 0.91 for all cases positive for UETG and UETS (Figures 10-7 and 10-8).

10.7 Discussion

10.7.1 Method validation

The object of the current study was to optimise and validate a sensitive and simple ZIC-HILIC-ESI-MS/MS method for direct determination of ETG and ETS in urine post-mortem samples. Previous methods using conventional RPLC were capable of separating ETG and ETS but both analytes were eluted near to the void volume and so may be affected by matrix effects. Matrix effects were absent using the ZIC-HILIC procedure in the current study due to the HILIC mobile phase which contained 70 to 90 % of organic solvent, which enhances ESI-MS/MS sensitivity The ZIC-HILIC procedure, in which the sample is deproteinated and reconstituted using acetonitrile, is a good alternative to RPLC.

The retention of polar analytes using HILIC phase is the opposite of that observed with RPLC. ETS is released easily from ZIC-HILIC. In contrast, a higher flow rate was needed to decrease the retention time of ETG. In contrast, ETG was eluted at lower organic modifier contents and low flow rates using RPLC followed by ETS.

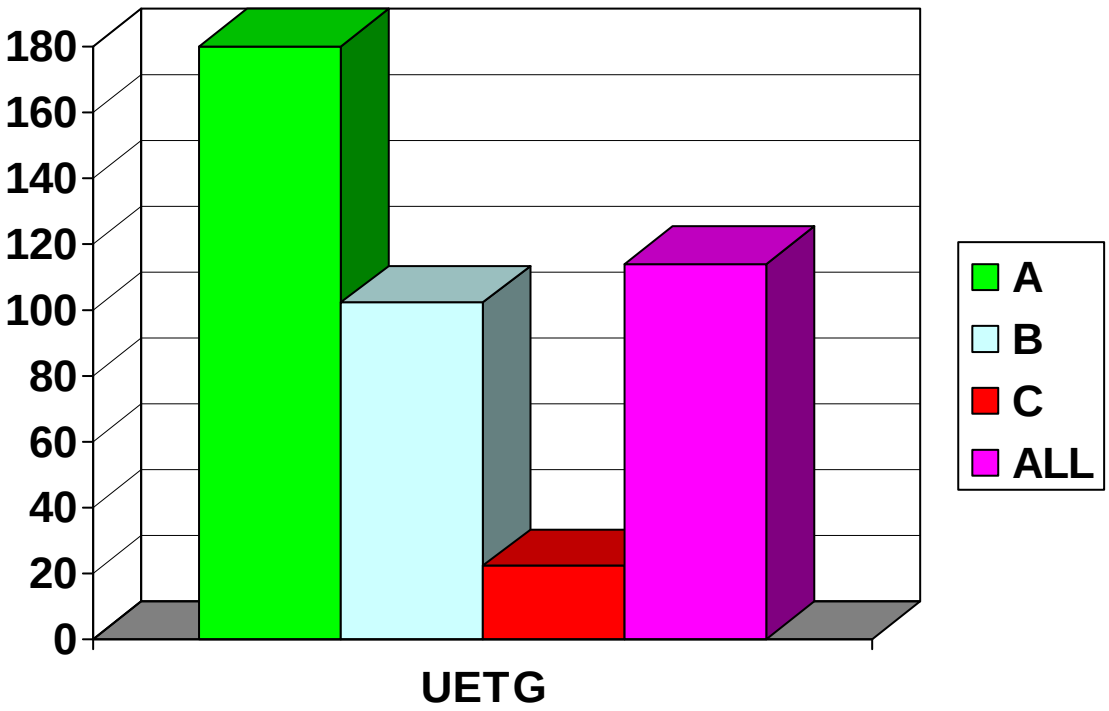


Figure 10-5: Median concentration of UETG in each group (µg/mL).

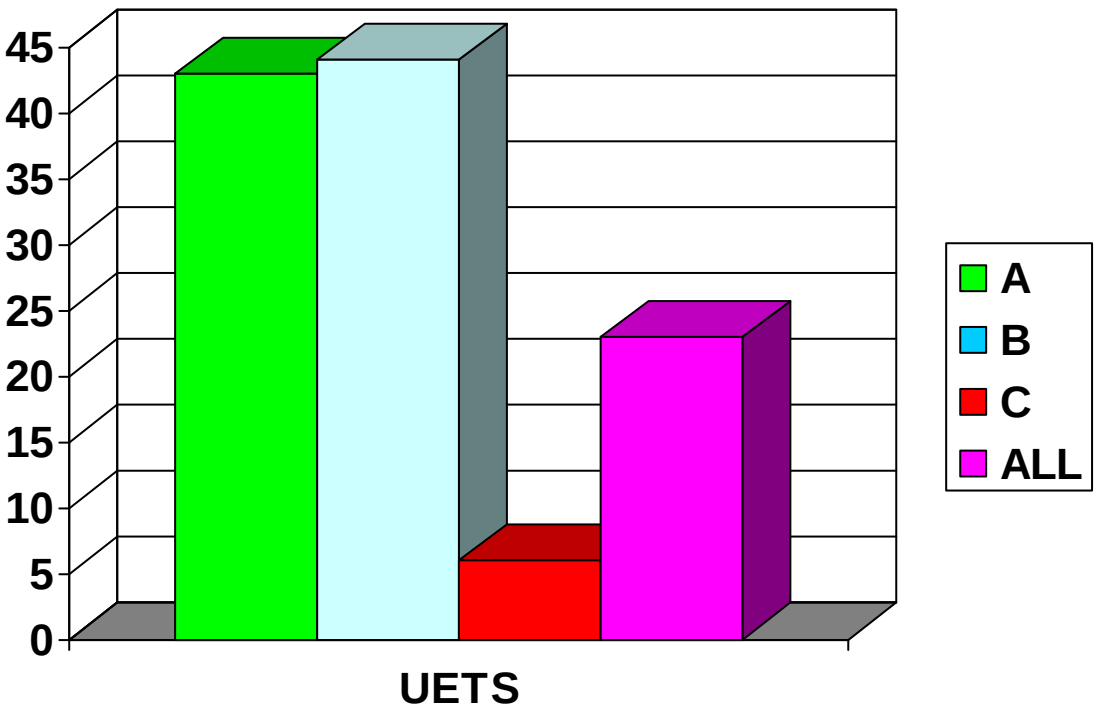


Figure 10-6: Median concentration of UETS in each group (µg/mL).

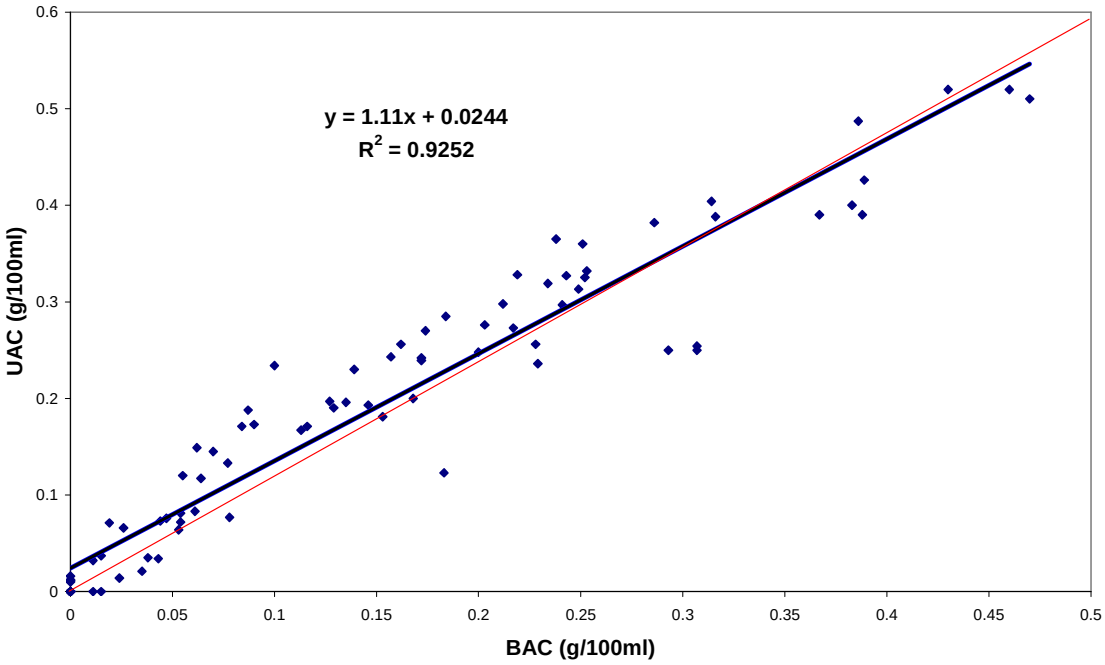


Figure 10-7: Scatter plot of UAC/BAC in 90 post-mortem cases.

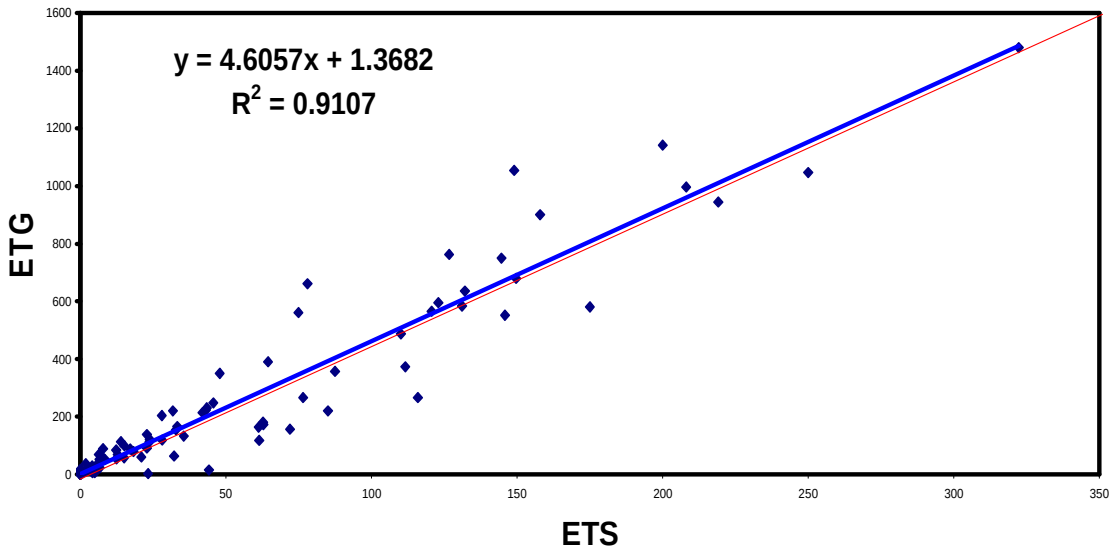


Figure 10-8: Scatter plot of UETG/UETS in 90 post-mortem cases.

In HILIC, very polar analytes are retained more strongly and buffer concentrations and pH have less of an effect compared to changing the percentages of organic modifier and buffer. The true HILIC phase is established with between 70 to 98 % of organic modifiers and less than that may be out of HILIC separation zone. This zone was found to be enough for the polar compounds included in this study to be separated.

As indicated earlier for the RPLC method for the determination of a wide range of opioids and their metabolites (chapter 5-9), not all glucuronide metabolites are eluted near to the void volume but some required high organic modifiers to be eluted from a conventional RP columns. For example, buprenorphine-3-glucuronide eluted at high organic modifier concentrations and norbuprenorphine-3-glucuronide, codeine-6-glucuronide and dihydrocodeine-6-glucuronide also eluted several minutes after morphine. Problems caused by matrix effects with encountered with early eluting metabolites such as M3G, DHM3G, M6G and DHM6G which required very low percentages of organic modifiers to be released. SPE followed by RPLC were capable of removing the matrix effects and achieved fine peak shapes in the previous Chapters using RPLC-MS/MS. In contrast, ETG and ETS are very polar compounds and clean methods of extraction for both metabolites simultaneously are not yet available as well as the use of negative mode for identification which is considered less sensitive than that of positive ion mode. Therefore, ZIC-HILIC provides more retention for these polar compounds as well as more organic modifiers percentage helps in enhancing ESI-MS response with very good shape peaks.

The effects of matrix on the ionisation of ETG and ETS in LC-ESI-MS/MS after protein precipitation or dilution have been investigated with RPLC methods. In all of these studies, matrix effects were present which required post-column addition of organic modifiers using RPLC, which was found to minimise the effects of matrices to a range of - 10% and - 20% [494,520,521,523]. This post-column addition device is not available in all laboratories. The alternative is to use HILIC phases which were found to minimise the matrix effects due to the use of high organic modifiers. However, in one previous study using SPE followed by HILIC-MS/MS for analysis of ETG in hair, matrix effects were reported to be between 20-50%. The authors attributed that to the hair colour and sample pre-treatment

procedure and the matrix effects were corrected by using deuterated internal standard [556].

In the present study, matrix effects were investigated using three quality control standards across the calibration range along with five different human urine sources. There were no matrix effects observed which was in agreement with most work that has been conducted with ZIC-HILIC-ESI-MS/MS procedures.

Although the LLOQs of the optimised method were very low, at 0.001 µg/mL, analyte cut-offs were established at 1 and 0.1 µg/mL for ETG and ETS, respectively, to avoid reporting a false positive result due to the possibility of consuming medicine and mouthwash containing alcohol. Politi *et al* [520] reported the presence of ETG and ETS in wine at concentrations of 0.15-3.97 and 0.21-39.14 µg/mL, respectively. ETG was detected at concentrations up to 3 µg/mL after alcohol exposure resulting from mouth wash but these results were questioned due to the use of excessive mouth wash [559]. Other research has reported that after accidental alcohol exposure from mouth wash the level of ETG ranged from 0.015 to 0.18 µg/mL [560]. Also, detectable levels of ETG in urine samples were reported after low doses of alcohol (0.5 and 1 g), in the range 0.15 -0.35 µg/mL [561].

Although there is no cut-off established yet for ETG and ETS, the use of 0.5 µg/mL as a cut-off has been suggested for forensic cases and 0.1 µg/mL as a cut-off for ETS was used in two studies [519,562]. In the United States of America, the cut-off for ETG in urine is in the range of 0.1-1 µg/mL[563]. In the current study, cut-offs of 0.1 and 1 µg/mL for ETS and ETG were used and found acceptable and positive cases detected in this study were higher than the ETS and ETG cut-offs.

Most LC-MS/MS instruments used for the quantification of both ETG and ETS were single or triple quadrupole mass analysers which provide a sensitive and selective method of determination. Until recently, there was only one report of an LC-ion trap-ESI-MS/MS used for the determination of ETG [539]. This method was published during the current study. The separation of ETG was achieved at 3 minutes by using Synegy Polar-RP (250 X 2 mm, 4 µl) column, i.e. ETG was within the range of the void volume. The authors reported no matrix effect problems. However, no post-column device addition of organic phase was used. The post-

column addition device has been used with the same size and type of column in order to enhance MS sensitivity. This method was capable of producing a linear calibration curve over a wide calibration curve range of 0.5-20 µg/mL and was then applied to real case samples. All method parameters were within the acceptable limits of validation [539].

In the current study, a Synergi Polar (150 X 2 mm, 4 µ) column was used at first but found not useful due to high matrix effects, which led to the use of ZIC-HILIC because no post-column device was available. The same ETG fragmentation was obtained as reported by Kaufmann and Alt [539]. ETG (M^+ 221.3) was broken down to many fragment ions at m/z 203,157,129, 113.4, 85 and 75. ETS (M^+ 125.4) was fragmented to only one product ion at m/z 97.3. Also, ETS-D5 (130.4) was fragmented to a single product ion at m/z 98.3. For greater specificity, the intensity ratios between two products ions at m/z 203 and 113.4, the major product ions for ETG, were calculated and used as quantifier ion. In the case of ETS, the major product ion and remaining precursor ion were used instead. Both ETG and ETS intensity ratios were constant with both calibration curve range and positive case samples which were both ± 20.

The first report of the quantification of ETG in serum and urine was a GC-MS method which had an LOD and LLOQ of 0.1 and 0.3 µg/mL, respectively. The use of LC-MS was found to decrease the LOD and LLOQ which, in most reported cases, were in the range 0.02 to 0.1 µg/mL [452,512,514,518,519,526,534]. Also, HPLC with electrochemical detection procedures was employed for the same purpose and reported a LOD in the range 0.01-0.03 µg/mL and 0.08 as LLOQ [76,538]. LC-MS/MS techniques were also applied for urine and blood analysis to determine ETG or ETS with an LOD and LLOQ in the range 0.025-0.1 and 0.1-0.3 µg/mL, respectively [444,453,454,494,509,520,521,523,527,535-537,539,564].

The ion trap mass analyser used in this work provided an accurate identification and clean MS/MS spectra. The combination of ZIC-HILIC-and ion trap mass analyser were sensitive and selective and provided a low LLOQ for ETG 300 fold less than a method reported with the same ion trap mass analyser [539]. This shows the great advantage of using ZIC-HILIC despite the differences in the type of matrix used in our study and the Kaufmann and Alt study [539]. This is a large improvement using HILIC phases taking into consideration that most LC-MS/MS

methods used triple quadrupole instruments, which are known to be more sensitive and selective than ion trap analyser. Precision, sensitivity, linearity and recovery of the optimised method were within the acceptable limits of method validation proposed by SOFT/AAFS guidelines.

10.7.2 Stability

Although ETG has been extensively studied and reported to be a reliable marker for alcohol recent consumption [449,450,452,509,512,526,533-537,539], there was increased concern about its stability and possibility of the risk of false negative results due to the degradation by bacteria. The use of UETG as evidence of ethanol intake alone has been not recommended by SAMHSA which could be due to false positive and negative results [451]. Although the use of a chemical preservative was found to stop ETG hydrolysis [565], hydrolysis of ETG to ethanol may occur in retained urine in bladder [517]. In this perspective, positive ETG results is considered as a marker of ante-mortem alcohol intake, but negative ETG should be interpreted with caution.

It is known that glucuronide conjugates are broken down to their free form by β-glucuronidase. This enzyme is present in *Escherichia coli* (*E. Coli*) which is the most common bacterium found in urinary tract infections. Although sulfate conjugates can be cleaved by β-sulfatase, this enzyme is not present in *E.Coli* which may explain the resistance of ETS against urinary tract infections and lead to the inclusion of ETS as complementary alcohol biomarkers [517].

Few studies have investigated the stability of ETG in post-mortem material to answer the question of whether ETG is stable. Schloegl *et al* [533] studied the degradation of ETG in urine, liver, skeletal muscles and blood obtained at autopsy. ETG decreased by 27.7% after 4 weeks at room temperature and ETG was detectable in all samples with concentrations higher than 1 μg/g. They also did not observe new formation of ETG in blood or liver specimens spiked with 0.1 g % of ethanol with storage at room temperature [514].

The disappearance of ETG during heavy putrefaction has been studied in blood samples under controlled conditions. Degradation of ETG was observed in non-preserved blood samples stored at 30-40 ºC; while no degradation or formation

of ETG were observed with preserved blood. They also reported the presence of ETG in urine while absent in blood [513].

The stability of ETG and ETS has been compared and ETS was found to be more stable and not degraded by bacteria [516,517]. ETG was reported to be degraded during putrefaction in the corpse but no formation or production of ETG post-mortem has been reported [513,523,528]. ETG and ETS were found stable after subjection to different storage conditions; after 24 hours in an autosampler tray at room temperature, four freeze/thaw cycles and up to 21 days at -20 ºC [494,521,524].

ETS was found stable under degradadative conditions in all studies [516,517]. In the current study, ETS was not detectable or was negative in some cases in which ethanol and ETG were present. Also, in one study, ETS was degraded by 84% after 6 days which should be taken into consideration when dealing with putrefied post-mortem cases [566]. In addition, the metabolism of ethanol via sulfation may be affected by consuming food or alcohol beverages containing ETS which would lead to a false positive result of ETS [520,542].

The stability of ETG and ETS were examined in the present work at different storage conditions. Both ethanol metabolites were stable which supported the previous reports mentioned above. The examined of the effect of urine tract infection on the stability was out of the scope of the present work.

10.7.3 Case samples

Since ethanol has a relatively short half-life [567], 50 % of alcoholics had negative BAC in one study [480]: alternative ethanol biomarkers are needed. Other alcohol markers have been used to detect alcoholics but found not to be useful in some circumstances. They required long periods of alcohol abuse with large doses, were influenced by diseases and not sensitive for single alcohol intake [525].

The combination of ingesting ethanol and other central nervous acting drugs can increase the risk of intoxication and death even with low drug concentrations. The BAC is important in assessing the role of ethanol in causing death where ethanol may increase toxicity as an additive factor, or be involved in cause of

death, or, at concentrations higher than 300 mg/100 mL, could be the cause of death[150,151,264].

As indicated earlier, the source of ethanol detected in blood after death can be interpreted in three different ways: short elapsed time between alcohol intake and death, ethanol is formed post-mortem, or a combination of both circumstances [456]. The phenomenon of alcohol post-mortem production results makes the interpretation of BAC more complicated especially when there is any sign of body putrefaction. In order to distinguish between ante-mortem and post-mortem alcohol, procedures such as testing UAC and vitreous humour alcohol concentration (VAC) were proposed [439]. The C3 alcohol n-propanol for example has been used as a marker of post-mortem alcohol synthesis. However, ethanol can also be formed subsequent to severe damage of the body, which provides suitable conditions for microorganism to form ethanol even in a living body [161].

Although many alcohol biomarkers have been used, ETG and ETS have received a great deal of interest due to their longer half-lives compared to ethanol, and due to their high sensitivity and selectivity. These alcohol biomarkers have also been found useful to confirm abstinence in alcohol treatment programs. Ethyl glucuronide (ETG) and ethyl sulfate (ETS) are direct non-oxidative ethanol metabolites and have been shown to be useful markers of alcohol consumption for several hours after death or when ethanol itself has been completely eliminated from the body. The presence of ETG and ETS in blood and urine give strong evidence of recent alcohol ingestion [452].

The correlation of BAC or UAC with both ETG and ETS was often poor, which makes the prediction of BAC from ETG or ETS impossible. Also, the rapid clearance of alcohol with differences in distribution and elimination, pharmacokinetic variation and activity of enzyme is responsible for the metabolism of both ethanol metabolites. A positive correlation between UETG and both BAC and UAC were reported with r^2 values of 0.549 and 0.617 [76]. Also, r^2 of 0.579 was reported between UAC and normalised UETG in other work [535]. Bicker *et al* [494] tested 50 post-mortem urine cases and found poor correlation between both ethanol biomarkers and BAC or UAC. In the present study, although positive correlations were obtained with group C between UAC and

both alcohol biomarkers, BAC was poorly correlated in all groups which was in agreement with the work of Bicker *et al* work [494].

In the current study, urine samples were divided into three groups depending on BAC in order to determine if there was any correlation between BAC and ETG and ETS levels in the urine samples. There was overlap between UETG and UETS levels in all three BAC groups using the median concentration. Higher UETG were observed with low BAC as well as median and high BAC groups which can be explained by a longer half-life in blood and urine than ethanol itself and also may depend on the number of drinking periods, which are difficult to detect.

Few studies have reported the concentrations of both UETG and UETS in post-mortem cases; most published cases were for living subjects and mostly after controlled administration of alcohol. Eighty six clinical urine samples tested positive for UETG and UETS, the mean (median) concentrations were 77 (7.52) µg/mL, and in the range of 0.13 -997 µg/mL for UETG; and were 18.5 (2.86) µg/mL and ranged from 0.05-264 µg/mL for UETS. In that study, 3 and 4 urine samples tested positive for only UETS and UETG, respectively [518].

Bicker *et al* [494] tested 50 post-mortem urine samples and found 48 cases positive for UETG using 0.1 µg/mL as the cut-off. The median UETG detected in this study was 98.4 µg/mL, ranging from 0.1-1348 µg/mL; they also reported a median concentration of 27.8, ranging from 0.1-220 µg/mL for UETS. A method using an electrochemical detector was applied for routine post-mortem analysis of 29 cases: BAC was higher than 70 mg/100 mL, and all cases tested positive for UETG with concentrations in the range of 47-1371 µg/mL [76].

In the current study, UETG and UETS were in the same range as the Bicker *et al* [494] study with overall median of 114 and 23 µg/mL and similar ranges, respectively. The ratios between UETG/UETS were reported previously. Bicker *et al* [494] reported a median of 2 and 1.7 was reported by Helander and Beck [518]. In recent work, Helander *et al* [519] reported mean UETG/UETS ratios of 2.5. In the present study, the median was 4.8 which was higher than that reported before (Figure 10-7). This could be interpreted by differences in analysis protocol or differences in population size. Also, the HILIC phase could be more sensitive for ETG than ETS.

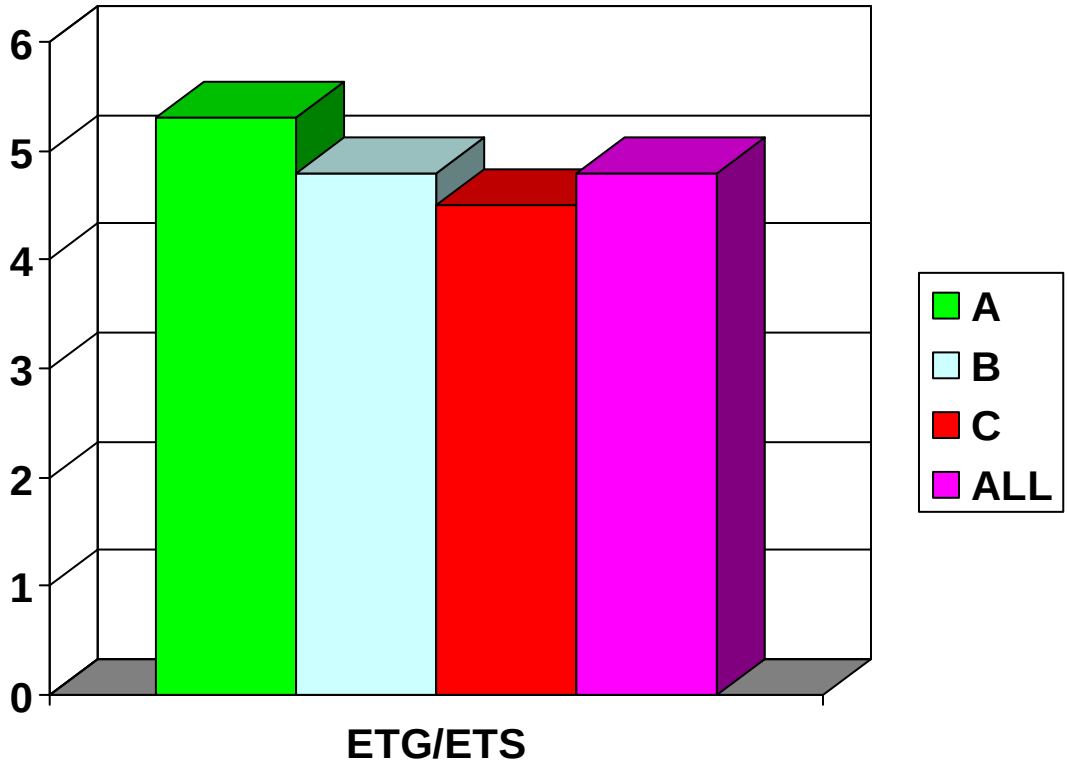


Figure 10-9: Comparison of the ratios of UETG/UETS between BAC groups.

10.8 Conclusions

A sensitive and selective method for direct determination of ETG and ETS in post-mortem urine samples was developed and validated. In the current study, ethanol was attributed to post-mortem synthesis in 9 out of 90 cases. It can be concluded that the risk of false positive ethanol results increased in the low ethanol concentration group as several cases tested negative for both biomarkers in group C. ETG was detected at low concentrations in some cases for which ETS tested negative, suggesting that ETG may have a longer half-life in urine or else ETS is unstable. False positive results for ethanol can be obtained at any BAC which makes interpretation of the presence of alcohol more difficult, especially with putrefied cases. In the present study, two putrefied cases tested positive for BAC and UAC but no ETG and ETS were detected in the urine samples. Although ETS is stable after being subjected to many stability conditions, the use of ETS as sole evidence of alcohol ingestion may lead to a false negative result as noticed in groups A and C in the present study. The use

of ETG is more a reliable ethanol biomarker. Both ethanol biomarkers should be determined in heavily putrefied cases and when the ethanol concentration in post-mortem blood is low, in agreement with previous studies. To the authors' knowledge, this is the first report of the determination of ETS using an LC-ESI-ion trap-MS/MS method, and of a HILIC-ESI-ion trap-MS/MS method for the simultaneous determination of ETG and ETS in post-mortem urine samples.

11 General Conclusions

The focus of the current work was to investigate the use of LC-MS/MS for different applications relating to forensic and clinical toxicology. Chapter 5 focused on an analytical method for multiple target analytes and the use of this method for the interpretation of the cause of death, survival time after last injection and identification of the source of opioid intake. In Chapter 6, a method for analysing trace levels of diamorphine metabolites in paediatric plasma following diamorphine administration was validated in order to study the pharmacokinetics of diamorphine following intravenous and intranasal administration. Chapter 7 studied the efficiency of the hydrolysis method of analysis and direct determination of opioids. Chapter 8 investigated case studies regarding current problems with oxycodone fatalities in the West of Scotland. Chapter 9 addressed identification criteria for opioids and their metabolites using a current LC-ion trap-ESI-MS/MS instrument. Finally in Chapter 10, the new trend of biomarker analysis was interpreted in this project with ethanol biomarkers and a procedure for polar glucuronide and sulfate metabolites of ethanol was validated and successfully used for separation of analytes using a HILIC phase.

Opioids are widely abused drugs due to their euphoria-producing properties and it is estimated that about 0.3% of the world's population misuse heroin each year. However, the use of opioids for pain management also continues to increase and deaths related to opioid abuse and medications are encountered regularly. The identification of illicit or licit opioid use depends on analytical toxicology findings which, until recently, involved tedious procedures for hydrolysis, extraction and derivatisation, and samples might need to be analysed several times to achieve these requirements. The arrival of LC-MS techniques in analytical toxicology has allowed these expensive, time-consuming procedures to be replaced by methods involving a single extraction and chromatographic analysis.

In Chapter 5, the aims were to develop a validated method for determining commonly encountered opioids and metabolites in unhydrolysed post-mortem specimens and apply it to interpreting the cause of death and source of the opioids detected. The stability was investigated in human whole blood of some

analytes which have not previously been studied, such as NAL3G, DHC6G, DHM3G, and DHM6G. In general, opioids and their metabolites were stable for the whole period of this stability study (Table 5-7). However, the concentration of 6-AC sharply decreased by 56% after 24 hours at 4 °C and by more than 77% after 48 hours. 6-MAM was stable up to 24 hours at 4 °C but decreased by 15% after 48 hours and continued decreasing over time by more than 30 % within the week of the stability study. MOR and COD concentrations increased after storage for 24 hours at 4 °C by 8 and 9% respectively and increased by 45% and 48%, respectively, after one month of storage as a result of the hydrolysis of 6-MAM and 6-AC to MOR and COD respectively. Increases in MOR and codeine concentrations after 24 hours at room temperature were similar to those observed after one month at 4 °C.

Deaths attributed to heroin use were classified into three types using the concentration of free morphine and its glucuronides (in blood: immediate death, sub-acute death (death in less than 3h), and delayed death (death after 3h). The poly-drug intoxication phenomenon, especially with ethanol, has been discussed in detail in this chapter as well as the presence of other central acting drugs and their role in heroin and other opioid related fatalities. The method was found useful for differentiating between users of heroin and other opioids, such as codeine and morphine, and for determining the survival time in deaths attributed to heroin use.

In addition to the value of using LC-MS techniques in forensic toxicology, Chapter 6 describes an application for clinical toxicology that has been developed for the analysis of diamorphine in paediatric samples; In recent years, intra-nasal diamorphine (DIM) has been recommended as an alternative to intravenous administration in children for the treatment of acute, severe pain because it is a less traumatic means of administering rapid, powerful analgesia to children in whom obtaining intravenous access may be technically difficult and distressing. This work was aimed at obtaining pharmacokinetic data for DIM and its metabolites in children following intravenous (IVDIM) and intranasal (INDIM) administration in a blind study. Since DIM is known to act as a pro-drug and to achieve analgesia via its metabolites, it was intended that the concentrations of active DIM metabolites would be used to evaluate whether or not INDIM can deliver rapid and efficient analgesia in children comparable to that obtained

with IVDIM. The need for a sensitive technique for the detection and quantitation of heroin and its metabolites is essential due to low concentrations of heroin and metabolites in children's plasma, as a result of the low dose of heroin given, and the limited sample volume obtained from children (0.25 mL or less). In addition, diamorphine can be easily hydrolysed to 6-monoacetylmorphine (6-MAM) during sample preparation and extraction, so this must be considered when developing a solid-phase extraction (SPE) method to prevent the hydrolysis of heroin.

The pharmacokinetics of DIM and its metabolites following IN and IV administration in children have been compared for the first time in this study, which confirmed that INDIM can achieve therapeutic plasma concentrations of active metabolites, although these are lower than those obtained with IVDIM and occur at later times after administration.

In Chapter 7, the efficiency of a hydrolysis method used for opioid analysis has been examined by comparison with direct analysis without the hydrolysis step, in the case of buprenorphine analysis for the first time using 21 real case samples. Results from the two methods correlated well, although the direct method gave slightly higher concentrations for BUP metabolites compared to the hydrolysis method. However, it was confirmed that the in-house hydrolysis procedure was effective in cleaving glucuronide conjugates at the range of concentrations encountered in cases in this study, which indicate buprenorphine abuse. Also, the hydrolysis rates of BUP3G and NBUP3G are different and it is important that hydrolysis methods are optimised using both glucuronide metabolites. The two methods used in this study gave the same ratios for TNBUP/TBUP. The ratios between conjugated metabolites and their free forms are of interest and can be helpful in the examination of enzymatic hydrolysis methods. In addition, ratios may be of value in interpretation of cases but additional work is required involving controlled administration studies to allow this to be evaluated.

The focus of Chapter 8 was to report fatalities involving oxycodone in the West of Scotland using an LC-ESI-MS/MS method developed for the determination of oxycodone and N- and O-demethylated metabolites, noroxycodone and oxymorphone, in unhydrolysed post-mortem specimens.

Ten oxycodone positive post-mortem cases were detected during the period July 2007-December 2008 in which nine deaths were drug related fatalities. Five of these were attributed solely to oxycodone intoxication and four cases to mixed drug intoxication. High levels of oxycodone in combination with low levels of noroxycodone and oxymorphone were identified in one case of suicide involving the deliberate ingestion of multiple tablets of OxyContin™. In four cases a number of undigested oxycodone tablets were identified in the stomach contents. Although there was overlap between blood oxycodone concentrations in deaths attributed to oxycodone only and poly-drug intoxication, the latter were associated with oxycodone concentrations less than 1 mg/L, while most cases in which oxycodone was the cause of death often had blood concentrations higher than 1 mg/L.

The role of parent drug in oxycodone fatalities has been fully studied but the role of the metabolites noroxycodone and oxymorphone in oxycodone fatalities was investigated in this report for the first time. Oxycodone was most commonly present in blood, urine and vitreous humour followed by noroxycodone. In some cases oxymorphone was not detected in blood but was found in urine. The ratio between parent drug and its N-demethylated metabolite is a useful tool for determining whether death occurred shortly after drug overdose ingestion or if it was delayed. It was found that the higher the ratio the shorter the elapsed time after ingestion before death occurred.

The current study (Chapter 8) is the first to report blood and urine noroxycodone and oxymorphone concentrations after acute oxycodone overdoses. It is also the first to report the vitreous humour level of noroxycodone following oxycodone intoxication in two cases. In addition, it is the first reported LC-MS/MS application to oxycodone related fatality cases in forensic toxicology. Also, stability of noroxycodone in blood and urine matrices and vitreous humour levels of noroxycodone following oxycodone intoxication was reported for the first time in this work. Oxycodone prescriptions have risen sharply in Scotland in recent years and the identification of ten oxycodone-related deaths in the past 18 months highlights the importance of including this drug in routine laboratory screening and confirmation procedures.

In Chapter 9, a complementary method using urine samples was developed and applied for routine opioid analysis together with blood samples. The results obtained by the optimised LC-MS/MS method in blood samples were compared with standard operation procedure routinely used for analysis of opioids; good correlation coefficients were observed between the concentrations obtained using the two methods for the free drug analytes methadone, MOR, COD and DHC. The use of two different chemical principle techniques is the method of choice for identification of drug in forensic toxicology. In addition, the identification criteria proposed by EU guidelines, FDA and SOFT/AAFS were also discussed in Chapter 9. A problem encountered with current LC-MS/MS instruments in providing sufficient identification points was discussed. It was discovered that two SRM transitions can be obtained for in most opioids with the exception of some polar metabolites, especially glucuronide metabolites which only fragment to one product ion. Therefore, the adjustment of collision energy voltages was used to obtain two SRM transitions which were found reliable and reproducible using both standard and case samples.

The interpretation of alcohol results in post-mortem specimens can be difficult due to the possibility of post-mortem production of alcohol, which may take place in the body or in the autopsy samples. Ethyl glucuronide (ETG) and ethyl sulfate (ETS) are primary ethanol metabolites and have been shown to be useful markers of alcohol consumption for several hours after death or when ethanol itself has been completely eliminated from the body. ETG and ETS are very polar metabolites requiring very low percentages of organic modifiers (less than 5%) for elution from a conventional reversed phase column, which results in poor retention, large matrix effects and low sensitivity in LC-MS. Post-column addition of organic solvent can enhance ESI-MS/MS response while preserving good chromatographic peak shapes. Recently, hydrophilic interaction chromatography (HILIC) has been introduced as an alternative to reverse phase LC separation of polar compounds. HILIC is suitable for ESI-MS as a high percentage of organic modifiers can be used, up to 95%, without reducing analyte retention.

In Chapter 10, a novel method using an ion trap mass analyser in combination with a hydrophilic interaction liquid chromatography column has been validated and applied for routine urine analysis for the first time. The second aim was to

employ the optimised method for the separation of these polar metabolites for analysis of urine samples obtained at autopsy. In the current study, urine samples were divided into three groups depending on BAC in order to determine if there was any correlation between BAC and ETG and ETS levels in the urine samples.

The method has been used for analysis of ETG and ETS in routine case samples and has been shown to be a useful tool to indicate recent ethanol consumption. It was found that a false positive BAC is more likely to be encountered when the BAC is low. The use of ETG as ethanol biomarker is more reliable than applying the method using ETS as sole evidence of alcohol ante-mortem consumption which may lead to false negative results. This was because ETG was found positive in cases negative for ETS but there were no positive ETS cases found negative for ETG. However, it is highly recommended to use both ethanol biomarkers in heavily putrefied cases and when the ethanol level in post-mortem blood is low. To the authors knowledge, this is the first report of the determination of ETS using an LC-ESI-ion trap-MS/MS method, and of a HILIC-ESI-ion trap-MS/MS method for the simultaneous determination of ETG and ETS in post-mortem urine samples

12 Future Work

LC-MS/MS has become the cornerstone in forensic and clinical toxicology investigations; this technique has moved from development stages to practical routine work [16]. In the current project, initial work was established using this technique for many applications to help toxicologists make decisions for identification and quantification of opioids and ethanol accurately.

However, the matrix effects phenomenon is still under investigation and still the major drawback in modern analytical methods using LC-MS/MS; a proper matrix effects investigation is necessary. A new trend in using fast extraction procedures or new materials for extraction and analyte separation has recently appeared. It is dependent upon the intended purpose of the method; liquid-liquid extraction is still in use due to enhanced sensitivity of LC-MS/MS instrumentation available. A direct injection procedure with simple sample pre-treatment is employed with clean matrices such as oral fluid and urine which need to be diluted in order to reduce the effects of matrices.

Solid phase extraction is the method of choice with new SPE materials appearing such as molecular imprinted SPE which is an inexpensive and can be used up to 100 times. However, it is limited to groups of drugs compared to that of conventional SPE cartridges such as mixed mode SPE. In addition, HILIC materials could be used in future for extraction of very polar metabolites.

The application of high organic modifier content in mobile phases is favoured for enhancing the response of ESI-MS/MS analysis. Few applications using HILIC phase for the separation of polar metabolites in forensic toxicology have been reported. Although these methods are efficient for very polar metabolite separation, it is still unsuitable for the simultaneous analysis of a wide range of drug and metabolite analysis. In addition, a recent shortage of acetonitrile has created urgency in finding an alternative solvent that can replace acetonitrile in both RPLC and HILIC phases without affecting method sensitivity and selectivity.

Highly accurate mass analyser instrumentation is now available and direct methods for drug and metabolite analysis should replace traditional hydrolysis method. Future work should entail simplifying the extraction method to be low

cost, fast and able to obtain as much information that could help on interpretation to avoid misinterpretation. An increase in the number of analytes included in each run is necessary to avoid overlooked analytes and false or positive results due to interference of isobaric fragment ions between analytes of interest.

At the time of the study reported in Chapter 9, few cases had been measured for both morphine and codeine as total and free drug. However, the laboratory method for general opioids screening was not optimised for analysis of total and free morphine, which reduces the value of comparing total morphine with heroin or codeine cases. In the routine method for analysis of heroin cases, an enzymatic hydrolytic method is used. As mentioned in the thesis with respect to buprenorphine, the laboratory enzymatic hydrolysis method may produce inaccurate results due to incomplete hydrolysis. For example, Wang *et al*. reported that enzymatic methods of hydrolysis are not accurate for the cleavage of morphine-6-glucuronide and codeine-6-glucuronide to their free form. They reported a negative result for morphine tested positive by immunoassay (400 ng/mL) and GC-MS method using acidic hydrolysis (271 ng/mL). Optimisation of the hydrolysis method was considered, but due to the limited time available, this work will need to be continued in future.

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14 Appendix 1: Publications in Support of this Thesis

Conferences

1. Method for Quantification of Opioids and their Metabolites in Autopsy Blood by Liquid Chromatography-Tandem Mass Spectrometry. Proceedings of The International Association of Forensic Toxicologists meeting, Ljubljana, Slovenia, 2006.
2. A Method for the Quantification of Heroin and its Metabolites in Plasma by Liquid Chromatography-Tandem Mass Spectrometry. Proceedings of The International Association of Forensic Toxicologists meeting, Seattle, USA, 2007.
3. Comparison between of a nonhydrolysis method and hydrolysis method for the detection of Buprenorphine metabolites in urine by LC-MS-MS. One day conference of Forensic Science Society.
4. Determination of 27 Opioids and Their Metabolites in Urine by LC-MS-MS. Proceedings of The International Association of Forensic Toxicologists meeting, Martinique, France West Indies, 2008.
5. Pharmacokinetics of Diamorphine in children following intravenous and intranasal administration. Proceedings of The International Association of Forensic Toxicologists meeting, Martinique, France West Indies, 2008.
6. Direct Determination of Ethyl Glucuronide and Ethyl Sulphate in Post-mortem Specimens using Hydrophilic Interaction Liquid Chromatography-ESI-MS. Proceedings of the Society of Forensic Toxicologists meeting, Pheonex, Arizona, United States of America, 2005.
7. Oxycodone Related Fatalities in the West of Scotland. Proceedings of the Society of Forensic Toxicologists meeting, Pheonex, Arizona, United States of America, 2005.
8. The Role of Oxycodone Metabolites in Oxycodone Related Fatalities in the West of Scotland. Proceedings of The International Association of Forensic Toxicologists meeting, Geneva, Switzerland, 2009.

Full Paper

1. Method for Quantification of Opioids and their Metabolites in Autopsy Blood by Liquid Chromatography-Tandem Mass Spectrometry. *Journal of Analytical Toxicology*. 31(7):394-408 (2007).
2. Comparison between of a nonhydrolysis method and hydrolysis method for the detection of Buprenorphine metabolites in urine by LC-MS-MS. *Journal of Analytical Toxicology*. 32(9): 744-753(2008).
3. Oxycodone Related Fatalities in the West of Scotland. *Journal of Analytical Toxicology*. 33(8): 423-432(2009).

Award

1. A young Scientists Award for Best paper published in 2006/07 during the International Association of Forensic Toxicologists meeting, Martinique, France West Indies (2008).

2. Distinguishing achievement Award during The third Saudi Conference held on Serri University, United Kingdom, June 2009.

Method for Quantification of Opioids and their Metabolites in Autopsy Blood by Liquid Chromatography-Tandem Mass Spectrometry.

Ahmed I. Al-Asmari and Robert A. Anderson; Forensic Medicine and Science Department, University of Glasgow,University Place, G12 8QQ, Scotland

Abstract
A method using LC/ESI/MS/MS was developed and validated for the determination of morphine, codeine, hydromorphine, dihydrocodeine, oxycodone, buprenorphine, and naloxone with their metabolites morphine-3-glucuronide, morphine-6-glucuronide, normorphine, 6-acetylmorphine, 6-acetylcodeine, codeine-6-glucuronide, norcodeine, hydromorphine-3-glucuronide, dihydrocodeine-6-glucuronide, norbuprenorphine, buprenorphine-3-glucuronide, norbuprenorphine-3-glucuronide, and naloxone-3-glucuronide in human whole blood. Polar metabolites (glucuronides) and other analytes were extracted by SPE using Bond Elut. Chromatographic separation was performed on a Synergi reverse phase column with gradient elution based on a mobile phase consisting of 10 mM ammonium formate adjusted to pH 3 (A) and acetonitrile (B) at flow rate 0.3 mL/min in the first 13 min, following by 0.2 mL/min for the next 13 min, after that the initial flow rate was applied until the end of analysis. The gradient conditions were: initially, 97% of solution A for 3 min, decreasing to 84.5% at 8 min, to 74% at 13 min and to 20% at 26 min. 5% of solution A was maintained for the next 3 min before returning to 97% for 7 min prior to the next injection. Intra-day and inter-day precision for all analytes were between 0.6 to 13.8% and recoveries were between 82% and 101.4%. Calibration curves were linear for all analytes over the concentration range 5-400 ng/mL and correlation coefficients (R2) were better than 0.999. Limits of detection and limits of quantitation were 0.16 - 1.2 ng/mL and 0.5 - 4.09 ng/mL, respectively. The method described consolidates previous work on opioids and their metabolite published in the literature and is the first to include the detection of naloxone-3-glucuronide. The method has been applied in routine post-mortem cases after opiate overdose with three.

Ai ms: it has been used to interpret the cause of death, to determine type of death (rapid or immediate death, sub-acute death, or delayed death) and to distinguish between heroin, morphine and codeine users.

A Method for the Quantification of Heroin and its Metabolites in Plasma by Liquid Chromatography-Tandem Mass Spectrometry

Ahmed I. Al-Asmari* and Robert A. Anderson
Department of Forensic Medicine& Science, University of Glasgow, UK

AIMS: In recent years, intra-nasal heroin has been recommended as an alternative to intravenous administration for the treatment of acute severe pain in children. This provides a rapid and less painful route of administration without decreasing the effectiveness of the analgesic properties. The need for a sensitive technique for the detection and quantitation of heroin and its metabolites is essential due to low concentrations of heroin and metabolites in children's plasma, as a result of the low dose of heroin given, and the limited sample volume obtained from children (0.25 mL or less). In addition, Heroin can be easily hydrolysed to 6-monoacetylmorphine (6-MAM) during sample preparation and extraction, so this must be considered when developing a solid-phase extraction (SPE) method to prevent the hydrolysis of heroin.
METHODS: 250 µl of plasma was added to 300 µl of 0.01 M ammonium carbonate, pH 9.3 and 25 µl of the internal standard working solution (1 µg/ml) was added. The mixture was vortex mixed. The supernatant was applied to a Bond Elut C18 SPE cartridge preconditioned with 2 ml methanol, 1 ml of deionised water, and 2 ml of 0.01 M ammonium carbonate (pH 9.3). The SPE cartridge was washed twice with 1 ml 0.01 M ammonium carbonate (pH 9.3), and then dried for 10 minutes. Retained drugs were eluted with 2 ml methanol, after which the eluate was evaporated to dryness under nitrogen at 50 ^{o}C. The extract was reconstituted with 80 µl of initial mobile phase and the 20 µl were injected into using a Thermo Finnigan LCQ DECA XP Plus ion trap instrument (Thermo Finnigan, San Jose, USA) equipped with a surveyor LC system interface. Chromatographic separation was achieved using a Synergy Polar RP column (150 x 2.0 mm, 4-µm particle size), protected by a guard column with identical packing material (4 x 2.0 mm, Phenomenex, Torrance, CA). Gradient elution was based on a mobile phase consisting of 10 mM ammonium formate adjusted to pH 3 (A) and acetonitrile (B) at a flow rate of 0.3 ml/min in the first 8 min, decreasing to 0.2 ml/min at 13 min for the next 13 min. After that, the initial flow rate was applied until the end of analysis. The gradient conditions were initially 97% of solution A for 3 min; decreasing to 84.5% at 8 min, to 74% at 13 min and to 20% at 26 min, 5% of solution A was maintained for the next 3 min before returning to 97% for 7 min prior to the next injection.
RESULTS: Intra-day and inter-day precision for all analytes were determined at three concentration 1, 5, and 25ng/ml and these were found to be 2.5-13.4% and 1.8-15% respectively. Recoveries of analytes of interest were between 81% and 109%. Calibration curves were linear for all analytes over the concentration range 0.1-50 ng/mL and correlation coefficients (R^2) were better than 0.999. Limits of detection and limits of quantitation were 0.08-0.37 ng/mL and 0.28-1.22 ng/mL respectively. **CONCLUSIONS:** A sensitive and specific method for the quantitation of heroin metabolites, namely heroin, 6-MAM, morphine, morphine-3-glucuronide, morphine-6-glucuronide and normorphine in human plasma was developed and validated. This method was developed for testing plasma samples obtained from children who are under treatment for acute severe pain and data of samples tested will be presented in the future.

Comparison between of a nonhydrolysis method and hydrolysis method for the detection of Buprenorphine metabolites in urine by LC-MS-MS.

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Abstract

BACKGRAOUND: Buprenorphine (BUP) is a semisynthetic opioid obtained from thebaine after a seven step chemical procedure. BUP has been used for the treatment of moderate to severe pain for more than 20 years for case in surgical and neoplastic origin [266], and in the treatment of opiate dependence, BUP has become an abused drug. Therefore, method for quantification BUP metabolites in urine was required due to the fact that most cases submitted for BUP analysis were urine samples. Although enzymatic hydrolysis method for quantification of total BUP and its main metabolites norbupenorphine (NBUP) were widely employed, hydrolysis method were found to be consuming time and inaccurate due to many limitation, including incomplete hydrolysis, introduction of interferences in the analysis. **AIMS**: the aims of this work was to develop and validate a method for the direct determination (DM) of buprenorphine (BUP), norbuprenorphine (NBUB), buprenorphine-3-glucuronide (B3G), norbuprenorphine-3-glucuronide (NBUP3G). This method was compared with in house enzymatic hydrolysis method (HM) using β-glucuronidase from *Helixa* which used routinely by Forensic Medicine and Science department, University of Glasgow for the determination of total buprenorphine (TBUP) and norbuprenorphine (TNBUP) using real positive BUP urine case sample. **METHODS:** Analytes of interest were extracted by Bond Elute C18 for DM, following by LC/MS/MS analysis using a Synergy Polar RP column (150 x 2.0 mm, 4 µm particle size). Gradient elution was based on a mobile phase consisting of 10 mM ammonium formate adjusted to pH 3 (A) and acetonitrile (B). **RESULTS :** Over one year of this study, all positive BUP cases were tested using HM and DM at the same time.17 cases were tested positive using DM and 14 case by using HM. The ratios of TNBUP/TBUP were calculated for both method and the ratios of free drugs over its glucuronides were also calculated using DM. **CONCLUSIONS:** The DM was more sensitive and all BUP metabolites were detected in most cases with comparing to HM. The comparison between the direct detection of buprenorphine metabolites including BUP, NBUP and their glucuronides obtaine by DM with TBUP and TNBUP using HM was reported for the first time in this work.

Determination of 27 Opioids and Their Metabolites in Urine by LC-MS-MS

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AIM: The aims were to develop a validated method for the determination of 27 commonly encountered opioids and metabolites in unhydrolysed post-mortem urine. and apply it to matched autopsy urine and blood samples, to assess the value of using both matrices in interpreting the cause of death and source of the opioids detected. **METHODS:** Following addition of 14 deuterated internal standards, analytes were extracted by SPE with Bond Elut C18® cartridges, followed by LC-MS-MS analysis using a Thermo-Finnigan LCQ Deca Plus instrument in the ESI SRM mode, fitted with a Synergy Polar RP column (150 x 2.0 mm, 4 µm). Gradient elution used a mobile phase with (A) 10 mM ammonium formate, pH 3 and (B) acetonitrile, at a flow rate 0.3 ml/min. Analytes were identified on the basis of their retention times and the relative intensities of their pseudo-molecular ions and two product ions.**RESULTS: Method validation**: acceptance criteria for linearity, precision, and recovery were achieved for all 27 analytes. Intra-day and inter-day precision were between 1-15 %. Calibration curves were linear for all analytes over the concentration range 5-250 ng/mL and correlation coefficients (R^2) were better than 0.999. LOD and LLOQ were 0.2-0.5 ng/mL and 0.5 – 1.6 ng/mL, respectively. Recoveries were 71-111%. No interference was detected with other common drugs. **Matrix effects**: matrix effects on analyte ionisation were investigated using five different human urine sources at two concentrations (5 and 100 ng/ml). Analyte responses were within ±20% of values obtained with unextracted standards. **Stability:** analytes were stable under different storage conditions, apart from 6-acetylmorphine (6-MAM) and 6-acetylcodeine (6-AC) at room temperature or at 4 °C. The rates of hydrolysis of 6-MAM and 6-AC in urine were low compared to blood samples. The stabilities of naloxone-3-glucuronide, dihydrocodeine-6-glucuronide, dihydromorphine-3-glucuronide and dihydmorphine-6-glucuronide in urine samples are reported for the first time. **Case Samples:** Matched blood and urine samples from 47 post-mortem cases and urine samples from 13 living subjects were analysed using the validated method for urine and our previously published method for autopsy blood. Total morphine to total codeine ratios were calculated to determine which opioid had been used. Also, survival time after last injection was investigated by calculating ratios between free drugs and metabolites, as described in our previous publication [*Al-asmari and Anderson (2007). J Anal Toxicol 31, 394-408*]. The role of opioids in combination with other drugs, including alcohol, cocaine and benzodiazepines, in opioid-related deaths is discussed. **CONCLUSIONS:** A sensitive and selective method was developed for the simultaneous determination of 27 opioids and metabolites in urine. The method could differentiate between users of heroin and other opioids, such as codeine and morphine. Furthermore, urine was the sample of choice for detection of buprenorphine and norbuprenorphine and their glucuronides.

Pharmacokinetics of Diamorphine in children following intravenous and intranasal administration

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In recent years, intra-nasal heroin has been recommended as an alternative to intravenous administration in children for the treatment of acute, severe pain because it is a less traumatic means of administering rapid, powerful analgesia to children in whom obtaining intravenous access may be technically difficult and distressing. **Aims**: This work was aimed at obtaining pharmacokinetic data for DIM and its metabolites in children following intravenous (IV-DIM) and intranasal (IN-DIM) administration in a blind study. Since DIM is known to act as a pro-drug and to achieve analgesia via its metabolites, it was intended that the concentrations of active DIM metabolites would be used to evaluate whether or not IN-DIM can deliver rapid and efficient analgesia in children comparable to that obtained with IV-DIM. **METHODS**: Plasma samples were obtained from twenty three children receiving DIM at the A & E department of a city-centre paediatric teaching hospital in Edinburgh. 13 children received IV-DIM (dose 0.1mg/kg) and 10 subsequent children had IN-DIM at the same dose in 0.2 ml normal saline dripped into both nostrils. The children were aged 3-13 years, with clinical diagnosis of isolated deforming limb fractures. Sequential blood samples were taken at 2, 5, 10, 20, 30 and 60 minutes post DIM administration. The blood tube was then centrifuged at 4000 rpm for 2 minutes and plasma was transferred to plain 'Ependorph' tubes that were immediately placed in a -70^{o}C freezer until analysed. Plasma samples were subsequently analysed for DIM, 6-monoacetyl morphine (6-MAM), morphine (MOR), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and normorphine (NMOR) using a method involving solid phase extraction and liquid chromatography-tandem mass spectrometry (LC-MS-MS) that was validated for this purpose. **RESULTS:** Concentrations of analytes were found to depend on the route of administration. The concentration of DIM metabolites using IN-DIM were lower than IV-DIM at the same dose and body weight, DIM concentrations were range of 190 to 2062 ng/ml and 2 to 43 ng/ml for IV-DIM and IN-DIM, respectively. The median peak concentrations of analytes of interest after IV-DIM were achieved at 2 min for DIM, 6-MAM and MOR, and at 20 and 60 min for M3G and M6G respectively. After IN-DIM, median peak concentrations were achieved for DIM at 2 min. However, DIM metabolites achieved different median peak concentrations after IN-DIM, it was achieved at 5 and again at 10 for 6-MAM, for MOR at 20 min and for both morphine glucuronides at 60 min. Ratios of concentrations (IV-DIM/IN-DIM) were calculated using the median peak concentrations and were 34, 26.4, 6.2, 3.8 and 2.9 for DIM, 6-MAM, MOR, M3G and M6G respectively. Following IV-DIM, the concentrations of DIM, 6-MAM and MOR decreased sharply after 2 min whereas the rate of decrease was slower after IN-DIM. Pharmacokinetic parameters i.e. C_{max}, T_{max}, $t_{½}$ and the area under the plasma curve were calculated for the two patient groups. These were compared with each other and with previous work in adult patients. **CONCLUSIONS:** The pharmacokinetics of DIM and its metabolites following IN and IV administration in children have been compared for the first time in this study, which confirmed that IN-DIM can achieve therapeutic plasma concentrations of active metabolites, although these are lower than those obtained with IV-DIM and occur at later times after administration.

Direct Determination of Ethyl Glucuronide and Ethyl Sulphate in Post-mortem Specimens using Hydrophilic Interaction Liquid Chromatography–ESI-MS.

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Background: The interpretation of alcohol results in post-mortem specimens can be difficult due to the possibility of post-mortem production of alcohol, which may take place in the body or in the autopsy samples. Ethyl glucuronide (ETG) and ethyl sulphate (ETS) are primary ethanol metabolites and have been shown to be useful markers of alcohol consumption for several hours after death or when ethanol itself has been completely eliminated from the body. ETG and ETS are very polar metabolites requiring very low percentages of organic modifiers (less than 5%) for elution from a conventional reversed phase column, which results in poor retention, large matrix effects and low sensitivity in LC-MS. Post-column addition of organic solvent can enhance ESI-MS-MS response while preserving good chromatographic peak shapes. Recently, hydrophilic interaction chromatography (HILIC) has been introduced as an alternative to reverse phase LC separation of polar compounds. HILIC is suitable for ESI-MS as a high percentage of organic modifiers can be used, up to 90%, without reducing analyte retention. **Aims**: This work was aimed at developing and validating a HILIC-ESI-ion trap-MS-MS method for identification and quantification of ETG and ETS as ethanol biomarkers and at employing this method for routine analysis of post-mortem samples. **Methods**: Following addition of pentadeuterated internal standards for ETG and ETS, 200 µl of acetonitrile was added to 0.1 ml of urine and centrifuged at 10000 rpm. The supernatant was then evaporated before reconstituting with 100 µl of initial mobile phase. Analytes of interest were separated on a ZIC-HILIC column (150 x 2.1 mm, 3.5 µm) (SeQuant, Umea, Sweden) connected to a Thermo-Finnigan LCQ Deca Plus LC-MS-MS instrument operated in the ESI-SRM mode. Gradient elution used a mobile phase with (A) 5 mM ammonium acetate and (B) acetonitrile. Analytes were identified on the basis of their retention times and the relative intensities of their pseudo-molecular ions and two product ions. **Case samples**: 90 urine case samples were divided into three groups depending on the ethanol concentration found in blood and analysed by the developed method: group A with post-mortem blood ethanol higher than 0.2 g/100 mL; group B with ethanol concentration in the range 0.08 to 0.2 g/100 mL and group C with ethanol concentration less than 0.08 g/100

mL. **Results:** ETG and ETS had high recoveries of 98-99 % and the HILIC column produced fine, sharp peak shapes and achieved baseline separation in less than 7 min. The calibration model was linear over a concentration range of 0.05-10 mg/L and correlation coefficients (R^2) were better than 0.999. In addition, a lower limit of quantification of 0.001 mg/L was obtained and no matrix effects were observed. The method has been used for analysis of ETG and ETS in routine case samples and has been shown to be a useful tool to indicate recent ethanol consumption. Both ethanol markers were detected in all groups with overall median concentrations of 113.6 mg/L and 23.2 mg/L for ETG and ETS, respectively. **Discussion and Conclusions:** It can be concluded that the risk of false positive ethanol results increased in the low ethanol concentration group as several cases tested negative for both biomarkers in group C. ETG was detected at low concentrations in some cases for which ETS tested negative, suggesting that ETG may have a longer half life in urine or else ETS is unstable. Our data were compared with previous studies and confirm that both ethanol biomarkers should be determined in heavily putrefied cases and when the ethanol level in post-mortem blood is low, which suggest the production of ethanol after death. To the authors' knowledge, this is the first report of the determination of ETS using an LC-ESI-ion trap-MS-MS method, and of a HILIC-ESI-ion trap-MS-MS method for the simultaneous determination of ETG and ETS in post-mortem urine samples.

Oxycodone Related Fatalities in the West of Scotland

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Objectives: In the United Kingdom, oxycodone is available on prescription in two forms, OxyContin (slow-release tablets) and OxyNorm (liquid/capsules). In Scotland, the number of prescriptions for oxycodone has risen by 430% since prescribing began in 2002 due to a shortage of diamorphine. Oxycodone misuse and related fatalities have been widely reported in the USA but it is hoped that stricter regulations will prevent a similar trend in the UK. The focus of this study is to review fatalities involving oxycodone in the West of Scotland using an LC-ESI-MS-MS method developed for the determination of oxycodone and its metabolites in post-mortem specimens. **Methods and Materials**: Four cases were identified where oxycodone was implicated in the death. Deuterated internal standards were added to blood samples collected post-mortem and analytes extracted using Bond Elut C18® cartridges. Separation was achieved using a Synergy Polar RP column (150 x 2.0 mm, 4 µm), gradient elution (mobile phase with (A) 10 mM ammonium formate, pH 3 and (B) acetonitrile), at a flow rate 0.3 ml/min using a Thermo-Finnigan LCQ Deca Plus instrument in the ESI SRM mode. **Results**: The developed method for oxycodone and its metabolites noroxycodone and oxymorphone was linear over the concentration ranges 5 - 250 ng/mL and 50 - 5000 ng/ml with correlation coefficients (R^2) greater than 0.999. Limits of detection and lower limits of quantification were 0.2 - 0.4 ng/mL and 1.0 - 1.2 ng/mL, respectively. Oxycodone, noroxycodone and oxymorphone were detected in three cases of oxycodone intoxication. In one case of poly-drug intoxication, oxycodone and noroxycodone were detected at low levels but oxymorphone was not present. **Conclusion:** A sensitive, selective and robust method for the determination of oxycodone, noroxycodone and oxymorphone was validated and applied to oxycodone intoxication cases. High levels of oxycodone in combination with low levels of noroxycodone and oxymorphone were identified in three cases of suicide involving the deliberate ingestion of multiple tablets of OxyContin. In all cases a number of undigested OxyContin tablets were identified in the stomach contents. Oxycodone prescriptions have risen sharply in Scotland in recent years and the identification of four oxycodone-related death in the past 10 months in the Strathclyde region of Scotland alone highlights the importance of including this drug in routine laboratory screening and confirmation procedures.

The Role of Oxycodone Metabolites in Oxycodone Related Fatalities in the West of Scotland

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Introduction: In the United Kingdom, oxycodone is available on prescription in two forms, OxyContin (slow-release tablets) and OxyNorm (liquid/capsules). In Scotland, the number of prescriptions for oxycodone has risen by 430% since prescribing began in 2002.
Aim: The focus of this study was to review fatalities involving oxycodone in the West of Scotland using an LC-ESI-MS-MS method developed for the determination of oxycodone and N- and O-demethylated metabolites, noroxycodone and oxymorphone, in unhydrolysed post-mortem specimens.
Methods: Deuterated internal standards were added to samples collected post-mortem and analytes extracted using Bond Elut C18® cartridges. Separation was achieved using a Synergy Polar RP column (150 x 2.0 mm, 4 μm), gradient elution (mobile phase with (A) 10 mM ammonium formate, pH 3 and (B) acetonitrile), at a flow rate 0.3 ml/min using a Thermo-Finnigan LCQ Deca Plus instrument in the ESI SRM mode.
Results: The developed method for oxycodone and its metabolites noroxycodone and oxymorphone was linear over the concentration ranges 5 - 250 ng/mL and 50 - 5000 ng/ml with correlation coefficients (R^2) greater than 0.999. Limits of detection and lower limits of quantification were 0.2 - 0.4 ng/mL and 1.0 - 1.2 ng/mL, respectively. Ten oxycodone positive post-mortem cases were detected during the period July 2007-December 2008 in which nine deaths were drug related fatalities. Five of these were attributed solely to oxycodone intoxication and four cases to mixed drug intoxication. High levels of oxycodone in combination with low levels of noroxycodone and oxymorphone were identified in one case of suicide involving the deliberate ingestion of multiple tablets of OxyContin™. In four cases a number of undigested oxycodone tablets were identified in the stomach contents. Although there was overlap between blood oxycodone concentrations in deaths attributed to oxycodone only and poly-drug intoxication, the latter were associated with oxycodone concentrations less than 1 mg/L, while most cases in which oxycodone was the cause of death often had blood concentrations higher than 1 mg/L. The role of parent drug in oxycodone fatalities has been fully studied but the role of the metabolites noroxycodone and oxymorphone in oxycodone fatalities was investigated in this report for the first time. Oxycodone was most commonly present in blood, urine and vitreous humor followed by noroxycodone. In some cases oxymorphone was not detected in blood but was found in urine. The ratio between parent drug and its N-demethylated metabolite is a useful tool for determining whether death occurred shortly after drug overdose ingestion or if it was delayed. It was found that the higher the ratio the shorter the elapsed time after ingestion before death occurred.
Conclusion: Oxycodone prescriptions have risen sharply in Scotland in recent years and the identification of ten oxycodone-related deaths in the past 18 months highlights the importance of including this drug in routine laboratory screening and confirmation procedures.