CRANFIELD UNIVERSITY

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FORENSIC TOXICOLOGY IN EMBALMED HUMAN REMAINS

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PhD Thesis
Academic Year: 2020 - 2021

Supervisors: Drs Karl Harrison, Hannah Moore and Nathalie Mai
April 2021

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ABSTRACT

Within the human body, vitreous humour, cerebrospinal fluid and synovial fluid are all contained within anatomical compartments that, by virtue of their relative avascularity, provide considerable protection from contamination during the embalming process. Analysing samples taken from embalmed medical school cadavers, and using an entirely novel headspace GC/MS method, the concentration of formaldehyde in all three fluids is shown to be significantly less than has previously been assumed in the literature. Using a newly-developed LC/Q-ToF method, the stability of fifteen drugs of forensic interest in representative concentrations of formaldehyde is then reported.

The study is prompted by the fact that United Kingdom nationals are dying abroad with increasing frequency. Following repatriation of their bodies, an autopsy may be undertaken before permission for a funeral is granted. Although toxicological screening may form an important part of the investigative processes, there exists a large degree of uncertainty surrounding the reactive nature of formaldehyde, the main constituent of embalming fluids, with substances commonly implicated in drug-related deaths. It is the case, therefore, that in repatriated bodies, all of which are routinely embalmed before leaving the country in which death occurred, the presence of formaldehyde can cause significant problems for the forensic toxicologist.

Although a number of papers in the past twenty years have investigated the challenges of drug detection in embalmed tissues, they have largely examined the problem from the perspective of histological samples preserved in formalin. While some of this work has, by implication, considered the analysis of samples taken from embalmed bodies, no studies have addressed directly the specific problems arising in the toxicological analysis of embalmed and repatriated bodies.

The studies reported within the thesis demonstrate that there is considerable merit in undertaking further work on the usefulness of these fluids in the toxicological analysis of embalmed and repatriated nationals.

Keywords:

Embalming, formaldehyde, repatriation, vitreous humour, cerebrospinal fluid, synovial fluid.

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The work presented in this thesis has come to fruition in no small part thanks to the advice, assistance, encouragement and inspirational teaching of a number of people.

The seeds for the project were sown during Dr Donald Peach's engaging neuropharmacology lectures during the course of the (now sadly defunct) 'DNAT' module of the Cranfield MSc FAA degree. The initial encouragement and guidance that I received from him in 2013 to pursue what was by any stretch of the imagination a relatively 'off-beat' topic, is something for which I am immensely grateful.

My primary supervisor in the earlier stages of the project, Dr Karl Harrison, has been the best sort of advisor that a part-time, 'mature' student could ever wish for. Trusting enough to allow me to pursue my own interests, but always willing to provide apposite and thought-provoking commentary, his mentoring has been an immense and hugely pleasurable educational experience. Dr Hannah Moore, latterly my primary supervisor, has been kind enough to allow me to follow my own instincts, and was instrumental in facilitating my access to Cranfield main site laboratories, thereby enabling much of the experimental work to take place. My greatest vote of supervisory thanks, however, must go to Dr Nathalie Mai, who has been with me since I first entered her laboratory, in 2013, as a complete analytical novice. Her patient teaching, boundless enthusiasm for my master's then doctoral projects, and constant generosity with her time have contributed probably more than anything else not only to the success of my work but also to the enjoyment of my time at Shrivenham.

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TABLE OF CONTENTS

ABSTRACT i
ACKNOWLEDGEMENTS iii
LIST OF FIGURES x
LIST OF EQUATIONS xii
LIST OF TABLES xiiii
LIST OF ABBREVIATIONS xv
GLOSSARY OF TERMS xvii
INTRODUCTION AND THESIS STRUCTURE 1
Introduction 1
Study Aims and Objectives 6
Thesis Structure 8
1 POST-MORTEM TOXICOLOGY 13
1.1 Introduction 13
1.2 Post-Mortem Toxicology 14
1.3 Post-Mortem Factors Affecting Toxicological Analysis 18
1.3.1 Toxicological matrices 18
1.3.1.1 Blood 18
1.3.1.2 Urine 20
1.3.1.3 Vitreous humour 22
1.3.1.4 Cerebrospinal fluid and synovial fluid 23
1.3.1.5 Gastric contents 25
1.3.1.6 Bile 26
1.3.1.7 Soft tissues 27
1.3.1.8 Hair 28
1.3.1.9 Bone, bone marrow and other matrices 28
1.3.1.10 Conclusions 29
1.3.2 Drug and sample stability 30
1.3.3 Post-mortem redistribution 37
1.4 Pre-Mortem Factors Affecting Toxicological Interpretation 43
1.4.1 Extent of drug absorption, distribution, metabolism and excretion before death 45
1.4.2 Drug tolerance 47
1.4.3 Physiology 49
1.4.4 Case history 51
1.4.5 Pharmacogenetics and pharmacogenomics 52
1.5 Conclusions 55
2 EMBALMING: PURPOSE, METHOD AND ASSOCIATED TOXICOLOGY LITERATURE 61
2.1 Introduction 61

2.2 Historical Basis of Embalming 62
2.3 Embalming Fluid Constituents 64
2.4 Embalming Method 66
2.4.1 Non-autopsy embalming method 67
2.4.2 Autopsy embalming method 68
2.4.3 Repatriation embalming method 69
2.5 Early Forensic Embalming Literature 70
2.6 Recent Embaling Forensic Literature 73
2.6.1 Benzodiazepines 73
2.6.2 Opiates and opioids 77
2.6.3 Stimulants 80
2.6.4 Antidepressants 84
2.6.5 Antipsychotics 87
2.6.6 Barbiturates 88
2.6.7 Other drugs and poisons 90
2.6.7.1 Volatile substances 90
2.6.7.2 Phenytoin 91
2.6.7.3 Paraquat 91
2.6.7.4 Heavy metals 92
2.6.7.5 Strychnine 92
2.6.7.6 Fenfluramine 93
2.6.7.7 Lidocaine 94
2.6.7.8 Sildenafil 94
2.6.7.9 Acetominaphen and salicylic acid 95
2.6.7.10 Aconitum alkaloids 95
2.6.7.11 Methamidophos 95
2.7 The Chemistry of Drug Degradation in the Presence of Formaldehyde 96
2.7.1 Antidepressants 96
2.7.2 Stimulants 98
2.7.3 Benzodiazepines 100
2.7.4 Barbiturates 101
2.8 Conclusions and Problems with Previous Work 101
3 CEREBROSPINAL FLUID, SYNOVIAL FLUID AND VITREOUS HUMOUR 111
3.1 Introduction 111
3.2 Cerebrospinal Fluid 113
3.2.1 Anatomy and physiology 113
3.2.2 Literature 115
3.2.3 Advantages and disadvantages 120
3.3 Synovial Fluid 121
3.3.1 Anatomy and physiology 121

3.3.2 Literature 123
3.3.3 Advantages and disadvantages 128
3.4 Vitreous Humour 129
3.4.1 Anatomy and physiology 129
3.4.2 Literature 131
3.4.3 Advantages and disadvantages 140
3.5 Conclusions 141
4 FORMALDEHYDE CONCENTRATIONS IN VITREOUS HUMOUR, SYNOVIAL FLUID AND CEREBROSPINAL FLUID OF EMBALMED HUMAN CADAVERS: AN EXPERIMENTAL STUDY 143
4.1 Introduction 143
4.2 Analytical Method 144
4.3 Sample Sourcing 147
4.4 Samples and Materials 149
4.4.1 Sample collection methods 150
4.4.2 Sample preparation 154
4.4.3 Stock and calibration solutions 154
4.5 Chromatographic Conditions 154
4.6 Method Validation 155
4.6.1 Selectivity, specificity, recovery and stability 156
4.6.2 Linearity 157
4.6.3 Instrument accuracy and precision 158
4.6.4 Lower limit of quantitation and lower limit of detection 159
4.6.5 Carryover 161
4.7 Results 161
4.7.1 'Hard-fix' cadavers 162
4.7.1.1 Vitreous humour 162
4.7.1.2 Synovial fluid 163
4.7.1.3 Cerebrospinal fluid 163
4.7.1.4 Stastistical analysis 164
4.7.2 'Soft-fix' cadavers 169
4.8 Discussion 170
4.8.1 Discussion of statistical results 175
4.8.2 Comparison between medical school embalming and repatriation embalming and the implications for results 179
4.8.3 Formaldehyde concentrations in 'soft-fix' cadavers 181
4.9 Conclusions 182
5 THE STABILITY OF DRUGS OF INTEREST IN FORMALDEHYDE 185
5.1 Introduction 185
5.2 Analytical Method and Development 190

5.3 Materials ... 191
5.3.1 Working, calibration, buffer and sample solutions ... 193
5.3.1.1 Working solutions ... 193
5.3.1.2 Calibration solutions ... 193
5.3.1.3 Buffered formaldehyde solutions ... 193
5.3.1.4 Sample solutions ... 194
5.4 Chromatographic and Mass Spectrometry Conditions ... 194
5.5 Method Validation ... 196
5.5.1 Selectivity, specificity, matrix effects and analyte stability ... 196
5.5.2 Linearity ... 197
5.5.3 Lower limit of quantitation and lower limit of detection ... 197
5.5.4 Carryover ... 197
5.5.5 Instrument accuracy and precision ... 198
5.6 Results and Discussion ... 198
5.6.1 Opiates and opioids results ... 199
5.6.1.1 6-MAM ... 199
5.6.1.2 Alfentanil ... 199
5.6.1.3 Carfentanil ... 200
5.6.1.4 Codeine ... 204
5.6.1.5 Fentanyl ... 204
5.6.1.6 Methadone ... 205
5.6.1.7 Morphine ... 206
5.6.2 Opiates and opioids discussion ... 211
5.6.3 Benzodiazepines and sedatives results ... 214
5.6.3.1 Diazepam ... 214
5.6.3.2 Flunitrazepam ... 215
5.6.3.3 Ketamine ... 215
5.6.3.4 Lorazepam ... 216
5.6.3.5 Midazolam ... 217
5.6.3.6 Zolpidem ... 217
5.6.4 Benzodiazepines and sedatives discussion ... 224
5.6.5 Stimulants results ... 231
5.6.5.1 Cocaine ... 231
5.6.5.2 BZE ... 231
5.6.6 Stimulants discussion ... 232
5.7 Conclusions ... 236
CONCLUSIONS AND FUTURE WORK ... 239
Conclusions ... 239
Limitations, Future Work and Implementation ... 241
REFERENCES ... 247

APPENDIX 1 - Summary of Chapter 1 literature review by subject and author 293
APPENDIX 2 - Published papers on the detection, stability and quantitation of drugs of abuse in embalmed tissue 296
APPENDIX 3 - Comparative post-mortem cerebrospinal fluid drug concentrations reported in the literature since 1984 305
APPENDIX 4 - Comparative post-mortem synovial fluid drug concentrations reported in the literature since 1993 312
APPENDIX 5 - Chromatogram and mass spectrometric data for formaldehyde 313
APPENDIX 6 - Preparation of working solutions 315
APPENDIX 7 - Preparation of calibration solutions 316
APPENDIX 8 - Preparation of buffer and buffered formaldehyde solutions 317
APPENDIX 9 - Preparation of sample solutions 318
APPENDIX 10 - Chromatographic, RT, [M+H]+, R, R2, LOQ, LOD and related data for all drugs 319
APPENDIX 11 - Instrument accuracy and precision 320
APPENDIX 12 - Analyte stability 321
APPENDIX 13 - Chromatogram and mass spectrometric data for 6-MAM 322
APPENDIX 14 - Chromatogram and mass spectrometric data for Alfentanil 323
APPENDIX 15 - Chromatogram and mass spectrometric data for BZE 324
APPENDIX 16 - Chromatogram and mass spectrometric data for Carfentanil 325
APPENDIX 17 - Chromatogram and mass spectrometric data for Cocaine 326
APPENDIX 18 - Chromatogram and mass spectrometric data for Codeine 327
APPENDIX 19 - Chromatogram and mass spectrometric data for Diazepam 328
APPENDIX 20 - Chromatogram and mass spectrometric data for Fentanyl 329
APPENDIX 21 - Chromatogram and mass spectrometric data for Flunitrazepam 330
APPENDIX 22 - Chromatogram and mass spectrometric data for Ketamine 331
APPENDIX 23 - Chromatogram and mass spectrometric data for Lorazepam 332
APPENDIX 24 - Chromatogram and mass spectrometric data for Methadone 333
APPENDIX 25 - Chromatogram and mass spectrometric data for Midazolam 334
APPENDIX 26 - Chromatogram and mass spectrometric data for Morphine 335
APPENDIX 27 - Chromatogram and mass spectrometric data for Zolpidem 336

LIST OF FIGURES

Figure 2-1 Chemical structure of formaldehyde 64
Figure 4-1 Scatterplots of Spearman's Rho test results showing correlations between formaldehyde concentrations detected in VH, SF and CSF 'hard-fix' samples 166
Figure 4-2 Scatterplots of Spearman's Rho test results showing correlations between volume of arterial embalming fluid injected and concentration of formaldehyde detected in each fluid 167
Figure 4-3 Scatterplots of Spearman's Rho test results showing correlations between time since embalming and concentration of formaldehyde detected in each fluid 168
Figure 4-4 Simple scatterplot of left vitreous humour formaldehyde concentrations by days since embalming 171
Figure 4-5 Simple scatterplot of right vitreous humour formaldehyde concentrations by days since embalming... 171
Figure 4-6 Simple scatterplot of left synovial fluid formaldehyde concentrations by days since embalming.. 172
Figure 4-7 Simple scatterplot of right synovial fluid formaldehyde concentrations by days since embalming.. 172
Figure 4-8 Simple scatterplot of cerebrospinal fluid formaldehyde concentrations by days since embalming.. 173
Figure 5-1 Structure and formula of 6-MAM 199
Figure 5-2 Structure and formula of alfentanil... 199
Figure 5-3 Structure and formula of carfentanil 200
Figure 5-4 Recovery (%) of 6-MAM in all concentrations of formaldehyde at 4°C...... 201
Figure 5-5 Recovery (%) of 6-MAM in all concentrations of formaldehyde at room temperature 201
Figure 5-6 Recovery (%) of alfentanil in all concentrations of formaldehyde at room temperature 202
Figure 5-7 Recovery (%) of alfentanil in all concentrations of formaldehyde at 4°C... 202
Figure 5-8 Recovery (%) of carfentanil in all concentrations of formaldehyde at room temperature 203
Figure 5-9 Recovery (%) of carfentanil in all concentrations of formaldehyde at 4°C 203
Figure 5-10 Structure and formula of codeine 204
Figure 5-11 Structure and formula of fentanyl 204
Figure 5-12 Structure and formula of methadone 205
Figure 5-13 Structure and formula of morphine 206

Figure 5-14 Recovery (%) of codeine in all concentrations of formaldehyde at room temperature 207
Figure 5-15 Recovery (%) of codeine in all concentrations of formaldehyde at 4°C ... 207
Figure 5-16 Recovery (%) of fentanyl in all concentrations of formaldehyde at room temperature 208
Figure 5-17 Recovery (%) of fentanyl in all concentrations of formaldehyde at 4°C... 208
Figure 5-18 Recovery (%) of methadone in all concentrations of formaldehyde at room temperature.. 209
Figure 5-19 Recovery (%) of methadone in all concentrations of formaldehyde at 4°C 209
Figure 5-20 Recovery (%) of morphine in all concentrations of formaldehyde at 4°C 210
Figure 5-21 Recovery (%) of morphine in all concentrations of formaldehyde at room temperature 210
Figure 5-22 Structure and formula of diazepam 214
Figure 5-23 Structure and formula of flunitrazepam 215
Figure 5-24 Structure and formula of ketamine.. 215
Figure 5-25 Structure and formula of lorazepam.. 216
Figure 5-26 Structure and formula of midazolam 217
Figure 5-27 Structure and formula of zolpidem 217
Figure 5-28 Recovery (%) of diazepam in all concentrations of formaldehyde at room temperature 218
Figure 5-29 Recovery (%) of diazepam in all concentrations of formaldehyde at 4°C 218
Figure 5-30 Recovery (%) of flunitrazepam in all concentrations of formaldehyde at room temperature.. 219
Figure 5-31 Recovery (%) of flunitrazepam in all concentrations of formaldehyde at 4°C... 219
Figure 5-32 Recovery (%) of ketamine in all concentrations of formaldehyde at room temperature 220
Figure 5-33 Recovery (%) of ketamine in all concentrations of formaldehyde at 4°C. 220
Figure 5-34 Recovery (%) of lorazepam in all concentrations of formaldehyde at room temperature.. 221
Figure 5-35 Recovery (%) of lorazepam in all concentrations of formaldehyde at 4°C 221
Figure 5-36 Recovery (%) of midazolam in all concentrations of formaldehyde at 4°C 222
Figure 5-37 Recovery (%) of midazolam in all concentrations of formaldehyde at room temperature.. 222
Figure 5-38 Recovery (%) of zolpidem in all concentrations of formaldehyde at room temperature 223
Figure 5-39 Recovery (%) of zolpidem in all concentrations of formaldehyde at 4°C . 223

Figure 5-40 Structure and formula of cocaine 231
Figure 5-41 Structure and formula of BZE... 231
Figure 5-42 Recovery (%) of cocaine in all concentrations of formaldehyde at room temperature 233
Figure 5-43 Recovery (%) of cocaine in 20,000μg/mL and 100μg/mL formaldehyde at 4°C and at room temperature 233
Figure 5-44 Recovery (%) of BZE in all concentrations of formaldehyde at room temperature 234
Figure 5-45 Recovery (%) of BZE in all concentrations of formaldehyde at 4°C 234

LIST OF EQUATIONS

Equation 2-1 Methylation of primary amines 97
Equation 2-2 Mathylation of secondary amines 97
Equation 2-3 Methylation of tertiary amines 97
Equation 2-4 Alkylation of secondary amines via the Eschweiler-Clarke reaction 97
Equation 2-5 Formation of addition products.. 98

LIST OF TABLES

Table 4-1 VH, SF and CSF 'hard-fix' donor conditions 151
Table 4-2 Total numbers of samples obtained 153
Table 4-3 GC/MS conditions 155
Table 4-4 Recovery of formaldehyde from spiked VH, SF and CSF 157
Table 4-5 Instrument accuracy and precision 159
Table 4-6 LOQ/LOD data for method ranging from 1-25µg/mL 160
Table 4-7 Formaldehyde concentrations detected in VH, SF and CSF 'hard-fix' samples 162
Table 4-8 Statistical descriptors of formaldehyde concentrations detected in VH, SF and CSF of 'hard-fix' samples 162
Table 4-9 Spearman's Rho test results showing correlations between formaldehyde concentrations detected in VH, SF and CSF 'hard-fix' samples 165
Table 4-10 Spearman's Rho test results showing correlations between formaldehyde concentrations detected in mean VH, SF and CSF 'hard-fix' samples 166
Table 4-11 Spearman's Rho test results showing correlations between volume of arterial embalming fluid injected and concentration of formaldehyde detected in each fluid 167
Table 4-12 Spearman's Rho test results showing correlations between time since embalming and concentration of formaldehyde detected in each fluid 168
Table 4-13 Formaldehyde concentrations detected in VH, SF and CSF 'soft-fix' samples 169
Table 5-1 Predicted likelihood of drug-drug interactions in the presence of formaldehyde 192
Table 5-2 Drug batches 193
Table 5-3 LC/Q-ToF conditions 195
Table 5-4 Recovery (%) of 6-MAM 201
Table 5-5 Recovery (%) of alfentanil 202
Table 5-6 Recovery (%) of carfentanil 203
Table 5-7 Recovery (%) of codeine 207
Table 5-8 Recovery (%) of fentanyl 208
Table 5-9 Recovery (%) of methadone 209
Table 5-10 Recovery (%) of morphine 210
Table 5-11 Recovery (%) of diazepam 218
Table 5-12 Recovery (%) of flunitrazepam 219
Table 5-13 Recovery (%) of ketamine 220
Table 5-14 Recovery (%) of lorazepam 221
Table 5-15 Recovery (%) of midazolam 222

Table 5-16 Recovery (%) of zolpidem 223
Table 5-17 Recovery (%) of cocaine 233
Table 5-18 Recovery (%) of BZE 234

LIST OF ABBREVIATIONS

3-TFMPP	3-Trifluoromethylphenylpiperazine
6-MAM	6-monoacetylmorphine
ACN	acetonitrile
ADME	absorption, distribution, metabolism and excretion (see Glossary)
ATP	adenosine triphosphate
BAC	blood alcohol concentration
BCSFB	blood-cerebrospinal fluid barrier
BRB	blood-retinal barrier
BZE	benzoylecgonine
BRB	blood-retinal barrier
BVB	blood-vitreous barrier
CNS	central nervous system
CE	cocaethylene
CSF	cerebrospinal fluid
cTHC	carboxy-tetrahydrocannabinol
cv%	coefficient of variation (see Glossary)
Da	dalton
DAD	diode array detection
DI	deionised
DNPH	2,4-dinitrophenylhydrazine
ECME	ecgonine methyl ester
EIC	extracted ion count
EMIT	enzyme multiplied immunoassay technique
ESI	electron spray ionisation
FID	flame ionisation detector
FPIA	fluorescence polarization immunoassay
GABA	gamma-aminobutyric acid
GC/MS	gas chromatography / mass spectrometry
GHB	gamma-hydroxybutyrate
H_2O	water
HPLC	high performance liquid chromatography
HTA	Human Tissue Authority
I-RIA	indirect radioimmunoassay
K_p	partition coefficient (see Glossary)
L	litre
LC/Q-ToF	liquid chromatography / quadrupole time-of flight spectrometry
LLE	liquid-liquid extraction
LOD	limit of detection

LOQ	limit of quantitation
LSD	lysergic acid diethylamide
M-3-G	morphine-3-glucuronide
M-6-G	morphine-6-glucuronide
MA	methamphetamine
MDDA	3,4-methylenedioxy-*N,N*-dimethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
mL	millilitre
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
NSAIDs	nonsteroidal anti-inflammatory drugs
PCR	polymerase chain reaction
PD	pharmacodynamics (see Glossary)
pH	potential of hydrogen
PK	pharmacokinetics (see Glossary)
pKa	-log of the acid dissociation constant (see Glossary)
Py/GC	pyrolysis/gas chromatography
RIA	radioimmunoassay
RT	room temperature
SAC	synovial alcohol concentration
SF	synovial fluid
SIM	selected ion monitoring
SPE	solid phase extraction
SSRI	selective serotonin reuptake inhibitor
t½	half-life (see Glossary)
THC	tetrahydrocannabinol
TIC	total ion count
t_R	retention time
UAC	urine alcohol concentration
µg/mL	micrograms per millilitre
UV	ultraviolet
V_D	volume of distribution (see Glossary)
VH	vitreous humour

GLOSSARY OF TERMS

Acetylation
A process of (Phase II conjugation) drug metabolism (*q.v.*) by which an acetyl functional group is added to a drug molecule. *cf.* methylation.

Acid dissociation constant
A quantitative measure of the strength of an acid in solution, usually expressed in mol/L.

ADME
A pharmacological abbreviation for absorption, distribution, metabolism and excretion, describing the physiological stages by which drug molecules pass through the body.

Agonal
Irregular and laboured breathing that commonly precedes death.

Anamnestic
Denoting the reaction of the body's immune system to an antigen that has been previously encountered.

Aphakic
An eye that does not contain a lens, whether as a result of congenital abnormality, pathology or surgical removal.

ATP-splitting
The biochemical energy-spending process by which the phosphate bonds with ATP molecules are broken, thus releasing energy.

Autolysis
Self-digestion; the process by which cells are destroyed by endogenous enzymes. In decomposition, usually observed in conjunction with the action of endogenous and exogenous bacteria.

Binding site
The specific region on a macromolecule (often a protein) to which a drug molecule attaches.

Biological matrix
In bioanalysis, any tissue containing target analytes, including, for example, blood and urine and, in the context of this thesis, VH, CSF and SF.

Coefficient of variation
In statistics, a standardised measure of the dispersion of a frequency or probability distribution, defined as the ratio of the standard deviation to the mean, and usually expressed as a percentage.

Competitive inhibition
In pharmacology, the state of one drug molecule binding to the target receptor, this preventing the binding of certain other molecules.

Conjugation
A pharmacological term describing the second phase of physiological drug metabolism, in which a metabolite is joined to another compound before excretion.

Cytochrome P450 system
A large group of enzymes responsible for the Phase 1 (oxidation and reduction) metabolism (*q.v.*) of many drug molecules.

Derivatisation
The process by which one chemical compound is changed to a new (though similar) compound that has properties more amenable to a particular analytical method.

Diuretic therapy
The administration of certain drugs (diuretics) that increase the rate (and, thus, the volume) of excretion of water and salts from the body. Used to treat a number of conditions, including high blood pressure as well as volume overload resulting from chronic heart failure.

Dose-death interval
The period of time between drug administration and death. Significant pharmacokinetic (*q.v.*) differences can be observed depending on the interval.

Dose-response curve
A linear graph demonstrating the relationship between drug dose (X-axis) and pharmacodynamic (*q.v.*) response (Y-axis)

Enzymatic induction
A pharmacological term describing the process whereby a drug molecule initiates or enhances the expression of a particular enzyme. *cf.* enzymatic inhibition.

Enzymatic inhibition
A pharmacological term describing the process by which the expression of an enzyme is reduced or prevented by a drug molecule, or the functioning of the enzyme is altered by the drug molecule.

Enzyme assay
A bioanalytical method for determining the presence and/or concentration of an enzyme in a sample. Used in the study of enzyme kinetics and inhibition.

Esterases
Hydrolase enzymes that, in conjunction with water, split esters into an acid and an alcohol.

First-pass metabolism
The anatomical/physiological process by which drugs that are administered orally (or rectally) are first metabolised in the liver (and gut wall) before passing into systemic circulation. In intravenous administration, by definition, drug molecules enter systemic circulation directly.

Genetic polymorphism
The discontinuous genetic variation of a particular DNA sequence.

Glomerular filtration rate
A figure commonly used to describe the efficiency (health) of the kidneys, and defined as the volume of blood passing through the filtrating glomeruli. Usually expressed in mL/min.

Half-life
A pharmacokinetic concept describing the amount of time taken for an administered drug dose to be reduced by half. Highly variable, depending on the drug molecule and certain physiological parameters.

Headspace GC/MS
A technique for sampling the volatile constituents of a liquid or solid, whereby the sample is heated until the vapour phase is in thermodynamic equilibrium with the sample.

Hepatic metabolism
Metabolism (*q.v.*) taking place specifically within the liver.

Hydrolysis
Along with oxidation and reduction, one of the Phase 1 modification reactions in drug metabolism (*q.v.*).

Immunoassay
A bioanalytical method of determining the presence and/or concentration of an analyte in solution by observing its response to the addition of an antibody or antigen.

Lipophilic / lipid-soluble
Drugs that generally diffuse rapidly due to the fact that the molecule is usually small and therefore passes easily across (lipid) cell membranes. *cf.* protein-binding.

Matrix effect
In an analytical method, the effect caused on the analyte by other components of the sample. In detection, matrix effects can result in ion suppression or enhancement and can thus affect the quality of the result.

Mediastinum
The central division of the thoracic cavity, bounded by the medial pleurae and containing the heart, great vessels and, posteriorly parts of the trachea and oesophagus.

Metabolite
A breakdown product of metabolism (*q.v.*).

Metabolism
In pharmacology, a collective term for the physiological and chemical processes by which the body breaks drug molecules into simpler compounds in order to aid elimination. Usually described in two phases, Phase 1 (modification) and Phase 2 (conjugation) (*q.v.*). Breakdown products are known as metabolites.

Methylation
A process of (Phase II conjugation) drug metabolism (*q.v.*) by which a methyl functional group is added to a drug molecule. *cf.* acetylation.

Octanol-to-water coefficient
See partition coefficient (*q.v.*).

Paracelsus
A Swiss physician (1493/4-1541), often known as the "Father of toxicology".

Parenchymal tissue
The functional tissue of an organ, as distinguished from its connective or supportive tissues.

Partition coefficient
In pharmacokinetics, the most commonly used term describing the lipophilicity (*q.v.*) of a drug molecule, and defined as the relationship (expressed as a ratio) between the concentration of a solute in a water-saturated (hydrophilic) phase and the concentration of the same solute in the lipophilic phase. The value, therefore, is less than one if the molecule is more water soluble, and greater than one if more lipid soluble. Also known as octanol-to-water coefficient.

Pharmacodynamic
A term referring to the mechanism of action of a drug molecule, *i.e.* the biochemical or physiological effect that the molecule induces when administered. *cf.* pharmacokinetic.

Pharmacokinetic
A term referring to the passage of a drug molecule through the body, and usually described in terms of the molecule's administration, distribution, metabolism and excretion (ADME). *cf.* pharmacodynamic.

(plasma) Protein binding
A pharmacokinetic term referring to the degree to which drug molecules attach to blood plasma proteins, and thereby the efficiency of the molecule to diffuse.

Receptor down-regulation
A term describing a decrease in the number of drug receptors on the surface of a cell, making the cell less sensitive (and responsive) to the drug molecule, often caused by chronic dosing.

Steady state
A pharmacokinetic concept describing the condition in which the amount of drug being absorbed and distributed is equal to the amount being metabolised and excreted. Usually reached after four to five half-lives (*q.v.*) of regular dosing.

Therapeutic dose
The amount of medication required to produce the desired pharmacodynamic response.

Therapeutic index/window
A pharmacological term describing the relative safety of a drug. Expressed as a ratio of the lowest dose that produces toxicity with the highest dose that produces the desired therapeutic response. Thus, the narrower the window, the greater the likelihood of inducing an adverse response.

Volume of distribution
A pharmacokinetic concept representing the propensity of a particular drug molecule to redistribute from the blood plasma to other tissue compartments. The numeric figure (usually given in L) is the theoretical volume that would be necessary to contain the entire amount of administered drug at the same concentration that is observed in the blood plasma.

Xenobiotic
An alternative name for a drug. A chemical substance within the body that is not endogenously produced. Naturally occurring substances (endobiotics) can also be defined as xenobiotics when they are detected in excessive concentrations.

INTRODUCTION

Introduction

Embalming, in the popular imagination, is the process by which Ancient Egyptians removed the internal organs of their pharaohs, soaking the bodies in curing solutions and then wrapping them in bandages, thus effecting a state of permanent preservation such that their rulers might be enabled to enter the afterlife in physical form. Perhaps obscured somewhat by the psycho-social mores of more recent times, however, neither the process of, nor the requirements for modern-day embalming are either widely known or widely discussed. As a method not only of enabling the temporary preservation of the body but also, and very importantly, as a means of enabling the best aesthetic presentation of a loved one to a grieving family, however, embalming has an important place in contemporary funereal practice (Bradbury, 1999).

In forensic practice, embalming is known to create difficulties and carries with it the potential for both confusion and erroneous reporting in cases where, subsequently, post-mortem examinations are undertaken. Incisional artifacts from arterial and venous access points associated with the common carotid arteries as well as, variously, the axillary, brachial, femoral, external iliac and radial arteries can mislead the unwary pathologist (Hanzlick, 1994). Incisions related to the removal of heart pacemakers, and puncture ‘wounds’ from the use of embalming trocars, as well as related damage to internal organs are also commonly found (Rivers, 1978). More recently, in a study of 80 embalmed cadavers, of the seven found to have a grossly identified aortic dissection, six were determined to be as a direct result of the physical embalming process (Rae et al., 2016).

In the case of toxicological analysis, furthermore, the effects of embalming can be highly problematic. It was recognised as long ago as 1980 that, because of its reactivity, formaldehyde, as the main preservative constituent of most embalming fluids, can oxidise, condense or destroy many drugs (Curran, McGarry and Petty, 1980). Indeed, in a set of guidelines written at around the same time for pathologists undertaking autopsies on exhumed and embalmed cases, it was said that the “toxicologic

examination of exhumed tissues is extremely difficult, and the number of drugs or poison (*sic*) which may be detected is quite limited" (Oxley, 1984, p.4). Some ten years after the publication of Oxley's guidelines, the position appears to have remained essentially unchanged, with Hanzlick, in his 1994 paper, stating that if toxicology is likely to form a crucial part of a particular case, the pathologist should consider whether target substances can be measured accurately and interpreted meaningfully in the fluids or tissues of a body that has been embalmed. Even after the turn of the millennium little progress had seemingly been made, with one study reporting that "there is a virtual lacuna of information on [the] decomposition of drugs in formaldehyde." (Suma, Shukla and Prakash, 2006b, p.692).

In the United Kingdom, bodies are not released to funeral directors for embalming until a cause of death has been established and a death certificate issued. In all these cases, therefore, toxicology on the bodies of what in 2018 were 4,359 drug-related deaths recorded in England and Wales, as well as 1,187 in Scotland and 137 in Northern Ireland (Office for National Statistics, 2018)[1] is undertaken before embalming is carried out.

In a typical year, however, around 6,000 United Kingdom nationals die abroad (Her Majesty's Foreign and Commonwealth Office, 2014; the most recent year for which statistics are available), and the majority of families elect to have the bodies of their loved ones returned to the UK for funeral services. Although many countries will investigate, with varying degrees of rigour, the death of non-nationals on their own soil, it the case that on return to the United Kingdom, the coroner of the county into which a body is repatriated must be able to certify a cause of death (Williams and Davison, 2014). In some instances, in such circumstances, the coroner will order a post-mortem examination in an attempt to confirm or determine the means by which death occurred.

[1] These figures include the involvement of controlled and non-controlled drugs and over-the-counter preparations, as well as deaths resulting from drug abuse and dependence, and also include accidents and suicides with a drug involvement, accidental poisonings as well as deaths due to the complications of drug abuse.

From a toxicological perspective, a very significant problem with this is that, for reasons of hygiene, all commercial airlines insist on bodies being properly embalmed before they can be transported (Rowland Brothers International, 2014, and personal experience as an embalmer and as the former owner of a funeral business handling frequent repatriations). Embalming, in its simplest form, involves the injection of a formaldehyde-containing preservative solution directly into the circulatory system, from which all soft tissues can be perfused, thus retarding decomposition. The problem as far as toxicology is concerned, however, is two-fold: firstly, that formaldehyde has been found to be highly reactive with many drugs, both pharmaceutical and illicit; secondly, although blood has traditionally been the matrix of choice for toxicological analysis, it is directly into the circulatory system that embalming fluid is introduced.

The problem of embalming has been recognised by toxicologists and pathologists for some years. It was described succinctly in the case of Harold Shipman, the British general practitioner convicted in 2000 of the murder of 15 of his patients. Of the nine bodies that were exhumed as part of the investigative process, five had been embalmed (Pounder, 2003; Kennedy, 2009). During Shipman's trial, it was stated by Julie Evans, the forensic toxicologist for the prosecution, that:

> "The effect of formaldehyde is that it may chemically change one drug to another and by reason of that affect the concentration of the drug ultimately detected."
>
> (*Regina v Harold Shipman* 1999 (Transcript of Trial, Day 21))

Mrs Evans further added that:

> "A drug concentration determined in formaldehyde affected tissue cannot be taken at face value as the real level of the drug in the tissue before formaldehyde was added is not actually known."

It was the aftermath of the death of Diana, Princess of Wales, however, that brought into clearer focus the difficulties first publicly highlighted in Shipman's trial. During the extensive coronial investigation into her death, carried out in 2007-2008, consideration was given to the matter of why Diana's body was embalmed in Paris within 16 hours of her death, when it was known that a post-mortem examination would almost certainly

be carried out following the repatriation of her body to the United Kingdom (Morgan 2011). In his evidence to the inquest, Dr Robert Chapman, the Home Office Pathologist who carried out the post-mortem examination of Diana's body when it arrived in the United Kingdom, offered the observation within his written statement that:

> "All of the available blood was contaminated or even largely replaced by the embalming fluid making any sample rather unreliable for toxicology testing."
>
> (Scott-Baker (2007): Robert Chapman, Witness Statement 24 Feb 07)

Chapman also gave verbal evidence to the inquest, adding to his earlier statement that:

> "The embalming procedure…certainly affects blood and perhaps urine and, to a lesser extent, the eye fluid, because of the use of chemicals, which will make toxicology difficult or indeed sometimes impossible."
>
> (Scott-Baker (2007): Robert Chapman, Witness Statement 26 Nov 07)

Later reports have further elucidated the problems that can be encountered in attempting to undertake toxicology on embalmed bodies, one study stating that:

> "Blood is usually entirely coagulated in a well-embalmed body and even when it can be obtained, the constituents of embalming fluid may confound toxicological interpretation. Typically the fluid contains ethanol, methanol and formaldehyde. The latter may react with some drugs, such as those containing a primary amine moiety, to give adducts, complicating the identification of drugs." (Allan and Roberts, 2008, p.37)

Indeed, it has been found, as will be explored further in Chapter 2, that formaldehyde has the capacity to hydrolyse, methylate or otherwise degrade a wide range of both pharmaceutical and recreational drug molecules. The result of such reactions can be not only the loss of the parent drug but also the appearance of compounds not present in the body before death; some known methylation reactions, moreover, can lead to the production of a non-prescribed parent compound from a demethylated ante-mortem ingested drug (Hoffman, Zedeck and Zedeck, 2012). Additionally, misleading toxicological findings can result from the presence of other compounds in the

embalming fluid: one particular case has reported the erroneous presence of a 10% ethanol concentration in the urine of an embalmed and repatriated body (Brinkmann, 1999).

Although the aftermath of the death of the Princess of Wales highlighted the problems associated with embalming and toxicology, it remains the case that toxicologists, pathologists and coroners continue to be challenged by the drug-degrading effects of formaldehyde (pers. comm. with several regional coroners, 2014; Williams and Davison, 2014). Although representing only a small percentage of the deaths they are required to investigate each year, coroners make the point that it is these deaths above many others that may by their nature be of particular forensic interest and which, therefore, deserve to be properly investigated. Indeed, of fatalities occurring abroad each year, around 40-50 that are subsequently repatriated to the United Kingdom for funeral services are suspected to have had drug-related deaths (pers. comm. with Professor Robert Forrest, former HM Senior Coroner for South Lincolnshire, 2014).

At least part of the problem would appear to be the varying degrees of diligence with which the deaths of non-nationals are treated abroad. In a series of repatriations handled by the Wales Institute of Forensic Medicine, for example, toxicology had been performed on only 11% of cases in the countries in which they had died (Williams and Davison, 2014). Despite the fact that, within Europe, repatriations are expected to be handled in accordance with the Agreement on the Transfer of Corpses (Conseil de l'Europe, 1975) and that, in 2000, a call was made for the 'harmonisation' of medico-legal autopsy requirements around the world (Conseil de l'Europe, 2000), it would appear that little progress has been made and practice varies considerably from country to country.

Study Aims and Objectives

As far as this thesis is concerned, its starting point was a question posited over 20 years ago by the American toxicologist, Timothy Rohrig. In a paper examining the comparative concentrations of fentanyl in fresh and embalmed liver samples, though speaking of the potential problems in the detection of any drug in the presence of formaldehyde, he asked:

> "Does the measured concentration of the embalmed tissues reflect the concentration at death, or does the embalming process artificially increase or decrease the measured concentration of drug?" (Rohrig, 1998, p.253)

In the intervening years since Rohrig posed this question, a number of researchers have undertaken work that has gone some way to providing an answer. On the whole, as will be explored in Chapter 2 of this thesis, the answer has been that the measured concentration of the embalmed tissues almost certainly does not reflect the concentration at death and that, furthermore, the embalming process can artificially increase the concentration of some drugs, while decreasing that of others. As a result of these findings, but in the absence of any reliable solution to the problem, the more nuanced question that has formed the basis of this thesis is, 'Are there any tissues in the body that are not affected by the embalming process, and are they likely to reflect drug concentration at the time of death if they can be analysed?'

Fortunately, in 2001, another American and extensively-published toxico-pathologist, Steven Karch, published a paper calling for the examination of other matrices for the post-mortem detection of drugs in bodies where, for whatever reason, blood may not be available. Although many tissues have subsequently been examined - many of which will be considered in Chapter 1 of this thesis - of particular interest to the central question being asked in this thesis is the viability of vitreous humour (VH) as a toxicological matrix. Long known for its utility in the determination of ante-mortem blood alcohol concentrations, the fluid has also been shown to be an excellent medium for the detection of a particularly wide range of pharmaceutical compounds. Within the context of an embalmed body, the potential of VH is considerable, primarily by virtue of

its anatomical location. Being separated from direct communication with the circulatory system by the blood-vitreous barrier and the blood-retinal barrier (the anatomy and physiology of these barriers is discussed in detail in Chapter 3 of this thesis), drug molecules can enter VH only by indirect mechanisms. As many of the - largely biochemical - processes that enable the passage of drug molecules across these barriers may, after death, cease to function, it is possible to postulate a hypothesis whereby, even in the embalmed body, the compartment containing VH becomes in essence an historical repository of ante-mortem toxicological information. Indeed, it is nearly 30 years since Professor Forrest stated that VH usually remains uncontaminated by the embalming process and may usefully be analysed for ethanol and for urea and creatinine (Forrest 1993). VH, however, is not the only biological fluid within the body to be protected from direct contact with blood. Although, when compared to VH, the literature concerning other avascular fluids is much more limited, a small number of studies have identified the passage of drug molecules across the membranes that separate the circulatory system from cerebrospinal fluid (CSF), the fluid that bathes the brain and spinal cord, and synovial fluid (SF), the fluid found in the synovial joints of the body. Both fluids are discussed in detail in Chapter 3.

The primary aim of this thesis, therefore, is to examine the viability of VH, CSF and SF for the detection of drugs of forensic interest in embalmed bodies. The subject of the study is timely, given the publication in recent years of several related studies. Addressing specifically the toxicology of embalmed samples, Nikolaou et al., (2013), Takayasu et al., (2013) and Lemaire-Hurtel, 2019, have all – while not providing any new methods – highlighted the difficulties that currently exist. A relatively recent PhD thesis, Yokchue (2016), has further highlighted the reactivity of formaldehyde with several drugs of forensic interest. It is encouraging, moreover, that two further forensic PhD projects have achieved success in detecting drugs in avascular fluids: Menshawi (2010), in part of his work, developed methods for the detection of several drugs of abuse in VH, and Rees (2011) compared the distribution of opiates and cocaine between post-mortem skeletal muscle and VH. An MPhil study, Griffiths (2012), has also reported encouraging results, when comparing VH drug concentration with those detected in

blood, and a more recent MSc thesis, Kennedy (2016), has reported correlations between blood, VH and brain tissue in the case of three compounds. Although none of these studies either addressed or examined embalmed tissues, they have all established, importantly, that there is merit in examining in greater detail the detection of drugs of abuse in avascular compartments.

The objectives of the study, designed specifically to consider a number of matters not yet addressed directly in the literature, were to:

i) Undertake a review of the literature in order to establish the extent of the current understanding of embalming toxicology;
ii) Undertake a review of the literature in order to establish the usefulness of VH, SF and CSF for the detection of drugs and their likely utility in cases of repatriation embalming;
iii) Develop a suitable analytical procedure for the simultaneous extraction, identification and quantification of formaldehyde in the of VH, SF and CSF of embalmed cadavers in order to establish whether the embalming process contaminates these fluids;
iv) Develop a suitable analytical procedure for the monitoring of the stability of a number of of drugs of forensic interest in conditions likely to be encountered in repatriation cases.

Thesis Structure

Of the five chapters comprising the thesis, the first three are detailed literature reviews, each of which, in different ways, paves the way for the two experimental chapters that follow. Thus, Chapter 1, as a general introduction to the thesis proper, is wide-ranging review of the literature pertaining to post-mortem toxicology. A number of comprehensive reviews of this nature can of course already be found in a number of monographs and papers. Where the current study differs from many of those already published, however, is that rather than taking as its starting point what it is that can be achieved in the field of post-mortem forensic toxicology, the starting point here, rather, is to provide a review of the particular difficulties that exist. Indeed, the analysis and

interpretation of post-mortem samples has for some time been regarded by many as one of the more challenging areas of toxicological endeavour. The provision, therefore, of a general review of the field will, it is hoped, provide a useful contextual basis to the premise of the thesis.

Chapter 2 is a comprehensive review of all formaldehyde-toxicology studies or case reports found in the mainstream literature since the first published mention of the problem in 1957. Much of this chapter is a prose summary of the literature, collated by drug class, but it is also complemented, in Appendix 2, by a tabular summary of concentrations of drugs detected in embalmed tissue, or else in laboratory conditions contrived to replicate those surmised to be encountered in embalmed bodies. Although much of the chapter is a narrative account, it ends with a critical review of the literature, from the perspectives both of the literature in its own right, as well as, more specifically, from that of the embalmed and repatriated body. From this particular perspective, the review seeks to highlight significant inconsistencies in the literature that it is suggested have led to incorrect assumptions being drawn concerning the toxicology of embalmed remains. These assumptions, it is further suggested, are not only incorrect, but have in part contributed to a position whereby many coroners, toxicologists and pathologists believe that useful toxicological analysis cannot be undertaken on embalmed remains. Drawing both on literature relating to the technical aspects of embalming practice, as well as on the author's professional experience as a qualified embalmer, the chapter proposes a redefinition of the basis on which previous assumptions have been made.

Having already signalled that the primary aim of the thesis is to evaluate the utility of VH, CSF and SF for the detection of drugs in embalmed bodies, Chapter 3 is a review not only of the anatomical and physiological basis by which drugs have been found to cross the respective barriers separating each of these fluids from the circulatory system, but also of all mainstream literature reporting drug molecules detected in these fluids. Drawing on a wide range of anatomical and toxicological literature, the chapter aims not only to review the mechanisms by which the distribution of a large number of both pharmaceutical and illicit drug molecules is known to take place, but also, and importantly, seeks to develop a theory relevant to post-mortem toxicology by which the

compartments in which the fluids are contained may become resistant to molecular ingress and egress following death. The theory, not previously explored within the context of analytical toxicology, is used to posit a hypothesis whereby the three fluids, by virtue of their protected environments, remain uncontaminated by the embalming process. Given the novelty of the experimental work reported in Chapters 4 and 5, it is Chapter 3, particularly, that seeks, therefore, to provide the secure theoretical foundation and justification for the work presented in later chapters.

Chapter 4 is an experimental study designed to test specifically the hypothesis of the thesis: that VH, CSF and SF may indeed be viable, and formaldehyde-free, toxicological matrices in embalmed bodies. Drawing on samples of all three fluids obtained from cadavers embalmed in UK medical school anatomy facilities using an arterial formaldehyde method, the chapter, believed to be the first study of its kind, presents a method, developed and optimised specifically for this study, that analyses and reports the formaldehyde concentrations in the VH, CSF and SF of 56 embalmed bodies. A headspace GC/MS method was developed with an LOQ of 0.4µg/mL, an LOD of 0.13µg/mL and a total run time of seven minutes; in all respects the method represents significant improvements on related methods reported previously in the literature.

The results of the study reported in Chapter 4, indicating – compared to what has previously been assumed in the literature – comparatively small concentrations of formaldehyde in the biological fluids analysed, paves the way for Chapter 5. Here, the stability of fifteen drugs of forensic interest, or their metabolites, are studied in concentrations of formaldehyde that, it is now proposed, are far more representative of those that are likely to be encountered in the VH, CSF and SF of embalmed and repatriated bodies. A further analytical method was developed for this study: utilising LC/Q-ToF, and a total run time of ten minutes per sample, it was possible to develop a single method, optimised specifically for all 15 drugs, which was both simple and highly sensitive. The results of the study suggest that the drug molecules studied exhibit considerable stability in the concentrations of formaldehyde likely to be encountered in the VH, CSF and SF of embalmed bodies.

Unfortunately, and as direct result of Coronavirus disease 2019 (COVID-19), the experimental phase of the project was abruptly curtailed when the Cranfield laboratories were closed with the imposition of the first national lockdown. At the time, work had just begun on the breakdown products associated with the degradation of the 15 drugs included in the study reported in Chapter 5 of this thesis. A small amount of analysis had been undertaken, and is reported in Chapter 5, but the majority of the planned work had to be abandoned and could not be restarted before submission of the thesis.

Additionally, it was not possible even to begin an extensive study that had been planned as a third experimental chapter. Over the course of several months, samples of VH, SF, CSF and fresh blood had been taken, prior to embalming, from cadavers that were brought to the Cambridge facility. These same cadavers were then re-sampled at varying intervals in the weeks following embalming. A specialist forensic toxicology laboratory based at one of the London teaching hospitals had agreed to analyse the samples. The samples, however, were taken to the laboratory just before the first national lockdown began, and, as the laboratory was also shut down, it was not possible to obtain the results in time to write them up into what was intended to be an important additional chapter. The significance of this additional work is addressed further in the 'future work' section of this thesis.

1 POST-MORTEM TOXICOLOGY

1.1 Introduction

The remit of toxicology, wide-ranging as it is, is in large part concerned with the health, as well as the legal conduct, of the living. In addition to its significant contribution to the development and testing of new drugs, often involving an assessment of the toxicity of new compounds using laboratory animals, toxicology is also commonly practised within the sub-specialities of clinical toxicology, employment drug testing and so-called human performance toxicology, including drink and drug driving (Hodgson, 2010; Negrusz and Cooper, 2013). Much toxicological endeavour is driven directly by clinical need. Thus, the development of new and increasingly sophisticated methods of detecting the presence and quantity of drugs in an increasingly wide range of bodily fluids and other tissues, for example, has in large part been motivated by the work of pharmacologists who continue to develop new drugs to treat a very wide range of medical conditions. Arguably, however, at least equal impetus has come from the requirements of law enforcement. Certainly, it has long been the case that the unwanted side effects of many medications administered within a clinical context are, in turn, actively sought in more recreational or proscribed milieux. Consequently, the forensic toxicologist might be expected to comment on a wide range of circumstances including, for example, the determination of substance abuse, the identification of drug-facilitated crime, as well as the important distinctions between accidental, suicidal and homicidal death. Indeed, one particularly useful, if broad, definition describes forensic toxicology as serving the individual, who may be a victim, an offender, a relative or a beneficiary, by providing information about accidental poisoning, malpractice, suicide and criminal offences (Vuori, 2009).

While the traditional autopsy is able to identify a very large number of disease states, as well as the failure of physiological processes that either directly cause death, or else may contribute to it, it is a procedure that is not, with only a very small number of exceptions, able to diagnose drug-mediated demise. As one study has reported, it remains the case that while histological examination can provide anamnestic and other circumstantial toxicologic indicators, it cannot definitively diagnose drug use (Passarino et al., 2005). Instead, methods

of analysing a range of body fluids and other tissues, largely based on clinical toxicology practice, have been adopted and developed over the course of a number of years.

1.2 Post-Mortem Toxicology

Toxicology has been described as the science that considers the adverse effects of chemicals and toxins on living cells (Rao, 2012) and indeed, until relatively recently, the determination of post-mortem drug levels was regarded by many as being a relatively simple process, analogous to the examination of concentrations observed in the living (Winek et al., 2001). On an assumed basis that once death occurred, drug concentrations in the body at the moment of death remained thereafter constant (largely as a result of the cessation of metabolic processes), it had been the case that, for many years, the amount of any drug detected in post-mortem samples would be compared directly to documented therapeutic and toxic levels in the living, and an opinion derived from this direct comparison would be provided (Drummer, 2004a).

It is now known, however, that post-mortem toxicology carries a number of challenges that are not encountered *in vivo*, while acknowledging nevertheless that the basis of meaningful reporting, as will be explored later in this chapter, is reliant on a number of pre-mortem factors (Flanagan and Connally, 2005a). The British forensic toxicologist, Robert Forrest, in an introduction to a paper dating from the early 1990s, wrote an excellent and concise summary, which is probably as valid today as when it was written nearly thirty years ago:

> "The human body can be regarded as a complex assembly of dynamic chemical systems. Maintaining the integrity of these systems is an affront to the second law of thermodynamics and requires the constant expenditure of energy. Once death takes place, the supply of energy from metabolic processes is dramatically reduced, the integrity of the different compartments within the body breaks down at differing rates; complex molecules tend to break down to their simpler subunits and to move down the concentration gradients that were maintained in life by the expenditure of metabolic energy. Obviously, these processes do not all occur at once. Thus, for a variable length of time after death the analysis of appropriate samples may yield useful information about the metabolic state of the person in the period immediately before death. Once death has taken place many drugs are released from their binding sites in tissue as pH decreases on death and as the processes of autolysis proceed. These phenomena can make the interpretation of drug concentrations after death less than straightforward." (Forrest, 1993, p.292)

Post-mortem toxicology is an area that, although perhaps the oldest of the toxicological disciplines, has received increasing medico-legal interest in recent years. It is also an area of practice, as Forrest's comments intimate so well, that carries inherent challenges, both in terms of analytical methods employed as well as in the subsequent interpretation of results. The challenge is in part due to the wide-ranging nature and quality of post-mortem tissue and fluid samples which can, looking from both ends of the temporal spectrum, include those taken shortly after death, as well as those taken from severely decomposed or exhumed bodies, all of which may contain different concentrations of drugs within the context of different chemical and biological environments (Byard and Tsokos, 2013a). Increasingly, it is also the case that drug-related intoxication deaths (whether accidental or intentional) involve the use of multiple drugs, and an awareness of the possible clinical implications of potential drug inter-reactions is necessary for the correct interpretation of analysis results (Wester et al., 2008). Indeed, it has been said that an inclusive toxicological investigation must encompass knowledge and understanding of the related case history, the correct specimen selection, the appropriate choice of drug extraction procedures and the application of the appropriate technique/s, as well as the meaningful interpretation of findings (Hoffman, Zedeck and Zedeck, 2012).

While informed toxicological reporting will take account of these several, variable and often inter-dependent factors, it is also the case that much is enabled – though is sometimes limited – by analytical and technological capabilities. Forensic toxicology has a long history. Indeed, the first recorded presentation of chemical tests in the British criminal court was in Oxford, in 1752, when Mary Blandy was tried for the murder of her father, having allegedly poisoned him with arsenic (Watson, 2004). During the course of the nineteenth century, techniques for extracting other metallic poisons, notably antimony, bismuth and mercury, were developed, along with the ability to extract alkaloids, such as nicotine, and other non-volatile organic compounds from bodily fluids (Flanagan et al., 2007). The twentieth century saw not only a significant increase in the number of compounds that could be identified, but was also witness to rapid advances in both the range and capability of analytical techniques. Colorimetric tests, microdiffusion and ultraviolet and infrared spectrophotometry were developed, alongside paper and ion-exchange column chromatographic techniques, followed by thin-layer

chromatography, as well as improved spectrophotometric methods (Flanagan et al., 2007). The major advances, however, were heralded by the development of gas chromatography (GC) in the mid-twentieth century, followed shortly afterwards by liquid chromatography (LC) (Flanagan et al., 2007). The refinement of GC and LC equipment and techniques, along with the development of highly-refined spectrometric technologies and immunoassay and enzyme-based assays, has increased analytical means greatly, enabling both qualitative and quantitative analysis to be performed. Indeed, such are the capabilities of state-of-the-art equipment, and their associated techniques, that almost any toxin can now be identified and quantified using one method or another (Levine, 2010).[2]

Given the range of analytical capability, however, it has also been said that a test result is only as good as the question it is asked to answer (Jenkins, 2008a). This is particularly relevant in the sphere of post-mortem toxicology, where it is acknowledged that our understanding of what happens to drugs in the body following death is still somewhat lacking in detail (Jones, 2011). Thus, while it is the case that for a very small number of drugs post-mortem alteration of one sort or another does not occur, for the majority of both prescription and illegal preparations it does not follow with any degree of certainty that what is detected post-mortem is conclusively indicative of the ante-mortem state. Indeed, in a more recent, if understated, re-articulation of Forrest's earlier warning, one writer has stated simply that "the concentrations measured at autopsy do not necessarily reflect those at the time of death" (Rees, 2011, p.1).

Although many of the difficulties associated with this area of work do not impinge directly on the experimental work reported later in this thesis, for the sake of completeness the remainder of this chapter is devoted to providing a summary review of the main trends reported in the post-mortem toxicology literature of the past twenty to thirty years. In PhD theses there is arguably neither the need nor the space to provide the sort of wide-ranging study that can already be found in published monographs. The account that follows,

[2] Concise and useful accounts of the historical development of toxicology and of analytical equipment can be found in Flanagan et al. (2007), Klaassen (2008) and Lappas and Lappas (2016), and of analytical methods in Druid, 2007; a particularly detailed technical report on all aspects of post-mortem toxicology practices in Europe can be found in European Monitoring Centre for Drugs and Drug Addiction, 2019.

therefore, aims to provide an up-to-date, but generalist, collation of the literature that is specific to the challenges encountered in post-mortem toxicology: a review that is currently lacking. More important, however, is the need to provide a context for both the results and the practical applications of the experimental work that is described later in this thesis. Even within this sort of review, however, there is limited space, and so in order keep the account to a manageable size, extensive comparisons of sample types are not provided, and analytical hardware and methods, furthermore, are deliberately addressed only in passing, as such accounts can be readily found in published studies, already cited. Instead, the survey almost exclusively restricts itself to collating the literature of what might be termed ‘problem areas’, some of which will be addressed again later in the thesis, when considering the practical applications of the experimental results presented. Given the wide-ranging and generalist approach adopted, many of the sections that follow end with suggestions for further reading, for the purposes of the interested reader.

Aside individual or small-group case studies, which together have formed probably the largest proportion of the post-mortem toxicology research output of recent years, subjects including sample selection and stability, as well as the phenomenon of post-mortem redistribution, have featured regularly in the literature. For the sake of organisational convenience, the review that follows considers these and other related matters under the broad heading of ‘post-mortem factors affecting toxicological analysis’. In comparatively more recent years, however, there has been an increasing interest in certain functional processes occurring in the living that, although sometimes difficult to account for, have significant bearing on the interpretation of post-mortem analytical results. These matters, which include factors such as drug absorption, distribution, metabolism, excretion and tolerance, as well as other physiological considerations, including the related and very much more recent areas of pharmacogenetics and pharmacogenomics, are subsequently reviewed under the heading of ‘pre-mortem factors affecting toxicological interpretation’. Following the review, a concluding summary seeks not only to draw together the most important strands to emerge, but, specifically for the purposes of this thesis, aims to define the context in which the later, experimental, chapters of the current study might be placed. A summary of papers reviewed, by topic, can be found in Appendix 1.

1.3 Post-Mortem Factors Affecting Toxicological Analysis

The literature has, in general terms, defined two broad areas in which challenges linking post-mortem drug concentrations to assumed ante-mortem conditions can be found. The first of these is degradation of the compound, either by enzymatic or microbial action, and the second, an alteration of the equilibrium of drug concentrations within the body in the period between death and sampling. The nature and, importantly, the extent of such degradation and alteration, however, varies according to the sample type analysed. In this section of the review, therefore, a brief survey is first undertaken of each of the matrices that has a place in toxicological analysis, following which a more detailed review of matters pertaining to stability and relative detectability is undertaken.

1.3.1 Toxicological matrices

1.3.1.1 Blood

Traditionally, blood has been both the clinical and the autopsy toxicological matrix of choice. This is not only because of the fact that it is relatively easy to collect from both the living and the dead, but also because on the whole it represents the dynamic state of drug distribution in the body, providing a generally reliable relationship to the state of the individual's pharmacologic condition, whether that be therapeutic, toxic or fatal (Jenkins, 2008a). As a result, by far the majority of data found in the literature are for blood and, because of this, comprehensive tables listing therapeutic, toxic and lethal concentrations have been compiled (see, for example, Musshoff et al., 2004, Baselt, 2017, Molina and Hargrove, 2018 and Schultz et al., 2020).

When it comes to providing forensic interpretation, however, it has long been acknowledged that for many drugs there can be considerable overlap in concentration outcome (Wennig, 2000) and, for other drugs, including not only oncological agents, but also, for example, many opioids, there is little or no distinction between therapeutic and toxic doses (Ferner, 2008). Indeed, one study states that, with the possible exception of paracetamol, there are no strictly defined boundaries that determine the distinction between therapeutic, toxic and fatal concentration ranges (Kennedy, 2010). Thus, although it has been said that while such data

tables may be useful, they should not be taken as absolute (Repetto and Repetto, 1997), and they do not in themselves provide a definitive aetiology of the death under investigation (Patel, 2012). Furthermore, and more recently, it has been suggested that the inter-individual variation in susceptibility to harm from drugs (which will be discussed in greater detail later in this chapter) is in fact so large that such tables should be used with particular caution (Ferner, 2012).

Despite these cautions there are, however, concentration factors above which there is consensus concerning morbidity and mortality. In the case of many drugs the literature generally agrees that blood concentrations found to exceed the therapeutic range by 10 to 20 times are consistent with toxicity or death. Closely aligned with this general maxim is that the higher the parent drug to metabolite ratio, the greater confidence there can be in determining acute intoxication, particularly, of course, when multiple drug analytes are detected. Equally, a result below a defined limit of detection for a particular drug can (usually) be safely interpreted as a lack of exposure to that drug (Hepler and Isenschmid, 2007).

As well as cautions concerning the use of concentration tables, however, there are a number of other recognised and forensically relevant difficulties in the interpretation of blood-drug concentrations. Aside cellular sedimentation, clot formation, contamination with tissue fluid and putrefaction (Flanagan, 2008a), which can be caused by endogenous as well as exogenous bacteria, one particularly well-reported challenge is in the interpretation of cardiac blood samples, where the drug concentration is found to be somewhere between the upper therapeutic limit, and the lower toxic or fatal limit. Whereas blood drug concentrations in the living, once the agent has been fully absorbed, are generally the same throughout the circulatory system, this is not the case in the body of a deceased person. It is well known that drug concentrations in cardiac blood tend to increase following death, due to a phenomenon known as post-mortem redistribution (which will be considered in more detail later in this chapter), whereas drug concentrations in peripheral blood samples – for example, those taken from the femoral or iliac veins – tend to remain constant (Ferner, 1996; Dinis-Oliveira et al., 2010). Cardiac blood, however, is technically much easier to obtain at autopsy than peripheral blood, and thus will generally tend to be the sample of choice.

Wide ranges of concentration ratios between heart blood and peripheral blood have been noted for many drugs. Basic drugs with large apparent volumes of distribution (V_D) generally exhibit ratio differences of up to 20 (although not all basic molecules with a large V_D follow the same pattern, and in individuals suffering from congestive heart failure, as well as certain acute conditions, the apparent V_D can be greatly reduced) (Skopp, 2004). It has, therefore, long been recognised that in 'borderline' therapeutic-toxic-fatal cases, attempts to provide definitive interpretations from cardiac blood samples alone could well be misleading without peripheral samples for contextual confirmation (Jones, 2011) (a table of central to peripheral post-mortem blood ratios for a number of drugs of forensic interest is given in Ferner, 2008). This is likely to be particularly important in cases of acute drug-related mortality, where a pharmacological steady state is unlikely to have been reached before death intervened.

A further interpretative problem with blood is that whereas clinical data found in the literature are often derived specifically from either the serum or plasma components, in post-mortem analysis it is often not possible to separate the individual components, and so results are commonly derived from the analysis of whole blood. This, however, can be problematic since it is well known that many drugs do not distribute evenly between the cellular and fluid components, leading to blood:plasma distribution ratios that can vary from drug to drug (Skopp, 2010). In the case of cannabinoids, furthermore, although blood to plasma ratios are generally found to be constant in the living, their distribution in post-mortem samples can be highly variable (Richardson, 2000). Additionally, some laboratories have recommended that greater attention is paid to the relationship between total and conjugated drug concentrations in blood, particularly in the case of morphine, suggesting that accurate reporting of this ratio may well assist in the subsequent interpretation of analytical results (Apple, 2011).

1.3.1.2 Urine

As a result of the physiological processes of metabolism and excretion, urine is generally considered to be an excellent matrix for the determination of drugs and their metabolites. Detection times, depending on the half-life of the drug concerned, of between 24 hours and around a month provide timescales which are certainly greater than the windows for the detection of respective drugs in blood (Hepler and Isenschmid, 2008). Helpfully, urine is

readily amenable to immunoassay and other spot tests due to the fact that it comprises more than 99% water. As a result of renal filtration, furthermore, urine contains comparatively few of the interfering proteins, lipids and other large molecules found in blood, and is therefore a relatively easy matrix to prepare for instrumental analysis, with many methods involving little more than straightforward dilution by way of sample preparation (Jones, 2011).

Additionally, the ability to detect often high concentrations of metabolites can provide a useful context for a range of other findings or interpretative suspicions (Dettmeyer, Verhoff and Schütz, 2014). Indeed, even in the complete absence of the parent compound, the urinary detection of the metabolite 6-MAM, for example, is incontrovertible evidence of the administration of heroin, and the presence of cocaethylene indicates that alcohol was ingested at around the same time that cocaine was taken (Kennedy, 2010). Different metabolites of the same parent compound can, furthermore, provide important circumstantial information. The detection of the morphine metabolite, morphine-6-glucuronide (M-6-G) is relevant not only because its presence can be used as positive confirmation that a degree of time has passed between the administration of morphine and the production of this metabolite (Thaulow et al., 2018) but, also, because it is pharmacologically active, it is the case that the metabolite itself may have contributed to an adverse outcome; morphine-3-glucuronide (M-3-G), by contrast, while also being a morphine metabolite, has no pharmacological action, and its detection may thus be interpreted differently (Ferner, 2012). Urine, more than blood, and by the nature of its property as the end product of metabolism, is an excellent matrix for such interpretative subtlety.

Like all analytical matrices, however, urine has its own disadvantages. Aside the fact that it is only available in around 50% of post-mortem cases - due to the bladder frequently being voided during the agonal phase of death - its main drawback is perhaps a paradox of its usefulness for metabolite detection. As drug molecules enter the urine only after they have passed through the physiological processes of metabolism and excretion, it follows that urine will not necessarily test positive in cases of acute, drug-related death (Baker and Jenkins, 2008), although a negative urine test in combination with a positive (high) blood concentration may, of course, provide strong evidence of rapid death. Another limitation is that, because of the metabolic processes through which most drugs pass before they are

excreted, urine cannot, compared to blood, be used to estimate either the time or the dose of drug ingested. Furthermore, it may be the case with many compounds that, depending on the length of time since administration, the parent drug may not be detectable at all, and thus specific analytical methods that are able to detect metabolites of particular drugs of interest must be developed and employed.

Perhaps one of its greatest drawbacks, however, is that urine, rather than being a circulating biological fluid, is a metabolic waste product (with its concentration being affected by several and varied factors, including not only rate of metabolism, but also fluid intake, pH and glomerular filtration rate); the consequence of this from a toxicological perspective is that the correlation between drug concentrations in urine and blood is extremely poor, even in clinical scenarios (Jones, 2011). Thus, the ability of urine to provide any meaningful context to a consideration of the pharmacological or toxicological effect of any particular drug is far from secure, and such indicative information is best sought from other matrices (Dinis-Oliveira et al., 2010).

1.3.1.3 Vitreous humour

Vitreous humour has long been recognised to be a useful matrix, primarily by virtue of the anatomically self-contained environment of the eye in which it is contained. Being separated from the rest of the body by the fibrous sclera, the liquid humours of the eye are less liable to either post-mortem redistribution or bacterial infiltration than many other biological matrices, often remaining sterile until the most advanced stages of decomposition (Jones, 2011). As a result of its protected environment, vitreous is extremely useful in the determination of ethanol concentration as it can be used successfully to differentiate post-mortem alcohol formation (a recognised phenomenon occurring in other biological matrices, particularly blood) from ante-mortem alcohol ingestion (Caplan and Levine 1990; O'Neal and Poklis, 1996).

One of the other significant advantages of VH over blood is that it lacks the esterase enzymes that are responsible for the, often problematic, hydrolysis of a number of forensically important drugs in blood, including 6-MAM, one of the principal metabolites of diamorphine, as well as cocaine. Vitreous, thus, is often regarded as the matrix of choice for the positive

determination of heroin and cocaine abuse (Jones, 2011). A further advantage from a practical application viewpoint is that, although it does contain some collagen, vitreous comprises over 99% water, and so, in common with urine, requires little preparation for analysis (Dinis-Oliveira et al., 2010).

The fact, however, that the compartment of the eye in which vitreous is contained does not have a direct blood supply means that there is a delay in the uptake of drugs (including alcohol) into the fluid, although conversely, and helpfully, there is probably also a delay in their excretion from it (Hepler and Isenschmid, 2008). Indeed, it has been suggested that drug concentrations detected in vitreous may represent corresponding circulating blood concentrations one to two hours before death (DiMaio and DiMaio, 2001).

A further disadvantage of vitreous is the relatively small size – a maximum of 3mL from each eye – of available sample, although the development of newer technologies and analytical methods makes this less of a problem (Jenkins, 2008a). Perhaps a greater problem in the forensic application of vitreous analysis, however, is the relative lack of comparative drug concentration data. Additionally, the comparative data that has been published often indicates significant variance in vitreous:blood concentration ratios. While it has been found, for example, that some drug molecules exhibit a vitreous to blood ratio that is close to unity, for others there is considerable variation. Highly lipid-soluble molecules including, for example, benzodiazepines, as well as highly protein-bound molecules, such as tricyclic antidepressants, appear to have much lower vitreous:blood ratios than either comparatively non-lipid-soluble or non-protein-bound molecules (Jones, 2011). While the utility of vitreous in post-mortem biochemical analysis is long established, the lack of comparative data limits to some degree its current usefulness as a toxicological matrix. The literature relating to the fluid is reviewed in greater detail in Chapter 3 of this thesis, where its potential usefulness in forensic analysis is explored further.

1.3.1.4 Cerebrospinal fluid and synovial fluid

The analysis of cerebrospinal fluid has a mainstream place in clinical practice, where for many years lumbar punctures have been utilised in, for example, the diagnosis of meningitis (Venkatesh, Scott and Ziegenfuss, 2000) and in determining the effectiveness of drug delivery

to the central nervous system (Shen, Artru and Adkinson, 2004). Perhaps by virtue of the fact that blood and VH are somewhat easier to collect during autopsy, CSF currently has a limited use in post-mortem toxicology. It has, however, been shown to have a number of advantages that lend itself to post-mortem analysis. As it comprises mostly water, it requires little sample preparation and readily amenable to most routine methods of analysis (Moffat, Osselton and Widdop, 2011). Moffat also suggests that it can be of particular use in in alcohol-related cases, especially when no VH is available and where post-mortem alcohol production may be suspected. Another advantage is that a number of drug molecules appear to pass quickly into CSF following intravenous administration (Jenkins and Lavins, 1998). A further advantage is that, like VH, CSF is contained within an anatomically isolated compartment and, compared to blood, is therefore comparatively protected from bacterial contamination (Palmiere et al., 2015). While disadvantages of CSF include the fact that correlations between blood and CSF may be inexact (Logan and Luthi, 1994), one of the main drawbacks is the limited quantity of reference data available, although one study, Engelhart and Jenkins (2007) reported the detection in CSF of nearly 300 drug molecules, and a more recent paper, Tominga et al. (2015), reported the detection of a wide range of drugs of which, for many, good blood:CSF correlations were found.

Serving as a lubricant for the majority of the articular joints within the body, the usefulness of synovial fluid, like CSF, originates in clinical practice, where it was first used in the diagnosis of rheumatological pathology (Kushner and Somerville, 1971). It has also been used in a wide range of biochemical diagnostic procedures, where, for example, sodium, potassium urea and glucose have all been quantified (Madea, Kreuser and Banaschak, 2001). The literature relating to its utility in post-mortem work is limited in scope, although the work that has been undertaken certainly points to the potential usefulness of the matrix. It has been found, for example, to be a reliable medium for the quantitation of ethanol, exhibiting a good correlation with blood (Winek et al., 1993b). More recently, a range of opiates as well as cocaine have been quantified and found to correlate well with blood (DeKing Hargrove and Molina, 2014). Like CSF, SF also has a high water content and therefore requires little pre-analytical sample preparation, although in common with CSF there are few comparative

reference data available, and so the use of the fluid as a toxicological matrix is currently somewhat limited.

Like VH, CSF and SF are both explored in much greater detail in Chapter 3 of this thesis, where literature relating not only to reported toxicological studies is reviewed, but also where the specific anatomical and physiological factors that make all three fluids especially relevant to the study are discussed.

1.3.1.5 Gastric contents

Although many drugs are abused intravenously, a large proportion of prescription drugs are administered orally, and evidence of drug overdose may thus be found in the stomach. This can be the case particularly when death has occurred before complete digestion of the swallowed medication has taken place, where the amount of drug detected in the stomach significantly exceeds the prescribed dosage regimen, and when corresponding blood concentrations support the likelihood of fatal ingestion. Additionally, and because of the fact that physiological metabolism does not take place until molecules reach the liver, drugs which in blood, urine or other matrices might only be detected as metabolites (or which, alternatively, may have been extensively distributed) can, in the stomach, be detected unchanged (Jones, 2011).

The point has been made many times, however, that gastric contents are rarely uniform in consistency and the chances, therefore, of inaccurate analysis are heightened unless the entire contents of the stomach are obtained and homogenised prior to analysis (Jones, 2011). It is also recognised that drugs are able to diffuse from the stomach into surrounding tissues (Pounder et al., 1996a) and, in a reverse process, the passive diffusion of certain drug molecules from blood to stomach can lead to misleading results being obtained from gastric samples alone (Skopp, 2004). A further interpretative challenge in the analysis of gastric matter is that the absence of high concentrations of drug in the stomach does not in itself rule out overdose, since it can take many hours for death to occur, during which time most or all of any ingested drug can have been passed from the stomach to the small intestine and been fully absorbed. A final - if only occasionally encountered - subtlety with this matrix is that although a molecule or metabolite may be detected in the stomach, it does not

automatically follow that the drug was ingested orally, since the gastric juice excreted into the stomach is formed from extracellular fluid which can contain readily detectable concentrations of basic drugs and their metabolites drawn from circulating blood (Jones, 2011).

1.3.1.6 Bile

Produced by the liver to aid the digestion of lipids, bile has been found to be useful for the qualitative determination of a wide range of drugs (DiMaio and DiMaio, 2001), though particularly for opiates, especially morphine, as well as cocaine and its metabolites (Agarwal and Lemoc, 1996). Due to the physiological process that controls the storage of bile in the gall bladder, concentrations of many drugs are found to be somewhat higher than corresponding blood concentrations. Opioids (especially buprenorphine and tramadol) and benzodiazepines are found in comparatively high concentrations in bile (Drummer, 2004a; Mercurio, Ceraso and Melai, 2019), and it is not unusual for cocaine and its metabolites to be detected in concentrations three to six times higher than the corresponding levels detected in blood (Agarwal and Lemoc, 1996). Furthermore, in the case of some drugs, including paracetamol, both the parent drug and the metabolite can be detected in bile for often significant periods of time after blood levels have fallen to what would otherwise appear to be inconsequential concentrations (Forrest, 1993), or where the drug is no longer detectable in blood (Vanbinst et al., 2002). Bile has also been shown to be a useful matrix for the detection of ethanol, particularly in the absence of urine (Caplan and Goldberger, 2014). A useful table of drugs found to concentrate in the bile, along with indicative bile to blood ratios, can be found in Leikin and Watson (2003).

In common with vitreous, however, the correlation between bile and blood concentrations is generally poor (Jones, 2011), even taking into account the concentrating mechanism that takes place in the gall bladder, and reliable quantitative interpretation is generally not considered to be possible; good correlations between blood and bile have, however, been reported in the case of some drugs, including amphetamines (Tominga et al., 2016). Additionally, it has been shown that bile drug concentrations may be affected by the redistribution of drug molecules from the liver and even the (anatomically distant) stomach (Hepler and Isenschmid, 2008), in a process that will be explored in greater detail later in this

chapter. A recent review paper that discusses, particularly, blood:bile ratios can be found in Ferner and Aronson (2018).

1.3.1.7 Soft tissues

Used often as supporting evidence for drugs detected in blood, soft tissue is routinely collected at autopsy for histological purposes, and large quantities of reference data exist from which helpful comparisons can be made. Liver, as the primary site of drug metabolism, has been found to provide an especially useful comparison with cardiac blood concentrations, and there are many reports in the literature of drug detection in this tissue. Liver is also a useful matrix for those drugs, including, for example, tricyclic antidepressants and phenothiazine-group neuroleptics, that have a large dose range but correspondingly low blood concentration (Jones, 2011). Additionally, and in part due to the direct movement of (orally ingested) drugs from the small intestine to the liver, via the hepatic portal system, concentrations of many, especially basic, drugs are found to be higher in the liver than in blood (Orfanidis et al., 2020). The liver has also long been recognised as being a useful matrix for the detection of drugs that are liable to post-mortem redistribution, furthermore, since concentrations in this tissue are generally found to be stable after death (Jones, 2011).

Other organs including kidney, brain and lung can also be used to provide confirmatory evidence, and certain tissues are regarded as being especially useful for the detection of particular, specific, compounds. Thus, lung tissue can, for example, be particularly helpful in the detection of volatile substances, including abused solvents, the spleen, as a particularly blood-rich organ, can aid the detection of haemoglobin-binding molecules, such as carbon monoxide and cyanide (Palmiere et al.,2018), heavy metals can often be detected in the kidney, where they tend to collect in elevated concentrations at the end of the excretion process (Hepler and Isenschmid, 2008) and carbon monoxide can be detected in a number of parenchymal tissues (Casali et al., 2015); finally, brain tissue can be a worthwhile analytical matrix for the identification of particularly lipophilic substances (including antidepressants, narcotics and halogenated hydrocarbons), as a result of its comparatively high fat content (Nedahl, Johansen and Linnet, 2019a), and it has been found to be especially useful for the detection of cocaine (Karch, 2009; Carvalho, 2017), amphetamine (Nedahl, Johansen and Linnet, 2019b), as well as volatile-related fatalities (Molina, Limfueco and Hargrove, 2015).

Brain tissue also benefits from the fact that, like vitreous, it is contained within a relatively enclosed environment and is therefore, to some degree, protected from post-mortem redistribution and bacterial decomposition. For tissues other than liver, however, the reference data are somewhat limited in terms of both number of cases reported, as well as attempts to correlate findings with respective blood concentrations, and their usefulness can thus only reliably currently extend as far as providing qualitative evidence of drug use.

1.3.1.8 Hair

In cases of advanced decomposition, where neither blood nor soft tissue remain, hair can be used for the qualitative determination of chronic drug use, as a large number of drug molecules are known to be incorporated into the vascular hair follicle, from where they are then effectively encapsulated as the hair grows (Henderson, 1993). The technique of segmental analysis can prove to be especially helpful, since it is known that hair generally grows at a uniform rate of approximately 1cm per month (Dettmeyer, Verhoff and Schütz, 2014) and, thus, using samples of head or pubic hair, it is possible to construct an historical 'timeline' of drug ingestion over months or even years, depending on the length of the individual's hair. Related to this is the fact that, compared to other matrices such as blood and urine, hair can be used to distinguish between single exposure and chronic abuse, although it is not diagnostic in cases of acute demise, where death will have intervened before drug molecules can be laid down in the follicle (Drummer, 2004a).

1.3.1.9 Bone, bone marrow and other matrices

Bone and bone marrow represent two of the least researched tissues to be explored in the post-mortem toxicology literature. Nevertheless, bone can be a useful source of toxicology information in burnt, putrefied and skeletonised cases, given that it can survive burial for hundreds, or even thousands, of years. Although some studies have pointed to the uptake of drugs into this matrix being to some extent a reversible process, bone (as well as teeth (Klima et al., 2016)), has been shown to be a reliable repository of a wide range of drug molecules, including opiates and opioids, benzodiazepines and amphetamines (Rubin, 2018; Vandenbosch et al., 2020; Franceschetti et al., 2021; Giordano et al., 2021). As far as bone marrow is concerned, in the absence of other, preferred, tissues, it has been used

qualitatively and successfully for several drug molecules (Lloyd and Evans, 2017). Given, furthermore, that it is a richly vascular tissue, some attempts have been made to correlate drug concentrations with those found in blood, though with little data available, it is fair to say that bone marrow is not as yet considered to be capable of yielding interpretatively reliable quantitative information.

There are varying quantities of literature for several other, disparate, tissue types that can be utilised in post-mortem toxicological investigations. These include skeletal muscle (often useful in cases of advanced decomposition (Watterson et al., 2010; Wyman et al., 2011; Hargrove and Molina, 2014), although an unequal perfusion of drugs into this tissue has been noted such that, in some cases, 20-fold variations have been recorded (Langford, Taylor and Pounder, 1998; Drummer, 2004a)), fly larvae (again, in cases of advanced decomposition (Kintz, Tracqui and Mangin, 1990; De Carvalho, 2010)), finger and toe nails (in the absence of fluids and soft tissues (Lloyd and Evans, 2017)), as well as other tissues as diverse as bodily fat (Levisky et al., 2001), earwax (Shokry et al., 2017), meconium (Wu, Marin and McMillin, 2017), umbilical cord tissue and blood (Kapur and Aleksa, 2020), oral fluid (Reisinger et al., 2019), pericardial fluid (Moriya and Hashimoto, 1999b), lung samples, including pleural effusions (Sims et al., 1999), amniotic fluid, breast milk and sweat (Jenkins, 2008a) and exhaled breath and fingerprints (Wolff, 2017). The quantity of data available, however, renders many of these matrices suitable only for simple, qualitative testing. A more detailed discussion of many of these alternative matrices will be found in Gallardo and Queiroz (2008), Hepler and Isenschmid (2008), Dinis-Oliveira et al., (2010) and Lloyd and Evans (2017). More comprehensive guidance can be found in the literature regarding the selection and benefits, as well as disadvantages, of each specimen type (see, for example, Druid (2007) and Jones (2011)), as well as the technical processes by which samples may be taken (see, for example, Forrest (1993), Flanagan, Connally and Evans (2005b) and Dinis-Oliveira et al. (2010)).

1.3.1.10 Conclusions

Even from this brief survey of biological fluids and soft tissues, it might be observed that in an ideal situation, given the significant variability of relative advantages and disadvantages, sample selection should be based not just on availability but also with reference to any known case history. Related to this, one toxicologist has commented that it can sometimes be helpful

to tailor the collection of specimens according to the types of substances expected (Drummer, 2004a). Sometimes, however, the choice of sample is limited by availability. For example, in the case of burns, exsanguination and advanced decomposition, as we have seen, only non-standard tissue types may be available, and from these only a qualitative indication of drug consumption may be possible. Indeed, with perhaps the exception of liver, reference data comparing blood and other tissue types have been reported infrequently (Skov, Johansen and Linnet, 2015).

In addition to sample type, however, two other particular factors, drug and/or sample stability and post-mortem redistribution, play significant roles in the determination and interpretative usefulness of analytical results. In turn, the literature of these subjects will now be considered.

1.3.2 Drug and sample stability

It is widely understood that the extent to which drugs can be detected in post-mortem samples does not in all cases remain constant over time. Opiates, for example, are well known to be unstable in blood samples stored at room temperature, with the principal diamorphine metabolite 6-MAM being significantly more unstable than either morphine or codeine (Huertas et al., 2019), and although the addition of sodium fluoride as a preservative increases the stability significantly, losses can still be great (Papoutsis et al., 2014). Even in post-mortem blood samples stored at -20°C, for example, losses of around 11% of codeine, 12% of morphine and 81% of 6-MAM have been recorded over extended periods of time, of up to nine years (Høiseth et al., 2014).

Xenobiotics can be lost from post-mortem samples due to straightforward aspects of their physical storage conditions, as well as by processes of biochemical degradation, either by endogenous enzymatic activity, or else by endogenous and exogenous bacterial activity.

It is unsurprising that volatile compounds, including aerosol propellants, ethanol, gaseous anaesthetics and organic solvents, as well as carbon monoxide, present storage challenges (Skopp, 2004). Of these compounds, ethanol is of course a frequent target analyte in many post-mortem investigations, and here it has been shown that although bacteria may play a part in sample instability, a simple but significant evaporation loss can occur due to excessive

ventilation between the liquid and gas phases in storage tubes, even at -18°C (Stojiljkovic et al., 2016). In the case of cyanide, loss of the analyte from blood has been shown to be the result of not only evaporation, but also complex mechanisms involving reaction with matrix components and thiocyanate formation (Ballantyne, Bright and Williams, 1974), although storage of samples below 4°C can reduce the loss (Ballantyne, 1976).

Analytical interference from the material with which the storage container is manufactured has also been reported. Studies have noted, variously, the detection of benzenes and zylenes, phthalate plasticisers and other solvents used in the production of Sarstedt Monovettes® that could inadvertently lead to a misdiagnosis of poisoning; other studies have identified significant decreases in concentrations of a number of therapeutic agents (particularly tricyclic antidepressants and antiepileptics) when in contact with the gel barrier used in certain vacutainers (Rohrig, 2019). A useful review article, Skopp (2014), discusses the effects of different methods of physical storage on sample stability, and a comprehensive review of the literature relating to storage stability is included in Dinis-Oliveira et al. (2010).

The metabolism of drug molecules in the living body is generally a very well understood process that forms the clinical basis of much pharmacological work. What is also well understood, moreover, is that death is not so much an event as it is a process, during which physiological functions shut down at different times, in some cases very much later than the point at which the more obvious physical signs of life, such as heartbeat and respiration, may have ceased to manifest themselves spontaneously. In the case of hepatic metabolism, it was first established some years ago that a number of drug metabolising enzymes continue to function for some time after death. Indeed, and importantly, since it is responsible for the clearance of a particularly wide range of pharmacologically active molecules, in the case of the cytochrome P450 system, the shift to a complete cessation of metabolic activity has been shown to take around 48 hours in rat livers (Yamazaki and Wakasugi, 1994). In the case of compounds with particularly short half-lives, and which are metabolised via the P450 system, the forensic implications of this fact for the analysis and interpretation of post-mortem samples could, of course, be significant.

Other work has noted that, as well as a delayed cessation of hepatic metabolic activity, endogenous enzymes may also be released in the early stages of decomposition (Paterson, 1993). Thus, the bioconversion of some drugs has been found to be mediated by a process whereby, via extra-hepatic hydrolysis, oxidation or reduction, a parent drug can be converted to metabolite (Skopp, 2004). Often, this degradation is found to come about as a result of esterases still operating in the sample matrix, and molecules containing ester moieties (including, for example, 6-MAM, cocaine and acetylsalicylic acid), photolabile compounds (including LSD, midazolam and phenothiazines), as well as compounds containing sulphur, and structures that are easily oxidisable or reducible, are all liable to be affected by this mechanism (Peters, 2007; Kerrigan, 2013). In some cases, cellular lysis can also result in the liberation of bound drug molecules (Butzbach, 2010). A useful account of the stability of heroin and its metabolites, as well as cocaine, in relation to esterase activity, is given in Rees (2011).

In some cases, variations in stability have been observed between different forms of the same compound. In the case of morphine, for example, in a phenomenon that formed part of the cross-examination of an expert witness at the well-reported trial of Dr Shipman (Pounder, 2003), the conjugated form can be almost completely converted to the free form within the post-mortem liver, although the rate of conversion is arrested at low temperatures; in blood however, the conjugated form appears to be entirely stable, even at 37°C over 10 days (Moriya and Hashimoto, 1997b). In a later piece of work, interestingly, Moriya's methodology was expanded in order to distinguish between fresh blood and post-mortem blood. In this study, although morphine was found to be relatively stable in fresh-frozen blood samples stored at 4°C, it was only stable in post-mortem blood when stored at -20°C (Skopp, 2001). Similar results were recorded in a study that examined the stability of M-3-G in blood (Carroll et al., 2000).

As well as enzymatic action, a process of bacterial transmigration is also known to play a significant role in xenobiotic breakdown. Here, following death, bacteria contained within the gastrointestinal tract in particular, but also in the lungs and the oral cavity, are able to multiply and penetrate tissue walls, ultimately entering the circulatory and lymphatic systems (Corry, 1978; Melvin et al., 1984). Unsurprisingly, an ambient or elevated temperature is likely to

hasten the movement of intestinal bacteria, although there is no clearly specified time frame in which this process proceeds (Morris, Harrison and Partridge, 2006).[3] The conversion in post-mortem blood of benzodiazepines (in particular clonazepam, flunitrazepam, and nitrazepam) to their 7-amino-metabolites, as well as the hydrolysis of heroin and cocaine, as a direct result of anaerobic bacterial action, are well-reported phenomena in this respect (Levine, Blanke and Valentour, 1983; Stevens, 1984; Robertson and Drummer, 1995; Robertson and Drummer, 1998; Moriya and Hashimoto, 2003). Significant – sometimes total – losses of antipsychotic compounds have also been reported from bacteria-laden blood, in some instances in as little as two days (Butzbach et al., 2013a), as well as losses of SSRI antidepressants from decomposing liver samples (Butzbach et al., 2013b). A particularly detailed and useful discussion of post-mortem bacteria and the processes by which they infiltrate the body can be found in Butzbach (2010).

Other work has reported the synthesis of compounds that were not present at death, occurring as autolysis proceeds and pH changes (Gibitz and Pluish, 2009), including the fermentative production of a range of volatile compounds such as 1-butanol, 1- and 2-propanol, acetaldehyde, acetic, butyric, isobutyric and propionic acids, acetone, isoamyl alcohol, isobutanol, and ethyl esters (Boumba, Ziavrou and Vougiousklakis, 2008). In some cases, the production of forensically significant molecules from otherwise relatively innocuous compounds has been noted, one particular instance being the conversion of thiocyanate to toxicologically significant amounts of cyanide (Egekeze and Oehme, 1980). Meanwhile, some recent work has identified the endogenous biosynthesis (Bodnar, 2016) as well as the exogenous source (commonly through diet) of a number of compounds, including a wide range of opiates and benzodiazepines, as well as carbon monoxide and cyanide (Rohrig, 2019), although the concentrations detected are generally likely to be of academic rather than forensic interest.

[3] A parallel can perhaps be drawn between the toxicologically significant processes of enzymatic action and bacterial transmigration, and human taphonomic processes, where enzymatic and putrefactive decay occur independently, yet competitively, and where the precipitating factors are difficult to quantify (Forbes, Perrault and Comstock, 2017).

While early work noted the bacterial biotransformation of a number of drugs, more recent studies have sought to identify the role of specific gastrointestinal bacteria in the process. In the case of benzodiazepines, for example, decreases of up to approximately 20% and 26% were recorded in diazepam samples inoculated with *Escherichia coli* and *Bacteroides fragilis,* respectively, and an almost total conversion of flunitrazepam to 7-aminoflunitrazepam was recorded in the presence of *Bacteroides fragilis* and *Clostridium perfringens* (Martindale, Powers and Bell, 2015). A criticism that might be levelled against this sort of work, however, is the methodologically isolated way in which research is sometimes conducted. Even without a specialised knowledge of gut flora, it could be argued that studying the stability of drugs exposed to only one bacterium is a contrived condition that is unlikely to be encountered in the reality of the post-mortem arena and is, therefore, of limited use. This is demonstrated quite clearly in the case of a number of drugs, the degradation of which can commonly be affected by more than one process. In the case of cocaine, for example, enzymatic degradation is known to precipitate the production of ecgonine methyl ester, while non-enzymatic (bacterial) hydrolysis results in the production of benzoylecgonine. Both processes, importantly, are known to occur simultaneously (Johanson and Fischman, 1989). A useful review of the literature concerning the bacterial degradation of drugs can be found in Castle et al. (2017), and recent and particularly detailed paper, Guo, Lee and Juong (2020), provides a helpful account of the role of gut bacteria in the reductive metabolism of a wide range of drugs.

As well as drug degradation, and the production of metabolites or even new compounds, it is also the case that increases in parent drug concentration can sometimes be observed. This has been noted in post-mortem blood samples where, for example, the formation of methaemoglobin has been found to lead to an apparent increase in carboxyhaemoglobin (Skopp, 2004). More rarely, a metabolite may be converted back to its parent compound: the conversion of certain labile metabolites, including *N*- and acyl-glucuronides, as well as sulphate conjugates, back to their parent compounds, for example, have been known to create misleading results (Skopp and Pötsch, 2002).

Of particular importance in forensic contexts, it is also known that, as decomposition progresses, putrefactive fermentation can lead to the production of ethanol (O'Neal and

Poklis, 1996; Kugelberg and Jones, 2007; Hanzlick, 2014; Quintas et al., 2017). Depending on the depositional environment, this well-recognised toxicological complication of decomposition can begin from between three and ten days of death (Caplan and Goldberger, 2014) and almost 100 different bacteria, fungi and yeasts have been found to generate exogenous ethanol in the decaying body (Kazmierczak and Azzary, 2008). Unsurprisingly, it is also known that the degree of post-mortem ethanol synthesis has been found to increase in proportion both to a rise in temperature, as well as to the interval between death and sampling (Petković, Simić and Vujić, 2005). A detailed account of the toxicological implications of the fermentative production of ethanol is given in Skopp, 2004; this paper also includes a helpful survey of toxicology in cases of advanced decomposition, including exhumation.

A similar process has been found to lead to the post-mortem production of gamma-hydroxybutyric acid, GHB, a drug that in recent times has been the subject of increasing recreational abuse (Elliott, Lowe and Symonds, 2004; Kintz et al., 2004; Beránková, Mutnanska and Balikova, 2006; Thomsen et al., 2017; Ha, Marta and Vargas, 2020). GHB interpretation, however, is perhaps even more complicated than that for ethanol, given that in life the body synthesises small amounts of the compound; work has sought to establish specific cut-off values in a range of biological matrices in order to distinguish ante-mortem ingestion from endogenous production (Andresen, Aydin and Iwersen-Bergmann, 2010), although distinction in the comparative relationship between ante-mortem endogenous production and post-mortem fermentative production is perhaps less clear (Castro et al., 2014; Kietzerow et al., 2020). Other work, however, has suggested that the post-mortem production of GHB occurs in barely significant quantities, especially when samples are stored at -20^{0}C (Busardò et al., 2014), and that using a cut-off to distinguish between endogenous production and exogenous use may, therefore, be unsafe (Korb and Cooper, 2014).

Temperature, as has already been intimated, has been found to play a major part in drug stability. One study of 46 drugs and metabolites in post-mortem femoral blood, for example, found that the majority were stable for one year at -20°C, although losses were noted for ethanol, desmethylmianserin, 7-amino-nitrazepam, THC and zopiclone, and increases were noted for ketobemidone and thioridazine (Holmgren et al., 2004). Relative stability has been noted in benzodiazepines stored at -20°C or lower (Melo, Bastos and Teixeira, 2012),

compared to room temperature where, in the case of diazepam, losses of up to 60% have been noted (Atanasov, et al., 2012). Other work has reported that the loss of a large number of parent compounds can in fact be reduced to between 10% and 20% if the samples are stored at -20°C and are analysed promptly; in many cases degradation may be completely inhibited if the samples are stored at -80°C (El Mahjoub and Staub, 2000; Suzuki and Watanabe, 2005). Temperature forms an important part of the experimental work reported later in this thesis, when considering the conditions in which bodies are stored during the repatriation process, and the consequences that differing conditions might have for the stability and subsequent detection of compounds of forensic interest.

The use of preservatives can also arrest degradation. In the case of ethanol, for example, although post-mortem *in vivo* fermentation cannot be entirely prevented, it has been found that the addition of 1.0% sodium fluoride to samples prevents subsequent production, even at 25°C (Lewis et al., 2004). In fact, it has been found that the addition of a preservative to biological samples will inhibit further endogenous enzymatic activity, as well as bacterial degradation, in the case of many drugs, from the time at which the sample is taken. In the case of the insecticide, malathion, however, sodium fluoride itself has been found to induce a breakdown of the parent compound (Byard and Butzbach, 2012).

As is often the case, however, there are exceptions. Cocaine and its metabolite, BZE, have been found to degrade over time even in the presence of fluoride/oxalate preservatives (Giorgi and Meeker, 1995) and even in urine stored at -37°C (Dugan et al., 1994). The loss of the cannabis constituent THC has also been reported from blood stored at -20°C (Drummer, Gerostamoulos and Chu, 2002b) and from frozen urine (Romberg and Past, 1994). The acid metabolite, cTHC, furthermore, is lost from urine whether it is stored at room temperature or frozen (Golding Fraga, Díaz-Flores Estévez and Díaz Romero, 1998). Additionally, one occasional, if important, cause of assumed sample loss, for which analytical and interpretative provision must be made, is the process of racemization, whereby, *in vivo,* an optically active compound can be chemically converted into a form that is optically inactive; the significance of this phenomenon to forensic analysis is important, since racemized molecules may be unrecognisable when using certain techniques including, for example, UV and fluorescence detection (Flanagan, 2008a).

Other, more recent, work has sought to determine the extent to which sample matrix effects may affect the ability of particular analytes to be detected. Although not strictly a matter of stability in the usual understanding of the term, so called matrix effects can have a significant influence on the extent to which some drugs can be detected by particular analytical methods. A comparatively recent piece of work, for example, has demonstrated that the ability to detect valproic acid (a very commonly prescribed anti-epileptic medication) in post-mortem whole blood declines by 85%, after storage for 28 days at room temperature; this is thought to be as a result of the high matrix dependency of the drug, as well as the progressively degraded nature of the blood samples (Kiencke et al., 2013). In this study, furthermore, Kiencke reported that the matrix effect appeared to be closely related to temperature, since the same study found that when the storage temperature was reduced to $4^{o}C$, the loss after 28 days was only 25%, and in samples stored at $-20^{o}C$, the drug was entirely recoverable.

In practice, of course, the underlying message within much of this literature is that a negative analytical finding is not definitive evidence of the ante-mortem absence of any particular drug molecule, in the same way that a positive finding is not definitive evidence of ante-mortem ingestion. As one toxicologist pithily notes, knowledge of degradation mechanisms and breakdown products is of exceptional importance in post-mortem toxicology (Skopp, 2004).

A table listing those molecules known to exhibit sample stability problems can be found in Leikin and Watson (2003); a useful (if now somewhat dated) overview can be found in Ellenhorn (1997), and, more recently, in Baselt (2017); finally, a table of drugs, metabolites and other poisons known to be unstable in whole blood or plasma can be found in Flanagan (2008b).

1.3.3 Post-mortem redistribution

The phenomenon known as post-mortem redistribution has been shown to be responsible for very significant time-dependent shifts in blood concentration in the case of some drugs; it can render the direct comparison of post-mortem concentrations with those encountered during therapeutic drug monitoring to be, at best, unreliable and, in many cases, wholly misleading (Davies, Johnston and Holt, 2016). First identified some 60 years ago, and in some

of the earlier literature termed 'necrokinetics' (Leikin and Watson, 2003), it has since been described as a toxicological nightmare (Pounder and Jones, 1990). Indeed, although the problem was originally identified in 1960 in relation to differences in barbiturate concentrations from blood obtained from central body cavities, compared to femoral samples, by the 1980s it was evident that the problem was also associated with many other drug molecules. Site-to-site variation in concentration by up to a factor of 10 has been reported (Pounder, 1993), and some studies have noted that those drugs exhibiting considerable post-mortem redistribution (usually defined as a high central blood to peripheral blood ratio) also commonly demonstrate high post-mortem to ante-mortem ratios (Cook, Braithwaite and Hale, 2000; Kennedy, 2015). Particularly significant, and forensically relevant, site-to-site variability in morphine and morphine glucuronide ratios in blood samples have been noted (Skopp et al., 1996). Different time-related patterns of distribution, furthermore, can emerge for different drugs. In the case of morphine, for example, although cardiac blood samples are consistently found to contain higher concentrations than peripheral samples, the ratio between the two does not appear to change over time (Logan and Smirnow, 1996; Crandall et al., 2006a); in the case of digoxin, however, whereas peripheral concentrations remain steady following death, cardiac concentrations increase markedly (Holt and Benstead, 1975).

Although still not fully understood, in its simplest form, the process of post-mortem redistribution is a very straightforward mechanism, whereby the concentration gradients resulting from the non-uniform distribution of drugs throughout the body during life, change in death, as the biochemical processes that maintain those concentration gradients cease to function (Ferner, 2012). The basis of this process may well be that described by Fick's first law of diffusion, which states that the rate of diffusion is proportional to the concentration gradient across the diffusion barrier (as well as the pH on either side of the membrane, and the drug molecule size, its ionisation state and its lipid solubility) (Enderle, 2012). It is also the case, however, that a physical transport of drugs can be brought about by the natural movement of blood within vessels. Such a process can come about as a result of, for example, pressure changes brought on by rigor mortis, resulting in a ventricular contraction that can move blood towards the superior vena cava and the subclavian veins, or else by putrefactive

gas formation that can move blood from the thoracic to the abdominal aorta, as well as from the inferior vena cava to the right atrium and the pulmonary veins (Pounder, 1993; Sastre et al., 2017). Early work supporting this physical transport theory of redistribution examined the post-mortem movement of morphine using rat models (Sawyer and Forney, 1988; Koren and Klein, 1992) as well as humans (Logan and Smirnow, 1996), identifying significant increases in blood concentration in the minutes following death. In a similar vein, the diffusion of drugs from the stomach to the left lobe of the liver has been found to occur purely as a result of gravity-driven blood flow (Hilberg et al., 1999), and it has been recommended, therefore, that if the liver is to be used for toxicological analysis, the sample should be taken from deep within the right lobe (Pounder et al., 1996b). Several opioids, furthermore, have been found to diffuse from the bladder into the femoral veins, via the iliac circulation (Moriya and Hashimoto, 2001). Other physical redistributive processes have been found to take place in the case of gastric contents being regurgitated into the lungs, either during the agonal phase, by passive relaxation of the oesopho-gastric sphincter during rigor mortis, or even as a result of manual handling of the supine body (Pélissier-Alicot et al., 2006), and from the lungs, drug molecules can be redistributed, in some cases via the pulmonary vessels, into cardiac blood (Marraccini et al., 1990; Pounder and Yonemitsu, 1991). Additionally, although perhaps not falling strictly within the usual definition of post-mortem redistribution, but a recognised cause of what might otherwise be regarded as unexplained localised differences in detected drug concentrations, is the case of transdermal opioid patches being left on bodies following death (Anderson and Muto, 2000), or automatic syringe drivers delivering cocktails of palliative drugs being left running (Richardson, 2000; Kerrigan, Honey and Baker, 2004).

As an extension to the physical transport theory, the chemical characteristics of some drugs make their liability to redistribute highly predictable. Thus, by a passive concentration-gradient driven diffusion, the propensity of, for example, highly lipid-soluble drugs to move from blood to surrounding muscle tissue has been observed (Drummer and Gerostamoulos, 2002a). Similarly, ethanol has been found to have diffused locally to pericardial fluid from the stomach when significant amounts have been ingested (Pounder and Smith, 1995). The passive diffusion of drug molecules from the gastric contents into the lower lobe of the left lung has also been noted (Pounder et al., 1996b), and other work has reported the often rapid

diffusion of basic drugs from the lungs to the left side of the heart in cases resting in the supine position (Shepherd, Lake and Camps, 1992; Gomez et al., 1995; Moriya and Hashimoto, 1999a). As lung tissue also accumulates high concentrations of basic and lipophilic molecules, redistribution of a number of psychiatric drugs to mediastinal structures has been observed (Boer, 2003; Rodda and Drummer, 2006). A further, if somewhat unusual, form of diffusion has also been observed in the movement of lidocaine from the trachea to heart blood, when the drug is used as a local anaesthetic for endotracheal intubation (Moriya and Hashimoto, 1997a) and also from the urethra into urine, during the course of transurethral procedures, including catheterisation (Tzortzis et al., 2009; Chan et al., 2014). Other work has identified the post-mortem preferential binding of some drugs, including cardiac glycosides, local anaesthetics, tricyclic antidepressants and opioids, to the myocardium (Bailey and Shaw, 1982), while a reverse diffusion from the myocardium back into the cardiac blood has also been noted (Prouty and Anderson, 1990; Dalpe-Scott et al., 1995). In some cases, furthermore, drugs detected in the heart may be present merely as an artefact of attempted cardiopulmonary resuscitation (Moriya and Hashimoto, 2000).

It has been suggested that drugs with an apparent volume of distribution greater than 3L/kg are the most likely to redistribute, via passive diffusion, from tissue into blood, and a useful table of some drugs known to exhibit this characteristic, along with approximate heart blood:peripheral blood ratios (and also a small list of those drugs believed not to redistribute) can be found in Leikin 2003. As with most aspects of post-mortem toxicology, however, there are exceptions. In the case of the antiarrhythmic medication amiodarone, for example, despite having a very large V_D of 66L/kg, the drug does not appreciably redistribute, yet desethylamiodarone, its pharmacologically very similar metabolite, can redistribute centrally at up to three times its peripheral concentration (Kennedy, 2010). In combination with high volumes of distribution, drugs which are basic, with a pKa of less than 7, and have a high octanol to water coefficient, and are thus highly lipophilic, are also more likely to exhibit redistributive characteristics (Zilg et al., 2017).

Physical transport, however, is not the only theory of post-mortem redistribution to have been postulated, and another important area of work has considered the breakdown of physiological and anatomical barriers, coming about as the result of autolytic changes to

cellular membranes. In this process, acidification, known to affect the pH of blood in the period of time following death before decomposition proper intervenes, is believed to cause damage to lysosomal membranes, alongside which enzymatic activity leads to the digestion of cell membranes. Thus, by these processes, drug molecules are redistributed into extra-cellular spaces as the permeability of the cell membranes and haemolysis progress (Skopp, 2010). Related to this, it has also been suggested that changes in ionisation, brought about by autolytic pH alteration, can lead to protein-bound drug molecules being released as a result of the ensuing shift in the octanol to water coefficient (Ferner, 2008).

Regardless of the breakdown of cellular membranes, however, with some drugs, even before death, it is the case that there is an uneven concentration distribution between plasma and red cells (Pounder, 1993). Although the rate at which blood initially clots and then subsequently lyses during the post-mortem period is unpredictable, it follows that the toxicological analysis of, for example, a clotted sample, may in the case of some drugs produce a significantly different result to the analysis of a non-clotted sample.

A detailed account of the mechanisms by which drugs are able to redistribute in the post-mortem period can be found in Pélissier-Alicot et al. (2003) and a wide-ranging review of the related literature can be found in Sastre et al. (2017).

The post-mortem redistribution literature is extensive, but one criticism that might reasonably be levelled against it is its seeming over-reliance on the reporting of individual case reports, the precise circumstances of which are often and clearly very different from one another. A limited number of studies have used animal models as a means of providing the sort of controlled environment that might serve to mitigate against the methodological inconsistency between individual case studies (see, for example, Drummer, 2007, where references for a number of animal studies can be found). Of course, animal studies are not without their methodological problems, and the fact that different studies have used different animals, regardless of the challenges of extrapolating the results of such studies, due to differences in anatomy as well as pharmacology, have been noted (Ferner, 2008).

Another practical fact, which appears to be surprisingly frequently overlooked in the literature, is that site-to-site variability in drug concentrations is a phenomenon that is by no

means unique to the post-mortem body. Following the introduction of a drug into the living body, there is a period of up to two hours, depending on the route of administration, during which time the dose is distributed (Buxton, 2011). During this period of distribution, it follows that the - sometimes significant - differences observed between arterial and venous blood concentrations may well also be reflected in post-mortem cases where death has occurred during the absorptive phase (Pounder, 1993). In this sense, while it is the case, as we have seen, that there are several means by which post-mortem redistribution can occur, the starting point for these processes may not be at all obvious if death has taken place within the context of an acute drug administration. Equally, and at the opposite end of the pharmacokinetic cycle, it has been observed, unsurprisingly, that during the elimination phase, the venous concentration of a number of drugs is greater than the arterial concentration (Pélissier-Alicot et al., 2003).

In practical terms, therefore, it has been suggested that a very simple way of establishing whether or not any particular drug may be subject to redistribution is to take samples from multiple sites and compare the results (Drummer, 2007). Another recent experimental method has been to undertake animal studies using pre-determined doses of a number of drug molecules, with subsequent comparative sampling being undertaken over a range of times (Gleba and Kim, 2020). Other studies, in an arguably more imaginative approach to the problem, have attempted to circumvent the challenges posed by post-mortem drug redistribution by assessing the viability of alternative or corroborating tissues. One particular area of interest has been in the viability of skeletal muscle, and some early work on this tissue was able to demonstrate that drugs detected in blood can also be detected in muscle, the concentration ratio in many cases being very close (Pounder, 1993). More recently, further work has examined blood from alternative anatomical locations to the usual central and peripheral sites, one particular study revealing that blood obtained from the popliteal vein of the leg demonstrates significantly less redistribution of diazepam, methadone and morphine than samples taken from cardiac, subclavian or femoral vessels (Lemaire et al., 2016). A further and even more recent study has suggested that intra-osseous fluid - or bone marrow - may be a matrix that is free of the redistributive problems encountered in routine blood samples (Rodda et al., 2018). Although more work remains to be done, the use of popliteal

blood or bone marrow in the context of forensic casework has the potential to be useful, and may certainly prove to be more helpful than skeletal muscle in acute drug-related deaths, given the likelihood of a faster distribution.

Other and equally recent work, however, rather than examining alternative matrices, has attempted to predict the likelihood of post-mortem redistribution based on a proposed ratio model of liver and peripheral blood concentrations (McIntyre, 2014). Although reporting consistent results, the method has only been able to predict whether the likelihood for redistribution is "little or no" or else is "significant". Extending this work, the same author has further developed his predictive model to enable an estimation of what many regard as the Holy Grail of post-mortem toxicology, namely, the precise calculation of the ante-mortem drug concentration. This, McIntyre calculates by dividing the peripheral post-mortem drug concentration (*P*) by what he calls a "PMR Factor (*F*)", which is defined as the liver to peripheral blood ratio described in his 2014 paper. Thus, in the form of an equation, $AM=P/F$, an ante-mortem concentration can be calculated (McIntyre, 2015; McIntyre, 2016). The method does not as yet appear to have been adopted to any wide extent in forensic practice.

In an attempt to identify the likelihood of a drug to undergo post-mortem redistribution, another relatively recent study has developed a novel, statistical, approach, using the physiochemical parameters of K_p and pKa, the structural parameters of hydrophobicity, size and surface area, and the molecular parameters of, among others, binding energy, dipolar movement, electronic energy, refractivity and Van der Waals surface area and volume. All of these factors are combined and manipulated in order to produce a predictive, so-called "quantitative structure-activity relationship", or QSAR (Giaginis, Tsantili-Kakoulidou and Theocharis, 2009; Giaginis, Tsantili-Kakoulidou and Theocharis 2014). While highly complex and time-consuming, it is a method which has shown promising accuracy when comparing predicted with actual extents of drug redistribution.

1.4 Pre-Mortem Factors Affecting Toxicological Interpretation

The past ten or so years have seen a developing interest in the literature of certain ante-mortem factors, a better understanding of which, it is now recognised, provides a fuller context in which analytical results can be interpreted. Increasing consideration has been

given, for example, to the role that multiple drug use may have in mortality, and the ways in which drug-drug interactions may lead to the enhancement of the physiologically harmful effects of individual drugs. Indeed, in the elderly, the medical practice of 'polypharmacy' (generally defined as the concurrent prescribing of at least five medications) can lead to unwanted, and sometimes fatal, drug-drug interactions (Scott et al., 2015).

Skopp, in a medical education review paper for pathologists, helpfully distinguishes between pharmacodynamic (PD) and pharmacokinetic (PK) drug-drug interactions, reminding us that PD interactions take place at receptor sites and can involve drugs with either similar or opposing actions, whereas PK interactions can either alter the absorption, distribution or excretion of a drug, or else, at a metabolic level, can result in enzymatic inhibition or induction (Skopp, 2010). Polydrug use, furthermore, may also competitively inhibit or induce metabolic enzymes, leading to additive, synergistic, potentiating, inhibitory or antagonistic pharmacological effects (Hoffman, Zedeck and Zedeck, 2012). One study has, in attractively simple terms, described the pharmacodynamic interactions of many drugs at the receptor level as being either additive (1+1=2), potentiating (0+1=2), synergistic (1+1=3) or antagonistic (1+1=0) (Mozayani and Raymon, 2004). The underlying message, however, is that at the interpretative stage of analysis, consideration must be given to the extent to which pharmacokinetic and pharmacodynamic variables may be relevant to the death (Davis, 2014).

In practice, and to quote a handful of examples from the literature, a synergistic cocaine-heroin interaction has been noted, in one case, along with the inhibition of heroin metabolism, as a result of the presence of ethanol, leading to reduced urinary and biliary excretion (Polettini et al., 2005). More recent work in this area has indicated that ethanol inhibits two different stages in the metabolism of heroin: the hydrolysis of the primary metabolite, 6-MAM to morphine, as well as the subsequent glucuronidation of morphine to its metabolites, M-3-G and M-6-G (Thaulow et al., 2014). In the context of forensic interpretation, the result of such inhibition is likely to be the circulatory retention of relatively elevated levels of heroin and its metabolites. Some particularly recent work has documented the reaction of alcohol with a number of drugs of forensic interest, including cannabis, cocaine, GHB methamphetamine, and several opioids (Singh, 2019), and another study has

identified synergistic interactions between new synthetic opioids and several classes of commonly-prescribed pharmaceutical drugs (Pérez-Mañá et al., 2018).

Other work has identified that the SSRI class of antidepressants can inhibit the metabolism of a number of benzodiazepines, opioids and other drugs, via the P4503A4 pathway, leading to temporally increased blood concentrations and an increased parent to metabolite ratio (Drummer, 2001). The co-ingestion of alcohol, as well as inhibiting the metabolism of heroin, can, furthermore, synergistically exacerbate the respiratory depressant effects of a number of drugs, including opioids. The result of this, with a number of centrally acting drugs, can be a fatal toxicity at what might otherwise be normally-tolerated doses (Koski, Ojanperä and Vuori, 2003; Flanagan, 2008b; Lennernäs, 2009). The literature has reported interactions known to take place between NSAIDs and opioids (Kolesnikov and Sõritsa, 2008), as well as benzodiazepines and opioids (McClure et al., 2017); it has reported the acetylation of morphine co-ingested with acetylsalicylic acid, the main pharmaceutical constituent of Aspirin (Naso-Kaspar et al., 2013), and it has noted interactions between the more recent, so-called 'Holy Trinity' mixture of benzodiazepines, opioids and muscle relaxants (Horsfall and Sprague, 2017).

Although the several and various pre-mortem factors that have bearing on the interpretation of analytical results do not operate in isolation, for the sake of providing a systematic review of the literature, they are considered individually below.

1.4.1 Extent of drug absorption, distribution, metabolism and excretion before death

The comparative extent to which the concentration of a drug might be observed in biological fluids and tissues is in large part driven by the pharmacokinetic parameters of absorption, distribution, metabolism and excretion (ADME). The degree to which these variable and inter-related factors might manifest themselves in analytical samples can have considerable bearing on the interpretation of analytical results. The range of this interpretative variability can be illustrated in three hypothetical cases of an orally administered drug.

In the first of these cases, a high concentration of an agent detected in the stomach contents, along with an appreciable concentration in the blood, but with no metabolites, would be

suggestive of death soon after drug administration. In the second case, however, a lower concentration in the stomach, along with a higher concentration in the blood and the detection of the agent's metabolites in the liver, would indicate that the drug had been well absorbed and distributed before death intervened. Finally, a complete absence of the parent drug in the stomach and blood, but with significant concentrations of metabolites detectable in the urine, might indicate that the drug that had been absorbed and fully distributed, as well as extensively metabolised and partly excreted before death took place.

These hypothetical cases demonstrate, although in somewhat simplistic terms, the need for context in post-mortem toxicology. Although the drug concentrations observed in the stomach, blood, liver and urine of these three imagined cases all have individual qualitative value (always assuming the absence of false positives), it is unlikely that meaningful quantitative interpretations can be made without the wider context of the drug's state of absorption, distribution, metabolism and excretion. Thus, the finding of a high concentration of a drug in the stomach of our first case, while interesting, does not tell us much more than that basic fact. Within the comparative context of little distribution and no metabolism, however, the toxicologist may well feel confident enough to state that death was almost certainly due to the acute ingestion of the drug detected in the stomach: a conclusion, importantly, that may not have been reached had there been no distribution of the drug from the stomach, or, conversely, had the drug been largely metabolised and excreted.

Reality, however, does not always follow the clarity of hypothetical constructs and, in practice, a number of factors can cloud interpretation. In controlled experimental conditions, even relatively small differences in the so-called dose-death interval, for example, have been shown to have significant bearing on the subsequent concentration of target analytes (Lafrenière and Watterson, 2010). ADME data, furthermore, are not always directly appropriate in the context of forensic analyses since illicit drugs are often administered by routes not generally used for therapeutic purposes including, for example, nasal insufflation and smoking, and for which pharmacological outcomes may be quite different to those encountered following more conventional routes of administration (Drummer, 2007; Allan and Roberts, 2008). Even where studies do exist, however, they are often derived from

biochemical processes that take place in the living, not the dead, and direct comparison therefore may not be definitively helpful.

The picture can be even more complicated when it comes to interpreting concentrations of drugs with low therapeutic indices. Here, not only is it difficult to discern what in itself might define the difference between a therapeutic, toxic or fatal dose, but the difficulty can be compounded by inherent uncertainties in analytical methodologies. Small and unaccountable variations in temperature, in volume measurement or reagent composition can result in two identical samples producing non-identical results in either intra- or inter-assay comparison. Although the analytical coefficient of variation acceptance (expressed as the variance of the standard deviation of assay results to the mean value), can commonly be around 10% - though can be appreciably higher - for those drugs for which only the smallest of differences of dose can mean the difference between morbidity and mortality, a cv% of 10% can be enough to preclude a definitive interpretation (Ferner, 2012). Related to this, pharmaceutical grade drug standards have occasionally been found to contain trace amounts of structural analogues that are assumed to have been inadvertently introduced during the process of manufacture (Rohrig, 2019). During the analytical process, furthermore, the in-vitro synthesis of 6-MAM from morphine has been found to occur when compounds containing an acetyl functional group are used for extraction or derivatizing purposes, and the acetylation of morphine has even been found to occur when it has been co-ingested with acetylsalicylic acid, the main pharmaceutical constituent of Aspirin (Naso-Kaspar et al., 2013).

1.4.2 Drug tolerance

The phenomenon of drug tolerance, and its associated problems, has been recognised for some time. At a pharmacodynamic level, chronic administration results in receptor down-regulation and the shift of the dose-response curve such that increasing amounts of the drug are required to induce the same pharmacological effects (Ferner, 2008). The result of this can be the (sometimes considerable) overlap of drug concentrations normally defined within therapeutic, toxic and fatal ranges (Jones and Holmgren, 2009; Launiainen and Ojanperä, 2014; Jones, Holmgren and Ahlner, 2016). Clinically, this has been found to be the case with patients following, for example, the long-term administration of oxycodone, morphine and

fentanyl, as well as in the post-mortem samples of chronic users of opioids and benzodiazepine-group drugs (Jung and Reidenberg, 2005). As one particular study has observed, the development of tolerance explains why otherwise healthy addicts receiving heroin-maintenance therapy have higher plasma morphine concentrations than others dying of heroin overdoses (Karch, 2001). A similar response is known to take place in alcoholics where, in one particular case, molecular down-regulation was found to be so marked that relative consciousness was possible even when serum ethanol concentrations were found to be three times greater than the generally accepted fatal value (Johnson, Noll and MacMillan, 1982). In addition to receptor down-regulation, furthermore, one recent study has stated that tolerance can also be achieved by means of an enhanced physiological induction of enzyme expression, or else through the co-ingestion of another drug that can cause the enhanced metabolism of the target drug (Rohrig, 2019). In both of these scenarios, the pharmacological effect is to decrease the half-life of the compound in question, in response to which the user may feel compelled to use more of the drug in order to experience the desired effects. Related to this, and possibly also effected through the induction of enzyme expression, is the phenomenon of cross-tolerance. Found particularly in relation to benzodiazepines, hallucinogens, phenethylamines and opioids, in this mechanism chronic exposure to one drug can lead to the relative tolerance of other compounds of structural similarity and, occasionally, to tolerance of those that are structurally unrelated (Rohrig, 2019).

Knowledge of the concept of tolerance can, clearly, be helpful in predicting the likely pharmacodynamic response to administered drugs, and it can, furthermore, be of significant relevance in the interpretation of drug involvement in the deaths of individuals (often heroin addicts) who, following a period of abstinence and subsequent re-equilibration of drug receptors, then return to their previous dose, with fatal results (Roberts and Buckley, 2007; Andrews and Kinner, 2012).

In practical terms, however, while some matrices – such as hair and nail - can be analysed to confirm suspected chronic drug use in the weeks or months preceding death (Pragst and Balikova, 2006; Paterson, Cordero and Stearns, 2009), there are currently no known reliable biochemical or histological markers that allow tolerance *per se* to be measured post-mortem. Some work in this area has, however, sought to identify reduced receptor numbers in the

brains of cocaine and opioid addicts (Karch, 2001; Ferrer-Alcon, La Harpe and Garcia-Sevilla, 2004) and the particularly recent development of 'metabolomic' studies is, among other things, seeking to identify biomarkers associated with the chronic administration of, particularly, opioids (Dinis-Oliveira, 2019; Brockbals et al., 2020). Although demonstrating potentially significant forensic usefulness, much more work remains to be undertaken in this area before accurate measurements of tolerance can be routinely reported.

1.4.3 Physiology

Physiology is known to have significant bearing on many pharmacological parameters, and knowledge of these factors may aid the interpretation of analytical results. Indeed, age-related degeneration, as well as acute disease states can predispose the users of certain drugs to clinical outcomes that might not be experienced by other groups of users (Gruszecki, Booth and David, 2007).

In advanced age - in a pharmacological context, usually defined as >75 years of age (Rowland and Tozer, 2011) - physiological changes can result in an increase in gastric pH, but slower gastric emptying times, as well as decreased gut motility and the reduction of intestinal absorption areas, all of which can impact analytical interpretation. The mechanisms by which reduced metabolism and clearance of drugs are experienced in the elderly are well understood, being related to a general decline in the efficiency of, respectively, the liver and kidneys (Drummer, 2013). In age-related liver degeneration, for example, it has been suggested that changes to the organ may be especially important considerations in post-mortem toxicology since a reduced liver size, as well as the reduction of hepatic blood flow, can limit the extent of first-pass metabolism and thereby result in higher-than-expected drug-plasma concentrations (Byard, 2013b). Earlier work on the liver in the elderly established that the metabolic capacity of the organ can be altered when, for example, oxidative pathways such as glucuronidation become more easily saturable (Jones, 2007; Jenkins, 2008b). As well as hepatic changes, age-related physiological decrease in kidney function in the elderly can lead to reduced clearance. In one study of the elderly, for example, although one third of the subjects appeared to have good kidney function, the remaining two thirds demonstrated renal clearance that was reduced in association with pre-existing cardiovascular disease or

other physiological risk factors (Shi and Klotz, 2011); the pharmacological implications of this are well recognised by medical practitioners, who commonly reduce the dosage of many medications in their renally-compromised patients in order to avoid excessive circulating concentrations.

Many studies have, in fact, established that in the elderly (as well as in acute and chronic conditions such as cirrhosis and acute viral hepatitis in those of 'normal' age), the combination of reduced hepatic and renal function may have a significant effect on the clearance of many drugs (Elbekai, Korashy and El-Kadi, 2004; Cardelli et al., 2012; Rohrig, 2019). Even something as seemingly insignificant as the alteration of the muscle to fat ratio that occurs within the ageing body can result in an alteration of the assumed volume of distribution of many drugs (Drummer, 2001). Indeed, common age-related physiological changes, including a reduction in body mass and total body water, but an increase in body fat, along with reduced serum albumin but increased α 1-acid glycoprotein, as well as reduced liver mass and hepatic blood flow and a reduced renal blood flow and glomerular filtration rate, can all contribute to changes in the expected pharmacokinetic parameters of many drugs (Byard, 2013b). Byard, in fact, goes on to suggest that the clinically commonly observed loss of homeostatic mechanisms in the elderly, including excess blood loss as a result of anticoagulant therapy, and dehydration and electrolyte imbalances caused as a result of chronic diuretic therapy, all contribute to a lower-than-expected resilience in this group to the pharmacological effects of drug molecules, in turn making post-mortem toxicological interpretation in such cases potentially extremely complicated.

In cases of critical illness too, the combination of haemodynamic and physiological imbalance commonly results in the slower clearance of many drugs: the half-life of midazolam, for example, in one study, has been shown to have increased from the usual two hours to up to ten in patients having undergone cardiac surgery (Drummer, 2001). In cardiac disease, furthermore, the rate of transport of molecules to the liver and kidney for excretion can be much reduced, and sepsis can greatly alter metabolic processes (Skopp et al., 2010).

Even in particularly obese people, furthermore, although the absorption of drugs is unchanged, there can be a very large increase in the apparent volume of distribution for many

drugs, particularly those that are highly lipid soluble: diazepam and midazolam V_D, for example, increasing by a factor of up to three (Drummer, 2001). Related to this, recent work has shown that bariatric surgery can result in markedly different rates of absorption to those observed in people with intact gastrointestinal tracts (Bishop-Freeman et al., 2019). Other work has sought to identify physiologically-based gender differences in drug toxicity (Nicholson, Mellor and Roberts, 2010).

One final area of interest has been in pharmacokinetic functioning in the time immediately preceding death. Decreased cardiac output and associated low blood pressure, along with impaired pulmonary ventilation, dehydration and associated electrolyte imbalance, as well as metabolic acidosis are all likely to alter the rate or degree of normal pharmacokinetic processes. It is known, for example, that peri-mortem hypoxia reduces intracellular pH and thereby induces an accumulation of basic - though not acidic or neutral - drugs (Skopp, 2010).

1.4.4 Case history

Of course, much of what has been discussed above, under the headings of 'drug tolerance' and 'physiology', can be regarded as case history. It is also the case that one of the important messages to have emerged from the recent literature, furthermore, is that it is with detailed circumstantial knowledge – case history - that the most nuanced analytical interpretations can be made.

It was said at the outset of this chapter that until relatively recently post-mortem toxicological analysis results would simply be compared to reference values obtained from the living, and an opinion would be presented on that basis. But possession of even a very simple case history might result in the presentation of a very different opinion. As one toxicologist states simply, while deaths following a period of coma may, when analysed, reveal drug concentrations consistent with therapeutic use, the same samples may have tested toxic hours earlier (Drummer, 2007). Extrapolating one of the three hypothetical cases described in 1.4.1, above, in the case of delayed hepatic toxicity caused by paracetamol poisoning, the complete absence of the parent drug in blood, and perhaps the detection only of metabolites in the urine, may lead to an incorrect interpretation without the context of case history (Vale and Proudfoot, 1995). Extending this analogy even further, it is the case that in paraquat

poisoning, death may not occur for some weeks after ingestion, by which time the drug may not be detectable, though contextually helpful pulmonary fibrosis may be found at autopsy (Ferner, 2008). Indeed, a significant number of autopsy findings can be indicative of intoxication or poisoning, and a useful table of these can be found in Skopp, 2010.

Sometimes, case history can be used to support a negative analytical finding in the case of an otherwise unexplained death: the toxicological absence of an anti-convulsant in a known epileptic patient suspected to have suffered a fatal fit, for example, would enable medication non-compliance to be proposed as a mechanism for the death (Flanagan, 2008b). At other times, knowledge of recent medical history can sometimes help distinguish between different forms of dose-related toxicity, whether acute, chronic, acute-on-chronic or self-poisoning (Flanagan, 2008a). In the case of Dr Shipman, for example, the finding of significant quantities of morphine in the skeletal muscles of several of his patients who, while otherwise well, had collapsed and died within short spaces of time of his visits to them, enabled case history to support his convictions for murder, since there appeared to be no alternative explanation for either the presence of morphine in their bodies or for their sudden deaths. (Ferner, 2012). Indeed, one study, although admittedly somewhat dated, has suggested that the accurate interpretation of post-mortem toxicological analyses is dependent on reference to case history in around 70% of cases (Harding-Pink and Fryc, 1991).

1.4.5 Pharmacogenetics and pharmacogenomics

Finally, the usefulness of pharmacogenetics and pharmacogenomics is something that has gained in currency in recent years, although it may be fair to say that interest currently remains more in the realm of academic interest, as well as in clinical application, than it does in forensic practice.

Work in this area, nevertheless, has developed as an emerging pharmacological sub-speciality with the specific aim of optimising drug therapy, where it is more widely known as 'personalised medicine'. Studied and reported with increasing frequency since the late 1990s, there has been some terminological distinction between 'pharmacogenetics' – the study of the whole spectrum of inherited differences in drug metabolism – and 'pharmacogenomics' - the association between an individual's genotype and their individual response to

xenobiotics. At a molecular level, it is known that the disposition of drugs can be altered by genetic polymorphisms that affect either metabolising enzymes, transport proteins or receptor, enzyme or other drug targets, and work in this area takes as its basis the premise that individual variations in drug response result from these differences in individual genetic makeup (Kupiec, Raj and Vu, 2006). Research, thus, seeks to define what these genetic differences in an individual may be, in order then to capitalise on these findings in order to improve clinical outcome.

As far as forensic toxicology is concerned, the greatest interest in the literature of recent years, unsurprisingly, has been in drug metabolising enzymes. Although the cytochrome P450 group is by far the best known, there are in excess of 30 groups of drug-metabolising enzymes, each able to metabolise different xenobiotics (with some molecules being metabolised by more than one enzyme); genetic mutations in any of these enzymes can result in entirely absent, reduced or, conversely, increased metabolic activity. (Rohrig, 2019). Inter-individual variations in any of these enzymes may, of course, result in different analytical outcomes, even when the same drug has been administered by the same route and in the same quantity. In the case of CYP2D6, for example, known to be the most polymorphic of all the CYP enzymes, and found to play a role in the metabolism of a large proportion of pharmaceutical compounds, up to 10% of the entire Caucasian population is known to be 2D6 deficient; compared to those expressing the enzyme, 2D6-deficient individuals can accumulate toxic concentrations of a number of drugs (Johansson and Ingelman-Sundberg, 2011). One recent study, furthermore, has linked five 2D6-polymorphisms to tramadol adverse reactions (Wendt et al., 2020). Other work has considered genetic variations that lead to the induction or inhibition of a number of other pharmacologically important enzymes (Lamba et al., 2012; Zanger and Schwab, 2013). Polymorphisms of the CYP3A4 enzyme, for example, have been shown to have contributed to methadone-related fatalities, due to a reduced rate of metabolism of the molecule (Richards-Waugh et al., 2014). This, in forensic terms, is of course potentially a very important fact, since a presumption of intent, in the case of an assumed fatal blood concentration of a particular drug, might change radically in light of what might alternatively be determined to be a polymorphism-mediated concentration.

Earlier in this chapter mention was made of drug-drug interactions and how, at a molecular level, such interactions can lead in changes to enzymatic activity, sometimes resulting in altered clearance. Much of our understanding of these reactions has come about as a direct result of pharmacogenetic research. It has been demonstrated, for example, that the commonly prescribed quinolone antibiotic, ciprofloxacin, is a very significant inhibitor of CYP3A4 (and 1A2), and significant medical complications have been reported when this drug has been administered along with the antipsychotic, clozapine (Bruwers et al., 2009). CYP2D6, furthermore, already absent in 10% of the Caucasian population, is known to be greatly inhibited by the antiarrhythmic, quinidine, as well as the SSRI antidepressant, fluoxetine; when taken along with codeine, the pharmacological response and clinical outcome can be very different for those in whom the enzyme has not been inhibited (Lam et al., 2014). Even grapefruit juice is known to inhibit the activity of CYP3A4, as a result of which the blood concentrations of any drugs that are substrates of this enzyme can be elevated: a fact that is well known in general practice in relation to the prescribing of certain statin-group medications.

Although the routine practical application of pharmacogenetics and pharmacogenomics to forensic toxicological work may seem some way off, recent success has been achieved in the assessment of the urinary ratio of 6ß-hydrocortisol to cortisol as a post-mortem marker of altered CYP3A activity (Lang and Linnet, 2014). As the CYP3A group of enzymes is believed to play a part in the metabolism of over 50% of drugs, relatively simple methods such as that proposed by Lang may, in due course, prove to be helpful at the interpretative stage of analytical work. Indeed, as long ago as 2002 it was recommended that possible genetic influences should be routinely considered in toxicological analysis, in a process sometimes referred to as "molecular autopsy" (Jannetto et al., 2002), and it is already several years since UK toxicology guidelines recommended the inclusion of metabolic profile consideration in the interpretation of results (Cooper, Paterson and Osselton, 2010).

A helpful introduction to pharmacogenetics and pharmacogenomics can be found in Wong (2011), detailed account of genetics and pharmacology (including a useful table of the frequency of genetic polymorphisms and affected drug substrates) can be found in Rowland and Tozer (2011), a discussion of the role of individual metabolic enzymes can be found in

Lappas and Lappas (2016), and case studies of toxicological applications can be found in White and Wong (2008).

1.5 Conclusions

The introduction to this chapter noted that, while the traditional autopsy is able to identify many pathological conditions, as well as the failure of many physiological processes, it is not, with only a small number of exceptions, able to identify drug-related death. Instead, ancillary techniques, focused on the analysis of a wide range of bodily fluids and other tissues, largely derived from clinical practice, have been developed in order to inform investigative processes. While, on the whole, providing acceptable qualitative proof, it has been acknowledged for some time, however, that the accurate interpretation of such analysis carries with it significant challenges that are not encountered in clinical toxicology: one study commenting that death renders the assumptions of clinical toxicology largely invalid (Ritter, 2008). Indeed, the challenges encountered in post-mortem toxicology have, in the case of some commentators, led them to opine that current methods have little to contribute to death investigation. Although writing in 2004, one eminent toxicologist, for example, has stated that “modern forensic toxicology – as paradoxical as it sounds – does not possess methods capable of proving that death has definitively been caused by poisoning” (Daldrup, 2004). Although in a number of circumstances his assertion may well hold true, many would argue that toxicology can in fact often prove more helpful than Daldrup suggests. One British clinical pharmacologist, for example, has stated that, in the best circumstances, toxicological analysis is able to detect drugs that should not be present, in concentrations that are unequivocally harmful, but that are, in the context of clinical history, consistent (Ferner, 2012).

What is perhaps implicit in these, and the many other, sometimes seemingly contradictory, commentaries that are to be found in the literature is that it is the interpretation of analytical results that is often the most difficult aspect of forensic toxicology. Here, arguably, there is also some paradox since, as another study has pointed out, determining whether drugs may have contributed to the death is the primary goal in the interpretation of post-mortem toxicology results (Leikin and Watson, 2003). As we have seen in the literature reviewed in

this chapter, however, as much as a negative finding does not, in itself, prove that a particular drug was not present at the time of death, so it does not automatically follow that the post-mortem detection of another drug is proof of its ante-mortem administration, or of its role in the subsequent death of the person concerned. Although there has, furthermore, been a steady stream of new literature in the field in recent years, much of which provides ever-refined techniques for the quantitative identification of many drugs, it is arguably a fact that despite providing the practitioner with much data, what research has also done is to widen the gulf between clinical toxicology and post-mortem toxicology. This in itself is not necessarily a bad thing and, as one analyst comments, is in some respects very welcome, although, as he subsequently adds, it arguably does little to add interpretive clarity to the outcomes of toxicological analysis (Teixeira, 2014).

As we have seen, the literature of the last two to three decades has examined and continues to address a number of factors, whether occurring before or after death, that either individually or in combination frustrate the interpretative stage of analysis. Indeed, and in the case of the post-mortem period, what can occur has been described by one toxicologist as a plethora of complexities (Kennedy, 2010). These, what might be termed, multi-layered difficulties begin with the analytical matrix. As we have seen, post-mortem toxicological endeavour has largely grown out of clinical pharmacology, where blood is the sample of choice. The variable nature of component degradation within this matrix after death, however, makes blood, in many respects, an inconsistent starting point in the majority of post-mortem analytical procedures. Despite this, and perhaps surprisingly, blood has, however, continued to form the backbone of much research, despite the fact that its use can invoke further interpretative difficulties.

As a result of endogenous enzymatic activity, for example, or as a result of endogenous or exogenous bacterial activity, a number of forensically important drugs, including opioids, benzodiazepines and cocaine, have been shown to be variably unstable in the post-mortem blood environment. The recommendation, therefore, has been that aspirated blood samples should be stored in preservative-enriched containers that are frozen to at least -20°C, if not -80°C. But even before enzymatic or bacterial degradation may have taken place, the phenomenon of post-mortem redistribution may have altered the localised concentration of

many drugs, particularly those with a high apparent volume of distribution, in the time between death and sampling. Although research into drug redistribution continues to be pursued, and may be helpful, it is tempting to conclude that the tri-fold problem-combination of blood, post-mortem redistribution and sample stability, and the variable nature that each of these factors can bring to bear, make it difficult to argue that analytical results can be interpreted with the degree of precision that might be preferred by the analyst and demanded by the investigative, legal process.

Paracelsus famously stated that while all substances possess poisonous qualities, it is the dose that defines whether or not the substance is an actual poison. In light of the research output of more recent times, however, a refinement of his maxim might also include reference to ADME conditions, drug-drug interactions, drug tolerance, physiology and pharmacogenetics. As we have seen, it is the case in many circumstances that the most meaningful analytical interpretation can only be presented if ante-mortem conditions have also been given adequate consideration. The literature of more recent years has begun to address a number of what might be termed pre-mortem factors that in some cases can have bearing on any subsequent analytical interpretation. The quantity and route of the administered drug, whether there was any degree of established tolerance to the drug in question, the amount of time that may have elapsed between administration of the drug and death, are all relevant matters in this respect. Additionally, age, gender, body weight and disease state may all play a part in the responsiveness of an individual to a given dose, such that a dose that might be tolerated by one individual could prove lethal to another. In many cases the picture can be further complicated by the fact that the death may be associated with the action, or indeed with the interaction, of more than one drug, including alcohol. Clarity in these matters, individually or collectively, can be crucially important within the context of a suspected acute overdose case.

The back-extrapolation of blood ethanol samples provides an excellent and reliable means of determining, with a good degree of accuracy, how much drink was in the system of a living subject at a particular time. The back-extrapolation of drug concentrations from autopsy samples, however, remains the elusive aspiration of post-mortem toxicology, although that is not, of course, to say that post-mortem analysis cannot provide helpful information. For all

but a few drugs, the process is not as straightforward as many courts might wish it to be, and opinions can rarely be reached by a simple reporting of analytical results. A considered assessment of post-mortem drug concentrations in the light of ante-mortem case history can, in many cases, provide a useful insight into the role that drugs may have played in unexplained deaths, and may certainly enable the provision of an opinion which, although only indicative, may well be better than none. The failure to take into account all relevant ante- and post-mortem factors, or a misinterpretation of the facts at any stage, can, as one toxicologist has stated clearly, have significant consequences for legal processes (Drummer et al., 2004b). Without context, it might be said, the identification of a range of molecules, in a range of concentrations, may lead to the drawing of erroneous conclusions. Perhaps in response to these difficulties, a number of recent studies have attempted to overcome what might be perceived as the subjective nature of analytical interpretation. It has been suggested, for example, that Bayesian statistics might be used to assess the likelihood of drug-related death, while taking into account pathological findings (Langford et al., 2015). More recently, the British forensic toxicologist Simon Elliott has proposed "toxicological significance scores", which take account of all available evidence, not just analysis results, and which might be used to indicate the likely role that a particular drug may have played in a death (Elliott, Dedefov and Evans-Brown, 2017).

Where some recent studies have sought to find objective ways of interpreting analytical data, others have sought to identify new analytical methods that by their nature remove some of the uncertainties that have been discussed in this chapter. The thrust of some of this work, moreover, aligns closely with the methods proposed in this thesis for the analysis of samples from embalmed bodies. Indeed, although VH, CSF and SF form the analytical basis of much of the work presented later in this thesis - specifically from the point of view of their usefulness in the toxicology of embalmed bodies - a number of writers have previously signalled the potential of these fluids as a means of bypassing some of the problems associated with blood. In some senses, furthermore, they might be seen as an alternative to the statistical approaches that have recently been recommended by the likes of Langford and Elliott. Levine, in his 2010 monograph, for example, noted the potential of VH, given its anatomically protected environment, as a matrix in which drug molecules would not be subjected to either

enzymatic or bacterial degradation to the same extent as they often are in blood. Levine, of course, was not original in his suggestion, the comparative sterility of VH having been first discussed as early as the late 1980s (Harper, 1989). It is perhaps the eminent toxico-pathologist, Steven Karch, however, who has been most vocal in his call for the forensic community to develop new methods as a direct means of solving old problems. As long ago as 2001, Karch wrote that "[as] post-mortem blood-drug concentration measurements are useful for the diagnosis of drug use and nothing more………", "pathologists should give serious thought to testing vitreous or spinal fluid instead of blood" (Karch, 2001, pp.394 and 395. Writing some 12 years later, in 2013, Karch was still calling for new methods. Summarising the contemporary literature, he stated that it was still very difficult to make any case for the quantitation of post-mortem blood concentrations, adding that the task becomes proportionately harder to make as the post-mortem interval increases. As an alternative he suggested examining brain concentrations. These, he says, are unlikely to change much after circulation has ceased, and [by virtue of the protected anatomical position of the brain] post-mortem redistribution is not a complicating factor. The same, of course, may be said for VH, SF and CSF, all of which, given their protected environments, as will be discussed further in Chapter 3, might be assumed to be less susceptible to either post-mortem redistribution or bacterial degradation. Admittedly, compared to blood, we have few data for VH, and even fewer for either SF or CSF, but one of the subtexts of Karch's call to action, surely, is that research has to begin somewhere. To avoid analysing new matrices simply because of the fact that we have few comparative data, Karch would no doubt argue, may be as paradoxical as it is unjustified. Indeed, one particular VH review paper of comparatively recent times has called directly on toxicologists to analyse the matrix and to publish their results in order "to enrich the literature of the subject." (Brunet and Mura, 2012).

Of course, part of the problem against which Karch and a small number of other toxicologists have recently railed may be the extent of sub-specialisation with which research is often conducted. One might argue, for example, that the significant and sustained post-mortem redistribution publication output of one particular British toxicologist has been misguided since, arguably, all it has succeeded in achieving is further to underline the fact that the phenomenon makes meaningful toxicological interpretation very difficult. Instead, Karch

might argue that this particular toxicologist may have made a more useful contribution had he directed some of his efforts into examining alternatives to blood. The same may be said for some experimental conditions reported in the literature which, it might be argued, are so removed from reality as to be rarely, if ever, encountered in properly-conducted forensic work. Reporting, for example, that up to 81% of 6-MAM can be lost from blood samples that are stored for up to eight years at -20ºC (*cf* Høiseth et al., 2014), or that a number of other compounds can remain stable for up to 18 years (Karinen et al., 2014) may be of academic interest, but it is unlikely to be of practical relevance in the majority of forensic laboratories. In this, and in other areas, one might argue that the degree of specialism espoused in some work is such that the proverbial wood is very much obscured by the trees.

As stated at the outset of this chapter, the slant of the literature review presented has in some ways tended towards highlighting the weaknesses inherent in the analysis of post-mortem samples. This focus has in the main been deliberate, as it has sought, as an end in itself, to collate recent research outcomes in a way that has not previously been attempted. The review has also been presented in this manner, however, as a means of paving the way for the subsequent chapters of this thesis. Although the toxicology of embalmed human remains may in many ways be an interesting subject, it is also a subject which is far from mainstream, and for which the literature is comparatively limited. In this sense, it was felt that there was compelling justification for providing a comprehensive and particularly wide-ranging context to what follows later in the thesis.

2 EMBALMING: PURPOSE, METHOD AND ASSOCIATED TOXICOLOGY LITERATURE

2.1 Introduction

The primary purpose of this chapter is to present a wide-ranging review as well as a critical appraisal of all the published literature relating to the toxicological analysis of human tissue preserved using formaldehyde. Some of the literature presents simple case studies, usually describing the toxicological findings in bodies that have been embalmed, buried, and subsequently exhumed for forensic examination. Much of the literature, however, describes laboratory experiments in which the methodological approach was to simulate the conditions to which drugs in the embalmed body might be presumed to be subjected. The literature, however, is in some respects quite disparate and is thus not always readily comparable. In many instances, methodological hypotheses differ, such that experimental variables do not always align. In other papers, uncertainty surrounding the precise conditions found within the embalmed body - whether chemical, physical, or a combination of the two – leads to the presentation of results that are, in many respects, little more than speculative. What this chapter aims to do, therefore, is to collate, for the first time, all published literature relating to the detection of drugs and poisons in formaldehyde. Importantly, furthermore, as well as first reviewing the existing literature, the later part of the chapter seeks to appraise it specifically within the context of the embalmed and repatriated body. Within this context, the review seeks to highlight a number of methodological concerns around which, it is proposed, much of the previous literature has been based. Chief among these is the matter of formaldehyde concentration used in experimental work, where it is suggested that a fundamental error made in one of the earliest papers in the modern-day literature, some thirty years ago, has been unquestioningly replicated ever since, with the result that much of the literature we have is based on what it is suggested is a false assumption. It is hoped that this novel approach to the subject will serve not only to present a detailed critique of past studies, but that it will also to afford the opportunity to posit a clear rationale for the experimental work presented in the later chapters of this thesis, rooted in a detailed understanding of the embalming process.

Embalming, however, is not a generally or widely understood subject. For the purposes of providing context to the discussion and critique that follows, therefore, consideration is first given to the nature of embalming fluid, including a description of the common constituents of commercial preparations. Following this, a brief account is given of the usual methods by which embalming is carried out on the human body. The review that follows then considers the formaldehyde-toxicology literature in detail. For the purposes of organisation, after an initial and brief survey of the early literature, this is presented by drug class, and generally chronologically; a list of publications discussed, also organised by drug class, are listed in Appendix 2. Finally, a concluding section seeks to draw out what might be regarded as the main problems with the literature, both in their own right as well as, specifically, from the perspective of the repatriation-embalmed body.

2.2 Historical Basis of Embalming

Embalming has been described as "the process of chemically treating the dead human body to reduce the presence and growth of microorganisms, to retard organic decomposition and to restore acceptable physical appearance" (Batra et al., 2010).

Although the modern-day requirement for embalming may be primarily driven by the aesthetic preferences of the living (Bradbury, 1999), in ancient times the need was very different. Many religious beliefs, including that of the Ancient Egyptians, believed that decomposition of the body would prevent the passage of the soul to the afterlife, and methods of long-term preservation were therefore commonly employed as a means of ensuring eternal life (McDermott, 2006). Natural preservation, whether by freezing, desiccation, by either dry cold or dry heat, came to be practiced in those parts of the world where climactic conditions presented the means by which such methods of what is often termed 'artificial preservation' could be utilised (Chamberlain and Parker Pearson, 2001). In Egypt, however, as early as the first dynasty, *c*.3200 BC, preservation by natural means – which, until this time, had been achieved by desiccating the body, through burial in the hot desert sands – was developed with the practice of abdomino-thoracic and cranial evisceration before a process of curing in natron (a solution of sodium salts) followed by the application

of herbs, oils, incense, gums, pitch, tree-derived resins (Aufderheide, 2003), and boric acid salts (Buckley, Clark and Evershed, 2004).

Later, in Europe, the surviving writings of several sixteenth-century physician-anatomists attest to methods seemingly practised for centuries on the bodies of royalty and nobles, involving evisceration and the complete immersion of the body in alcohol, followed by the insertion of preservative herbs into the cavities, after which the body would be wrapped in tarred or waxed sheets (Brenner, 2014).

It was not until the seventeenth century, however, following William Harvey's work demonstrating the circulatory system of the human body, that the means of vascular embalming that constitutes the forerunner of today's methods began to be developed (Aird, 2011). Although early arterial injections often included oils, such as oil of turpentine, oil of chamomile and oil of lavender, as well as mixtures containing, variously, alcohol, arsenic, creosote, glycerine, mercury and phenol, each of which possessed limited abilities to arrest decomposition, it was not until the discovery of formaldehyde, in 1869, by the German chemist August von Hofmann, that effective vascular preservation could be achieved (Mayer, 2006; detailed accounts of the history of embalming can be found in Mayer, 2006 and Brenner, 2014). Formaldehyde-based embalming fluids that were developed in the second half of the nineteenth-century constitute the basis of the fluids still used today, worldwide, by funeral homes and medical schools. In the context of a funeral home, a typical commercial embalming fluid is formulated to give the body a natural, life-like colour, as well as, ideally, to maintain tissue flexibility (Ajmani, 1993) and will contain a number of substances. Although there is variation in the precise proportions used by different manufacturers, the constituents of embalming fluids are essentially universal (O'Sullivan and Mitchell, 1993), consisting of disinfectants, modifying agents (including buffers, anticoagulants, surfactants and humectants), dyes, perfuming agents and diluents (Balta et al., 2015). Although detailed descriptions of the ingredients of commercial embalming fluids can be found in Mayer, 2006 and Brenner, 2014 (the latter also contains a number of embalming fluid 'recipes'), the following, from Mayer, 2006, unless otherwise stated, is a summary of the main constituent compounds.

2.3 Embalming Fluid Constituents

Formaldehyde – a gas, produced by the oxidation of methyl alcohol, first synthesised by Aleksandr Butlerov in 1859 and identified as an aliphatic aldehyde by August van Hofmann in 1867 (Vulimiri et al. 2011). It has a chemical formula of CH_2O, a simple structure (see Figure 2-1) and a relative molecular mass of 30.03. It is readily soluble in water, when it is known as formalin, containing approximately 37% formaldehyde by weight and 40% by volume, and in commercial preparations is commonly formulated with 10-15% methanol, added as a stabiliser to prevent polymerisation (Brown, 1999). It oxidises easily, leading to the formation of formic acid, and can be reduced to methanol; above 150°C, it forms methanol and carbon monoxide (Yokchue, 2016).

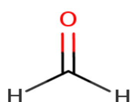


Figure 2-1 Chemical structure of formaldehyde

Formaldehyde is an effective bactericide, with excellent disinfectant and preservative properties. It eliminates putrefactive micro-organisms and reacts with tissue proteins to form cross-linked resins that are highly resistant to decompositional processes. When used as an embalming fluid, formaldehyde reacts with proteins, lipids and nucleic acids (Hopwood, 1969). One of the primary reactions is a protein cross-linking effected by the insertion of a methylene bridge (-CH_2-) between the nitrogen atoms of adjacent proteins, amines and associated nucleotides, causing the molecules to fix (Brenner, 2014). Thus, the main benefit of formaldehyde in embalming is that is causes the cross-linking of tissue proteins which, in turn, makes the environment less accessible to micro-organisms, thus slowing natural decay processes (Titford, 2012).

The degree of fixation is variable, and although many protein couplings are stable, they can be reversed by acid hydrolysis; the stronger the concentration of formalin injected, however, so the greater is the resultant degree of acid-resistant fixation (Bedino, 2003).

Formaldehyde has disadvantages, however, as it coagulates blood, which can impede the embalming process, and it creates greying as well as dehydrating effects in bodily tissues

when it mixes with blood. Although highly effective against bacteria, furthermore, its fungicidal and insecticidal properties are less pronounced, and even heavily embalmed bodies remain vulnerable to moulds or maggot infestations (Ajmani, 1993).

Although the Biocidal Products Directive 98/8/EC (European Parliament and Council, 1998), threatens to prevent the use of formaldehyde, it remains for the time being the primary preservative substance in the majority of commercially available embalming fluids.

Phenol – a disinfectant, commonly produced from coal tar or petroleum by the partial oxidation of isopropylbenzene. Its ability to affect cell permeability and to deactivate intra-cellular enzymes makes it an extremely effective bacteriostatic at a concentration as low as 0.2%, and at 1.0-1.5% concentration its lipophilic character allows it to denude cells of their walls, and to denature and precipitate proteins, making it a useful bactericidal and fungicidal (Bedino, 1994). Its ability to denature proteins, however, can result in the discolouration and dehydration of embalmed tissue (Richins, Roberts and Zeilmann, 1963).

Solvents – water is the primary solvent, with most diluted embalming fluids containing over 90% by volume. Additionally, many embalming fluids also contain methanol as an additional solvent, at between 1% - 3% by volume. Methanol, additionally, has disinfectant properties, being toxic to many micro-organisms, and it also acts as a refrigerant and prevents the polymerisation of formaldehyde, as well as coagulating albumin and maintaining the optimal density of the embalming fluid (Bradbury, 1978).

Wetting agents / surfactants – the most commonly used of which is sodium laurel sulphate, a non-ionising surfactant which reduces the surface tension of the embalming fluid, allowing better penetration through the vascular bed and into the tissues (Macdonald and Macgregor, 1997). Glycerin can also be used, although it is hygroscopic and can cause tissue dehydration (Ajmani, 1993).

Anticoagulants – usually sodium citrate or sodium oxalate and used to aid the distribution of the fluid throughout the body by dissolving blood clots, which are one of the main challenges to effective embalming.

Metallic salts – typically magnesium and sodium sulphates are used, like wetting agents, for their osmotic properties in solution, in addition to their ability to counteract the dehydrating effects of other constituent substances.

Oils – typically lanolin, used to aid tissue penetration and to reduce post-embalming tissue dehydration.

Humectants – usually sorbitol or glycerine and used, like oils, to reduce post-embalming dehydration. Additionally, there is some evidence to suggest that glycerine may improve the efficiency of formaldehyde (Bradbury, 1978), although other evidence suggests that sorbitol may be a better, creating less darkening or 'browning' of the embalmed tissues (Brenner, 2014).

Dyes – usually eosin or ponceau (though can also include erythrosine, amaranth, acid fuchsin, toluidine red or rhodamine), and used to stain tissues in order to give a life-like appearance to the body (Ajmani, 1993).

Perfumes – typically including oils of cloves, lavender and rosemary, as well as benzaldehyde, and used to mask the pungent odour of the formaldehyde and phenol.

Buffers – typically a mixture of either boric acid and sodium borate or disodium phosphate and sodium bicarbonate, and used in order to maintain the slightly alkaline pH (pH ~9) that best stabilises the formaldehyde within the embalming fluid. In addition to acting as buffers, borax salts also contribute to the prevention of bacterial tissue decomposition, as well as protecting against the development of mould (Macdonald and MacGregor, 1997). Boric acid, furthermore, has insecticidal properties, and has been used as a preservative in some recently reformulated embalming fluids (Thiel, 2002; Majewski et al. 2003).

2.4 Embalming Method

Unlike earlier methods of body preservation, the primary focus of modern-day, funeral-home embalming is the outward appearance of the corpse (Trompette and Lemonnier, 2009), allied to which is the disinfection of the body and the short-term preservation of tissue (Davidson and Benjamin, 2006). Additionally, there is an ongoing need for the preservation of bodies for educational purposes, where university medical schools

may wish to retain cadavers for two or three years rather than for the two or three weeks required by funeral homes, and where different embalming fluids – based on much greater concentrations of formaldehyde than those used in funeral homes – are employed (Brenner, 2014).

The British Institute of Embalmers (BIE), the registrant body of UK qualified embalmers, states in its trainee textbook that the purpose of modern embalming is threefold: preservation (to retard the process of decay), presentation (to restore a more life-like appearance) and sanitation (to prevent danger to public health) (British Institute of Embalmers, 2005). There is a particular emphasis on the appearance of the body, especially in cases of sudden or traumatic death when, psychologically, there is often a desire for family members to view a body that, as a result of careful embalming, presents as closely as possible a realistic appearance of the deceased (Bradbury, 1999). Modern-day embalming involves the introduction of a formaldehyde-based preservative fluid into the circulatory system, by means of a pressurised system, that enables all tissues of the body to be perfused, with the simultaneous removal of blood. The precise method of embalming varies, depending primarily on whether or not the body has been subject to an invasive autopsy examination, since such a procedure causes widespread disruption of the circulatory system and thus requires a specific embalming method.

2.4.1 Non-autopsy embalming method

The embalming of a non-autopsied body takes advantage of the fact that the whole of the circulatory system is interconnected and the entire body, thus, can be embalmed via one injection point. Typically, a small incision will be made above the right clavicle or in the right groin (vascular anatomy is located slightly more superficially on the right-hand side of the human body compared to the left and is therefore easier to locate and access) and the common carotid or femoral arteries, respectively, will be identified and isolated. The artery wall is opened and a cannula is secured within the lumen. The cannula is attached via tubing to a tank of embalming fluid and an electric pump. Alongside the arterial cannula, a drainage catheter is secured within the anatomically-related vein - the internal jugular or femoral vein - through the same supra-clavicular or groin incision that is used to access the respective

artery. When the pump is activated, embalming fluid passes around the circulatory system displacing blood, via the vein drainage tube, into a collection bottle for later disposal. Although the amount of fluid injected will vary according to several factors – including not just the size of the body, but also the degree of dehydration present at death, as well as the general condition of the body and the length of time for which the body needs to be preserved prior to disposal – a typical, short-term, funeral-home embalming will introduce between two and five litres of fluid into the circulatory system. Although arterial embalming is a highly effective means of penetrating deep tissues, it cannot effectively treat the frequently infective contents of the lungs or gut. At the conclusion of the arterial injection, therefore, a small incision is made below the xiphoid process of the sternum, through which a long, narrow and hollow instrument, known as a trocar, is introduced. Directed into the abdomino-thoracic cavities, the trocar is fitted with a very sharp, arrow-shaped tip which, by a vigorous thrusting movement, is used to pierce organs; the other end of the trocar is attached via tubing to a suction pump and, thus, liquid contents of the lungs, stomach and intestines can be aspirated and discarded. Next, the trocar is used to introduce approximately 1 litre of highly concentrated embalming fluid into the aspirated organs, thus ensuring their thorough disinfection. Finally, the arterial cannula, vein drainage tube and trocar are removed, vessels are closed and incision points are sutured (Frederick and Strub, 1974; Ajmani, 1993; British Institute of Embalmers, 2005; Mayer, 2006; author's professional experience as an embalmer).

2.4.2 Autopsy embalming method

Routine autopsy practice involves the evisceration and examination of the entire contents of the abdomino-thoracic cavities via a large vertical incision from the top of the sternum to the pubis, in addition to which the brain may also be removed via a wide occipital craniotomy. The massive circulatory disruption created by this process precludes embalming by the method described above and, instead, a more invasive procedure is required. First, the incisions through which the abdomino-thoracic and cranial eviscerations were effected are re-opened and the internal organs (which have usually been placed inside a plastic viscera bag in the abdomen) are removed. Limbs are then injected individually from within the cavity: arms via the subclavian arteries and legs via the common iliac arteries. The head is injected

via both common carotid arteries, which are accessed within in the neck cavity, the tracheo-oesophageal structures having been removed for examination during autopsy. Blood displaced from the associated veins drains directly into the cavities, including the base of the skull, from which it can be directly aspirated. Finally, the internal organs are treated topically with concentrated embalming fluid, the viscera bag is returned to the abdomen, and all incisions are re-sutured. (Frederick and Strub, 1974; Ajmani, 1993; British Institute of Embalmers, 2005; Payne-James et al., 2001; Mayer, 2006; author's professional experience as an embalmer).

2.4.3 Repatriation embalming method

As already noted in the Introduction, all commercial airlines require a comprehensive embalming process to be carried out before transportation can take place. The primary goal of this embalming is sanitation and, following the procedure, the body is placed within an hermetically-sealed, zinc-lined coffin, which in turn is placed within a sealed packing case filled with hessian; there can, thus, be no risk of other cargo becoming contaminated.

Although custom varies from country to country, and although some embalmers will prepare a repatriation body according to the non-autopsy method described in 2.4.1, above, the majority of embalmers will carry out what is generally known as a 'six-point embalming.' In this process, incisions are made above both clavicles and the head is injected via both the left and the right common carotid arteries. The insertional direction of the arterial cannulae can then be reversed so that abdomino-thoracic areas receive fluid, following which both axillary arteries are commonly raised, via incisions in the axillae, so that both arms can be individually injected, following which both femoral arteries can be isolated, enabling the individual injection of each leg (if the body has undergone an autopsy, then the method described above in 2.4.2 may be followed). Following this, cavities will be aspirated and filled with concentrated embalming fluid according to the process already described and, finally, all incisions will be sutured, before the body is sealed within its coffin, and moved, accompanied by an embalming certificate confirming the adequate sanitary state of the body (British Institute of Embalmers, 2005; Mayer, 2006; pers. comm. with Rowland Brothers International; author's commercial experience in the funeral industry).

2.5 Early Forensic Embalming Literature

The problem of detecting drugs in previously-embalmed bodies was first signalled in the literature in a 1957 paper, where it was noted that at some significant time after an autopsy had taken place, the question of whether or not a chemical agent had caused or contributed the death could occasionally be raised (Sunshine and Hackett, 1957). An actual case that unfolded in exactly this way, concerning the pre-mortem ingestion of drugs, prompted the author to undertake some long-term studies on the stability of barbiturates in formalin-fixed tissues, where it was found that initial drug concentrations decreased significantly within the first two months of storage, but then remained relatively stable thereafter and were still detectable at 36 months.

The subject of toxicology in embalmed or formalin-fixed tissue is then entirely absent from the literature until the mid-1970s, when a renewed interest in forensic problems created by the embalming process came about following a blunder by a mortuary employee at the San Bernardino Medical Examiner’s Office, in California. The mistake arose when a senior executive of a large corporation was killed in a traffic accident while on duty. For a number of reasons, it was important to determine whether or not he had been drinking at the time of the accident and toxicological analysis of his body was undertaken. Despite established mortuary protocol, the mortuary technician unfortunately failed to take a blood sample before proceeding with the embalming. As soon as the error became apparent, samples of intravascular blood, urine, brain and vitreous humour were obtained. Fortunately, the embalming fluid used did not contain ethanol, and as none was detected in any of the fluids analysed, it was determined that embalming had not affected the analytical process (Scott, Root and Sanborn, 1974).

The case, nevertheless, provoked thought into how the laboratory might have dealt with a scenario whereby the embalming fluid used had contained ethanol and, thus, the results of any toxicology undertaken on the usual matrices may have been compromised. The laboratory, therefore, undertook a study on 38 embalmed cases. In the experiment, samples of VH were first aspirated from the eyes of all 38 cases. Next, arterial embalming was performed on each body, following usual mortuary procedures. Finally, a second sample of

VH was aspirated from one or both eyes and subject to GC analysis. Scott found a good correlation between pre- and post-embalming ethanol VH concentrations in all cases, concluding that post-embalming VH analysis, particularly if undertaken within four days of embalming, would give an accurate determination of pre-mortem blood-alcohol concentration. He also found, however, that methyl alcohol from the embalming fluid had diffused into the VH of 19 of the 38 cases, although the degree of diffusion did not appear to correlate with the post-embalming blood methyl alcohol concentration. Although Scott's paper represents a useful early insight into some of the forensic implications of embalming, no account is given of the precise nature of the embalming method employed, nor of the composition or concentration of the embalming fluid used in the process, and it is thus difficult to assess the significance or comparative relevance of his findings.

Scott's paper was followed shortly afterwards by a wide-ranging comparative examination of vitreous chemistry, with samples, like Scott's, being tested before and after arterial embalming (Coe, 1976). Although the primary focus in this analysis of 35 cases was post-mortem biochemistry, the paper also described ten cases whose samples were found to contain alcohol. The study noted that in all cases there was a dilutional factor in the post-embalming VH concentrations which, while deemed to be insignificant in eight cases, was as high as 0.1% in two.

The 1980s began with a paper (Fransioli, Szabo and Sunshine, 1980) which reported the simple qualitative detection of two opioids, methadone and propoxypyhene, 19 and 24 months respectively after formalin fixing in the laboratory, although the primary experimental aim of the work was in fact to compare GC and EMIT analytical techniques.

Two years later, and in response to a suspicion that the depolarising neuromuscular blocking drug succinylcholine was implicated in a number of homicidal poisonings, an American-led team undertook a study of the recovery of the drug from overdosed rats that were subsequently embalmed, stored, then analysed (Forney et al., 1982). Although little detail is provided of the experimental conditions – other than stating that kidney, liver and muscle samples of the rats were stored at 8°C for six months – Forney reports the successful detection of the compound at the end of the study period.

In 1988, a case report on the detection of ethchlorvynol - a GABA-enhancing sedative and hypnotic, developed in the 1950s, though discontinued in many countries in the late 1990s – was published, noting the presence of embalming fluid in the bile, pericardial fluid, vitreous humour and bone marrow of a case, some 52 hours after embalming had taken place (Winek et al., 1988). Although suggesting that the ethchlorvynol concentration was probably higher prior to embalming, Winek was nonetheless able to determine a toxic – and very close to lethal – level in the body.

Finally, in 1993, another American team reported the development of a GC/MS method for the detection of carbon monoxide in decomposed, embalmed, exhumed, formalin-fixed and some "fire-dried" cases (Middleberg et al., 1993). In a small number of forensic cases, they were able to report the successful detection of carbon monoxide in the recently embalmed spleen of one case, the formalin-fixed spleens of two others and, finally, the embalmed skeletal muscle of one exhumation case.

The early literature, it might be observed, is a disparate body of papers. Experimental aims and methods, where stated, differ widely, almost exclusively, and many papers report little more than the simple and qualitative determination of individual drugs. Although these criticisms, and others besides, can also, as will be seen, be levelled at some of the more recent literature, it is the case that what has followed has been an increased interest in the subject of toxicology in embalmed and formalin-fixed tissues, aligned with an increasing ability to report ever more refined results as analytical methodologies and equipment have developed.

What follows, therefore, is an account of the literature that has been published since 1990 which, for the sake of organisation, has been grouped by drug class, each listed class containing a chronological survey of the published literature. Although much of the account is descriptive, some comparative remarks are made, where direct comparison is possible, and a more detailed critique relating the literature to the subject matter of the current thesis then follows later in the chapter. Accompanying the following account, in Appendix 2, is a comprehensive summary of studies carried out on the stability of drugs and poisons in formaldehyde since 1980 that can be read alongside the commentary for each drug class. The table has been presented in a way that illustrates the summary findings from each paper for

each of the experimental conditions examined. Like the review that follows, the table has also been arranged by drug class. In the case of some papers, drugs from multiple classes are examined; in such cases, papers are referenced within each individual section of the table, according to the drug described. Many of the early papers include little, if any, reference to control samples, and the results of degradation studies are presented in unqualified terms. Although many of the more recent studies do report the comparative stability of control samples, for the sake of adopting a consistent approach in the review that follows, and in keeping with the general essence of the chapter, all results are presented in absolute terms.

2.6 Recent Forensic Embalming Literature

2.6.1 Benzodiazepines

The 1990s, it might be observed, heralded a new approach in the study of toxicology formalin-fixed samples. Rather than simply presenting simple case reports, as had generally been the case until this point, now comparative studies began to be undertaken and reported. This change in emphasis almost certainly came about in response to an increasing forensic awareness, hand-in-hand with, and facilitated by, new and developing analytical capabilities. The embalming literature from this time was also clearly prompted by what several authors of the time describe as a paucity of information relating to compounds in a fixed or embalmed environment.

Some of the first compounds to be examined were benzodiazepines, and the earliest of these papers, Winek, Esposito and Cinicola (1990), describes the experimental aim as being to determine the usefulness of exhumation after a period of time, arguing that if a compound degrades to the point that it can no longer be detected, then the justification threshold for the exhumation of a body may not be met. Using blood samples spiked with diazepam, this paper was one of the earliest to report not only the degrading effect of formaldehyde on drug molecules, but also the fact that greater concentrations of formaldehyde were linked directly to greater degrees of drug loss. Thus, after 30 days in 5% formalin (given that, as was earlier stated, formalin is a solution containing approximately 37% formaldehyde by volume, 5% formalin solution contains around 1.85% formaldehyde) Winek reported a 41.4% loss of

diazepam, compared to a much greater loss of 56.3% in an 8% formalin (containing approximately 2.95% formaldehyde) solution.

Five years later, a Japanese laboratory working on diazepam expanded the findings of Winek by considering the influence that pH might have on the degradation of the drug. Reporting markedly different results, Nishigami et al. (1995) recorded losses of up to 32% in samples of tissue fixed in 10% formalin (3.7% formaldehyde) buffered to pH 7.4 for 28 days, losses of up to 52% in non-buffered solution, and losses of up to 96% in samples fixed in 4% paraformaldehyde solution. There were, however, some significant disparities in Nishigami's results. Whereas a loss of 96% diazepam was recorded in samples of skeletal muscle fixed in 4% paraformaldehyde buffered to pH 7.4, for example, the same muscle fixed in a 10% formalin solution buffered to the same pH appeared to record a gain in diazepam concentration of some 71%. Similarly, although liver samples fixed in 10% non-buffered formalin recorded losses approaching 50%, the same samples fixed in 10% formalin buffered to pH 7.4 recorded an increase in concentration of around 45% within the first 24hrs, followed by a return to the original concentration by day 3, after which the concentration increased again by around 35% by day 7, returning to the original concentration by day 15, where it remained until the end of the experiment on day 28. Nishigami was unable to explain with any certainty the reason for these anomalies, stating that while contamination is unlikely to be the cause, the inconsistencies may be due to a disproportionate or uneven distribution of diazepam within the respective tissue samples.

Other than a single case report, detailing the detection of midazolam in the liver of an exhumed body that had been embalmed some 24 months earlier (Rohrig, 1998), it was to be a further six years until the next paper examining the stability of benzodiazepines in formaldehyde was published. In this study, Tracy 2001, the author introduced the paper by signalling the need for a detailed study because of the substantial abuse potential of benzodiazepines; related to this, he noted the frequency with which benzodiazepines were implicated in forensic casework, including an increasing incidence of requests for post-embalming toxicology. With an explicit aim of establishing the suspected variable influences of formaldehyde concentration, pH and duration of exposure, Tracy examined the stability of ten benzodiazepines in a range of laboratory conditions. His main findings were that the

breakdown of alprazolam, diazepam, midazolam, prazepam and triazolam is accelerated greatly in an acidic environment, as well as in higher concentrations of formaldehyde. Not all results, however, followed this pattern. Tracy found that flunitrazepam and lorazepam, for example, degraded fastest in an alkali environment, remaining stable in both neutral and acidic formalin solutions, whereas diazepam and flurazepam degraded to an appreciable extent in all pH conditions, independent of formaldehyde concentration. Of the ten compounds studied, diazepam and prazepam were the most resistant to formaldehyde-induced degradation. Although all ten drugs examined are chemically related, the results are clearly inconsistent, and Tracy concludes his paper by stating that although the presence of formaldehyde can have profound effects on the breakdown of these drugs - and although the degradation is in most cases influenced by both the concentration of the formalin solution and its pH - the degree of acceleration of breakdown process is very much compound dependent.

Having so far considered formaldehyde concentration, pH and duration of exposure as experimental variables, the next paper to examine the stability of benzodiazepines in formalin solutions was a Japanese study that included work on the effect of temperature on the rate of degradation. In this study, Suzuki and Kaneko (2009), the experimental design was similar to previously published methods: 10% and 20% formalin (3.7% and 7.4% formaldehyde) solutions were used, with one set of samples buffered to pH 7.4 and the second set un-buffered, and both sets of samples observed over a period of 90 days. The novel investigative parameter in this paper, however, was that the samples were examined in duplicate, with one set being stored at room temperature, and the other set refrigerated at 4°C. Although the study Suzuki undertook included only one benzodiazepine (bromovalerylurea, a bromoureide-class sedative and hypnotic marketed in Asia), his results identified two interesting trends. First was the fact that degradation of the compound was faster as well as ultimately greater in the formalin solutions buffered to pH 7.4, than it was in the un-buffered solutions. In the room temperature samples, for example, the drug was completely undetectable after approximately seven days and ten days in, respectively, the 10% and 20% buffered formalin solutions; this finding contrasted with losses of approximately 30% and 50%, respectively, in the un-buffered samples after approximately the same period of time.

Second, and unsurprisingly, although formaldehyde concentration still played a part, compound degradation was retarded in those samples that were refrigerated. In the un-buffered samples, where the distinction was most marked, there was only a marginal loss of the parent compound from both concentrations of formalin solution refrigerated at 4°C, where around 95% of the starting concentration could still be detected even after 90 days. In the buffered samples, approximately 20% of the starting concentration could still be detected in the 10% formalin solution after 90 days stored at 4°C, and approximately 10% could be recovered from 20% formalin in the same conditions.

Subsequently, a Chinese paper examining the stability of estazolam in samples of heart, liver, kidney and brain from a sacrificed dog, fixed in 4% formaldehyde solution (Yuan, Wang and Yun, 2011), recorded losses of 99.2%, 98.3%, 99% and 97.8% of the compound from each of the respective tissues. Its experimental design, however, arguably fails to capitalise on any of the previously noted variables that commonly play a part in drug stability in formalin-fixed solutions. Only one concentration of formaldehyde was used, for example, with no buffering, and no inclusion of temperature studies, and with a concluding note stating simply, and somewhat unhelpfully, that biological samples containing estazolam should not be preserved in formaldehyde solution.

Other than a 2015 paper examining the stability of a number of drug classes in formaldehyde (Uekusa, Hayashida and Ohno, 2015), reporting a loss of 33.4% of bromazepam from a 15% un-buffered formalin solution, there have only two other benzodiazepine studies. The first, Ameline, Raul and Kintz (2019), in a simple experimental approach, records the loss of around 73% midazolam from drug-spiked blood in 4% formalin solution, buffered to pH 7.0, and stored at 4°C for 21 days. Finally, a very recent study reports the stability of ten psychotropic drugs in 6% formaldehyde, buffered to pH 7.0-7.5, and stored at room temperature for six months (Asano et al. 2020). The experimental method was not one that had been used previously, and involved the drug-spiking of liver homogenates, followed by the addition of formalin solution, then storage at room temperature for six months. The approximate recovery rates after six months for brotizolam, diazepam, estazolam, etizolam and triazolam were, respectively, 5%, 40%, 0%, 5% and 85%.

2.6.2 Opiates and opioids

As has been noted already, there had been some early interest in opiates in formalin-fixed tissues, with Fransioli, Szabo and Sunshine reporting the detection of methadone and propoxyphene as long ago as 1980. Following this, however, it was to be well over a decade until opiates reappeared in the embalming literature, in a paper examining the concentration of morphine detectable in the body of a nursing home resident, exhumed three weeks after burial (Levine et al., 1994). The body had been embalmed and the toxicology laboratory of the Chief Medical Examiner of Maryland was able to determine that the liver morphine concentration was within the range that could have proved fatal and, presumably as a result of this conclusive fact, no attempt was made to examine the extent to which the embalming process may have altered the concentration of morphine detectable.

An unusual exhumation case only four years later, however, provided the opportunity for a direct comparison between embalmed and un-embalmed liver fentanyl concentrations (Rohrig, 1998). In this case, toxicology was carried out as part of the initial autopsy investigations, and the body was subsequently embalmed and buried. Approximately two years later, however, the body was exhumed and examined for a second time (for reasons that are not stated in the paper), where it was found that the liver fentanyl concentration was 26% lower than at the time of the first analysis. Furthermore, although concentrations for other drugs known to have been administered to the patient before death (including diphenhydramine, fluoxetine, lidocaine, meperidine, midazolam, norfluoxetine, normeperidine, promethazine and methohexital) were either not reported or not quantitated at the first autopsy, they were all found, at the second analysis, to be well within expected concentrations for the recorded pre-mortem administered doses. Although no other experimental studies were carried out as part of the investigation, Rohrig's study was notable in that it presented the first recorded direct comparison between un-embalmed and embalmed samples from the same case.

In 2001, two Italian university departments of legal medicine collaborated in a study which broadened the experimental scope of Rohrig's work. In this research (Cingolani et al., 2001), which was carried out at the same time as Tracy was examining the stability of

benzodiazepines in formalin solutions, the concentration of morphine in the liver and kidney samples of five autopsy cases was first determined, after which the samples were fixed in 10% formalin at pH 7.0, stored at room temperature for 12 weeks, then re-analysed. Although only 29.41% of the originally quantified morphine could be recovered from the fixed kidney samples, and 36.29% from the fixed liver samples, Cingolani, in a novel experimental approach, then analysed the formalin solution in which the individual samples had been stored. The finding was that a further 42.17% of the original morphine could be recovered from the formalin in which the kidney samples had been stored and 74.93% from the liver formalin. What this implied (notwithstanding the fact that the sum of the concentrations recovered from the liver samples and the formalin solution in which they were stored exceed the starting concentration) was that significant quantities of morphine had redistributed from the tissue samples into the formalin solution, and that if both the fixed samples and the solution in which they had been stored were analysed, then the resulting total would be very near the concentration originally detected in the pre-embalmed specimens. Cingolani was able to conclude, therefore, that although the quantitative values of morphine obtained from fixed tissues were very different to those obtained from the original, non-embalmed, specimens, morphine nevertheless exhibits good stability in biological specimens, and that, importantly, the aggregate of morphine concentrations from the fixed sample, as well as from the formalin solution in which they were stored, would provide a good indication of the original morphine concentration prior to fixation.

In the same year as Cingolani's paper, a study by the Institute of Forensic Science at the Ministry of Justice in Shanghai (Xiang et al., 2001) reported very different results from what was, methodologically, a closely-related experiment. Tissue came from rabbits that had been dosed with morphine and subsequently sacrificed, with Xiang taking duplicate samples of liver, lung, kidney and heart, one set of which was immediately frozen at -20°C, and the other fixed in 10% formalin solution; both sets of samples were then stored for four months before being analysed. Compared to the subsequent morphine recovery from the frozen samples, a mean of only 4.13% could be recovered from the formalin-fixed samples, with only a further 0.84% being recovered from the formalin solution in which the samples had been stored. A total, therefore, of only 4.97% of the morphine from the fixed samples was detected, when

compared to the concentration recovered from the frozen samples; this is a result that is clearly very different to that obtained by Cingolani. In the same experiment, Xiang also measured the comparative concentrations of meperidine (better known in the United Kingdom as the opioid analgesic, pethidine) in formalin-fixed and frozen tissue samples. In contrast with his morphine findings, Xiang was able to detect a mean of 16.46% of the starting concentration of the meperidine in the fixed samples, and a further 38.53% in the formalin solutions in which they had been stored, making a total recovery of 54.99% when compared with the frozen samples.

Although Xiang's morphine results are very different to those reported by Cingolani, closer comparative examination of the experimental conditions reveals one interesting difference, namely, that although Cingolani's formalin solution was buffered to pH 7.0, Xiang's solution was unbuffered. We have already seen that pH can be a significant influence on the stability of benzodiazepines in formalin solution, and although Tracy reported the opposite trend to that observed by Xiang (namely, that an increasingly acidic pH resulted in a faster rate of degradation), the difference in experimental pH in the methods of Cingolani and Xiang may explain the very different results.

Next, the laboratory of the Médecine Légale in Nice, in 2003, reported the case of a national who had died in Thailand and was embalmed and repatriated to France, whereupon an autopsy was performed (Alunni-Perret et al., 2003). Although the paper is a simple case report, and appears very much to follow the method reported in Levine's 1994 paper, the author was able to detect a concentration of morphine (as a metabolite of heroin), within the fatal range, in the bile of the deceased, as well as in a liver sample, some nine days following embalming.

Completing the chronological survey of opiates and opioids in the embalming literature, Ameline, Raul and Kintz (2019) reported a very rapid loss of oxycodone in drug-spiked blood samples stored in 4% formaldehyde solution, buffered to pH 7.0, after 21 days at 4°C. Recovery was only 15% at Day 1, had fallen further, to 0.5% by Day 7 and was undetectable by Day 14.

2.6.3 Stimulants

The earliest paper examining the stability of stimulants in formaldehyde came from a Japanese team responding to the increased incidence of methamphetamine (MA) in forensic work, following the prohibition of its possession, use and distribution in their country (Takayasu et al., 1994a). Although including one human, MA-positive, embalmed case in the study, Takayasu's primary experimental approach was to take samples of brain, lung, liver, kidney and skeletal muscle from rabbits that had been dosed with MA, in an approach that was later to be followed by Xiang when studying morphine, as already discussed. The samples were fixed in 10% non-buffered formalin, stored at room temperature and analysed at 1, 3, 7, 14 and 28 days. The loss of MA from each tissue type was marked, with a recovery of only 3.1% from brain, 0.3% from lung, 2.3% from liver, 1.4% from kidney and 1.6% from skeletal muscle at Day 1; by day 28 the recovery rates were 0.4%, 0.04%, 0.2%, 0.04% and 0.1% from each respective tissue.

In response to what was said to be the increasing recreational use of stimulants, Cingolani, who three years earlier had examined the stability of morphine, published a study examining the detection of cocaine and its metabolites in fixed tissues (Cingolani et al., 2004). In this study, samples of liver taken from forensic post-mortem cases found to be positive for cocaine were fixed in a solution of 10% formalin buffered to pH 7.0 and were analysed after 28 days. Given the well-established fact that cocaine is quickly metabolised following administration ($t½$ = 0.7-1.5h (Johanson and Fischman, 1989)), the target analyte in the analysis was BZE, one of the primary metabolites of cocaine. After 28 days, the BZE concentration detected in the fixed samples was only 12.31% of that found in the original autopsy samples. Following the experimental approach adopted in his morphine paper, Cingolani then additionally analysed the solution in which the fixed liver samples had been stored, reporting that an additional 84.47% of the original BZE could be recovered. With a total recovery, therefore, of 96.78%, Cingolani concluded, first, that formalin has a particularly marked extraction capability and, second, that BZE has good stability and does not react chemically with formaldehyde.

A paper published the following year by the School of Pharmacy at West Virginia University (Tirumalai et al., 2005) set out to develop further the work undertaken on MA by Takayasu some 11 years earlier. Where the previous author's study recorded the recovery of the drug in 10% un-buffered formalin, Tirumalai set out to examine its stability in a wider range of conditions as well as, in a novel approach, aiming to identify the conversion compounds of the reaction. In a further development of Takayasu's method, Tirumalai's experiment did not use any human or animal tissue, instead simply reacting MA in solution with formalin. Mixtures of MA with 5%, 10% and 20% formalin solution, at pH 3.5, 7 and 9.5 were analysed over the course of 30 days at room temperature. A very clear relationship emerged between both the rate and the extent of MA decomposition as the concentration of formalin increased and, additionally and independently, as the pH became more basic. Differing rates of decay were observed, from an almost complete recovery in 5% formalin at pH 3.5 at Day 30, to a total loss of parent compound in 20% formalin at pH 9.5 after only 24 hours. Additionally, the presence of *N*-methyl-methamphetamine as a breakdown product was noted, its production being approximately inversely proportional to recorded degradation of the parent compound. Thus, Tirumalai's concluding recommendation was that in cases of post-embalming forensic analysis in cases of suspected MA administration, the target of analysis should be expanded to include the *N*-methyl derivative. This was, for obvious reasons, an important analytical recommendation to note, and one which was to be adopted subsequently by other analysts.

Tirumalai stated in his MA study that his laboratory's aim was to examine, over the course of several papers, the stability of a range of forensically-implicated drug molecules in formalin solution. The second of these studies, in the name of one of his earlier co-authors, followed later in the same year (Shakleya et al., 2005), and reported the stability of 3,4-methylenedioxymethamphetamine (better known as MDMA or ecstasy) in formalin-fixed liver samples. In this brief paper the authors demonstrated that within 30 minutes of commencing the reaction, MDMA began to be converted to its methylated derivative, 3,4-methylenedioxy-*N,N*-dimethylamphetamine (MDDA), and that after 24 hours only approximately 10% of the original quantity of MDMA was recoverable.

The following year, Shakleya published a follow-up paper to Tirumalai's earlier MA study, in which he presented a method for the detection and identification of the methylated

degradation product of the reaction (Shakleya et al., 2006). Although employing the same limited experimental method as he used in his 2005 study of MDMA (human liver samples injected with 20% formalin at pH 6.0, kept at room temperature for 24 hours and analysed), Shakleya was nevertheless not only able to confirm Tirumalai's findings, but was also able to develop a sensitive MS-MS technique for the qualitative determination of *N,N*-dimethylamphetamine. Additionally, given that he was able to demonstrate the complete loss of the parent compound after a period of time, Shakleya made the new and forensically important point that the analysis of formalin-fixed tissues for methamphetamine can lead to false negative findings.

The next paper examining the stability of stimulants in reaction with formaldehyde followed in 2009, when an Italian forensic laboratory published a study charting the degradation of cocaine (Viel et al., 2009). Appearing to develop the methodology reported in Cingolani's 2001 study, Viel spiked cocaine-positive human brain and liver samples with 10% formalin buffered to pH 7.4, and found that cocaine is readily hydrolysed to BZE, with only approximately 20% of the parent compound remaining after 15 days. Although this finding was substantially similar to that of Cingolani, what Viel did, like Tirumalai, was to examine the stability of the compound in a number of different conditions, some of which produced very different results. In an un-buffered 10% formalin solution, pH ~3.5, for example, he was able to demonstrate that cocaine is relatively stable, even after 30 days. This finding led Viel to conclude that formalin does not appear to play an active role in the hydrolysis of cocaine, at least not under acidic conditions. What Viel was additionally able to determine was that although both cocaine and BZE were readily extracted into formalin solution, the extraction process was faster and greater from the liver samples (up to 80% extraction at 30 days) than it was from the brain samples (up to 60% extraction at 30 days). This, he suggests, is probably because of the comparatively greater lipophilicity of brain tissue.

As well as Viel's study, 2009 also saw the publication of a further paper on the stability of cocaine and BZE in formalin-fixed tissues (Hilal et al.,2009). In this – compared to what had gone before - methodologically limited study, liver, lung, kidney and brain tissue from drugged rats was fixed in 10% formalin solution, buffered to pH 7.0, and stored at 25°C for 30 days. Although not reporting any substantially new findings, this paper does confirm Viel's finding

that cocaine is rapidly converted to BZE in these particular conditions; also, that of the four tissue types examined, brain demonstrated the slowest redistribution of BZE from the tissue itself into the formalin solution in which it was stored, with liver demonstrating the fastest redistribution.

A third and final paper to be published in 2009 came from a Japanese team who studied the reaction of MA in formalin, alongside the reaction of molecules from several other drug classes (Suzuki and Keneko, 2009). Their experimental method was, like that of Hilal, arguably somewhat limited, using only two concentrations of formalin solution, 10% and 20%, both un-buffered. Unlike previous formalin studies on stimulant drugs, however, Suzuki examined the influence of temperature on the kinetics of the reactions taking place (as has already been noted at 2.6.1, where Suzuki's linked benzodiazepine study was discussed), comparing samples stored at room temperature with those kept at 4°C. Not surprisingly, and in agreement with the previous work of both Takayasu and Tirumalai, it was found that there was a total loss of MA in 20% formalin after 90 days at room temperature. What Suzuki also reported, however, over and above previous findings, was an almost complete recovery of MA from both 10% and 20% formalin solutions stored at 4°C. Although perhaps unsurprising, this is nevertheless an important finding, confirming and demonstrating very clearly the influence that temperature can play on the kinetics of formalin reactions

MDMA was examined once again in Maskell et al. (2013), alongside the stability of two novel, so-called 'designer drugs', 4-methylmethcathinone (better known as mephedrone) and 3-triflouromethylphenylpiperazine (also known as 3-TFMPP). Increasing the range of reaction conditions adopted by Shakleya in his 2005 study of MDMA, Maskell included three concentrations of formalin, 5%, 10% and 20%, in three different pH environments, unbuffered (~3.5), 7.0 and 9.5, at room temperature, for 60 days. In agreement with Shakleya's observations, Maskell found that MDMA (as well as mephedrone and 3-TFMPP) degraded fastest in the most alkaline environment, reporting a recovery of only 50% of 3-TFMPP, 5% of MDMA and 4% of Mephedrone after 28 days at pH 9.5, compared to 78% of MDMA, 74% of 3-TFMPP and 63% of Mephedrone in the un-buffered, pH~3.5, reaction mixtures. In a refinement of Shakleya's work, furthermore, Maskell was able to demonstrate that an

increasing concentration of formaldehyde leads to increase in the rate of drug degradation, although in the case of these three compounds pH is nevertheless a more significant driver.

Finally, a paper examining the stability of several classes of central nervous system-targeted drug classes (Uekusa, Hayashida and Ohno, 2015), noted a 100% recovery of MA and MDMA from certain refrigerated solutions of formalin, while recording significant losses from room temperature solutions.

2.6.4 Antidepressants

Although not regarded as typical drugs of abuse, antidepressants have been implicated in forensic casework for many years, having a well-documented history of being used in suicidal overdose. Tricyclic-group antidepressants, though nowadays prescribed less commonly than the newer-generation antidepressants, can be fatal at comparatively low doses, and so were the focus of early research into their stability in formalin-fixed tissues.

In one of the earliest studies (Dettling et al., 1990), the stability of nortriptyline was monitored over seven days, firstly in un-buffered solutions containing 1, 5, 10, 20 and 40% formaldehyde and, secondly, in 20% formaldehyde buffered to pH 2.0, 4.0 and 9.5. Dettling found that, as a general rule, most of the degradation took place within the first 24 hours, with concentrations remaining relatively stable thereafter. Following the trend that has already been discussed in relation to some benzodiazepines, opiates and stimulants, Dettling also noted that the rate of degradation increased as the concentration of formaldehyde increased; the trend was especially notable in increasingly alkaline solutions. Furthermore, Dettling was able to identify the primary breakdown product of the reaction, noting that in pH 9.5 solutions, the formation of amitriptyline was directly proportional to the loss of nortriptyline; in other pH environments, directly proportional relationships could not be established.

The next published paper to examine the stability of tricyclic antidepressants reported the recovery of amitriptyline, desipramine, imipramine and nortriptyline from the formalin-fixed liver samples of several forensic cases (Winek, Zaveri and Wahba, 1993a; following preliminary findings noted in Winek, Esposito and Cincola, 1990). Comparing samples that had been fixed in 10% formalin solution, buffered to pH 6.9-7.1, for between 7-22 months,

with samples that had been frozen without first being fixed, Winek reported a general trend of significant loss of the parent compound from the liver samples. In an approach that, as we have already seen, was to be adopted by Cingolani some ten years later, he also noted good recovery from the formalin solutions in which the samples had been stored. In the case of desipramine, for example, while only between 2.6% and 5.6% of the drug could be recovered from the various fixed liver samples, the recovery rate from the formalin solutions in which the samples had been fixed ranged from 56.3% to 95.2%. The disparity was even more marked in the case of imipramine samples, where recovery from the liver samples was between around 6% and 21%, while up to complete recovery was recorded when including the formalin solutions. Although demonstrating clearly the extent to which drugs can leach from tissue into formalin solution, Winek was unable to establish consistent ratios for any of the drugs examined.

Five years later, a team led by the Japanese toxicologist Takayasu – who had previously, as we have already seen, undertaken related work on stimulants – published a paper examining the same four tricyclics that had been studied by Winek (Takayasu et al., 1998). Here, however, Takayasu's method differed significantly from that published in his earlier paper, as well as from that of Winek, in that he used no tissue, but instead monitored the reaction of the four drugs in solutions of 10% formalin buffered to pH 3, 5, 7, 9 and 11. Despite qualitatively corresponding with Winek's findings, many of Takayasu's results differ quantitively, often markedly so. Whereas Winek recorded amitriptyline losses of up to 18.2%, for example, Takayasu's corresponding loss was 97.3%, which in itself contrasts with the losses of 63-87% recorded by Dettling in his earlier study of the same drug. The contrast between studies in the case of imipramine was even more marked, where Takayasu noted a recovery of only 2.7% in contrast to Winek's near 100% recovery of the same drug. Takayasu also noted a novel finding, in that he recorded significant losses of desipramine in pH 3, 9 and 11. Thus, the degradation of this particular compound in formaldehyde, while appearing less marked in relatively neutral solutions, appeared to increase in both acid and alkali conditions, unlike any other previous finding.

Following the development of newer anti-depressants, two subsequent papers in the embalming-toxicology literature concern the drug class known as Selective Serotonin

Reuptake Inhibitors, or SSRI's. Despite having a wider therapeutic window than their tricyclic predecessors, SSRI's, by virtue of the fact that they are used to treat a range of psychiatric conditions, including post-traumatic stress disorder, depression and anxiety, are a common finding at autopsy. This fact was noted in the first of the two papers (Suma and Prakash, 2006a) who, following Takayasu's method of excluding tissue from the experimental method, monitored the degradation of sertraline in 5%, 10% and 20% formalin solutions at pH 3.0, 7.0 and 9.5 over the course of 30 days (conditions very similar to those used by Tirumalai in his paper published the previous year). Suma reported an almost 100% loss of the parent compound at the end of the study, irrespective of formalin concentration or pH, although there was a trend for the rate of decomposition to accelerate in proportion to increasing formaldehyde concentration and increasing alkalinity. In alkali conditions, for example, losses of between 96-98% were recorded in all formalin concentrations after 14 days, compared to only 60-70% losses in acid conditions. Even in acid conditions, however, whereas around 60% of the drug was lost after 30 days in the 5% formalin reaction mixture, in the 20% formalin mixture, none of the parent compound could be recovered after the same period of time. Suma was able to identify the degradation product of the reaction as the *N*-methyl derivative of the parent drug and, like Dettling's work on amitriptyline, was able in some conditions to quantify a rate of recovery of the derivative that was almost directly proportional to the loss of the parent compound.

The second paper concerning SSRI's was also published by Suma (Suma, Shukla and Prakash, 2006b), at around the same time as his sertraline study. Here, the stability of fluoxetine (better known as Prozac) was examined, using exactly the same formalin concentrations and pH conditions used in the sertraline study. Although 50% of the parent drug was converted to its *N*-methyl derivative within one hour in all reaction mixtures, the subsequent rate of decay became greater as the formalin concentration increased: thus, after four days, at pH 3.0, the conversion had reached 52%, 67% and 75% in, respectively, 5%, 10% and 20% formalin, reaching 100% in all cases by day 30. In the case of alkali reaction mixtures, the rate of decay was further accelerated, in all cases reaching 100% by the end of the study. Like his work on sertraline, Suma noted an almost proportional recovery of the *n*-methyl derivative in relation to the loss of the parent compound.

Finally, Asano et al. (2020) notes the 20% loss of the tricyclic amitriptyline after six months in 6% formaldehyde at room temperature, and the 90% loss of the SSRI paroxetine in the same conditions.

2.6.5 Antipsychotics

As well as papers on sertraline and fluoxetine, Suma published a further study in 2006 (Suma, Kosanam and Prakash, 2006c), this time examining the stability of bupropion, an atypical antidepressant, and olanzapine, an atypical antipsychotic. Using similar experimental conditions to those used in the sertraline study (and copying exactly the experimental conditions employed by Gannett, as will be discussed presently), Suma noted a 100% loss of bupropion in 20% formalin solution at pH 9.5, with a corresponding 100% recovery of the *N*-methyl derivative of the drug. Conversely, with olanzapine, a loss of only 2%, was recorded at the end of the study, with a corresponding production of the *n*-methyl derivative.

Although Nishigami, in his 1995 study of diazepam, as we saw earlier, also observed the stability of the antipsychotic chlorpromazine – noting losses of up to 97% - the next paper to consider the stability of antipsychotics in formaldehyde was Uekusa, Hayashida and Ohno's 2015 study, already discussed in relation to its study of MA and MDMA. Uekusa's methodology involved the fixing of liver and kidney samples from a forensic case in 15%, non-buffered (pH 4.5-5.0), formalin solution at 4ºC, as well as at room temperature, with analysis taking place at 1, 3, 6 and 13 months; additionally, tissue-free, drug-formalin mixtures were also analysed. The recoveries recorded were variable. In the case of chlorpromazine, for example, although a recovery of 84.8% could be made from the drug-formalin solution, only 66.8% could be recovered from the fixed kidney samples and only 54.3% from the fixed liver samples. Inconsistencies were also noted in the recovery of levomepromazine and promethazine.

Finally, the recent study of ten psychotropic drugs, Asano et al. (2020), already mentioned in the context of benzodiazepines and antidepressants, reported the recovery of approximately 25% quietapine and only around 1% levomepromazine from drug-spiked liver homogenates, stored in 6% formaldehyde solution, buffered to pH 7.0-7.5, at room temperature and for six months.

2.6.6 Barbiturates

Although less commonly prescribed nowadays, barbiturates had for many years an important clinical role as central nervous system depressants, and were widely administered for their anxiolytic, hypnotic and anticonvulsant properties. Their therapeutic window, however, is generally much narrower than other classes of drugs used to manage the same clinical complaints, such as benzodiazepines, and toxic as well as lethal doses can be comparatively small (Stone and Darlington, 2000). As a result, this class of drugs was frequently implicated in certain unexplained deaths.

The first in a small number of papers to consider the stability of barbiturates in formaldehyde came from a Greek forensic laboratory (Tsoukali-Papadopoulou, 1987). This was a simple forensic case report that identified phenobarbital, at a concentration of 10µg/ml, in the brain tissue of a child that had been placed in formalin some fifteen months earlier.

Winek, Esposito and Cincola, in their 1990 study (which, as has already been discussed, examined the stability of diazepam) also examined the recovery of phenobarbital. Unlike his diazepam experimental conditions, however, where blood samples were spiked with the drug before adding formalin, in the case of phenobarbital, Winek took liver samples from a positive autopsy case and immersed them in 5% and 8% formalin solutions. Losses of 62.36% and 67.46% in the 5% and 8% formalin solutions, respectively, were recorded after 28 days. In an experimental approach that was, as we have seen, to be later adopted later by several laboratories, Winek then analysed the formalin solutions in which the liver samples had been stored, finding that the 5% solution was to yield a further 17.26% of the original concentration of drug, and the 8% solution, a further 11.10%. Total recoveries of phenobarbital, therefore, were 54.90% from the 5% formalin solution and 43.65% from the 8% solution.

There was then a period of some 11 years before the publication of the next barbiturate paper (Gannett et al. 2001b). In this study, and prompted by what the authors claim was the implication of barbiturates in suicide and murder, the stability of pentobarbital, phenobarbital and secobarbital in 5%, 10% and 20% formalin in un-buffered solutions, as well as in solutions buffered to pH 7.0 and 9.5, was monitored over the course of 30 days (in experimental conditions that replicated very closely the same conditions applied by Tracy et

al. in their 2001 study of benzodiazepines, and in which Gannett was a named author). Despite all three drugs being chemically related, the results of Gannett's experiments were particularly inconsistent. In the case of phenobarbital, for example, an increasingly alkaline reaction mixture and an increasing concentration of formaldehyde were found, independently, to accelerate decomposition by a rate of between three and ten-fold, such that in an un-buffered (pH~3.5) solution of 5% formalin, there was virtually no loss of parent drug after 30 days; by contrast, in a solution of 20% formalin at pH 9.5, only approximately 50% of the parent drug could be recovered after the same period of time. Pentobarbital, however, was found to decompose to a lesser extent, although the influence of pH was once again evident: in the acidic and neutral mixtures there was found to be little appreciable decomposition in any concentration of formalin, whereas in pH 9.5, loss of parent compound was recorded as being up to approximately 30% in 20% formalin. Finally, secobarbital was found to be remarkably stable in all solutions, with recovery never being less than 97%, even in 20% formalin at pH 9.5.

The final barbiturate paper was published some three years after Gannett's work (Cingolani et al., 2005) by the same laboratory that had previously examined morphine and cocaine stability. Adopting a similar experimental approach to his earlier work, Cingolani fixed phenobarbital and butalbital-positive liver samples from autopsy cases in 10% formalin buffered to pH 7, analysing the tissue samples, as well as the formalin solutions in which they were stored, over a period of six months. Although only able to recover 57.11% of the initial concentration of phenobarbital, and 21.66% of the butalbital, from the fixed liver samples, Cingolani, like Winek, was able to recover further amounts of the drug from the formalin solutions in which the samples had been stored: in this case, 30.84% from the phenobarbital solution and 66.56% from the butalbital solution. In total, therefore, Cingolani was able to recover a total of 87.95% phenobarbital and 88.22% butalbital from tissue and solution combined.

In the case of barbiturates, compared to those drug classes already considered in this review, it is unusual that there is one particular drug, phenobarbital, that has been addressed within three individual studies: those of Winek, Gannett and Cingolani. A comparison between the results of these studies appears, at least initially, to be interesting, since the results of all three

are markedly different. Winek, as we have seen, recorded losses of around 62-60% (though only around 45-56% when also including the formalin solution in which the samples had been stored), Gannett recorded losses of up to 50%, and Cingolani, losses of around 43% (though only around 12% when also including the formalin storage solution). Examination of the methods employed in the three studies, however, reveals differences that are almost certainly significant enough to make the meaningful comparison of results very difficult. Gannett's study, for example, involved the simple reaction of phenobarbital in formalin solutions, whereas both Winek and Cingolani used drug-positive liver samples that were stored in solution, for different periods of time, and with no preliminary study of the extractability of these drugs from the respective tissues. While Winek's report, furthermore, fails to state the pH in which his formalin solutions were buffered, Cingolani fails to exploit Gannett's earlier – and clearly important - finding that established the critical significance of pH in the degradation of barbiturates. Direct comparison of results, therefore, given what we have already seen of the importance that variables including formalin concentration, pH and temperature can have on the recovery of drugs, is very difficult. It is a difficulty that, unfortunately, is encountered regularly in the embalming literature.

2.6.7 Other drugs and poisons

As well as the studies described above that have monitored the stability of related molecules within respective drug classes, a number of papers have examined the recovery of individual compounds of forensic interest. These are now considered in turn.

2.6.7.1 Volatile substances

In an experiment that was methodologically very similar to his work on MA, which has already been discussed (Takayasu et al., 1994a), Takayasu et al. (1994b) examined the stability of several volatile substances commonly encountered in forensic work. Drawing on the premise that formalin-fixed histology samples were increasingly required for subsequent toxicological analysis, Takayasu subjected rabbits to a vapour mixture of chloroform, diethylether and toluene, as well as intravenous administration of ethanol. Brain, kidney, liver, lung and skeletal muscle samples were then fixed in un-buffered (pH 5.1) 10% formalin for 14 days. Chloroform, diethylether and ethanol concentrations all reduced significantly after only 24

hours and the loss was considerable after 14 days, the total loss of ethanol, for example, being between 87.00% and 95.67% from among all the tissues studied. There were some exceptions, however, particularly in the case of toluene, where the total loss varied between only 11.74% and 39.80% and also in the case of skeletal muscle, where the loss of all substances was less in this tissue than in all others. Takayasu was able to conclude that formalin-fixed tissue samples can be used qualitatively for the detection of volatile substances administered ante-mortem for a period of at least 14 days following fixation.

2.6.7.2 Phenytoin

In the same study in which he examined the stability of diazepam, desipramine and phenobarbital, Winek, Esposito and Cincola (1990) also charted the degradation of phenytoin, a commonly prescribed anti-epileptic, finding that formaldehyde concentration had little part to play. Whereas 35.7% of the drug was found to be lost after 30 days in a 5% formalin solution, for example, a slightly lesser amount of 33.7% was lost from the 8% formalin solution. Using drug-positive liver samples, furthermore, only 3.1% of the starting concentration was lost after fixing in 5% formalin for 28 days.

2.6.7.3 Paraquat

In the first of three papers to examine the stability of the highly toxic herbicide, paraquat, in formaldehyde, a Chinese toxicology laboratory published a spectroscopic method (Kuo and Kuo, 1988) for the quantification of the compound in formalin-fixed, rat liver samples and in two forensic cases of paraquat poisoning, using formalin-fixed organs. In the second paper, a Japanese team published a further spectroscopic method that was able to detect the compound in the formalin-fixed organs of two forensic cases that had been stored for 1.5 and 6.5 years, respectively (Minakata et al., 1989). Finally, Kuo published a second paraquat paper, further developing the method published in his 1988 study. In this study, Kuo (1990), he examined liver, kidney and lung samples from an actual forensic case. Comparing concentrations of paraquat detected in these three tissues at autopsy, Kuo re-analysed them after two months of formalin fixation (concentration and pH not stated), finding that total recovery had decreased by 46.30%, 56.78% and 45.72% for each of the tissues, respectively. He also found that there had been a significant transference of the compound from tissue to

formalin solution, in one case noting that a greater concentration of paraquat could be detected in the formalin solution than could be recovered from the fixed liver sample. The practice of analysing the formalin solution in which tissue samples had been stored was a methodological approach that, as has already been discussed, came to be adopted by a number of laboratories in their own work on formalin-fixed tissues.

2.6.7.4 Heavy metals

In the only published study of the stability of heavy metals in formaldehyde, an American team noted that formalin fixation had little effect on the recovery of the majority of elements (Bush et al., 1995). Using brain, kidney, liver, heart, hair and skeletal muscle samples from 30 forensic cases, the laboratory compared concentrations detected at the time of autopsy with those recoverable after 1 week, 6 months and 12 months of fixation. Although stating neither the concentration of the formalin in which the samples were fixed, nor the pH at which the solutions were buffered, the study reports 12-month recovery rates of at least 99% for the "essential elements" calcium, copper, iron, magnesium and zinc. The "toxic elements", cadmium, arsenic, mercury and lead, similarly, were almost entirely recoverable from fixed tissues after 12 months. The only exceptions in the study were manganese and aluminium. In the case of manganese, a significant decrease in concentration was recorded over time: although relatively stable at 1 week, after 6 months the concentration has reduced from 6µg/g to 4.5µg/g, reducing further to 3.8µg/g after 12 months. Although he did not analyse the solution in which tissues had been stored, Bush assumed the loss to be due to leaching. In contradiction to his manganese findings, in the case of aluminium, Bush reported that the mean concentration detectable in fixed samples increased over time, from 0.9 µg/g at time zero, to 1.5µg/g after six months and 2.3µg/g after 12 months. No explanation was offered for the apparent increases in concentration.

2.6.7.5 Strychnine

An Italian team, at the University of Ancona's Institute of Legal Medicine, published a paper in the late 1990s that was to be the first of several stability studies of drugs in formaldehyde (Cingolani et al., 1999). In this study, the recovery was reported of the alkaloid poison strychnine, a potent central nervous system stimulant and convulsant, from the kidney and

liver of a forensic case that had been fixed in 10% formalin solution buffered to pH 7.0. The paper reported significant losses of strychnine after eight weeks. Compared to concentrations measured at the original autopsy, recovery from the kidney was only 36.57%, and from the liver was 23.80%. Adopting the method that was to be repeated in his later work, however, Cingolani then analysed the formalin solutions in which the tissue samples had been stored, finding that an additional 26.95% of the original strychnine concentration could be recovered from the liver formalin solution and 41.42% from the kidney formalin solution. Cingolani notes the higher concentration of strychnine in the formalin solutions than in the tissues themselves, stating what was to become a repeated explanation that this is probably due to the extraction capability of the formalin solutions. He concludes, unsurprisingly, by stating that the interpretation of quantitative data obtained from fixed tissues requires considerable care.

2.6.7.6 Fenfluramine

Gannett, as has already been discussed, carried out an extensive study of the degradation of barbiturates in formaldehyde in 2001. In the same year he also published a paper reporting the stability in formaldehyde of fenfluramine, an anorectic, or appetite suppressant (Gannett et al., 2001a); this is a drug that has in fact now been withdrawn from use in many countries. Using exactly the same experimental conditions (5%, 10% and 20% formalin solutions, un-buffered (pH~3.5) and pH 7.0 and 9.5 for 30 days) a, by now, familiar pattern emerged from the study: the more concentrated the formalin solution, the greater (and faster) the loss of fenfluramine, and the more alkali the solution, then so too the greater (and, again, faster) recorded loss of the parent compound was noted. Formalin concentration and pH appeared, furthermore, to work synergistically, and thus the greatest loss was recorded in the 20% formalin solution buffered to pH 9.5, where a 100% loss of parent compound was recorded on Day 1. On the other hand, and in the opposite condition, a loss of only 10.5% was recorded at Day 30 in the 5% formalin un-buffered mixture. In a further finding, Gannett reported that in virtually all of the reaction mixtures, the amount of *n*-methyl derivative recovered was almost exactly proportional to the percentage loss of the parent compound.

2.6.7.7 Lidocaine

In an unfortunate medical accident in a Japanese hospital, a patient suffering from ventricular arrhythmia was given a 5mL intravenous injection of 10% lidocaine hydrochloride (500mg) instead of 2.5mL of a 2% solution (50mg), after which he suffered complete cardiorespiratory arrest and died. Toxicological analysis was not carried out until 40 days after autopsy, using samples of the victim's brain, liver, kidney and skeletal muscle that had been preserved in formalin solution (Kudo et al., 2004). Although showing considerable variation in concentration of lidocaine, the drug was recoverable from all tissues, as well as from the formalin solutions in which the samples had been stored. In order to ascertain whether the formaldehyde was likely to have caused any loss of lidocaine from the samples, the laboratory carried out an experiment using rat tissue. After administration of lidocaine and euthanasia, samples of brain, liver, kidney and skeletal muscle were stored in 10% formalin solution, buffered to pH 7.0, for four weeks. Although there were some variations between tissues, a loss of around 80% of the original concentration was reported by the end of the study, with most of the loss having occurred within the first two weeks.

2.6.7.8 Sildenafil

In an Italian case of sudden death, toxicological analysis carried out at the time of autopsy revealed the presence of sildenafil – used for erectile dysfunction, and better known as Viagra – in several tissues and fluids. In response to what was said to be an increase in unexpected mortality following the administration of sildenafil, a study was undertaken into the stability of the drug in formalin solution, for those cases where the need for toxicology was not identified until after the autopsy had taken place (Pagani et al., 2005). Using the same samples of brain, heart, kidney, liver, lung and spleen that had been analysed at the time of the original autopsy, the laboratory then fixed them in formalin solution for four weeks. While stating neither the concentration of the formalin solution, nor the pH to which it was buffered, the study reports that although there was considerable loss from the fixed tissues (ranging between approximately 60% in liver to approximately 81% in lung), significant amounts of the drug could be recovered from the formalin solutions in which the samples had been stored (ranging from almost 60% in the liver formalin solution, to over 77% in the lung formalin solution). Analysing both the tissue and the formalin solution in which the tissue had been

stored, the lowest overall recovery was for the spleen samples, at just over 88%, and the highest overall recovery was for liver samples, at almost 99%. Taking into account the need to include tissue and fluid, Pagani concludes that the drug shows good stability in biological specimens submitted to fixation.

2.6.7.9 Acetaminophen and salicylic acid

Suzuki and Kenko, in their 2009 paper that examined the degradation of bromovalerylurea and MA in formaldehyde, also reported the stability of acetaminophen (the analgesic better known in the United Kingdom as Paracetamol) and salicylic acid (used to treat skin conditions, including acne). In the case of acetaminophen, Suzuki reported the importance of pH as well as formalin concentration and temperature on the stability of the drug, finding that whereas a 10% formalin solution buffered to pH 7.4 at 4°C had very little effect (recovery being almost 100%), an un-buffered, 20% formalin solution at room temperature resulted in a loss of around 40% of the parent compound. In the case of salicylic acid, however, Suzuki reported essentially complete recovery of the drug in all conditions.

2.6.7.10 Aconitum alkaloids

A Japanese homicide was reported that involved poisoning with *Aconitum* (a genus of flowering plants belonging to the Renunculaceae family, used in traditional Chinese and Japanese medicine, and a highly potent neurotoxin) that was mixed into the jam of a cake and fed to the unsuspecting victim, whose body was then dumped in a river. Following autopsy, the cause of death was given as drowning, but some five years later evidence emerged of the true nature of the death. Liver, kidney and lung samples had been retained, preserved in 10% formalin buffered to pH 7, and these samples were analysed qualitatively, revealing the presence of benzoylaconine and benzoylmesaconine (Miyaguchi and Sekine, 2010).

2.6.7.11 Methamidophos

In response to frequent accidental or deliberate pesticide poisonings in developing countries, a Chinese laboratory studied the stability of the organophosphorous insecticide, methamidophos, in post-mortem blood and liver samples, in several conditions, including

10% formalin at room temperature. Utilising samples obtained from dosed and sacrificed dogs, the experiment determined that formaldehyde increased markedly the rate of degradation of the compound, the half-life in 10% formalin stated as being two days, in contrast with the stated half-lives of 4.1, 9.8 and 17.8 days in blood stored at, respectively, room temperature, 4^oC and -20^oC (Wei et al., 2017).

2.7 The Chemistry of Drug Degradation in the Presence of Formaldehyde

Although a number of papers discussed in the course of this chapter make reference to the degradation or decomposition of drug molecules in the presence of formaldehyde, it is only a relatively small number of studies that include any discussion of the chemistry of such decomposition, and even fewer that seek either to identify (or quantitate) the degradation products. Those papers that discuss the chemistry of the suspected reactions are now considered; like the preceding section, for the sake of organisational convenience, this is undertaken by drug class.

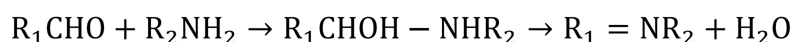
2.7.1 Antidepressants

The merit of studying the breakdown products of drugs in reaction with formaldehyde was first signalled in the 1990 paper of Dettling et al. that reported on the synthesis of amitriptyline from the reaction of nortriptyline with formaldehyde. Dettling reported the increasing methylation of the parent drug when reacted with increasing concentrations of formaldehyde, although the conversion was not always directly proportional to the recorded loss of the parent drug.

Building on Dettling's work, Winek, Zaveri and Wahba's 1993 study of tricyclic antidepressants then noted that although during *in vivo* metabolism, the tertiary amine amitriptyline is demethylated to form nortriptyline and, likewise, imipramine is metabolised to desipramine, in reaction with formaldehyde the reverse of this process takes place. Here, a variable degree (depending on the concentration of formaldehyde) of methylation occurs, nortriptyline converting to amitriptyline, and desipramine to imipramine. Winek observes that the process by which these conversions take place is the reaction of amines with formaldehyde, leading

to the production of Schiff bases, which then undergo reduction to form the methylated analogues.

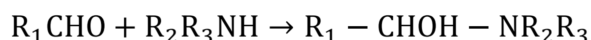
Takayasu et al. (1998) considers the chemistry of amine methylation in greater detail. In the case of primary amines, he states that their addition to formaldehyde (or to any aldehyde) produces *N*-substituted hemiaminals which then lose water to produce stable Schiff bases:



$$R_1CHO + R_2NH_2 \rightarrow R_1CHOH - NHR_2 \rightarrow R_1 = NR_2 + H_2O$$

Equation 2-1 Methylation of primary amines

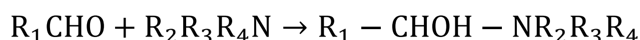
When secondary amines are added to aldehydes, Takayasu notes, there is an initial formation of *N,N*-disubstituted hemiaminals though, unlike primary amines, they are unable to lose water:



$$R_1CHO + R_2R_3NH \rightarrow R_1 - CHOH - NR_2R_3$$

Equation 2-2 Methylation of secondary amines

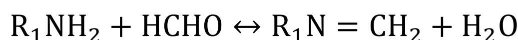
Tertiary amines, Takayasu says, can only give salts:



$$R_1CHO + R_2R_3R_4N \rightarrow R_1 - CHOH - NR_2R_3R_4$$

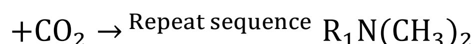
Equation 2-3 Methylation of tertiary amines

As far as tricyclic antidepressants are concerned, however, Takayasu goes on to state that when secondary amines are treated with formaldehyde in the presence of hydrogen and a hydrogenation catalyst (or another reducing agent), reduction-alkylation takes place. Importantly, if the formaldehyde solution contains formic acid (formalin solution ordinarily does contain formic acid due to the air oxidation of formaldehyde (Tirumalai et al., 2005)), then secondary amines are reductively-alkylated with formaldehyde and formic acid, via the Eschweiler-Clarke reaction, to form, respectively, their *N*-methyl and *N,N*-methyl derivatives:



$$R_1NH_2 + HCHO \leftrightarrow R_1N = CH_2 + H_2O$$

$$R_1N = CH_2 + HCOOH \leftrightarrow R_1NHCH_3$$



$$+CO_2 \rightarrow^{\text{Repeat sequence}} R_1N(CH_3)_2$$

Equation 2-4 Alkylation of secondary amines via the Eschweiler-Clarke reaction

Indeed, according to Takayasu, Winek's assertion that secondary amines react with formaldehyde to form Schiff bases is probably incorrect and that, instead, the methylated products of formaldehyde's reaction with nortriptyline and desipramine come about almost certainly as a result of the Eschweiler-Clarke reaction.

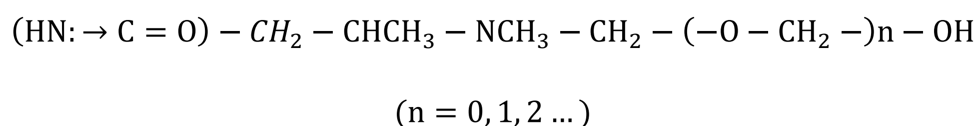
The Eschweiler-Clarke reaction was also the mechanism by which Gannett et al. (2001a) noted the methylation of fenfluramine to *n*-methyl fenfluramine in the presence of formaldehyde, recording the almost exactly proportional recovery of the *N*-methyl derivative compared to the loss of the parent compound.

Although not explicitly highlighted by either Winek nor Takayasu, the forensic implications of any process by which metabolites might be converted back to the parent compound are, of course, potentially very significant.

The chemistry of SSRI antidepressant reactions with formaldehyde has also received some attention. Suma and Prakash's 2006 study of sertraline, described the production of *n*-methyl sertraline, noting that as a molecule containing a secondary amine group, like the tricyclic antidepressants, the mechanism of conversion is likely to be the Eschweiler-Clarke reaction. Similarly, in their 2006b paper, Suma, Shukla and Prakash established that the Eschweiler-Clarke reaction was also likely to be responsible for the methylation of fluoxetine, in the presence of formaldehyde, leading to the production of *n*-methyl fluoxetine.

2.7.2 Stimulants

Another relatively early study to consider the chemistry of formaldehyde reactions was the methamphetamine paper, Takayasu et al. (1994), where it was speculated that the nitrogen atom of the drug molecule and the carbon of the aldehyde react nucleophilically and form addition products:



$$(\mathrm{HN{:}} \rightarrow \mathrm{C=O}) - CH_2 - \mathrm{CHCH_3} - \mathrm{NCH_3} - \mathrm{CH_2} - (-\mathrm{O} - \mathrm{CH_2} -)\mathrm{n} - \mathrm{OH}$$

$$(\mathrm{n} = 0, 1, 2 \ldots)$$

Equation 2-5 Formation of addition products

In their 2005 study of MA, however, Tirumalai et al. proposed a simple methylation process, leading to the production of *N*-methyl-methamphetamine, in the same way that *N*-methyl derivatives were found to form from tricyclic and SSRI antidepressants. The same process of methylation was proposed by Shakleya et al. in their 2006 study of MA, as well as in their earlier, 2005, study of MDMA, where its reductive methylation to MDDA, via the Eschweiler-Clarke reaction, was noted. As recently as 2013, in their stability study of MDMA, mephedrone and 3-TFMPP, Maskell et al. also proposed that it was via the Eschweiler-Clarke reaction that all three molecules were methylated in the presence of formaldehyde. Although noting only a minimal dependence on the concentration of the formaldehyde solution, Maskell did, however record very different rates of methylation between the three drugs, with mephedrone being the fastest to degrade (82% loss after 24hrs; nearly complete loss after 60 days), followed by MDMA (49% loss after 24hrs; nearly complete loss after 60 days) and finally 3-TFMPP (0% loss after 24hrs; loss of approximately 70% after 60 days). Maskell infers that the reason for these variable rates of decay may be due to the influence of the side chain on the aromatic rings in each of these compounds. Mephedrone and MDMA both have aliphatic chains of two carbon atoms attached to the secondary amine, and the faster degradation observed in the case of mephedrone, Maskell suggests, may be as a result of stability of the iminium ion produced during the Eschweiler-Clarke reaction, due to the presence of a carbonyl group. In the case of 3-TFMPP, however, the secondary amine is within the aliphatic ring structure, and it may well, thus, be the position of the amine group either within or without the aliphatic ring structure that determines the stability of amine compounds in formaldehyde. Indeed, both Winek, Esposito and Cincola (1990), reporting on the stability of phenytoin, and Suma, Kosanam and Prakash (2006c), in their study of bupropion and olanzapine, suggest that the reason for the comparative stability of these drugs in formaldehyde under certain conditions may be because of the difficulty of an intermediate iminium ion formation in the Eschweiler-Clarke reaction, due to the presence in all of these drugs of an aliphatic ring structure.

Yokchue's doctoral work on the reaction of several drug molecules with formaldehyde identified the conversion of amphetamine to methamphetamine; this, she proposes, was also probably via the Eschweiler-Clarke reaction, amphetamine being a primary amine. In 10%

formalin solution, she recorded the conversion as being 0.66%, 3.06% and 12.58% on days 0, 1 and 7, respectively. In reacting methamphetamine with 10% formalin, Yokchue identified a very limited conversion of the parent compound to *N,N*-dimethylamphetamine, the rate of conversion being 0.76%, 0.78% and 0.83% at days 0, 1 and 7. Finally, in reaction with 10% formalin, and in agreement with the findings of both Shakleya and Maskell, MDMA was found to convert to MDDMA, at the rate of 2.4%, 2.2% (sic) and 5.7% by days 0, 1 and 7. Interestingly, however, it appeared to be the case that neither amphetamine, MA nor MDMA were completely converted to their *N*-methyl derivatives. In the case of amphetamine, for example, the sum of the peak areas of the parent drug and its methylated conversion product detected at days 1 and 7 comprised approximately 42% and 30%, respectively, of the peak area of the parent drug at the start of the experiment; in the case of MA and its conversion product, the respective sums were 54% and 59% and, for MDMA and its conversion product, 68% and 70%. These significant discrepancies in peak areas, Yokchue suggests, indicate that other (as yet, unidentified) chemical reactions take place alongside the Eschweiler-Clarke reaction, resulting in the synthesis of additional breakdown products.

2.7.3 Benzodiazepines

Tracy et al. (2001), charting the stability of ten benzodiazepines, considers the chemistry of their reactions with formaldehyde, surmising that the likely pathway is one of either acid or base hydrolysis, followed by the Eschweiler-Clarke reaction. Regardless of the hydrolytic conditions or the site of cleavage, the result will always be the methylation of a nitrogen ring. In acid conditions, Tracy suggests that the imine bond of the ring is the most likely site of hydrolysis; in base conditions, the amide bond of the ring is the most likely site. Although such hydrolysis reactions are common for benzodiazepines, as well as being readily reversible, Tracy predicts that in the presence of formaldehyde, the Eschweiler-Clarke reaction causes the methylation of the now available primary amine or aniline, thereby preventing re-closure of the ring and, thus, resulting in the production of new compounds. Subsequent work has established the high sensitivity to hydrolysis of the diazepinone ring in all benzodiazepines (Cabrera, de Waisbaum and Nudelman, 2005), Tracy having already determined that hydrolysis pathways following the 1,2-position cleavage appear to occur under basic conditions, whereas acidic conditions are more likely to induce a 4,5-position cleavage.

Although Tracy concludes by stating that work was already underway to identify conclusively these reaction products in order to aid forensic toxicology analysis, the results have not as yet been published.

2.7.4 Barbiturates

Gannett's 2001b paper, as well as recording the rate of degradation of three barbiturates in the presence of formaldehyde, also aimed to determine the chemical processes by which decomposition products were formed. He suggested that barbiturates are susceptible to hydrolytic decomposition, the rate of hydrolysis being dependent upon pH, with the rate of decay typically increasing as pH increases. Gannett's assumption was that re-cyclization of the ring structure may be inhibited by reaction with the 1,6-cleavage product, leading to synthesis of the methylol-amide and the imine, by simple condensation with formaldehyde. Alternatively, a completely blocked re-cyclization could lead to the production of methylated products, brought about by the Eschweiler-Clarke reaction. Although successfully identifying 2-phenylbutyric acid as the major decomposition product of phenobarbital in formaldehyde, Gannett suspected that there may be up to five additional compounds, though none of these were definitively identified.

2.8 Conclusions and Problems with Previous Work

Compared to many other areas of forensic research, the toxicology of embalmed human remains has received little attention. The literature we do have, furthermore, represents a wide range of methodological approaches. Whereas some papers, for example, are simple and single case reports offering little more than indicative conclusions, a range of other approaches have included findings from multiple drug-positive forensic cases, as well as the results of drug-spiked human tissue experiments, drug-formalin mixture reactions, in addition to several animal studies. While the comparatively limited embalming literature has been beneficial for the particularly wide range of drugs and poisons it has considered, one of the arguably consequent problems of the way in which work in the area has developed is that results have often been somewhat equivocal and, at times, seemingly contradictory. This is coupled with the fact that although a number of toxicologists have been keen to highlight inherent problems, few have suggested solutions. Thus, some might argue that this has been

an area of research that would appear sometimes to be without clear direction. In this respect, of course, the literature that we do have is not by any means unique in its deficiencies; that conclusions may be drawn on a lack of clear evidence is a charge that has been levelled against post-mortem toxicology more generally, and on more than one occasion (Drummer, 2004a; Guzelian et al., 2005). In the case of the embalming literature, perceived problems are relevant not only from the point of the view of the criticisms they invite in their own right, but also because they also raise very particular concerns that are relevant to the current study and which, therefore, must be considered explicitly.

Of the several problems there are with the literature, one is the seemingly disparate nature of the target drug molecules reported. Even within the same class of drug, it is only occasionally the case that more than one study has been undertaken on any one particular drug, and thus the drawing of trends, let alone conclusions, is difficult. In the case of Winek, Zaveri and Wahba (1993a) and Takayasu et al. (1998), moreover, although both studied the exact same four tricyclic antidepressants, their experimental conditions and analytical methods are so markedly different as to make a direct comparison of results very difficult, if not impossible.

Implicit in some of the early papers is an indication of other difficulties associated with toxicology and formaldehyde. Rohrig, in his 1998 fentanyl case report, for example, noted that embalming may cause a dilutional effect on systemic drugs, may lower the extractability or recovery of drugs, and may also chemically react with target compounds. In their 2001 paper examining the stability of benzodiazepines, Tracy et al. also noted the many variables that come into play when drugs come into contact with formalin solution. In the ten compounds tested, as we have seen, it was found that although the decomposition of all but one was accelerated by formaldehyde, the rate of decay varied from compound to compound, and was in many cases time, formalin-concentration and pH dependent, each of these variables contributing individually, as well as perhaps synergistically, to the rate of decay. The inherent difficulties associated with formaldehyde were further highlighted in Skopp’s 2004 post-mortem toxicology review paper, where the author commented that drug instability [in a formalin environment] may derive from physical, chemical or bacterial processes. As Nikolaou et al. summarized, in their 2013 review of the toxicology-embalming

literature, "the fixation or embalming process may actually mask, alter, destroy or sequester many compounds, so that the stability of a substance in [a] formalin environment should always be considered." (p.314)

Aside the fundamental issue concerning the seemingly variable reactivity of formaldehyde itself, however, there are a number of methodological problems that make a usefully comparative assessment of the literature difficult to achieve. Chief among these problems is that many of the published studies appear to treat all tissue samples, however anatomically diverse, as being pharmacologically equal, without giving any consideration to the important and very real matters of drug absorption, distribution, metabolism and excretion reviewed in Chapter 1. It might seem surprising that such little reference is made to these critically important pharmacological principles, since the bearing they bring to experimental results was highlighted clearly in Nishigami, Takayasu and Ohshima (1995). Here, it was noted that diazepam and chlorpromazine are both highly lipophilic drugs with particular tissue accumulation properties, and that the distribution of chlorpromazine to lung tissue in rabbits is up to 64 times greater than the commensurate concentration detected in plasma. Nishigami also points out, however, that the subsequent decrease in chlorpromazine concentration, in the presence of formaldehyde, was more conspicuous in fixed lung tissue than in brain, kidney, liver or skeletal muscle samples. He asserts, therefore, that the degradation of drugs may well be influenced not only by the concentration of the formalin solution used to fix the tissues, and the pH to which it is buffered (or not), but also by the nature of the tissue itself. Associated with this, Nishigami noted that the tendency for increased diazepam concentrations in lung tissue and skeletal muscle may be related to the fact that the partition coefficients of diazepam in these two tissues are higher than in brain tissue and – particularly since the drug was also detected in the formalin solution in which the samples were stored – it is possible that diazepam eluted from the lung and skeletal muscle samples may have subsequently reaccumulated or been redistributed within the tissues. These are basic, long-understood and important pharmacological principles which appear to have been overlooked by a number of analysts who have published in the field.

Related to this, another methodological problem to emerge from the literature centres around the different matrices that are examined in different papers, such that, again, direct

and meaningful comparison of results between studies is very difficult. Whereas a number of papers, for example, follow a simple experimental method of monitoring the degradation of a drug solution in a formalin solution, others place drug-positive human and animal tissue in formalin solutions, while others first spike tissues with drugs before soaking them in formalin. Although many of the early papers do little more than plot the decay of the target drugs over time, Cingolani et al. (2001 and 2004) strongly advocate the analysis of not only the tissue itself, but also the solution in which the tissue was stored, noting the seemingly high extraction capabilities of formaldehyde. Indeed, for some drugs, as we have seen, the entire original quantity can be recovered from an analysis of not only the tissue, but also the formalin solution in which it was stored. Such a recommendation is, of course, easily achievable with histological samples, where small amounts of tissue are stored in small amounts of formalin. This methodology, however, is clearly not readily transferable to the setting of an embalmed and repatriated body, where the same practical considerations of discrete samples do not apply.

Despite many papers simply plotting the decay of target analytes, as early as 1993, Winek et al. had demonstrated that there are chemical processes other than simple leaching taking place. This observation had been paved by Kuo in his 1990 study of paraquat and was further developed in Nishigami, Takayasu and Ohshima's 1995 study of chlorpromazine and diazepam. Both papers noted that during the fixation process, xenobiotics are extracted into the surrounding formalin solution at differing rates, depending on the hydrophilicity index of both the substance itself and the particular tissue under fixation. This forensically important observation was confirmed and reinforced in the cocaine studies reported in Viel et al. (2009) and Hilal et. al. (2009). It is the case, however, that many other subsequent analysts have failed to take account of this potentially very significant fact.

Other work took as its focus the fact that rather than simply leaching the parent drug from the tissue in question, in a number of instances formaldehyde caused chemical reactions that led to the production of new compounds. Identified as early as 1990, by Dettling et al., the finding came with a warning that it was not always possible to identify directly proportional relationships between the breakdown of the parent compound and the formation of secondary molecules; depending on the concentration of formaldehyde present, the pH and

the duration of exposure, furthermore, the ratio can be extremely difficult to predict. In the case of methamphetamine, Tirumalai et al. (2005) goes so far as to suggest that the target analyte in the post-embalming analysis of suspected cases should in fact be the *N*-methyl derivative of the parent compound, since MA itself will almost certainly be undetectable given its very high rate of decomposition in the presence of formaldehyde. Indeed, in their 2009 cocaine study, Hilal et al. say that knowledge of the reactivity of drugs with formaldehyde needs to be considered carefully when performing post-embalming toxicological analysis and that the determination of degradation products will lead to accurate conclusions being reached. The relevance of this from a forensic perspective was highlighted clearly in the work of Winek and Takayasu, both of whom identified the conversion of tricyclic antidepressant metabolites back to the parent compound, in the presence of formaldehyde. The concomitant risk of obtaining misleading forensic results is obvious, though it is disappointing that relatively few subsequent studies have attached much, if any, importance to identifying either the products of formaldehyde-induced drug degradation, or the chemical processes by which such products are synthesised.

As far as the current project is concerned, however, any emphasis in the literature of identifying breakdown products may be something of a proverbial red herring, since the matter of formaldehyde concentration is the one factor that may be of pivotal importance to this study. A number of studies, as we have seen, have noted the significant influence that formaldehyde concentration can have on the rate of degradation of many drugs. It is the case, however, that aspects of this work appear be based on early and incorrect statements that have been followed by subsequent researchers. The first error was in the 1990 nortriptyline study of Dettling et al., where it was stated that tissue exposed to 20% formaldehyde "is a circumstance often observed in well-embalmed tissue." (p.326) Then, in their 2001 benzodiazepine paper, Tracy et al., quoting a personal communication with a funeral home in Morgantown, West Virginia, stated that, "generally, the final formaldehyde concentration of the embalming fluid used [for funeral home embalming] is between 5% and 20%, although lower concentrations can be used." (p.167) Consequently, Tracy et al. set their experimental conditions at 5%, 10% and 20% formaldehyde concentrations. These same concentrations are also used by Gannett et al. in their 2001a fenfluramine study, where the experiment is

described as being “under typical embalming conditions,” (p.89) and these exact concentrations are then repeated in almost every subsequent paper in the embalming-toxicology, up to and including Yokchue’s doctoral thesis. Although 10% formalin (containing, therefore, approximately 3.7% formaldehyde by volume) is commonly used in the fixing of pathology samples for histological examination (Burnett and Crocker 2005; Waters, 2009), Dettling and Tracy are completely incorrect in their assertions that embalming is typically carried out using final formaldehyde concentrations of 5-20%. In reality, the concentration of formaldehyde used in funeral home embalming is almost always between 1% and 2%, and this fact is well supported in mortuary literature, as well as in common embalming practice.

In the seminal study of its time, *The Disposal of the Dead,* it is stated that “the formaldehyde concentration of arterial embalming fluid rarely exceeds 2%” (Polson, 1962). In another important instructional volume, *The Principles and Practice of Embalming,* the authors recommend that the arterial solution strength should be 1% (Frederick and Strub, 1974). More recently, in what is widely regarded by mortuary practitioners to be the ‘embalmers’ bible’, *Embalming History, Theory and Practice,* Mayer writes that the formaldehyde concentration in typical fluids is between 1% and 2% (Mayer, 2006). Further confirmation comes from an author who states that embalming commonly involves treatment with a formaldehyde solution of 2% concentration (Gunn, 2009), and from a French embalming review paper which states that the final concentration of diluted fluid used in embalming is 0.5-1.5% (Anger et al., 2008). These comments very much accord with the advice of the British Institute of Embalmers, whose students are taught that “the final concentration of formaldehyde solution should be around 1-2%, depending on the condition of the body to be embalmed, as well as the length of time before the funeral service is due to take place” (British Institute of Embalmers 2005, E1). A final formaldehyde concentration of 1-2% also accords with common practice (pers. comm. with several embalmers and professional experience as a BIE-qualified embalmer).

The reason for this fundamental error is alluded to in Anger et al. (2008). In this French review paper of toxicology and embalming, it is explained that in order to prepare a fluid for injection, the embalmer takes a commercially-formulated “index” and dilutes it in water. The “index”, Anger goes on to explain, refers to the formaldehyde concentration of the commercially

available fluid, which the embalmer will choose according to the condition of the body. Thus, a range of fluids are made available to the embalmer that are already partially diluted, in differing concentrations. Dodge, one of the largest worldwide manufacturers of embalming fluids, list in their catalogue a selection of injection fluids with indexes that range from 5-30 (Dodge, 2019), the numerical indicator being the concentration of formaldehyde, as a percentage, by volume, in that particular fluid. Lower concentrations are chosen when only a relatively 'light' embalming is required, and higher concentration fluids are used when a greater degree of preservation is required (pers. comm. with several embalmers and personal, professional experience). The embalmer then, simply, selects the appropriate index and adds the same volume of whichever index is chosen to water (usually 100mL of index to every 900mL water) to create the final fluid, thus avoiding having to calculate in each case whatever volume of fully-saturated solution would need to be diluted in order to create the desired final concentration in each case.

Tracy's paper was written nearly 20 years ago, and it has not been possible to investigate further the source of his information, from the personal conversation he is stated to have had with a funeral home in Morgantown. 'Index' embalming fluids, however, have formed the basis of commercial preparations for longer than living memory (pers. comm. with several embalmers and personal experience), and it is almost certainly in this respect that the misunderstanding came about, with Tracy failing to appreciate that the index fluid will be further diluted.

However the misunderstanding came about, the significance of this fundamental error cannot be over-emphasised for its implications on the toxicology of embalmed human remains. Experimental work carried out on the stability of morphine in formalin solutions more representative of the concentrations encountered in funeral home embalming produced results that contrast markedly with published studies based on non-representative concentrations (Lloyd, 2014). In this study, a loss of 62.62% of the original concentration of morphine was recorded in a 2% formaldehyde solution buffered to 7.2 for nine weeks, and a reduced loss of 54.21% in 1% solution over the same period of time. These results are very significantly different to the losses of up to 70.59% recorded by Cingolani et al. in 10% formaldehyde and up to 95.87% recorded by Xiang et al. in their 10% formaldehyde study of

morphine stability. If one considers the results of the studies that have been undertaken on the stability of a large number of drugs, as summarised in Appendix 2, using particularly concentrated formalin solutions, the implications of these facts are brought into sharp focus. Instead of assuming that embalmed bodies cannot successfully be examined for drugs, due to their rapid degradation, it may well be the case that many drugs remain detectable for greater periods of time following embalming.

As well as formaldehyde concentration, another significant determinant on the rate of drug degradation to emerge from the toxicology-embalming literature is pH. In my 2014 study of morphine, an increasingly basic pH was found to accelerate the rate of decay, the loss of 47.28% in 1% formaldehyde at pH 6.2 after 63 days, for example, increasing to 54.21% in a pH 7.2 solution and 65.22% in pH 8.2. Although this pattern, as has been seen, has been noted by many other analysts, a significant number of contradictory findings have also been reported. Tirumalai et al. (2005) for example, found that MA degraded more rapidly in neutral or basic conditions, and Tracy et al. (2001) observed the enhanced decomposition of certain benzodiazepines, including alprazolam and midazolam, under acidic conditions, whereas Takayasu et al. (1998) noted the accelerated breakdown of desipramine in both acid and alkali solutions, though not in neutral conditions. Tracy, moreover, intimates that there may be variable rates of decay as a result of changes in post-mortem pH, due to the usually acidic pH of formalin (pH ~3.5) being modified to the slightly alkaline as protein breakdown begins. Furthermore, although an alkaline environment commonly develops in the later stages of autolytic decomposition, the release of carbon dioxide during cellular lysis in the early stages of decomposition, leads to an acidic shift in the pH of blood (Haglund and Sorg, 2002). With circulating blood in the living having a pH of approximately 7.3 – 7.4 (Provan and Krentz, 2002), this point, in itself, may have important toxicological forensic implications.

As far as the current project is concerned, there are two points that are relevant to any discussion of pH. First, is the fact that compared to formalin-fixed histology samples, the pH in embalmed bodies is likely to be far less of a variable factor than might be suggested by the literature. This is because of the fact that, unlike histological formalin, commercial embalming fluids are always buffered to approximately pH 9.0 in order to stabilise the formaldehyde within the solution and prevent its precipitation (Mayer, 2006). Second, and perhaps more

importantly, is the fact that if the main hypothesis of this thesis can be proven, and it can be shown that formaldehyde does not infiltrate those anatomical compartments containing VH, SF and CSF, then the focus of the narrative will need to move from the stability of drugs in formaldehyde to the stability of drugs in biological fluids. Several studies, as discussed in Chapter 1, have alluded to the potential instability of certain drugs in the post-mortem body, independent of the presence of formaldehyde, a subject that has received no consideration in the embalming literature. The matter of pH will be further discussed in the introduction to Chapter 5.

Temperature is the third and final factor that may have important bearing on the work of the current project. Despite discussing the stability of drugs in various concentrations of formaldehyde, and in different conditions of pH, many analysts fail to emphasise the very considerable kinetic influence that temperature has in many chemical reactions. Suzuki and Keneko, in their 2009 study, demonstrated with great clarity the significant impact that temperature changes can have on reactions, reporting the almost complete recovery of methamphetamine from 20% formalin solution at 4°C, compared to a 100% loss in the very same solution at room temperature. Not one paper has drawn explicit attention to the potential relevance of temperature to post-mortem toxicology in embalmed remains. It is the case, however, that in the majority of funeral homes bodies are commonly stored in refrigeration units and thus the "room temperature" results reported in much of the literature may well be unrepresentative of the likely scenarios to be observed in actual bodies, rather than in stylised laboratory experiments. The matter of temperature will be further addressed in the introduction to Chapter 5.

In summary, there are a number of problems in the literature that would benefit from being re-examined within the context of embalmed and repatriated bodies. Chief among these is formaldehyde concentration, where existing studies have, with some ambiguity, seemingly apportioned relevance not only to histological fixed samples, but also to embalmed and to exhumed human remains. As has been explained, however, it is the case that funeral-home embalming is routinely effected using significantly less concentrated solutions of formaldehyde than has previously (wrongly) assumed to be the case. Given what we know about the markedly reactive nature of formaldehyde with many compounds, the relevance

of embalming undertaken with much less concentrated formalin solutions may well be critically relevant to subsequent toxicological findings. It is highly likely that the extent of the decomposition of many drugs may be significantly less than the existing literature would have us believe, both in terms of the quantity of the loss, as well as the rate at which this loss takes place. Alongside formaldehyde concentration, the important kinetic variables of pH and temperature have considerable relevance to the current study, and both factors, in embalmed and repatriated bodies, may be likely to produce results that differ markedly from much of the published literature, again with associated and important forensic relevance. If research undertaken in those conditions relevant explicitly to embalmed bodies can be brought to bear on the existing literature, then it may well be that in future a much more accurate and, therefore, forensically robust toxicological examination of embalmed and repatriated bodies can be undertaken.

3 CEREBROSPINAL FLUID, SYNOVIAL FLUID AND VITREOUS HUMOUR

3.1 Introduction

As was noted in Chapter 1, blood has traditionally been the sample of choice for toxicological analysis in post-mortem forensic work. The important reason for this, simply, is that blood is the easiest tissue or fluid to collect and, during autopsy, the major vessels can be readily tapped for samples (Forrest, 1993). Additionally, given that drugs by definition enter vascular circulation immediately following intravenous injection, and often within a relatively short period of time following other forms of administration, blood provides a useful medium through which the pharmacologic state of the peri-mortem individual can be established. Despite its ease of collection, and the large body of pharmacokinetic data that has been collected over many years, blood does, however, have a number of drawbacks as a matrix for forensic analysis. Earlier, the problems of post-mortem redistribution were considered, as well as the fact that a number of commonly prescribed - as well as abused - drugs undergo degradation in blood, often as a result of enzymatic metabolism or bacterial invasion. Sample preparation can be difficult, furthermore, and analytical methods that are to provide meaningful results must be able to remove proteins and lipids from the matrix, as well as other interfering biomolecules which may be present in significant quantities (Margalho et al., 2011).

Given that, as has been established, post-mortem processes can significantly affect the ability of toxicologists to state with any degree of accuracy the concentration of drugs present within tissues, especially blood, it follows that fluids contained within closed compartments that have no direct blood supply may well provide more helpful evidence. This is not only because of the potential for a diminished likelihood of post-mortem drug redistribution, but also due to the expectation of reduced bacterial invasion, as a result of the anatomic isolation of the fluids (Levine, 2010). Within the human body, there are three particular tissues that have for several years been flagged for their potential usefulness in this respect. By far the greatest amount of literature relates to vitreous humour (VH), the fluid found within the eyeball, behind the lens. Second, is cerebrospinal fluid (CSF), the liquid contained within the cranium

and spinal column that bathes the brain and the spinal cord. The final fluid, synovial fluid (SF), is a lubricating liquid found within all the synovial joints of the body.

As long ago as 1990 the potential usefulness of CSF in the detection of cocaine was noted (Rowbotham et al., 1990) and more recently, Steven Karch, at the time Assistant Medical Examiner for San Francisco, and himself a well-published forensic toxicologist, has argued that alternatives to blood testing are clearly needed. “Pathologists”, he argues, and as was cited earlier, “should give serious thought to testing vitreous or spinal fluid instead of blood.” (Karch, 2001, p.394) When considering embalmed bodies, furthermore, Robert Forrest, formerly Senior Coroner for South Lincolnshire, and Honorary Professor of Forensic Toxicology at the University of Sheffield, writing in 1993, signaled clearly the merit in considering analysis of avascular fluids, stating that VH remains uncontaminated by the embalming process and may be usefully analysed for ethanol, as well as for urea and creatinine (Forrest, 1993). Forrest’s position has more recently been reinforced by Karch, who writes that vitreous, by virtue of its protected environment, may provide the only opportunity to establish an ante-mortem ethanol concentration in embalmed bodies (Karch, 2008) and by Allan, who has written that analysis of vitreous humour in embalmed and repatriated cases may provide useful data (Allan and Roberts, 2008).

From the perspective of the current project, the greatest attraction of all three fluids is the fact that none of them receive a direct blood supply. Instead, as will be explored later in this chapter, molecules are only able to enter (and leave) all three fluids through tightly-arranged cellular membranes that are, by virtue of their structure, size-selective, allowing only particularly small molecules to cross. The physiological and biochemical processes by which molecules are able to cross the respective membranes protecting all three fluids, furthermore, appear to be driven by energetic reactions that terminate on the cessation of metabolism, following death. It follows, therefore, that it is possible to posit a hypothesis whereby, following death, the anatomical structures containing CSF, SF and VH become historical repositories of toxicological evidence that are protected not only from bacterial invasion and post-mortem redistribution but also, importantly, from contamination with embalming fluid.

The forensic literature pertaining to avascular fluids is limited when compared to that for blood, in terms of both volume and scope. With the exception of VH, where an early identification of the usefulness of the fluid in determining time since death has served to catalyse an on-going interest in the toxicological possibilities of its analysis, consideration of the utility of CSF and SF has been sporadic and, with little exception, the literature that we do have is largely disparate, as well as being, until very recently, arguably simplistic in its aims. What follows for the remainder of this chapter, therefore, is a review of the literature relating to the post-mortem utilisation of CSF, SF and VH, which aims both to survey and summarise what has been published, as well as to highlight individual methodological trends. Although the primary focus of this review is the toxicological use of all three fluids, in the interests of providing a comprehensive account, work examining the biochemical usefulness of the fluids is also included.

3.2 Cerebrospinal Fluid

3.2.1 Anatomy and physiology

Cerebrospinal fluid, sometimes referred to simply as spinal fluid, is a clear, watery fluid contained within the subarachnoid space and circulating in the ventricles of the brain as well as in the central canal of the spinal cord (Martin, 2010). The choroid plexus of the brain, located within the ventricular cavities, is the principal site of CSF formation, and the fluid enters circulation through what is known as the blood-cerebrospinal fluid barrier (BCSFB) (Davson and Segal, 1996). Around two-thirds of circulating CSF originates in the choroid plexus, with the remaining third being brain interstitial fluid, formed by the oxidation of glucose within the cells of the brain, as well as plasma movement from extra-choroidal brain vasculature; this fluid enters circulation via what is known as the blood-brain barrier (BBB) (Shen, Artru and Adkinson, 2004). The total volume of circulating CSF in healthy human beings is in the range of approximately 100-160 mL, accounting for around 18% of the total brain/spinal cord volume (Luders, Steinmetz and Jancke, 2002). It is produced at a rate of around 25-40 mL/h, a corresponding volume being reabsorbed at the same rate, giving a total volume turnover time of 3-6 hours (Redzic et al., 2005).

Although the literature describing the anatomy and physiology of the central nervous system is extensive and wide ranging, the most important fact about both the BBB and BCSFB, for the purposes of the present study, is that certain large molecules, including blood, cross neither barrier. Instead, the tight cellular junctions at both barriers contribute to a mechanism whereby solutes flux across the capillary walls (Hawkins and Davis, 2005). In the case of the BCSFB, for example, endothelial cells lining the capillaries of the choroid plexus are structurally fenestrated, allowing the filtration of plasma out of the vasculature and into the stromal space (Rosenberg, 1990). Importantly, however, epithelial cells adjacent to the choroid plexus vasculature contain tight, apical junctions, having the effect of restricting the direct passage of plasma from the stroma into the CSF (Bradbury, 1989). Thus, given the physiological mechanisms by which CSF is produced and the cellular processes by which it passes into circulation, drugs may enter the CSF either directly through the BCSFB at the choroid plexus, or indirectly, across the BBB, via the brain's interstitial fluid.

Polarised biochemical transport systems - including basic, as well as dicarboxylic and neutral amino acids, monocarboxylic acids, monosaccharides, nucleobase and nucleoside, peptides and certain amines - facilitate the movement of essential nutrients, physiological substrates, as well as xenobiotics across both the BBB and the BCSFB (Lee et al., 2001). It is an important fact, however, that drugs (as well as nutrients and other substrates) have differing degrees of lipid solubility, as well as differing affinities for influx transporters, and the extent to which they enter the CSF is determined by these two factors (Oldendorf, 1974). It follows, therefore, that the more lipid soluble the xenobiotic in question, and/or the greater its affinity for the biochemical transporters that effect movement across the BBB and the BCSFB, then the greater (and faster) will be its passage into circulating CSF (Robinson and Rapoport, 1992). Smaller and non-polar molecules, furthermore, move across the capillary membranes of both the BBB and the BCSFB more readily than do larger and polar molecules (Rowland and Tozer, 2011). The tight junctions of the vasculature, however, also carry a high electrical resistance (Greig, 1992). Many molecules require energy in order to overcome this resistance and pass through the barrier and it has been demonstrated that such energy requirements are coupled to cellular metabolism, including ATP-splitting (Breen et al., 2002). Collectively, through the physical restrictions of the non-fenestrated cells, as well as through the biochemical

processes of specific efflux and metabolic conversion activities, the central nervous system is protected from potentially toxic waste products of normal metabolism, as well as from many xenobiotics (Miller, Lowes and Pritchard, 2005).

The relative impermeability of both the BBB and the BCSFB is a well-known problem in the development of new pharmaceutical compounds that are required to penetrate the CNS (for example in the treatment of meningitis and depression), and the development of molecules capable of crossing the plasma membrane on one side of the cell, moving through the cytoplasm of the cell, then crossing the plasma membrane on the other side, presents significant challenges to pharmacologists (Partridge, 2003). Indeed, whereas very many drugs are transported to target sites within the body by binding themselves to proteins within the blood, compared to whole-blood plasma, the concentration of protein within the CSF is only 0.3-0.6%, and this means of drug delivery is, therefore, unviable (Pragst et al., 1994). That said, when molecules are able to cross the barriers, they can be readily detected: a pharmacologic study, examining the pharmacokinetic behaviour of acetaminophen (Paracetamol), for example, found that the drug could be detected in CSF after only a single dose, delivered intravenously, orally, or rectally (Singla et al., 2012); unsurprisingly, intravenous introduction resulted in higher plasma and CSF concentrations than did either oral or rectal administration, though consistent plasma:CSF concentration ratios were established between the subjects.

Of particular relevance to the current study, it has been found that as production of ATP is known to cease rapidly after death, the process of active transport is consequently blocked (Pélissier-Alicot et al., 2003). If it follows, therefore, that the xenobiotic contents of the CSF are thereby protected, drug molecules being unable either to enter or leave the fluid, then the sampling of the fluid as an historical repository of ante-mortem pharmacologic state alluded to earlier in the discussion, may well be possible.

3.2.2 Literature

In the living, analysis of CSF is diagnostically useful in the treatment of several pathological states, including meningitis (Venkatesh, Scott and Ziegenfuss, 2000) and Alzheimer’s Disease (Blennow, 2005; Formichi et al., 2006), as well as other degenerative disorders (Botez and

Young, 2001; Lescuyer et al., 2004). It can also be analysed to determine the effectiveness of drug delivery to the central nervous system (Shen, Artru and Adkinson, 2004) when treating a range of chronic conditions including, for example, intractable epilepsy (Rambeck et al., 2006) and HIV in the neurological compartment (Antinori et al., 2005).

Following death, CSF culture techniques can be used to establish a diagnosis of meningococcal sepsis, meningococcal disease, as well as meningitis (Ploy et al., 2005; Ventura et al., 2013); it also has an early history of being used for the post-mortem determination of both diabetes and uraemia (Naumann, 1949) and of diabetic coma (Sydow et al., 2018). Recently, CSF has also been used to identify polymorphisms leading to higher expressions of catecholamines in certain users of methamphetamine (Matsusue et al., 2017). Another recent study, however, has demonstrated different concentrations of certain biochemical markers, depending on the site of sampling, whether subarachnoid space or lumbar puncture, although there is as yet no indication of whether drug concentrations might be similarly affected (Garland et al., 2018).

Importantly, furthermore, PCR analysis has demonstrated that many bacteria do not infiltrate the CSF, even in cases of advanced decomposition (Palmiere et al., 2015), and that analysis of the sample is not usually not hindered by handling problems. A 1994 study, comparing morphine concentrations in CSF with those in blood, for example, noted that, of the 23 cases examined, blood drawn at the time of each respective autopsy had already clotted, lysed and had begun to putrify; CSF samples, on the other hand, remained in generally good condition (Logan and Luthi, 1994). Being a clear fluid, comprising mainly water, CSF, furthermore, requires very little sample preparation and is amenable to most routine analytical methods (Moffat, Osselton and Widdop, 2011).

In Logan's study, mentioned above, significantly, morphine was detected in the CSF of all 23 cases, even when the corresponding levels in both blood and urine were very low, suggesting that death had occurred only a very short time after drug administration. Importantly, an earlier study (Moore et al., 1984) demonstrated peak CSF concentrations within two minutes of a single intravenous dose of morphine, and in at least one of the cases examined by Logan, the concentration of morphine detected in both the urine and CSF was so low as to suggest that death followed very shortly after administration, and before the drug had distributed

freely. Logan points out, however, that one of the drawbacks of using CSF is the inexact correlation between blood and CSF morphine concentrations. He was, nevertheless, able to reconcile the ratio (which was found to be approximately 2:1) to the extent of being able to determine, with confidence, that a CSF morphine concentration greater than 0.02 g/mL signaled, in every case, brain morphine concentrations high enough to result in the degree of respiratory depression associated with morphine-induced death, and other equally encouraging work on CSF:blood ratios for several drugs certainly serves to pave the way for further research into this matrix.

In the same year as Logan published his paper, a study was undertaken that examined the detection of heroin and its metabolites in several tissue types (Goldberger et al., 1994). In this study, drawing on earlier research (Way, Young and Kemp, 1965, and Barrett, Dyssegaard and Shaw, 1992) that had demonstrated the extreme lability of heroin in biological fluids, Goldberger was able to show that the concentration of 6-MAM (as the primary metabolite of heroin) in CSF, and also in brain and spleen samples, was significantly higher than in blood, as well as in liver, lung and kidney.

Despite Logan and Goldberger's seemingly promising findings, there appears to have been little immediate interest in pursuing CSF as a viable toxicological matrix. It was not, in fact, until some four years later, in 1998, that the County Coroner's Office of Cleveland, Ohio, conducted a similar study to that of Logan's, in this instance comparing blood and CSF for the presence of 6-MAM in 65 forensic cases (Jenkins and Lavins, 1998). The hypothesis in this study was based on established facts, in that the author had already determined that heroin has a very short half-life and is particularly unstable in aqueous media (Jenkins et al., 1994). Jenkins demonstrated that, when either smoked or injected intravenously, heroin would be metabolised to 6-MAM within five and nine minutes, respectively. Given, therefore, the speed both at which heroin is metabolised, and CSF is produced, it could be reasonably hypothesised that 6-MAM might be detected in CSF. This position had been established by work in the 1970s (Oldendorf et al., 1972), which had demonstrated the delivery of heroin to brain tissue within 10-15 seconds of intravenous administration. Jenkins' and Lavin's 1998 study was successful in detecting 6-MAM in 60 of the 65 examined cases. Importantly, furthermore, of the 60 cases where it was detected in CSF, 6-MAM could not be detected in the blood of 30

of those cases, leading Jenkins to conclude that had CSF not been analysed in all 65 cases, then heroin-related deaths in 46% of the cohort would not have been identified. A similar study undertaken in Berlin a year later (Pragst et al., 1999) also demonstrated the primacy of CSF (and VH) for determining heroin administration in a number of cases, where the corresponding urine analyses were negative for 6-MAM.

Following Pragst's paper, it was not until 2004 that any further work on the usefulness of CSF as a toxicological matrix appeared in the literature. In that year, toxicologists from the County Coroner's Office in Dayton, Ohio, published a paper examining several tissue types for evidence of heroin administration (Wyman and Bultman, 2004). 6-MAM was detected in the CSF of 16 of the 25 cases examined, compared with 13 cases where the metabolite was detected in femoral blood (it will also be seen, later in this chapter, that the same study also identified 6-MAM in the vitreous of all 25 cases). As Karch was later to point out, and reinforcing Jenkins' earlier position, given that the metabolite is known to be rapidly hydrolysed to morphine in blood, CSF may well be an especially useful matrix for the detection of 6-MAM and, thus, for determining heroin use (Karch, 2008).

A wide-ranging study published in 2007, comparing the concentration of a number of drugs and their metabolites in the blood and CSF of 282 cases (Engelhart and Jenkins, 2007), determined that a large range of an anaesthetics, anticonvulsants, antidepressants, antihistamines, benzodiazepines, opioids and sedatives were readily detectable in CSF. Engelhart's study provided confirmation of some of Oldendorf's earlier work, reinforcing the fact that lipophilic drugs appear to cross into the CSF more readily that hydrophilic compounds. Engelhart also reported that, as a general principle, drug concentrations in CSF were lower than in blood though, in the case of the xenobiotics targeted in his own study, with no consistent ratio in the case of any particular target molecule.

A number of published case reports have also recorded the presence of drugs in CSF. One report (Pragst et al., 1994) detected a wide range of prescription as well as over-the-counter drugs, where the average CSF:blood ratio was found to be 0.74, and a ratio of <0.3 was suggested as being indicative of a short survival time following administration. In the case of an apparent suicide, by ingestion of a quantity of carbamate-derived insecticide, Moriya and

Hashimoto (2005) reported a blood concentration of less than 10 ng/mL, but CSF concentration in excess of 2000 ng/mL; the low concentration in blood being explained by the esterase activity and alkaline environment of the matrix. Horak and Jenkins (2005) reported the detection of the SSRI antidepressant citalopram in CSF and blood, with a ratio of 0.2, although another SSRI antidepressant, olanzapine, was undetectable in CSF, despite being identified in blood. Felby, Neilsen and Thomsen (2008) demonstrated a good correlation between blood and CSF, in 105 cases, for the presence of the ketones, acetone, acetoacetate and β-hydroxybutyrate, all being important post-mortem indicators of both diabetic and alcoholic ketoacidosis; Felby notes the usefulness of CSF for this diagnosis when neither blood nor urine are available for analysis, reporting the finding of ketone bodies in the CSF samples of all 105 cases, though he does acknowledge that the results of previous work (Pounder, Stevenson and Taylor, 1998b and Kadiš, Balžic, and Ferlan-Marolt, 1999) are more circumspect than his own findings. More recent work on ketone bodies (Palmiere, Mangin and Werner, 2014a) has reported lower concentrations in CSF, compared to either blood or urine. This study, however, presents an arguably more considered view than many other papers, in that it looks to the physiology of the blood-brain barrier for a possible explanation of decreased CSF concentrations: while the BBB does contain a biochemical transporter for short-chain monocarboxylic acids, including ketones, Palmiere notes that ketones are markedly hydrophilic and, as such, are likely to be relatively impenetrable across the BBB.

Only one published study has examined the usefulness of CSF for the post-mortem identification of ethanol (Gelbke et al., 1978). Comparing blood and CSF concentrations in 509 cases, Gelbke reported an excellent correlation coefficient (r = 0.943), as well as a useful regression equation from which, it was claimed, an accurate blood alcohol level could be calculated.

More recently, in 2015, a Japanese team published what is, to date, the most comprehensive review of post-mortem drug concentrations in CSF (in this instance, compared with concentrations detected in blood and pericardial fluid). This study, Tominga et al. (2015), reports toxicology findings for a particularly wide range of drugs, in 103 cases, though acknowledges the inherent difficulties of predicting the extent to which a drug might be expected to cross the blood-brain barrier, noting the potential influence of pharmacokinetic

variables, including the degree of protein binding of drugs, as well as their volume of distribution within the body. Despite these variables, Tominga reported consistent correlations between blood and CSF for a number of drugs, including acetaminophen, phenobarbital and caffeine, noting an expected lower concentration of phenobarbital in CSF, given that up to 60% of the drug circulating in blood is present in a protein-bound form. Orally-administered psychotropic preparations, including promethazine and chlorpromazine (as well as oral cold remedies, including chlorpheniramine and dihydrocodeine) showed significantly lower concentrations in CSF, a factor that could be predicted by the preferential protein binding of these drugs, as well as their hydrophilicity. Benzodiazepine CSF:blood correlations were equivocal, though this fact may be due to the comparatively small number of benzodiazepine cases included in the study (n=10). Both methamphetamine and amphetamine, however, are highly lipophilic and have large volume of distribution and, as such, would be expected to pass readily across both the BCSFB and the BBB; Tominga notes almost equivalent correlations between CSF and blood (as well as pericardial fluid) for both drugs, whether administered orally or intravenously. Finally, although not utilizing CSF for direct toxicological analysis, one recent study has examined the fluid for post-mortem evidence of increased hormonal prolactin concentrations as markers of ante-mortem drug use; a good correlation was found between serum and CSF in the case of the dopamine antagonists, chlorpromazine, levomepromazine and olanzapine (Tani et al., 2019).

A summary of the principal published papers on drug detection in CSF is given in Appendix 3. In an attempt to draw comparison between relative blood and CSF concentrations, and in a novel means of presentation, where data have been published, the blood concentrations are presented alongside the CSF concentrations, following which blood:CSF ratios have been calculated in each case.

3.2.3 Advantages and disadvantages

Although the number of studies examining the usefulness of CSF in the detection of compounds of forensic interest is limited in number, of those studies that have been undertaken, importantly, all report the fluid to be qualitatively useful. The relative speed at which certain drugs have been demonstrated to enter the CSF lends considerable weight to

the potential suitability of the fluid in forensic contexts and, in the case of 6-MAM, CSF has been shown to be superior to blood in the determination of heroin abuse. Given, furthermore, that CSF has been shown to be protected from post-mortem bacterial infiltration, and that the biochemical and metabolic processes that serve to transport blood-borne molecules across both the BCSFB and the BBB cease following death, the anatomical region in which CSF circulates during life appears, after death, to function as a static environment in which the passage of molecules, probably in either direction, does not take place.

Several important factors – including, for example, the lipophilicity/hydrophobicity and volume of distribution of individual xenobiotics, in conjunction with questions surrounding drug absorption and metabolism – currently preclude the possibility of any useful quantitative assessment of drug concentrations in CSF. As we have seen, however, some recent and encouraging work on CSF:blood ratios for several drugs certainly serves to pave the way for further research into this matrix.

3.3 Synovial Fluid

3.3.1 Anatomy and Physiology

Synovial fluid is found within synovial joints, the most common of the human articular joints (Ateshian and Mow, 2005). A synovial joint consists of a cavity filled with SF, surrounding which is articular cartilage covering the bony surfaces of the opposing bones, as well as a fibrous capsule containing the synovium, across which nutrients, enzymes and other metabolites are able to cross by diffusive and convective transport systems. (Hui et al., 2012). SF is, in healthy subjects, a clear-to-straw-coloured, viscous liquid, and approximately 1mL of SF can be found in each knee joint, the knee being the largest synovial joint in the human body (Ropes, Rossmeisl and Bauer, 1940).

The composition of SF is complex, though it is essentially a blood-plasma dialysate, combined with lubricant molecules, including hyaluronan and proteoglycan 4, secreted by cells which line and are within the joint space (Schumacher et al., 1994; Jay, Britt and Cha, 2000); additionally, SF contains soluble molecules, including cytokines, growth factors and morphogens which, collectively, mediate cellular communication within the joint (McInnes

and Schett, 2007). One of the major components of SF, derived from the plasma dialysate, is proteins, although the process of membranous filtration is such that large protein molecules are unable to enter the fluid easily. The total protein concentration in SF is only approximately one third of that found in blood plasma, and of this amount, approximately one half is albumin (Levick, 1981).

It has been known for some time that the synovium is highly vascular and has a dense endothelial barrier that prevents blood from entering the synovial space (Knight and Levick, 1983). The capillaries within the synovium, Knight reports, provide a continuous flow of plasma filtrate, including nutrients and drugs, to the joint, which is of particular importance for the homeostasis of the (avascular) articular cartilage. One particular - and potentially significant - difference between the synovial membrane, compared with both the BBB and BCSFB, however, is that the diffusion of xenobiotics into the SF does not appear to be governed by drug lipophilicty; indeed, the very lipid-insoluble aminoglycoside antibiotics, for example, appear to enter the SF readily (Baciocco and Iles, 1971).

When considering the potential of SF as a matrix for toxicological analysis, therefore, it is helpful to think of the synovium simply as a size-selective, semi-permeable membrane. Due to the selective permeability of the membrane, molecules of high molecular weight within the blood plasma are unable to pass easily across the synovial membrane and are either absent or found only in low concentrations in normal SF. It is the case, therefore, that normal SF is relatively acellular when compared to whole blood: erythrocytes are virtually absent, and only around 3% of the number of leucocytes ordinarily found in blood are detected in healthy SF (Sabaratnam et al., 2005).

Although the molecular size of synovial proteins is ordinarily small, in cases of localized pathology the number, as well as the size of proteins is seen to increase due to changes in the permeability of the synovium (Kushner and Somerville, 1971). In rheumatoid arthritis, for example, the particularly large molecules of globulins and glycoproteins, unknown in healthy joints, are found in high concentrations, and large plasma proteins, including, for example, α2 glycoprotein, β1 lipoprotein and β2 macroglobulin, are found in much higher concentrations than in normal fluid (Schur and Sandson, 1963). In rheumatoid arthritis, as well as an

increased synovial permeability to large protein molecules, it is also the case that, conversely, the permeability of smaller molecules including, for example, glucose and urea, is decreased, due to a combination of synovial hyperplasia, increased vascular permeability and cellular infiltration (Hui et al., 2012); the same decrease in small molecule permeability is seen to a much smaller extent in osteoarthritis (Pejovic, Stankovic and Mitrovic, 1995). The forensic implications of such disease states on the passage of xenobiotic molecules into the SF are yet to be addressed in the literature.

3.3.2 Literature

Although SF has an important place and a long-standing history in the rheumatology literature (see, for example, Kushner and Somerville (1971)), and has recently been shown to have a strong correlation with blood, for the detection of a number of biochemical substrates, including sodium, potassium, glucose, lactate, urea, uric acid and creatinine (Strettabunjong, Thongphap and Chittamma, 2019 and 2020), with the exception of a small number of mainly alcohol-related papers from the late 1960s and early 1970s it has not been not until much more recently that the forensic possibilities of the fluid have begun to be explored.

Compared to CSF, where several studies have considered the usefulness of the fluid for detecting medical conditions in the living, little work has been conducted on the use of SF in this context. Indeed, the only publication of note records an extensive study published in 1999, examining the pharmacokinetics of non-steroidal anti-inflammatories (NSAIDs) in SF (Day, et al., 1999) and the role of albumin as a binding protein for this class of drug. Day found, interestingly, that the endothelial fenestration within the synovium allows access of both albumin-bound, as well as unbound, or free, drug molecules to the SF. In the case of most NSAIDs, however, a steady state within the SF was found to be approximately 60% of that found in blood plasma, a difference explained by the proportionately lower concentration of albumin in the synovial compartment and a finding that may have bearing when attempting to predict the extent to which other drugs may be able to penetrate the synovial membrane.

The first mention in the literature of SF as a matrix for post-mortem drug detection is a 1965 paper recording the detection of alcohol concentrations (Aurdlicky and Pribilla, 1966). A number of drugs, including ethyl alcohol, barbiturates and anxiolytics, as well as opiates and

opioids, were then qualitatively identified in SF (Šita et al., 1971a). Šita published a second paper 1971, this time demonstrating the presence of 11 drugs in SF and noting that those drugs with a greater affinity for albumin were detected in higher concentrations (Šita et al., 1971b).

Virtually nothing further addressing the analysis of SF for the presence of drugs then appears in the literature for over 20 years. In 1993, however, the Allegheny County Department of Pittsburgh, Medical Examiner's Office, published a study in which ethanol concentrations in blood and SF were compared (Winek et al., 1993b). Winek's laboratory analysed the ethanol concentration in 28 post-mortem cases and found what they described as a good distribution ratio of 0.99 ± 0.29 between blood and SF, noting that the time elapsed between dosing and death, as well as the time to achieve equilibrium between blood ethanol concentration and synovial ethanol concentration, are two significant factors likely to be responsible for the wide variation in ratio.

Importantly, Winek notes that SF has an average water content of 96.6% and, as such, is a suitable biological fluid for post-mortem ethanol determination. He also points out that SF does not contain alcohol dehydrogenases, the enzymes found in significant quantities in the liver and also in blood, that oxidise alcohol. Thus, he claims, importantly, that the concentration of alcohol detected in SF should reflect the actual concentration at the time of death.

Four years later, in 1997, a Japanese team published the results of a similar study to that of Winek. Their paper (Ohshima et al., 1997) expanded the scope of Winek's study, however, by comparing not only blood-alcohol concentrations (BAC) with synovial-alcohol concentrations (SAC), but also by comparing urine-alcohol concentrations (UAC) with corresponding synovial concentrations, urine being a commonly analysed sample in cases of drink driving. His study examined 12 cases and in its regression analysis found that the BAC and UAC had a fairly linear relationship with the SAC: the BAC:SAC ratio for all 12 cases fell within the range 0.60-0.94 and UAC:SAC ratios were 0.90-1.21. What Ohshima then did, however, was to compare actual BACs with those calculated from the average BAC:SAC ratio for all 12 cases, finding a difference of 11.8 ± 9.4%. Comparing actual UACs with those calculated from the average

UAC:SAC ratio, he also noted an average difference of 7.9% ± 6.0%. In summary, Ohshima concludes that although further work is necessary, the prediction of blood and urine alcohol concentrations from synovial alcohol concentration, within a range, should be possible. Post-mortem alcohol concentration in the SF, he suggests, reflects very much its distribution in the body at the time of death. As a secondary finding, Ohshima also noted that SF appeared to be much less subject to bacterial invasion than either blood or urine and that, given its anatomical isolation, drugs within the fluid are comparatively far less likely to be subject to post-mortem redistribution.

A further study examining the usefulness of SF for the prediction of blood alcohol concentrations was published by a Turkish team in 2009 (Büyük et al., 2009). In his examination of 50 cases, Büyük found a closer BAC:SAC ratio than Ohshima's earlier study, reporting a ratio of 0.90 ± 0.07. Büyük's regression analysis, furthermore, with a correlation coefficient of 0.984, demonstrated a close linear relationship between the BAC and the SAC. Büyük, however, is careful to point out that significant differences in the BAC:SAC ratio can occur, depending on when precisely in the metabolic phase death takes place. Thus, a BAC:SAC ratio of 1.5 was found in a case where death was known to have taken place during the alcohol absorption phase, whereas a ratio range of 0.76-0.97 was recorded when death occurred during the later elimination phase. Chapter 1 of this dissertation discussed the challenges that ante-mortem pharmacokinetic processes pose to accurate post-mortem toxicology, and if ever a case were needed to illustrate the degree to which absorption, distribution, metabolism and excretion phases can affect the analytical finding, then Büyük's study clearly demonstrates the extent to which knowledge of timeline can greatly assist the interpretation of results.

As will be discussed later, potassium analysis in vitreous as a means of determining time since death has been in use for many years. It was not until 2001, however, that a comparative and comprehensive biochemical examination of SF was published (Madea, Kreuser and Banaschak, 2001), although a number of earlier studies (More and Castellano Arroyo, 1985a and 1985b; Alybaeva, 1987; Sahoo and Mohanty, 1998) arguably paved the way for this later work. Madea compared the sodium, potassium, calcium, chlorine, urea, creatinine and glucose concentrations in the vitreous and synovial fluids of 74 cases, finding comparable

levels in both fluids and, importantly, determining the timelines of both glucose and potassium to be very similar in both fluids. Although differences were found in calcium (synovial concentrations were much higher than vitreous), creatinine (again, higher in synovial) and chlorine (this time, higher in vitreous than in synovial), concentrations of potassium and especially sodium, were found to be comparable between the two fluids. The particularly interesting and important finding, however, was that the post-mortem concentration of potassium increased in both fluids at a near parallel rate. In cases, therefore, where vitreous fluid is not available - whether because of traumatic injury or other reason - Madea suggests that synovial potassium levels can be used with a very good degree of accuracy to estimate time since death.

An Indian study in 2007 confirmed Madea's findings, reporting not only a steady rise of potassium concentration in SF following death but also demonstrating a good correlation with time lapse, to the extent that an equation for the computation of death for up to 48 hours post-mortem was suggested (Sheikh, 2007).

A further comparative study of the biochemical properties of SF and VH was published in 2011 by the Departments of Forensic Medicine at two government medical colleges in Nagpur, India (Tumram, Bardale and Dongre, 2011). In this study where, helpfully, the times of death were known for all 154 cases, analysis of calcium, chlorine, creatinine, glucose, sodium, potassium and urea was undertaken. Like Madea's earlier study, this paper also reported that the average concentrations of calcium, creatinine, sodium and urea do not change significantly after death, in either synovial or vitreous, although, contrary to Madea's findings, they found a significant difference in potassium concentration between the two fluids. Tumram does, furthermore, like Sheikh and Madea, report that potassium increases in both fluids at a relatively linear rate. Indeed, such is the strength of linearity in the increase in potassium concentration in SF, compared to VH, that Tumram confidently proposes synovial concentrations as being more confirmatory than vitreous in the determination of death interval. He also presents a formula that allows a calculation, with an accuracy degree of one hour, up to a maximum of 36 hours following death.

A further Indian study (Siddhamsetty et al., 2014) sought to examine whether a semi-arid environmental climate could have any effect on post-mortem biochemical concentrations in SF, in 210 cases recovered in Delhi at varying times following death. The results of this study, similar to those of Madea, Sheikh and Tumram, report a positive correlation between potassium concentration and time of death (as well as reporting similar findings for other electrolytes), though they generally report a higher mean concentration at all time intervals. They conclude by stating that changes in synovial potassium and sodium concentrations have a 71% predictability of time of death up to 72 hours in the semi-arid environment of Delhi, though beyond 72 hours linearity was too erratic to be a useful indicator.

A 2014 study (Palmiere and Werner, 2014b) sought to compare the concentration of β-hydroxybutyrate in the SF and blood of 30 cases, as a means of determining ante-mortem hypothermia, a condition commonly suspected when raised βHB levels are detected in blood. In known cases of hypothermia, increased βHB concentrations were found in both blood and SF, though wide blood/SF ratios of 0.54 to 2 were reported. Palmiere concludes by stating that SF could be useful for the diagnosis of hypothermia if other biological fluids usually collected at autopsy are unavailable.

With the exception of Aurdliky and Šita's early reports in the late 1960s and early 1970s, it was not until 2014 that a paper appeared in the literature considering the use of SF as a toxicological matrix for anything other than alcohol (DeKing, Hargrove and Molina, 2014). Reviewing previous literature, this paper, published by toxicologists at the Bexar County Medical Examiner's Office in San Antonio, reminds us of the fact that SF has been shown to be an acceptable alternative to VH for electrolyte testing, in the determination of time since death and, as such, is deserving of further attention to assess its toxicological potential. The basis of DeKing's assertion was a study of 98 post-mortem cases, where the usefulness of SF, compared to blood, VH and urine, was assessed for the determination of cocaine, as well as opiate and opioid concentrations. The results are extremely encouraging, demonstrating excellent synovial correlation with other matrices for many drugs. Linear regression analysis comparing blood:SF ratios for morphine, hydrocodone, oxycodone and BZE (as the principal metabolite of cocaine) showed strong associations and high degrees of predictability in all cases, although the relationship was less clear for cocaine, and SF did not appear to be as

sensitive for 6-MAM as either vitreous or urine. In contradiction of the earlier work of Sabaratnam 2005, however, demonstrating the non-permeability of the synovium to large molecules, DeKing's study found that the larger molecules of 6-MAM and oxycodone appeared to enter the SF as readily as the smaller molecules of BZE and morphine. No consideration of the pathological state of the knee joints of the individuals included in the study appears to have been undertaken, however, and the conflicting findings in DeKing's report could well be explained by those earlier studies that demonstrated clearly the increased permeability of the membrane in arthritic states. DeKing concludes her paper by reiterating the promise of SF as a viable alternative to blood for toxicological testing, and she calls for further study, including method development, for a wider range of drugs.

Since DeKing's paper, the only other study to have examined the use of SF from a toxicological perspective is a report of a GC/MS method able to detect morphine, 6-MAM, codeine, cocaine, BZE and ECME (Petrochilou et al., 2019). Helpfully, although the paper reports findings from only six forensic cases, it includes comparative blood as well as SF concentrations, and calculations of blood:SF ratios. Petrochilou concludes her paper by stating that SF can be a useful alternative biological specimen in forensic toxicology, and calls for the wider reporting of authentic cases, so that the matrix can be more widely recognized for its use.

Appendix 4 presents comparative drug concentrations for SF and blood reported in the forensic literature since 1993. Like the corresponding CSF table presented in Appendix 3, where comparative data is published, blood concentrations are noted alongside SF concentrations, following which the SF:blood ratio is given.

3.3.3 Advantages and disadvantages

Like CSF, SF is an essentially avascular fluid, protected from direct blood circulation by a cellular membrane. Much of the synovial literature has focused on the use of the fluid as an aid to estimating time of death. Other work, however, has demonstrated the usefulness of the fluid in the qualitative determination of alcohol, a number of studies finding particularly close correlations between blood alcohol and synovial alcohol concentrations. These alcohol studies, additionally, have demonstrated that drugs can enter the SF within a short enough

space of time to enable the fluid to be a forensically useful matrix. Further work has shown that drug entry into SF may not be as closely governed by lipophilicity as is the case with CSF, and this, in conjunction with the fact that SF has a greater protein content than CSF, may have important implications in the utility of the fluid for detecting a wider range of drugs than can be observed in CSF. Furthermore, although only relatively small molecules are able to cross the healthy synovial membrane, it has been demonstrated that in cases of localised pathology the membrane becomes permeable to significantly larger molecules. While such states may well allow a larger range of drug molecules to cross the synovial membrane, it is not clear whether the post-mortem membrane is likely to remain impervious to embalming fluid. The recent and extremely promising study by DeKing, however, suggests that – if SF remains uncontaminated after embalming – then there is likely to be considerable merit in undertaking further work on this matrix.

3.4 Vitreous Humour

3.4.1 Anatomy and physiology

Vitreous humour is a colourless liquid located between the lens and the retina, having a viscosity approximately two times that of water and constituting approximately 80% of the eye; it has a typical volume of 4mL per in each eye, comprising approximately 99% water, with the remaining 1% consisting of sugars, salts, phagocytes, collagen fibres (Tripathi and Tripathi, 1984) and as many as 1205 different forms of protein (Murthy et al., 2014).

The eye has a dual blood supply: at the posterior aspect the retinal circulation is supplied by the central artery; at the anterior, the uveal circulation comprises a series of ciliary arteries that also supply the tissue of the ciliary body, the site of VH production (Cole, 1984). In the same way that closely-packed cellular membranes have been shown to prevent blood from crossing over into the CSF and the SF, so too, a similar anatomical structure is found in the eye – the blood-vitreous barrier (BVB) – consisting of a vascular endothelium on a basement membrane, or stroma, and two layers of ciliary epithelium (Levine and Jufer, 2008). Movement across the BVB has been shown to occur by several mechanisms, including convection (high-molecular weight substances and colloidal particles), diffusion (low-molecular weight substances) and active transport, as well as both hydrostatic and osmotic

pressure. A similar membrane associated with the retinal circulation – the blood-retinal barrier (BRB) - also exists, where an endothelium of retinal blood vessels (the inner BRB) is layered alongside an epithelium of retinal pigment (the outer BRB), movement across the membrane being facilitated by similar mechanisms to those operating across the BVB (Cunha-Vaz 2004).

Although VH is produced by tissues that are supplied by the ciliary arteries, it has been shown that xenobiotic molecules are able to penetrate the vitreous through both the uveal and the retinal circulations (Cunha-Vaz, 2004). In the field of clinical ophthalmology, however, the pharmacological challenges of penetrating the ocular barriers are well known (Thrimawithana et al., 2011), and it is the case that many molecules cannot be introduced into the body of the eye by any means other than by a needle injection directly into the globe (Cholkar et al., 2013). Related to this fact, forensic work has established that, due to the relatively low protein content of VH compared to blood, highly protein-bound drug molecules cross the BRB to only a limited extent in their free form (Holmgren et al., 2004; Lin and Lin 2005). Further work has established that, akin to the passage of xenobiotics from blood to CSF, the passage of drug molecules across the eye membranes is dependent on their size and shape, as well as their charge, lipophilicity and protein-binding potential (Cunha-Vaz, 2004). This passage is further governed by concentration differences across the membrane, linked to hydrostatic and osmotic pressure (Schmidt et al., 1997). Thus, in the case of the BRB (and probably also the BVB), although drugs may be transported across the barrier either by passive diffusion or active transport, in general, the greater the degree of hydrophilicity and the higher the molecular weight of the compound, then the greater the likelihood that passage is facilitated by active transport mechanisms (Mannermaa, Vellonen and Urti, 2006). Moreover, in an extensive piece of research examining the permeability of the BRB to beta-blockers, it was found not only that the most lipophilic molecules had permeability coefficients that were seven to eight times greater than the most hydrophilic molecules, but also that, although hydrophilic molecules demonstrated similar rates of uptake and efflux, penetration was found to be faster than outflow for lipophilic molecules, such permeation asymmetry being due, almost certainly, to active transport mechanisms (Pitkanen et al., 2005).

In the same way that the blood-synovial membrane has been shown to be permeable to larger-sized molecules in cases of localized pathology, so too, it has been demonstrated that the vascular membranes of the eye exhibit a greater permeability (in terms of both entry and exit) in cases of infection and inflammation, as well as in aphakic eyes; age-related macular degeneration, as well as diabetic retinopathy have been shown to impair the selectivity of the membranes (Cummings and Cunha-Vaz, 2008), furthermore, and it is believed that other factors including systemic disease, patient age and xenobiotic interaction with bacteria may all affect the entry and exit of drugs to and from the vitreous (Gardner, 1987).

VH shares other characteristics with CSF and SF which make it attractive as a forensic matrix. One particular point of interest is that because the water content of VH is replaced every 10 to 15 minutes (Sebag, 1989), drugs that are able to cross the protective membranes can be expected to be detectable within a short period of time following administration. Like CSF and SF, VH, being contained within an isolated compartment protected by the osseous anatomy of the orbits, is furthermore relatively well protected from bacterial entry and putrefaction (Dinis-Oliveira et al., 2010). Indeed, when compared to blood, VH has been shown to be far less likely to be the subject of bacterial contamination (Harper, 1989). More than simply being protected from bacterial access, however, it is also known that VH actively inhibits bacterial growth (Egger et al., 1997), this being an important factor in the determination of alcohol in decomposing bodies where putrefactive processes are known to contribute to the production (or degradation) of ethanol in blood samples (Corry, 1978; Caplan and Levine, 1990).

3.4.2 Literature

Vitreous humour has, when compared to CSF and SF, an extensive literature, for which there are two likely reasons. First, the sample is extremely easy to obtain from the deceased (although the sampling of CSF and SF are not especially difficult procedures). Second, and perhaps more significant, however, is the fact that some of the earliest VH literature established the fluid as an excellent matrix on which to undertake alcohol analysis, as well as electrolyte and biochemical analysis and, given the importance of these particular areas in forensic work, a useful precedent was established.

Early papers described the usefulness of VH analysis in the detection of electrolyte concentration irregularities for the post-mortem diagnosis of a number of disease states, for example, in cases of unexpected infant death, due to hypernatraemia and uraemia (Emery, Swift and Worthy, 1974). Subsequent work, concentrating on metabolic pathology, demonstrated the primacy of VH for the diagnosis of, among other conditions, hyperglycaemia (Zilg et al., 2009) as well as the usefulness of the fluid in the determination of ante-mortem blood glucose levels in the post-mortem diagnosis of fulminant type 1 diabetes (Takata et al., 2016). Other work, however, has highlighted the fact that, as with SF, localised disease states may lead to erroneous biochemical and toxicological results (Parsons, Start and Forrest, 2003), and one study has reported significant differences in potassium concentrations between the two eyes (Pounder et al., 1998a), although this may simply be due to either evaporation of VH through the cornea prior to sampling, leading to altered residual concentrations (Blana et al., 2011) or else as a result of sample handling problems (Gagajewski et al., 2004).

VH is an established sample in post-mortem biochemistry where it is perhaps best known for its utility in the determination of post-mortem interval, and in this context changes in the concentration of vitreous potassium have been reported since the early 1960s (Jaffe, 1962, Sturner and Gantner, 1964, and Hansson et al., 1966, for example). In addition to recording significant concentration changes in potassium, other early studies documented the comparative stability of other analytes, including chloride, sodium, creatinine and lactate (Coe, 1969 and 1972 and Leahy and Farber, 1967, for example). Many other studies have followed, and useful and comprehensive bibliographies of the VH literature, from biochemical perspectives, are given in Thierauf, Musshoff and Madea, 2009, Palmiere and Mangin, 2012, Rohrig, 2019 and Pigaiani et al., 2020.

In the toxicology literature, the first paper to be published (Sturner and Coumbis, 1966) established gas chromatography as a reliable means of quantifying ethyl alcohol in VH, demonstrating a correlation between blood and VH of 1:1. A number of other papers soon followed which sought either to refine Sturner's GC method (Leahy, Farber and Meadows, 1968), or else to test further the correlation between alcohol concentrations in VH and blood (Coe and Sherman, 1970), in VH, blood and muscle (Felby and Olson, 1969a), in VH, blood and

liver (Felby and Olson, 1969b), or else to report the feasibility of new analytical methods (Sturner and Garriott, 1975). Since then, numerous papers have compared the concentrations of alcohol in blood and VH, and an extensive bibliography of this literature is provided in Kugelberg and Jones (2007).

To a much greater extent than work on CSF and SF – again, almost certainly due to the correspondingly earlier and successful use of the fluid in post-mortem biochemical analysis – the VH literature of the past four decades reports the development of new and increasingly accurate analytical hardware, with associated method development work. There are also comparative quantitative studies which seek to contrast the concentration of an increasingly wide range of pharmaceutical and illicit drugs found in VH, with corresponding concentrations detected in other bodily fluids and tissues, often using methods that appear not to have been widely tested on either CSF or SF.

Ziminski et al. (1984), for example, reports the success of radio-immunoassay techniques for the determination of morphine, methadone and barbiturate concentrations in VH, comparing concentrations with those detected in blood, brain, liver and kidney and noting that drugs that are least affected by protein binding, as well as those with greater lipid solubility, and greater water solubility, appear to cross the BVB more readily than those compounds without these chemical characteristics. The use of liquid chromatography column switching when attempting to identify cocaine and BZE in VH is reported in Logan and Stafford, 1990 and Bogusz, Maier and Driessen (1997), which describes the successful use of liquid chromatography coupled to atmospheric pressure chemical ionization-mass spectrometry for the detection of heroin and its metabolites in a number of cases.

More recently, time-of-flight mass spectrometry coupled to liquid chromatography has been used for quantifying 45 named parent compounds and 24 metabolites (Pelander, Ristimaa and Ojanperä 2010) and a hybrid triple-quadrupole linear ion trap LC method has been used to report the detection of some 700 drugs of interest (Dresen et al., 2010). A fully validated, quantitative, GC/MS method for the simultaneous detection of amphetamines, cannabinoids, cocaine and opiates in VH has been developed, that finds VH to be an entirely suitable alternative to blood, particularly when detecting amphetamines and cocaine, as well as

opiates and their metabolites (Peres et al., 2014). An electrospray ionisation-liquid chromatography-tandem mass spectrometry (ESI/LC/MS-MS) technique has also been developed, in this case for the identification and quantification of 24 drugs of wide-ranging forensic interest (Arora et al., 2014).

In terms of the drugs themselves, a large number of wide-ranging papers have reported techniques for the detection of a considerable range of both pharmaceutical and illicit molecules in VH, including alcohol (Hardin, 2002), amphetamines (Clauwaert et al., 2000; De Letter et al., 2000, 2002 and 2004; Decaestecker et al., 2001; Ishikawa et al., 2018), antidepressants (Evenson and Engstrand, 1989; Ntoupa et al., 2020), barbiturates (Ojanperä et al., 1986), benzodiazepines (Scott and Oliver, 2001; Teixeira et al., 2004; Bazmi et al., 2016), cannabinoids (Jenkins and Oblock, 2008c), cocaine (Mackey-Bojack, Kloss and Apple, 2000; Chronister, Walrath and Goldberger, 2001; Duer, Spitz and McFarland, 2006; Fernández et al., 2006, 2011 and 2012), LSD and its metabolites (Favretto et al., 2007), opiates (Bermejo et al., 1992; Lin et al., 1997; Pragst et al., 1999; Wyman and Bultman, 2004; Antonides, Keily and Marinetti, 2007; Knittel et al., 2009; Kovatsi et al., 2011) and synthetic opioids (Chesser et al., 2019), as well as individual drugs including, for example, 4-chloromethcathinone (Nowak, Szpot and Zawadzki, 2020), GHB (Kintz et al., 2004), meprobamate (Bévalot et al., 2011) and phencyclidine (Cox et al., 2007), in addition to broad-based screening techniques that are able to detect several classes of drug (Metushi, Fitzgerald and McIntyre, 2016). An extremely comprehensive table listing the concentrations of over 100 drugs reported in more than 300 case reports is given in Bévalot et al. (2016), and a helpful review paper that discusses in detail correlation studies between VH and other biological matrices in given in Wójtowicz, Wietecha-Posłuszny and Snamina (2020).

As was noted in Chapter 1, work on the post-mortem redistribution of drugs began to be reported in the literature in the early 1980s. Although the majority of studies sought to track and, subsequently, predict the rate of diffusion of a number of xenobiotics from one blood-based site to another, a small number of analysts sought to investigate the potential of VH as a fluid that might not be affected by drug redistribution to the same extent as blood, given its relative anatomical isolation within the ocular compartment (there are no corresponding published studies testing the capacity of drugs to redistribute either to or from CSF or SF).

One of the earliest studies of VH for its non-distributive potential (McKinney et al., 1995) reported the dosing of juvenile swine with toxic concentrations of cocaine, following which the animals were euthanized, VH and blood then being periodically sampled and compared. If some of the findings were equivocal, the study did nevertheless serve to flag the potential of VH as a matrix for meaningful toxicological analysis.

During the 1990s, however, toxicology work on blood established that the interpretation of post-mortem cocaine concentrations, compared to most other drugs, was particularly complicated. It was found not only that there were there significant discrepancies in the concentration of cocaine and its principal metabolite, BZE, between sites of collection (Hearn et al., 1991a), but that, furthermore, the concentration of BZE did not appear to increase in proportion to the rate of cocaine metabolism (Logan, Smirnow and Gulbery, 1997), as would be expected. This made it difficult to align, with any meaningful degree of certainty, post-mortem concentrations of cocaine with pre-mortem toxicity (Karch, 1998).

As analytical and study methodology improved, however, further cocaine studies were undertaken with what appeared to be more encouraging results. A comparatively large study (Mackey-Bojack, Kloss and Apple, 2000), when compared to many of the earlier sample sizes, examining 62 cases, found no difference between mean concentrations of vitreous cocaine, CE (cocaethylene, a further cocaine metabolite) and ethanol, compared to corresponding concentrations detected in blood. Mackey-Bojack's study was also the first to attempt any detailed regression analysis, comparing VH to blood, reporting a linear relationship between the two, with respect to cocaine and BZE. What the study also revealed was a significant statistical difference between the mean values of BZE in VH and blood, and an imprecise correlation in the regression analysis of CE and ethanol between VH and blood.

By the time Mackay-Bojack undertook his study, it had already been established that CE is formed through the simultaneous metabolism of cocaine and ethanol (Jatlow et al., 1991). Cocaine users commonly also drink alcohol (Jatlow, 1993), and CE, furthermore, as an active metabolite, can amplify as well as prolong the effects of cocaine, thus, effectively, increasing its toxicity (McCance-Katz et al., 1993; Hearn et al., 1991b). Mackay-Bojack's paper was particularly successful in drawing together these previous findings, along with a number of

other disparately reported strands, taking account, for example, of the fact that cocaine appears to continue to undergo metabolic change after death, resulting in the formation of BZE and EME, that cocaine's V_D of 2L/kg means that there is a greater probability of post-mortem release from tissues in which it is stored, and that the method of sample collection and preservation can have a significant bearing on subsequent enzymatic degradation. Although not able to provide definitively diagnostic data, Mackey-Bojack's study was important in its attempt – to a much greater extent than any previous post-mortem toxicology paper – to reconcile theoretical principles with analytical findings and, as such, provides useful pointers to other laboratories undertaking similar work.

It was not for a further seven years, however, that another significant comparative study of VH was undertaken, this time by the Montgomery County Coroner's Office, in Dayton, Ohio, comparing VH and blood concentrations for a range of opiates, including codeine, hydrocodone, morphine and 6-MAM, as well as cocaine and its metabolite, BZE. This paper, Antonides, Kiely and Marinetti (2007), was arguably overdue, since the previous notable comparative study that had included opiates, Lin 1997, had been published some 10 years earlier, and had reported only simple correlations between codeine and morphine concentrations in VH, blood and urine. Antonides' study was, however, on a significantly broader scale, analyzing 52 opiate cases, 40 cocaine cases, and 15 opiate-and-cocaine-combined cases, and concluding that the concentration relationship between blood and VH concentrations for many of these compounds is not linear. Hydrocodone, cocaine, oxycodone and 6-MAM, for example, were found to have higher VH than blood concentrations in 71%, 72%, 96% and 100% of the cases, respectively, whereas blood specimens recorded higher BZE concentrations in 78% of cases. Morphine and codeine concentrations, furthermore, appeared to show a greater variance between VH and blood, compared to other opiates. A potentially useful finding was that, in a number of cases, both cocaine and 6-MAM were detectable in VH, when they could not be detected in blood, suggesting that VH may offer a longer window of detection for these and other drugs with comparatively short half-lives. A parallel with Jenkins' work showing 6-MAM to be more readily detectable in CSF, compared to blood, might be observed. Recent work, furthermore, has shown that from a particularly

wide range of sample types analysed from authentic forensic cases, VH demonstrated better 6-MAM detectability than any other alternative matrix (Maskell et al., 2019).

Like Mackay-Bojack, Antonides' study also moves away from what might be seen as the purely one-dimensional methodologies that dominated the early toxicology literature, where analytical results were routinely reported in some sort of pharmacokinetic stasis, with little, if any, attempt to contextualize findings within the framework of physiological and post-mortem affect. Antonides includes in her work consideration of the fact that VH is lacking in esterases - enzymes, it will be recalled from Chapter 1, abundant in blood, that are responsible for the hydrolysis of a number of drugs, including cocaine and opiates. In this context, the fact that many of the target drugs of Antonides' study were detected in greater concentrations in VH than in blood is unsurprising, and the corollary, as she points out, is that detection in VH should be possible for a longer period of time than in blood, a potentially useful fact when attempting purely qualitative analysis. Caution may, however, be needed on this point since other – and earlier - work, which Antonides appears to have overlooked, has shown that VH appears to mediate some degree of esterase activity (Behar-Cohen et al., 2001).

It might be observed that of all the literature on the toxicology of CSF, SF and VH thus far discussed, there have been no studies from the United Kingdom. Toxicology in the UK has traditionally been based around practices of operational casework to a much greater degree than it has ever been research driven. There are a small number of university departments supporting work in this area of forensic science, and those that do undertake research are very nearly all of relatively recent foundation. Linked to this is the fact that commercial laboratories have, unsurprisingly, made little provision for research, and any work that has been undertaken in recent years has tended to concentrate on the analysis of so-called designer drugs. A very real obstacle to post-mortem toxicology research in many parts of the United Kingdom, furthermore, has been the Human Tissue Act 2004, which makes it extremely difficult for researchers to obtain appropriate samples in statistically meaningful quantities.

The University of Glasgow has, however, benefitted from a research-active toxicology department for a number of years and, being based in Scotland, is not subject to the restriction of the Human Tissue Act. The department has for some time had an interest in alternative matrices under the direction of Karen Scott (who has now moved to work in the USA), and it was it was from this unit that the first UK post-mortem drug study to consider VH was published. In this study, Scott and Oliver (2001), a comparison of 27 cases was made between the VH and blood concentrations of temazepam and diazepam (as the most commonly abused benzodiazepines in the West of Scotland), along with desmethyldiazepam, an important metabolite of diazepam. The study found that all three compounds could be detected in VH, and that there was a reasonable correlation between blood and VH for temazepam and diazepam, though not for desmethyldiazepam. Although VH can be used with an acceptable degree of certainty to determine the presence of these three benzodiazepines, Scott concludes that it cannot be used with any confidence to estimate pre-mortem concentrations.

When reviewing the CSF literature, earlier in this chapter, consideration was given to Jenkins' 1998, paper, which concluded that 6-MAM, as evidence of heroin administration, was more readily detectable in this matrix than in blood. More recently, it has also been suggested that VH may be a preferred matrix, even when blood is available (Rees, Pounder and Osselton, 2013). Comparing VH with (femoral) blood, in 70 cases, Rees, in her doctoral work at Bournemouth University, was able to demonstrate a significantly extended window of detection for 6-MAM, the principal metabolite of heroin. Confirming Antonides's earlier work, Rees suggested that this was almost certainly due to the lack of esterase activity in VH. A further study published in 2013, comparing blood, brain and VH, and again comparing esterase activity, also noted the superiority of vitreous, over blood, for the detection of cocaine and its metabolites, again due specifically to the lack of enzymatic activity within the medium (Carvalho et al., 2013).

If Mackay-Bojack's study of cocaine in VH and blood was worthy of note for its novel consideration of physiological and post-mortem influences on analytical interpretation, then so too is the work of several laboratories that have used animal analogues in an attempt to better inform the prediction of post-mortem pharmacokinetic parameters in humans.

Perhaps unsurprisingly, these are experimental approaches which currently - given the ethical challenges of undertaking such work - have only limited literature. Although early animal studies, using rabbits, sought only to evaluate the usefulness of VH for the quantitative determination of ethanol (Fernández et al., 1989), MDMA (De Letter et al., 2000) and diazepam (Teixeira et al., 2004), without any reference to corresponding blood concentrations, latterly, a small number of experiments have sought to determine the distribution of heroin and its metabolites in the brains of mice (Andersen et al., 2009) and rats (Gottås et al., 2013 and 2014), and at several collection sites in a pig (Crandall et al., 2006b), all at particular fixed times, following administration.

These experimental models have been further developed by a French team who, more recently, have sought to begin defining the reality of pharmacokinetic parameters in practice, rather than simply acknowledging them in only theoretical terms. In this study, Bévalot et al., 2015, undertaken by an established forensic laboratory in Lyon, post-mortem toxicology results from cases where in many instances time of drug administration and time of death were both known, were compared with results obtained from closely-controlled animal analogues. Taking as a starting point the fact that most post-mortem studies are based on autopsy populations of disparate composition - and that, in each individual study population, there is wide intra-variability of the many non-controlled parameters discussed in Chapter 1, including, for example, drug ingestion pattern, drug dose, the interval between dosing and death, and the interval between death and sampling - Bévalot's study compares a series of autopsy cases alongside an *in vivo* animal study, contrasting xenobiotic distribution in blood with VH (and bile), for a small number of specific molecules (caffeine, citalopram, cyamemazine, diazepam, meprobamate and morphine), using GC/MS-MS. By dosing and euthanizing rabbits under closely controlled experimental conditions, the generally 'unknown' pharmacokinetic variables that are inherent in post-mortem studies could be standardised before being compared to autopsy data. Although this was only one study, the results, when compared to autopsy data concerning, especially, dose-death interval, pointed to VH as providing a longer window of detection than blood for all six molecules and, importantly, were strongly suggestive of a significant quantitative correlation between blood and VH concentrations, again for all six drugs.

In a related experimental model, finally, a Norwegian laboratory undertook a study comparing the pharmacokinetics of heroin and its metabolites in blood and VH, using a pig model (Gottås et al., 2016). Here, ocular microdialysis was utilized to acquire VH dialysate, and venous catheters for blood, in order to obtain samples over a wide timeframe, without the need to euthanize the animals. Useful comparative data were generated concerning, particularly, the slower transport of heroin metabolites into VH. Indeed, such was the degree of correlation observed between drug concentrations in blood and VH, that Gottås was able to state that if the approximate time frame between intake and death is known, then an entirely accurate ante-mortem blood concentration could be calculated, based on post-mortem VH concentration, and given the known stability of drugs in VH.

3.4.3 Advantages and disadvantages

Like cerebrospinal fluid and synovial fluid, vitreous humour is contained within a protected environment that, even in death, is free from both blood and bacteria, bacterial growth being further actively inhibited by protective substances within the fluid itself. Moreover, VH, when compared to blood, may induce far less esterase activity, making it a matrix of significant interest in forensic cases. Several laboratories have found VH to provide a longer window of detection than that found in blood for several xenobiotics including, importantly, 6-MAM and cocaine. This, in combination with the fact that the majority of drugs of forensic interest can be detected in VH, and that many of these compounds appear to be transported into the VH very quickly following administration, has led some studies to conclude that VH is the preferred matrix for analysis, even when blood is available. From an extraction and sample preparation point of view, too, VH has advantages in that it is easy to collect, requires very little treatment and has few interfering compounds when examined using either LC or GC.

VH certainly has a greater representation in the literature than either CSF or SF, and the interest it has attracted has provided the scope for a wider-ranging set of hypotheses to be tested. Thus, not only has VH been analysed for the qualitative determination of a large number of molecules, but studies comparing VH concentrations with those detected in blood have also been undertaken, a number of which have begun to address some of the important pre-mortem pharmacokinetic parameters that can have very significant effects on the post-

mortem detection of drugs. Uniquely, when compared with CSF and SF, VH has been the subject of living animal experimental models which have sought to define precisely the extent to which a number of drugs are able to enter the fluid, with the goal of enabling quantitative assessments to be made of pre-mortem drug administration, as well as estimations of survival time, following administration. Although these studies have begun to illuminate our understanding of what, in forensic casework, are usually unknown parameters, they have, as yet, been unable to correlate to an acceptable degree of certainty either VH:blood concentration ratios, or blood concentration extrapolations. Thus, attempts at quantitative interpretation using VH are currently somewhat limited in their usefulness. Ongoing work into the processes by which drug molecules are transported across the ante-mortem ocular membranes, however, alongside other work that is currently examining the distribution of xenobiotics within the ocular tissues, as well as continually improving pharmacokinetic modelling methods, may soon enable more definitive interpretations to be made.

3.5 Conclusions

Steven Karch ended his 2001 paper, 'Alternate strategies for postmortem drug testing', with a clear challenge to the *status quo*. "Progress in post-mortem toxicology", he said, "depends on the willingness of pathologists and toxicologists to consider the introduction of new, and very different, diagnostic techniques." (p.395) "The greatest impetus for change", he concludes, "will come when the forensics community finally comes to grips with the fact that post-mortem blood-drug concentration measurements are useful for the diagnosis of drug use and nothing else."

The analysis of vitreous humour and, to a much greater extent, cerebrospinal fluid and synovial fluid, goes some way to meeting Karch's challenge. Certainly, with perhaps the exception of VH, the use of these fluids does not currently fall within the normal battery of toxicological testing, although there is paradox in pathologists' reluctance to request such tests, while citing the lack of comparative reference data. This is a particularly important, if subtle, point since it is the case that although many forensic determinations do necessitate the provision of precise, quantitative data, the requirements of other cases are simply for qualitative confirmation. Indeed, in the case of Dr Shipman, for example, the qualitative

determination alone of heroin metabolites in a number of exhumed bodies was, when compared with those patients' prescribing records, enough to form the basis of a criminal conviction.

Both Karch and Forrest have written of the usefulness of VH as a forensic matrix in embalmed cases, and other literature considered in this chapter has highlighted some of the advantages of VH over blood and urine, including, for example, its amenability to analysis, its reduced esterase activity, its bacteria-free composition and its qualitative reliability. As this review of the literature has demonstrated, however, CSF and SF are also contained within similarly-structured, protected environments to that which appears to prevent VH from being contaminated during the embalming process. If, indeed, it is the case that all three fluids remain free from formaldehyde, then this paves the way for the development of toxicological methods that may well be helpful in such cases.

This review, furthermore, has highlighted some important differences in the pre-mortem biochemical and physiological processes by which xenobiotics are able to enter the three compartments. Lipophilic molecules, for example, appear more likely to penetrate the membranes enclosing CSF, whereas protein-bound drugs are found more readily in SF, and it is likely to be the case, therefore, that account will need to be taken of the particular matrix in which a particular drug is detected, post-mortem: a provision that may well direct the nature of the analysis undertaken. The important point, however, is that the inherent differences between the permeation characteristics of all three fluids are likely to present the opportunity for the detection of a commensurately wider range of drugs.

Compared to many other areas of forensic science, the volume and scope of publications relating to post-mortem toxicology, using matrices other than blood or urine, is limited. When placed within the broader context of the other literature considered in this chapter, however, clear and promising indictors begin to emerge by which the possibility of being able to undertake meaningful toxicological analysis on bodies that have been previously embalmed seems increasingly likely.

4 FORMALDEHYDE CONCENTRATIONS IN VITREOUS HUMOUR, SYNOVIAL FLUID AND CEREBROSPINAL FLUID OF EMBALMED HUMAN CADAVERS: AN EXPERIMENTAL STUDY

4.1 Introduction

In the words of one toxicologist, formaldehyde can mask, alter, destroy or sequester many compounds (Nikolaou et al., 2013). As we saw in Chapter 2, a wide range of methodological approaches have been applied to research conducted over a number of years in this field; methods that have sought to demonstrate both the rate and the processes by which drug molecules are altered, destroyed or sequestered in a formaldehyde environment. Of particular relevance to the current study is the wide range of formaldehyde concentrations that have been used in previous experimental work. In respect of embalmed bodies, the formaldehyde concentrations reported in the literature, it is suggested, are incorrect: experimental formaldehyde concentration ranging, typically, from 5% to 20% contrast markedly with the final formaldehyde concentration of 1% to 2% commonly used in repatriation embalming. Thus, the literature we have, recording, as it does, the widespread and rapid loss of many pharmaceutical compounds in the presence of comparatively very high concentrations of formaldehyde, may not be applicable in cases of repatriation embalming. Moreover, given both the variability and inconsistency of experimental methods, as discussed in Chapter 2, as well as the results reported from them, it can be argued that the time is ripe for the investigation of new methods that might be more representative of the conditions likely to be encountered in embalmed and repatriated bodies.

Chapter 3 reviewed the literature relating to the anatomy and physiology of cerebrospinal fluid, synovial fluid and vitreous humour, then considered the extent to which these fluids are known to be useful repositories of drug molecules, and, in conclusion, proposed that they may be viable fluids for the forensic detection of drugs in embalmed bodies. We have seen that VH was reported in a small number of papers some years ago as being a potentially useful matrix in the toxicological examination of embalmed bodies; we have also seen that post-mortem toxicology is certainly possible in VH and in CSF, and the literature is suggestive of the fact that the same is likely to be the case for SF.

On the basis that VH, SF and CSF are all contained within avascular compartments, the working hypothesis that might be drawn from Chapter 3 is that, as these fluids may well not be contaminated during the embalming process, they may consequently be viable matrices for toxicological examination in fixed bodies. Drawing on the closing comments in Chapter 2, furthermore, it may be possible to add to the working hypothesis that even if these fluids are contaminated during the embalming process, the concentration of formaldehyde entering them may well be considerably less than those concentrations assumed in the existing literature. This study seeks to test this hypothesis by reporting an experimental assessment of the extent to which VH, SF and CSF are contaminated by the embalming process, using samples of all three fluids obtained from embalmed cadavers.

4.2 Analytical method

The risks to human health from exposure to formaldehyde have long been recognised and, in consequence, many methods have been developed that quantify the molecule in a wide range of analytes. Given that the Environmental Protection Agency of the United States of America determined in 1999 that a daily human intake of formaldehyde exceeding 0.2mg/kg body weight would likely be harmful to health (EPA, 1999), and that the International Agency for Research on Cancer determined in 2004 that the substance is considered to be a dangerous human carcinogen (IARC, 2004), many of the published methods - whether quantifying the substance in building materials or agricultural products, foodstuffs or pharmaceuticals - have been developed for what might be broadly termed environmental purposes.

Many published methods recognise the potential difficulties of analysing formaldehyde. It was noted some years ago that the formaldehyde molecule is not readily ionisable and cannot, therefore, by easily analysed by MS (Soman, Qui and Li, 2008). Further analysts have commented that methodological robustness problems deriving from the thermal instability of the molecule make direct quantification by GC/MS difficult and lead to poor LOQ and LOD values (Lobo et al., 2014). Daoudy et al. (2018), furthermore, notes the analytical challenges posed by the high chemical reactivity of the molecule, as well as its low UV detector sensitivity and specificity, adding that, as the molecule is soluble in water and in organic solvents, it is difficult to extract and analyse directly in a specific, accurate and sensitive way. For all of these

reasons, and due to the small and polar nature of the molecule, Backe, 2017 notes that formaldehyde is usually derivatised before analysis, typically using 2,4-dinitrophenylhydrazine (DNPH) (see, for example, Soman, Qui and Li, 2008, Yeh et al., 2013, Lobo et al., 2014, Hu and Wang, 2015, Story et al., 2015, Wahed et al., 2016, Yilmaz et al., 2016 and Rezende et al., 2017). A particularly detailed account of published formaldehyde methods is given in Su and He, 2017.

While many of the published methods achieve separation using HPLC, a number utilise GC, of which several, contrary to the majority of methods, describe the analysis of underivatized formaldehyde. One of the earliest of these papers, Sharp (2001), reports a headspace, FID, method for the detection of over 40 volatile organic compounds, including formaldehyde, separation being achieved with dual-phase elution on J&W Scientific DB-1 and DB-WAX columns. Headspace sampling was achieved after 30 minutes at 70°C, with a split of 20:1, and the programme commenced at 40°C, held for nine minutes, before ramping to 150°C at 10°C per minute, with no hold, giving a total run time of 20 minutes. Formaldehyde, on the DB-1 column eluted at 15.42 minutes and, on the DB-WAX column, at 2.38 minutes. Quantitative linearity data for formaldehyde are not given.

Several commercial Application Notes describing the GC analysis of underivatized formaldehyde have also been published. An Agilent note (Agilent, 2015/16), for example, details the use of a DB-WAX column and an FID detector, using direct sampling, a split ratio of 1:100 and an isothermal 35°C oven temperature, although the note does not state any quantitative linearity data.

A 2018 Restek Application Note takes advantage, like Sharp's earlier paper, of the volatile nature of formaldehyde, utilising headspace sampling, and using a thermal conductivity detector. Employing a Rt-U-BOND column, and a split of 10:1, the sample is heated in the headspace oven at 60°C, and is then separated using a six-minute programme, beginning at 100°C (held for one minute) then ramped to 150°C at 25°C per minute (held for three minutes) (Restek, 2018). Like the Agilent method, the Restek application note does not, however, present any quantitative linearity data.

A 2018 Phenomenex application note (ID No: 21516) also uses a headspace method (60°C for ten minutes), followed by separation using a ZB-1 column in a 50°C isothermal programme, utilising a split of 5:1 and FID detection (Phenomenex, 2018). Again, no quantitative data are given.

A much earlier Tekmar-Dohrmann application note for the analysis of underivatized formaldehyde in water-soluble polymers, however, provides considerably more method detail (Tekmar-Dohrmann, 1999). Designed to quantify the release of formaldehyde during the drying and curing of latex binders, the method uses a full evaporation technique of a 20µL sample, equilibrated at 150°C for 30 minutes, following which the sample is separated on a J&W Scientific DB-WAX column, following a programme that begins at 40°C (held for three minutes), after which the temperature is ramped to 230°C at 20°C per minute, giving a total run time of approximately 12 minutes, detection being achieved using a quadrupole mass spectrometer, operated in the single ion mode. The method is said to be optimised over the range 200-1000ppm formaldehyde.

For the current experiment, the requirement to identify formaldehyde in biological fluids obtained from embalmed cadavers was such that a much more sensitive method was required than any reported in the literature. Taking as its starting point the Tekmar-Dohrmann application note, however, it was possible to develop and optimise a method that was not only universally suitable for the analysis of CSF, SF and VH, but which also had a considerable degree of sensitivity.

During the method development stage, a wide range of gradient programmes as well as incubation times and temperatures were tested, with the goal of creating the most sensitive and robust method. Sensitivity, however, was sometimes achieved at the expense of robustness, and *vice versa*, and the work to achieve an acceptable balance was extensive. It was found, for example, that considerable sensitivity could be achieved following high temperature headspace equilibration. When warmed for too long and at too high a temperature, however, the instability of the formaldehyde molecule noted in Lobo et al. (2014) was observed. When warmed for only short periods, or at comparatively lower temperatures, although the repeatability of the method was much improved, the degree of

sensitivity was much lower. At length, an acceptable compromise was reached, whereby 1mL samples (a larger sample size than that used in the Tekmar-Dohrmann method) would be heated at 95°C for 20 minutes (as opposed to 150°C for 30 minutes). The newly-developed method furthermore, employed a split of 1:30, as opposed to the 1:15 split used in the Tekmar-Dohrmann method.

Of considerable importance to this method, furthermore, was the total run time, given the large number of samples requiring analysis. Many different gradient programmes were tried in order to reduce the run time of the Tekmar-Dohrmann method. Using the same column as the Tekmar-Dohrmann method, a J&S Scientific DB-WAX, it was in fact possible to achieve a clean separation, with a formaldehyde retention time of 0.87min and a total run time of only 7min.

The final method was able to achieve an LOQ of 0.4µg/mL and LOD of 0.13µg/mL across all three sample types, within acceptable validation parameters. The optimal conditions that achieved the best separation, along with hardware and software specifications, and all validation parameters, are detailed below in Table 4-3.

4.3 Sample sourcing

In an ideal scenario samples for this study would have been obtained from authentic embalmed and repatriated bodies. This, however, was difficult to achieve on several levels. The main constraint was related to the fact that, in England, Wales and Northern Ireland, the use of post-mortem samples is regulated by the Human Tissue Authority (HTA). Specifically, with the exception of samples that are taken expressly for the purpose of determining cause of death, samples can only be used for research purposes if permission has been obtained from the executors of the deceased, or if the deceased has given advance permission. Following personal conversations with several regional coroners, it quickly became apparent that, within the timescales involved, and with the resources available, this would not be a viable option for the numbers of samples that would be required to undertake a statistically significant study.

Under the aegis of the HTA, however, a number of university medical schools are licensed to carry out anatomical dissection and research using cadavers properly consented and donated for these express purposes. In agreement, first, with the University of Bristol, and then with the University of Cambridge, it was possible to obtain samples of VH, SF and CSF from donated cadavers that had first been embalmed, prior to subsequent student dissection. Although samples from embalmed and repatriated cadavers may have been preferable, there were in fact a number of distinct advantages to obtaining samples from medical school cadavers. Chief among these was the fact that bodies donated to medical schools are embalmed using a clearly defined protocol. As a result, when obtaining the samples, it was also possible to ascertain precise details concerning the concentration of formaldehyde used, the volume of fluid injected, and the amount of time that had elapsed between embalming and sampling; facts that are frequently not available for repatriation-embalmed cadavers.

The advantages, however, and with some degree of paradox, brought with them three particular drawbacks of using medical school cadavers. Firstly, because of the fact that medical schools generally require much longer-term preservation than that needed for repatriation purposes, the concentration of formaldehyde used in their embalming fluids is usually much more concentrated than that used for repatriation embalming. In the case of the University of Cambridge, for example, the final formaldehyde concentration of the fluid used for routine embalming, commonly referred to as 'hard-fix embalming', is 4.2%. This contrasts markedly with the final formaldehyde concentration of 1% to 2% used in repatriation embalming, as discussed in Chapter 2. Second, in the case of medical school cadavers, and again because of the requirement for comparatively longer-term preservation, considerably larger volumes of arterial embalming fluid are injected than is typically the case with repatriation embalming. Indeed, in the 43 Cambridge cadavers included in the study, an average of over 26L of fluid was injected in each case, compared – as will be discussed in greater detail later in this chapter – with somewhere in the region of 2L to 5L used in repatriation embalming. Finally, as a result of logistical arrangements with the Human Anatomy Centre at the University of Cambridge, the period of time between embalming and sampling was in many cases significantly greater than the timescales encountered in repatriation scenarios; this matter is also discussed in greater detail later in this chapter.

Fortunately, however, both Bristol and Cambridge occasionally undertake 'soft-fix embalming'. For the purposes of, for example, laparoscopic surgical training, for which the body needs to be preserved in a less rigid and more life-like manner than the hard-fix technique commonly used for long-term preservation, a lesser volume of a weaker concentration of formaldehyde can be injected. For the eight soft-fix cases that it was possible to include in the current study, although precise details of neither the volume of fluid injected nor the final formaldehyde concentration were recorded by the technical staff carrying out the embalming, it is understood that in each case the volume injected was approximately 10L of a commercial formalin solution diluted to a final formaldehyde concentration of approximately 2%. Furthermore, the samples in each case were obtained between three and seven days following embalming and were, therefore, more closely aligned to the timescales encountered in repatriation cases.

Thus, samples for the study were obtained from a total of 51 cadavers, 43 of which had been embalmed using the hard-fix method, and eight using soft-fix. Although the majority of samples included in the study were therefore obtained from cadavers that had been embalmed using much greater volumes of significantly more concentrated solutions of formaldehyde than those used in repatriation embalming, this in itself is not necessarily a wholly bad thing. Indeed, from one important perspective, it can of course be argued that these samples have, therefore, been obtained from cadavers that represent the very worst-case scenarios: no repatriated body is likely to have been embalmed using such quantities of fluid containing such concentrations of formaldehyde. With the addition of the small number of soft-fix samples obtained, however, it should be possible to provide nuance to the study by reporting indicative results that might be more closely matched to those likely to be obtained from repatriated cadavers.

4.4 Samples and materials

Samples of VH, SF and CSF were obtained from cadavers donated to the Human Anatomy Centre, Department of Physiology, Development and Neuroscience, at the University of Cambridge. All cadavers included in the study had been properly consented for research purposes in accordance with current Human Tissue Authority practice and the study was

approved by both Cranfield University Research Ethics Committee (CURES/1734/2016) and the NHS Research Ethics Committee (IRAS Project ID 128934; REC Reference 17/NI/0034).

All 'hard-fix' cadavers were embalmed within seven days of death, and within two days of arriving at the facility, using a commercially prepared embalming fluid, purchased from Vickers Laboratories Ltd, containing 4.2% (42,000µg/mL) formaldehyde, 38.0% ethanol, 1.5% methanol and 56.3% water by volume. Embalming was carried out using either the common carotid or femoral artery. Following embalming, cadavers were placed in refrigerated units where they remained for up to two years before being utilised for student or research anatomical dissection. The 43 hard-fix cadavers included in the study had been embalmed over a range of timescales, ranging from 9 days to 22 months prior to sampling. Details of the time between embalming and sampling, as well as volume of embalming fluid injected in each case are given in Table 4-1.

Additionally, eight 'soft-fix' cadavers were included in the study. Samples were obtained from either the Human Anatomy Centre at the University of Cambridge (cadaver numbers 2 and 7) or else from the Centre for Comparative and Clinical Anatomy at the University of Bristol (cadaver numbers 1, 3, 4, 5, 6 and 8) . As discussed above, approximately 10L of a commercial formalin solution diluted to a final formaldehyde concentration of approximately 2% (20,000µg/mL) was injected in each case, using the common carotid or femoral artery. VH, SF and CSF samples were in all cases taken between three and seven days following embalming.

4.4.1 Sample collection methods

Although previously published VH and SF studies have pooled VH from both eyes, as well as pooling SF from both knees, in the current experiment VH was collected separately from both eyes, and SF was collected separately from both knees, for the additional purpose of being able to undertake an assessment of potential intra-donor variability. VH and SF were collected using 22-G sterile needles attached to 5mL sterile Leur-lock syringes. When aspirating VH, as described generally in the literature (Forrest, 1993, Flanagan, Connally and Evans, 2005b, and Dinis-Oliveira et al., 2010), the sclera of each eye was punctured close to the lateral canthus, aiming the needle towards the centre of the globe. At a depth of approximately 2cm, gentle suction (in order to avoid aspirating retinal fragments) would usually yield 2-3mL fluid from

Table 4-1 - VH, SF and CSF ‘hard-fix’ donor conditions

Donor identification number	**Volume of embalming fluid used in fixation (L): Total volume (arterial volume + local volume)**	**Time from embalming to sampling**
1	39 (32+7)	22 months
2	40 (26+14)	21 months
3	35 (30+5)	22 months
4	34.5 (29+5.5)	21 months
5	43.5 (24+19.5)	21 months
6	37 (32+5)	20 months
7	Not known	20 months
8	37.5 (30+7.5)	20 months
9	31.5 (28+3.5)	19 months
10	32.5 (21+14.5)	19 months
11	27 (22+5)	18 months
12	Not known	18 months
13	35 (15+20)	16 months
14	35 (28+7)	16 months
15	29.5 (25+4.5)	16 months
16	36.5 (30+6.5)	15 months
17	33 (28+5)	15 months
18	32 (20+12)	14 months
19	Not known	14 months
20	40 (33+7)	13 months
21	39 (30+9)	14 months
22	32.5 (29+3.5)	12 months
23	34 (27+7)	12 months
24	33 (20+13)	11 months
25	45 (25+20)	11 months
26	33 (28+5)	11 months
27	35.5 (27+8.5)	11 months
28	31 (23+8)	10 months
29	39.5 (27+12.5)	10 months
30	34.5 (27+7.5)	9 months
31	34.5 (29+5.5)	9 months

Donor identification number	Volume of embalming fluid used in fixation (L): Total volume (arterial volume + local volume)	Time from embalming to sampling
32	31.5 (29+2.5)	9 months
33	30.5 (26+4.5)	9 months
34	33 (29+4)	9 months
35	35 (25+10)	8 months
36	Not recorded	5 weeks
37	31 (24+7)	31 days
38	33 (23+10)	10 days
39	38 (26+12)	7 days
40	39 (18+21)	36 days
41	31 (24+7)	28 days
42	Not recorded	17 days
43	35 (28+7)	9 days

Key:
Local donor identification = Cambridge-assigned cadaver identity number
Volume of embalming fluid = the first figure is the total volume used (L); this is then broken down by total arterial volume via initial arterial injection, and subsequent volume (L) given by local hypodermic injection.
Time from embalming to sampling = time elapsed between embalming and storage, and subsequent sampling.

each eye, which would then be transferred to a suitably-labelled sample container and refrigerated. Following the approach described in DeKing, Hargrove and Molina (2014), SF was aspirated from the knee joint, the largest synovial joint in the human body. Directed to pierce the skin at an angle of approximately 60°, the needle would enter the joint from the infero-lateral edge of the patella. Passing through the synovial membrane, at a depth of around 1-2cm, it would then usually be possible to aspirate approximately 1mL of fluid from each joint space. The sample would then be transferred to a suitably-labelled sample container and refrigerated.

CSF was collected according to the method described in Gong, Yu and Yuan (2017), using 13-G sterile needles attached to 10mL sterile Leur-lock syringes. Briefly, the apex of the mastoid

process was palpated, and from here the intended puncture site was located 1cm posterior to and 1cm inferior to that point. The needle was inserted in a perpendicular plane, parallel to the imaginary line joining both external auditory meatuses. At a depth of around 5.5-6.5cm, perforation of the atlanto-occipital posterior membrane, followed by perforation of the dura mater, would be felt, at which point CSF could usually be freely aspirated and would then be transferred to a suitably-labelled sample container and refrigerated.

For some cadavers, technical reasons restricted or precluded the aspiration of some samples. In the case of SF, for example, cases having undergone total knee replacement surgery would be found to have little or no fluid (due to the frequent excision of the synovial membrane during the procedure). In other cases, an insufficient volume of SF was available to meet the sample preparation protocol, which required at least 0.5mL. In the case of VH, like SF, it was not always possible to aspirate the necessary volume of fluid; in one case, for example, corneal harvesting immediately after death precluded the aspiration of fluid from either eye. Finally, some CSF samples had to be discarded when the vertebral artery was inadvertently damaged when inserting the 13-G needle, as a result of which the fluid became contaminated with local blood; this was particularly the case with some of the earlier samples, when the technique of atlanto-occipital puncture was being perfected. All samples were stored at 4°C prior to analysis; preservatives were not used. The total numbers of hard-fix samples taken for the study from the 43 cadavers are given in Table 4-2. Soft-fix samples are discussed later in this chapter, at sections 4.7.2 and 4.8.3.

Table 4-2 - Total numbers of samples obtained

Sample type	Number of samples obtained
Vitreous humour (left eye)	34
Vitreous humour (right eye)	41
Synovial fluid (left knee)	27
Synovial fluid (right knee)	29
Cerebrospinal fluid	33
TOTAL NUMBER OF SAMPLES	**164**

4.4.2 Sample preparation

A very simple sample preparation step was optimised for the analytical method. The sample was vortex mixed, following which 0.5mL was pipetted into a 50mL beaker and diluted with 24.5mL deionised water (DI H_2O). The diluted sample was stirred, following which 1.0mL was pipetted into a 10mL (22x45mm), clear-glass, headspace vial, fitted with a magnetic, screw-thread cap, for immediate analysis. As the sample preparation stage involved adding 0.5mL of each sample to 24.5mL H_2O, the analytical result for each sample would subsequently be multiplied by a factor of 50 to give the final analyte concentration.

4.4.3 Stock and calibration solutions

Analytical-grade formaldehyde in water (37% v/v) was purchased from Sigma Aldrich and DI H_2O was obtained from the Millipore Synergy UV system. All glassware was grade A standard.

1% (10,000µg/mL) formaldehyde stock solution was prepared daily by pipetting 2.7mL formaldehyde standard solution into a 100mL volumetric flask, which was made up to 100mL with DI H_2O.

The stock solution was then diluted with DI H_2O to prepare the formaldehyde calibration standards at 25, 10, 5, 2.5 and 1 µg/mL prior to GC/MS analysis.

4.5 Chromatographic Conditions

A simple, selective and highly-sensitive GC/MS method was developed and validated for the detection of underivatized formaldehyde in samples of VH, SF and CSF obtained from embalmed cadavers.

As formaldehyde is known to be highly reactive with many drug molecules, and in order, therefore, to develop the most sensitive method that was able to detect the lowest concentrations of formaldehyde in later forensic application work, the MS was operated in single-ion monitoring mode (SIM). Formaldehyde has ion abundances of 28, 29 and 30, but as 28 and 29 are both also contained within air, SIM m/z 30 was selected as the qualitative indicator of the molecule. Precise chromatographic conditions are listed below in Table 4-3. Formaldehyde chromatographic and mass spectrometric data are reproduced in Appendix 5.

Table 4-3 – GC/MS conditions

GC/MS system	Agilent Technologies 7890B GC System, equipped with PAL auto-sampler, linked to Agilent Technologies 5977A quadrupole MS
Headspace Oven	95°C/20min
Oven Agitator	10sec on/2sec off at 500rpm
Syringe Temp	100°C
Injection split	15:1
Injection volume	100µL at 500µL/sec
Injection temperature	140°C
Carrier Gas	Helium, 1.2mL/min
Column	J&W Scientific DB-Wax-52CB 60mx0.32mmx2µm
Gradient	Starting at 40°C and hold for 2min, then ramp to 180°C at 60°C per min and hold for 2min. Total run time 7min.
Transfer Line	150°C
Detection	MS
Detector Temperature	Source 220°C Quad 150°C
PC Software	Agilent Mass Hunter data analysis software with National Institute of Science and Technology (NIST) spectral library

The method was validated for selectivity and specificity, linearity, lower limits of quantification and detection, and method precision, details of which follow below.

4.6 Method Validation

Chapter 1 of this thesis spoke of the necessity for analytical accuracy in forensic work. The interpretation of the analytical results of samples containing drugs with narrow therapeutic windows is especially dependent on the use of a method that is not only optimised for the target analytes, but is also validated for parameters including selectivity, specificity, linearity, lower limits of detection and quantitation, accuracy and precision. Although the requirements of the current experiment do not demand such a high standard of validation, for the purposes of presenting results that are as meaningfully accurate as possible all normal validation parameters have been assessed and are described below.

4.6.1 Selectivity, specificity, recovery and stability

Selectivity is generally defined as the ability of the method to recognise the analyte of interest, free of interference, and can be determined by injecting blank samples to check that no peaks can be observed at the same retention time as the target analyte. Specificity, or recovery, may be defined as the ability of the method to differentiate the analyte unequivocally from any other matrix component, and can be determined by injecting samples of the sample matrix that are spiked with the analyte of interest.

Ten to twenty blanks are recommended for the assessment of selectivity, and the number of spiked samples required for the assessment of specificity can be tailored to the needs of the method. A lack of response within the range of the calibration solutions confirms the selectivity and specificity of the method, although at the LOQ a degree of interference is permissible within an acceptability criterion of ±20% (Wenclawiak and Hadjicostas, 2010).

Recovery may be defined as the extent (expressed as %) to which the method is able to extract and detect the analyte of interest. It is commonly assessed at low, medium and high concentrations by comparing the response of known reference values with the response of extracted blank matrices to which the analyte is added at the same nominal concentration, and is calculated as follows:

Recovery (%) = spiked blank matrix response/known reference value response*100

For selectivity confirmation for the current experiment, 10 VH blanks (from five cadavers), 10 SF blanks (from five cadavers) and 10 CSF blanks (from 10 cadavers), all obtained from cadavers prior to embalming, were analysed.

No interference was detected in any sample at or around t_R 0.86 (the t_R of formaldehyde using the optimised method), and no corresponding analyte ions were detected by MS (run in full mass-scanning mode), confirming that the method is appropriately selective.

For confirmation of method specificity, three VH blanks were spiked with formaldehyde in order to obtain final concentrations of 5, 10 and 25µg/mL; three SF blanks and three CSF blanks were spiked according to the same protocol. Samples were then prepared for analysis using the optimised method.

The VH, SF and CSF spiked sample containers were then closed with screw caps, wrapped with parafilm, and refrigerated for six months at 4ºC in order to test long-term stability. After six months the samples were prepared using the method described in Section 4.4.2 above. 6-month stability was calculated as follows:

Stability (%) = 6-month recovery/initial recovery*100

Recoveries of the target analyte in all three matrices are given in Table 4-4. At 25µg/mL recovery from all three matrices was within 5% of the nominal concentration, at 10µg/mL was within 3%, and at 5µg/mL was within 1% in SF and CSF and within 9% in VH. At six months, recovery in all matrices was less than the initial recovery: with the exception of CSF 25µg/mL, where a loss of 7.27% was found after six months, the six-month recovery of all other concentrations and analytes was within 4.4% of the initial recovery.

Table 4-4 – Recovery of formaldehyde from spiked VH, CSF and SF

Matrix and formaldehyde concentration (µg/mL)	Initial recovery (%)	Recovery after 6 months (%)	6-month stability (%)
VH: 5	108.27	103.49	95.59
SF: 5	99.04	97.23	98.17
CSF: 5	99.92	99.28	99.36
VH: 10	102.46	99.67	97.28
SF: 10	96.27	92.62	96.20
CSF: 10	98.69	98.91	100.22
VH: 25	100.82	100.54	99.72
SF: 25	97.69	93.72	95.94
CSF: 25	104.71	97.10	92.73

4.6.2 Linearity

Linearity may be defined as the ability of the method to obtain a corresponding response that is proportional to the concentration of the analyte. For this experiment, a five-point calibration curve was used, giving a linearity range of 1-25µg/mL. Although matrix-matched calibration solutions have been recommended when analysing biological samples (Cooper

2010), a number of published methods have used water-based solutions for VH, SF and CSF, on the basis that the three fluids are comprised largely of water; see, for example, Wyman and Bultman (2004), which uses water-based calibration solutions for VH and CSF, and DeKing, Hargrove and Molina (2014), which uses acetonitrile-based calibration solutions for SF (personal conversation with lead author). For the current experiment, calibration standards were prepared in DI H_2O. A five-point calibration curve was prepared in triplicate on each of the analysis days.

The regression equation was formulated in the usual way (Nahler, 2009 and Catalano, 2013) using Microsoft Excel (2016, v.16.0), and the correlation coefficients, R and R^2, were calculated as 0.99 demonstrating a good linear relationship between the formaldehyde concentration and the GC/MS response. Analytical results were subsequently processed within Microsoft Excel (2016, v.16.0) using the usual method of linear regression.

4.6.3 Instrument accuracy and precision

Instrument performance may be measured by undertaking 'trueness' tests for systematic error, and 'precision' tests for random error.

Trueness, or accuracy, describes the closeness of agreement between an analytical result and a known reference value, and may be expressed as the percentage deviation of the analytical test result from that of the accepted reference value, with an acceptable range of ±20% (Cooper, Paterson and Osselton, 2010).

To test accuracy, samples, *n*=3 each, of 5, 10 and 25µg/mL formaldehyde in DI H_2O were analysed and the response was measured against the daily calibration curve. Accuracy was then calculated as the percentage deviation of the mean result from that of the nominal concentration: (nominal concentration – mean measure concentration)/nominal concentration*100. The results, all of which are within acceptable limits, are given in Table 4-5.

As described in Danzer (2007), precision can be assessed, firstly, by undertaking tests of intra-assay precision, or 'repeatability', and defined as the ability of the method (and hardware) to reproduce the same result from multiple and successive replicates of the same sample, and,

secondly, by testing inter-day precision, or 'reproducibility', defined as the precision of the method (and hardware) to reproduce the same result from multiple replicates of the same sample over an extended period of time.

Repeatability and reproducibility tests are commonly undertaken using samples of low, medium and high concentrations derived from the method working range, and assessment may be expressed as the percentage relative standard deviation (%RSD), with an acceptable range of ±20% at low concentrations and ±15% at high concentrations (Flanagan et al., 2007): (standard deviation of measured mean/measure mean) *100.

For repeatability assessment, samples, *n*=5, of 5, 10 and 25µg/mL formaldehyde in DI H_2O were analysed in immediate succession and were measured against the daily calibration curve. For reproducibility assessment, samples, *n*=5, of 5, 10 and 25µg/mL formaldehyde in DI H_2O were analysed on three different days during the experiment and were measured against the daily calibration curve in each case. The results, all of which are within acceptable limits, are given in Table 4-5.

Table 4-5 – Instrument accuracy and precision

Reference concentration (µg/mL	Accuracy (% deviation)	Repeatability (%RSD)	Reproducibility (%RSD)
5	4.95	3.19	4.90
10	5.23	3.86	5.06
25	5.76	5.43	5.26

4.6.4 Lower limit of quantitation and lower limit of detection

The limit of quantitation of the instrument is generally defined as the lowest concentration of a target molecule that can be quantified within the acceptable range of precision and accuracy. The limit of detection may be defined as the lowest concentration at which a molecule can be reliably detected, though not quantified, and thus is usually somewhat lower than the limit of quantitation. Although both limits can be determined according to the signal-to-noise ratio, an alternative method is to derive both values from the calibration curve data (Reichenbächer and Einax, 2011). Using the calibration curve data, the root mean square error (RMSE) method, described in Corley, 2003, and applicable when a linear relationship exists

between analyte concentration and detector response, can be employed. Applying the RMSE method, the Instrument Quantification Limit (referred to hereafter as the LOQ) and the Instrument Detection Limit (referred to hereafter as the LOD) were calculated as follows:

LOQ = 10*RMSE/Slope

LOD = 3.3*RMSE/Slope

where RMSE is defined as SQRT((SUMSQresiduals)/(no. of calibration points -2)).

Using these calculations, the method was shown to have an LOQ of 0.40µg/mL and an LOD of 0.13µg/mL, as shown in Table 4-6, below. These values represent a very significantly increased degree of sensitivity to the Tekmar-Dohrmann Application Note, from which the current method was developed, and where, although precise LOQ and LOD values are not stated, the method is said to be optimised only to a minimum of 200ppm (200µg/mL).

In order to provide maximum analytical flexibility for the potential subsequent forensic application of the method, two additional calibration curves, along with LOQ and LOD data, were also plotted and calculated with maximum calibration points of 50µg/mL and 500µg/mL. The 50µg/mL calibration curve (R=0.99) provided an LOQ of 0.36µg/mL and an LOD of 0.12µg/mL. The 500µg/mL calibration curve (R=0.99) provided an LOQ of 6.38µg/mL and an LOD of 2.11µg/mL.

Table 4-6 – LOQ/LOD data for method ranging from 1-25µg/mL

Formaldehyde concentration (µg/mL)	Area measured	Area calculated	Residual
25	39256.20	39275.74	-19.54
10	15757.91	15737.21	20.70
5	7955.45	7891.04	64.41
2.5	3984.56	3967.95	16.61
1	1531.91	1614.10	-82.19
SUMSQres			11990.04

RMSE	63.22	***n=***	5
Intercept	44.86	**Slope**	1569.24
R	0.99	**R^2**	0.99
LOQ	0.40µg/mL	**LOD**	0.13µg/mL

4.6.5 Carryover

Carryover may be defined as the appearance in the chromatogram of an analyte contained within a previous injection matrix. The phenomenon can occur, particularly, when especially large concentrations of target analyte are injected.

To test the method for carryover interference, the same nine samples that were prepared for the specificity tests were re-injected, with a blank (DI H_2O) added into the sequence after each spiked injection (additionally, and for the purposes of the extended-range methods described earlier, additional samples of VH, SF and CSF were spiked with formaldehyde in order to obtain final concentrations of 50 and 500µg/mL and were injected with corresponding blanks after each sample). The chromatogram of each blank was then examined around the pre-determined retention time of formaldehyde, and the MS data were checked in SIM *m/z* 30.

No carryover was observed in any blank sample.

4.7 Results

The purpose of the experiment reported in this chapter was, very simply and solely, to analyse samples of VH, SF and CSF for the presence of formaldehyde. In consequence of this, the results section is both brief and straightforward. In the case of each fluid, the number of samples analysed is reported, along with mean, minimum and maximum formaldehyde concentrations detected, a summary of which is presented in Table 4-7. In Table 4-8, additionally, and in order to give comparative context to the results, the standard deviation, Percentile 25, Percentile 75 and the median values from each sample set are reported. Results from the hard-fix cadavers are first reported, followed by statistical commentary, after which results of the soft-fix samples are presented.

Table 4-7 - Formaldehyde concentrations detected in VH, SF and CSF 'hard-fix' samples

Matrix / (*n*)	Mean formaldehyde concentration (µg/mL)	Maximum formaldehyde concentration (µg/mL)	Minimum formaldehyde concentration (µg/mL)
VH left and right (75)	589	1403	170
VH left (34)	595	1369	256
VH right (41)	584	1403	170
SF left and right (56)	590	1454	165
SF left (27)	608	1290	220
SF right (29)	573	1454	165
CSF (33)	420	863	101

Table 4-8 – Statistical descriptors of formaldehyde concentrations detected in VH, SF and CSF 'hard-fix' samples

Matrix	Mean conc. (µg/mL)	St. Dev. (µg/mL)	Percentile 25 (µg/mL)	Median conc. (µg/mL)	Percentile 75 (µg/mL)	Min. conc. (µg/mL)	Max. conc. (µg/mL)	*N*=
VH (L)	595.77	280.23	359.79	518.98	700.45	256.28	1368.91	34
VH (R)	583.59	289.44	392.79	499.76	733.55	170.06	1402.69	41
SF (L)	608.22	302.68	403.64	547.29	702.43	220.45	1290.18	27
SF (R)	573.12	301.08	364.50	500.47	664.57	165.18	1453.83	29
CSF	420.06	211.44	246.05	360.78	599.95	101.44	863.24	33

Key:
Mean conc. = mean concentration of all samples
St. Dev. = Standard deviation of all samples
Percentile 25 = concentration below which 25% of the analysed results were detected
Median conc. = median concentration of all samples
Percentile 75 = concentration below which 75% of the analysed results were detected
Min. conc. = minimum concentration detected in all samples
Max. conc. = maximum concentration detected in all samples

4.7.1 'Hard-fix' cadavers

4.7.1.1 Vitreous humour

75 samples of VH were analysed, and all contained formaldehyde. Rounding to the nearest whole number, the mean formaldehyde concentration from all VH samples was 589µg/mL, with a maximum concentration in any one sample of 1403µg/mL and a minimum of 170µg/mL.

Of left eye only samples, of the total analysed (*n*=34), the mean formaldehyde concentration was 595µg/mL, with a maximum of 1369µg/mL and a minimum of 256µg/mL.

Of the right eye only samples, of the total analysed (*n*=41) the mean formaldehyde concentration was 584µg/mL, with a maximum of 1403µg/mL and a minimum of 170µg/mL.

The VH results are summarised in Table 4-7.

Statistical data for VH results are presented in Table 4-8.

4.7.1.2 Synovial fluid

56 samples of SF were analysed, and all contained formaldehyde. Rounding to the nearest whole number, the mean formaldehyde concentration from all SF samples was 590µg/mL, with a maximum concentration in any one sample of 1454µg/mL and a minimum of 165µg/mL.

From analysis of samples taken from the left knee, of the total analysed (*n*=27), the mean formaldehyde concentration was 608µg/mL, with a maximum of 1290µg/mL and a minimum of 220µg/mL.

From the right knee, of the total analysed (*n*=29) the mean formaldehyde concentration was 573µg/mL, with a maximum of 1454µg/mL and a minimum of 165µg/mL.

The SF results are summarised in Table 4-7.

Statistical data for SF results are presented in Table 4-8.

4.7.1.3 Cerebrospinal fluid

33 samples of CSF were analysed, and all contained formaldehyde. Rounding to the nearest whole number, the mean formaldehyde concentration from all CSF samples was 420µg/mL, with a maximum concentration in any one sample of 863µg/mL and a minimum of 101µg/mL.

The CSF results are summarised in Table 4-7.

Statistical data for CSF results are presented in Table 4-8.

4.7.1.4 Statistical analysis

In order to test both the reliability and the strength of the results presented, statistical analysis was undertaken.

Spearman's Rho tests (using the IBM SPSS Statistics (v.25) package) were performed on a number of variables in order to determine whether relationships exist between the data sets produced by the study. The Spearman's Rho test measures the strength as well as, importantly, the direction of association between two variables, where a correlation coefficient $r = 1$ indicates a perfect positive correlation, $r = 0$ indicates no association, and where $r = -1$ indicates a perfect negative correlation. When interpreting the degree of relationship, results of ± 0.50-1 may be assumed to indicate a high degree of correlation, ± 0.30-0.49, a moderate degree of correlation, and a result below ± 0.29, a low degree of correlation; thus, the closer that *r* is to zero, so the weaker is the relationship between the two variables (Field, 2018). The strength of correlation, however, must always be considered alongside the probability (*p*) of the correlation being statistically significant. Statisticians generally consider a correlation to have meaningful weight if the *p* value is 0.05 or below, where 0.05 indicates that there is only a 5%, or one in 20, chance of the correlation being the result of random association. It follows that the lower the calculated *p* value, so the stronger is the evidence of a non-random association; thus, a *p* value of 0.01, indicates a one in a hundred chance of the correlation being random, and a *p* value of 0.001, a one in a thousand chance (Field, 2018).

The Spearman's Rho test was used to determine, firstly, whether there are any comparative relationships between the concentrations of formaldehyde detected in each of the fluids within each individual cadaver. In this test, therefore, left and right VH were compared with each other, left and right SF were compared with each other, and all four fluids were then compared with CSF. Secondly, the mean of the left and right VH formaldehyde concentrations was compared with the mean of the left and right SF concentrations, and both means were also compared with CSF concentrations. The results for the individual fluids are reported in Table 4-9 and for the mean fluid concentrations in Table 4-10; comparative scatterplots are reproduced in Figure 4-1 and discussion of the statistical results follows at Section 4.8.1.

Table 4-9 –Spearman's Rho test results showing correlations between formaldehyde concentrations detected in VH, SF and CSF 'hard-fix' samples

		VH (L)	VH (R)	SF (L)	SF (R)	CSF
VH (L)	Correlation coefficient	1.000	0.667**	0.504*	0.295	0.430*
	p-value (2-tailed)	N/A	<0.001	0.014	0.162	0.025
	N	34	34	23	24	27
VH (R)	Correlation coefficient	0.667**	1.000	0.613**	0.356	0.230
	p-value (2-tailed)	<0.001	N/A	0.001	0.058	0.205
	N	34	41	27	29	32
SF (L)	Correlation coefficient	0.504*	0.613**	1.000	0.369	0.309
	p-value (2-tailed)	0.014	0.001	N/A	0.076	0.142
	N	23	27	27	24	24
SF (R)	Correlation coefficient	0.295	0.356	0.369	1.000	0.436*
	p-value (2-tailed)	0.162	0.058	0.076	N/A	0.026
	N	24	29	24	29	26
CSF	Correlation coefficient	0.430*	0.230	0.309	0.436*	1.000
	p-value (2-tailed)	0.025	0.205	0.142	0.026	N/A
	N	27	32	24	26	33

Key:
Correlation coefficient: Spearman's Rho test result (range -1 to +1)
p-value (2-tailed): two-tailed probability value
N: number of samples compared
***:** correlation is significant at the 0.05 level (2-tailed)
****:** correlation is significant at the 0.01 level (2-tailed)

Tests were also performed in order to establish whether any relationship exists between the volume of arterial formaldehyde injected into each cadaver (as detailed in Table 4-1) and the concentration of formaldehyde detected in each of the fluids, both individual left and right VH and SF values, as well as means. The results of these tests are presented in Table 4-11, scatterplots are shown in Figure 4-2 and discussion follows at 4.8.1.

Finally, tests were performed in order to establish whether any relationship exists between the time since embalming (as detailed in Table 4-1) and the concentration of formaldehyde detected in each of the fluids, again, both individual and mean. The results of these tests are presented in Table 4-12, scatterplots are shown in Figure 4-3 and discussion follows at 4.8.1.

Table 4-10 – Spearman's Rho test results showing correlations between formaldehyde concentrations detected in mean VH, mean SF and CSF 'hard-fix' samples

		VH (L+R)	SF (L+R)	CSF
VH (L+R)	Correlation coefficient	1.000	0.353	0.295
	p-value (2-tailed)	N/A	0.126	0.135
	N	34	20	27
SF (L+R)	Correlation coefficient	0.353	1.000	0.302
	p-value (2-tailed)	0.126	N/A	0.172
	N	20	24	22
CSF	Correlation coefficient	0.295	0.302	1.000
	p-value (2-tailed)	0.135	0.172	N/A
	N	27	22	33

Key:
Correlation coefficient: Spearman's Rho test result (range -1 to +1)
p-value (2-tailed): two-tailed probability value
N: number of samples compared

Figure 4-1 – Scatterplots of Spearman's Rho test results showing correlations between formaldehyde concentrations detected in VH, SF and CSF 'hard-fix' samples

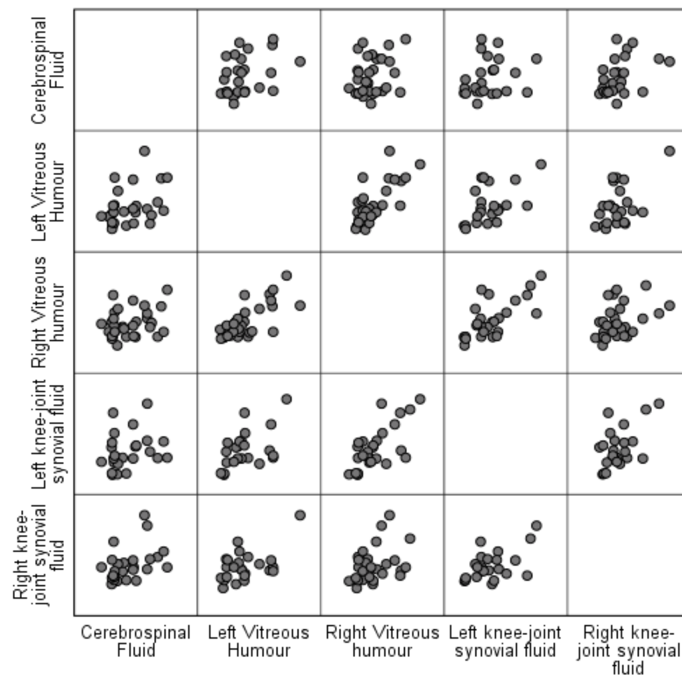


Table 4-11 - Spearman's Rho test results showing correlations between volume of arterial embalming fluid injected and concentration of formaldehyde detected in each fluid

	Correlation coefficient	p-value (2-tailed)	*N*
VH (L)	-0.035	0.857	29
VH (R)	-0.061	0.729	35
VH (L and R)	-0.107	0.579	29
SF (L)	-0.187	0.404	22
SF (R)	-0.007	0.972	25
SF (L and R)	-0.018	0.937	21
CSF	0.272	0.153	29

Key:
Correlation coefficient: Spearman's Rho test result (range -1 to +1)
p-value (2-tailed): two-tailed probability value
N: number of samples compared

Figure 4-2 – Scatterplots of Spearman's Rho test results showing correlations between volume of arterial embalming fluid injected and concentration of formaldehyde detected in each fluid

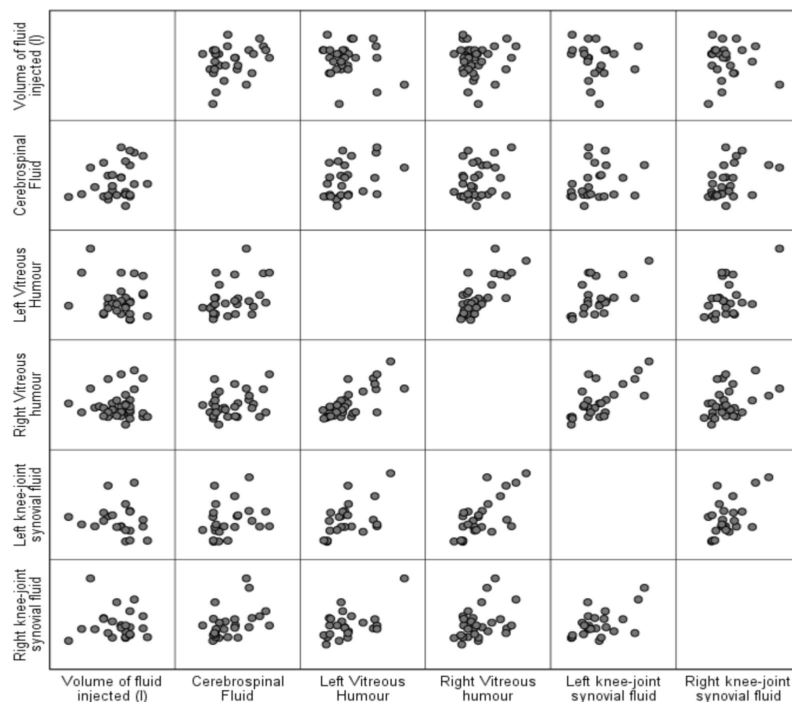
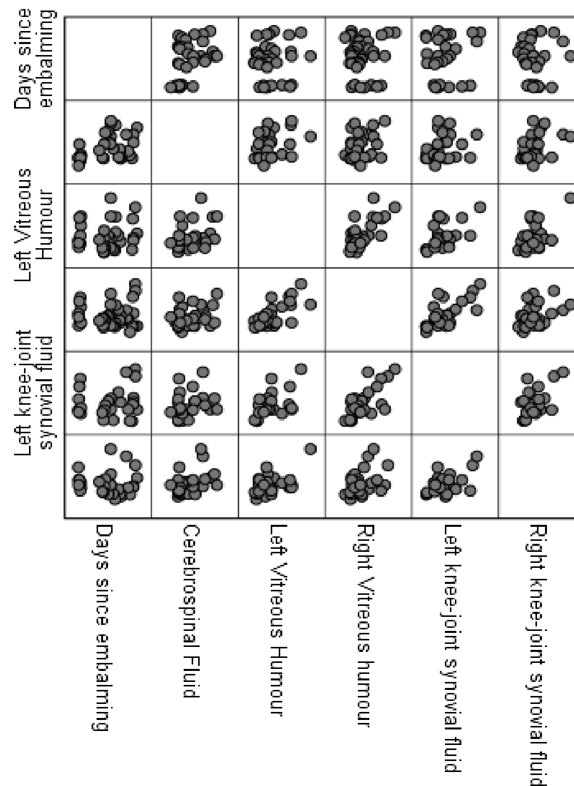


Table 4-12 – Spearman's Rho test results showing correlations between time since embalming and concentration of formaldehyde detected in each fluid

	Correlation coefficient	**p-value (2-tailed)**	***N***
VH (L)	0.160	0.366	34
VH (R)	0.004	0.979	41
VH (L and R)	0.065	0.716	34
SF (L)	0.197	0.324	27
SF (R)	-0.138	0.476	29
SF (L and R)	-0.053	0.804	24
CSF	0.369	0.035*	33

Key:
Correlation coefficient: Spearman's Rho test result (range -1 to +1)
p-value (2-tailed): two-tailed probability value
N: number of samples compared
*****: Correlation is significant at the 0.05 level (2-tailed)

Figure 4-3 – Scatterplots of Spearman's Rho test results showing correlations between time since embalming and concentration of formaldehyde detected in each fluid



4.7.2 'Soft-fix' cadavers

Although it was not possible to obtain samples of all fluids from all eight soft-fix cadavers, results of the formaldehyde analysis undertaken on the samples included in the study are presented in Table 4-13.

Of the seven cadavers from which VH was analysed (total number of samples = 13), no formaldehyde was detected in four. Of the three cadavers in which VH formaldehyde was detected, the maximum concentration in any sample was 14.70µg/mL, and the minimum was 0.48µg/mL.

Of the five cadavers from which SF was analysed (total number of samples = 8), no formaldehyde was detected in two. Of the three cadavers in which SF formaldehyde was detected, the maximum concentration in any sample was 6.60µg/mL, and the minimum was 1.52µg/mL.

Of the three cadavers from which CSF was analysed, no formaldehyde was detected in one. Of the remaining two cadavers, one had a CSF formaldehyde concentration of the 4.90µg/mL, and the other, 1.22µg/mL.

Table 4-13 Formaldehyde concentrations detected in VH, SF, CSF 'soft-fix' samples

Cadaver No.	Time from embalming to sampling	VH (L) formaldehyde concentration (µg/mL)	VH (R) formaldehyde concentration (µg/mL)	SF (L) formaldehyde concentration (µg/mL)	SF (R) formaldehyde concentration (µg/mL)	CSF formaldehyde concentration (µg/mL)
1	7 days	1.59	N/A	N/A	N/A	4.90
2	7 days	Not detectable	Not detectable	N/A	Not detectable	N/A
3	7 days	Not detectable	Not detectable	1.52	Not detectable	Not detectable
4	6 days	14.70	10.14	N/A	6.60	N/A
5	3 days	N/A	N/A	N/A	N/A	1.22
6	5 days	Not detectable	Not detectable	1.66	Not detectable	N/A
7	4 days	Not detectable	Not detectable	Not detectable	Not detectable	N/A
8	5 days	Not detectable	0.48	N/A	N/A	N/A

Key:
N/A: sample not available for analysis
Not detectable: result below optimised LOD

4.8 Discussion

This is the first reported study that has examined, explicitly, the detection of formaldehyde in the tissues of embalmed human cadavers. Although previous experimental studies monitoring the stability of drug molecules have included the use of widely-varying formalin conditions, they have always been based on assumed concentrations of formaldehyde in biological samples. In this study, however, for the first time, it has been possible to analyse the concentrations of formaldehyde detected in three different fluids in authentic embalmed bodies. Within the context of the existing literature, as reviewed in Chapter 2, the results of the current study are revealing and important.

Before discussing the results in detail, however, it is necessary to consider one particularly important factor that may affect their reliability. Table 4-1 provides details of the 43 cadavers that formed the basis of the study. From the table, it will be seen that the periods of time between embalming are sampling are wide-ranging: from 9 days to 22 months. There are no published studies on the stability of formaldehyde in cadavers, let alone in the biological fluids included in the current study. In order to examine the kinetics of formaldehyde over time in the cadavers studied, however, it is possible to construct simple graphs, plotting formaldehyde concentration against time since embalming. Figure 4-3 is a summary scatterplot showing formaldehyde concentration detected against time since embalming for all fluids. In closer detail, Figures 4-4 to 4-8 are the individual scatterplots for, respectively, left VH, right VH, left SF, right SF and CSF. What each of these scatterplots demonstrates is that there appears to be no relationship between the length of time since embalming and the concentration of formaldehyde detected. Thus, formaldehyde would appear to neither dissipate nor accumulate over time in the fluids examined.

Although the starting concentration of formaldehyde injected into each cadaver was uniform (4.2%, or 42,000µg/mL) these scatterplots may, however, represent a somewhat crude assessment of stability, since, as Table 4-1 also shows, the volume of (arterial) embalming fluid injected into each cadaver differed, ranging from 15L to 33L. It is not inconceivable that larger volumes of injected embalming fluid may subsequently result in greater concentrations

Figure 4-4

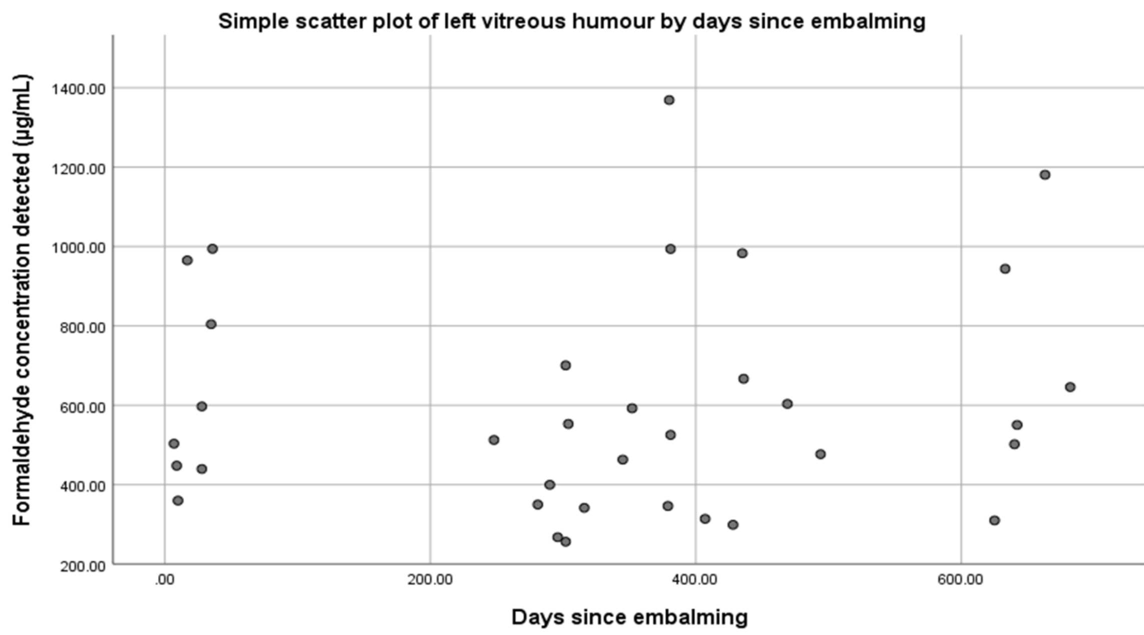


Figure 4-5

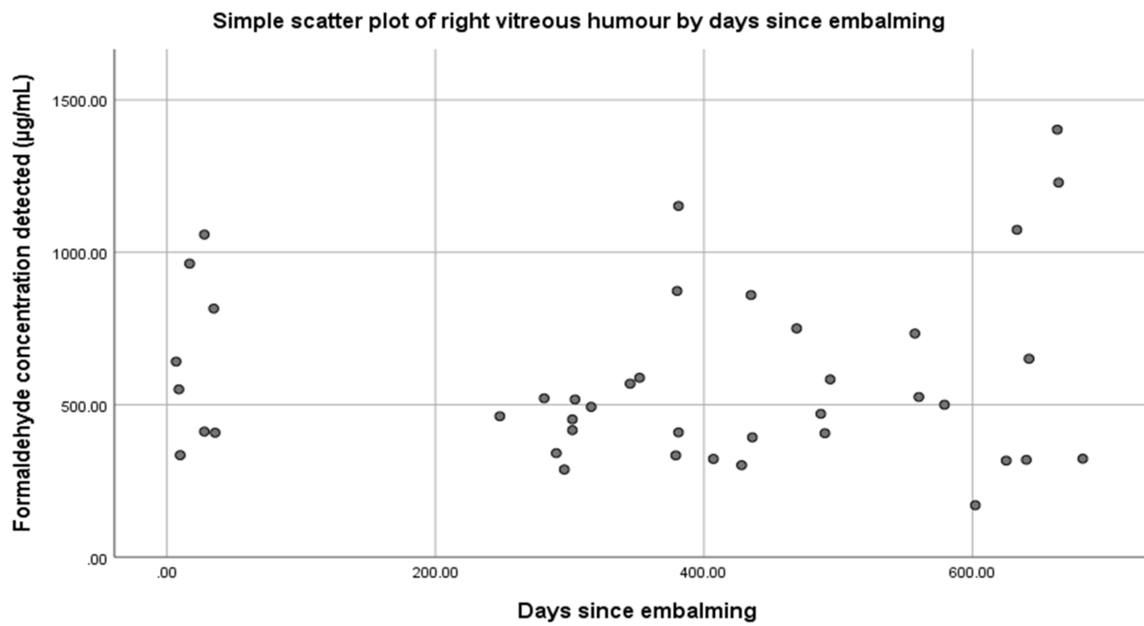


Figure 4-6

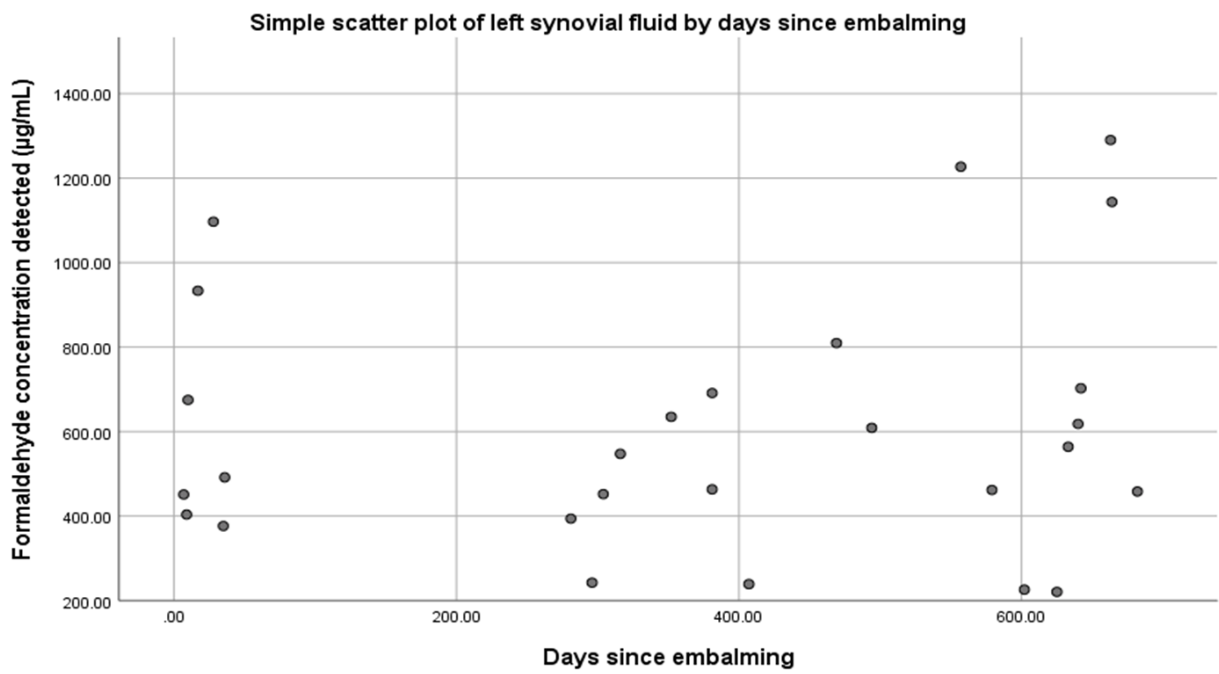


Figure 4-7

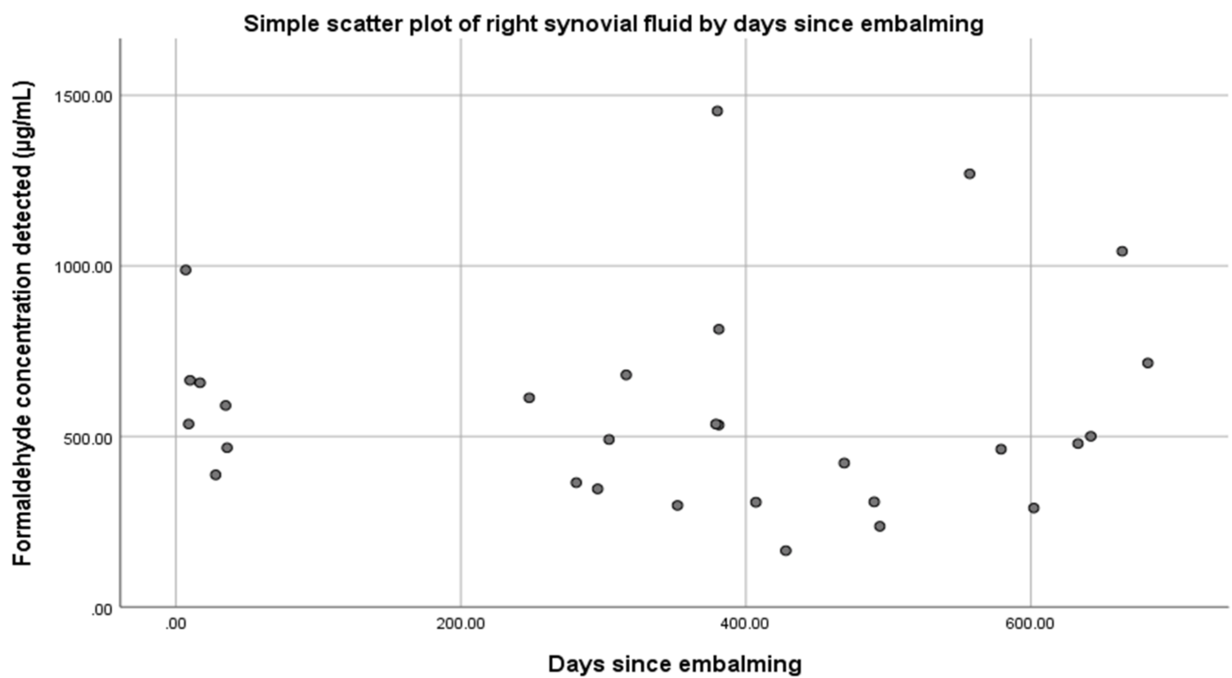


Figure 4-8

of formaldehyde being detected in the biological fluids; this will be discussed in more detail presently. In the meantime, however, it is possible to examine further the kinetics of formaldehyde concentration detected against time since embalming by undertaking a statistical examination of the results. Using Spearman's Rho tests, it was possible to compare individual formaldehyde concentrations in all fluids against each other, over time. Thus, it is possible to determine whether or not there are any particular trends in the concentration of formaldehyde detected over the whole range of sampling times included in the study. The results of these tests, presented in Table 4-12, are revealing, as they demonstrate that with the exception of CSF there appear to be no correlations. Thus, as far as general trends are concerned, and as intimated by the scatterplots, formaldehyde neither accumulates nor dissipates over time. In the case of CSF, a correlation of 0.369 (p = 0.035) shows a moderate degree of positive association, significant at the 5% level. Thus, in the case of this fluid, it would seem that there may be a degree of accumulation of formaldehyde within CSF as time increases; this result will be discussed in greater detail later in the chapter. For all VH and all SF results, as well as, with a degree of caution, all CSF results, what the scatterplots and the Spearman's Rho tests demonstrate is that, despite the samples coming from cadavers that

had been embalmed over wide periods of time, the results are essentially comparable and can thus be treated equally.

In terms of the analytical results themselves, of principal note are the relatively low concentrations of formaldehyde detected in all fluids sampled from the 43 hard-fix cadavers. Although detectable in every sample, the concentration in all 164 samples analysed never exceeded 1,454µg/mL, and was as low as 101µg/mL. These concentrations of formaldehyde are highly significant with respect to the embalming-toxicology literature. In Chapter 2, it was shown that much of the literature has based experimental formaldehyde concentrations on an apparent misunderstanding, first noted in a 2001 paper, that has been repeated ever since. This error has led to the execution of experiments using formalin solutions typically containing final formaldehyde concentrations of between 5% and 20% (50,000 – 200,000µg/mL) which, while perhaps apposite in the context of histological sample fixation, are clearly not appropriate within the context of embalmed and repatriated cadavers, where the final formaldehyde concentration of the 'neat' arterial fluid that is injected is usually somewhere between 1% and 2% (10,000 – 20,000µg/mL). But even concentrations of 1% to 2% assume direct contact between the 'neat' embalming fluid and the tissue containing the drug molecule of interest.

What more than anything the results from the hard-fix cadavers provide is a significant corroboration of the hypothesis posited in Chapter 3: namely, that by virtue of the fact that VH, SF and CSF are contained within essentially avascular compartments, they appear to be provided with a significant degree of protection from the effects of the embalming process. The embalming fluid used in the Cambridge facility contains a final formaldehyde concentration of 4.2% (42,000µg/mL). In the case of the analysis results presented in this experiment, however, the mean VH, SF and CSF formaldehyde concentrations were, respectively, only 589µg/mL, 590µg/mL and 420µg/mL, equating to 0.0589%. 0.059% and 0.042%. Of the total 164 samples analysed, the highest detected formaldehyde concentration, in a sample of VH, 1,454µg/mL, equates to 0.1454%, and the lowest, in a sample of CSF, of 101µg/mL equates to only 0.0101%. It would appear, therefore, that only a very small fraction of the formaldehyde that is injected into the circulatory system of the Cambridge hard-fix cadavers passes across either the blood-vitreous or blood-retinal barriers,

in the case of VH, the synovium, in the case of SF, or the blood-brain or blood-cerebrospinal fluid barriers in the case of CSF. Indeed, the mean formaldehyde concentrations of around 590µg/mL detected in the VH and SF of the 43 cadavers amounts to only 1.4% of the 42,000µg/mL injected, and the mean concentration of 420µg/mL detected in the CSF of these cadavers amounts to only 1.0% of the concentration injected. These results, therefore, very much accord with the theories concerning the biochemical permeability of these barriers, as discussed in Chapter 3 and, particularly, with the theory that the ability for molecules to cross each of these barriers may be reduced following death.

4.8.1 Discussion of statistical results

Data sets were analysed in order to define a number of possible relationships between the concentrations of formaldehyde detected in each of the three fluids, VH, SF and CSF, both in terms of their relationship with each other, as well as any relationship with the volume of embalming fluid injected into each cadaver, and the amount of time elapsed since embalming.

When comparing formaldehyde concentrations between individual fluids, several statistically significant associations were identified. With reference to Table 4-9, in the case of VH, a correlation of 0.667 (p = <0.001) between the concentrations detected in both eyes of the 34 cadavers from which VH was available in both eyes can be regarded as strong relationship, particularly in light of an extremely good probability. A strong association was also identified between left VH and left SF (n=23), where a correlation of 0.504 (p=0.014), was found, and the correlation of 0.613 (p=0.001) between right VH and left SF (n=27) was even better. Finally, moderately strong correlations of 0.430 (p=0.025) were identified between left VH and CSF (n=27), and of 0.436 (p=0.026) between right SF and CSF (n=26). All other correlations were generally poor: thus, no statistically significant correlation was found between formaldehyde concentrations in both knees, and associations with CSF were not especially strong.

Before discussing the potential significance of these correlations, a note of caution should first be sounded. In the case of all the associations examined, the number of samples within each set, with only a small number of exceptions, did not exceed 30. Thirty, as statisticians

commonly caution, however, is the lowest sample set size from which it might reasonably and safely be expected to infer trends. In terms of the relatively small sample set sizes analysed in the current experiment, therefore, the prediction let alone the establishment of trends from the data reported in this chapter might be regarded as being, at best, provisional or indicative.

While holding this caution in mind, it is, however, possible to draw on the literature in order to provide some possible context to these associations, although previous studies in this respect are limited, since there are no published studies comparing formaldehyde concentrations in different biological fluids. As discussed in Chapter 3, however, while a small number of papers have reported comparative drug concentrations between left and right VH, and even fewer comparing left and right SF, very little in the literature reports directly on the passage of drug molecules, let alone any other type of molecule, either into or out of the biological fluids in the post-mortem period. A small number of studies, however, provide some indication of why differences may be observed. In the case of VH, three particular factors may contribute. First, a well-known warning, published in technical guides to obtaining post-mortem samples, is that great care must be observed when aspirating VH, since not only is the retina a delicate structure, but it also receives a blood supply; the inclusion in the sample of retinal fragments, therefore, could lead to an artificially high analytical result (Parsons, Start and Forrest, 2003). Secondly, and more recently, in a study of post-mortem vitreous biochemistry, it has been observed that potassium can leak from the retina into the VH (Belsey and Flanagan, 2016). The process by which potassium leakage takes place does not appear to be fully understood but, as far as this study is concerned, if potassium can leak into the VH, then what might be inferred is the possibility of drug molecules also leaking into the VH, leading to analytical discrepancies. Finally, Blana et al. (2011) notes that VH can evaporate through the cornea of the eye, leading to artificially elevated concentrations of analytes within the fluid.

In the current study, the sampling of VH was conducted carefully, and the accidental aspiration of retinal fragments, therefore, seems an unlikely explanation of analytical discrepancy, although it cannot be discounted entirely, as samples were occasionally obtained by different technical staff whose individual methods may have differed slightly.

However, as was detailed in Table 4-1, the Cambridge hard-fix embalming method involves the injection of particularly large volumes of fluid into each cadaver. Although there is nothing in the literature to support this supposition, it may well be the case that replacing what is in the average living person 5 litres of blood (Martin, 2010) with, on average, over 26 litres of embalming fluid, may result in the forcing of fluid into the VH through either the blood-vitreous barrier or the blood-retinal barrier. Additionally, as the eyelids of the Cambridge cadavers are not glued closed or held shut in the way that those of a funeral home embalming subject would ordinarily be, so the evaporation of VH through the cornea, as described by Blana, and leading to altered formaldehyde concentrations, cannot be discounted.

In order to examine further the possibility of raised analyte concentrations being caused by the forcing of embalming fluid through the membranes of the eye, statistical tests were undertaken comparing the volume of embalming fluid injected and the concentration of formaldehyde detected in the biological fluids of each cadaver. As the statistical analysis presented in Table 4-11 and the scatterplot shown in Figure 4-2 both illustrate, tests revealed very poor correlations in not just VH samples, but also in SF and CSF samples. These results would appear to indicate, notwithstanding the statistically small sample set sizes, that there is no direct relationship between the volume of fluid introduced into the cadaver during the embalming process and the concentration of formaldehyde subsequently detectable in any fluid.

In Chapter 3, some consideration was given to the biochemical and physiological processes that allow the passage of drug molecules across the membranes separating VH, SF and CSF from the circulatory system. Although many of these mechanisms are said to be energy driven to greater or lesser extents, it is unclear whether certain processes are able to operate even in the absence of energy. If indeed such processes are able to operate in the absence of energy, then this may explain some of the inconsistencies between formaldehyde correlations in the biological fluids studied. Of the processes discussed, it would seem unlikely that hydrostatic pressure played a significant part in the transference of formaldehyde across each of the membranes in the case of the current experiment, since if this were the case, then the statistical tests examining the relationship between volume of fluid injected and

formaldehyde concentration detected might have been expected to produce stronger results, assuming all samples are comparable.

Osmotic pressure, however, is one process by which it is known that molecules can cross the membranes protecting the VH, SF and CSF (as discussed in Chapter 3), and although the literature is lacking in any consideration of the extent to which the mechanism may continue in the post-mortem environment, it may be the process that, at least to some degree, explains the passage of formaldehyde into each of the fluids. Using the Spearman's Rho test, it was possible to undertake a crude assessment of whether osmotic pressure may be responsible for the passage of formaldehyde into the three fluids, by examining the relationship between the concentration of formaldehyde detected in each fluid and the period of time that had elapsed since embalming had taken place. The results of the tests, as detailed in Table 4-12, and the scatterplot shown in Figure 4-3, however, reveal that all relationships were extremely weak with the exception of CSF (n=33) , where a correlation of 0.369 (p=0.035) demonstrated a moderate degree of association. While on the one hand failing to confirm that osmotic pressure may be related to the formaldehyde concentrations detected in each of the fluids (notwithstanding the inherent lack of certainty with which it might be said that the test does in actual fact examine osmotic pressure), the results, nevertheless are particularly important incidental confirmatory findings, demonstrating that formaldehyde appears neither to accumulate nor be lost from either VH or SF in the extended period following embalming. In the case of CSF, however, a weak degree of relationship reveals a trend for increasing concentrations of formaldehyde in direct proportion to an increasing amount of time since embalming. There may, thus, over time be a passage of formaldehyde across either the blood-cerebrospinal fluid barrier or the blood-brain barrier, or across both.

One potential reason for the differences in formaldehyde concentration correlations observed between the fluids included in the current study may be something very simple that was flagged in Madea and Musshoff's 2007 paper on post-mortem vitreous chemistry. Here, the researchers suggested that the reason for concentration differences observed between eyes may in fact be related simply to technical problems created by the variable viscosity of the fluid and, thus, the ability of the analyst to sample accurately and consistently. On the basis that the pipetting of viscous fluids is known to be problematic and requires particular

care, Madea implied that variable degrees of viscosity may lead to analytical inconsistencies. While the literature surveyed in Chapter 3 described CSF as being clear and watery, VH was defined in one study as having a viscosity approximately two times that of water, and the significant viscosity of SF is defined by the fact that the synovial joint space and membrane contain lubricant-excreting molecules for the purpose of reducing friction between opposing bones. Evaporation of VH through the cornea, furthermore, could conceivably increase the viscosity of the remaining fluid. Although tips designed specifically for pipetting viscous liquids are available, a failing of this study was that these were not used, and it is certainly possible, therefore, that intra-eye or intra-knee variation may at least in part have arisen as a result of sample preparation errors. Some further weight may be added to this theory from the generally poor statistical correlations associated with SF samples in the study.

4.8.2 Comparison between medical school embalming and repatriation embalming, and the implications for results

The method of embalming performed on the cadavers sampled for the study reported in this chapter carries some important differences to the process utilised in the preparation of remains for repatriation, as described in Chapter 2. Although the mechanical process of arterial injection is essentially identical, there are significant differences in both the formaldehyde concentration and in the volume of the fluid injected.

As discussed in Chapter 2, the final formaldehyde concentration used in funeral home embalming fluid is, depending on the condition of the body, and the degree of preservation required, usually somewhere between 1% and 2%. In the case of all the hard-fix cadavers sampled for the study reported here, however, the embalming fluid in every case contained 4.2% formaldehyde. In terms of the quantities of fluid injected, in the case of funeral home embalming this is perhaps more of an art than a science, since one of the primary concerns in this context, as was discussed earlier, is the presentation of the body. Thus, the total amount of fluid injected is judged on a case-by-case basis, and is based on the visual observations of the embalmer, as the procedure progresses. Some texts, however, do give indications of the approximate amounts of fluid injected. Polson, Brittain and Marshall (1962), for example, suggests that the approximate volume of fluid that will be required is around

one pint (0.47L) for every stone (6.35kg) of body weight, plus an additional one pint; a 75kg body, therefore, might be expected to take approximately six litres of embalming fluid, on Polson's approximation. Ajmani, 1993, on the other hand, states that the average volume of fluid required for arterial embalming will be around 10L, whereas Anger et al. (2008) indicates that the usual volume will be 4-6L. Other sources state differing volumes, although in all cases acknowledging that differences in body condition make it impossible ever to state precisely the quantity of fluid necessary to undertake an effective embalming. In practical terms, most embalmers agree that 2L-5L of fluid will be sufficient to ensure a short-term, presentation-driven treatment, and that 5L-10L will produce a very much more robust fixation (pers. comm. with several embalmers, and own professional experience as an embalmer). In the case of the Cambridge hard-fix cadavers sampled for this study, however, the volume of fluid injected directly into the circulatory system ranged from - for those cadavers for which the information was recorded (see Table 4-1) – 15L to 33L, with an average injection volume of 26.21L. These are quantities of fluid that are clearly very significantly in excess of those used in the type of embalming that will be encountered in repatriation cases.

Although the caveat accompanying the statistical analyses of the results presented in this chapter advises interpretative caution, there was, nevertheless, some suggestion of the fact that, at least in the case of CSF, there may be some degree of formaldehyde diffusion across one or both of the membranes separating this fluid from the circulatory system. This diffusion may be enhanced by the concentration of the formaldehyde, as well as by the volume of fluid injected, as a result of increased concentration and pressure gradients across the membranes. Although it would be experimentally unsafe to extrapolate this suggestion directly onto the overall working hypothesis of the thesis, it is at least suggestive, nevertheless, that in the case of repatriation cases, the concentration of formaldehyde entering the VH, SF and CSF may well be less than the concentrations detected in the Cambridge cadavers. Indeed, as a minimum, what probably can be assumed with a good degree of certainty is that the formaldehyde concentrations detected in the fluids of the Cambridge cadavers are likely to represent the worst-case scenarios, and that formaldehyde concentrations detected in repatriated bodies are, therefore, very unlikely to be higher.

4.8.3 Formaldehyde concentrations in 'soft-fix' cadavers

Although it was extremely difficult – for reasons of both ethical approval and logistical arrangement – to obtain biological samples from actual repatriated bodies, it has been possible to investigate preliminarily the extent to which formaldehyde might penetrate the VH, SF and CSF of authentic repatriation cases by analysing samples taken from a small number of cadavers embalmed using 'soft-fix' techniques. As described above, at 4.3, cadavers are occasionally utilised for surgical training purposes, for which hard fixation using high concentrations of formaldehyde and large volumes of fluid is not appropriate. Instead, a weaker concentration of formaldehyde and a lesser volume of fluid will ensure the short-term preservation of a cadaver, the tissues of which, in terms of their rigidity, will be much more life-like than their hard-fix counterparts. Although detailed embalming records were not compiled for any of the eight soft-fix cadavers included in the study, it is understood from the technical staff concerned that approximately 10L of fluid containing a final formaldehyde concentration of approximately 2% (20,000µg/mL) was injected into each cadaver. In respect of both formaldehyde concentration and volume, therefore, the conditions are certainly more representative of repatriation embalming than the large volume, high concentration method on which many of the results reported in this chapter have been obtained.

For technical reasons, it was not always possible to obtain a full set of samples for analysis. Nevertheless, of the 24 samples analysed from the eight cadavers included in this study, 15 were found to contain no formaldehyde above the LOD of 0.13µg/mL. Of the nine samples that tested positive for formaldehyde, the lowest concentration of formaldehyde detected was 0.48µg/mL, the highest was 14.70µg/mL, and the mean was 5.29µg/mL. When including in the calculation those samples containing no formaldehyde, the mean concentration was found to be 1.78µg/mL. These results contrast markedly with the formaldehyde concentrations detected in the hard-fix cadavers where, from the 164 samples analysed, the lowest was 101µg/mL, the highest was 1,454µg/mL, and the mean was 549µg/mL. In respect of the lowest concentrations detected, the hard-fix result was approximately 200 times greater than the corresponding soft-fix result. In the case of both the mean and the highest concentrations detected, the hard-fix results were both around 100 times greater than the corresponding soft-fix results.

Although the number of samples included in the study was small, it is helpful that they nevertheless came from eight separate cadavers. Indeed, although the sizes of the sample sets are not great enough to lend themselves to meaningful statistical analysis, they are arguably broad enough, in terms of the number of cadavers from which the samples were taken, at least to provide some indication of the likely results that might be observed in authentic embalmed and repatriated bodies. Indicatively, therefore, it is possible to infer two conclusions from the soft-fix results. Firstly, it might be suggested the results of this analysis certainly lend weight to the earlier hypothesis that lesser volumes of weaker formaldehyde solution may well manifest themselves in the embalmed body in smaller concentrations of analyte detectable in VH, SF and CSF. Even in the case of the soft-fix cadavers, however, their embalming method is still excessive, when compared to that used in repatriation embalming, where the volume of fluid injected, as described earlier, is generally no greater than 5L. This, therefore, leads to the second inferred conclusion from the soft-fix results. Namely, that in the case of authentic embalmed and repatriated bodies, it may be the case that the three biological matrices considered in the current study remain entirely uncontaminated by the embalming process.

4.9 Conclusions

From a methodological point of view, the work presented in this chapter is novel. No previously published GC method for underivatized formaldehyde has reported detection at the concentrations developed for the current work. And no published studies, furthermore, report the detection of formaldehyde in post-mortem biological fluids. The Tekmar-Dohrmann Application Note, on which the current method was developed, reports detection at 200μg/mL. After considerable development, however, the current method was validated with a LOQ of 0.40 μg/mL and an LOD of 0.13 μg/mL for VH, CSF and SF.

From the point of view of the hypothesis of the thesis, however, significant conclusions can be drawn from the work. The literature reviewed in Chapter 3 highlighted the fact that, in the living, CSF, by virtue of the epithelial barriers separating it from the circulating blood, is protected from the potentially toxic waste products of normal metabolism, and from many pharmaceutical compounds. Those molecules that are able to cross the barriers require

biochemical processes to do so, and many of these processes are energy driven. It has long been known that the production of ATP, the source of energy required to enable these biochemical processes to function, ceases rapidly after death and that, as a result, active transport may thus be consequently blocked. Other work has described similar anatomical as well as similar physiological characteristics in the membranes separating VH and SF from the circulatory system. The results of the current study certainly appear to lend weight to the theory that these membranes may indeed prevent, or at least very much reduce, the passage of molecules across them: the fact that formaldehyde injected into the arterial system is able to penetrate tissues of the body to the extent that it can arrest decomposition for considerable periods of time, but can be detected in VH, CSF and SF in only very low concentrations (and in concentrations that are many times lower than the starting concentration) is suggestive that the membrane separating each respective fluid from its local vascular system is a barrier to formaldehyde transport. If it follows that, as well as preventing transport into each respective fluid, each membrane also prevents transport out of these fluids, and that the xenobiotic contents of each fluid is thereby protected, then the sampling of VH, CSF and SF as historical repositories of the ante-mortem pharmacologic state becomes an attractive proposition.

In the specific case of embalmed bodies, the results presented in the current study are potentially significant and very much complement this 'historical repository' theory. As was mentioned in the introduction to this thesis, it has been that case that, for many years, coroners have commonly and deliberately overlooked undertaking toxicological analysis on embalmed and repatriated bodies, in the belief that results were unlikely to be unhelpful, given the rates of degradation of many compounds reported in the literature. The corollary of the results presented in the current experiment, however, point in a very different direction. They are suggestive of the fact that, at the sort of concentrations at which formaldehyde has been detected in VH, SF and CSF, drugs that have entered these fluids may be comparatively safe from degradation, at least for a period of time that would enable meaningful analysis to take place. Indeed, when considered alongside the concentrations of formaldehyde used in many of the studies reviewed in Chapter 2, degradation may, at concentrations of around 549µg/mL (the mean formaldehyde concentration detected in the

hard fix cadavers sampled for the current study), be significantly retarded. At concentrations of 5.29µg/mL (the mean concentration detected in the formaldehyde-positive soft-fix cadavers), or 1.78µg/mL, when including in the mean those samples found to contain no formaldehyde, degradation may well be entirely absent. It is the case, furthermore, that, by virtue of the large volumes of comparatively highly concentrated embalming fluid that are used to preserve the Cambridge cadavers, the concentrations of formaldehyde detected in the VH, CSF and SF sampled for the current study may represent what is above the upper limit of what might reasonably be expected to be found in the same fluids from repatriation-embalmed cases.

While Chapter 2 presented a comprehensive review of the stability of a wide range of drug molecules in a variety of formaldehyde concentrations, no studies have been published that examine the stability of drugs of abuse in concentrations of formaldehyde as low of those detected in the samples analysed in the current study. Predictions that little or no degradation is likely to take place are, therefore, little more than conjecture and need to be tested. With this in mind, the final experimental chapter of the thesis examines the stability of 15 common drugs of abuse, or their metabolites, in a range of formaldehyde concentrations, in conditions that aim to replicate those that might be encountered in repatriated bodies.

5 THE STABILITY OF DRUGS OF FORENSIC INTEREST IN FORMALDEHYDE

5.1 Introduction

The results reported in Chapter 4 have served to establish the concentration of formaldehyde detected in the VH, SF and CSF of heavily embalmed medical school cadavers, and have, furthermore, given some indication of the concentrations that may be expected to be detected in cases of repatriation embalming. The chapter concluded by suggesting that the existing embalming literature may, as far as embalmed bodies are concerned, be misleading, and that, by implication, the current coronial expectation of being unable to achieve meaningful analytical results from such bodies may therefore be misplaced.

The purpose of Chapter 5 is to test directly this last statement by examining the stability of a number of drugs of forensic interest in the concentrations of formaldehyde detected in the VH, SF and CSF of the Cambridge/Bristol embalmed cadavers. Fifteen drugs were selected for the study. A number of drug molecules were chosen because of their existing place in the literature and, therefore, the opportunity to present some degree of comparison. Others were selected either because of the frequency with which they are reported to be encountered in forensic practice or, in the case of drugs such as carfentanil, because of their comparative novelty and thus their corresponding lack of attention in the literature.

In selecting experimental conditions for this experiment, consideration was given to methods reported previously in the literature, in light of not only the results reported in Chapter 4, but also with regard to the conditions that are likely to be found in embalmed and repatriated remains. The three most important experimental condition variables considered in the literature, as discussed at length in Chapter 2, are formaldehyde concentration, pH and temperature.

As far as formaldehyde concentration is concerned, previous methods have commonly employed final formaldehyde concentrations of between 5% and 20%, equating to 50,000-200,000µg/mL. Given, however, the fact that Chapter 4 reported formaldehyde concentrations ranging from only 1,454µg/mL down to 101µg/mL in the Cambridge hard-fix

cadavers, and from 14.70μg/mL down to 0.48μg/mL or less in the soft-fix cadavers, it quickly becomes evident that the higher concentrations used previously would not be appropriate in the current experiment. It was therefore decided to use concentrations of 1,000μg/mL and 100μg/mL formaldehyde, these concentrations being indicative of what might reasonably be encountered in repatriation cases. Additionally, as the concentrations of formaldehyde used in repatriation embalming, being approximately 10,000-20,000μg/mL, are not currently reported in any of the literature, it was decided to include these concentrations in the experiment from a 'worst case scenario' perspective. Indeed, although Chapter 4 has provided strong evidence of the fact that the membranes separating VH, SF and CSF from the circulatory system do prevent extensive formaldehyde contamination, an indication of the stability of drugs in the 'neat' concentrations of fluid used in embalming, may provide useful and supplementary points of reference, particularly in light of the experimental novelty of the approach. Thus, the final formaldehyde concentrations used in the current experiment are 20,000, 10,000, 1,000 and 100μg/mL.

Much of the literature reviewed in Chapter 2 reported the enhanced degradation of a number of drug molecules in particularly alkaline environments, and of some in acid conditions. The extremes of pH reported in the literature, however, are not appropriate to the current experiment, for the simple fact that they are highly unlikely to be encountered in repatriated bodies. Tracy, as we have seen, speculated that the somewhat acidic pH of formalin (pH ~3.5) may be modified to a more alkaline pH in the post-mortem body, as decomposition begins and proteins break down. As was noted in the concluding comments in Chapter 4, however, it is the case that, unlike laboratory formalin solutions, which are commonly un-buffered, preparations formulated for funeral home use are commonly buffered to around pH 9.

What, additionally, the literature reviewed in Chapter 3 has shown is that the morphologically compartmentalising nature of the membranes separating VH, SF and CSF from the vascular system are such that the fluids are not affected by the early stages of bodily decomposition in the same way as, for example, blood; the compartments, at least for a time, appear to remain bacteria free. Although the pH of blood, following death, has been found to become more alkaline, the literature provides little indication of what the comparative situation is within VH, SF and CSF. Given the relative anatomical and circulatory isolation of these fluids,

compared to blood, however, it may be reasonable to assume that the pH is likely to remain unchanged in the early stages of decomposition.

In three studies summarized in Chirila and Hong (2016), the pH of VH in the living was determined as being, variously, 7-7.3, 7.4-7.52, or 7.5. Devgun and Dunbar (1986) reported that the pH of post-mortem VH can range between 7.3-9.1. In the case of SF, an early study, Cummings and Nordby (1966), found that the pH in the healthy knee joints of a number of subjects ranged between pH 7.341-7.644, with a mean of pH 7.434; in patients suffering from rheumatoid arthritis, the mean pH was 7.207, and in osteoarthritis was 7.475. An earlier study, Jebens and Monk-Jones (1959), found the pH of normal SF to be 7.768 ±0.044, and in osteoarthritic cases to be 7.549 ±0.040. No study of pH in post-mortem SF appears to have been undertaken. In the case of CSF, Siesjö (1972) cites twelve earlier studies examining the pH of this fluid in the living, where it was found to range from 7.307 to 7.345, with a mean of 7.326; Turitto and Slack (2016) gives a range of pH 7.35-7.70. No studies appear to have been undertaken on the pH of post-mortem CSF.

As it was felt to be logistically unwieldy to experiment with solutions in more than one pH, and as the pH of circulating blood, as described earlier, is approximately 7.4, a figure that coincides with pH values observed in the VH, SF and CSF of the living, it was decided that all formaldehyde-drug reaction solutions would be buffered to pH 7.4

Finally, temperature, as was discussed in Chapter 2, is a variable that has received little attention in the embalming literature. Suzuki and Keneko, in their 2009 study, reported the very much greater degradation of methamphetamine-formaldehyde solutions stored at room temperature, when compared to those stored at 4°C. Other than Suzuki's study, however, little comparative attention has been paid to what might potentially be a significant catalyst in the degree of reaction exhibited in drug-formalin solutions. In the case of embalmed bodies, however, the reality of body storage facilities, as well as the physical process by which bodies are repatriated, are somewhat variable in provision. Hospitals and funeral directors in many - though certainly not all - countries around the world routinely refrigerate bodies once they are received, although there is no international and, in many countries, not even any national standard in this respect (pers. comm. with Rowland Brothers International). After

embalming, some funeral directors will refrigerate bodies; others will not. Transporting a body to an airport is, in most countries, likely to be undertaken in non-refrigerated vehicles, and the hold in which the coffin is carried is unlikely to be refrigerated in any sort of controlled manner. Thus, of the three variables of formaldehyde concentration, pH and temperature, it is the latter of these for which it is very difficult to replicate experimentally the reality of the process of repatriation.

What can be accounted for experimentally however, is the reactivity of drugs in formalin solutions at either end of the range of temperatures to which the body may be exposed in the period between death and subsequent post-mortem examination. The degradation of all 15 drugs included in the experiment, therefore, was monitored in solutions stored at 4°C, as well as in those stored at room temperature.

In summary, therefore, all 15 drugs were studied in eight different conditions over the period of the experiment: 20,000, 10,000, 1,000 and 100µg/mL formaldehyde, all buffered to pH 7.4, with sets of samples stored at both 4°C and at room temperature. It was decided, furthermore, that the duration of the experiment would be 35 days. While this period of time is significantly greater than the length of time between death and the repatriation of a body in the majority of cases (pers. comm. with Rowland Brothers International)[4], at 35 days it is possible to make comparison with the existing embalming literature, where many of experiments are reported as having been conducted over approximately 30 days. All samples were analysed in triplicate on Days 0, 2, 4, 7, 14 and 35 of the experiment.

A simple and sensitive LC/Q-ToF method was developed and validated for the detection of 15 common drugs of abuse, or their metabolites. The compounds analysed included opioids (alfentanil, carfentanil, codeine, fentanyl, methadone and morphine, and the primary heroin metabolite, 6-MAM), benzodiazepines (diazepam, flunitrazepam, lorazepam and midazolam,

[4] See, also, Williams 2014, where the mean time from death to UK autopsy in a series of 44 repatriation cases was 13 days (range 7-41) where no autopsy was conducted abroad, and 16-17 days (range 6-72) in cases where an autopsy was first conducted abroad. Holz 2020, furthermore, reports a retrospective analysis of 151 repatriations to the Institute of Legal Medicine, Frankfurt am Main, from 56 different countries, where the median interval between death and German autopsy was 11 days (range 2-603).

as well as the pharmacologically related zolpidem), the stimulant, cocaine, and its primary metabolite, benzoylecgonine (BZE), and the sedative, ketamine.

In addition to the primary stated aim of this experiment, it was originally intended that the work reported in this chapter would extend into an analysis of the drug-formaldehyde breakdown products in the case of each of the 15 drug molecules included in this study, in order to develop the preliminary work reported in Yokchue (2016). Much of the literature reviewed in Chapter 2 of the current study recommends that the ability to be able to identify conversion products may be useful in cases where the degree of formaldehyde-induced degradation is such that the parent compound can no longer be detected. Yokchue, as part of her doctoral thesis, was able to identify certain breakdown products of a number of amphetamines, opiates and benzodiazepines, in reaction with formaldehyde, using an optimised LC/MS-MS protocol. The current experiment examines the stability of a number of drugs that have not been included in previous studies and, thus, provides an excellent opportunity to develop further Yokchue's work. With the availability of LC/Q-ToF equipment at Cranfield University, furthermore, it was decided that this would be a more precise platform than LC/MS-MS with which to attempt identification.

After method development work had been completed, however, and the experiment proper was underway, it quickly became apparent that, with only a very small number of exceptions, the stability in formaldehyde of the fifteen compounds examined was such that work on the identification of conversion products was almost certainly completely unnecessary, at least within the broader conceptual framework of the thesis. With the exception, in certain conditions, of cocaine and methadone, all compounds were shown to have very good stability; all parent compounds could be expected to be identified and quantified within the timescales that are commonly encountered in repatriation embalming. The identification of conversion products, therefore, at least as far as the implications of the current project are concerned, is probably unnecessary. It remains the case, however, that soft tissue removed at autopsy is routinely preserved in concentrated solutions of formalin, and may occasionally be required for subsequent toxicological analysis (it is this premise, of course, that forms the basis of much of the existing embalming-toxicology literature). For this reason, therefore, it

was decided to pursue this aspect of the experiment, and attempt identification work on those compounds included in the study that demonstrated any degradation, however small.

Unfortunately, this part of the work coincided with the increasing threat of COVID-19 in the United Kingdom, and the first day of chromatographic analysis proved to be the last, as the Cranfield laboratories were then closed following Government directives. The amount of data gathered, thus, was limited and the results that can be reported are an incomplete representation of what may have been achieved. It was not possible, for example, to increase the run time of each injection beyond the 10-minute programme used for the main experiment, and provision could not therefore be made for any comparatively late-eluting compounds. It was, nevertheless, possible to discern the presence of a small number of potential conversion products, from a selected number of drugs; these findings are reported later in the chapter.

5.2 Analytical Method and Development

Perhaps the greatest challenge in developing the method was balancing the implications of the well-known and well-reported reactivity of formaldehyde alongside conducting an experiment that, within the limits of a single chapter within a doctoral thesis, was logistically and physically manageable. In order to eliminate entirely the possibility of drug-drug inter-reactions in a formaldehyde environment, the experimental preference would have been to react and analyse each of the 15 drugs in isolation. However, with four different concentrations of formaldehyde, in two different temperatures, analysed on six separate days, with each sample being analysed in triplicate, the workload necessary to satisfy this approach would have been considerable on many levels.

It was decided, therefore, to reduce the number of samples by 'batching' the drugs into groups. Consideration was given to the likelihood of drug reactions and drug-drug interactions between the compounds studied, in the presence of formaldehyde. Formaldehyde is well known to bring about the condensation and polymerisation of nucleophiles; in the case of primary amines, a second step, involving dehydration, is also known to occur (Brown, 1999). Although none of the molecules included in the experiment are either primary or secondary amines, a number of the compounds contain hydroxyl groups which, in the presence of

formaldehyde, can result in the formation of *M*+30 adducts, as well as leading to a dimerization reaction resulting in 2*M*+12 adducts (Kamps et al., 2019 and Metz et al., 2004). Although fentanyl and its analogues, along with the majority of the benzodiazepines, were expected to be relatively unreactive, it was predicted that the opioids may have been liable to produce adducts. Additionally, the two ketone compounds, ketamine and methadone, might be expected to react in the same way as formaldehyde because of their carbonyl group, and might additionally lead to the production of hydrate adducts, *M*+12 (Carey, 2002). Finally, the two tropanes, cocaine and BZE, are known to transfer their benzoyl group, and 6-MAM, its acetyl group (Carey, 2002).

Fortunately, with the collaboration of Dr Jeff Pons, a pharmaceutical chemist now working at Cranfield University, it was possible to predict the likelihood of drug-drug inter-reactions in formaldehyde, and from this information to plan a method that would eliminate the probability of such reactions. The predicted inter-reactions from which the method was developed are reproduced in Table 5-1, below. From this table, it was therefore possible to 'batch' drugs into groups that were deemed unlikely to exhibit inter-reaction.

During the LC/Q-ToF method development stage it became necessary to make changes to the initial batching plan in order to account for some very close retention times resulting from the preferred chromatographic conditions. It was possible, however, to formulate batches, none of which comprised molecules predicted to be likely to undergo inter-reaction. The drug batches are listed in Table 5-2.

5.3 Materials

6-MAM and lorazepam (both 1mg in 1mL acetonitrile), alfentanil, BZE, cocaine, codeine, diazepam, fentanyl, flunitrazepam, ketamine, methadone, midazolam, morphine and zolpidem (all 1mg in 1mL methanol) and carfentanil (100µg in 0.5mL methanol) pharmaceutical standards, along with formaldehyde (37% by weight, in H_20, with 10-15% methanol as a stabilizer (to prevent polymerisation)), acetonitrile and formic acid (both HPLC grade) were purchased from Sigma Aldrich, all drug standards being supplied by Supelco. Ultra-pure water (18.2 Ω/cm) was obtained from the PURELAB® Ultra System.

Table 5-1 - Predicted likelihood of drug-drug interactions in the presence of formaldehyde

	6-monoacetylmorphine	Alfentanil	Carfentanil	Codeine	Fentanyl	Methadone	Morphine	Diazepam	Flunitrazepam	Lorazepam	Midazolam	Benzoylecgonine	Cocaine	Ketamine	Zolpidem
6-monoacetylmorphine															
Alfentanil															
Carfentanil															
Codeine															
Fentanyl															
Methadone	X			X											
Morphine						X									
Diazepam															
Flunitrazepam															
Lorazepam						X									
Midazolam															
Benzoylecgonine															
Cocaine															
Ketamine	X			X			X			X					
Zolpidem															

Key:
LIKELY (red and 'x') UNLIKELY (green)
POSSIBLE (yellow) SAME MOLECULE (blue)

Table 5-2 – Drug batches

Batch	Drugs
Batch 1	6-MAM Diazepam Zolpidem
Batch 2	Codeine Flunitrazepam
Batch 3	BZE Carfentanil Midazolam
Batch 4	Cocaine Ketamine Methadone
Batch 5	Alfentanil Morphine
Batch 6	Fentanyl Lorazepam

5.3.1 Working, calibration, buffer and sample solutions

5.3.1.1 Working solutions

Using A-grade volumetric glassware in all cases, three sets of working solutions were prepared from the drug standards:

i) Basic working solution: 1mg in 10mL (all drugs except carfentanil)
100µg in 5mL (carfentanil)

ii) 10,000ng/mL working solution (all drugs)

iii) 1,000ng/mL working solution (all drugs)

Final working solutions were stored in 1.5mL HPLC vials and frozen at -20°C when not in use. Precise details of working solution preparation are given in Appendix 6.

5.3.1.2 Calibration solutions

On each analysis day, calibration solutions were prepared in batches, as listed in Table 5-2. For each batch, 1 to 6, seven concentrations, totaling 42 calibration vials, were prepared in dilutions of 500ng/mL, 250ng/mL, 100ng/mL, 50ng/mL, 25ng/mL, 10ng/mL and 5ng/mL. Precise details of calibration solution preparation are given in Appendix 7.

5.3.1.3 Buffered formaldehyde solutions

For the experiment proper, drugs were prepared in 20,000, 10,000, 1,000 and 100µg/mL formaldehyde solutions, all buffered to pH 7.4, using potassium phosphate monobasic and dibasic. Precise details of buffer and formaldehyde solution preparation are given in Appendix 8.

5.3.1.4 Sample solutions

As already described, drugs were batched for analysis into groups containing molecules that were thought likely to be chemically unreactive with each other. In order to limit analytical workload, replicates were not prepared, though each sample was analysed in triplicate on each day. Within each batch, sample solutions were prepared in formaldehyde buffered to concentrations of 20,000, 10,000, 1,000 and 100µL/mL. Two sets of sample solutions were prepared for each batch, one set to be refrigerated and the other to be kept at room temperature. In addition to the range of samples prepared in buffered formaldehyde, a set of control samples for each batch was prepared and refrigerated. Precise details of sample solution preparation are given in Appendix 9.

Immediately following preparation, each Batch A, B, D, F and H solution was refrigerated at 4°C, and each C, E, G and I solution was stored at room temperature.

For the degradation study - the part of the experiment designed to identify the breakdown products of drug-formaldehyde reactions - individual reaction mixtures of all fifteen compounds were prepared in 20,000µg/mL formaldehyde: 500ng/mL mixtures were prepared by adding the appropriate volumes of 20,000µg/mL formaldehyde solution buffered to pH 7.4 to individual drug standards. The reaction mixtures were stored at room temperature for 14 days and were then analysed using the same LC method and conditions as reported for the main experiment.

5.4 Chromatographic and Mass Spectrometry Conditions

Helpfully, a recently published method for the screening of a large number of compounds in blood, utilising LC/Q-ToF, Partridge et al (2018), includes 12 of the drugs to be examined in the current study. From Partridge's method it was possible, with further and considerable

development work, as discussed in 5.2, to optimize a method for the particular requirements of the current experiment.

Details of the final method are reproduced in Table 5-3. Chromatographic and mass spectrometric data for all drugs are reproduced in Appendices 13-27.

All samples were analysed in triplicate. Primary analytes were identified by comparison with the retention times of concurrently run calibration standards and were confirmed from spectral ion data using Q/ToF MS operated in full scan mode. Data were acquired and quantified utilizing the extracted-ion count (EIC) method within the Agilent ChemStation software. Retention times and $[M+H]^+$ masses used to identify primary analytes are recorded in Appendix 10.

Table 5-3 – LC/Q-ToF conditions

LC/Q-ToF system	Agilent Infinity 1290 LC attached to Agilent 6540 UHD Q-ToF/MS
LC injection volume	0.4µl
Mobile phase	0.1% formic acid in H_20 (A) 50:50 Acetonitrile:H_20 (B)
Column	Thermo Scientific Hypersil Gold 50x2.1mm, 1.9µm
Programme	Commence at 95% (A), reducing to 60% (A) by 7.50 min, reducing to 5% (A) by 8.00 min, returning to 95% (A) by 8.50 min, followed by 1.5 min post-run; total run time, 10 min.
Column temperature	30ºC
Column flow rate	0.4mL/min
Source gas temperature and flow rate	200ºC at 8L/min
Nebuliser pressure	40psig
Sheath gas temperature and flow rate	350ºC at 10L/min
Capillary voltage	400v
Nozzle voltage	100µL @ 500µL/sec
Fragmenter voltage	175v
Q-ToF calibration solution	Agilent ESI-L Low Concentration Tuning Mix
PC Software	Agilent ChemStation data analysis software

5.5 Method Validation

The validation parameters described in Chapter 4, in relation to the previous experiment, were applied in exactly the same manner in the current experiment, unless specifically noted otherwise below.

5.5.1 Selectivity, specificity, matrix effects and analyte stability

The selectivity of the method was assessed by analysing blanks of all four concentrations of the buffered formaldehyde solutions, as follows:

i) 20,000µg/mL formaldehyde in 50:50 ACN:H_2O buffered to pH 7.4, x3 replicates;

ii) 10,000µg/mL formaldehyde in 50:50 ACN:H_2O buffered to pH 7.4, x3 replicates;

iii) 1,000µg/mL formaldehyde in 50:50 ACN:H_2O buffered to pH 7.4, x3 replicates;

iv) 100µg/mL formaldehyde in 50:50 ACN:H_2O buffered to pH 7.4, x3 replicates.

Using total ion current (TIC) chromatograms, no responses were detected at any of the method retention times, as recorded in Appendix 10, for any of the compounds of interest. Furthermore, using EIC chromatograms, with reference to the ion values stated in Appendix 10, no drug analyte or decomposition ions were detected, confirming that the method is appropriately selective.

In order to assess specificity, recovery, and any possible interference related to the well-reported LC matrix effects phenomenon, first, drug standards at concentrations of 50, 250 and 500ng/mL (in 50:50 ACN:H_2O, buffered to pH 7.4) were individually injected, in triplicate, and the peak areas measured against the daily calibration curves. Next, drugs were batched in the groups used in the experiment proper, as described above, at concentrations of 50, 250 and 500ng/mL (in 50:50 ACN:H_2O, buffered to pH 7.4), and these were also analysed in triplicate, and the data was measured against the calibration curves. The peak areas of the batched injections were then compared to those obtained from the individual injections. Differences in peak area between individual and batched injections were found to be within the %RSD for precision (repeatability), as recorded in Appendix 11, in the case of every molecule at each of the three concentrations. The method was therefore deemed to be appropriately specific.

In order to assess analyte stability, 500ng/mL batched drug solutions were prepared in 50:50 ACN:H_2O buffered to pH 7.4, as described earlier, and were refrigerated at 4°C. Samples were analysed in triplicate on each analysis day. The initial recovery and subsequent stability of each compound is recorded in Appendix 12.

5.5.2 Linearity

Calibration solutions were prepared according to the method detailed above at 5.3.1.2. Solutions were analysed in triplicate, following which six or seven-point calibration curves were constructed for each compound. R^2 values for all compounds, ranging between 0.9992and 1, demonstrated good linear relationships between all drug concentrations and the LC/Q-ToF responses. Retention time, calibration points(*n*), standard error, slope, intercept, R and R^2 data for each curve are recorded, by drug batch, in Appendix 10.

5.5.3 Lower limit of quantitation and lower limit of detection

The LOQ and LOD, as defined in Chapter 4, were calculated from each calibration curve. Data for each drug are recorded in Appendix 10.

5.5.4 Carryover

To assess the method for carryover, 50, 250 and 500ng/mL samples (*n*=5 of each concentration) of batched drugs, batched according to the protocol already described, were prepared in 50:50 ACN:H_2O buffered to pH 7.4. Each sample was successively injected, with blanks (50:50 ACN:H_2O buffered to pH 7.4) inserted after each sample, following which the chromatogram of each blank was examined. No peak area greater than the respective LOD for each drug, as detailed in Appendix 10, was detected in any case. In order further to protect the data from any risk of carryover, on each of the six analysis days of the experiment proper, additionally, a blank (50:50 ACN:H_2O buffered to pH 7.4) was included in the run after every six test samples. All blanks were analysed, and none were found to contain carryover greater than the defined LOD.

5.5.5 Instrument accuracy and precision

To assess instrument accuracy, 250, 500 and 750ng/mL samples of each batch (*n* =3 of each concentration of each batch) were analysed and the response was measured against the daily calibration curve. Accuracy was then calculated as the percentage deviation of the mean result from that of the nominal concentration. The results, all of which are within acceptable limits, are given in Appendix 11.

Precision tests were performed to test both the repeatability and reproducibility of the method, using the method described in Chapter 4.

For the repeatability test, 50, 250 and 500ng/mL samples (*n*=5 of each concentration) of batched compounds (batched according to the usual protocol), and prepared in 50:50 ACN:H_2O buffered to pH 7.4, were analysed in immediate succession and were measured against the daily calibration curve. Calculated %RSDs ranged from 0.48 to 12.78 and were therefore considered appropriately precise according to the criteria described previously. Repeatability data for all drugs are recorded in Appendix 11.

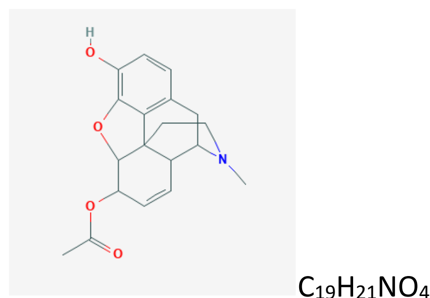
For the reproducibility test, 50, 250 and 500ng/mL samples (*n*=5 of each concentration) of batched compounds (batched according to the usual protocol), and prepared in 50:50 ACN:H_2O buffered to pH 7.4, were prepared on three separate days and were analysed and compared. Calculated %RSDs ranged from 2.35 to 20.84 and were therefore considered appropriately precise according to the criteria described previously, with the one exception of 6-MAM, with a recorded reproducibility %RSD of 20.84. Reproducibility data for all drugs are listed in Appendix 11.

5.6 Results and Discussion

In the results section that follows, absolute recoveries are reported in all cases and are recorded by calculating the mean of the triplicate analysis undertaken in each case. In order to estimate relative stability, however, the control data are also reported alongside the recovery from each individual condition, and the 4°C figures that follow also include the control stability samples for comparative purposes.

5.6.1 Opiates and opioids results

5.6.1.1 6-MAM

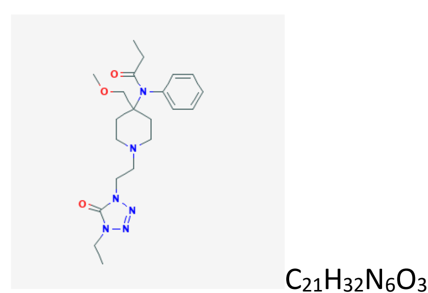


$C_{19}H_{21}NO_4$

Figure 5-1 – Structure and formula of 6-MAM

Losses of 6-MAM were observed in all formaldehyde solutions by Day 35 of the experiment. The greatest loss was recorded in the 20,000µg/mL RT solution, where the recovery was 46% at Day 35. In the 100µg/mL formaldehyde solutions, 91% and 96% recoveries were made from the RT and the refrigerated solutions, respectively; from 1,000µg/mL formaldehyde, 56% and 58% recoveries were made from the same conditions after 35 days. Although losses were greater in RT solutions, compared to those that were refrigerated, the pattern of degradation between both was very similar. The control sample remained relatively stable, being 96% recoverable by Day 35. Details of the recovery of 6-MAM in all conditions are summarized in Table 5-4. Illustrations of the degradation patterns in 4°C and RT samples are given in Figures 5-4 and 5-5, respectively.

5.6.1.2 Alfentanil

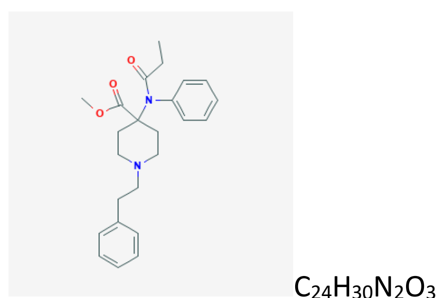


$C_{21}H_{32}N_6O_3$

Figure 5-2 – Structure and formula of alfentanil

Alfentanil underwent very little degradation in any condition. It was entirely stable in 100µg/mL and 1,000µg/mL formaldehyde, at both 4°C and at RT. In both 10,000µg/mL and 20,000µg/mL formaldehyde, small losses were noted by days four and seven, after which it remained stable so that, at Day 35, it was still possible to recover 97% from the 10,000µg/mL RT solution and 95% from the 20,000µg/mL RT solution. The control solution remained entirely stable throughout the experiment. A summary of alfentanil recovery is given in Table 5-5. An illustration of the stability of the compound in RT conditions is given in Figure 5-6 and, in 4°C conditions, in Figure 5-7.

5.6.1.3 Carfentanil



$C_{24}H_{30}N_2O_3$

Figure 5-3 – Structure and formula of carfentanil

Carfentanil demonstrated good stability in all conditions. 94% of the drug was recoverable from 20,000µg/mL formaldehyde at RT after 35 days, and 95% from 10,000µg/mL formaldehyde at RT after the same period of time. In 1,000µg/mL formaldehyde, at RT, 93% of the drug was recoverable after 35 days, and, in 100µg/mL formaldehyde the slightly lower recovery rate of 91% was recorded at day 35. The control solution remained stable throughout the experiment, being 98% recoverable on Day 35. The results of the carfentanil reactions are summarized in Table 5-6, and the patterns of stability for all concentrations of the drug in RT and refrigerated conditions are depicted in Figures 5-8 and 5-9, respectively.

Table 5-4 – Recovery (%) of 6-MAM
showing % recovery (standard deviation) on each day for each condition

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (2.69)	71 (2.16)	71 (2.16)	83 (1.93)	83 (1.93)	86 (4.51)	86 (4.51)	100 (1.44)	100 (1.44)
2	100 (2.49)	69 (2.05)	68 (4.13)	68 (1.52)	68 (1.27)	73 (4.61)	73 (1.97)	100 (0.55)	100 (1.56)
4	100 (1.81)	66 (0.62)	66 (1.79)	68 (2.20)	68 (1.72)	72 (1.28)	70 (0.87)	100 (1.81)	100 (0.74)
7	99 (1.12)	64 (4.41)	56 (1.31)	68 (1.52)	62 (4.13)	61 (3.09)	60 (2.46)	100 (1.90)	100 (0.85)
14	98 (1.69)	62 (2.55)	55 (0.79)	57 (3.15)	55 (1.11)	59 (1.26)	57 (0.57)	97 (4.92)	97 (5.14)
35	96 (1.26)	53 (0.30)	46 (0.90)	57 (0.81)	54 (1.08)	58 (0.44)	56 (0.81)	96 (0.31)	91 (0.52)

Figure 5-4 – Recovery (%) of 6-MAM in all concentrations of formaldehyde at 4°C

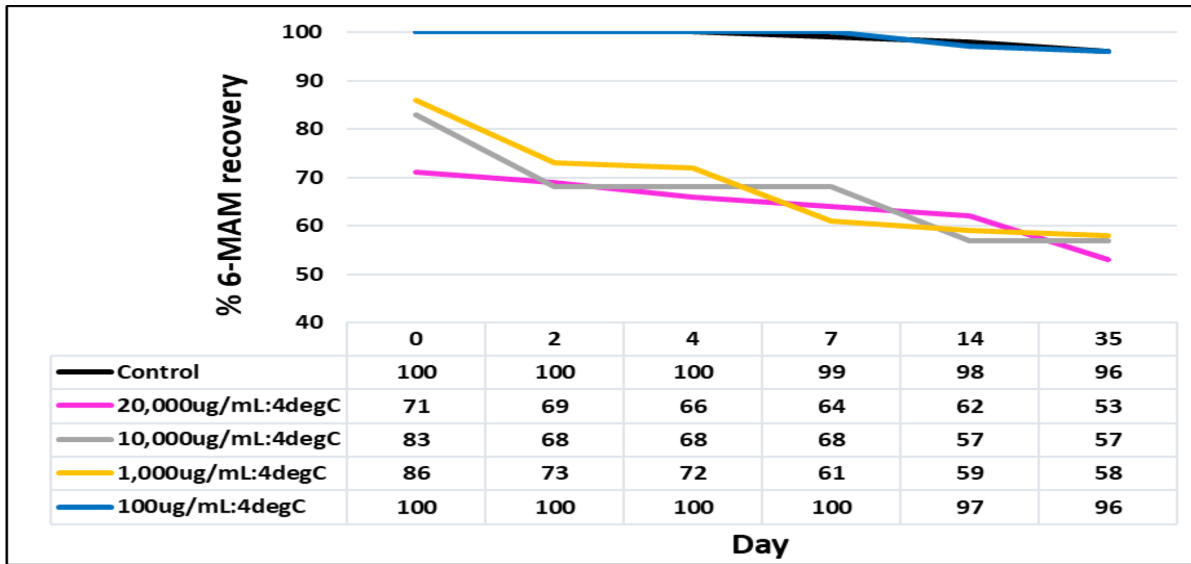


Figure 5-5 – Recovery (%) of 6-MAM in all concentrations of formaldehyde at room temperature

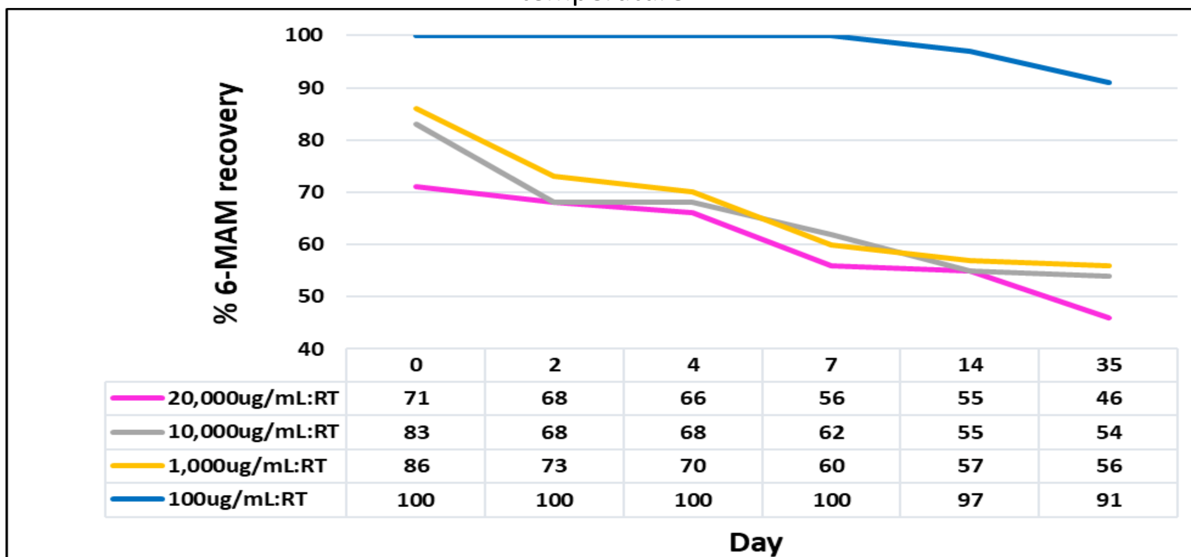


Table 5-5 – Recovery (%) of alfentanil

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (1.10)	100 (0.31)	100 (0.31)	100 (0.72)	100 (0.72)	100 (0.42)	100 (0.42)	100 (0.31)	100 (0.31)
2	100 (1.21)	100 (0.42)	100 (0.67)	100 (0.53)	100 (0.76)	100 (0.46)	100 (0.64)	100 (0.65)	100 (0.56)
7	100 (0.90)	98 (0.31)	96 (0.42)	99 (0.23)	97 (0.31)	100 (0.12)	100 (0.42)	100 (0.31)	100 (0.87)
14	100 (0.23)	98 (0.20)	96 (2.31)	99 (2.51)	97 (2.50)	100 (0.70)	100 (0.80)	100 (0.68)	100 (0.99)
35	100 (0.27)	98 (0.46)	95 (1.03)	99 (1.51)	97 (0.50)	100 (0.57)	100 (0.58)	100 (0.76)	100 (0.54)

Figure 5-6 – Recovery (%) of alfentanil in all concentrations of formaldehyde at room temperature

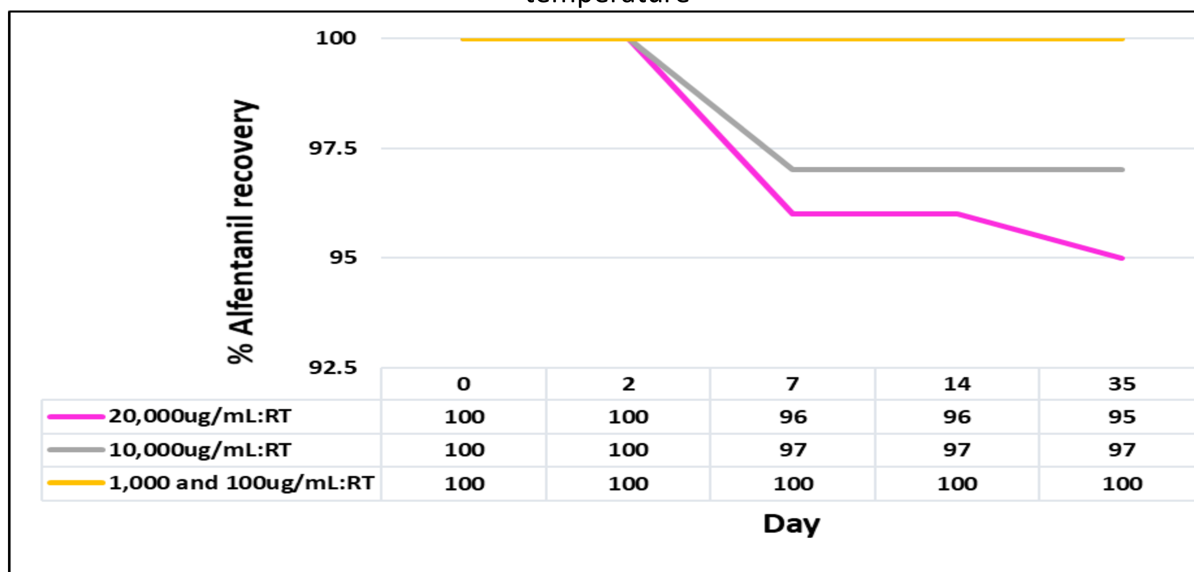


Figure 5-7 – Recovery (%) of alfentanil in all formaldehyde concentrations at 4°C

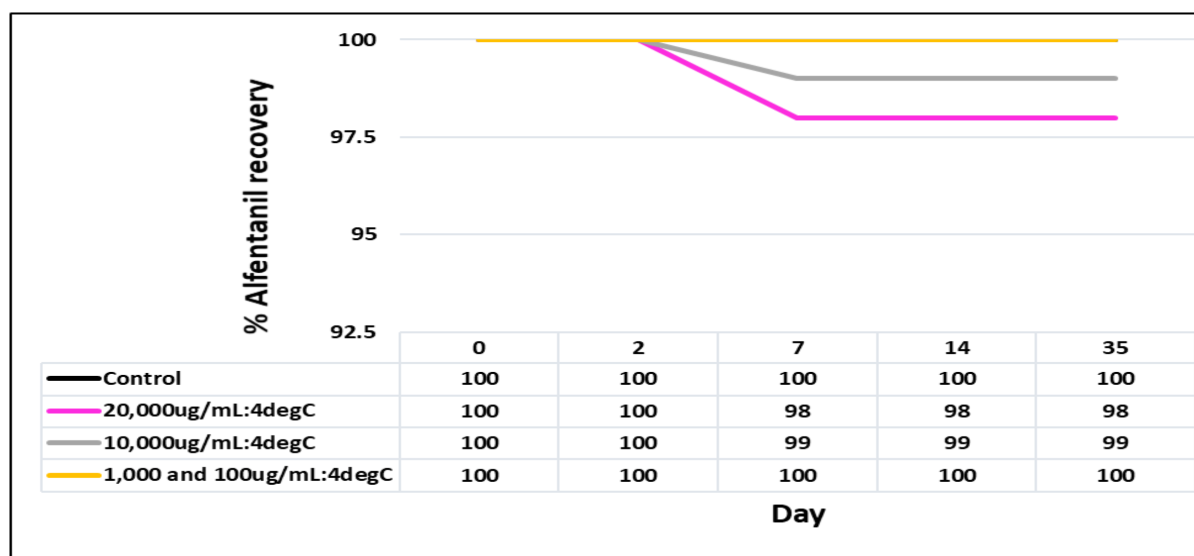


Table 5-6 – Recovery (%) of carfentanil

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (0.12)	101 (0.31)	101 (0.31)	102 (0.81)	102 (0.81)	102 (0.12)	102 (0.12)	102 (1.53)	102 (1.53)
2	100 (0.70)	101 (1.03)	104 (1.40)	101 (0.60)	100 (1.25)	99 (1.47)	100 (0.42)	95 (1.80)	99 (0.90)
4	100 (0.72)	98 (1.03)	97 (0.69)	99 (0.72)	98 (0.64)	96 (0.50)	96 (1.60)	95 (0.99)	96 (0.20)
7	99 (0.81)	97 (0.46)	95 (0.20)	98 (0.23)	98 (0.81)	96 (0.42)	94 (0.58)	94 (0.23)	94 (0.35)
14	99 (0.42)	96 (0.81)	95 (0.58)	98 (0.81)	96 (1.15)	95 (1.30)	93 (0.90)	92 (0.46)	92 (0.81)
35	98 (0.83)	95 (1.10)	94 (1.20)	98 (1.10)	95 (1.36)	95 (0.72)	93 (0.83)	91 (0.40)	91 (0.99)

Figure 5-8 – Recovery (%) of carfentanil in all concentrations of formaldehyde at room temperature

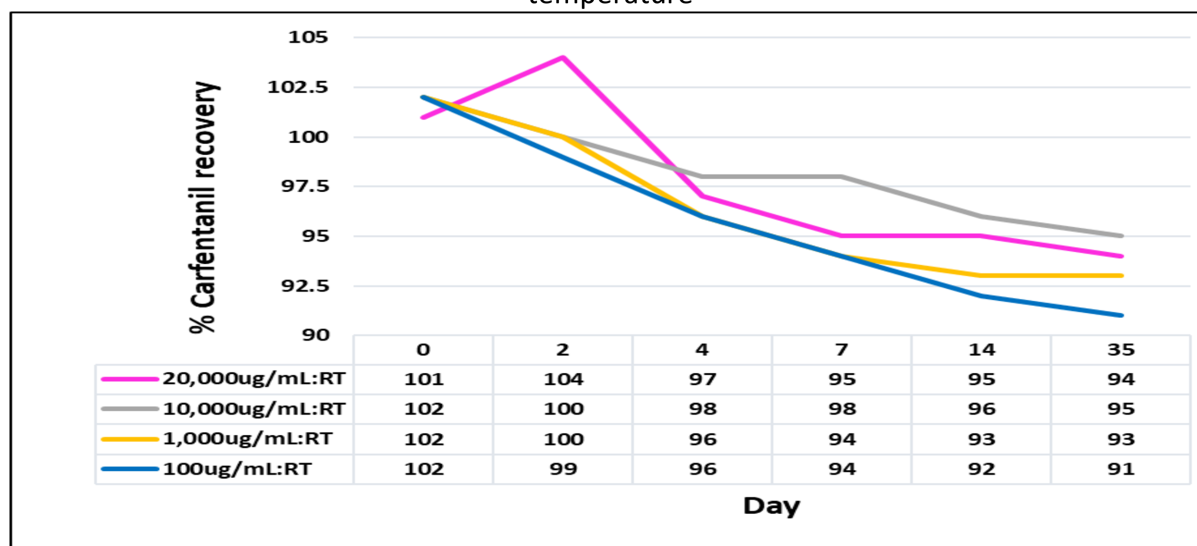
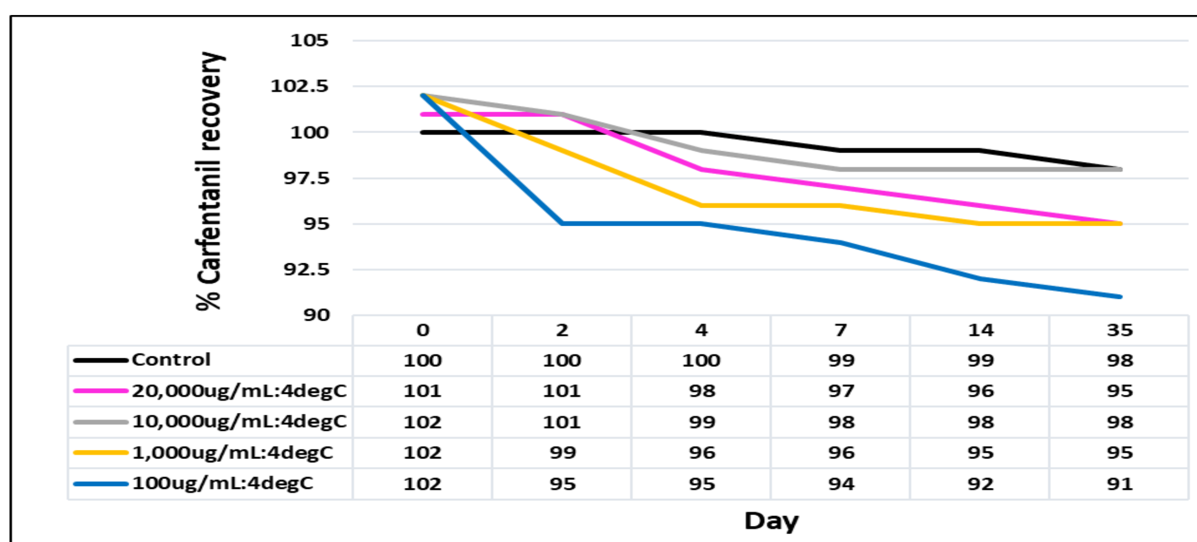


Figure 5-9 – Recovery (%) of carfentanil in all formaldehyde concentrations at 4°C



5.6.1.4 Codeine

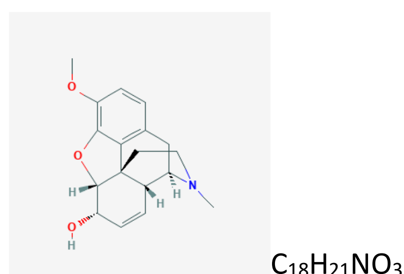

$C_{18}H_{21}NO_3$

Figure 5-10 – Structure and formula of codeine

At least 91% of the starting concentration of codeine was recoverable from all conditions by Day 35. Apparent increases in drug concentration above the 500ng starting concentration were observed in all conditions. This observation was most marked in the 10,000μg/mL formaldehyde solution at RT, where the apparent concentration of codeine was 148% of the starting concentration at day 4 (this phenomenon will be discussed further at 5.6.4, below), although in all cases the concentration recoverable had fallen below 100% by Day 14. The control sample did not exhibit the same increase in concentration but underwent some degree of degradation and was 74% recoverable by Day 35. A summary of the codeine stability results can be found in Table 5-7. Illustrations of the recovery of codeine from all RT and refrigerated concentrations are shown respectively in Figures 5-14 and 5-15, both illustrating the apparent initial increase in drug detection in each condition, followed by subsequent reductions.

5.6.1.5 Fentanyl

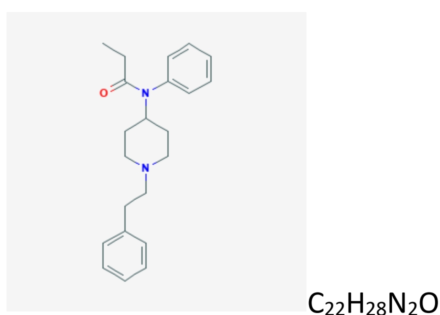
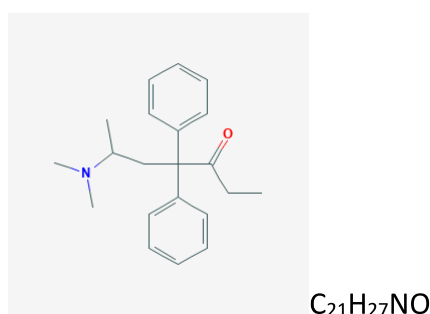

$C_{22}H_{28}N_2O$

Figure 5-11 – Structure and formula of fentanyl

Fentanyl analysis demonstrated apparent increases in concentration of up to 10% in all conditions in the first two days, following which decreases were observed in all conditions. By day 35, the compound was recoverable in all formaldehyde solutions to within 3% of the of the starting concentration. The control solution remained entirely stable for the duration of the experiment. The results for all conditions are summarized in Table 5-8. Illustrations of the comparative concentrations of fentanyl detected in all RT and all refrigerated formaldehyde conditions are presented in Figures 5-16 and 5-17 respectively.

5.6.1.6 Methadone



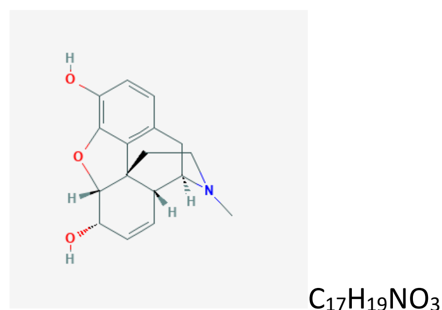
$C_{21}H_{27}NO$

Figure 5-12 – Structure and formula of methadone

Methadone was 95% recoverable from the refrigerated 100µg/mL formaldehyde solution after 35 days, and 85% recoverable in the same concentration of formaldehyde at RT. In refrigerated 1,000µg/mL formaldehyde solution, 86% of the starting concentration was recoverable at Day 35. In most other conditions significant losses were recorded. In 20,000µg/mL refrigerated formaldehyde, while 91% methadone could be recovered on Day 2, and 63% on Day 7, the recovery rate fell to 38% by Day 14 and to 26% by Day 35. In 20,000µg/mL formaldehyde at RT, the respective recovery rates on Days 2, 7 and 14 were 64%, 6% and 1%. Significant losses were also recorded in 10,000µg/mL formaldehyde solution at RT, where only 15% could be recovered by Day 7, 2% by Day 14 and 1% by Day 35. In refrigerated 10,000µg/mL formaldehyde solution, significantly greater recovery was observed, the respective percentages for Days 7, 14 and 35 being 72%, 50% and 42%. The control solution remained relatively stable throughout the experiment, being 97% recoverable on Day 35. A summary of the results in all conditions is given in Table 5-9. Figure

5-18 illustrates the recovery of methadone in all concentrations of formaldehyde at RT, and Figure 5-19 illustrates the smaller losses observed in refrigerated conditions.

5.6.1.7 Morphine



$C_{17}H_{19}NO_3$

Figure 5-13 – Structure and formula for morphine

Significant losses of morphine were observed in 10,000µg/mL and 20,000µg/mL formaldehyde solutions. In 20,000µg/mL formaldehyde, at RT, the total amount of morphine recovered after 35 days was 17%, and in the corresponding 10,000µg/mL formaldehyde solution was 56%. In the corresponding 1,000µg/mL and 100µg/mL formaldehyde solutions, the amounts recovered after 35 days were 88% and 96%, respectively. In all refrigerated conditions, the total amounts of morphine recoverable at Day 35 were marginally greater than in the corresponding RT conditions, being 20%, 63%, 89% and 99% in 20,000, 10,000, 1,000 and 100µg/mL formaldehyde solutions, respectively. An apparent increase in morphine concentration that exceeded the starting concentration by up to 19% was noted in all but the 20,000µg/mL solutions in the first two days of the experiment. The control solution remained relatively stable for the duration of the experiment, and was 96% recoverable by Day 35. Results for all conditions are reproduced in Table 5-10. Illustrations of the stability of morphine in refrigerated conditions given in Figure 5-20, and for RT conditions in Figure 5-21.

Table 5-7 – Recovery (%) of codeine

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	98 (2.19)	118 (14.38)	118 (14.38)	122 (6.89)	122 (6.89)	97 (16.89)	97 (16.89)	107 (6.70)	107 (6.70)
2	93 (4.81)	134 (3.61)	132 (5.87)	147 (10.41)	129 (25.87)	133 (1.03)	142 (25.52)	No data	131 (28.67)
4	89 (19.37)	137 (32.01)	144 (13.80)	148 (8.62)	113 (25.12)	133 (26.33)	101 (20.81)	99 (8.73)	101 (15.15)
7	87 (0.81)	97 (0.46)	120 (0.20)	97 (0.61)	99 (0.81)	99 (0.42)	96 (0.58)	95 (0.23)	94 (0.35)
14	85 (0.42)	96 (0.81)	94 (0.58)	96 (0.81)	99 (1.15)	96 (1.30)	94 (0.90)	92 (0.46)	92 (0.81)
35	74 (0.83)	95 (1.10)	94 (1.20)	96 (1.10)	99 (1.36)	95 (0.72)	93 (0.83)	91 (0.40)	91 (0.99)

Figure 5-14 – Recovery (%) of codeine in all formaldehyde concentrations at room temperature

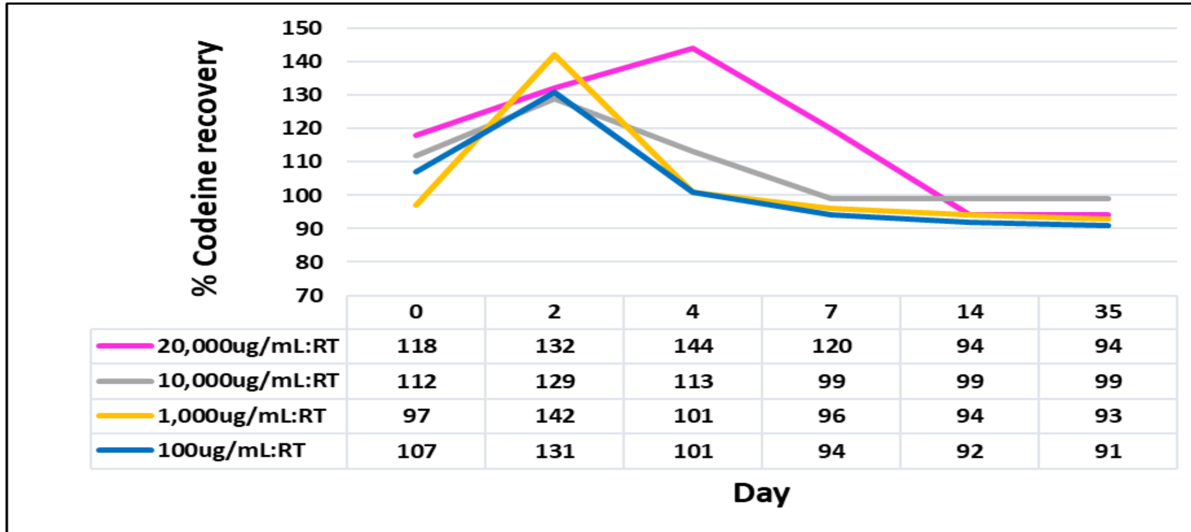


Figure 5-15 – Recovery (%) of codeine in all formaldehyde concentrations at 4°C

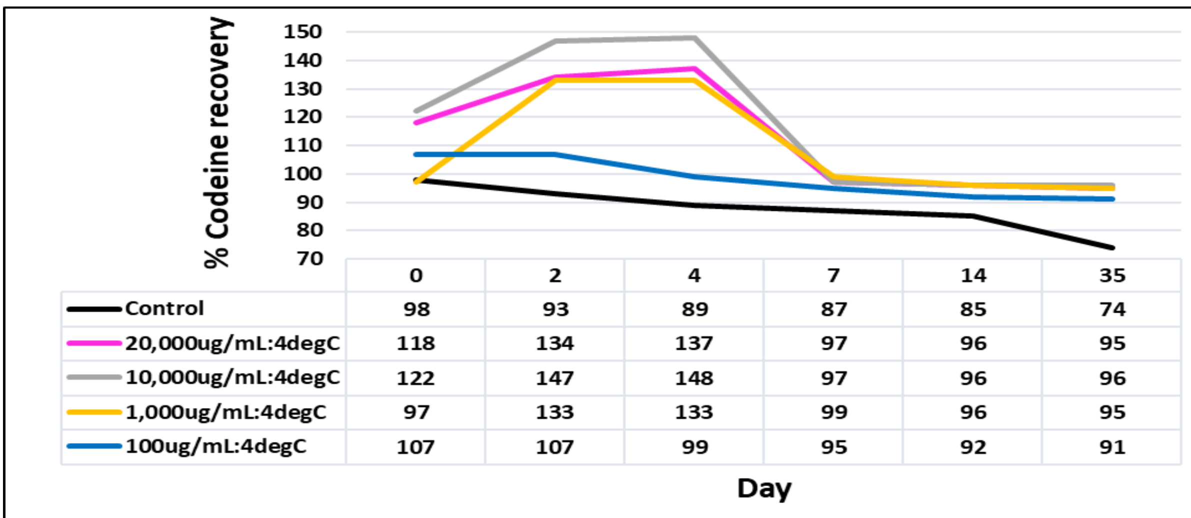


Table 5-8 – Recovery (%) of fentanyl

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (1.31)	100 (1.04)	100 (1.04)	100 (0.42)	100 (0.42)	100 (0.64)	100 (0.64)	100 (0.20)	100 (0.20)
2	100 (0.81)	104 (0.35)	108 (0.12)	104 (0.58)	110 (0.42)	102 (0.23)	108 (0.35)	103 (0.42)	107 (0.12)
7	100 (0.23)	103 (0.64)	103 (0.42)	106 (0.61)	104 (0.87)	104 (1.17)	105 (0.31)	106 (4.88)	106 (0.35)
14	100 (0.61)	102 (0.50)	101 (3.21)	105 (0.42)	103 (0.92)	104 (0.92)	103 (0.42)	106 (0.20)	106 (0.12)
35	100 (0.81)	101 (0.72)	101 (0.31)	103 (0.20)	101 (0.31)	100 (0.87)	101 (0.50)	104 (0.23)	103 (0.81)

Figure 5-16 – Recovery (%) of fentanyl in all formaldehyde concentrations at room temperature

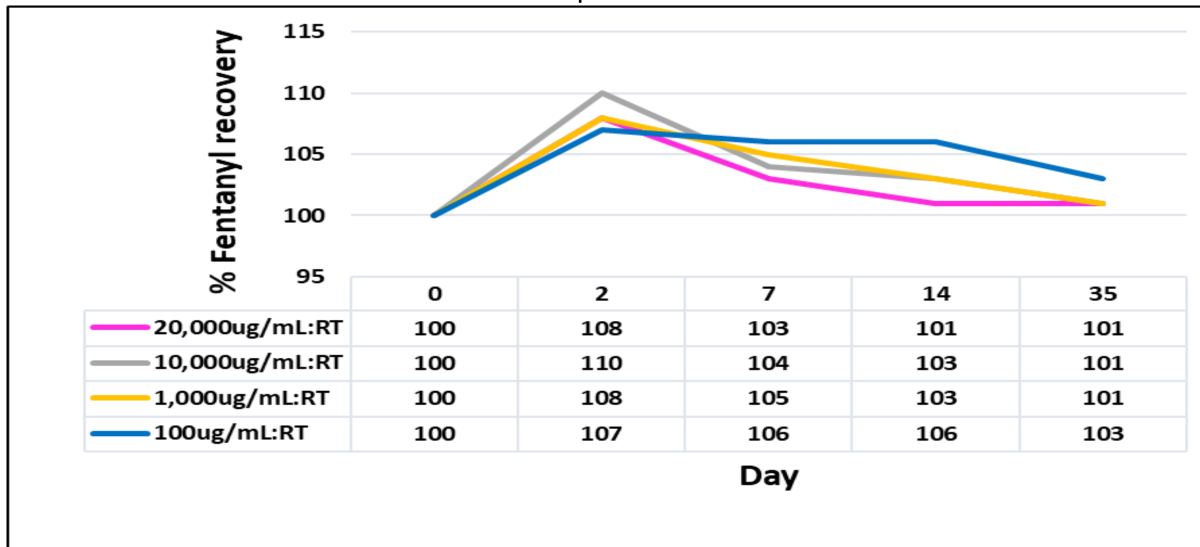


Figure 5-17 – Recovery (%) of fentanyl in all formaldehyde concentrations at 4°C

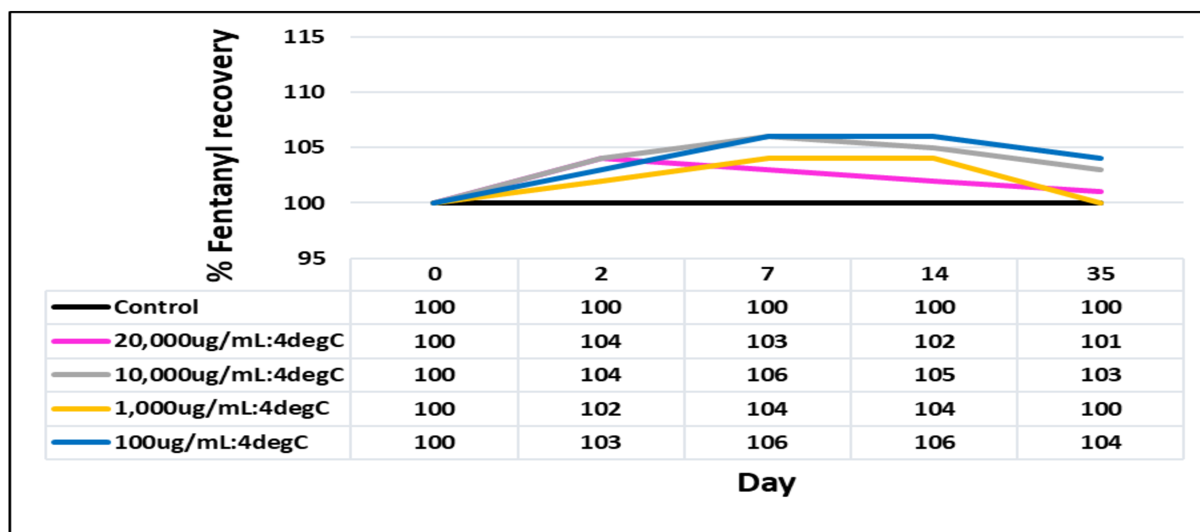


Table 5-9 – Recovery (%) of methadone

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (0.20)	96 (0.58)	96 (0.58)	97 (0.23)	97 (0.23)	97 (0.20)	97 (0.20)	98 (0.31)	98 (0.31)
2	100 (0.72)	91 (0.42)	64 (7.51)	95 (7.51)	72 (0.12)	90 (2.09)	88 (0.50)	99 (0.40)	92 (0.42)
7	100 (0.12)	63 (0.50)	6 (0.12)	72 (3.88)	15 (0.81)	90 (0.40)	74 (0.53)	97 (0.20)	92 (0.23)
14	99 (0.23)	38 (0.50)	1 (0.00)	50 (3.75)	2 (0.12)	No data	56 (0.42)	97 (0.23)	87 (0.00)
35	97 (0.64)	26 (0.31)	1 (0.00)	42 (0.20)	1 (0.00)	86 (0.12)	33 (0.42)	95 (0.35)	85 (0.20)

Figure 5-18 – Recovery (%) of methadone in all formaldehyde concentrations at room Temperature

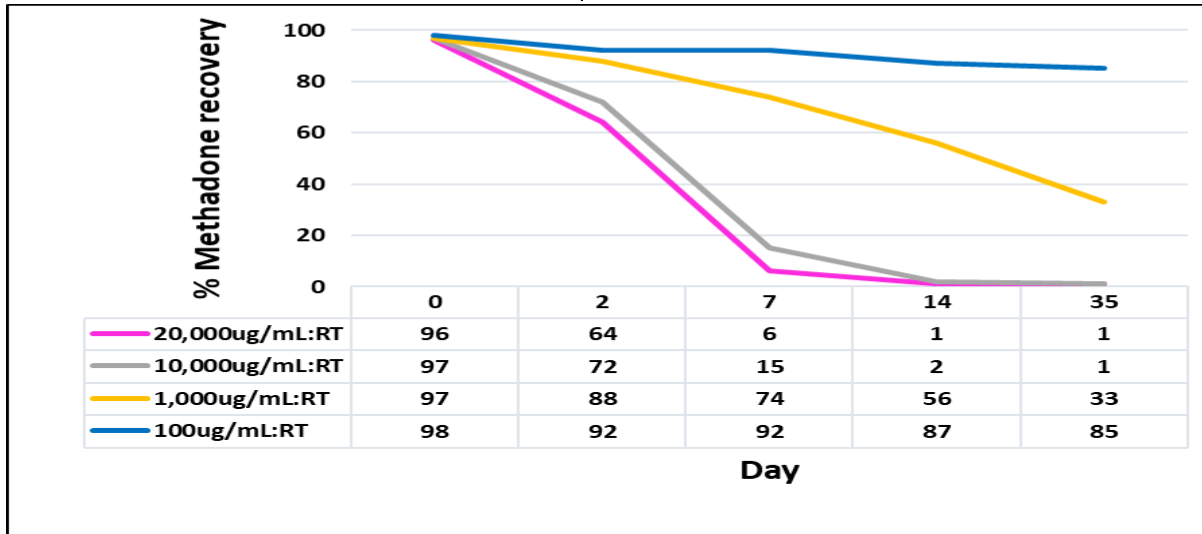


Figure 5-19 – Recovery (%) of methadone in all formaldehyde concentrations at 4°C

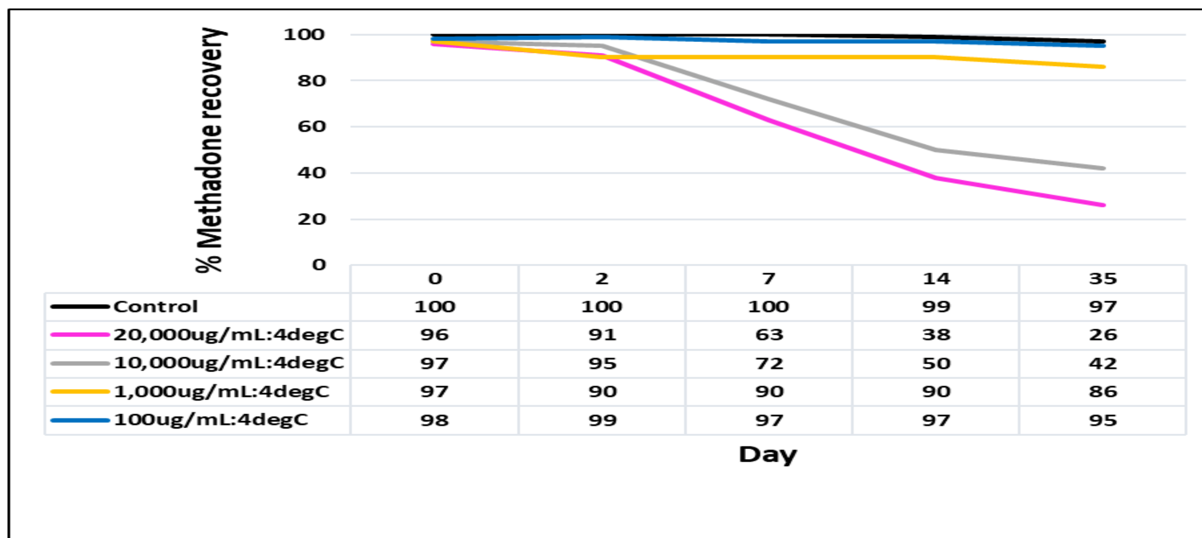


Table 5-10 – Recovery (%) of morphine

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (1.91)	62 (1.83)	62 (1.83)	114 (6.41)	114 (6.41)	115 (4.36)	115 (4.36)	111 (3.40)	111 (3.40)
2	100 (1.21)	62 (0.40)	50 (0.57)	109 (9.02)	103 (12.29)	119 (8.29)	114 (9.09)	115 (6.41)	108 (7.53)
7	99 (2.27)	44 (2.19)	41 (2.87)	85 (5.02)	78 (1.40)	106 (3.87)	102 (0.99)	108 (4.31)	104 (2.23)
14	98 (4.41)	36 (0.35)	31 (1.53)	71 (1.33)	69 (0.42)	106 (6.82)	97 (3.14)	106 (7.70)	101 (7.30)
35	96 (3.30)	20 (2.83)	17 (3.54)	63 (4.53)	56 (2.95)	89 (0.57)	88 (4.80)	99 (1.94)	96 (2.55)

Figure 5-20 – Recovery (%) of morphine in all formaldehyde concentrations at 4°C

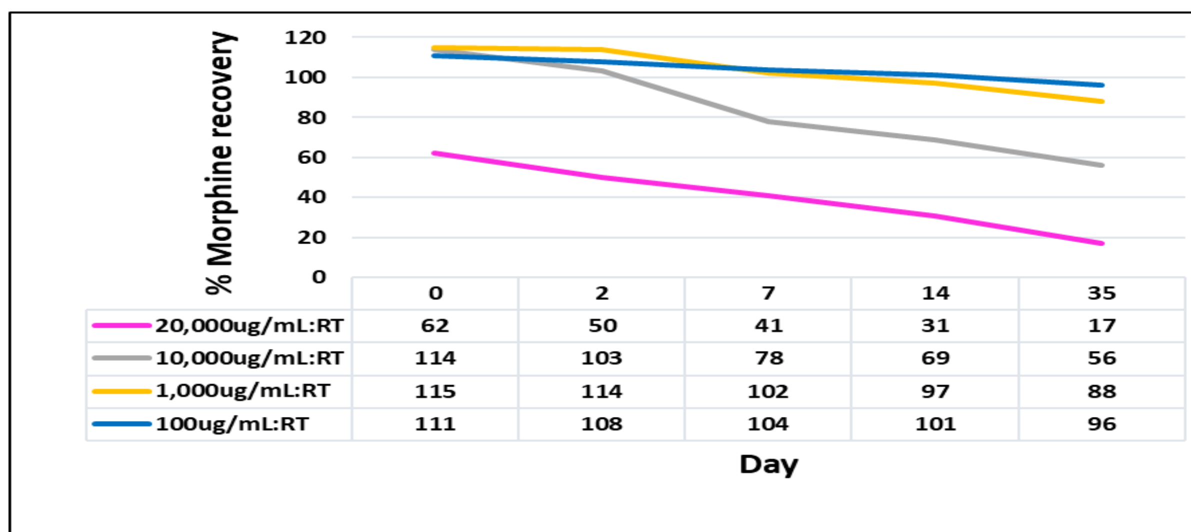
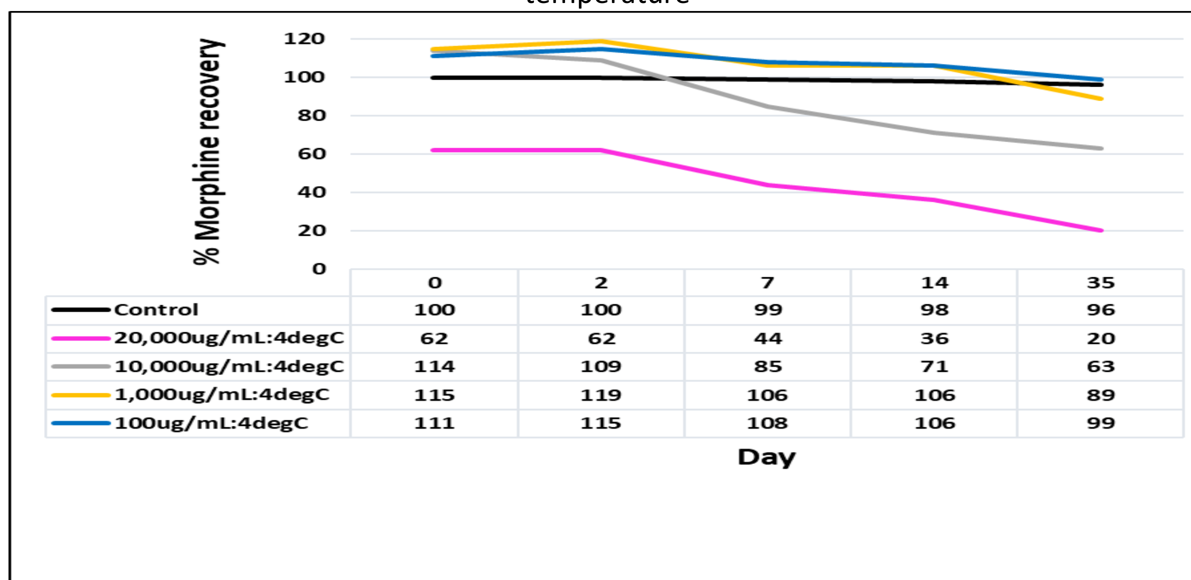


Figure 5-21 – Recovery (%) of morphine in all formaldehyde concentrations at room temperature



5.6.2 Opiates and opioids discussion

As mentioned above, alfentanil was extremely stable in all conditions. Indeed, in 1,000µg/mL and 100µg/mL formaldehyde solutions – the range of concentrations detected in the Cambridge hard-fix cadavers discussed in Chapter 4 – 100% of the starting concentration was recoverable at Day 35 in RT as well as in refrigerated conditions (the control sample exhibited 100% recovery on Day 35). Carfentanil, similarly, exhibited considerable stability, more than 91% of the starting concentration being recoverable in all conditions by Day 35 (control recovery: 98%), and fentanyl, as already mentioned, remained essentially unchanged in all conditions for the duration of the experiment (control recovery: 100%).

Neither alfentanil nor carfentanil are addressed in the existing embalming literature. In the case of fentanyl, however, Rohrig (1998) reports that in a body exhumed some 24 months after embalming, the concentration of the drug detected in the liver was 33.6ng/g, compared to 45.4ng/g at autopsy, representing a loss of almost 26%. In the current experiment, although apparently small initial increases in fentanyl concentration (this phenomenon is discussed further at 5.6.4, below) were noted in all conditions, by the end of the experiment no losses were recorded in any concentration of formaldehyde. Although not directly comparable, the results of the current experiment would at least appear to accord with the relative stability of the compound reported by Rohrig in his single case report. It is difficult, however, to make a direct comparison, since Rohrig reports nothing of the either the concentration of formaldehyde used in the embalming, or of the condition of the exhumed body. Although increasing concentrations of formaldehyde commonly result in greater degrees of drug degradation, bacterial decomposition, as discussed in Chapter 1, can also have a profound effect on stability, and the physical process of autolysis can in itself cause a dilutional effect that would be sufficient to cause significant, artifactual anomalies on analysis. Direct comparison is thus not possible.

6-MAM was comparatively less stable, although even in the refrigerated 20,000µg/mL formaldehyde solution, 53% of the starting concentration was still recoverable by Day 35, and 46% in RT conditions. In weaker concentration of formaldehyde, however, better recoveries were recorded. In 1,000µg/mL formaldehyde, for example, nearly 60% of the starting

concentration was recoverable on Day 14, and in 100µg/mL formaldehyde, almost 100% was recoverable on Day 14 (control recovery: 98%). These are important findings, since 1,000µg/mL is the indicative concentration of formaldehyde detected in the hard-fix Cambridge cadavers, and 100µg/mL, the indicative maximum concentration in soft-fix and, by extension, repatriation embalming. Given, furthermore, that the majority of repatriations are completed within around two weeks of death, it seems likely that 6-MAM, as the principal metabolite of diamorphine, and, thus, definitive evidence of heroin use, will be readily detectable in repatriation-embalmed bodies.

Methadone was very unstable under a number of conditions, but very much more stable in those likely to be encountered in repatriation cases. At one end of the results spectrum, in 20,000µg/mL and 10,000µg/mL RT formaldehyde solutions, only 1% and 2%, respectively, of the starting concentrations were detectable at Day 14. In the same concentrations of formaldehyde at 4°C, the amounts recoverable increased to 38% and 50% (control recovery: 99%). In 1,000µg/mL formaldehyde, by contrast, 56% of the starting concentration of methadone was recoverable at Day 14 in RT conditions. In 100µg/mL formaldehyde, this figure increased to 87% at RT and 97% when refrigerated. Like 6-MAM, therefore, it might be reasonably concluded that methadone should be readily recoverable from repatriation-embalmed bodies.

As discussed in Chapter 2, the literature contains an early paper, Fransioli, Szabo and Sunshine (1980), that provides qualitative confirmation of the presence of methadone in a 10% formalin solution (37,000µg/mL formaldehyde) refrigerated at 4°C for 19 months. Although reporting concentrations of between 0.51-2.3µg/g in the various tissues that were analysed, Fransioli did not establish starting concentrations in any tissue, merely presenting his findings as a case study proving the detection of methadone. Comparison of Fransioli's results with those of the current experiment is thus not possible.

In the degradation study undertaken as part of the current experiment, the methadone-formaldehyde mixture appeared to lead to the production of two conversion products: one with an t_R of 5.298 and an $[M+H]^+$ *m/z* of 340.2275, and the second with an t_R of 5.653 and an $[M+H]^+$ *m/z* of 370.2381. The first of these conversion products, with a Da of exactly 30 more

than the parent compound (methadone $[M+H]^+$ m/z = 310.166), can probably be assumed to be the mono formaldehyde adduct: $[M+HCHO+H]^+$ (formaldehyde $[M+H]^+$ m/z = 30.031). The second product, with a Da of 60 more than the parent compound, is probably the bis formaldehyde adduct: $[M+2HCHO+H]^+$.

Like 6-MAM and methadone, the morphine results in the current experiment were also equivocal, depending on the particular condition. The smallest amount recoverable was 17% at Day 35 in the 20,000µg/mL formaldehyde, RT, condition (control recovery: 96% at 4°C). In conditions that are likely to be more representative of repatriation-embalmed bodies, however, the amount of drug recovered is in each case was much greater. In 1,000µg/mL formaldehyde, for example, around 100% of the compound was recoverable at Day 14, in both refrigerated and RT conditions, and by Day 35, the amount recoverable at both temperatures was still only slightly less than 90%. In 100µg/mL formaldehyde, furthermore, the compound appeared to be almost entirely stable until Day 14, only falling very slightly below 100% by Day 35. There is obvious forensic significance in these findings.

Of the opiates and opioids examined in the current experiment, morphine has received the greatest attention in the embalming literature. In its earliest mention, in Levine et al. (1994), a single case report details merely the detection of the compound in a body exhumed approximately three weeks after death, embalming and burial. Similarly, the most recent report of morphine in the literature, Alunni-Perret et al. (2003), is another case report, detailing the quantitative findings in bile and liver samples taken from a body nine days after embalming. Cingolani et al. (2001) and Xiang et al (2001), however, both report on the stability of morphine in conditions which, although somewhat different to those employed in the current study, do allow for some degree of comparison. In the case of Cingolani, he reported recovery levels of approximately 30% in kidney samples, fixed in 10% formalin solution (37,000µg/mL), buffered to pH 7.0, and stored at RT for 12 weeks. When also analysing the formalin in which the tissue samples had been stored, however, Cingolani reported an almost 100% recovery of morphine from the tissue and solution combined. By contrast, Xiang reported recovery rates of between around only 5% and 55% from several tissue types fixed in 10% un-buffered formalin for four months prior to analysis. Finally,

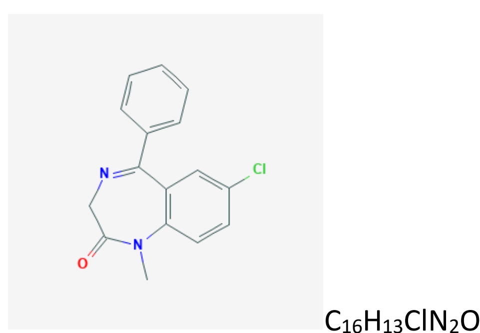
Yokchue, in her 2016 thesis, reported a recovery of around 65% from a 10% formalin solution, buffered to pH 7.4 and kept at RT for 30 days.

In the current experiment, the closest condition to those found in the morphine literature, given that both Cingolani and Xiang use 10% formalin is the 20,000µg/mL formaldehyde solution. Here, after 35 days, the respective recovery rates from RT and refrigerated samples were very similar, at 17% and 20%. Although in marked contrast to Cingolani's findings, and certainly lower than the recovery reported by Yokchue, the results of the current experiment are consistent, to the extent that direct comparison can be usefully made, with those of Xiang.

The only other opiate studied in the current experiment to have received previous mention is codeine, the stability of which was reported in Yokchue (2016). Here, a recovery of 71% was made from a solution of 10% formalin, buffered to pH 7.4, after 30 days at RT. In the current experiment, 94% of the starting concentration of codeine was recovered from the 20,000µg/mL formaldehyde solution at RT after 35 days (control recovery: 74% at 4°C). This recovery, if somewhat greater than that reported by Yokchue, confirms nevertheless that the molecule is largely stable in formaldehyde over relatively short periods of time.

5.6.3 Benzodiazepines and sedatives results

5.6.3.1 Diazepam



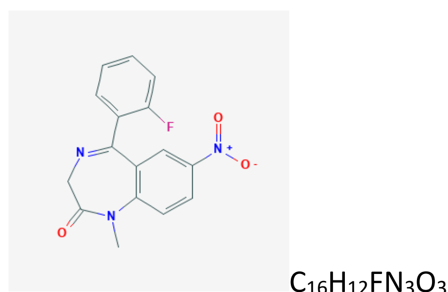
$C_{16}H_{13}ClN_2O$

Figure 5-22 – Structure and formula of diazepam

Diazepam was highly stable in all concentrations of formaldehyde within both refrigerated and RT conditions. In 1,000µg/mL and 100µg/mL solutions, the drug exhibited no degradation within the first seven days, and remained 98% recoverable by Day 35 in each case. In 10,000µg/mL solutions 96% was recoverable in the Day 35 RT solution, and 98% in the

refrigerated solution. In 20,000µg/mL formaldehyde, the respective recoveries were 95% and 98%. The control solution remained entirely stable throughout the experiment. Results for all conditions are shown in Table 5-11, and the relative stability of diazepam in each concentration of formaldehyde at RT and at 4°C is illustrated in Figures 5-28 and 5-29, respectively.

5.6.3.2 Flunitrazepam



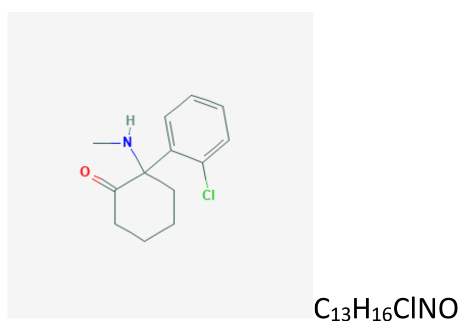
$C_{16}H_{12}FN_3O_3$

Figure 5-23 – Structure and formula of flunitrazepam

Recovery of flunitrazepam was at least 89% after 35 days. In both 1,000µg/mL and 100µg/mL formaldehyde, recovery was never lower than 100% until Day 14, and was never lower than 92% at Day 35. In 20,000 µg/mL formaldehyde, the respective recoveries from room

temperature and refrigerated solutions were 89% and 98%. The control sample remained relatively stable throughout the experiment, being 97% recoverable on Day 35. Results from all conditions are given in Table 5-12. Illustrations of the stability of flunitrazepam in all RT and refrigerated concentrations of formaldehyde are given in Figures 5-30 and 5-31, respectively.

5.6.3.3 Ketamine

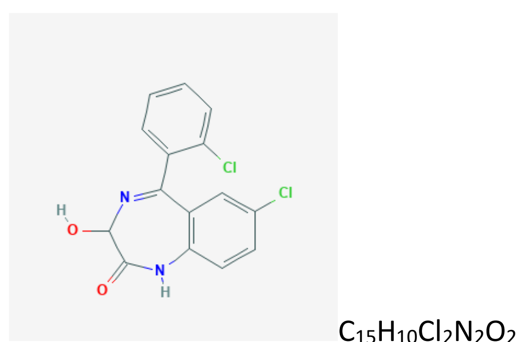


$C_{13}H_{16}ClNO$

Figure 5-24 – Structure and formula of ketamine

Ketamine remained relatively stable in all conditions. In the refrigerated 20,000µg/mL formaldehyde solution, 92% of the drug was recoverable at Day 35; in the corresponding RT solution, the recovery was 88%. In 100µg/mL and 1,000µg/mL formaldehyde solutions, the recovery rates were at least 91% in both refrigerated and RT conditions. The control solution remained relatively stable throughout the experiment, being 96% recoverable at Day 35. Results of recovery from all conditions are given in Table 5-13, and illustrations of the stability of the drug in all concentrations of formaldehyde in RT and refrigerated conditions are given in Figures 5-32 and 5-33, respectively.

5.6.3.4 Lorazepam

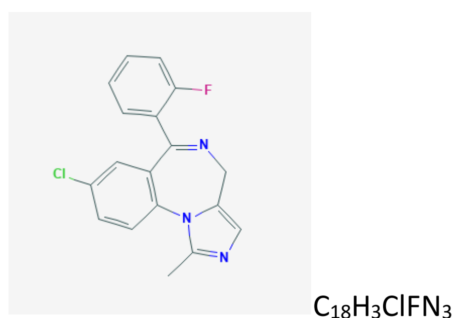


$C_{15}H_{10}Cl_2N_2O_2$

Figure 5-25 – Structure and formula of lorazepam

Lorazepam was relatively stable in 1,000µg/mL and 100µg/mL formaldehyde solutions, where the Day 35 recovery rates in RT conditions were 83% and 84%, respectively. In 10,000µg/mL and 20,000µg/mL formaldehyde, the respective RT recovery rates were 68% and 60%. Recovery was proportionately greater in each refrigerated condition, being 73%, 81% and 87% in, respectively, 20,000, 10,000 and 1,000µg/mL formaldehyde solutions (no data were recoverable for the 100µg/mL formaldehyde RT condition for days 2, 7, 14 and 35 of the experiment). The control solution remained stable throughout the experiment, being 95% recoverable on Day 35. Results for the recovery of lorazepam in all conditions are given in Table 5-14, and illustrations of the stability of the drug in all concentrations of formaldehyde at RT are given in Figure 5-34, and at 4°C in Figures 5-35.

5.6.3.5 Midazolam

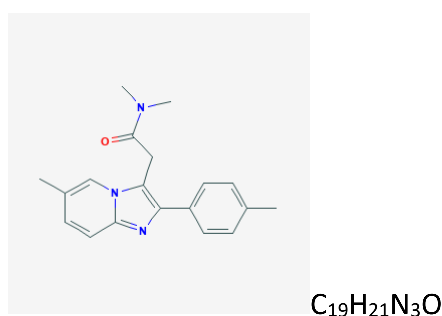


$C_{18}H_3ClFN_3$

Figure 5-26 – Structure and formula of midazolam

Near 100% recovery of midazolam was recorded in all concentrations of formaldehyde in refrigerated and RT conditions. At Day 35, 100% recovery was noted in both 20,000µg/mL formaldehyde solutions, and in the refrigerated 10,000µg/mL formaldehyde solution. In 1,000µg/mL and 100µg/mL formaldehyde solutions, the recovery at Day 35 was between 96% and 98%. The control solution remained entirely stable for the duration of the experiment, being 100% recoverable on Day 35. Results from all conditions are shown in Table 5-15. Figures 5-36 and 5-37 illustrate the comparative stability of the compound in all conditions.

5.6.3.6 Zolpidem



$C_{19}H_{21}N_3O$

Figure 5-27 – Structure and formula of zolpidem

Zolpidem was relatively stable in all conditions. In all concentrations of formaldehyde, both refrigerated and RT, recovery was never less than 95% by Day 35. In many conditions, there was an apparent increase in concentration between Day 0 and Day 4, in excess of the starting concentration, before concentrations reduced from Day 7. Recovery rates in refrigerated and RT samples in each respective concentration of formaldehyde were very similar. The control solution remained relatively stable throughout the experiment, and was 96% recoverable on

Day 35. The results for all conditions are given in Table 5-16, and illustrations of the recovery in all formaldehyde concentrations at RT and at 4°C are given, respectively, in Figures 5-38 and 5-39.

Table 5-11 – Recovery (%) of diazepam

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (0.85)	100 (2.46)	100 (2.46)	100 (0.42)	100 (0.42)	100 (1.10)	100 (1.10)	101 (0.85)	101 (0.85)
2	100 (1.97)	100 (2.72)	99 (12.43)	100 (1.01)	100 (3.5)	100 (2.66)	100 (0.28)	101 (1.55)	101 (2.03)
4	100 (0.64)	100 (1.20)	99 (2.32)	100 (0.95)	99 (0.60)	100 (0.70)	100 (0.20)	100 (1.27)	101 (1.71)
7	100 (0.61)	100 (0.50)	98 (0.20)	100 (0.50)	99 (1.22)	100 (0.58)	100 (0.35)	100 (0.31)	101 (0.23)
14	100 (0.42)	99 (0.40)	96 (0.64)	98 (0.12)	97 (2.66)	99 (0.61)	99 (2.20)	99 (0.40)	99 (1.80)
35	100 (0.42)	98 (0.31)	95 (0.81)	98 (0.83)	96 (1.12)	98 (0.42)	98 (0.72)	98 (0.20)	98 (0.58)

Figure 5-28 – Recovery (%) of diazepam in all formaldehyde concentrations at room temperature

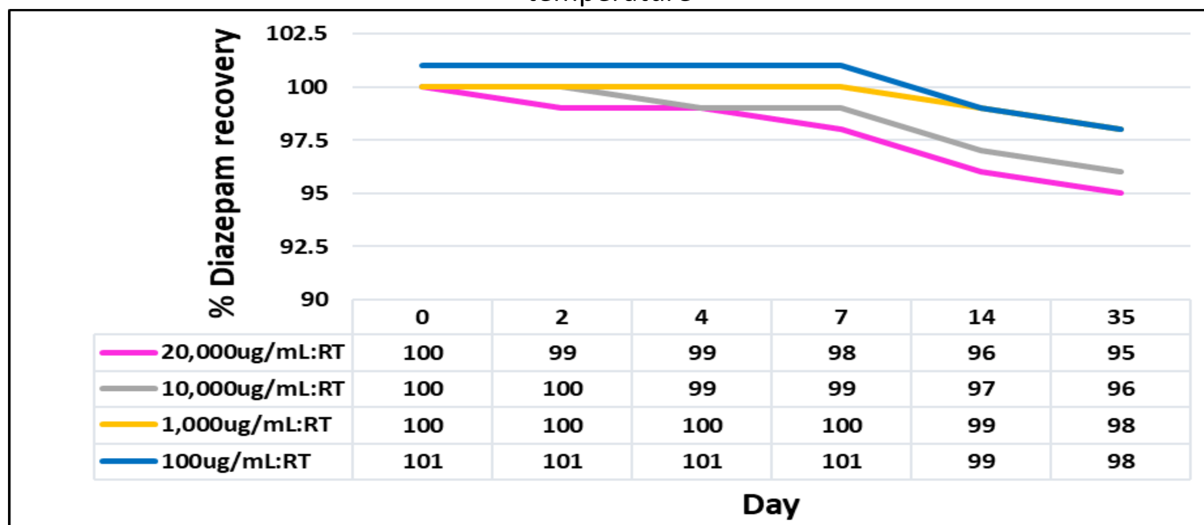


Figure 5-29 – Recovery (%) of diazepam in all formaldehyde concentrations at 4°C

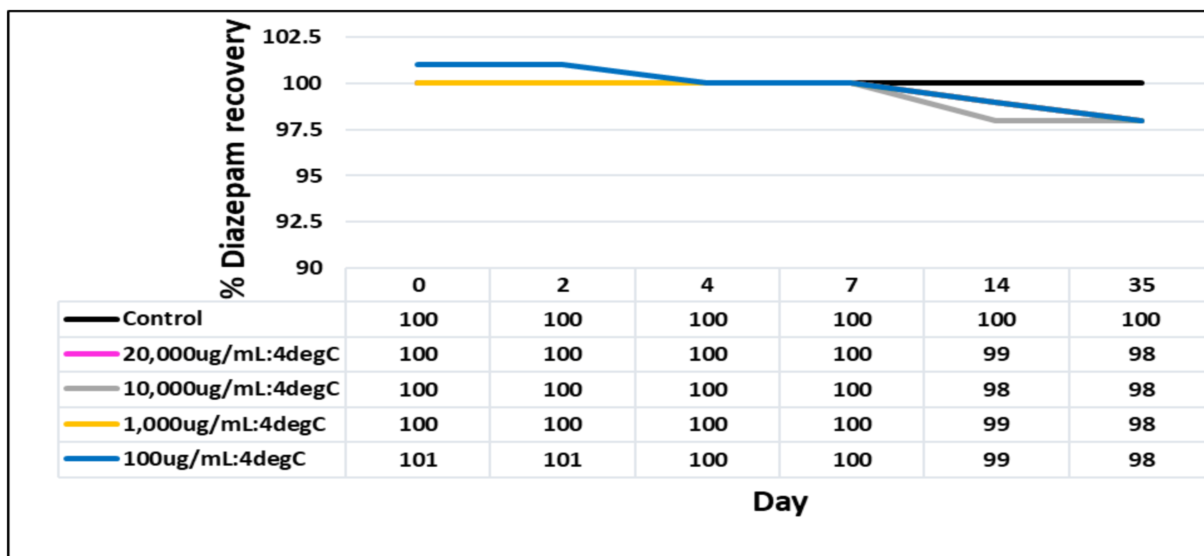


Table 5-12 – Recovery (%) of flunitrazepam

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (0.61)	100 (0.20)	100 (0.20)	100 (0.58)	100 (0.58)	100 (0.50)	100 (0.50)	100 (1.03)	100 (1.03)
2	100 (2.62)	100 (1.25)	104 (0.72)	106 (12.89)	104 (13.12)	106 (12.25)	121 (0.72)	No data	114 (13.17)
4	100 (0.81)	101 (0.31)	96 (0.72)	104 (1.50)	103 (0.31)	104 (0.64)	110 (0.99)	100 (0.83)	103 (10.74)
7	100 (1.81)	101 (0.40)	95 (3.26)	107 (0.35)	105 (0.50)	102 (0.42)	105 (0.35)	100 (0.50)	102 (0.46)
14	99 (2.26)	100 (0.20)	92 (0.46)	104 (0.50)	100 (0.90)	99 (0.23)	99 (0.61)	95 (0.31)	99 (1.14)
35	97 (0.42)	98 (0.81)	89 (0.20)	101 (0.64)	96 (0.83)	95 (0.42)	95 (0.60)	92 (0.42)	95 (0.31)

Figure 5-30 – Recovery (%) of flunitrazepam in all formaldehyde concentrations at room temperature

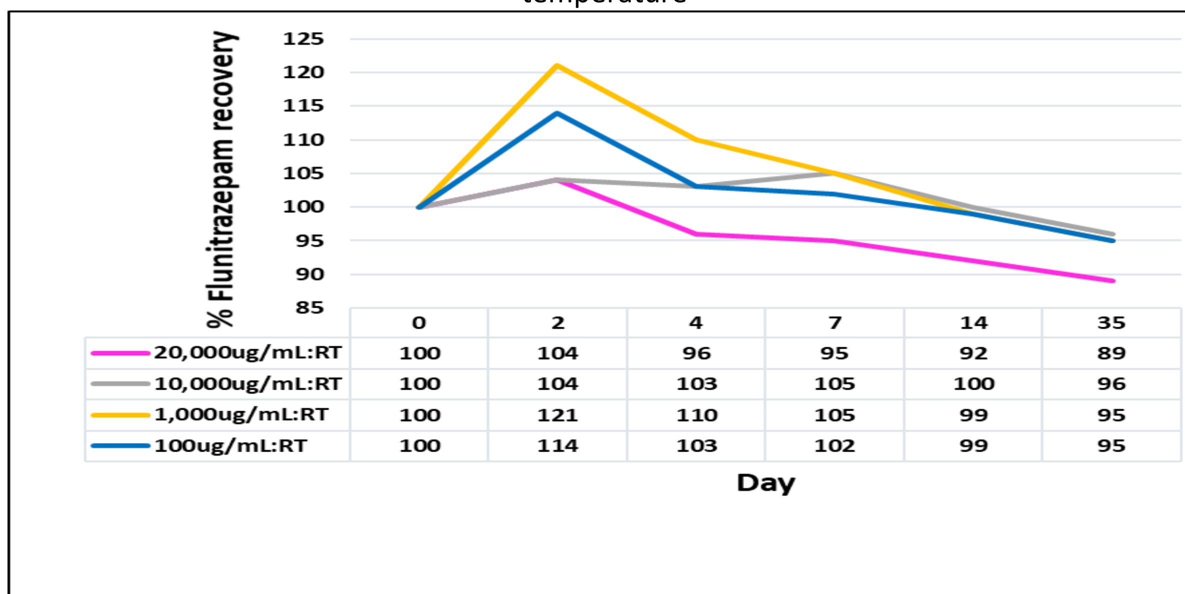


Figure 5-31 – Recovery (%) of flunitrazepam in all formaldehyde concentrations at 4°C

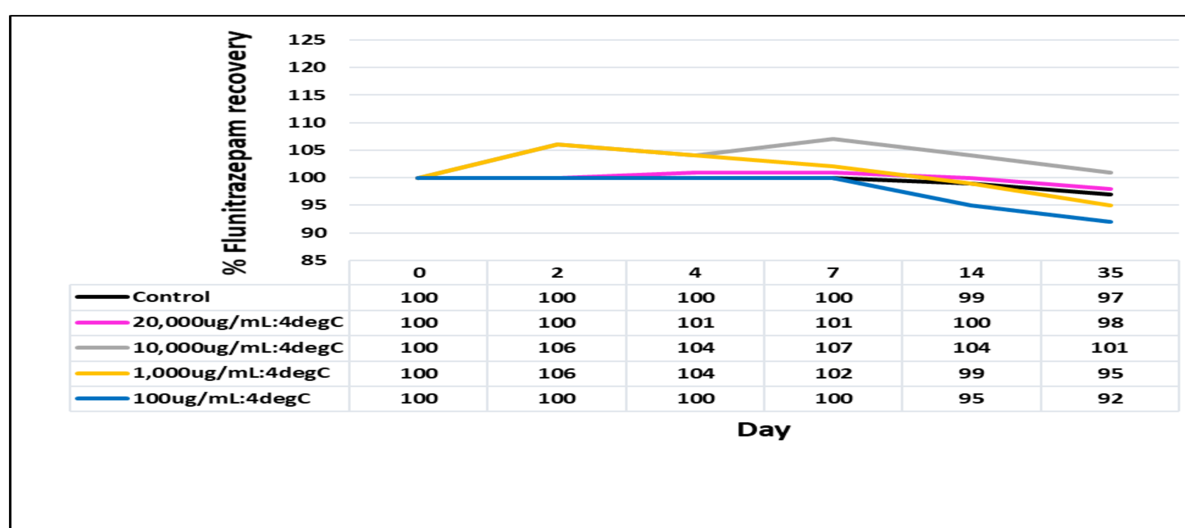


Table 5-13 – Recovery (%) of ketamine

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (0.42)	98 (0.87)	98 (0.87)	100 (0.35)	100 (0.35)	100 (0.60)	100 (0.60)	100 (0.58)	100 (0.58)
2	100 (0.35)	97 (1.22)	102 (1.31)	102 (0.46)	101 (0.40)	101 (5.56)	100 (1.17)	99 (0.40)	100 (1.42)
7	99 (1.22)	97 (0.61)	92 (0.70)	99 (10.82)	99 (9.22)	101 (0.40)	97 (1.39)	99 (0.58)	100 (0.83)
14	99 (0.31)	96 (1.75)	91 (0.20)	97 (5.15)	97 (3.56)	No data	95 (0.81)	97 (1.25)	97 (1.03)
35	96 (0.76)	92 (1.01)	88 (0.60)	94 (1.10)	91 (0.61)	94 (0.12)	91 (0.23)	93 (0.20)	94 (0.72)

Figure 5-32 – Recovery (%) of ketamine in all formaldehyde concentrations at room temperature

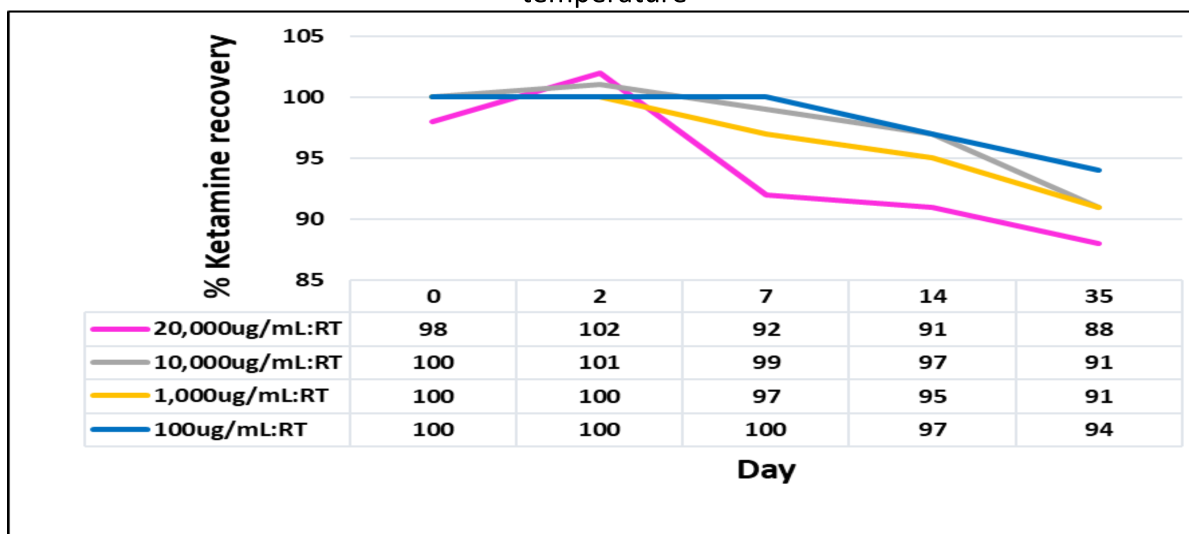


Figure 5-33 – Recovery (%) of ketamine in all formaldehyde concentrations at 4°C

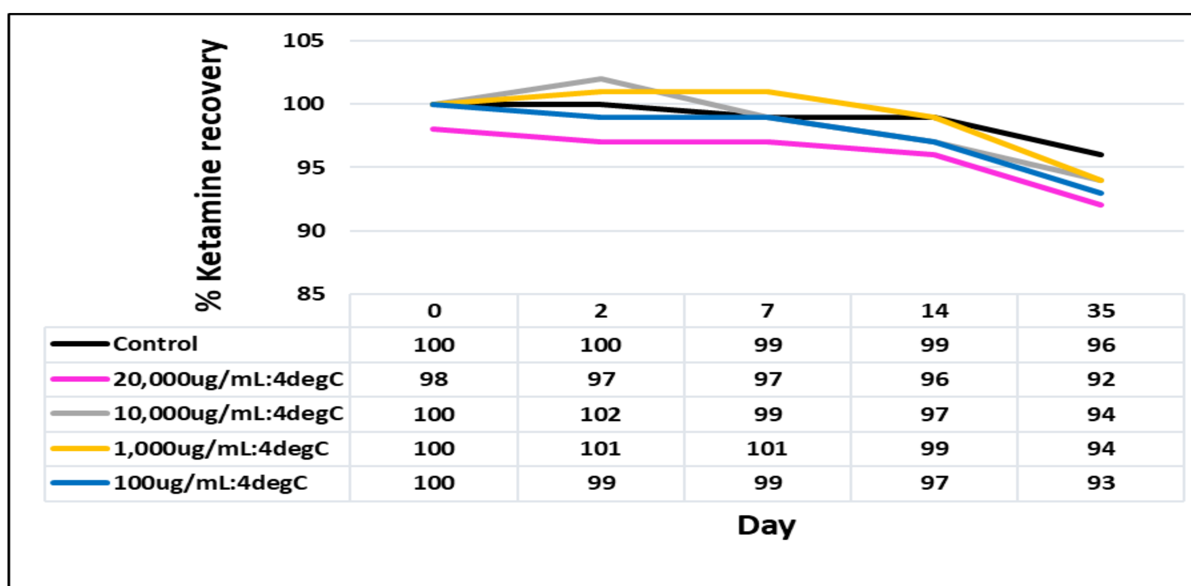


Table 5-14 – Recovery (%) of lorazepam

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (2.37)	88 (1.79)	88 (1.79)	89 (0.50)	89 (0.50)	98 (1.06)	98 (1.06)	98 (1.51)	98 (1.51)
2	100 (1.30)	83 (0.60)	70 (0.90)	85 (0.81)	84 (1.29)	97 (0.46)	93 (1.21)	No data	92 (2.95)
7	99 (0.58)	79 (0.58)	68 (0.90)	82 (0.72)	78 (0.61)	97 (0.95)	91 (0.64)	No data	92 (0.99)
14	98 (1.21)	77 (1.14)	67 (0.60)	81 (0.46)	75 (0.72)	95 (0.31)	87 (1.03)	No data	87 (0.50)
35	95 (1.14)	73 (0.20)	60 (0.61)	81 (1.03)	68 (0.83)	87 (1.80)	83 (0.76)	No data	84 (0.50)

Figure 5-34 – Recovery (%) of lorazepam in all formaldehyde concentrations at room temperature

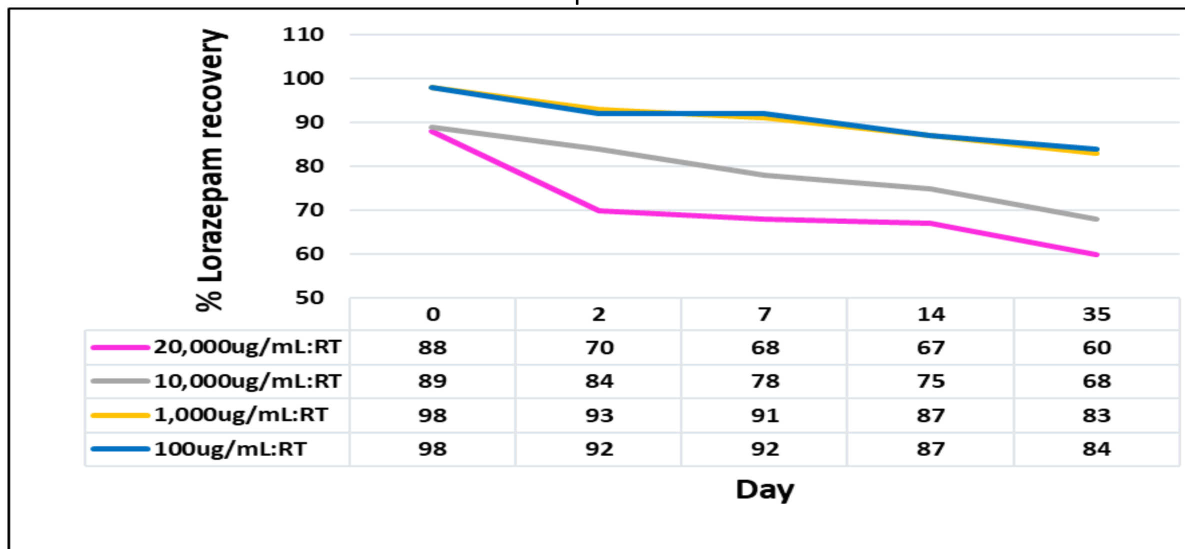


Figure 5-35 – Recovery (%) of lorazepam in all formaldehyde concentrations at 4°C

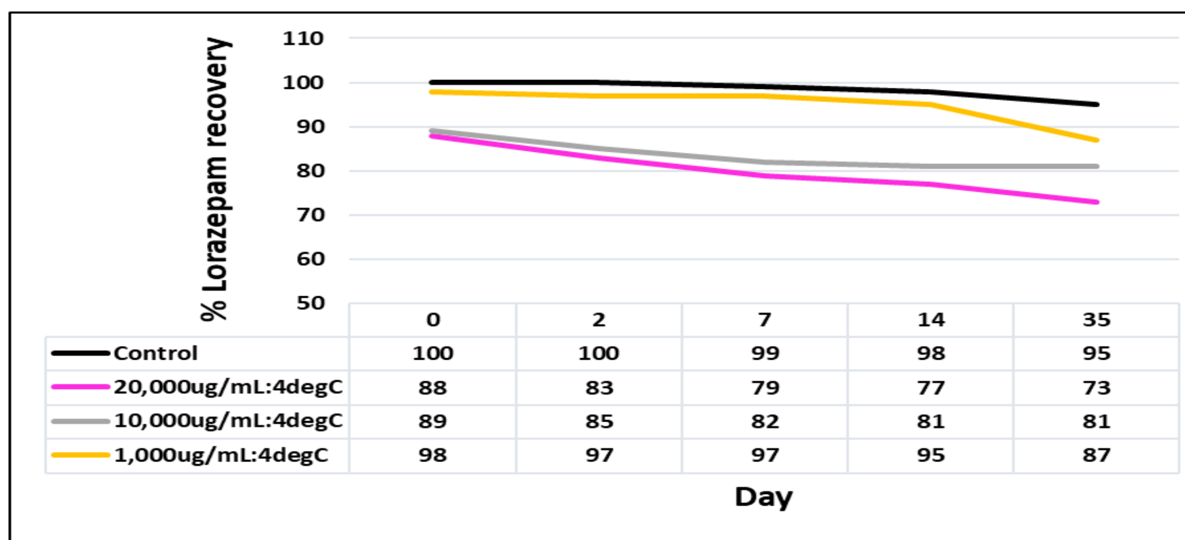


Table 5-15 – Recovery (%) of midazolam

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (0.61)	100 (0.72)	100 (0.72)	101 (0.46)	101 (0.46)	100 (0.20)	100 (0.20)	102 (1.68)	102 (1.68)
2	100 (0.12)	101 (0.81)	101 (0.53)	100 (0.31)	102 (0.40)	100 (0.61)	101 (0.85)	99 (0.31)	100 (0.20)
4	100 (0.61)	100 (0.53)	100 (1.10)	100 (0.58)	100 (0.50)	98 (0.46)	98 (1.68)	99 (0.69)	98 (1.11)
7	100 (0.70)	101 (1.06)	100 (1.33)	101 (1.04)	101 (0.42)	101 (0.60)	98 (1.62)	99 (0.64)	98 (0.60)
14	100 (0.12)	100 (1.06)	100 (0.80)	100 (0.80)	100 (0.31)	98 (0.42)	98 (0.12)	98 (0.31)	98 (0.12)
35	100 (0.53)	100 (0.23)	100 (1.25)	100 (0.61)	99 (0.81)	96 (0.35)	97 (2.54)	97 (0.64)	98 (0.50)

Figure 5-36 – Recovery (%) of midazolam in all formaldehyde concentrations at 4°C

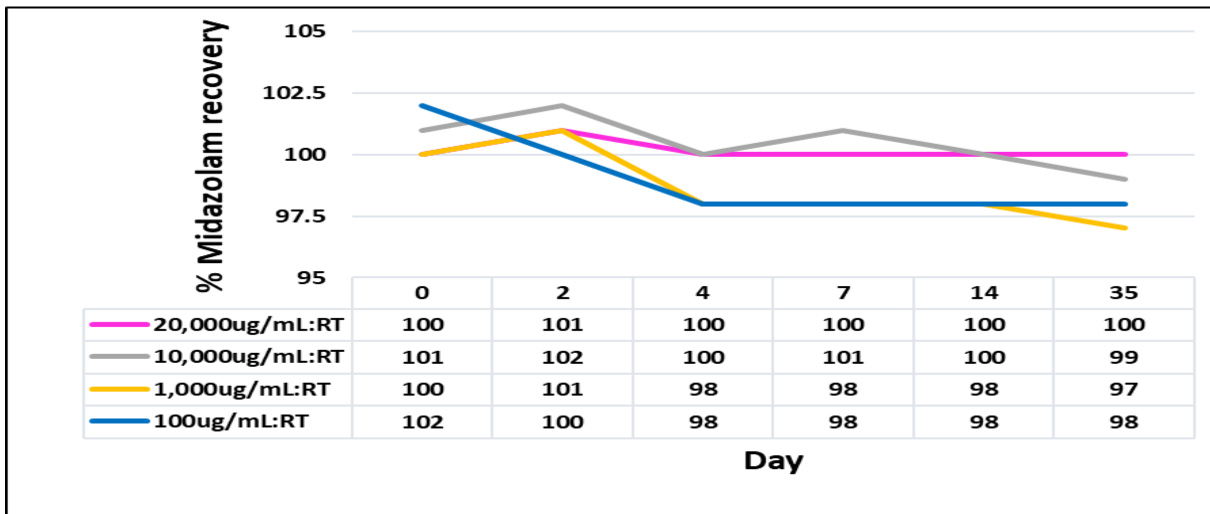


Figure 5-37 – Recovery (%) of midazolam in all formaldehyde concentrations at room temperature

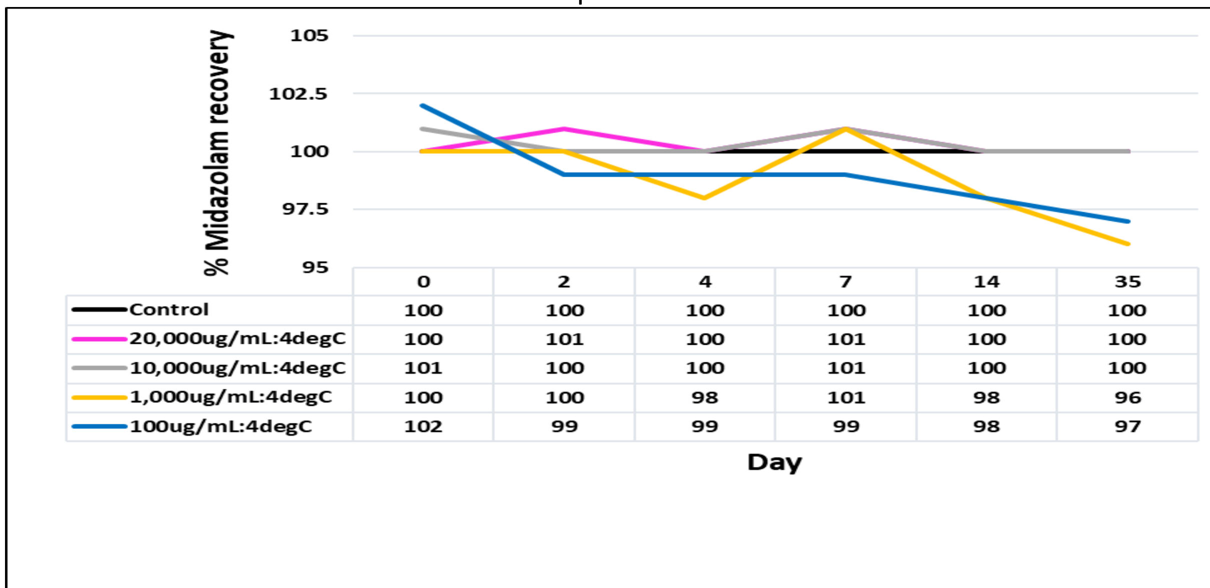


Table 5-16 – Recovery (%) of zolpidem

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (0.20)	100 (0.70)	100 (0.70)	99 (0.12)	99 (0.12)	100 (0.61)	100 (0.61)	100 (0.35)	100 (0.35)
2	100 (0.35)	100 (0.53)	100 (0.46)	100 (0.40)	100 (0.71)	100 (0.12)	100 (0.42)	101 (0.35)	101 (0.81)
4	100 (0.64)	100 (1.40)	103 (0.23)	103 (0.00)	107 (0.80)	104 (0.50)	104 (0.53)	103 (0.42)	103 (0.61)
7	99 (0.31)	100 (0.12)	100 (0.31)	100 (0.46)	104 (0.12)	102 (0.70)	102 (0.42)	101 (0.00)	102 (0.50)
14	98 (0.31)	100 (0.64)	97 (1.33)	97 (0.12)	104 (1.94)	96 (0.42)	98 (1.62)	97 (0.20)	98 (1.79)
35	96 (0.23)	97 (0.72)	95 (0.70)	95 (0.50)	99 (11.66)	95 (0.31)	98 (0.50)	96 (0.46)	97 (0.42)

Figure 5-38 – Recovery (%) of zolpidem in all formaldehyde concentrations at room temperature

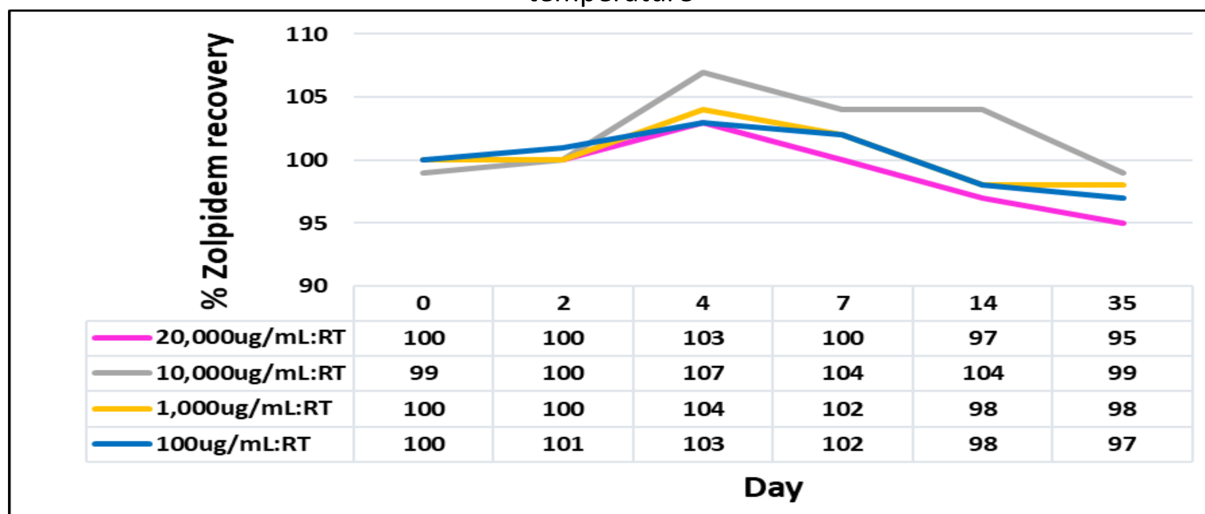
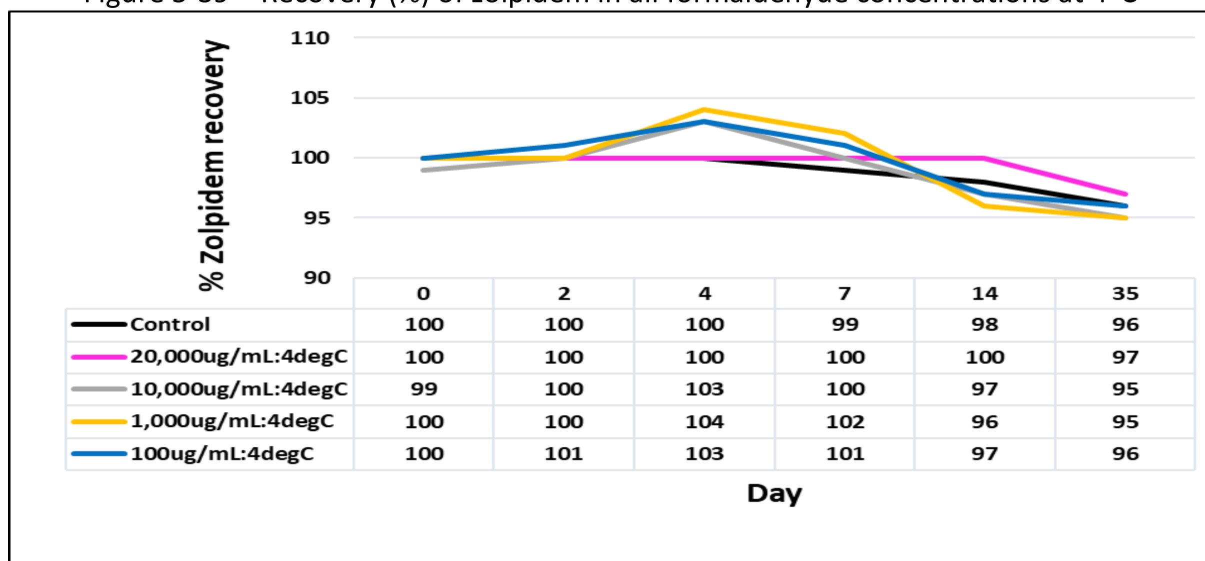


Figure 5-39 – Recovery (%) of zolpidem in all formaldehyde concentrations at 4°C



5.6.4 Benzodiazepines and sedatives discussion

Of the five benzodiazepines included in the current study, including the pharmacologically-related compound zolpidem, all but lorazepam demonstrated excellent stability in all conditions.

The recovery of zolpidem, at Day 35, was never less than 95% in any condition (control recovery: 96%). In the case of diazepam, similarly, the recovery was excellent in all conditions. In 20,000µg/mL formaldehyde, at RT, 95% of the starting concentration was still recoverable on Day 35 (control recovery: 100%). In 10,000µg/mL formaldehyde, the corresponding recovery was 96%, and this increased to 98% in both 1,000µg/mL and 100µg/mL formaldehyde solutions. As discussed in Chapter 2, diazepam has received previous attention in the embalming literature. First mentioned in Winek, Esposito and Cincola's 1990 study, losses of 41% were reported in drug-positive blood samples spiked with 5% formalin (approximately 18,500µg/mL formaldehyde) and 56% in the same samples spiked with 8% formalin (approximately 29,500µg/mL formaldehyde), all after 30 days. The losses recorded by Winek are very significantly greater than those recorded in the current study, although his paper does not state either the pH to which the formaldehyde solution was buffered or the temperature at which the experiment was conducted, and thus direct comparison is not possible. Aside formaldehyde concentration and pH, bacterial degradation, however, may be a relevant factor in Winek's results. Winek does not discuss this possibility, but literature reviewed in Chapter 1 reported losses of up to 26% of diazepam in blood samples containing certain bacteria. Closely aligned to this is the matter of temperature, and further literature discussed in Chapter 1 reported losses of up to 60% of diazepam in blood samples stored at RT: bacterial degradation is enhanced by increasing temperature. Thus, the experimental differences between Winek's and the current experiment make meaningful comparison very difficult.

Comparison with the study reported in Nishigami, Takayasu and Ohshima (1995) is, however, of potentially greater interest since one of the several experimental conditions investigated was 10% formalin buffered to pH 7.4, for 28 days. Although the concentration of formaldehyde in Nishigami's study – approximately 37,000µg/mL - is greater than any of

those used in the current experiment, the solution is buffered to the same pH, and the timescale over which the experiment is conducted is also similar. In Nishigami's study, brain, lung, liver, kidney and skeletal muscle were examined, and although the results after 28 days were somewhat variable, the lowest recovery from any tissue was 68%. Like Winek's study, however, it is difficult to make direct comparisons between Nishigami's results and those of the current experiment. Nishigami states that the samples included in his study came from forensic-positive cases, though no further detail is given of the condition of the bodies from which the samples were taken. Conceivably, therefore, bacterial degradation be partly responsible for the losses reported, regardless of either formaldehyde concentration or pH, given the susceptibility of benzodiazepines to such instability.

The work reported in Tracey et al. (2001) may, however, be more directly comparable, as the experimental conditions here use drug-spiked formalin samples, rather than blood or other tissue samples. Employing what was described as a 5% formaldehyde solution (50,000µg/mL), buffered to pH 7.0, Tracy recorded a diazepam recovery of around 85% of the starting concentration after 30 days; this decreased to around 75% recovery in 10% formaldehyde (10,000µg/mL) and around 60% in 20% formaldehyde (200,000µg/mL), both over the same period of time. Tracy also reports diazepam as being the most stable in formaldehyde of the ten benzodiazepines included in the study, stating that the methyl substituent on the amide nitrogen of its benzodiazepine ring stabilizes the ring, thus hindering hydrolysis (Asano et al. (2020) also reports diazepam as being the most stable of the five benzodiazepines studied in this experiment, being 40% recoverable from 6% formaldehyde (60,000µg/mL) buffered to pH 7.0-7.5 after six months). What Tracy also points out, however, is that diazepam degradation increases greatly, both in terms of rate of decay and in absolute loss, as formaldehyde concentration increases and as pH decreases. The 95% recovery recorded in the current experiment, in conditions of 20,000µg/mL formaldehyde may seem reasonable when compared to Tracy's 85% recovery recorded in conditions of 50,000µg/mL formaldehyde at pH 7.0.

Like the experimental conditions applied by Tracy, those reported in Yokchue (2016) are also more closely aligned to those of the current experiment. Using a drug-spiked, 10% formalin solution (37,000µg/mL) buffered to pH 7.4, Yokchue reports a 90% diazepam recovery after

30 days. This is a figure that is close to the recoveries reported in the current experiment, and is only 5% less than the recovery reported by Tracy when using 50,000µg/mL formaldehyde solution buffered to pH 7.0. The recoveries reported by Tracy and Yokchue, as well as those in the current experiment, are all significantly better than those reported by either Winek or Nishigami, suggesting that conditions in blood or other soft tissues are inherently different to those used in simulated experiments. The implications of this finding may need to be explored further.

What Nishigami's paper also reported was that for some tissues spiked with 10% formalin, buffered to pH 7.4, the recovery after 28 days was, in several cases in excess of 100%; in the case of skeletal muscle, for example, a recovery of 171% was recorded. These findings are relevant, since in the current experiment, in the case of a small number of compounds, recoveries in excess of 100% were also recorded, at various points. The recovery of codeine, for example, was found to increase from the start of the experiment until Day 2 in 100µg/mL and 1,000µg/mL formaldehyde solutions, and until Day 4 in 10,000µg/mL and 20,000µg/mL solutions, before subsequently decreasing. Morphine recovery appeared to follow a similar pattern, though here the greatest rises were observed in the less concentrated solutions of formaldehyde. In the case of flunitrazepam, RT solutions of all concentrations of formaldehyde exhibited apparent initial increases above 100%, before decreasing, though the concentration rises observed in refrigerated solutions were proportionately smaller.

Nishigami's study of diazepam is the only paper in the formaldehyde-toxicology literature to address this phenomenon directly and, although unable to provide a definitive explanation, does provide some indicators of what might underlie its occurrence. One interesting point that Nishigami highlights in his own study is that although recoveries in excess of 100% were recorded from tissues fixed in 10% buffered formalin, increases were never found to occur in the same tissues fixed in non-buffered formalin (pH 5.1). Some years previously, Dennis (1972) reported that in aqueous solution, the major fixative effects of formaldehyde are mediated by the carbonium ion ($^{+}CH_2OH$). As an increase in pH results in a proportionate decrease in the production of carbonium ions, Nishigami, drawing on Dennis's work, reasoned that buffered formaldehyde solutions produce weaker degrees of fixation than non-buffered solutions. Although this theory would, of course, explain why smaller concentrations of

diazepam were recovered from those tissues fixed in non-buffered formalin solutions, it does not explain directly why some recoveries were greater than 100%. In their 2001 study of benzodiazepines, Tracy et al. also reports apparent increases in the recovery above the starting concentration. In 5% formaldehyde buffered to pH 7.0, for example, a recovery of around 110% is recorded at the beginning of the experiment, following which the concentration falls below 100%, although the control solution is noted as being around 115% of the starting concentration on Day 0 and remains above 100% for the 30-day duration of the experiment. Tracy's results also state that flunitrazepam (50,000µg/mL formaldehyde, unbuffered), lorazepam (50,000, 100,000 and 200,000µg/mL formaldehyde, all pH 9.5), flurazepam (50,000 and 100,000µg/mL formaldehyde, un-buffered, as well as controls in un-buffered, buffered to pH 7.0 and buffered to pH 9.5 solutions) and oxazepam (50,000µg/mL formaldehyde, unbuffered) all exhibit apparent increases in concentration, variously, above 100%. These rises, very much like those observed in the current work, usually occur near the beginning of the 30-day experiment, before decreasing, (although in the case of the flurazepam control solution in Nighigami's study, there was a gradual and sustained increase throughout the course of the experiment). Tracy offers no explanation as to why such increases may have occurred.

Rohrig, in his 1988 paper, discusses the phenomenon of formaldehyde altering the recovery or extractability of drugs, but here he is talking about extracting drugs from biological matrices, which is clearly a very different situation to the method employed in the current experiment. It seems unlikely, furthermore, that carryover can explain the apparent excessive recoveries, as this part of the method was appropriately assessed in the development stage and in any event, blank samples were included regularly in the experiment proper, and no excessive carryover was noted in any of these.

Although Tracy cannot account for the apparent increased in concentration observed in his own experiment, there are some clues in the results of the current experiment that may provide some explanation. The first point to note is the comparative size of the standard deviation in the recoveries that were recorded as being in excess of 100%. In the case of codeine, where some of the largest apparent excess recoveries were recorded, the standard deviation between the three samples analysed is up to 32.01. In the case of morphine, the

standard deviation in those samples recording a recovery above 100% is up to 12.29, and is up to 13.17 in the case of similar flunitrazepam samples.

As stated earlier, however, the recoveries that are recorded above 100% all appear to occur within the first few days of the experiment, and certainly by Day 7, after which more expected results are recorded. One explanation may be a sampling or hardware error. With reference to the 'batching' of drugs that was carried out, as detailed in Table 5-2, it will be seen that codeine and flunitrazepam were not only batched together, but were the only two compounds in Batch 2. Morphine, by contrast, was included in Batch 5, along with alfentanil. Whereas the results for alfentanil are unremarkable, those for fentanyl, the first compound in Batch 6, and the compound to be analysed immediately after morphine, are at times irregular, and some have slightly larger than expected standard deviations. It seems possible, therefore that alterations in hardware performance may provide an explanation for the anomalies noted in the current experiment, although the fact that calibration standards were not run between the injection of each drug makes it difficult to assess this theory any further.

Leaving the phenomenon of recoveries in excess of 100%, and moving to the degradation study, in the diazepam reaction mixture, an apparent conversion product was identified with a t_R of 1.322 and a $[M+H]^+$ *m/z* of 118.0862. Some previous work has discussed the breakdown of diazepam. One study, Huang and Moody (1995), identified the *N*-dealkylation at the *N*-1 position, as well as hydroxylation at the 3 position. In the case of the *N*-dealkylation pathway, this leads to the production of nordiazepam (*N*-desmethyldiazepam), with an $[M-H]^+$ *m/z* of 271.0633, whereas hydroxylation leads to the production of temazepam ($[M-H]^+$ *m/z* of 301.0738), followed by oxazepam ($[M-H]^+$ *m/z* of 287.0582). A further study, Maślanka et al. (2013), reported its hydrolysis to open-ring diazepam, 2-glycyloamino-5-chloro-benzophenone with an $[M-H]^+$ *m/z* of 303.3. Yokchue, in her work, identified traces of three conversion products, with $[M-H]^+$ ions of *m/z* 303, 315 and 375. The finding in the current experiment of a compound with an $[M+H]^+$ *m/z* of 118.0862 is clearly not in agreement with any previous finding. Unfortunately, however, it was not possible to obtain any further spectral data that might have provided further confirmation, before the laboratory was closed due to COVID-19.

Like zolpidem and diazepam, midazolam was also very stable in all conditions, recovery being never less than 98% at Day 14 and 96% at Day 35 (control recovery: 100%). In the embalming literature, the compound was first noted in Rohrig (1998) in the case of liver samples analysed from a body exhumed approximately two years following embalming and burial. Although detected at a concentration of 0.15µg/g, quantitative analysis had not been undertaken at autopsy, and it was thus not possible to assess the relative stability of the compound. Tracy's 2001 study of benzodiazepines, however, did monitor the stability of the compound over 30 days. Here, in 50,000µg/mL formaldehyde, buffered to pH 7.0, Tracy recorded recovery of approximately 85% and, in 200,000µg/mL formaldehyde, recovery of around 80%. Tracy also notes that, compared to diazepam, midazolam lacks the methyl substituent on the amide nitrogen of the benzodiazepine ring and may, thus, be more susceptible to hydrolysis. Indeed, it was the case that in un-buffered formaldehyde, Tracy recorded the almost total loss of midazolam over the 30 days of the experiment, although in buffered solutions, as described above, the loss was more modest. By contrast, Ameline, Raul and Kintz (2019) recorded a loss of around 73% of the drug in 4% formalin (approximately 14,000µg/mL formaldehyde) buffered to pH 7.0, at 4°C and after 21 days. The results of the current experiment are in general keeping with the finding of good stability noted by Tracy in buffered formaldehyde.

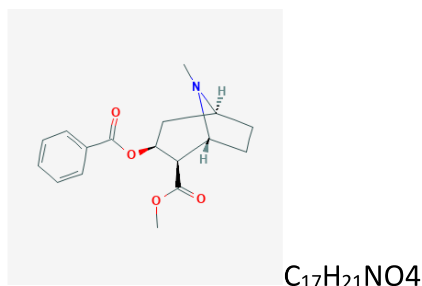
Flunitrazepam also exhibited very good stability in the current study. At Day 14, at least 92% of the starting concentration was recoverable in all conditions (control recovery: 99%), and by Day 35, the lowest recovery, in 20,000µg/mL formaldehyde solution, stored at RT, was 89% (control recovery: 97%). Flunitrazepam was also included in Tracy's 2001 study, where, in a 50,000µg/mL formaldehyde solution buffered to pH 7.0, a recovery of around 110% was recorded after 30 days. In 100,000µg/mL and 200,000 µg/mL formaldehyde solutions, the respective recoveries were in the order of 105% and 95%. Losses in formaldehyde buffered to pH 9.5 were, however, much greater, being up to approximately 70% in 200,000µg/mL formaldehyde. This, Tracy attributes to an electron withdrawal, via resonance, of the nitro substituent located on the phenyl ring, adjacent to the benzodiazepine ring, thus allowing hydrolysis. The slightly greater losses detected in the current experiment may, therefore, be due to the use of a less basic pH than that of Tracy, although it was not possible to identify degradation products that may have provided support to this theory.

Lorazepam was the only benzodiazepine included in the current study to have exhibited comparative instability, although the degradation of the compound was by no means as extensive as that observed in some of the opiates. The best recoveries were made from the weaker solutions of formaldehyde. In the 100µg/mL RT solution, for example, an 87% recovery was made at Day 14 (control recovery: 98%), and this had reduced only slightly to 84% by Day 35 (control recovery: 95%). In 1,000µg/mL formaldehyde, 87% and 95% could be recovered from RT and refrigerated solutions, respectively, at Day 14; by Day 35, the respective figures were still 83% and 87%. In 20,000µg/mL formaldehyde, by contrast, 67% and 77% of the drug was recoverable from RT and refrigerated solutions on Day 14, though even by Day 35 the recovery rates had fallen only slightly to 60% and 73% in each respective condition. In their 2001 study of benzodiazepines, Tracey et al. recorded a recovery of approximately 85% after 30 days from both 50,000µg/mL and 100,000µg/mL formaldehyde solutions, buffered to pH 7.0, and a recovery of around 80% from a 200,000µg/mL formaldehyde solution. Tracy notes that in the case of this molecule, the hydroxyl group located one carbon away, at the C_3 position of the benzodiazepine ring, could exert electron-withdrawing properties, thus enabling preferential hydrolysis of the amide bond. Although the recoveries reported in the current experiment are somewhat lower than those reported by Tracy, they do nevertheless confirm that the compound can be recovered from formaldehyde solutions, even after 35 days.

In the degradation study included in the current experiment, the lorazepam reaction mixture revealed the presence of one apparent conversion product, with a t_R of 5.151 and an $[M+H]^+$ *m/z* of 459.1686. Without additional spectral data, identification of this compound has not been possible.

5.6.5 Stimulants results

5.6.5.1 Cocaine

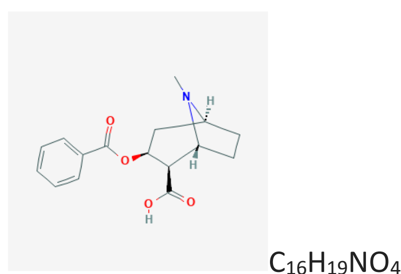


$C_{17}H_{21}NO4$

Figure 5-40 – Structure and formula of cocaine

Cocaine was unstable in all RT solutions. By Day 7, in all concentrations of formaldehyde, at RT, recovery had fallen to around 50%; this dropped further, to around 25%, by Day 14, and to around 5% by Day 35. The rate and degree of degradation were smaller in refrigerated conditions: in all concentrations of formaldehyde, around 90% of the starting concentration of cocaine was recoverable at Day 14; by Day 35, this had fallen in each case to around 75%. The stability of the control solution mirrored closely that of the refrigerated formaldehyde solutions. The results for all conditions are reproduced in Table 5-17. Figure 5-42 illustrates the similar degradation pattern of all RT solutions of formaldehyde. Figure 5-43 illustrates the difference in rates of decay between 20,000µg/mL and 100µg/mL formaldehyde solutions in both refrigerated and RT conditions.

5.6.5.2 BZE



$C_{16}H_{19}NO_4$

Figure 5-41 – Structure and formula of BZE

BZE was relatively stable in 100 µg/mL and 1,000 µg/mL formaldehyde solutions, with losses no greater than 10% over 35 days in any condition. In more concentrated formaldehyde conditions, the degree of degradation was greater, although in 20,000µg/mL formaldehyde, at RT, it was still possible to recover 82% of the compound on Day 35. The control solution remained relatively stable throughout the experiment, being 97% recoverable on Day 35. Results of recovery in all conditions are reported in Table 5-18. The patterns of degradation in RT and refrigerated formaldehyde conditions are illustrated in Figures 5-44 and 5-45, respectively.

5.6.6 Stimulants discussion

In the current experiment, cocaine was found to be unstable in RT solutions of formaldehyde, but relatively stable when refrigerated. The degradation, furthermore, appeared to be unrelated to the concentration of formaldehyde. By Day 7, for example, the amount of cocaine recoverable from RT solutions was slightly more than 50% in all concentrations of formaldehyde. This figure fell to around 25% in all concentrations of RT formaldehyde at Day 14, and to around 5% by Day 35. In a similar pattern, degradation in refrigerated solutions also appeared to be unrelated to formaldehyde concentration, although here the rate of loss was significantly retarded. By Day 7, for example, around 95% of the starting concentration was recoverable (control recovery: 95%), and this reduced to around 90% in all conditions by Day 14 (control recovery: 89%), and to around 75% by Day 35 (control recovery: 73%).

Viel et al. (2009), as discussed in Chapter 2, undertook a detailed study on the stability of cocaine in formaldehyde, in an un-buffered (pH 3.5) solution of 40,000µg/mL formaldehyde, as well as in the same concentration buffered to pH 7.4, over a period of 30-days. Although the compound was found to be stable in the un-buffered solution, a recovery rate of around only 20% was recorded in the solution buffered to pH 7.4 after 15 days, leading Viel to conclude that formaldehyde does not play an active role in the degradation of cocaine; no further work has examined, specifically, the stability of cocaine in formaldehyde. Using first-order regression calculations, however, Viel further concluded that the half-life of cocaine, in a buffered formalin solution, and independent of the starting concentration, is around 7 days.

Table 5-17 – Recovery (%) of cocaine

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (0.50)	101 (1.31)	101 (1.31)	102 (0.23)	102 (0.23)	103 (0.12)	103 (0.12)	101 (0.12)	101 (0.12)
2	98 (0.12)	98 (2.82)	90 (0.50)	100 (1.29)	88 (0.12)	101 (3.78)	84 (1.74)	100 (0.20)	84 (0.53)
7	95 (0.31)	95 (0.87)	53 (0.23)	96 (1.81)	54 (1.30)	97 (10.52)	54 (0.12)	96 (2.27)	54 (0.35)
14	89 (0.23)	91 (0.95)	26 (0.81)	91 (1.1)	27 (0.23)	No data	28 (0.40)	91 (0.31)	27 (0.31)
35	73 (0.61)	74 (0.70)	4 (0.31)	75 (2.20)	5 (0.87)	76 (0.61)	5 (0.23)	75 (1.17)	5 (0.31)

Figure 5-42 – Recovery (%) of cocaine in all concentrations of formaldehyde at room temperature

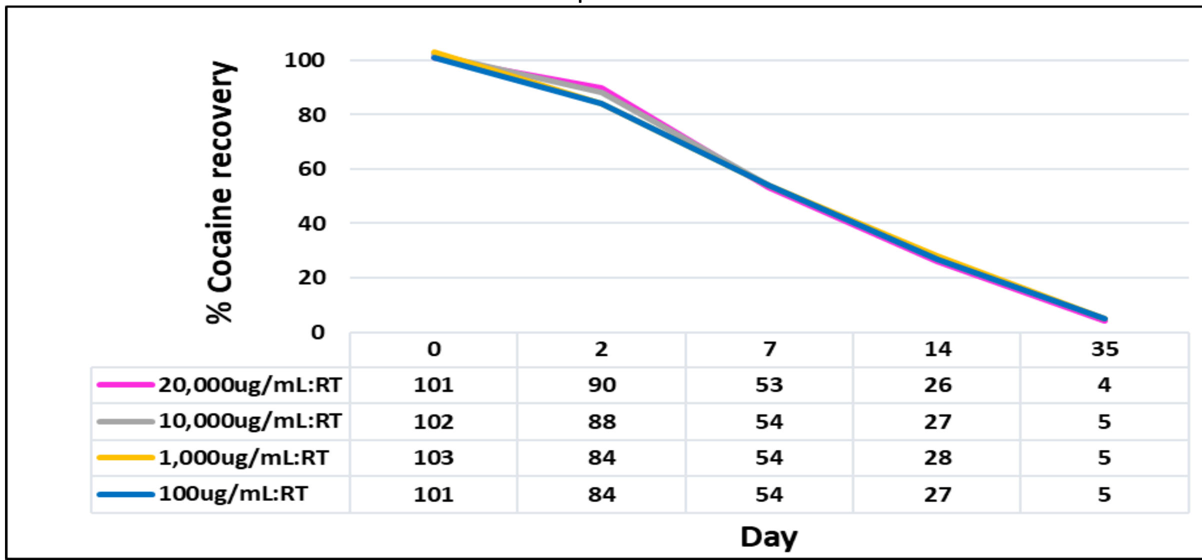


Figure 5-43 – Recovery (%) of cocaine in 20,000µg/mL and 100µg/mL formaldehyde at 4°C and at room temperature

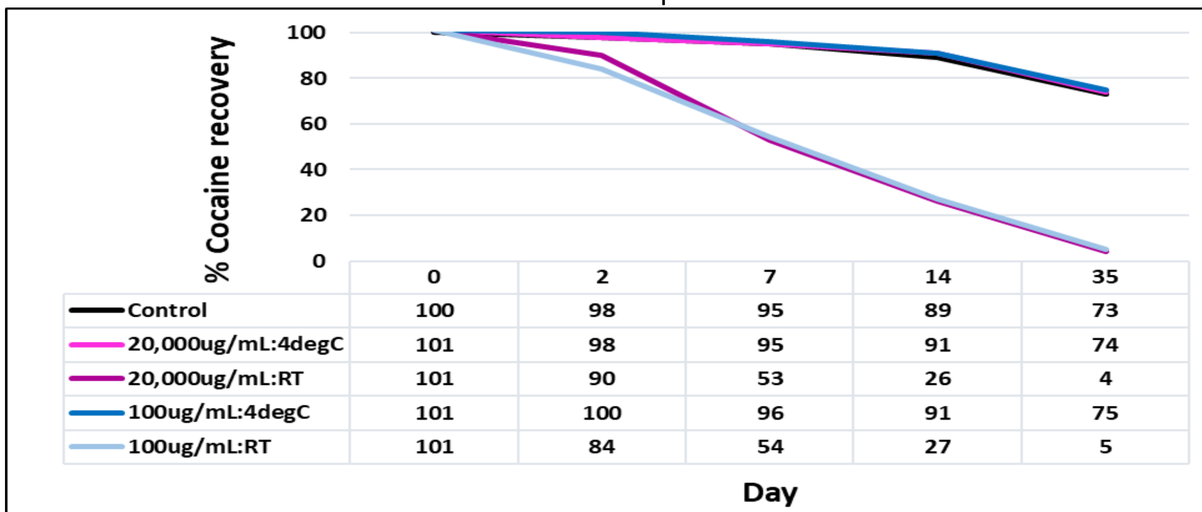


Table 5-18 – Recovery (%) of BZE

Day	Control	20,000µg/mL-4oC	20,000µg/mL-RT	10,000µg/mL-4oC	10,000µg/mL-RT	1,000µg/mL-4oC	1,000µg/mL-RT	100µg/mL-4oC	100µg/mL-RT
0	100 (0.60)	100 (0.23)	100 (0.23)	100 (0.84)	100 (0.84)	100 (0.21)	100 (0.21)	100 (0.12)	100 (0.12)
2	100 (0.95)	92 (0.31)	87 (0.60)	93 (0.42)	95 (0.72)	95 (0.12)	94 (0.28)	99 (0.42)	99 (0.31)
4	100 (0.81)	90 (1.29)	85 (0.46)	91 (0.23)	93 (0.83)	95 (0.42)	92 (0.92)	97 (0.99)	96 (0.20)
7	100 (0.31)	90 (1.30)	85 (1.17)	88 (1.27)	90 (3.64)	92 (2.91)	92 (2.03)	95 (1.14)	94 (1.39)
14	99 (0.42)	88 (0.60)	84 (5.18)	87 (1.50)	89 (1.14)	92 (2.60)	90 (1.33)	95 (2.37)	92 (1.40)
35	97 (1.56)	86 (4.61)	82 (2.99)	87 (1.42)	85 (1.56)	90 (3.68)	90 (0.28)	95 (1.84)	92 (0.90)

Figure 5-44 – Recovery (%) of BZE in all concentrations of formaldehyde at room temperature

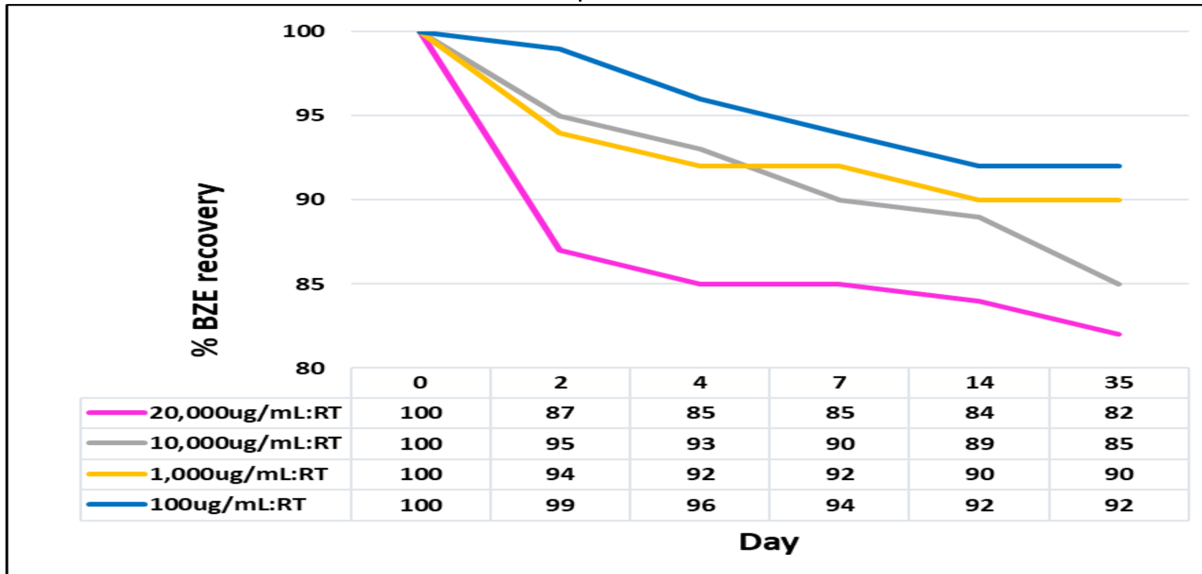
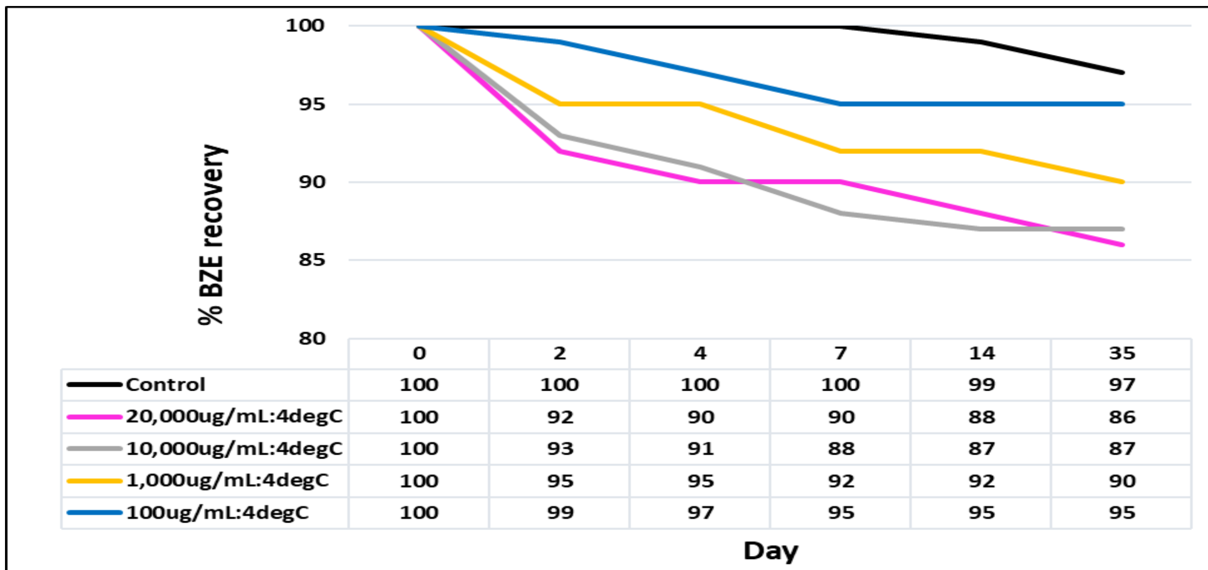


Figure 5-45 – Recovery (%) of BZE in all formaldehyde concentrations at 4°C



The findings of the current experiment accord very closely with this finding. In the room-temperature solutions, all buffered to pH 7.4, the recovery of cocaine at Day 7 in 20,000, 10,000, 1,000 and 100µg/mL formaldehyde was, respectively, 53%, 54%, 54% and 54%. This compares to an expected recovery of around 50%, with a half-life of seven days. Over the remaining course of the experiment, a seven-day half-life would indicate expected recoveries of around 25% at Day 14, and around 3% at Day 35. In the RT solutions in the current experiment, the actual Day 14 recovery was between 26% and 28%, and the Day 35 recovery between 4% and 5%. The findings of the current experiment are, therefore, in very strong agreement with those of Viel.

Five years earlier, the 2004 study of Cingolani et al. had identified the rapid hydrolysis of cocaine in several biological fluids, recommending that forensic analysis should, instead, seek to identify the presence of BZE, as the primary metabolite, as evidence of cocaine use. Indeed, in Cingolani's study, based on actual autopsy samples, cocaine was detected in only one sample of fresh tissue, whereas in all other fresh tissue, as well as in fixed samples, it was BZE that was detected. As was discussed in Chapter 1, cocaine is highly susceptible to hydrolysis in certain cholinesterase-laden biological fluids including, particularly, blood. As Cingolani further notes, the hydrolysis of cocaine into BZE is increased in basic media, but can be slowed by refrigeration and freezing, as well as by reducing the pH. The results of the current experiment certainly confirm the significant retardation of decay of the compound in refrigerated conditions. As we have seen, by Day 7, in all refrigerated formaldehyde solutions, the recovery of cocaine was between 95% and 97%. By Day 14, the recovery was between 90% and 92% in all refrigerated solutions, and was between 74% and 76% by Day 35; this contrasted markedly with the much poorer recoveries made from RT solutions. The current experiment, furthermore, has been able to confirm that the hydrolysis of cocaine can take place in non-esterase fluids.

When examining the stability of BZE, Cingolani compared concentrations in liver samples tested at the time of autopsy, with re-analysis of the same samples after they had been stored in 10% formalin buffered to pH 7.0 for 28 days. A 12.31% recovery was noted from the samples of liver stored in formalin, although a further 84.47% was recovered from the formalin solution in which the samples had been stored, giving a total recovery, therefore, of

96.78%. This, unsurprisingly, led Cingolani to conclude that BZE has good stability and does not react chemically with formaldehyde. The relative quantities of BZE recoverable in the current experiment were not quite as large as those recorded by Cingolani, although significant stability was still demonstrated. By Day 14, for example, 84% of the starting concentration was recoverable in the 20,000µg/mL formaldehyde solution stored at RT, and this only fell slightly, to 82%, by Day 35. In increasingly weaker concentrations of formaldehyde, the recovery of BZE was proportionately greater, such that in the 100 µg/mL solutions, 92% of the starting concentration was recoverable at Day 14 at RT, and 95% when refrigerated; neither 100µg/mL formaldehyde solution underwent any further degradation between days 14 and 35. The findings reported in the current experiment are in general agreement with the very good stability noted by Cingolani.

Cocaine and BZE were the last of the compounds in which it was possible to undertake any degradation product identification work. In the case of cocaine, a peak was observed with an identical retention time to BZE. The compound, when analysed by MS, had a $[M+H]^+$ *m/z* of 290.13, identical to that of the BZE standard. Although quantitation was not possible within the time available, the identification of BZE within the cocaine chromatogram appears to add further clarification to Cingolani's 2004 report of the rapid hydrolysis of cocaine: the identification of BZE in the current experiment suggests that cocaine can be hydrolyzed even without the presence of esterase-containing biological fluids, a fact that has also been recognized elsewhere (see, for example, Baselt, 2017).

In the case of BZE, a compound was identified at t_R 0.928, with a $[M+H]^+$ value of 224.1285. Without further spectral data, it has not been possible to identify this compound. No other peaks of interest were noted.

5.7 Conclusions

Of the 15 drugs or metabolites studied and reported in this chapter, their stability in formaldehyde have varied, depending on experimental condition. Some compounds, such as alfentanil, fentanyl, diazepam, midazolam and zolpidem appeared to be extremely stable in all concentrations of formaldehyde, remaining virtually undegraded even after 35 days. The stability of other compounds, such as 6-MAM and lorazepam, however, appeared to be highly

dependent on the concentration of the formaldehyde solution in which they were stored, and clear patterns have been identified that link increasing concentrations of formaldehyde with proportionately poorer recoveries of the compound of interest. Factors other than formaldehyde concentration, however, have been shown to play a part in the stability of some drug molecules. In the case of cocaine, for example, temperature has been shown to have a significantly greater bearing on the stability of the compound than does formaldehyde concentration. In the cases of methadone, morphine and, to a lesser extent, a number of other compounds, moreover, it was an inter-relationship between both formaldehyde concentration and temperature that determined the stability of the compound.

For reasons already discussed at length, the methodological approach in the current study has been markedly different to those adopted in previous work, and direct comparison of results is, in consequence, difficult. Useful comparisons, nevertheless, have been made, and in the case of several compounds it has been possible to confirm general agreement with trends reported previously. Most importantly, however, and where the current study stands apart from existing work, is that it has been possible, for the first time, to examine the stability of a small number of compounds of forensic interest in the conditions that might realistically be expected to be encountered in embalmed and repatriated remains. Taking the concentrations of formaldehyde that were detected in the hard-fix and soft-fix cadavers, and reported in Chapter 4, all fifteen drugs included in the study demonstrated very good rates of recovery in 100µg/mL and 1,000µg/mL concentrations of formaldehyde. Even methadone, as seemingly the most formaldehyde-sensitive of all the compounds studied, was 87% recoverable in 100µg/mL RT formaldehyde at Day 14, and 56% recoverable in 1,000µg/mL formaldehyde after the same period of time; by Day 35, the respective recoveries from both concentrations were still 85% and 34%.

In conclusion, therefore, as an indicative study, and directed by the findings reported in Chapter 4, it is certainly possible to postulate that all fifteen compounds included in the experiment may be detectable in repatriated bodies, 14 days after embalming and, with the possible exception of cocaine, even after 35 days. The current study has a number of limitations, and the results carry with them some reservations which will be addressed in the concluding comments that follow in the final chapter. Notwithstanding these cautions,

however, results such as those reported in this chapter are strongly suggestive of the fact that, contrary to the position assumed in much of the previous literature, there may be ways, previously unconsidered, of undertaking meaningful forensic toxicological examination in embalmed human remains.

Conclusions and Future Work

Conclusions

The work presented in this thesis demonstrates the theoretical and practical means by which drugs of forensic interest may be recoverable from embalmed bodies, and thus the means by which toxicologists may in future be able to provide meaningful analytical commentary in ways that are not currently possible.

One of the first objectives of this project, as stated in the Introduction, was to review the existing literature, assessing its applicability to the specific circumstances of repatriation embalming. It was proposed in Chapter 2 that the limited literature we have, while on the whole appropriate for histological samples preserved in highly concentrated solutions of formalin, cannot be directly applied to samples from embalmed and repatriated bodies. While some of the previous literature has attempted to extrapolate such findings directly onto scenarios involving embalming and exhumation, misunderstandings concerning the process by which embalming is commonly undertaken have led to incorrect inferences being made.

A further objective of this project, however, was to examine biological fluids other than blood for their utility as suitable toxicological matrices in embalmed cases. For the first time, work presented in this thesis has demonstrated that it is possible to examine certain biological fluids that are barely affected by the embalming process. As has been demonstrated in Chapter 4, even in cadavers that are embalmed using much greater volumes of much more concentrated embalming fluids than those commonly used in repatriation work, it has been possible to establish that three biological fluids contained within anatomically avascular compartments – vitreous humour, synovial fluid and cerebrospinal fluid – remain largely protected from formaldehyde contamination during and after the process of embalming. In the hard-fix cadavers sampled and analysed for the current study, the mean concentration of VH formaldehyde detected was 589µg/mL; in SF, it was 590µg/mL and, in CSF, was 420µg/mL. The formaldehyde concentrations of between 5% and 20% (50,000 – 200,000µg/mL) commonly used in the studies reported in the earlier literature thus, clearly and markedly, contrast with the concentrations detected in the VH, SF and CSF of actual embalmed bodies. In the case of the eight soft-fix cadavers also analysed, a maximum concentration detected

among all samples of VH, SF and CSF of 14.7µg/mL, along with a mean concentration of 5.29µg/mL and, in many cases, the detection of no formaldehyde, further and compelling evidence is provided not only of the deficiencies in the current literature, but also, and importantly, of the potential merits of examining these avascular fluids in embalmed bodies.

An important objective of the project was to assess the stability of a representative group of drug molecules in the concentrations of formaldehyde detected in the biological fluids of embalmed cases, as opposed to the concentrations of formaldehyde surmised in previous studies. Building on the work presented in Chapter 4, it has thus been possible to monitor the stability of a number of drugs of forensic interest in actual concentrations of formaldehyde detected in the VH, CSF and SF of embalmed cadavers. Many of the drug molecules demonstrated excellent rates of recovery, even in the more concentrated solutions of formaldehyde used. Although formaldehyde concentration was certainly shown to play a part in the degradation of a number of compounds, temperature, unsurprisingly, was also shown to have significant bearing on the kinetics of the drug-formaldehyde reactions in many cases. It was significant, however, that in reaction with the concentrations of formaldehyde detected in the embalmed cadavers reported in the Chapter 4, all fifteen compounds studied were recoverable by Day 35 of the stability study.

Bringing together the findings reported in Chapters 4 and 5, with the literature review of the utility of VH, SF and CSF as toxicological matrices, as presented in Chapter 3, it becomes possible, for the first time, to propose the means by which it may now be possible to undertake meaningful toxicological examination on embalmed and repatriated bodies. In the very limited amount of literature we currently have examining SF for the presence of drug molecules, four of the fifteen compounds examined in Chapter 5 (6-MAM, BZE, cocaine and morphine) have already been successfully identified in the fluid. In the case of the CSF literature, the corresponding number of compounds already identified increases to seven (6-MAM, BZE, cocaine, diazepam, midazolam, morphine and zolpidem). The VH literature is already extensive, and many reports chart the quantitation of a very wide range of opioids, benzodiazepines, anti-depressants and stimulants, as well as many other drug classes; indeed, one paper alone, Dresen et al. (2010), reports the qualitative identification of 700 compounds. Thus, while Chapters 4 and 5 have demonstrated the practical basis on which

VH, SF and CSF might be utilised in the toxicological investigation of embalmed and repatriated bodies, it is the case that Chapter 3 provides a sound theoretical basis on which such analyses might be undertaken.

Limitations, Future Work and Implementation

Although the results reported within this thesis are certainly promising, some of the premises on which the work is grounded, and on which the concluding recommendations are made, would benefit from both clarification and further development.

Chief among these requirements is the need to better define the concentrations of formaldehyde detectable in bodies that are embalmed using methods more closely aligned to those employed in repatriation cases. The majority of the samples analysed and reported in the current study came from bodies that had been embalmed with very significantly greater volumes of very much more concentrated fluid than is ever used in repatriation cases. In the majority of instances, furthermore, the cadavers sampled for the current study had been embalmed very many months prior to sampling. With statistical analysis, it has been possible to demonstrate the likely stability of formaldehyde concentrations in the months following embalming. By virtue of the fact that the concentration of formaldehyde used in the embalming of the Cambridge cadavers, furthermore, is so comparatively high, it can also be argued that the samples included in the study represent the very worst-case scenarios that might ever be encountered in actual repatriation cases. The eight soft-fix cadavers that were also included in the study, however, were far more representative of what is encountered in actual repatriation cases. These samples only became available towards the end of the study, and the results obtained from them, while very encouraging, come from such a small sample size that further confirmatory work is needed. Obtaining research samples from actual repatriation cases within England, Northern Ireland or Wales – the territorial extent of the Human Tissue Authority – is likely to be difficult. It should, however, be possible to gather more soft-fix samples from university medical schools, and it may be possible to undertake collaborative work with a centre that is not bound by the research-restrictive covenants of the HTA 2004.

Additionally, it would be beneficial to repeat the Chapter 4 experimental work, as several methodological anomalies became apparent after the work had been completed that may have influenced the results. The importance of the physical storage conditions of samples, for example, was not appreciated. Here, there was an inconsistency with the extent of the ventilation space between liquid and gas phases in storage tubes, as well as with the need to seal the screw tops of tubes with parafilm before freezing the samples as quickly as possible. Other potential sources of error, as discussed in Chapter 4, include, in the case of VH, retinal leakage, the accidental aspiration of retinal fragments during sampling and the evaporation of the matrix through the cornea, when eyelids are not closed. Importantly, and particularly in the case of SF and CSF, as viscous fluids, is the need to use positive displacement pipetting. This was a technique that unfortunately was not employed in the current study and which, in itself, may have contributed a degree of inaccuracy to the results. Finally, it would also be beneficial to obtain a greater number of samples than those used in the current study in order to lend greater statistical significance to the trends inferred.

Related to a requirement to seek further confirmation of the extent to which VH, SF and CSF are contaminated by the embalming process is the need to test the basic supposition of this thesis and examine whether the theory that has been developed works in practice. Here, it is frustrating that the COVID-19 national lockdowns prevented this part of the planned work being tested. As was discussed in the Introduction, the project plan included the analysis of a number of samples, taken from drug-positive cases both before and after embalming. The results would have been revealing on several levels. First, as it was possible to obtain pre-embalming blood samples, it would have been informative to establish which of the drugs detected in this matrix could also be detected in VH, SF and CSF (many of the donors from which samples were obtained were included in the study because they came to the facility via palliative care, and could be expected, therefore, to have had pharmacological steady states of a number of opioids and benzodiazepines in their fluids). As was discussed in Chapter 3, there is much in the literature concerning the detection of drugs in VH, though less concerning CSF, and very little indeed as far as SF is concerned, and this part of the work, therefore, may have provided a useful addition to earlier published findings.

Beyond that, however, would have been the opportunity to test the same fluids again, but after embalming had taken place. Aside related considerations concerning, particularly, the stability of drugs of forensic interest in biological fluids (regardless of the presence of formaldehyde), it would of course have been extremely useful to have had the analytical results of this final experiment in order to provide some preliminary evidence of the efficacy of the hypothesis of the thesis. Although the fundamental theory that has been developed – particularly in respect of the experimental work reported in Chapter 4 and Chapter 5 – provides strong evidence of the expected worth of the method, it would have been conceptually beneficial to have been able to provide demonstrable evidence of this, and it is hoped to be able to pursue further this aspect of the work as a matter of priority.

Even if these results had been available, however, and even if they had succeeded in providing definitive evidence of the efficacy of the central thesis of this study, the results would still have carried with them the need for subsequent qualification. At best, the accompanying conclusion would have been that VH, SF or CSF might provide definitive qualitative evidence of the presence of certain drugs of forensic interest. Any attempt at comparative quantification, however, would have to be tempered by the current paucity of reference data, particularly in the case of CSF, and most especially in relation to SF. Meaningful quantitative interpretations of drug concentrations in VH, SF and CSF cannot currently be made to standards that could be regarded as being forensically safe. Medical school cadavers, however, provide a potentially valuable source of comparative data. While it is admittedly the case that such cadavers will be unlikely to provide information on those drugs that are used for purely illicit purposes, it is the case that the majority of donors are older, often hospital, hospice or home-bound patients, who are regular users of a range of pharmaceutical compounds. They often, furthermore, come to university facilities with well-documented medical histories, from which a clear understanding of pre-mortem drug administration can be readily obtained. The opportunity, therefore, to undertake comparative research, contrasting pre-embalming drug concentrations of a range of drugs in blood, alongside those detected in VH, SF and CSF is considerable. Related to this is the fact that few studies have been published that report the stability of drugs in these alternative matrices. Even in the case of VH, for which the greatest amount of comparative data has been published, very little

is known of the stability of drugs of forensic interest. Although there have been assumptions that many drugs are stable in these fluids – mainly as a result of the protection they receive due their relative anatomical isolation – it is clear that studies need to be undertaken before there can be any attempt at providing reliable quantitative interpretation of analytical results within the context of actual casework. The need for this work was clearly signaled in Rees's PhD thesis (Rees, 2011) though, nearly ten years later, studies are yet to be reported.

There is, moreover, wider merit in undertaking further and more wide-ranging comparative studies of VH, SF and CSF. Bévalot et al., in their 2016 review of VH literature, suggest that work should be undertaken to assess the viability of the fluid for predicting ante-mortem blood concentrations. Rohrig, furthermore, in a recent review of the literature relating to post-mortem redistribution, has suggested that more work should be undertaken on specimen types that may be resistant to passive diffusion. Alongside popliteal blood, intraosseous fluid and brain tissue, he recommends the investigation of VH, CSF and SF as possible samples that may not be subject to the post-mortem redistribution of drug molecules (Rohrig, 2019). Additionally, there is a need to undertake comparative work on the detection merits of each of the three fluids in relation to a range of drug molecules. The literature, as we have seen, has found CSF to be especially useful for lipophilic molecules, whereas, in the case of SF, the accumulation of xenobiotics may be governed by a simple diffusion process that is likely to allow larger protein-bound molecules to cross the synovial membrane. Related to this is the fact that all three fluids are contained within relatively protected environments that, by virtue of their anatomical structure, arrest bacterial contamination for some time following death; they also appear to be devoid of the esterase enzymes that can cause so many problems with the detection of a range of drugs in blood. Thus, there is justification in undertaking further work on the three fluids, not just for the purpose of extending the study reported in this thesis, but also as a means of addressing several other, related, toxicological matters, in order to assess further the potential use of these fluids as alternatives to blood, even in non-embalmed cases.

Finally, although the work on conversion products, presented at the end of Chapter 5, was also limited in its extent by the consequences of COVID-19, it is an area of work which would benefit from further attention. While the experimental results presented in this thesis are

suggestive of the fact that it may in due course be possible to undertake meaningful toxicological analysis in embalmed human remains, it is the case that samples may occasionally need to be analysed that do not fall within the 35-day window provided by the current work. This may be a particularly relevant point in the case of exhumations which, however infrequently undertaken, are nevertheless usually of significant forensic importance. Depending on the time that has elapsed between burial and exhumation, furthermore, it may well be the case that samples of VH, SF or CSF are unobtainable, or else are contaminated as a result of decomposition. If soft tissues are removed for analysis, therefore, the provisions made in this thesis are unlikely to apply, and other methods will be needed. Further work on the conversion products of drug-formaldehyde reactions may, therefore, prove beneficial in such cases.

The work presented here, particularly the experimental concepts, as well as the methodological approaches underpinning each of the experiments, are novel and untried in the forensic arena. It was suggested earlier in the Conclusions that modifications to the experimental approach utilised in Chapter 4 should be made and the work repeated. It is not expected that the outcome of such a modified experiment will change to any significant degree the underlying premise of the work, or that it will have any significant impact on the conclusions drawn from the stability study reported in Chapter 5. It will, however, provide a more robust basis on which the work can be presented to the forensic community. It was also suggested earlier that the work originally conceived as an additional experimental chapter – analysing the VH, SF and CSF from cases both before and after embalming – should be undertaken as a matter of priority as a 'proof of concept' test. Assuming that the outcome of this work confirms the trends reported in Chapter 5, it is hoped that the theories and methods developed during the course of this doctoral project can be implemented as a viable means of undertaking meaningful qualitative toxicological analysis in embalmed and repatriated cadavers, and that the work will have succeeded in advancing laboratory capabilities in this comparatively understudied, but important, field of forensic science.

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Appendix 1 - Summary of Chapter 1 literature review by subject and author

Subject	Pages	Authors cited
1.3.1 Toxicological matrices 1.3.1.1 *Blood*	20-31 20	Apple (2011), Baselt (2017), Dinis-Oliveira et al. (2010), Ferner (1996, 2008 and 2012), Flanagan (2008a), Hepler and Isenschmid (2007), Jones (2011), Kennedy (2010), Molina and Hargrove (2018), Musshoff et al. (2004), Patel (2012), Repetto and Repetto (1997), Richardson (2000), Schultz et al. (2020), Skopp (2004 and 2010) and Wennig (2000)
1.3.1.2 *Urine*	22	Baker and Jenkins (2008), Dettmeyer, Verhoff and Schütz (2014), Dinis-Oliveira et al. (2010), Ferner (2012), Hepler and Isenschmid (2008), Jones (2011), Kennedy (2010) and Thaulow et al. (2018)
1.3.1.3 *Vitreous humour*	24	Caplan and Levine (1990), DiMaio and DiMaio (2001), Dinis-Oliveira (2010), Hepler and Isenschmid (2008), Jenkins (2008a), Jones (2011) and O'Neal and Poklis (1996)
1.3.1.4 *Cerebrospinal fluid and synovial fluid*	25	DeKing, Hargrove and Molina (2014), Engelhart and Jenkins (2007), Jenkins and Lavins (1998), Kushner and Somerville (1971), Logan and Luthi (1994), Madea, Kreuser and Banaschak (2001), Moffat, Osselton and Widdop (2011), Palmiere et al. (2015), Shen, Artru and Adkinson (2004), Tominga et al. (2015), Venkatesh, Scott and Ziegenfuss (2000) and Winek et al. (1993b)
1.3.1.5 *Gastric contents*	27	Jones (2011), Pounder et al. (1996a) and Skopp (2004)
1.3.1.6 *Bile*	28	Agarwal and Lemoc (1996), Caplan and Goldberger (2014), DiMaio and DiMaio (2001), Drummer (2004a), Ferner and Aronson (2018), Forrest (1993), Hepler and Isenschmid (2008), Jones (2011), Leikin and Watson (2003), Mercurio, Ceraso and Melai (2019), Tominga et al. (2016) and Vanbinst et al. (2002)
1.3.1.7 *Soft tissues*	28	Carvalho (2017), Casali et al. (2015), Hepler and Isenschmid (2008), Jones (2011), Karch (2009), Molina, Limfueco and Hargrove (2015), Nedahl, Johansen and Linnet (2019a and 2019b), Orfanidis et al. (2020) and Palmiere et al. (2018)
1.3.1.8 *Hair*	30	Dettmeyer, Verhoff and Schütz (2014), Drummer (2004a) and Henderson (1993)
1.3.1.9 *Bone, bone marrow and other matrices*	30	De Carvalho (2010), Dinis-Oliveira et al. (2010), Druid (2007), Drummer (2004a), Flanagan, Connally and Evans (2005b), Forrest (1993), Franceschetti et al. (2021), Gallardo and Queiroz (2008), Giordano et al. (2021), Hargrove and Molina (2014), Hepler and Isenschmid (2008), Jenkins (2008a), Jones (2011), Kapur and Aleksa (2020), Klima et al. (2016), Kintz, Tracqui and Mangin (1990), Langford, Taylor and Pounder (1998), Levisky et al. (2001), Lloyd and Evans (2017), Moriya and Hashimoto

cont'd		(1999b), Reisinger et al. (2019), Rubin (2018), Shokry et al. (2017), Sims et al. (1999), Vandenbosch et al. (2020), Watterson et al. (2010), Wolff (2017), Wu, Marin and McMillin (2017) and Wyman et al. (2011)
1.3.2 Drug and sample stability	32-39	Andresen, Aydin and Iwersen-Bergmann (2010), Atanasov et al. (2012), Ballantyne, Bright and Williams (1974), Ballantyne (1976), Baselt (2017), Beránková, Mutnanska and Balikova (2006), Bodnar (2016), Boumba, Ziavrou and Vougiousklakis (2008), Busardò et al. (2014), Butzbach (2010), Butzbach, et al. (2013a and 2013b), Byard and Butzbach (2012), Caplan and Goldberger (2014), Carroll et al. (2000), Castle et al. (2017), Castro et al. (2014), Corry (1978), Dinis-Oliveira et al. (2010), Drummer, Gerostamoulos and Chu (2002b), Dugan et al. (1994), Egekeze and Oehme (1980), Ellenhorn (1997), Elliott, Lowe and Symonds (2004), El Mahjoub and Staub (2000), Flanagan (2008a and 2008b), Forbes, Perrault and Comstock (2017), Gibitz and Pluish (2009), Giorgi and Meeker (1995), Golding Fraga, Díaz-Flores Estévez and Díaz Romero (1998), Guo, Lee and Jeong (2020), Ha, Mata and Vargas (2020), Hanzlick (2014), Høiseth et al. (2014), Holmgren et al. (2004), Huertas et al. (2019), Johanson and Fischman (1989), Kazmierczak and Azzazy (2008), Kerrigan (2013), Kiencke et al. (2013), Kietzerow et al. (2020), Kintz et al. (2004), Korb and Cooper (2014), Kugelberg and Jones (2007), Leikin and Watson (2003), Levine, Blanke and Valentour (1983), Lewis et al. (2004), Martindale, Powers and Bell (2015), Melo, Bastos and Teixeira (2012), Melvin et al. (1984), Moriya and Hashimoto (1997b and 2003), Morris, Harrison and Partridge (2006), O'Neal and Poklis (1996), Papoutsis et al. (2014), Paterson (1993), Peters (2007), Petković, Simić and Vujić (2005), Pounder (2003), Quintas et al. (2017), Rees (2011), Robertson and Drummer (1995 and 1998), Rohrig (2019), Romberg and Past (1994), Skopp et al. (2001), Skopp and Pötsch (2002), Skopp (2004 and 2014), Stevens (1984), Stojiljkovic et al. (2016), Suzuki and Watanabe (2005), Thomsen et al. (2017) and Yamazaki and Wakasugi (1994)
1.3.3 Post-mortem redistribution	39-44	Anderson and Muto (2000), Bailey and Shaw (1982), Boer (2003), Buxton (2011), Chan et al. (2014), Cook, Braithwaite and Hale (2000), Crandall et al. (2006a), Dalpe-Scott et al. (1995), Davies, Johnston and Holt (2016), Drummer and Gerostamoulos (2002a), Drummer (2007), Enderle (2012), Ferner (2008 and 2012), Giaginis, Tsantili-Kakoulidou and Theocharis (2009 and 2014), Gleba and Kim (2020), Gomez et al. (1995), Hilberg et al. (1999), Holt and Benstead (1975), Kennedy (2010 and 2015), Kerrigan, Honey and Baker (2004), Koren and Klein (1992), Leikin and Watson (2003), Lemaire et al. (2016), Logan and Smirnow (1996), Marraccini et al. (1990), McIntyre (2014, 2015 and 2016), Moriya and Hashimoto (1997a, 1999a, 2000 and 2001), Pélissier-Alicot et al. (2003 and 2006), Pounder and Jones (1990), Pounder and Yonemitsu (1991), Pounder (1993), Pounder and Smith (1995), Pounder et al. (1996b), Prouty and Anderson (1990), Richardson (2000), Rodda and Drummer (2006), Rodda et al. (2018), Sastre et al. (2017), Sawyer and Forney (1988), Shepherd, Lake and Camps (1992), Skopp et al. (1996), Skopp (2010), Tzortzis et al. (2009) and Zilg et al. (2017)
1.4 Pre-mortem factors affecting toxicological interpretation	45-55	Davis (2014), Drummer (2001), Flanagan (2008b), Hoffman, Zedeck and Zedeck (2012), Horsfall and Sprague (2017), Kolesnikov and Sõritsa (2008), Koski, Ojanperä and Vuori (2003), Lennernäs (2009), McClure et al. (2017), Mozayani and Raymon (2004), Naso-Kaspar et al. (2013), Pérez-Mañá et al. (2018), Polettini et al. (2005), Scott et al. (2015), Singh (2019), Skopp (2010) and Thaulow et al. (2014)

1.4.1 Extent of drug absorption, distribution, metabolism and excretion before death	46-48	Allan and Roberts (2008), Drummer (2007), Ferner (2012), Lafrenière and Watterson (2010), Naso-Kaspar et al. (2013) and Rohrig (2019)
1.4.2 Drug tolerance	48-50	Andrews and Kinner (2012), Brockbals et al. (2020), Dinis-Oliveira (2019), Ferner (2008), Ferrer-Alcon, La Harpe and Garcia-Sevilla (2004), Johnson, Noll and MacMillan (1982), Jones and Holmgren (2009), Jones, Holmgren and Ahlner (2016), Jung and Reidenberg (2005), Karch (2001), Launiainen and Ojanperä (2014), Paterson, Cordero and Stearns (2009), Pragst and Balikova (2006), Roberts and Buckley (2007) and Rohrig (2019)
1.4.3 Physiology	50-52	Bishop-Freeman et al. (2019), Byard (2013b), Cardelli et al. (2012), Drummer (2001 and 2013), Elbekai, Korashy and El-Kadi (2004), Gruszecki, Booth and Davis (2007), Jenkins (2008b), Jones (2007), Nicholson, Mellor and Roberts (2010), Rohrig (2019), Rowland and Tozer (2011), Shi and Klotz (2011) and Skopp (2010)
1.4.4 Case history	52-53	Drummer (2007), Ferner (2008 and 2012), Flanagan (2008a and 2008b), Harding-Pink and Fryc (1991), Skopp (2010) and Vale and Proudfoot (1995)
1.4.5 Pharmacogentics and pharmacogenomics	53-55	Bruwers et al. (2009), Cooper, Paterson and Osselton (2010), Jannetto et al. (2002), Johansson and Ingelman-Sundberg (2011), Kupiec, Raj and Vu (2006), Lam et al. (2014), Lamba et al. (2012), Lang and Linnet (2014), Lappas and Lappas (2016), Richards-Waugh et al. (2014), Rohrig (2019), Rowland and Tozer (2011), Wendt et al. (2020), White and Wong (2008) and Zanger and Schwab (2013)

Appendix 2 - Published papers on the detection, stability and quantitation of drugs in embalmed tissue (after Nikolaou et al., 2013)

Drugs analysed	Sample type	Conditions	**Results** (↓ = decrease in concentration, ↑ = increase in concentration, ↔ = stable concentration)	Literature reference
<u>Benzodiazepines (and related anxiolytics/hypnotics):</u>				
Diazepam	Spiked human blood	Added to 5% and 8% formalin, for 30 days	↓41.4% in 5% formalin ↓56.3% in 8% formalin	Winek, Esposito and Cincola (1990)
Diazepam	Formalin-fixed brain, lung, liver, kidney and skeletal muscle	Samples from sacrificed rabbits, fixed for 28 days in: 1) 10% formalin solution (buffered pH 7.4); 2) 10% formalin solution (non-buffered, pH 5.1); 3) 4% paraformaldehyde solution (buffered, pH 7.4)	1) ↓up to 32%, but ↑71% in skeletal muscle 2) ↓up to 52%, but ↔ in some tissues 3) ↓up to 96%	Nishigami et al. (1995)
Midazolam	Embalmed human liver	Body exhumed 24 months after embalming	Detected at 0.15µg/g	Rohrig (1998)
Alprazolam, Chlordiazepoxide, Diazepam, Flunitrazepam, Flurazepam, Lorazepam, Midazolam, Oxazepam, Prazepam, Triazolam	Drugs in formalin solution	30 days at room temperature: 1) Formaldehyde solution 5%/10%/20% (non-buffered) 2) Formaldehyde solution 5%/10%/20% (pH 7) 3) Formaldehyde solution 5%/10%/20% (pH 9.5)	1) ↓highly variable, up to 100% 2) ↓highly variable, up to approx. 95% 3) ↓highly variable, up to approx. 80%	Tracy et al. (2001)
Bromovalerylurea	Drugs in formalin solution	90 days at 4°C / room temp: 1) Formalin solution 10%/20% (non-buffered) 2) Formalin solution 10%/20% (pH 7.4)	1) ↓up to 5% at 4°C ↓up to50% at room temp. 2) ↓up to 90% at 4°C ↓up to 100% at room temp.	Suzuki and Kaneko (2009)

Drugs analysed	Sample type	Conditions	Results (↓ = decrease in concentration, ↑ = increase in concentration, ↔ = stable concentration)	Literature reference
Estazolam	Formalin-fixed heart, liver, kidney and brain	Samples from sacrificed dog, fixed in 4% formaldehyde solution	↓up to 99.2%	Yuan, Wang and Yun (2011)
Bromazepam	Drugs in formalin solution and formalin-fixed liver and kidney samples from forensic case	15% formalin solution, unbuffered (pH 4.5-5.0) for 13 months at 4°C and room temperature	↓up to 33.4%	Uekusa, Hayashida and Ohno (2015)
Midazolam	Spiked blood in formalin solution	4% formalin solution buffered to pH 7.0 for 21 days at 4°C	↓73.1%	Ameline, Raul and Kintz (2019)
Brotizolam, Diazepam, Estazolam, Etizolam and Triazolam	Drug-spiked liver homegenates	6% formaldehyde buffered to pH 7.0-7.5 at room temperature for six months	Brotizolam ↓<95% Diazepam ↓<60% Estazolam ↓<100% Etizolam ↓<95% Triazolam ↓<15%	Asano et al. (2020)
<u>Opiates and Opioids</u>:				
Methadone and Propoxyphene	Brain, kidney, liver and spleen samples from forensic cases	Fixed in 10% formalin solution at 4°C for 19 months (Methadone) and 24 months (Propoxyphene	Samples positive for both drugs at end of study	Fransioli, Szabo and Sunshine (1980)
Morphine	Liver sample from embalmed body	Body exhumed 3 weeks after death	Sample positive for Morphine	Levine et al. (1994)
Fentanyl, Meperidine and Normeperidine	Embalmed human liver	Body exhumed 24 months after embalming	Fentanyl ↓26% compared to conc. at time of death Meperidine detected at 0.18µg/g Normeperidine detected at 0.14µg/g	Rohrig (1998)
Morphine	Formalin-fixed liver Formalin-fixed kidney	Fixed in 10% formalin solution, pH 7, room temp. for 12 weeks	↓63.71% after 12 weeks ↓70.59% after 12 weeks (near total recovery, however, when formalin solution also analysed)	Cingolani et al. (2001)

Drugs analysed	Sample type	Conditions	Results (↓ = decrease in concentration, ↑ = increase in concentration, ↔ = stable concentration)	Literature reference
Morphine	Formalin-fixed liver, lung, kidney and heart	Samples from sacrificed rabbits, fixed in 10% formalin solution, non-buffered, for 4 months	↓95.87% compared to frozen samples (↓95.03% when formalin solution also analysed) ↓83.54% compared to frozen samples (↓45.01% when formalin solution also analysed)	Xiang et al. (2001)
Morphine (as a metabolite of Heroin)	Bile and liver sample from embalmed body	Analysis undertaken 9 days after embalming	Samples positive for Morphine	Alunni-Perret et al. (2003)
Oxycodone	Spiked blood in formalin solution	4% formalin solution buffered to pH 7.0 for 21 days at 4°C	↓100%	Ameline, Raul and Kintz (2019)
Stimulants:				
Methamphetamine	Formalin-fixed brain, lung, liver, kidney and skeletal muscle	Samples from sacrificed rabbits, fixed in 10% formalin, stored at room temperature for 28 days	↓from 99.6% to 99.96%	Takayasu et al. (1994)
Cocaine, Benzoylecgonine	Formalin-fixed liver samples	Samples from forensic cases, fixed in 10% formalin, pH 7, for 4 weeks	↓87.69% after 4 weeks (↓3.22% when formalin solution also analysed)	Cingolani et al. (2004)
Methamphetamine	Drug in formalin solution	5%, 10% and 20% formalin solution at pH 3.5, 7 and 9.5 for 30 days	↓variable, depending on formalin % and pH, but up to 100%	Tirumalai et al. (2005)
MDMA and MDDM	Formalin-fixed human liver samples	Fixed in 20% formalin solution, unbuffered, for 24hrs	↓approximately 90%	Shakleya et al. (2005)
Methamphetamine	Formalin-fixed human liver samples	Formalin solution 10%/20% (non-buffered)	Samples positive for Methamphetamine and derivative after 24hrs	Shakleya et al. (2006)
Cocaine, Benzoylecgonine	Human brain and liver samples in formalin solution	Fixed in 10% formalin solution, unbuffered (pH~3.5) and buffered to pH 7.4	↓up to 80% in liver samples ↓up to 60% in brain samples	Viel et al. (2009)
Cocaine, Benzoylecgonine	Rat brain, liver, lung and kidney samples	Fixed in 10% formalin solution buffered to pH 7.0, at 25°C, for 30 days	Samples positive for methylated derivative	Hilal et al. (2009)

Drugs analysed	Sample type	Conditions	Results (↓ = decrease in concentration, ↑ = increase in concentration, ↔ = stable concentration)	Literature reference
Methamphetamine	Drugs in formalin solution	90 days at 4°C / room temp: Formalin solution 10%/20%, non-buffered and buffered to pH 7.4 for 90 days at 4°C and room temp	↓variable, depending on formalin % and pH, but up to 100%	Suzuki and Kaneko (2009)
MDMA, Mephedrone, 3-TFMPP	Drugs in formalin solution	5%, 10% and 20% formalin in unbuffered pH~3.5, pH 7.0 and 9.5, 60 days at room temp.	↓26% to↓37% in pH~3.5 ↓50% to↓96% in pH9.5	Maskell et al. (2013)
Methamphetamine, MDMA	Drugs in formalin-fixed liver and kidney samples from forensic case	15% formalin solution, unbuffered (pH 4.5-5.0) for 13 months at 4°C and room temperature	MA ↓between 0% and 37.6% MDMA ↓ between 0% and 40.4%	Uekusa, Hayashida and Ohno (2015)
SSRI Anti-depressants:				
Fluoxetine and Norfluoxetine	Embalmed human liver	Body exhumed 24 months after embalming	Fluoxetine detected at 27.4µg/g Norfluoxetine detected at 20.1µg/g	Rohrig 1998
Sertraline	Drugs in formalin solution	5%, 10% and 20% formalin solution at pH 3.0, 7.0 and 9.5 for 30 days	↓variable, but up to 100% in 20% formalin / pH 9.5	Suma and Prakash (2006a)
Fluoxetine	Rat liver samples in formalin solution	Samples injected with Fluoxetine and fixed in 5%, 10% and 20% formalin solution at pH 3.0, 7.0 and 9.5 for 30 days	↓variable, but up to 100% in 20% formalin / pH 9.5	Suma, Shukla and Prakash (2006b)
Paroxetine	Drug-spiked liver homogenates	6% formaldehyde buffered to pH 7.0-7.5 at room temperature for six months	↓<90%	Asano et al. (2020)
Tricyclic Anti-depressants:				
Nortriptyline	Formalin-fixed liver samples	1, 5, 10, 20 and 40% v/v formaldehyde solution, non-buffered, and buffered to pH 2.0, 4.0 and 9.5 for 7 days	↓variable, up to 89.2% in 20% formaldehyde at pH 9.5	Dettling et al. (1990)
Desipramine	Drug in formalin solution Formalin-fixed liver	Fixed in 5% and 8% formalin solution for 30 days	↓71.66% in 5% formalin ↓71.56% in 8% formalin	Winek, Esposito and Cinicola (1990)

Drugs analysed	**Sample type**	**Conditions**	**Results** (↓ = decrease in concentration, ↑ = increase in concentration, ↔ = stable concentration)	**Literature reference**
Amitriptyline, Desipramine, Imipramine, Nortriptylene	Formalin-fixed forensic liver samples	Fixed in 10% formalin, buffered to pH 6.9-7.1 for 7-22 months	↓highly variable but up to 97.4% in liver samples, though in some cases ↓0% when formalin solution also analysed	Winek, Zaveri and Wahba (1993a)
Amitriptyline, Desipramine, Imipramine, Nortriptylene	Drugs in formalin solution	3.7% formalin solution buffered to pH 3, 5, 7, 9 and 11	Amitriptyline ↓variable, up to 97.3% Desipramine ↓variable, up to 36%% Imipramine ↓variable, up to 73%% Nortriptyline generally ↔	Takayasu et al. (1998)
Amitriptyline	Drug-spiked liver homogenates	6% formaldehyde buffered to pH 7.0-7.5 at room temperature for six months	↓<20%	Asano et al. (2020)
<u>Anti-Psychotics</u>:				
Chlorpromazine	Formalin-fixed brain, lung, liver, kidney and skeletal muscle	Samples from sacrificed rabbits, fixed for 28 days in: 1) 10% formalin solution (buffered pH 7.4); 2) 10% formalin solution (non-buffered, pH 5.1); 3) 4% paraformaldehyde solution (buffered, pH 7.4)	1) ↓up to 52% 2) ↓up to 88% 3) ↓up to 97%	Nishigami, Takayasu and Ohshima (1995)
Olanzapine, Buproprion	Drugs in formalin solution	5%, 10% and 20% formalin solution at pH 3.0, 7.0 and 9.5 for 30 days	Bupropion ↓up to 100% in 20% formalin at pH 9.5 Olanzapine ↓only up to 2%	Suma, Kosanam and Prakash (2006c)
Chlorpramazine, Levomepromazine, Promethazine	Drugs in formalin solution and formalin-fixed liver and kidney samples from forensic case	15% formalin solution, unbuffered (pH 4.5-5.0) for 13 months at 4°C and room temperature	Chlorpromazine ↓up to 45.7% Levomepromazine ↓up to 51.7% Promethazine ↓up to 48%	Uekusa, Hayashida and Ohno (2015)
Levemepromazine and Quetiapine	Drug-spiked liver homogenates	6% formaldehyde buffered to pH 7.0-7.5 at room temperature for six months	Levemepromazine ↓<99% Quetiapine ↓<75%	Asano et al. (2020)

Drugs analysed	Sample type	Conditions	Results (↓ = decrease in concentration, ↑ = increase in concentration, ↔ = stable concentration)	Literature reference
<u>Barbiturates:</u>				
Phenobarbital	Brain tissue from forensic case	Fixed in formalin solution for 15months (conditions not stated)	Phenobarbital detected at 10μg/ml	Tsoukali-Papadopoulou (1987)
Phenobarbital	Formalin-fixed liver samples from forensic case Drugs in formalin solution	Fixed in 5% and 8% formalin solution (unknown pH) for 28 days Formalin solution 5%/10%/20% in non-buffered and Ph 7.0 and 9.5 for 30 days	↓62.36% in 5% formalin, but ↓45.10% when formalin solution also analysed ↓67.46% in 8% formalin, but ↓56.36% when formalin solution also analysed	Winek, Esposito and Cinicola (1990)
Methohexital	Embalmed human liver	Body exhumed 24 months after embalming	Detected at 0.17μg/g	Rohrig (1998)
Pentobarbital, Phenobarbital, Secobarbital	Drugs in formalin solution	5%, 10% and 20% formalin in unbuffered pH~3.5, pH 7.0 and 9.5 for 30 days	Pentobarbital ↓up to 30% Phenobarbital ↓up to 50% Secobarbital ↓up to 3%	Gannett et al. (2001b)
Butalbital and Phenobarbital	Formalin-fixed liver samples from autopsy cases	Fixed in 10% formalin buffered to pH 7.0 for 6 months	Butalbital ↓78.34%, but ↓11.78% when formalin solution also analysed Phenobarbital ↓42.89%, but ↓12.05% when formalin solution also analysed	Cingolani et al. (2005)
<u>Volatile Organic Compounds:</u>				
Ethchlorvynol	Bile and pericardial fluid from forensic case	Samples taken 52hr after embalming	112mg/L in bile; positive (not quantitated) in pericardial fluid	Winek et al. (1988)
Toluene, Ethanol, Diethylether, Chloroform	Formalin-fixed brain, lung, liver, kidney and skeletal muscle	Samples from sacrificed rabbits, fixed in 10% formalin, non-buffered (pH 5.1) stored at room temperature for 14 days	↓56.73-88.00% chloroform ↓56.03-87.96% diethylether ↓87.00-95.67% ethanol ↑8.57-↓39.80% toluene	Takayasu et al. (1994b)
<u>Other drugs and substances:</u>				
Succinylcholine (neuromuscular blockading agent)	Spiked kidney, liver and muscle samples from rats	Fixed in formalin solution at 8ºC for 6 months (other conditions not stated)	All tissues positive for Succinylcholine after 6 months	Forney et al. (1982)
Phenytoin (anti-epileptic)	Spiked blood	Added to 5% and 8% formalin, for 30 days	↓35.7% in 5% formalin ↓33.7% in 8% formalin	Winek, Esposito and Cinicola (1990)

Drugs analysed	Sample type	Conditions	Results (↓ = decrease in concentration, ↑ = increase in concentration, ↔ = stable concentration)	Literature reference
Paraquat (herbicide)	Formalin-fixed organs from forensic case, formalin-fixed rabbit kidney, liver and lung, and formalin-fixed rat liver	Human case 1 tested 2 days after fixation, human case 2 tested approximately 1 month after fixation, rabbit and rat samples fixed in 10% formalin solution (unknown time)	All human and animal samples tested positive for paraquat	Kuo and Kuo (1988)
Paraquat (herbicide)	Formalin-fixed kidney, liver, lung and spleen from two forensic cases	Samples stored in formalin for 1.5 years (case 1) and 6.5 years (case 2)	0.02-0.08 µg/g case 1 0.02-0.06 µg/g case 2	Minakata et al. (1989)
Paraquat (herbicide)	Formalin-fixed liver, kidney and lung samples from three forensic cases	Variously, fixed for 2 days to 2 months (concentration and pH not stated)	↓up to 56.78%, but significant recovery also possible from formalin solution in which samples stored	Kuo (1990)
Carbon monoxide (as product of fire)	Formalin-fixed spleens, embalmed spleen and embalmed and exhumed skeletal muscle from 4 forensic cases	Embalmed or fixed tissues (precise conditions not stated)	4.6µg/g detected in exhumed muscle 12µg/g in embalmed spleen 6.0 and 22µg/g in formalin-fixed spleens	Middleberg et al. (1993)
Heavy metals	Formalin-fixed brain, kidney, liver, heart, hair and skeletal muscle samples from 30 forensic cases	Fixed in formalin (concentration and pH not stated) for 12 months	↓<1% for calcium, copper, iron, magnesium, zinc, cadmium, arsenic, mercury and lead ↑155% for aluminium ↓36.67% for manganese	Bush et al. (1995)
Diphenhydramine and Promethazine (antihistamine) Lidocaine (antiarrhythmic)	Embalmed human liver	Body exhumed 24 months after embalming	Diphenhydramine detected at 4.7µg/g Promethazine detected at 3.2µg/g Lidocaine detected at 8.7µg/g	Rohrig (1998)
Strychnine (poison)	Formalin-fixed liver and kidney samples from forensic case	Fixed in 10% formalin buffered to pH 7.0 for 8 weeks	↓up to 63.43% in kidney (but ↓22.01% when formalin solution also analysed) ↓up to 72.20% in liver (but ↓49.25% when formalin solution also analysed)	Cingolani et al. (1999)

Drugs analysed	Sample type	Conditions	Results (↓ = decrease in concentration, ↑ = increase in concentration, ↔ = stable concentration)	Literature reference
Fenfluramine (anorectic)	Drugs in formalin solution	5%, 10% and 20% formalin in unbuffered pH~3.5, pH 7.0 and 9.5 for 30 days	↓highly variable, depending on formalin % and pH, but up to 100% in 20% formalin at pH 9.5	Gannett et al. (2001a)
Tetramine (rodenticide)	Formalin-fixed liver, lung, kidney and heart	Samples from sacrificed rabbits, fixed in 10% formalin solution, non-buffered, for 4 months	↓92.87% compared to frozen samples (↓88.89% when formalin solution also analysed)	Xiang et al. (2001)
Lidocaine (antiarrhythmic)	Formalin-fixed brain, liver, kidney and skeletal muscle from rats	Fixed in 10% formalin solution buffered to pH 7.0 for 4 weeks	↓variable between tissues, but up to 80%	Kudo et al. (2004)
Sildenafil (anti-hypertensive)	Formalin-fixed brain, heart, kidney, liver, lung and spleen samples from forensic case	% concentration of formalin solution not stated, nor pH. Samples stored for 4 weeks	↓variable between tissues up to 81.05% (but between ↓1.11% and 11.61% when formalin solution also analysed	Pagani et al. (2005)
Acetaminophen (analgesic) Salicylic acid (dermatologic)	Formalin-fixed brain, heart, kidney, liver, lung and spleen samples from forensic case	10% and 20% formalin solution, non-buffered and buffered to pH 7.4	Acetaminophen ↓variable, depending on formalin % and pH <60% Salicylic acid ↔in all solutions	Suzuki and Keneko (2009)
Aconitum alkaloids (poisons)	Liver kidney and lung samples from forensic case	Stored in 10% formalin solution buffered to pH 7.0 for 5 years	~10pg/g of benzolyaconine and benzolylmesaconine detected	Miyaguchi and Sekine (2010)
Methamidophos (organophosphorous insecticide)	Blood samples from dosed and sacrificed dogs	10% formalin solution (pH not stated)	↓100% after 10 days	Wei et al. (2017)
Diphendydramine (antihistamine)	Drug-spiked liver homogenates	6% formaldehyde buffered to pH 7.0-7.5 at room temperature for six months	↓<5%	Asano et al. (2020)

Key:

Drug analysed: listed by drug class, corresponding to categories used in main text
Sample type: description of matrix basis of experiment whether forensic case, experimental tissue or drugs in formalin solution
Conditions: length of time since embalming and/or description of main experimental conditions
Results: quantitative results for exhumation cases; stability results at end of experimental period in other cases

Appendix 3 - Comparative post-mortem cerebrospinal fluid drug concentrations reported in the literature since 1994

Drug	Analysis Method	Blood Concentration and source of sample (number of cases)	CSF Concentration (number of cases)	CSF:Blood ratio	Literature Reference
Morphine	EMIT/FPIA/RIA GC/MS	0.021-0.294 µg/mL (23)	0.023-0.14 µg/mL (23)	0.18-1.82	Logan and Luthi (1994)
Morphine Amitriptyline Caffeine Carbamazepine Clomethiazole Diazepam Flurazepam Phenazone Phenobarbital Primidone	I/RIA HPLC/DAD	0.12-0.53 µg/mL (154) 2.1µg/mL (1) 1.1-3.0µg/mL (2) 7.2µg/mL (1) 194µg/mL (1) 0.4-0.6µg/mL (2) 19µg/mL (1) 3.2µg/mL (1) 7.3-88µg/mL (2) 3.3µg/mL (1)	0.08-0.2 µg/mL (103) 0.05µg/mL (1) 1.1-1.4µg/mL (1) 7.8µg/mL (1) 22µg/mL (1) 0.06-0.4µg/mL (1) 0.6µg/mL 1.0µg/mL (1) 5.1-35µg/mL (2) 2.7µg/mL (1)	0.45-1.12 0.02 0.37-1.27 1.08 0.11 0.15-0.68 0.03 0.13 0.40-0.69 0.82	Pragst et al. (1994)
6-monoacetylmorphine Morphine	SPE GC/MS	11.3/16.2ng/mL (2) 207.8/81.7ng/mL (2)	58.0/38.5ng/mL (2) 40.0/36.6ng/mL (2)	2.37-5.13 0.19-0.45	Goldberger et al. (1994)
6-monoacetylmorphine Morphine	LLE GC/MS	6-90ng/mL (33) 30-1580ng/mL (65)	1-320ng/mL (60) 5-1580ng/mL (61)	0.17-3.55 0.17-1.00	Jenkins and Lavins (1998)
6-monoacetylmorphine Morphine	SPE GC/MS	2-1600ng/mL (24) 0.03-5.8ng/mL (29)	1-30ng/mL (15) 0.024-0.43ng/mL (22)	0.02-0.5 0.07-0.8	Pragst et al. (1999)

Drug	Analysis Method	Blood Concentration (number of cases) and source of sample	CSF Concentration (number of cases)	CSF:Blood ratio	Literature Reference
6-monoacetylmorphine	SPE GC/MS	0.001-0.098mg/mL (13)	0.001-0.406mg/mL (16)	2.7-17.3	Wyman and Bultman (2004)
Morphine		0.01-0.57mg/mL (13)	0.01-0.38mg/mL (16)	0.25-1.56	
Codeine		0.01-0.04mg/mL (13)	0.01-0.04mg/mL (16)	0.55-2.00	
Benzodiazepines					
Diazepam	LLE GC/MS	Not Reported	Not Reported	0.36 (43)	Engelhart and Jenkins (2007)
Nordiazepam				0.37 (51)	
Alprazolam				0.85 (4)	
Chlordiazepoxide				0.24 (1)	
Anticonvulsants					
Phenytoin				0.33 (23)	
Phenobarbital				0.63 (8)	
Carbamazepine				0.4 (5)	
Sedatives					
Meprobamate				1.09 (21)	
Carisoprodol				0.96 (10)	
Butalbital				0.35 (2)	
Secobarbital				0.29 (1)	
Antidepressants					
Amitriptyline				0.3 (20)	
Nortriptyline				0.06 (17)	
Imipramine				0.21 (5)	
Desipramine				0.04 (4)	
Doxepin				0.3 (5)	

Drug	Analysis Method	Blood Concentration (number of cases) and source of sample	CSF Concentration (number of cases)	CSF:Blood ratio	Literature Reference
Sertraline					
Fluoxetine		Not	Not	0.27 (6)	Engelhart and Jenkins (2007)
Citalopram		reported	reported	0.02 (6)	cont'd
Venlafaxine				0.38 (6)	
Trazodone				0.68 (5)	
Mirtazapine				0.11 (5)	
Bupropion				0.19 (4)	
Erythro (*sic*)				0.38 (5)	
Threo (*sic*)				0.25 (7)	
Morphol (*sic*)				0.38 (7)	
				0.73 (6)	
Opioids					
Propoxyphene				0.33 (21)	
Methadone				0.33 (8)	
Meperidone				0.41 (7)	
Tramadol				0.21 (4)	
Oxycodone				0.72 (3)	
Anaesthetics				1.19 (45)	
Lidocaine				0.17 (7)	
Phencyclidine				0.47 (2)	
Ketamine					
Antihistamines					
Diphenhydramine				0.34 (25)	
Chlorpheniramine				0.32 (3)	
Doxylamine				0.14)	
NSAIDS					
Ibuprofen				0.02 (4)	

Drug	Analysis Method	Blood Concentration (number of cases) and source of sample	CSF Concentration (number of cases)	CSF:Blood ratio	Literature Reference
Others Dextromethorphan Caffeine Cyclobenzaprine Diltiazem Clozapine Guiafenisin		Not reported	Not Reported	 0.49 (10) 0.96 (10) 0.79 (5) 0.3 (5) 0.2 (3) 2.11 (1)	Engelhart and Jenkins (2007) cont'd
Cocaine	SPE GC/MS	3,210.60ng/mL (1) Femoral venous blood 970.40ng/mL (1) Femoral arterial blood	3,132.50ng/mL (1)	0.97 3.33	Alvear et al. (2014)
Benzoylecgonine (BZE)		19,847.00ng/mL (1) Femoral venous blood 3,031.70ng/mL (1) Femoral arterial blood	537.4ng/mL (1)	0.03 0.18	
Psychostimulants Methamphetamine	LLE/SPE GC/MS	0.06-1.84µg/mL Cardiac (19) 0.07-1.7µg/mL Iliac (19)	0.06-1.03µg/mL (23)	0.17-1.37 Cardiac 0.14-2.41 Iliac	Tominga et al. (2015)
Amphetamine		0.02-1.58 µg/mL Cardiac (17) 0.02-2.29µg/mL Iliac (12)	0.01-1.58µg/mL (18)	0.56-3.00 Cardiac 0.32-4.54 Iliac	
Narcotics/ Antitussive analgesics Acetaminophen		0.01-25.0µg/mL Cardiac (5) 0.47-13.2µg/mL Iliac (2)	0.01-25.0µg/mL (7)	0.52-1.55 Cardiac 0.60-0.63 Iliac	

Drug	Analysis Method	Blood Concentration (number of cases) and source of sample	CSF Concentration (number of cases)	CSF:Blood ratio	Literature Reference
Caffeine		0.10-10.9µg/mL Cardiac (15) 0.7-8.28µg/mL Iliac (11)	0.10-10.9µg/mL (15) 0.02-1.77µg/mL (6)	0.57-2.33 Cardiac 0.68-1.55 Iliac	Tominga et al. (2015) cont'd
Dihydrocodeine		0.02-1.77µg/mL Cardiac (5) 0.06-1.02µg/mL Iliac (4)	0.01-2.43µg/mL (6)	0.38-0.87 Cardiac 0.56-0.77 Iliac	
Chlorpheniramine		0.01-2.43µg/mL Cardiac (7) 0.01-1.03µg/mL Iliac (6)	0.09-52.8µg/mL (21)	0.28-0.67 Cardiac 0.42-0.67 Iliac	
Barbiturates					
Phenobarbital		0.09-52.8µg/mL Cardiac (21) 0.25-33.9µg/mL Iliac (12)	0.17-2.25µg/mL (3)	0.19-1.02 Cardiac 0.41-1.07 Iliac	
Secobarbital		-----	0.04-3.44µg/mL (17)	-----	
Phenothiazine derivatives					
Promethazine		0.04-3.44µg/mL Cardiac (16) 0.02-4.01µg/mL Iliac (12)	0.05-1.79µg/mL (8)	0.10-0.52 Cardiac 0.11-1.00 Iliac	

Drug	Analysis Method	Blood Concentration (number of cases) and source of sample	CSF Concentration (number of cases)	CSF:Blood ratio	Literature Reference
Chlorpromazine		0.01-2.43µg/mL Cardiac (7) 0.01-1.03µg/mL Iliac (6)	0.17-0.94µg/mL (4)	0.28-0.67 Cardiac 0.42-0.67 Iliac	Tominga et al. (2015) cont'd
Levomepromazine		-----	0.04-1.74µg/mL (7)	-----	
Benzodiazepines					
Midazolam		0.04-1.74µg/mL Cardiac (7) 0.04-0.43µg/mL Iliac (5)	0.11-0.18µg/mL (2)	0.05-0.57 Cardiac 0.22-0.79 Iliac	
Estazolam		-----	0.42µg/mL (1)	-----	
Flunitrazepam		-----	0.06-0.24µg/mL (3)	-----	
Hypnotics					
Zolpidem		-----	0.06-0.24µg/mL (3)	-----	
Antidepressants					
Olanzapine		-----	0.15-0.82µg/mL (4)	-----	
Mianserin		-----	0.11-0.54µg/mL (2)		
Mirtazapine		-----	0.11µg/mL (1)		
Anaesthetics					
Lidocaine		0.03-4.07µg/mL Cardiac (46) 0.01-2.21µg/mL Iliac (29)	0.03-4.07µg/mL (49)		
Thiamylal			0.45-22.3µg/mL (3)		

Drug	Analysis Method	Blood Concentration (number of cases)	CSF Concentration (number of cases)	CSF:Blood ratio	Literature Reference
Propofol Ketamine **Others** Diphenhydramine Methylphenedrine Isoniazid			3.73µg/mL (1) 0.96µg/mL (1) 0.05-0.89µg/mL (4) 0.27µg/mL (1) 10.5µg/mL (1)	----- ----- 0.10-3.67 Cardiac 0.02-12.18 Iliac	 Tominga et al. (2015) cont'd

Key:

Drug analysed: listed by drug class
Analysis method: analytical basis of method used
Blood concentration: analytical result or range, along with number of cases reported and, where listed, anatomical source of sample
CSF concentration: analytical result or range, along with number of cases reported
CSF:Blood ratio: calculated from analytical results, where reported for, CSF and blood concentrations from the same case

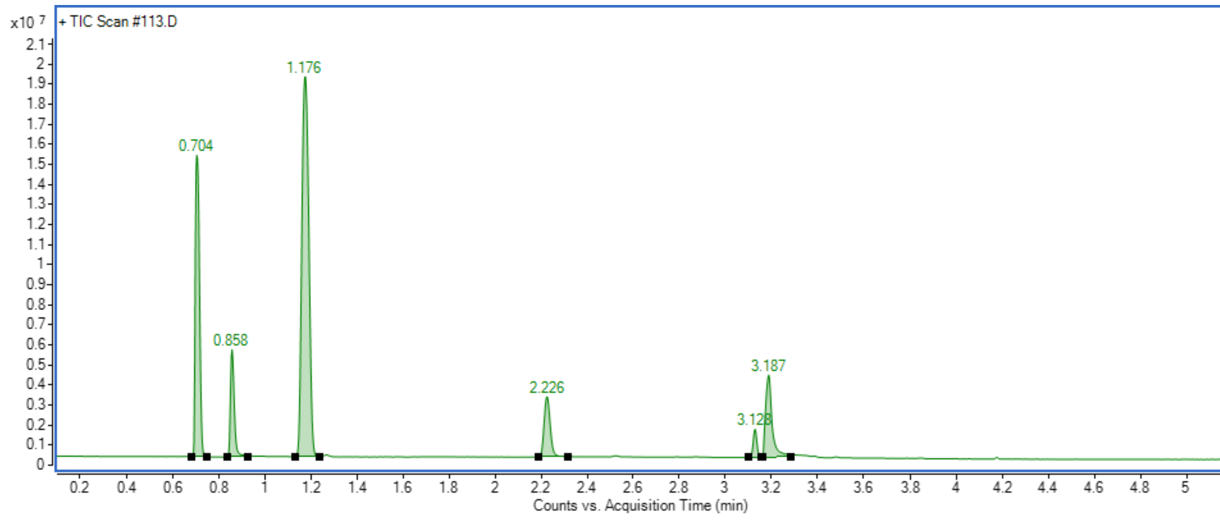
Appendix 4 – Comparative post-mortem synovial fluid drug concentrations reported in the literature since 1993

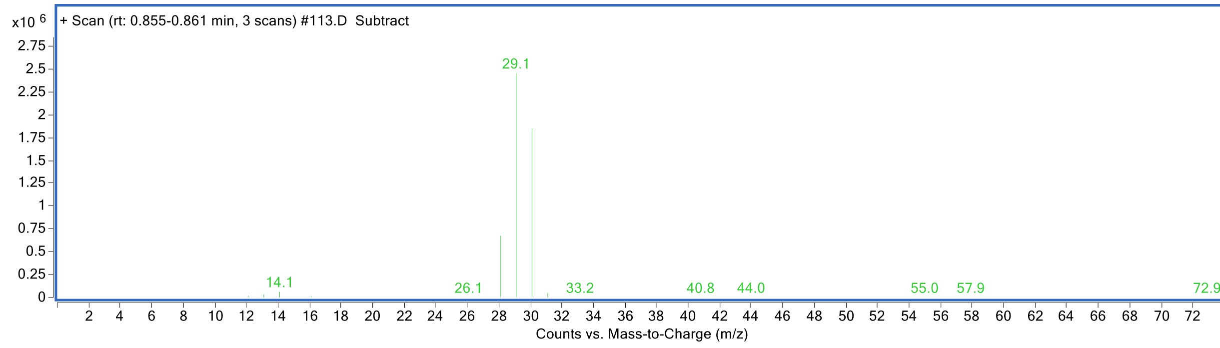
Drug	Analysis Method	Blood Concentration (number of cases)	SF Concentration (number of cases)	Blood:SF ratio	Literature Reference
Ethanol	GC/MS	20-323mg% (28)	38-3736mg% (28)	0.40-1.72 (Mean = 0.99 ± 0.29)	Winek et al. (1993b)
Ethanol	Py/GC	0.26-5.12mg/mL (12)	0.71-7.07mg/mL	0.60-0.94 (Mean = 0.76 ± 0.12)	Ohshima et al. (1997)
Ethanol	Headspace GC	243.1mg/dL ±140.3 (13)	270.5mg/dL ± 160.7 (13)	0.95 ± 0.07	Büyük et al. (2009)
Cocaine	SPE GC/MS	Mean = 0.16mg/L (20)	Mean = 0.07mg/L (17)	0.43-4.3 (Mean = 2.1)	DeKing, Hargrove and Molina (2014)
BZE		Mean = 0.78mg/L (23)	Mean = 0.54mg/L (23)	0.8-2.2 (Mean = 1.5)	
Morphine		Mean = 0.22mg/L (43)	Mean = 0.1mg/L (37)	0.3-8.6 (Mean = 2.5)	
6-MAM		-------	Mean = 0.03mg/L (13)	-------	
Hydrocodone		Mean = 0.14mg/L (18)	Mean = 0.07mg/L (18)	0.9-3.5 (Mean = 1.4)	
Oxycodone		Mean = 0.31mg/L (7)	Mean = 0.12mg/L (7)	0.0-3.7 (Mean = 1.9)	
Morphine	GC/MS	15.3-276ng/mL (5)	7.2-75.8ng/mL (5)	1.89-3.65 (mean = 2.71)	Petrochilou et al. (2019)
Codeine		9.1-21.2ng/mL (3)	5.6-7.5ng/mL (3)	1.63-3.12 (mean = 2.49)	
BZE		16.2-584ng/mL (3)	15.3-362ng/mL (3)	1.06-2.77 (mean = 1.81)	
EME		50.1-178ng/mL (3)	16.6-28.8 ng/mL (3)	2.61-6.32 (mean = 3.98)	
Cocaine		96.6ng/mL (1)	67.4ng/mL (1)	1.43	
6-MAM		Not detected	5.6ng/mL	N/A	

Key:

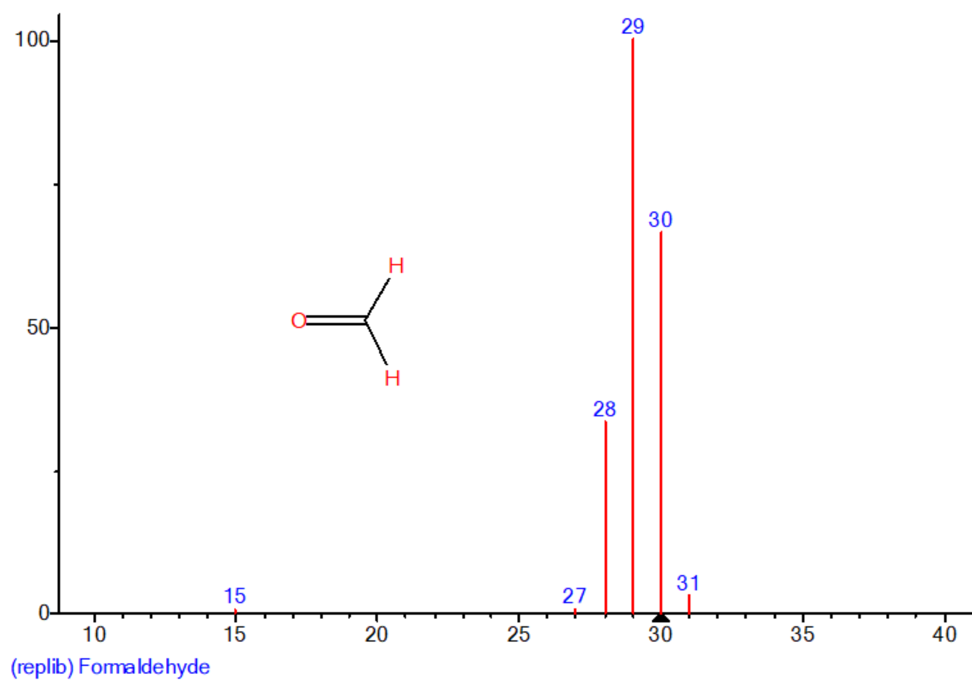
Drug analysed: listed by drug class
Analysis method: analytical basis of method used
Blood concentration: analytical result or range, along with number of cases reported and, where listed, anatomical source of sample
SF concentration: analytical result or range, along with number of cases reported
Blood:SF ratio: calculated from analytical results, where reported for SF and blood concentrations from the same case

Appendix 5 – Chromatogram and mass spectrometric data for formaldehyde

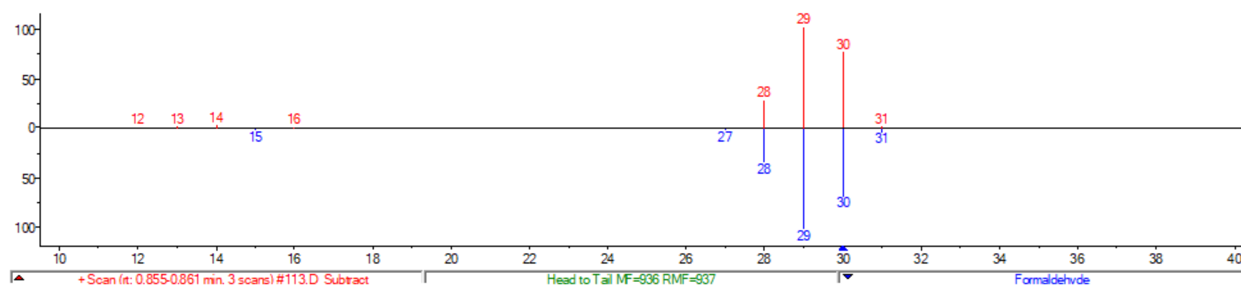




NIST Library spectrometric data



Comparison with NIST library
Red is MS from formaldehyde standard used in experiment and blue is MS from NIST library



Appendix 6 – Preparation of working solutions

Step	Starting solution	Dilution	End solution	Storage conditions
1a	1mg in 1mL drug standards (all drugs except carfentanil)	Each individual standard made up to 10mL with 50:50 ACN:H_2O	**1mg in 10mL** **'Basic working solution'** **(100,000ng/mL)**	10mL flasks sealed with parafilm and frozen at -20^oC
1b	100µg in 0.5mL drug standard (carfentanil)	Made up to 5mL with 50:50 ACN:H_2O	**100µg in 5mL** **'Basic working solution'** **(100,000ng/mL)**	5mL flask sealed with parafilm and frozen at -20^oC
2a	1mg in 10mL Basic working solution (all drugs except carfentanil)	100µL of each 'basic working solution' made up to 1mL with 50:50 ACN:H_2O	**10,000ng/mL working solution**	1.5mL HPLC vials frozen at -20^oC
2b	100µg in 5mL Basic working solution (carfentanil)	'Basic working solution' already diluted to 10,000ng/mL	**10,000ng/mL working solution**	1.5mL HPLC vial frozen at -20^oC
3	10,000ng/mL working solution (all drugs)	100µL of each 10,000ng/mL working solution made up to 1mL with 50:50 ACN:H_2O	**1,000ng/mL working solution**	1.5mL HPLC vials frozen at -20^oC

Appendix 7 – Preparation of calibration solutions

Step	Starting solution	Dilution	End solution
1	50µL of each 10,000ng/mL working solution	Made up to 1mL with 50:50 ACN:H_2O	**500ng/mL calibration solution**
2	100µL of each 500ng/mL calibration solution (1)	Made up to 200µL with 50:50 ACN:H_2O	**250ng/mL calibration solution**
3	100µL of each 500ng/mL calibration solution (1)	Made up to 500µL with 50:50 ACN:H_2O	**100ng/mL calibration solution**
4	100µL of each 500ng/mL calibration solution (1)	Made up to 1mL with 50:50 ACN:H_2O	**50ng/mL calibration solution**
5	200µL of each 50ng/mL calibration solution (4)	Made up to 400µL with 50:50 ACN:H_2O	**25ng/mL calibration solution**
6	100µL of each 100ng/mL calibration solution (3)	Made up to 1mL with 50:50 ACN:H_2O	**10ng/mL calibration solution**
7	50µL of each 50ng/mL calibration solution (4)	Made up to 1mL with 50:50 ACN:H_2O	**5ng/mL calibration solution**

Appendix 8 – Preparation of buffer and buffered formaldehyde solutions

Step	Starting solution	Dilution	End solution
1	N/A	0.35g potassium phosphate monobasic (KH_2PO_4) and 1.27g potassium phosphate dibasic (K_2HPO_4) made up to 1L with 50:50 ACN:H_2O	**pH 7.4 phosphate buffer 0.01M**
2	10.81mL formaldehyde standard (370mg/mL)	Made up to 200mL with pH 7.4 phosphate buffer (1)	**20,000µg/mL buffered formaldehyde solution**
3	5.40mL formaldehyde standard (370mg/mL)	Made up to 200mL with pH 7.4 phosphate buffer (1)	**10,000µg/mL buffered formaldehyde solution**
4	540µL formaldehyde standard (370mg/mL)	Made up to 200mL with pH 7.4 phosphate buffer (1)	**1,000µg/mL buffered formaldehyde solution**
5	54µL formaldehyde standard (370mg/mL)	Made up to 200mL with pH 7.4 phosphate buffer (1)	**100µg/mL buffered formaldehyde solution**

Appendix 9 – Preparation of sample solutions

Batch Name / Number	Starting solutions	Dilution	End solution
Starting solutions (all drugs except carfentanil	500µL each of the 1mg in 10mL drug working solutions	Made up to 5mL with 50:50 ACN:H2O	**10,000ng/mL starting solutions**
Starting solution (carfentanil)	100µL in 0.5mL fresh drug standard	Made up to 5mL with 50:50 ACN:H2O	**10,000ng/mL starting solutions**
Batch 1A	500µL each of the 6-MAM, diazepam and zolpidem starting solutions	Made up to 10mL with pH 7.4 phosphate buffer	**500ng/mL control solutions (for refrigeration)**
Batch 1B	500µL each of the 6-MAM, diazepam and zolpidem starting solutions	Made up to 10mL with 20,000µg/mL buffered formaldehyde	**500ng/mL in 20,000ng/mL buffered formaldehyde (for refrigeration)**
Batch 1C	500µL each of the 6-MAM, diazepam and zolpidem starting solutions	Made up to 10mL with 20,000µg/mL buffered formaldehyde	**500ng/mL in 20,000ng/mL buffered formaldehyde (for room temperature)**
Batch 1D	500µL each of the 6-MAM, diazepam and zolpidem starting solutions	Made up to 10mL with 10,000µg/mL buffered formaldehyde	**500ng/mL in 10,000ng/mL buffered formaldehyde (for refrigeration)**
Batch 1E	500µL each of the 6-MAM, diazepam and zolpidem starting solutions	Made up to 10mL with 10,000µg/mL buffered formaldehyde	**500ng/mL in 10,000ng/mL buffered formaldehyde (for room temperature)**
Batch 1F	500µL each of the 6-MAM, diazepam and zolpidem starting solutions	Made up to 10mL with 1,000µg/mL buffered formaldehyde	**500ng/mL in 1,000ng/mL buffered formaldehyde (for refrigeration)**
Batch 1G	500µL each of the 6-MAM, diazepam and zolpidem starting solutions	Made up to 10mL with 1,000µg/mL buffered formaldehyde	**500ng/mL in 1,000ng/mL buffered formaldehyde (for room temperature)**
Batch 1H	500µL each of the 6-MAM, diazepam and zolpidem starting solutions	Made up to 10mL with 100µg/mL buffered formaldehyde	**500ng/mL in 100ng/mL buffered formaldehyde (for refrigeration)**
Batch 1I	500µL each of the 6-MAM, diazepam and zolpidem starting solutions	Made up to 10mL with 100µg/mL buffered formaldehyde	**500ng/mL in 100ng/mL buffered formaldehyde (for refrigeration)**
2A-E	500µL each of the codeine and flunitrazepam starting solutions	Following the same protocol as 1A-E	**As 1A-E**
3A-E	500µL each of the BZE, carfentanil and midazolam starting solutions	Following the same protocol as 1A-E	**As 1A-E**
4A-E	500µL each of the cocaine, ketamine and methadone starting solutions	Following the same protocol as 1A-E	**As 1A-E**
5A-E	500µL each of the alfentanil and morphine starting solutions	Following the same protocol as 1A-E	**As 1A-E**
6A-E	500µL each of the fentanyl and lorazepam starting solutions	Following the same protocol as 1A-E	**As 1A-E**

Appendix 10 – Chromatographic t_R, $[M+H]^+$, R, R^2, LOQ, LOD and related data for all drugs

Batch 1

Drug	Retention time (m)	$[M+H]^+$	Calibration points (*n*)	SE	Slope	Intercept	Calibration curve R	Calibration curve R^2	LOQ (ng/mL)	LOD (ng/mL)
6-MAM	1.880	328.1540	6	10,021.41	7,789.31	357,254.37	1.00	1.00	12.87	4.25
Zolpidem	3.883	308.1763	7	216,734.97	64,264.46	297,402.76	0.9999	0.9998	33.73	11.13
Diazepam	6.910	285.0792	7	24,689.95	29,258.57	841,725.80	1.00	1.00	8.44	2.78

Batch 2

Drug	Retention time (m)	$[M+H]^+$	Calibration points (*n*)	SE	Slope	Intercept	Calibration curve R	Calibration curve R^2	LOQ (ng/mL)	LOD (ng/mL)
Codeine	1.454	300.1593	7	4,877.61	11,706.91	4,140.04	1.00	0.9999	4.17	1.37
Flunitrazepam	6.427	314.0929	6	68,942.00	15,176.52	74,896.68	0.9999	0.9997	45.43	14.99

Batch 3

Drug	Retention time (m)	$[M+H]^+$	Calibration points (*n*)	SE	Slope	Intercept	Calibration curve R	Calibration curve R^2	LOQ (ng/mL)	LOD (ng/mL)
BZE	2.521	290.1390	7	10,858.53	27,601.03	17,946.51	0.9999	0.9997	3.93	1.30
Midazolam	5.074	326.0860	6	1,165,803.59	188,568.84	997,326.77	0.9996	0.9992	61.82	20.40
Carfentanil	5.611	395.2341	6	42,546.43	27,168.19	367,736.47	1.00	1.00	15.66	5.17

Batch 4

Drug	Retention time (m)	$[M+H]^+$	Calibration points (*n*)	SE	Slope	Intercept	Calibration curve R	Calibration curve R^2	LOQ (ng/mL)	LOD (ng/mL)
Ketamine	2.478	238.0983	7	28,834.46	19,494.17	41,251.76	1.00	1.00	14.79	4.88
Cocaine	3.575	304.1535	7	60,963.62	41,284.03	17,100.10	1.00	1.00	14.77	4.87
Methadone	6.681	310.2156	6	42,625.91	44,566.45	485,814.28	1.00	1.00	9.56	3.16

Batch 5

Drug	Retention time (m)	$[M+H]^+$	Calibration points (*n*)	SE	Slope	Intercept	Calibration curve R	Calibration curve R^2	LOQ (ng/mL)	LOD (ng/mL)
Morphine	0.712	286.1438	6	5,122.88	2,945.07	129,328.01	1.00	1	17.39	5.74
Alfentanil	4.926	417.2608	6	50,492.91	50,117.32	77,505.90	0.9998	0.9996	10.07	3.32

Batch 6

Drug	Retention time (m)	$[M+H]^+$	Calibration points (*n*)	SE	Slope	Intercept	Calibration curve R	Calibration curve R^2	LOQ (ng/mL)	LOD (ng/mL)
Fentanyl	4.987	337.2278	6	29,700.67	31,242.30	1,803,205.62	1.00	1.00	9.51	3.14
Lorazepam	6.231	321.0194	6	34,391.93	38,813.03	-584,675.40	1.00	1.00	8.86	2.92

Appendix 11 – Instrument accuracy and precision

Compound	Ref. conc. (ng)	Accuracy (% deviation)	Repeatability (%RSD)	Reproducibility (%RSD)
Batch 1				
6-MAM	50	13.66	9.30	20.84
	250	3.38	7.32	19.87
	500	2.26	4.57	17.96
Zolpidem	50	3.00	0.70	6.53
	250	1.00	1.25	8.63
	500	1.70	3.81	9.45
Diazepam	50	9.90	1.98	12.09
	250	2.10	2.08	9.57
	500	2.70	8.45	6.18
Batch 2				
Codeine	50	8.46	2.39	11.57
	250	3.04	1.74	12.44
	500	0.94	6.00	12.38
Flunitrazepam	50	4.40	2.85	7.41
	250	3.30	2.19	12.24
	500	0.50	6.56	11.22
Batch 3				
BZE	50	6.60	1.76	10.6
	250	3.60	3.91	14.51
	500	1.30	12.78	14.80
Midazolam	50	6.50	7.39	11.67
	250	7.70	3.47	14.83
	500	3.85	3.58	12.96
Carfentanil	50	13.46	2.05	3.00
	250	3.19	1.54	9.81
	500	4.60	4.42	9.61

Compound	Ref. Conc. (ng/mL)	Accuracy (% deviation)	Repeatability (%RSD)	Reproducibility (%RSD)
Batch 4				
Ketamine	50	8.06	3.48	2.35
	250	4.60	0.67	3.74
	500	1.30	0.48	3.4
Cocaine	50	5.40	0.70	3.52
	250	6.44	2.35	8.28
	500	1.56	4.63	7.86
Methadone	50	0.00	2.20	4.50
	250	0.10	1.25	7.04
	500	5.30	3.66	6.01
Batch 5				
Morphine	50	14.08	2.23	10.2
	250	3.20	3.06	16.57
	500	1.00	7.27	8.94
Alfentanil	50	8.40	1.79	12.67
	250	4.07	0.65	7.68
	500	3.20	4.32	8.93
Batch 6				
Fentanyl	50	12.90	1.33	3.37
	250	0.70	1.03	6.85
	500	0.90	3.93	6.63
Lorazepam	50	11.90	10.45	3.58
	250	2.40	0.75	8.45
	500	0.40	6.74	8.98

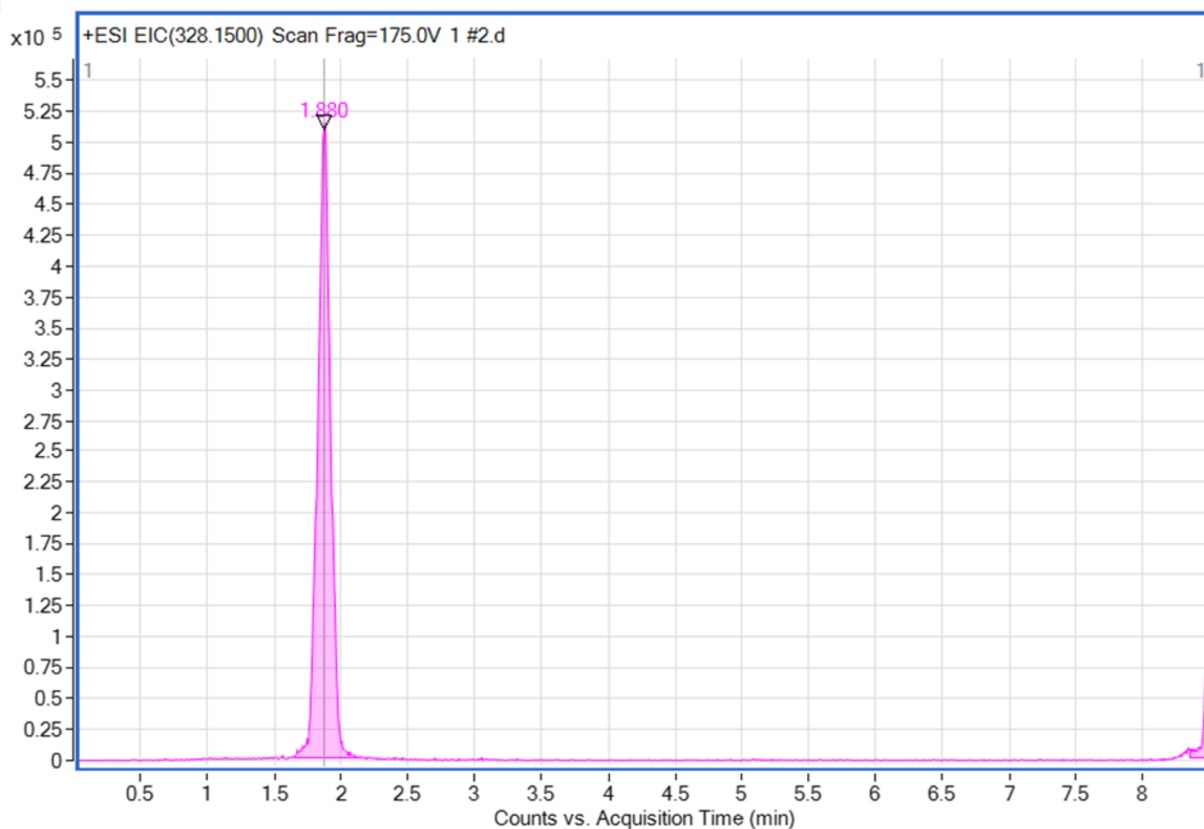
Appendix 12 – Analyte stability

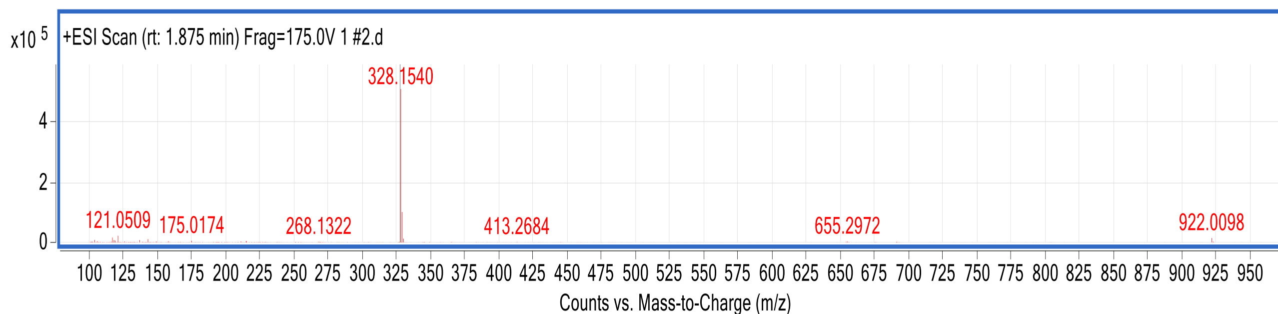
Showing the recovery (as a percentage, rounded to the nearest whole number) on each analysis day

	Day 0 Recovery (%)	**Day 2 Recovery (%)**	**Day 4 Recovery (%)**	**Day 7 Recovery (%)**	**Day 14 Recovery (%)**	**Day 35 Recovery (%)**
6-MAM	100	100	100	99	98	96
Alfentanil	100	100	100	100	100	100
BZE	100	100	100	100	99	97
Carfentanil	100	100	100	99	99	98
Cocaine	100	98	No data	95	89	73
Codeine	98	93	89	87	85	74
Diazepam	100	100	100	100	100	100
Fentanyl	100	100	No data	100	100	100
Flunitrazepam	100	100	100	100	99	97
Ketamine	100	100	No data	99	99	96
Lorazepam	100	100	No data	99	98	95
Methadone	100	100	No data	100	99	97
Midazolam	100	100	100	100	100	100
Morphine	100	100	No data	99	98	96
Zolpidem	100	100	100	99	98	96

Appendix 13 - Chromatogram and mass spectrometric data for 6-MAM

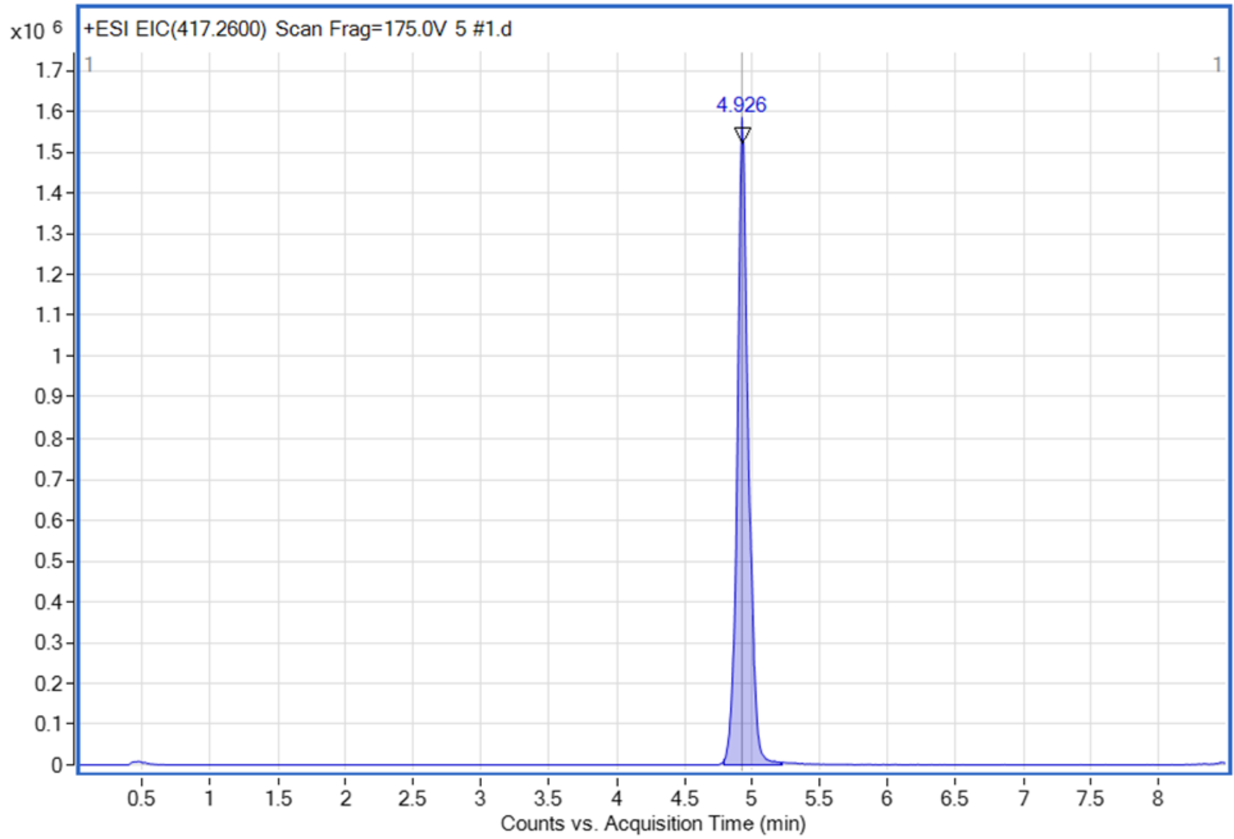
t_R = 1.880

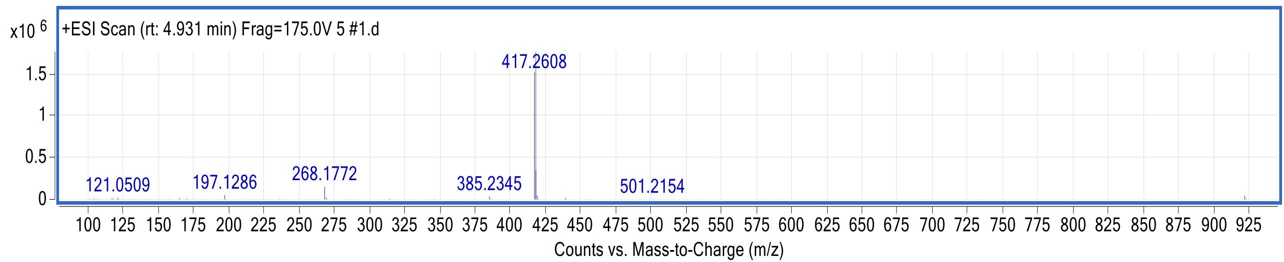




Appendix 14 - Chromatogram and mass spectrometric data for Alfentanil

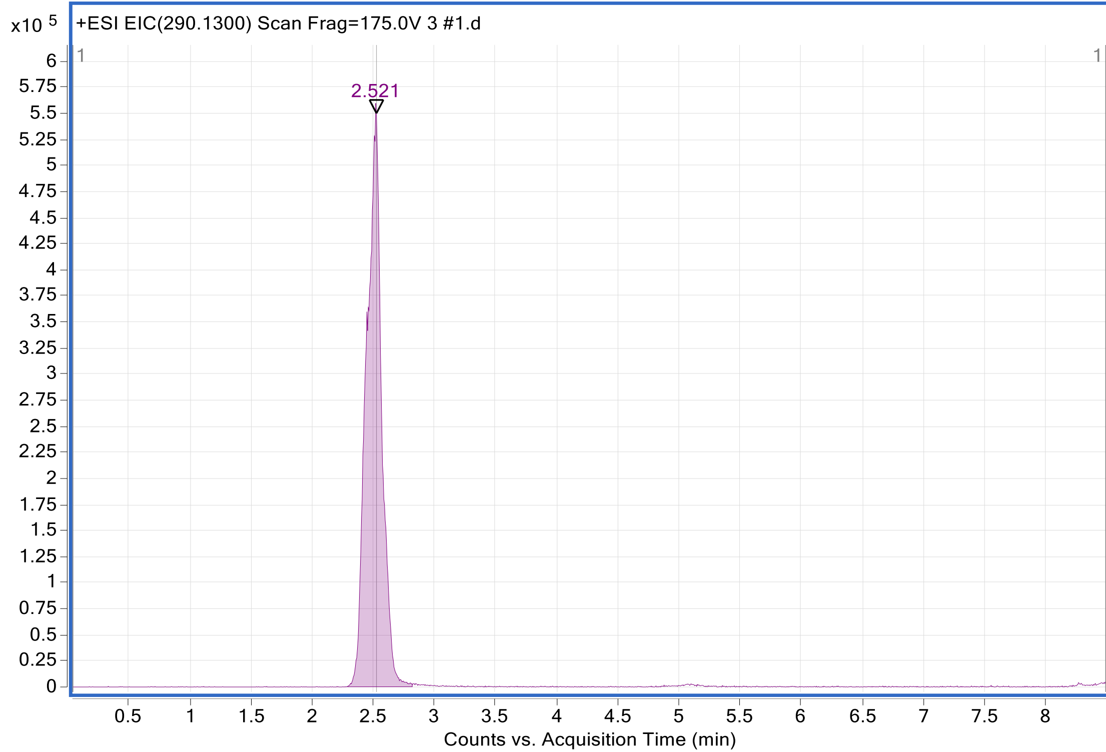
t_R = 4.926

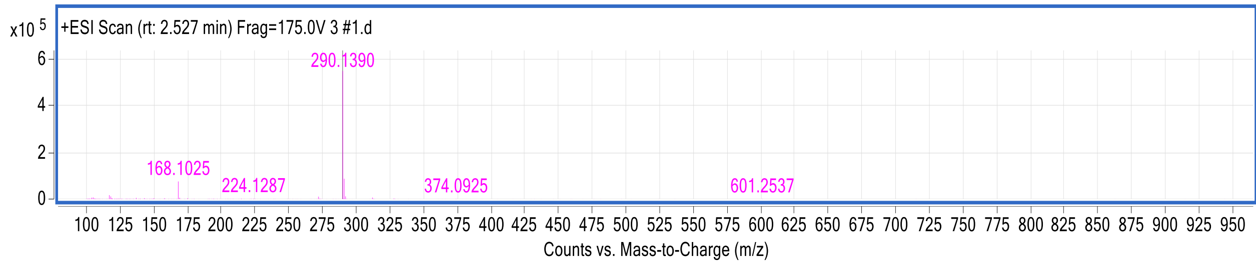




Appendix 15 - Chromatogram and mass spectrometric data for BZE

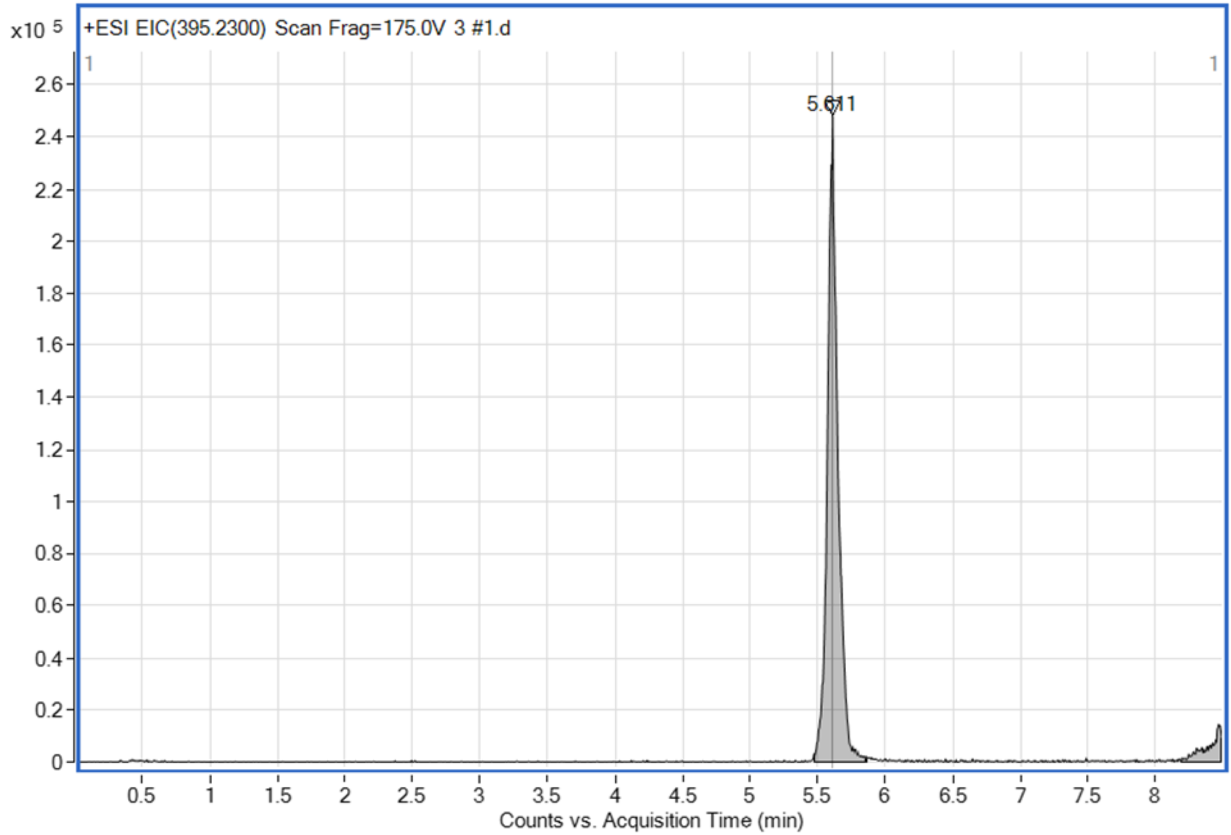
t_R = 2.521

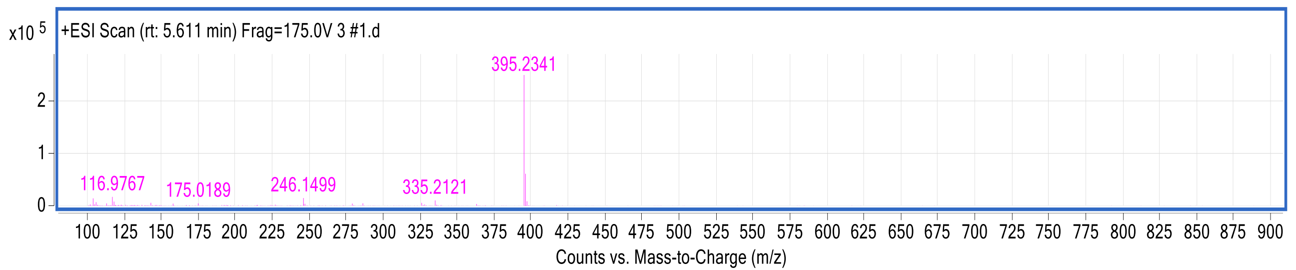




Appendix 16 - Chromatogram and mass spectrometric data for Carfentanil

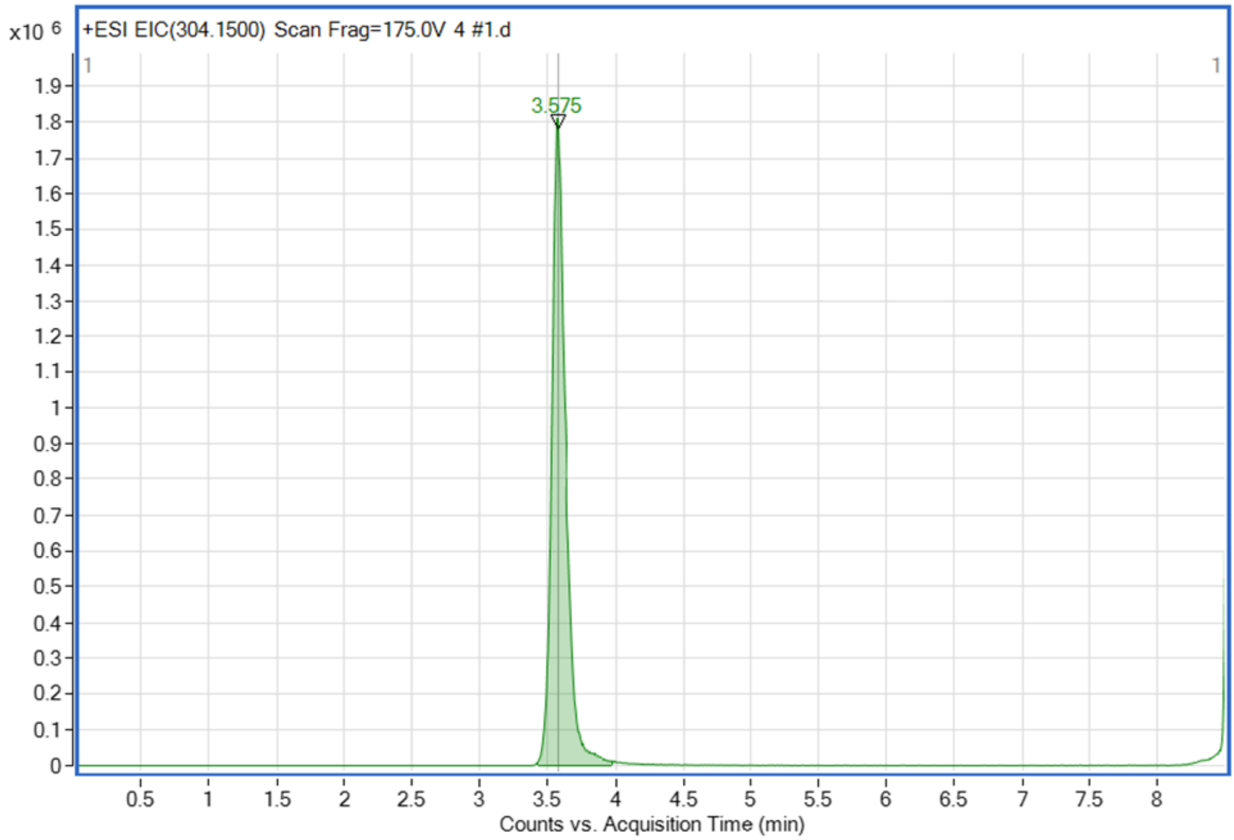
t_R = 5.611

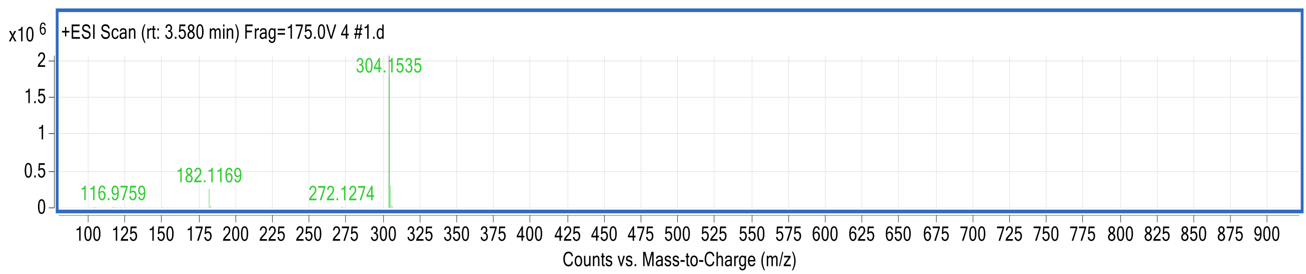




Appendix 17 - Chromatogram and mass spectrometric data for Cocaine

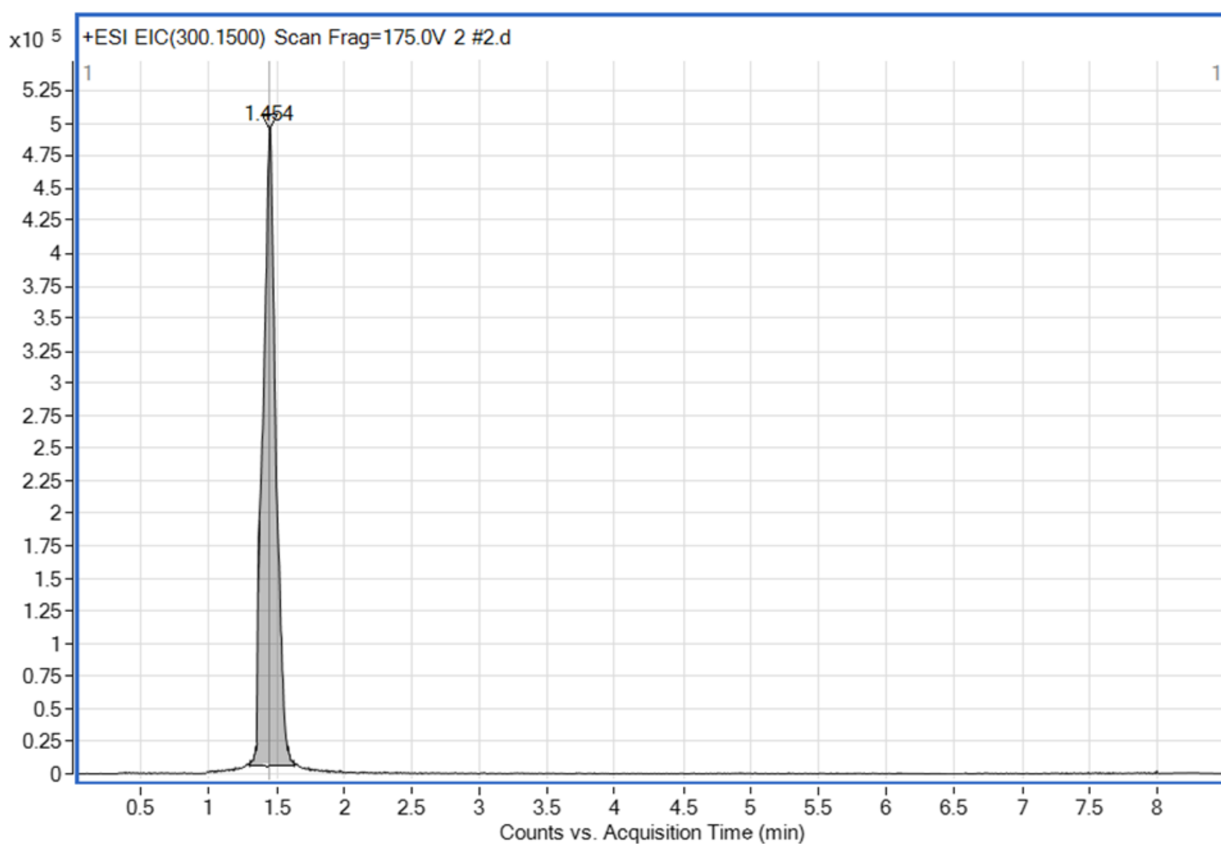
t_R = 3.575

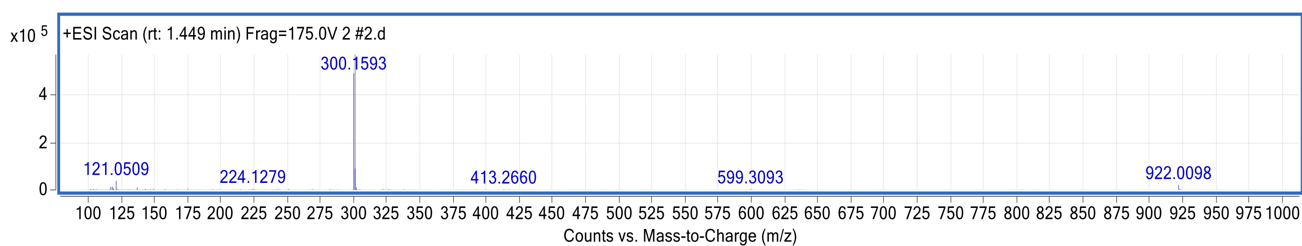




Appendix 18 - Chromatogram and mass spectrometric data for Codeine

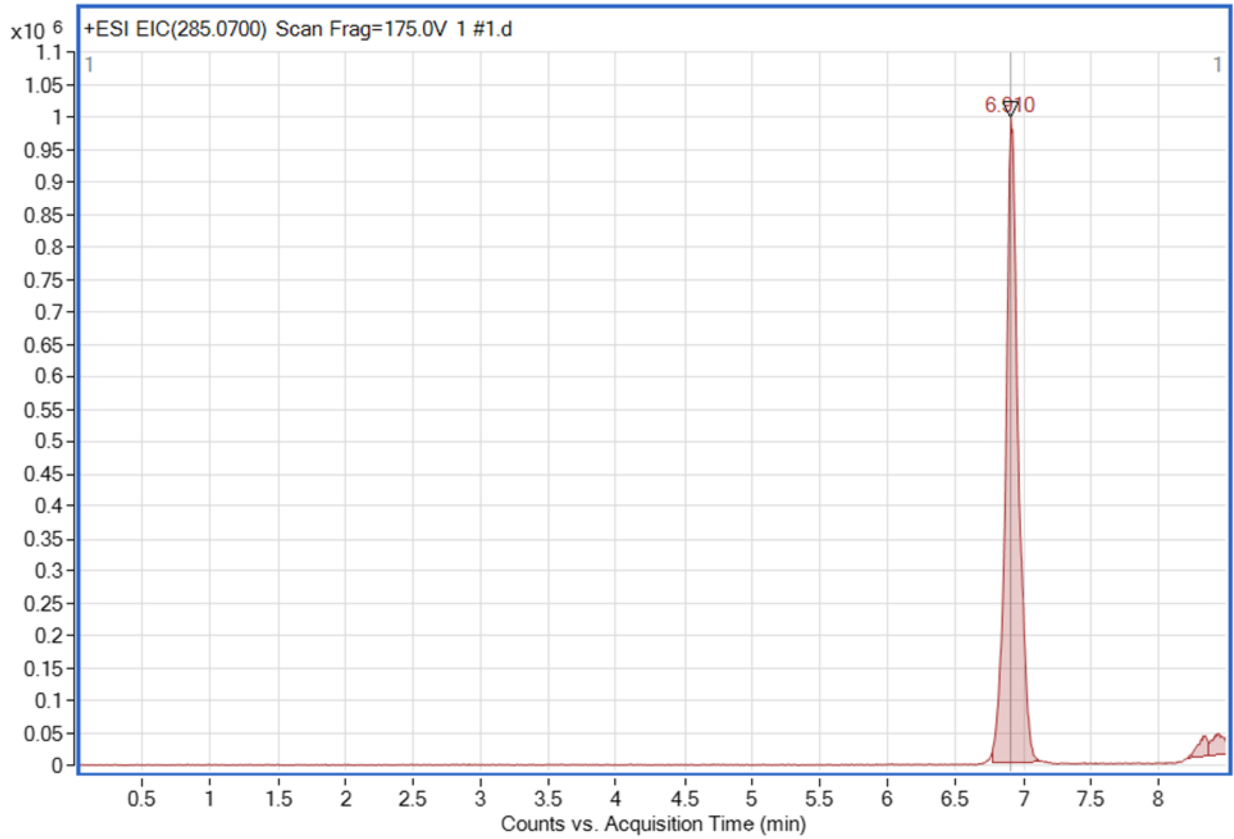
t_R = 1.454

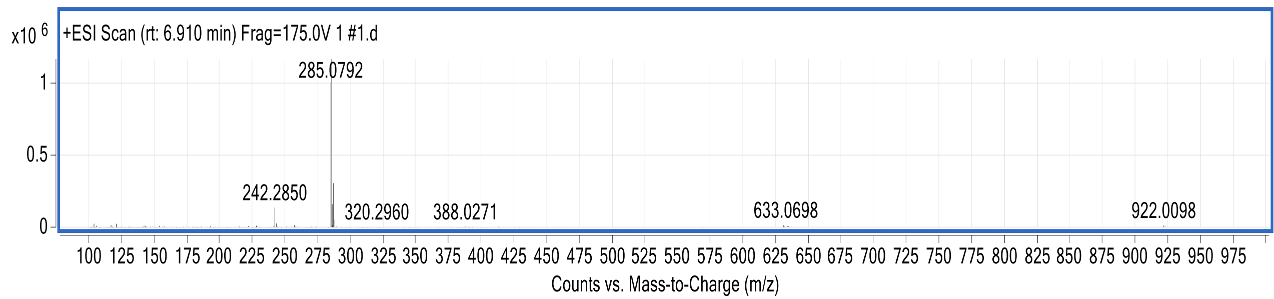




Appendix 19 - Chromatogram and mass spectrometric data for Diazepam

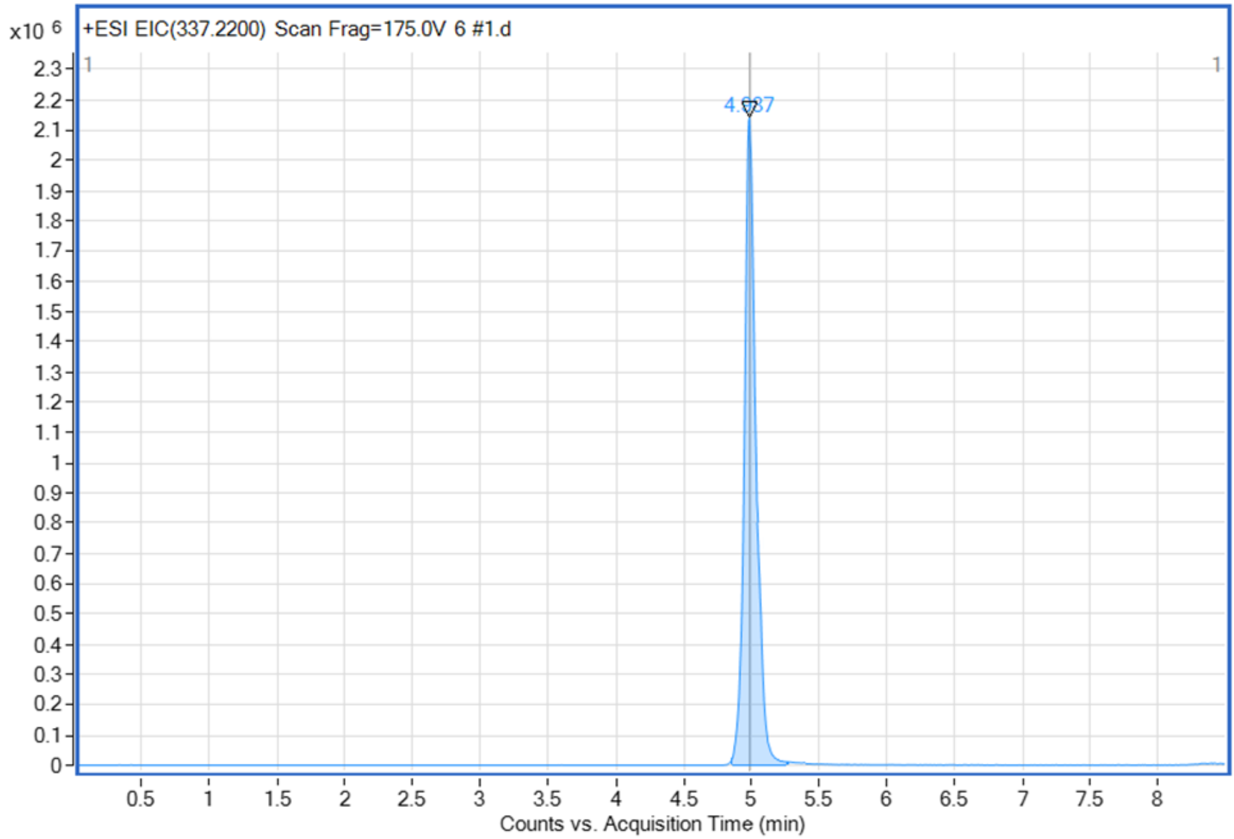
t_R = 6.910

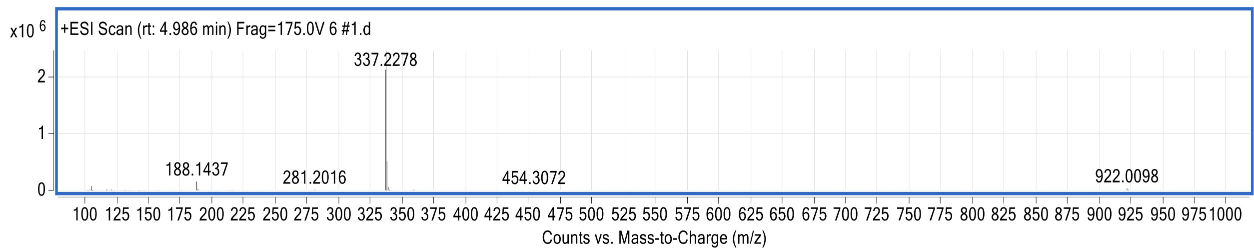




Appendix 20 - Chromatogram and mass spectrometric data for Fentanyl

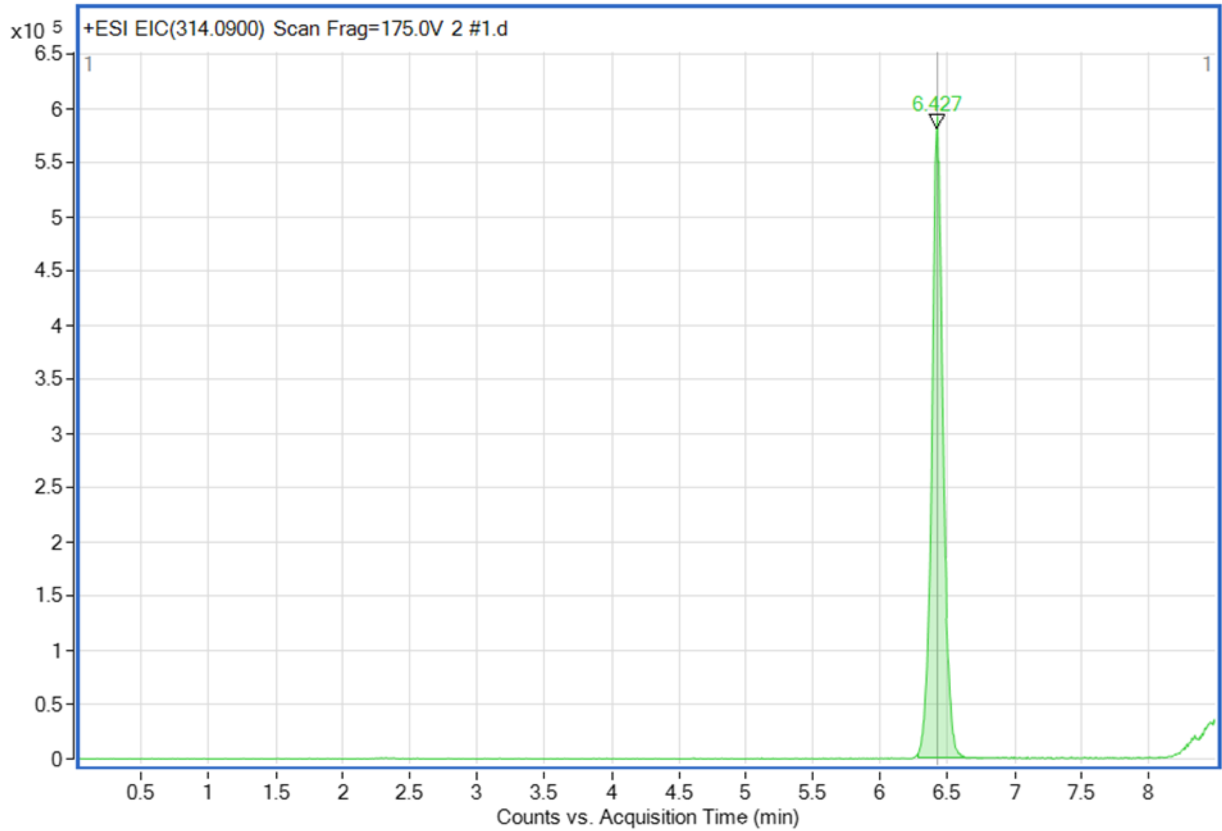
t_R = 4.987

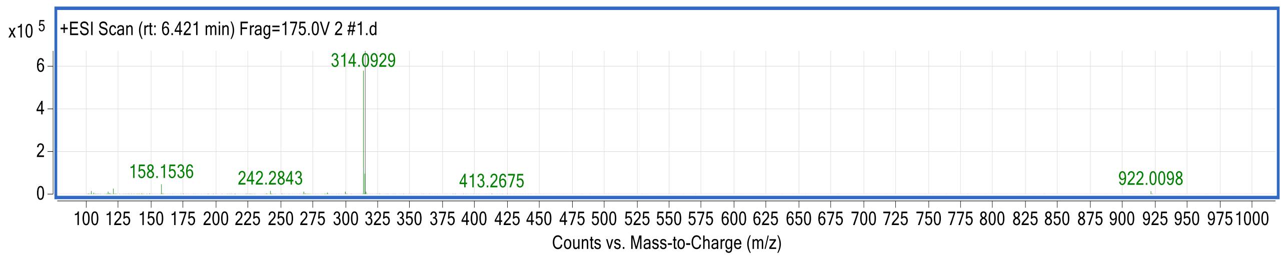




Appendix 21 - Chromatogram and mass spectrometric data for Flunitrazepam

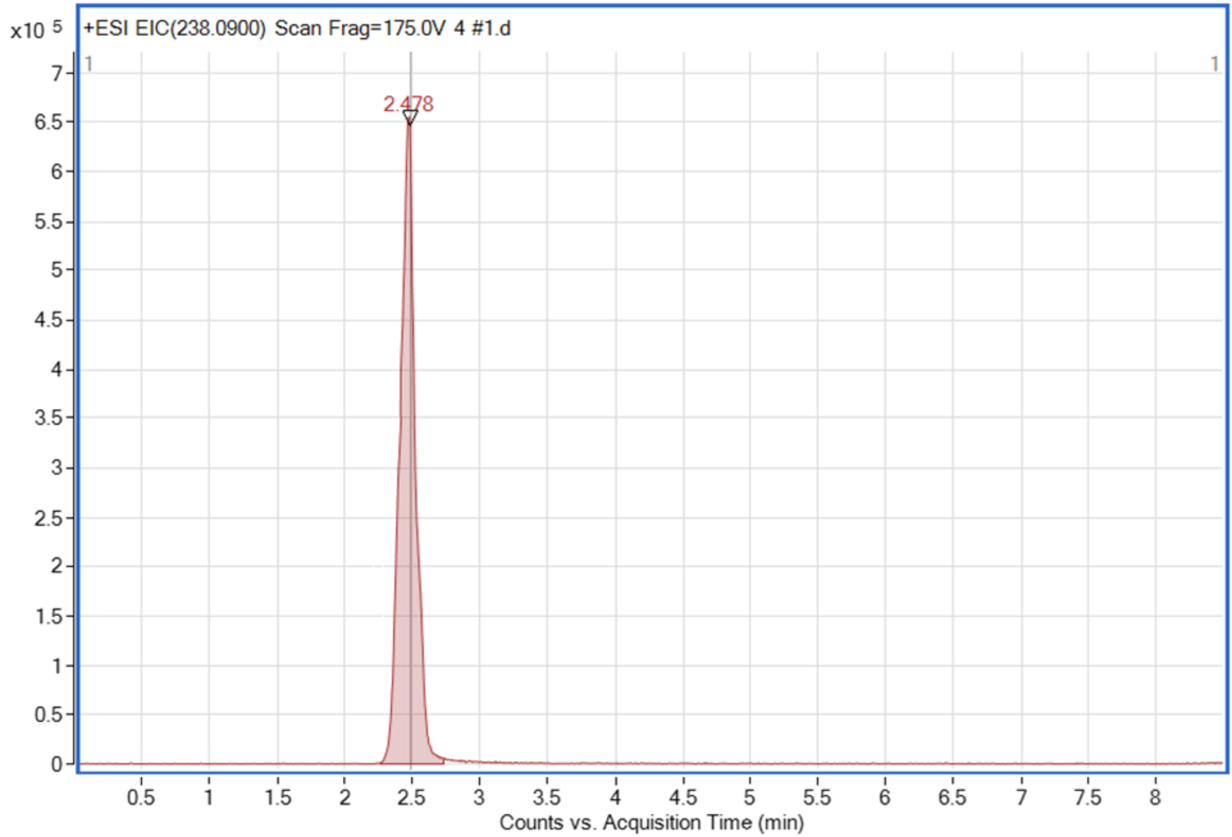
t_R = 6.427

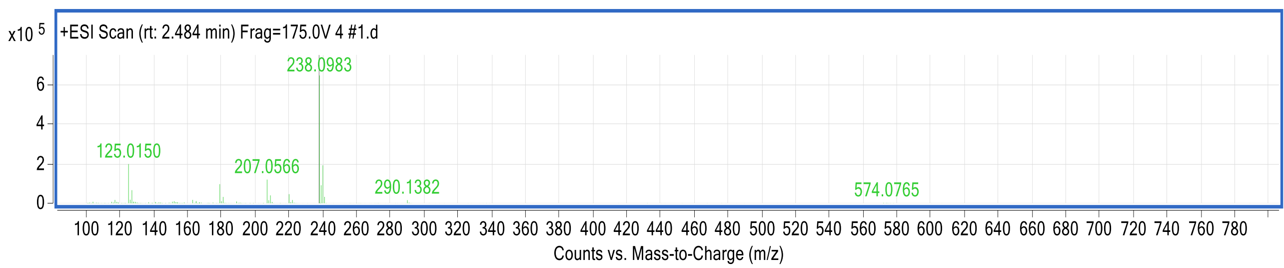




Appendix 22 - Chromatogram and mass spectrometric data for Ketamine

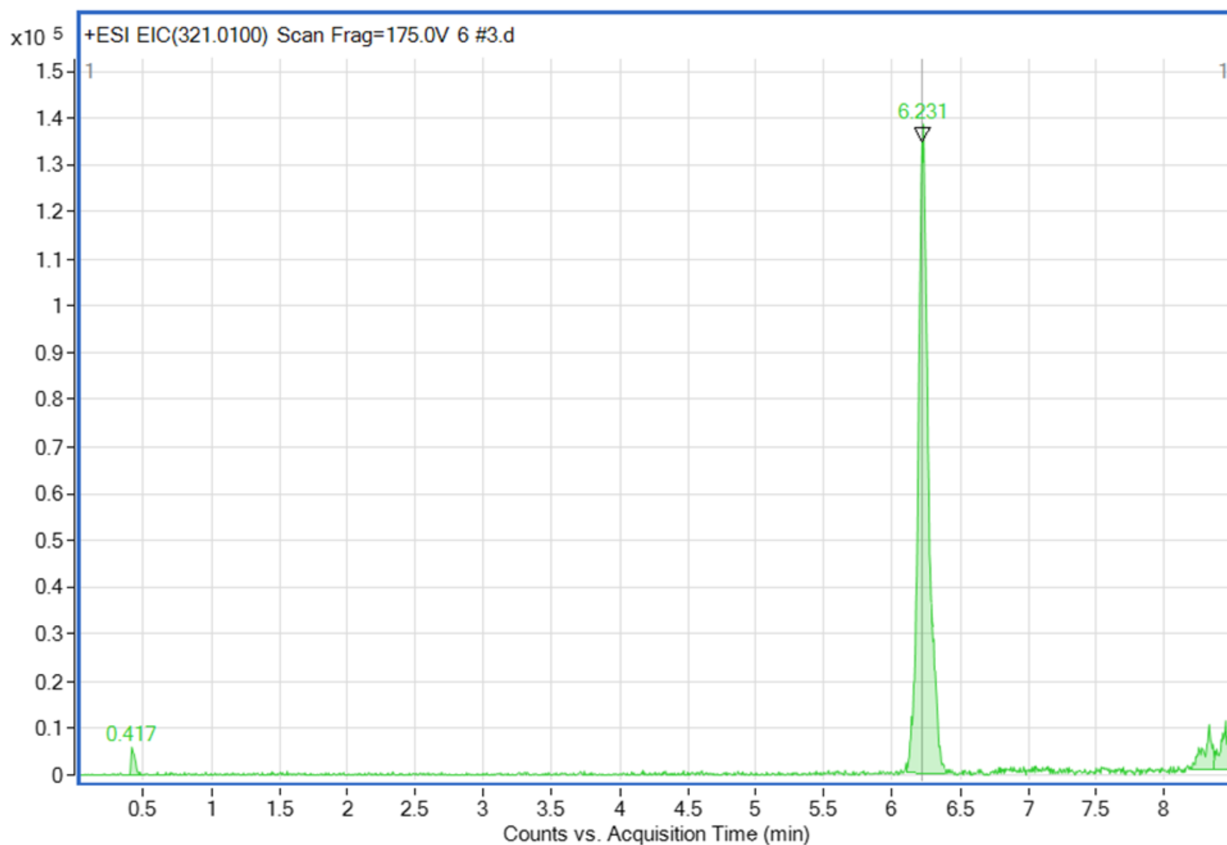
t_R = 2.478

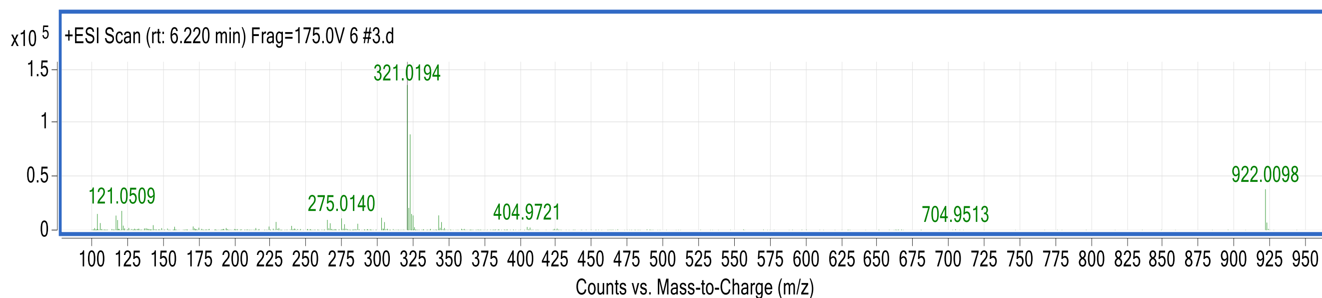




Appendix 23 - Chromatogram and mass spectrometric data for Lorazepam

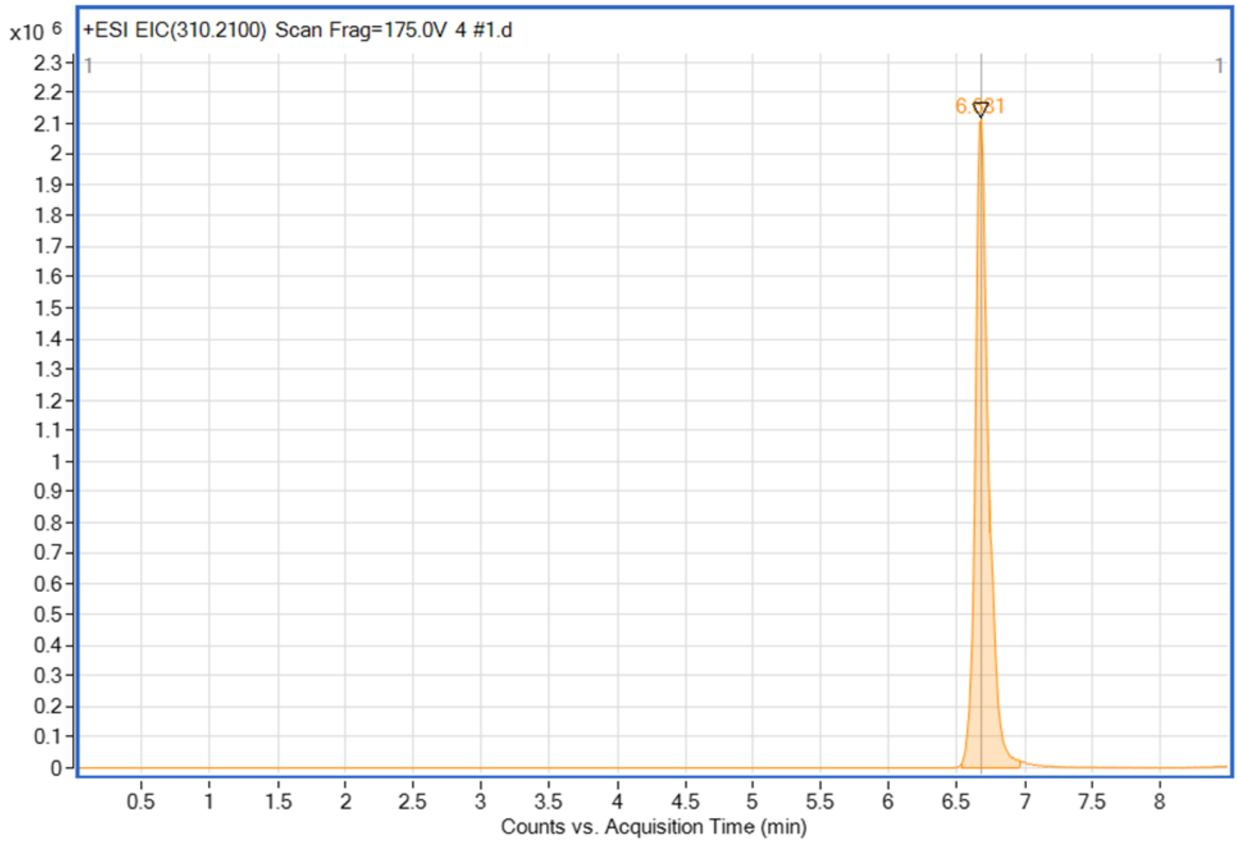
t_R = 6.231

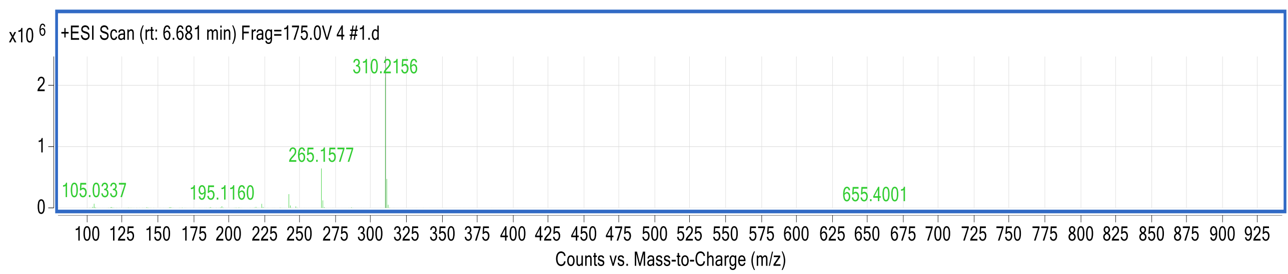




Appendix 24 - Chromatogram and mass spectrometric data for Methadone

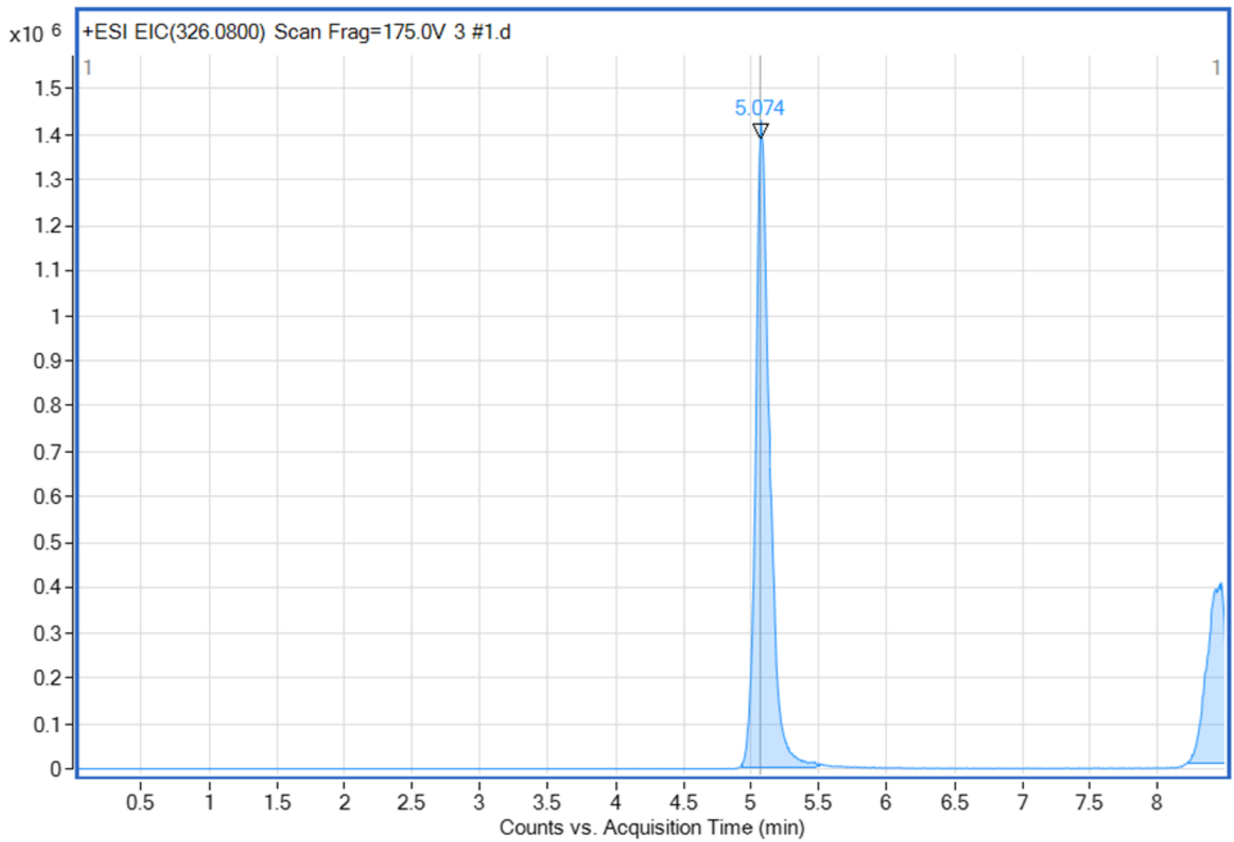
t_R = 6.681

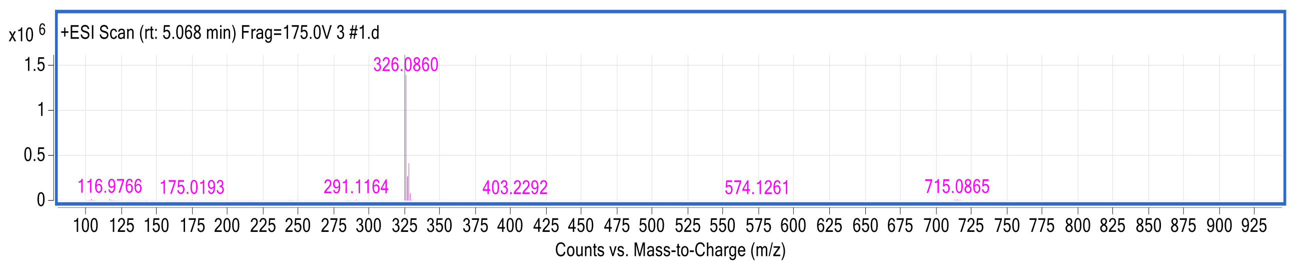




Appendix 25 - Chromatogram and mass spectrometric data for Midazolam

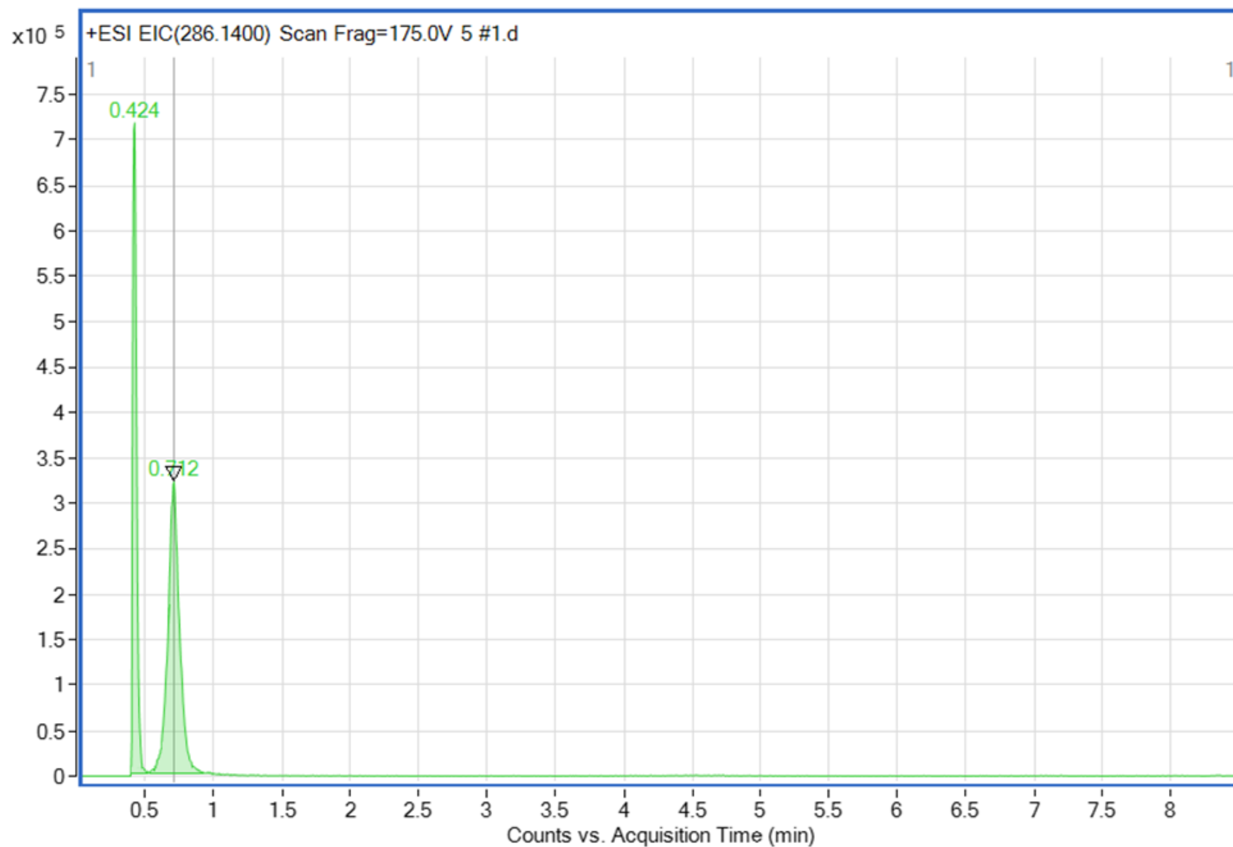
t_R = 5.074

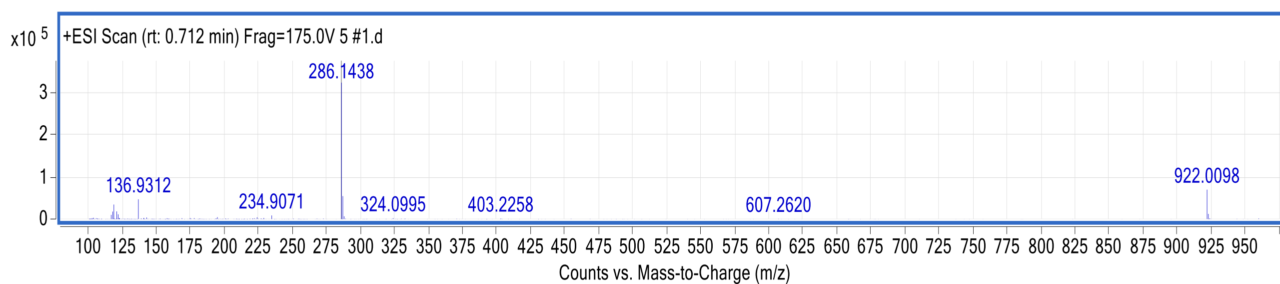




Appendix 26 - Chromatogram and mass spectrometric data for Morphine

t_R = 0.712





Appendix 27 - Chromatogram and mass spectrometric data for Zolpidem

t_R = 3.883