Novel Approaches to the Analysis of Benzodiazepines and Morphine-Related Analytes in Forensic Samples

by

Rebecca Webb

A thesis
submitted for the degree of
Doctor of Philosophy

Department of Chemistry, Materials and Forensic Science
University of Technology, Sydney
2005

Dedicated to the memory of Sir Joseph Swan (1828 – 1914) – pharmacist, physicist, inventor, chemist, and distant ancestor whose genetic influence has fuelled my passion for science.

Certificate of Authorship/Originality

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree.

I also certify that the thesis has been written by me. To the best of my knowledge, this thesis contains no material previously published or written by another person, except as fully acknowledged within the text. All information sources and literature used are indicated in the thesis. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged.

Rebecca Webb

Production Note:
Signature removed prior to publication.

Acknowledgements

It's been a long, often difficult (but fulfilling) journey, made all the more easier thanks to a number of very special people who were always willing to offer me support and guidance when I most needed it. To my supervisors, Assoc Prof Michael Dawson and Dr Philip Doble – thank you for your ideas, encouragement and understanding. With your help, I have gained experience in so many different areas of research and the confidence to master any obstacle. I would also like to thank the staff from the Division of Analytical Laboratories (DAL), particularly Tania Prolov and Alan Hodda for facilitating my access to the necessary samples, and Jenny Easson for her assistance in the lab. Many thanks to all the staff and students in the Department of Chemistry, Materials and Forensic Science who helped me at various stages throughout my research; in particular, a very big thank you to Ronald Shimmon, Dr Kamali Kannangara, Dr Frank Cordaro, Mark Tahtouh and Tristan Rawling for their assistance with the synthetic aspects of my project. To all my fellow PhD students (past and present), especially Tam, Al, Katherine, Sonia, Matt, Jane, Daniel, Karen, Garry, Tristan, Mark – you've all been such great friends, and I know I would have gone crazy without you – thanks for all the good times! Finally, and most importantly, I have been lucky enough to have been blessed with wonderful supportive family and friends – too many to name individually, but you all know who you are. Dad, thanks for putting up with your "boomerang kid" for the past 18 months, and all the associated work! I'm forever grateful. Mum, you may live over 2000 km away, but you're still only a phone call or a short flight away. Thank you for always being there for me. To Jim – I would not be where I am today without you. You've helped me to realise what's important in life and what it takes to achieve it. Thank you for inspiring me every day of my life.

List of Publications

Type of Publication	Number	Reference
Papers submitted to refereed journals	3	[1, 2, 3]
Poster presentations at International Meetings	4	[4, 5, 6, 7]

[1] **Webb, R.**, Doble, P., Dawson, M., 2005, "A rapid capillary zone electrophoresis method for the analysis of benzodiazepines in spiked beverages", submitted for publication in Journal of Forensic Sciences. *(Chapter 3)*

[2] **Webb, R.**, Doble, P., Dawson, M., 2005, "Optimisation of HPLC Gradient Separations Using Artificial Neural Networks (ANNs): Application to Benzodiazepines in Post-Mortem Samples", submitted for publication in Journal of Chromatography B. *(Chapter 4)*

[3] **Webb, R.**, Dawson, M., Doble, P., Eason, J., Prolov, T., 2005, "Simultaneous Quantification of Morphine, Morphine-3-Glucuronide, Morphine-6-Glucuronide and Codeine in Post-Mortem Blood Samples", submitted for publication in Journal of Forensic Sciences. *(Chapter 5)*

[4] **Webb, R.**, Dawson, M., Kelly, T., Doble, P., 2002, "Analysis of morphine glucuronides", ANZFSS 16th International Symposium on the Forensic Sciences, Canberra, May, 2002. *(Chapter 2)*

[5] **Webb, R.**, Dawson, M., Kelly, T., Doble, P., 2002, "Analysis of morphine glucuronides", Interact 2002, Sydney, July, 2002. *(Chapter 2)*

[6] **Webb, R.**, Doble, P., Dawson, M., 2003, "Artificial neural networks for the optimisation of benzodiazepine analysis", TIAFT 41st International Meeting, Melbourne, November, 2003. *(Chapter 4)*

[7] **Webb, R.**, Doble, P., Dawson, M., 2003, "Artificial neural networks for the optimisation of benzodiazepine analysis", ANZFSS 17th International Symposium on the Forensic Sciences, Wellington, March, 2004. *(Chapter 4)*

List of Abbreviations

3MAM	3-monoacetylmorphine
6MAM	6-monoacetylmorphine
7-NH_2-CLO	7-aminoclonazepam
7-NH_2-FLU	7-aminoflunitrazepam
7-NH_2-NIT	7-aminonitrazepam
Ac	acetyl
AC	acetylcodeine
ACN	acetonitrile
ADI	adinazolam
AIC	Australian Institute of Criminology
ALC	alcohol
ALP	alprazolam
ANN	artificial neural network
ANZFSS	Australia and New Zealand Forensic Science Society
APCI	atmospheric pressure chemical ionisation
B	blood
BBB	blood brain barrier
βCD	beta-cyclodextrin
BGE	background electrolyte
Bi	bile
BLOQ	below limit of quantification
BRO	brotizolam
BROM	bromazepam
bs	broad singlet
bt	broad triplet
C6G	codeine-6-glucuronide
CAR	carbamazepine
CD	cyclodextrin

CE	capillary electrophoresis
CEC	capillary electrochromatography
CEDIA	cloned enzyme donor immunoassay
CHL	chlordiazepoxide
C_L	clearance
CLB	clobazam
CLO	clonazepam
CLOR	clorazepate
CLOT	clotiazepam
CLOX	cloxazolam
cm	centimetre
CMC	critical micelle concentration
CNS	central nervous system
COD	codeine
CSF	cerebrospinal fluid
CV	coefficient of variation
CZE	capillary zone electrophoresis
d	doublet
dt	double triplet
DAD	diode array detector
DAL	Division of Analytical Laboratories
DCM	dichloromethane
DEAE	*N*-diethylaminoethylene
DEL	delorazepam
DEM	demoxepam
DHC	dihydrocodeine
DHM	dihydromorphine
DIA	diazepam
DMβCD	heptakis-(2,3-di-*O*-methyl)-β-cyclodextrin
DS	dextran sulfate

E	erythrocytes
ECD	electrochemical detection
EIA	enzyme immunoassay
EKC	electrokinetic chromatography
ELISA	enzyme linked immunosorbent assay
EOF	electroosmotic flow
ESI	electrospray ionisation
ESP	electrospray
EST	estazolam
ETI	etizolam
EtOAc	ethyl acetate
F	fluorescence
FLU	flunitrazepam
FLUD	fludiazepam
FLUM	flumazenil
FLUR	flurazepam
FPIA	fluorescence polarisation immunoassay
GABA	gamma (γ)-aminobutyric acid
GC	gas chromatography
gluc	glucuronide
H	hair
HAL	halazepam
HALO	haloxazolam
HC	hydrocodone
HER	heroin
HM	hydromorphone
HPLC	high-performance liquid chromatography
hr or hrs	hour(s)
IPA	isopropyl alcohol
ISF	ideal separation function

K_a (or pK_a)	acid dissociation constant
KET	ketazolam
kg	kilogram
kV	kilovolts
L	litre
L_{eff}	effective length
LLE	liquid-liquid extraction
LOD	limit of detection
LOP	loprazolam
LOQ	limit of quantification
LOR	lorazepam
LORM	lormetazepam
LPME	liquid-phase microextraction
L_{tot}	total length
M	molar
m	multiplet
m/z	mass to charge ratio
M3G	morphine-3-glucuronide
M6G	morphine-6-glucuronide
μ_{app}	apparent mobility
Mec	meconium
MED	medazepam
μ_{eff}	effective mobility
MEKC	micellar electrokinetic chromatography
μ_{EOF}	electroosmotic flow mobility
MeOH	methanol
MEX	mexazoram
mg	milligram
µg	microgram
MID	midazolam

min	minutes
mL	millilitre
MLP	multi-layer perceptron
mM	millimolar
mm	millimetre
μm	micrometre
mmol	millimole
MOR	morphine
MRM	multiple reaction monitoring
MS	mass spectrometry
MSA	methanesulfonic acid
mV	millivolts
ng	nanogram
$-NH_2-$	amino
NIT	nitrazepam
nM	nanomolar
NMR	nuclear magnetic resonance spectroscopy
nor-CHL	norchlordiazepoxide
nor-CLB	norclobazam
nor-DIA	nordiazepam
nor-FLU	norflunitrazepam
nor-FLUD	norfludiazepam
NOS	noscapine
OC	oxycodone
OD	overdose
-OH-	hydroxy
OXA	oxazepam
P	plasma
PAP	papaverine
PAR	paracetamol

PDA	photodiode array
PDDAC	Poly(diallyldimethylammonium chloride)
PRA	prazepam
PR_s	product resolution
QSRR	quantitative structure-retention relationship
RBF	radial basis function
RIA	radioimmunoassay
R_{min}	minimum resolution between consecutive peaks
RP	reversed phase
RRA	radioreceptor assay
S	serum
s	singlet
S/N	signal to noise ratio
SC	sodium cholate
SDC	sodium deoxycholate
SDS	sodium dodecyl sulphate
sec	seconds
SFE	supercritical fluid extraction
SIF	supervised injection facility
SIM	selected ion monitoring
SPE	solid-phase extraction
SPME	solid-phase microextraction
SRM	selected reaction monitoring
STA	systematic toxicological analysis
t	triplet
$t_{1/2}$	half-life
TCA	tricyclic antidepressant
TEA	triethylamine
TEAP	triethylammonium phosphate
TEM	temazepam

TET	tetrazepam
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIAFT	The International Association of Forensic Toxicologists
TLC	thin layer chromatography
TMβCD	heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin
TOF	tofisopam
TOF-SIMS	time of flight-secondary ion mass spectrometry
TRI	triazolam
TSP	thermospray
U	urine
UDP	uridine diphosphate
UDPGT	UDP-glucuronosyltransferases
UV	ultraviolet
V	volts
V_D	volume of distribution
VH	vitreous humor

Abstract

This research has focused on the development of analytical methods for the quantification of benzodiazepines and opiates in forensic samples. Both these classes of drugs are regularly encountered in forensic toxicology, especially in cases of fatal heroin overdose, and thus quantitative methods for their analysis are particularly relevant.

Chapter 1 outlines the nature of the heroin problem in Australia and the different drug policies currently employed to help reduce the problems associated with heroin use and overdose. The involvement of metabolic factors and CNS depressants, such as benzodiazepines, in heroin overdose is discussed. An introduction to post-mortem drug analysis, as well as a comprehensive review of previously published methods for the analysis of heroin, its metabolites and benzodiazepines in biological samples, is presented.

Chapter 2 describes procedures for the synthesis of commonly encountered morphine and benzodiazepine metabolites as an alternative to purchasing these metabolites commercially. Yields of 48, 25, 74 and 70% were obtained for M3G, M6G, 7-aminonitrazepam and 7-aminoclonazepam respectively.

Chapter 3 documents the development of a rapid capillary zone electrophoresis (CZE) method for the analysis of nine benzodiazepines using dynamically prepared doubly-coated capillaries, consisting of a polycation of poly(diallyldimethylammonium chloride) (PDDAC) and a polyanion of dextran sulfate (DS). The addition of cyclodextrins to the background electrolyte (BGE) was also investigated as a means of improving analysis time. The validated method was successfully applied to the analysis of spiked beverages, with run times of less than 6.5 minutes.

Chapter 4 describes the optimisation of a gradient HPLC separation of nine benzodiazepines using artificial neural networks (ANNs) in conjunction with experimental design. Various outputs and types of training data were investigated to yield the most appropriate ANN, which gave predictive errors of less than 5% for six of the nine analytes studied. A novel chromatographic function was also developed as a means of assessing the quality of chromatographic separations. The optimised method was validated for blood and successfully applied to authentic post-mortem samples. The limits of detection of the method ranged from 0.0057 to 0.023 μg/mL, and recoveries were in the order of 58 – 92%.

Chapter 5 details the development of a simple and rapid HPLC method for the simultaneous determination of morphine, M3G, M6G and codeine. Following SPE, the analytes were determined in post-mortem blood samples taken from heroin-related deaths. Cases involving the use of benzodiazepines in conjunction with heroin were found to have lower ratios of M6G/MOR and M3G/MOR, suggesting rapid death following heroin administration. M6G/M3G ratios were calculated to investigate the possible contribution of M6G towards heroin overdose with respect to its potential analgesic activity. M6G/M3G ratios were higher in cases involving the use of heroin only.

Table of Contents

List of Figures

List of Tables

List of Equations

Chapter One

Introduction

1 Introduction

1.1 Summary

Heroin (diacetylmorphine) use may be regarded as a significant illicit drug problem in Australia. Heroin users are at a greater risk of premature mortality than their non-heroin-using peers, and although there are a number of factors that may contribute towards this excess mortality, overdose remains the leading cause. The repercussions of heroin overdose extend far and wide, and are not merely limited to heroin users themselves. Public health and economic implications affect the entire community, rendering prevention of overdose a concern for the whole of society. In this introductory chapter, the nature of the heroin problem in Australia is discussed, as are the various drug strategies employed by the Australian government to help reduce the problems associated with heroin use and overdose.

While heroin overdose is widely regarded as a cause for concern, the reasons for fatal heroin overdoses are frequently unclear from the analysis of opioids present in post-mortem blood. In a significant number of heroin-related fatalities, the morphine concentrations found are no greater than many non-fatal overdoses and, in some cases, may even be below toxic levels. In addition, the blood morphine concentrations found in heroin overdoses exhibit a large amount of variation between cases. One reason for these observations may be the ambiguous reporting of either free morphine or total morphine (morphine plus the glucuronide metabolites) concentrations, which limits the interpretation of heroin-related fatalities. Interpretation is further confounded because morphine-6-glucuronide (M6G) is pharmacologically active, while morphine-3-glucuronide (M3G) has been shown to antagonise opioid effects. These two metabolites may therefore play a vital role in the mechanism of heroin overdose. Numerous methods have been published for the simultaneous analysis of these analytes, however there is limited data available concerning their concentrations and ratios in the post-mortem blood of heroin-related fatalities. A method capable of quantifying morphine, M3G and M6G in post-

mortem samples may help to establish a relationship between heroin concentration and effects, and allow the possible contribution of M3G and M6G to be explored in more detail.

Cases in which morphine is the only drug detected at autopsy represent a minority of heroin overdoses. Estimates suggest that over a third of injecting drug users currently use benzodiazepines and, as a result, they are frequently encountered in addition to morphine in heroin-related fatalities. The concomitant use of heroin and benzodiazepines has a significant impact on the likelihood of an overdose. Since the depressant effects of benzodiazepines and heroin are additive, the co-administration of these two drugs may increase the likelihood of a fatal heroin overdose through the potentiation of respiratory depression. As a result, less heroin may be needed to produce a fatal outcome in the presence of benzodiazepines. Given the importance of benzodiazepines and morphine-related analytes in forensic toxicology, there have been numerous methods developed for their analysis in biological specimens, and an extensive review of these methods is presented in this chapter. While the analysis of benzodiazepines has been studied extensively, it has primarily focused on HPLC separations, and novel approaches employing emerging analysis and optimisation techniques are rarely explored. Further research into these areas could enable significant advantages in method development, optimisation and sample throughput to be realised.

1.2 Origin of Heroin Production

Opiates are naturally occurring alkaloid analgesics obtained from the opium poppy *papaver somniferum*, and include morphine and codeine [1]. The opium poppy flourishes in dry, warm climates and is found in the Middle East, Southeast Asia and parts of Central and South America. Morphine, the principal component of opium, was the first alkaloid to be isolated in pure form [2]. Natural and semisynthetic alkaloids prepared from opium are classified as opioids. Also included under this classification are synthetic compounds whose pharmacological effects, rather than structure, are similar to those of morphine [1]. Many of these semisynthetic opiate

derivatives are made by relatively simple modifications of morphine. For example, heroin can be obtained synthetically by acetylation of the hydroxyl groups of morphine at the 3- and 6-positions with acetic anhydride as shown in Figure 1-1.

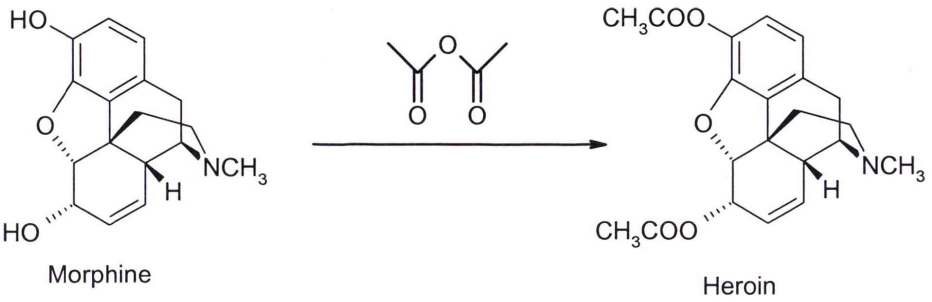


Figure 1-1 Synthesis of heroin from morphine

The first synthesis of heroin was in 1874 by C.R. Wright [3]. Finding that heroin did not produce many of the common morphine side effects, it was marketed as an analgesic by the Bayer pharmaceutical company in 1898 as a "less addicting morphine substitute" [4]. However, while heroin was a more effective analgesic than morphine, over time it was found to possess greater toxicity. In particular, heroin was found to have a more pronounced respiratory depressant effect, and physical dependence and tolerance were produced more rapidly with heroin than with morphine [5].

In Australia, the Standard for the Uniform Scheduling of Drugs and Poisons classifies drugs and poisons into nine Schedules, with Schedule 1 drugs subjected to minimal regulation and control, and the maximum level of control for Schedule 9 drugs. Heroin is classified as a Schedule 9 drug: "poisons which are drugs of abuse, the manufacture, possession, sale or use of which should be prohibited by law" [6]. Prior to 1953, heroin was available on prescription in Australia as a treatment for pain, until pressure from the World Health Organization resulted in its prohibition [7]. Since then, the use of heroin in Australia has been prohibited and confined to the domains of the illicit drug user. Overseas, legislation prohibits the use of heroin for

recreational purposes, however some countries (e.g. Canada and the United Kingdom) allow exceptions for its clinical use in the management of chronic pain in terminally ill patients [1]. UK legislation also allows for the prescription of heroin to treat heroin dependence.

As a result of the manufacturing process, illicit heroin may contain several related opium alkaloids and synthetic artefacts, including acetylmorphine, acetylcodeine, codeine, papaverine, and morphine [1]. Before being sold on the street, heroin is usually "cut" with various diluents, such as sugars, to add bulk. Other additives may include caffeine and paracetamol [8]. Illicit heroin is produced in three major regions, as shown in Figure 1-2: the Golden Triangle, which encompasses Myanmar, Laos and Thailand; the Golden Crescent, which includes Afghanistan, Turkey and Pakistan; and Central and South America, of which the two major opium-producing countries are Colombia and Mexico. Australia has traditionally been a Golden Triangle customer, and Myanmar is the most important source of heroin for the Australian drug market. The majority of drugs not consumed within the Golden Triangle are transported into Australia through Malaysia, Singapore and Vietnam [9]. In 2002, Myanmar produced over 800 tonnes of heroin, and Afghanistan, another major supplier of heroin to Australia, produced more than 3000 tonnes [10]. In comparison with Afghanistan and Myanmar, Colombia and Mexico are minor producers of opium, with average annual productions of about 130 tonnes [10]. Due to its close proximity to the United States, drug trafficking routes from this region are focused on the North American continent, and very little Central and South American heroin reaches Australia.

a) b) c)

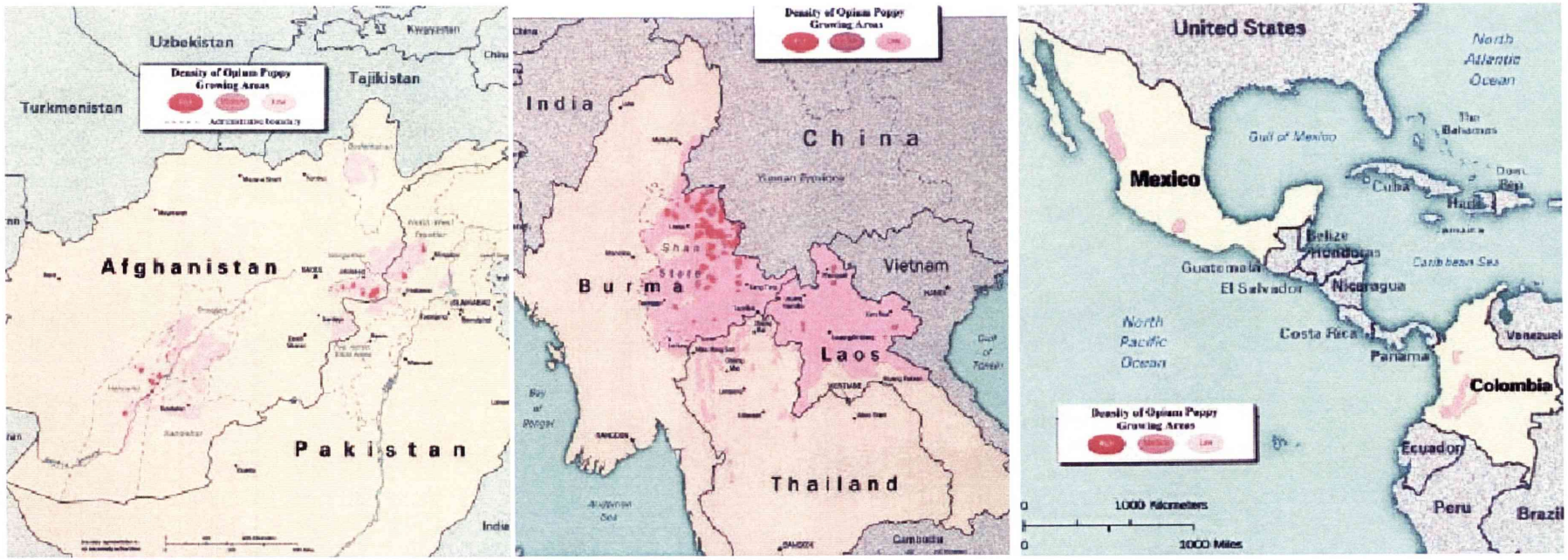


Figure 1-2 Geographical origin of heroin divided into a) Golden Crescent b) Golden Triangle c) Central and South America

1.3 Nature and Extent of the Heroin Problem

There have been estimated to be between 67 000 and 92 000 dependent heroin users in Australia, who are typically daily, or near-daily injectors of heroin and of other opioid and sedative drugs when heroin is not available [11]. Heroin users are at a greater risk of premature mortality than their non-heroin-using peers, with yearly mortality rates estimated at between 1% and 3%. This translates into a rate six to twenty times greater than that expected among peers of the same age and gender [12]. Although there are a number of factors that may contribute towards this excess mortality, such as HIV, hepatitis and violence, overdose remains the leading cause of death among heroin users.

In the decade to 1999, deaths from heroin overdose increased steadily, from 302 in 1989 to 960 in 1999, representing a tripling of the overdose rate [13]. Since reaching a peak in 1999, there has been a decline in the number of heroin fatalities in Australia (refer to Figure 1-3), which may in part be due to a reduction in the availability of heroin over this period. This so-called heroin 'drought' was thought to be caused by failing crops in Asia and Afghanistan, the low value of the Australian dollar, the success of policing aimed at reducing local and overseas supply, or the cyclical end to the heroin 'glut' that was experienced pre-1999 [14]. Official reports, based on market indicators and interviews with heroin users, suggest that since 2002, heroin consumption has again been on the rise, yet still remaining lower than pre-1999 levels [15]. In NSW, the price of heroin fell from $320/g in 2001 to $300/g in 2002 [15], suggesting that the rise in consumption may be the result of an increase in supply on the Australian drug market. Along with this escalation in the availability and consumption of heroin in Australia, come fears that the number of heroin-related fatalities will again increase.

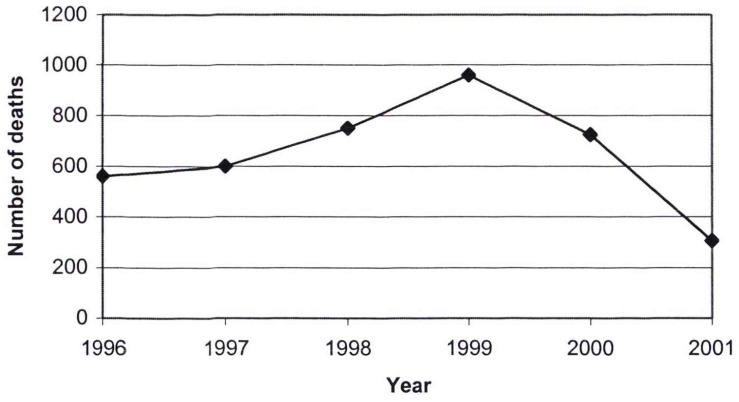


Figure 1-3 Number of opioid overdose deaths in Australia among 15-44 yr olds, 1996-2001

Source: Special Counterdrug Summary [9]

For every fatal overdose, there are estimated to be another 31 that have a non-fatal outcome [16]. Interviews with clients of the Sydney Medically Supervised Injecting Centre found that 45% had experienced at least one non-fatal heroin overdose [17]. The reason why some overdoses result in death and some do not remains unclear. In some instances, an otherwise fatal overdose can be avoided with prompt medical treatment and the administration of the antagonist naloxone. Its high lipid solubility means it is readily absorbed, allowing it to rapidly enter the CNS and normalise respiration within 1 – 2 minutes [4].

The repercussions of heroin use and overdose are not merely limited to the heroin users themselves, with public health, crime and economic implications affecting the entire community. In the period 1988 to 1992, health service costs rose from $908 million to $1248 million [18], due to an increase in demand for hospital and ambulance services by illicit drug users. Despite a recent reduction in the number of heroin-related deaths, health costs still remain high, as the number of non-fatal heroin overdoses attended by ambulance continues to greatly outnumber fatalities [19]. In addition to economic considerations, the spread of blood-borne viruses, such as HIV and hepatitis, is a social concern for the community. Between 60 – 65% of

heroin-related fatalities test positive for hepatitis C [20, 21], and this figure is probably similar for current users. As a consequence of its illicit nature, heroin use is invariably associated with crime in society. The total cost of heroin-related crime in Australia has been estimated to be between $535 million and $1.6 billion per annum, with property crime losses, as a consequence of illicit drugs, accounting for 32% of the total cost of law enforcement [22]. A survey of heroin users in Sydney indicated that illegal activities, predominantly acquisitive property crime and the sale and distribution of illicit drugs, accounted for 82% of the sample's income [22].

1.4 Drug Policy

Each country is responsible for its own drug policy, which dictates its legal standpoint regarding drug use, outlines strategies to achieve its overall goal, and provides remedies if the law is contravened. The prohibition model, favoured by countries such as the United States and Sweden, regards drug use as a criminal and moral issue, rather than one of public health. The strategies implemented under this zero-tolerance approach are abstinence-based programs, and the issue of heroin overdose is addressed through law enforcement strategies that are directed towards influencing the supply of the drug [23]. However, there is little evidence to suggest that supply-based law enforcement strategies have any marked difference on the availability of heroin [14, 24, 25]. Reducing supply causes an increase in price, which may only increase revenue to drug dealers, prompt users to commit crimes to fund their addiction, or increase demand for other potentially more harmful drugs [26]. At the other end of the spectrum is drug legalisation or decriminalisation. Some countries take this approach and legalise or decriminalise certain recreational drugs which society consider to be "soft", such as alcohol, tobacco, and in some instances cannabis, whilst continuing to prohibit the "harder" recreational drugs. For example, the consumption of small amounts of cannabis is legal in The Netherlands, however consumption and possession of heroin is still illegal. Opponents of this approach believe that legalisation would lead to increased drug consumption, resulting in reduced productivity, and spread of disease. However, supporters believe that drug

consumption would not increase, since society is tending to move away from the use of other harmful but legal drugs such as tobacco.

Somewhere in between these two extremes lies the harm minimisation approach, which has been Australia's official drug policy since 1985. Rather than focusing on the elimination of drugs from society, the goal of harm minimisation is to reduce the adverse consequences associated with drug use for both the community and the individual drug user. Illicit drug use is not encouraged under harm minimisation, however governments acknowledge that where drug use is going to occur, they have a responsibility to implement public health and legal strategies to minimise the harm associated with this practice. For this reason, drug use is viewed as a public health, rather than criminal or legal issue.

Strategies aimed at educating the community about the consequences of drug use, in an endeavour to deter this practice, are a key component of the government's harm-minimisation approach. Substitution-based programs are another important strategy. These programs aim to reduce illicit drug use through the provision of legal substitutes, which include the opioid agonist methadone, the partial opioid agonist buprenorphine and the opioid antagonist naltrexone. Supervised injecting facilities (SIFs) are another strategy aimed at preventing overdose and reducing the risk of death or serious injury by allowing drugs to be injected in a supervised environment where appropriate medical treatment can be sought in time. As evidence of this, the Sydney Medically Supervised Injecting Centre, which ran from May 2001 [17] to April 2003, managed 450 heroin overdoses on site with no fatalities [27]. SIFs also enable appropriate disposal of needles and syringes, ensuring a safer environment for the community, and the provision of clean injecting equipment to reduce the spread of blood-borne viruses. Finally, support groups, such as Family Drug Support, may assist families isolated by the stigma of drug-related deaths and advocate various support and treatment options [28].

1.5 Contributing Factors in Heroin Overdose

The primary mechanism involved in heroin overdose is opioid-induced depression of respiration with resulting hypoxia and death [29]. Although the mechanism of overdose is clear, speculation surrounds the exact cause of heroin overdose. An overdose is often described as being the result of a quantity or quality (purity) of heroin in excess of the person's current tolerance to the drug [30, 31]. However, post-mortem observations suggest that this definition may be over-simplistic. Overdose deaths resulting from a large, or highly pure intake of heroin should correspond to relatively high blood morphine concentrations at autopsy. Yet, a large proportion of overdose cases possess morphine concentrations no greater than those found in non-fatal overdoses [32], with some cases even reported to be below toxic levels [30, 31]. This large variation could be a result of incomplete metabolism of morphine due to differing survival times after heroin injection and ambiguous reporting of the morphine concentration data. However, it may also be attributed to hypersensitivity reactions, heroin contaminates, metabolic variations, drug interactions, reduced tolerance and systemic dysfunction.

1.5.1 Hypersensitivity, Contamination and Batches

It is often suggested that death is not a consequence of any pharmacological action of heroin, but is due to the presence of toxic contaminants in low purity heroin [30]. Heroin users themselves believe that variations in purity are the major cause of non-fatal and fatal overdoses [31], however most additives in street-grade heroin are relatively harmless, and some even enhance the drug effect [8]. For example, caffeine can increase the bioavailability of heroin hydrochloride when smoked. A study conducted between 1993 and 1995 [8] found the mean purity of street-grade heroin samples to be 66%, with 85% of samples having an average purity of at least 50%. The main adulterants were pharmacologically inactive diluents to add bulk, or pharmacologically active adulterants used enhance drug effect. Similar results were found for another study, which found the purity of police heroin seizures to range between 62 and 71% [33].

Similarly, there is little evidence to support the claim that overdoses are caused by hypersensitivity reactions. While anaphylaxis may occur in a small number of cases, large-scale studies into heroin-related fatalities have shown no evidence of anaphylactic shock [20]. There is also little support for the theory that a particular batch of heroin may be responsible for overdose deaths. Ambulance officers, witnesses and drug and alcohol workers have consistently observed that a batch of heroin, which resulted in a fatal outcome for one person, did not do so for another [34].

1.5.2 Systemic Dysfunction

A number of systemic diseases may be responsible for an increase in susceptibility to heroin overdose. Hepatic disease has been shown to slow the clearance of morphine in patients receiving morphine therapy. It is therefore possible that heroin users suffering substantial liver damage may experience prolonged periods of heavy intoxication, thereby increasing their risk of respiratory depression [13]. Opioid users with reduced pulmonary function may be at greater risk of overdose as they are more vulnerable to fatal respiratory depression. Heroin users are particularly at risk of suffering from impaired pulmonary function as a result of smoking and increased susceptibility to infection [13].

1.5.3 Reduced Tolerance

Tolerance is an important, but complex factor to consider when examining the cause of heroin overdoses. Research has shown that fatal heroin overdoses are more prevalent in cases of low tolerance, where considerably less heroin was being used in the months preceding death [13]. Tagliaro *et al.* [35] measured morphine concentrations in the hair of heroin overdose victims to determine their degree of tolerance. They found that most fatal overdoses had hair morphine contents lower than those found in the hair of active consumers, suggesting that a fatal overdose was more likely when tolerance was low. Similar findings were also found by Kronstrand *et al.* [36]. This effect is also observed in a clinical setting during chronic pain therapy, whereby a patient may require doses of several grams of morphine in

divided doses each day, which would be a life-threatening dose to an opioid-naïve subject [37]. Apart from periods of abstinence, there are other situations that may cause a reduction in tolerance. Tolerance may be modified by learning or conditioning, so that administration of an opioid in an environment not previously associated with administration of the drug will be associated with a lower degree of tolerance [38-42]. In a study by Gutiérrez-Cebollada *et al.* [32], it was found that 52% of non-fatal overdose subjects had administered the drug in an unusual setting prior to the overdose. The risk of overdose is also higher following transition periods when tolerance is low, e.g. starting and temporarily discontinuing methadone treatment [43], and release from prison [13].

On the other hand, overdoses have also been shown to occur in older, more experienced users who presumably have a high tolerance to the drug [31]. One explanation is that tolerance to the respiratory depressive effect of heroin may be incomplete and slower to develop than tolerance to the euphoric effects [13, 29]. An experienced heroin user, who increases the dose as tolerance develops, may be at a higher risk of overdose since the dose sufficient for respiratory depression is less than the dose required to achieve the desired effects [13].

1.5.4 Metabolic Variations

Heroin is first metabolised to the pharmacologically active 6-monoacetylmorphine (6MAM) and morphine, before uridine diphosphate-glucuronosyltransferase (UDPGT) enzymes metabolise it further to the main metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), of which only M6G is an agonist. The pharmacological action of morphine and M6G and their effects are discussed in detail in a subsequent section. Since morphine and M6G possess analgesic activity, it is possible that high levels of these two metabolites may be associated with increased CNS depression and a greater risk of respiratory failure. This idea is supported by clinical studies, which attribute inter-patient variations in analgesia to wide differences in plasma concentrations of morphine and M6G in patients receiving morphine for pain [44-49]. To further confound matters, the most

prevalent metabolite, M3G, may even antagonise the respiratory depressant and analgesic effects of M6G and morphine [44, 50, 51]. The combination of several active metabolites and one antagonist translates into a considerable potential for inter-individual and possibly intra-individual differences in drug effect due to genetic variations in the amount of each metabolite produced. The contribution of M3G and M6G towards heroin overdose could be explored in greater detail through the post-mortem analysis of the individual glucuronide metabolites. Unfortunately, in many cases the morphine concentration reported is ambiguous, as the concentration may be that of total morphine (morphine plus M3G and M6G) or unconjugated free morphine only.

1.5.5 Drug Interactions

Cases in which morphine is the only drug detected at autopsy represent a minority of heroin fatalities [12], with one Australian study finding drugs in addition to morphine in 86% of cases [20]. Of particular concern is the co-administration of other depressant drugs, such as benzodiazepines and alcohol, which can increase the likelihood of a fatal overdose due to potentiation of the respiratory depressant effects of heroin [30]. The mechanism by which this is likely to occur involves the major excitory neurotransmitter of respiration, glutamate, and the major inhibitory neurotransmitter, gamma (γ)-aminobutyric acid (GABA). Benzodiazepines and alcohol facilitate the inhibitory effect of GABA, and alcohol decreases the excitory effect of glutamate [29].

Benzodiazepines are used clinically in the treatment of anxiety, insomnia, epilepsy, and as muscle relaxants [52]. Estimates suggest that greater than one third of injecting drug users currently use benzodiazepines [53], with diazepam and temazepam being the most common [53, 54]. Since the depressant effects of benzodiazepines and heroin are additive, less heroin may be needed to produce a fatal outcome in the presence of benzodiazepines. The co-administration of benzodiazepines with heroin has a significant effect on the likelihood of an overdose, and the following proportions of heroin fatalities that were positive for

benzodiazepines have been reported: 33% [31], 26% [55], 44% [56], 28% [57], 45% [20], and 71% [21]. The most common reason cited for benzodiazepine use by heroin users is to enhance the feeling of high from the opiates, while a small proportion use benzodiazepines to reduce opiate withdrawal symptoms [58]. Far from being obtained illegally, they are often prescribed, with approximately 30% of heroin users obtaining benzodiazepines through legal prescriptions [59]. Around 26% of heroin users who had ever used benzodiazepines are deemed to be benzodiazepine dependent [60], and are therefore at a greater risk of experiencing a fatal heroin overdose.

The combination of heroin with alcohol is another common post-mortem observation, with the following proportions of heroin fatalities testing positive for alcohol being reported: 33% [31], 74% [61], 45% [55], 41% [56], 50% [57], 36% [20], 33% [21], and 38% [62]. In combination with alcohol, heroin can cause respiration to slow or even cease, and a smaller than usual dose may be required to exert a fatal effect [63].

1.6 Post-mortem Drug Analysis

In systematic toxicological analysis (STA), the major task is to detect and identify unknown substances in a given biological matrix. Since the number of potentially toxic drugs and chemicals exceeds seven million [64], a rational chemical-analytical approach is necessary. The drug-screening process can be divided into two stages – sample preparation and analysis. In a post-mortem setting, putrefactive compounds and the decomposed nature of specimens limit the applicability of clinically validated assays [65]. As a result, special consideration must be given to sample preparation and analysis when post-mortem specimens are involved.

1.6.1 Specimen Selection

Post-mortem specimens are often selected based upon availability and apparent cause of death, and can range from commonly encountered specimens, such as blood, bile and liver, to less common specimens, such as bone. While most methods validated

for plasma, serum or urine are interchangeable, post-mortem blood can present problems in most of these methods due to its higher viscosity and often variable condition [65]. So, although blood is generally a readily available specimen, it may not be the specimen of primary choice. Liver is the specimen most frequently used in post-mortem toxicology, and often the drug test results supplement blood toxicology data [65]. Urine is often a screening specimen, however bile may be used when urine is not available, as it contains high concentrations of a number of drugs, including morphine and benzodiazepines [65]. Vitreous humor (VH) is an easily prepared sample, as it contains very little protein, and is often analysed to supplement blood alcohol concentrations [65]. To determine exposure to drugs over a period of weeks or months, hair and nail specimens can be collected and analysed, since they offer a wider window of detection than other biological specimens.

1.6.2 Extraction

The purpose of an extraction step is to selectively remove the compound of interest from the biological matrix and leave behind unwanted materials [66]. Prior to chromatographic determination of drugs in blood, cells and proteins have to be removed. The traditional method for isolating a range of basic and neutral drugs from biological specimens is a single-step liquid-liquid extraction (LLE) extraction from basic pH. Protein precipitation, by the use of organic solvents, inorganic salts, metallic ions or acids, is also a common method [67]. The relative order of effectiveness of organic solvents in precipitating proteins is acetonitrile > acetone > ethanol > methanol, which is roughly inversely related to their polarity [66].

Solid-phase extraction (SPE) applications have traditionally been limited to plasma, serum and urine, with difficulties arising with the use of SPE for whole or post-mortem blood since red blood cells tend to clog the column [68]. As many drugs are bound to red blood cells, direct application of blood onto the SPE column often results in variable recoveries, so pre-treatment (e.g. protein precipitation) is often a necessary step to release drugs from the biological matrix [67]. Factors that may affect the behaviour of drugs during SPE include sorbent, pH of the sample and

extraction system, the clean-up step, properties and volume of eluent and flow rate of the eluent [67]. SPE has a number of advantages over LLE, including higher selectivity, cleaner extracts, no emulsions, more reproducible results, reduced solvent usage and the ability for automated analysis [67].

1.6.3 Analysis

Immunoassay screening techniques can be used in post-mortem toxicology, however where putrefaction has occurred, false negative results may be obtained. False positive results for amphetamines may also occur due to the presence of amine putrefaction products [65]. Although immunoassays have the advantage of low cost, high throughput and simplicity, problems with cross-reactivity restrict their application to screening for drugs of abuse.

Confirmation can be achieved by GC with MS or DAD detection, however hydrolysis of conjugated metabolites and derivatisation prior to extraction is necessary, resulting in a time-consuming analytical procedure. Reversed-phase HPLC on octyl- or octadecyl-silica with gradient elution is the most popular technique used in STA [64] since it reduces the need for time-consuming sample preparation steps, and allows the separation of both hydrophilic and lipophilic analytes. MS offers selectivity by the use of selective ionisation techniques. Soft ionisation methods, such as electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI), as well as tandem MS methods, can enable LOQs in the pg – ng/mL range to be achieved [69]. However, the relatively high cost of MS means that other detectors, such as UV or DAD, are still widely used.

1.6.4 Post-mortem Considerations

Extended post-mortem periods before specimen collection and/or inadequate or prolonged storage before analysis can present problems concerning stability and redistribution. Many drugs are known for their unstable nature e.g. nitrobenzodiazepines, however, when specimens are stored at -20°C or lower and analysed promptly, post-mortem changes of many drugs can be minimised.

Morphine is relatively stable in unpreserved specimens when frozen, but shows significant losses when stored at 4°C or higher for more than a few days [65]. Its glucuronides are also relatively stable in blood [70, 71]. Since the extent of chemical change in the post-mortem interval, or post-mortem metabolism may affect the interpretation of results, it is important to know the stability of drugs in biological tissues.

Disruption of cellular membranes following death causes increased drug concentrations in blood due to diffusion from other sites. This process of redistribution is significant for highly lipid soluble drugs, or drugs with a high tissue concentration relative to blood. Cardiac blood is the most affected collection point, so to reduce the variability in post-mortem drug concentrations, it has been recommended that femoral blood be collected where possible to provide a more representative concentration at the time of death [65].

1.7 Drugs of Interest

Benzodiazepines and morphine-related analytes are regularly encountered in forensic toxicology, especially in cases of fatal heroin overdose. The involvement of heroin metabolites and benzodiazepines in heroin overdose warrants investigation, thus analytical methods for their analysis are highly relevant. For this reason, the drugs of interest in this research were the metabolites of heroin, specifically M3G and M6G, and benzodiazepines.

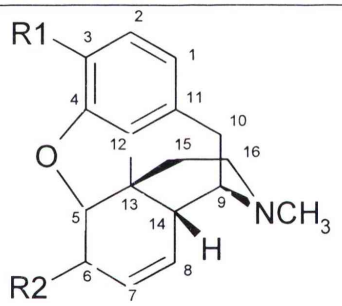
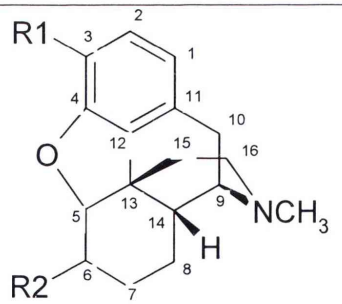
1.7.1 Heroin and Metabolites

1.7.1.1 Structural Features of Heroin and Related Compounds

Heroin and other structurally related opioids are phenanthrene derivatives, consisting of 5 fused ring systems. C1-4, 11 and 12 form an aromatic ring, with the *O* bridge between C4 and C5 forming a furanoide-type ring. The methylamine chain connects C9 and C13 to form an *N*-methylpiperidine ring across the phenanthrene skeleton [5]. Variation of the substituent at the 3- and 6-positions affords a variety of related

opioids with different receptor binding properties and analgesic activity. Some of these structurally related opioids are depicted in Table 1-1. It has been demonstrated that the free phenolic hydroxyl group at position 3 is an essential requirement for opioid receptor binding [72, 73]. This is supported by studies which have found that compounds with a substituent at the 3-position, such as M3G, have little affinity of for opioid receptors [73, 74]. However, decreasing the length of the alkyl chain of those substituents at position 3 may increase receptor-binding affinity [75, 76]. The chemical group at position 6 appears to have little effect on binding [73, 75], but it may affect the receptor selectivity [72].

Table 1-1 Structures of some compounds chemically related to heroin

Compound	R_1 (C3)	R_2 (C6)	
Heroin	$OCOCH_3$	$OCOCH_3$	
Morphine	OH	OH	
Morphine-6-glucuronide	OH	$OC_6H_9O_5$	
Morphine-3-glucuronide	$OC_6H_9O_5$	OH	
Codeine	OCH_3	OH	
Codeine-6-glucuronide	OCH_3	$OC_6H_9O_5$	
Dihydrocodeine	OCH_3	OH	
Dihydrocodeine-6-glucuronide	OCH_3	$OC_6H_9O_5$	
Hydrocodone	OCH_3	=O	
Hydromorphone	OH	=O	

The tertiary amino group accounts for the basicity of opioids, with typical pK_a values ranging between 7.6 and 8.9. This ensures a degree of protonation at physiological pH, which is believed to facilitate the interaction with receptors [1].

1.7.1.2 Pharmacological Action and Effects

Heroin is usually administered as the hydrochloride salt by intravenous or subcutaneous injection, or nasal insufflation [3]. The salt is generally not smoked since it has a relatively high melting point and tends to decompose, thereby reducing its bioavailability. By comparison, the free base form is often smoked since it will vaporise readily without decomposition at lower temperatures than heroin hydrochloride [8]. A recovery rate of only 17% has been reported when heroin hydrochloride was smoked, compared to 62% for heroin base [8].

The analgesic effects of heroin are attributed to the combined effects of 6MAM and morphine [1]. Following heroin injection, users often report feeling a "rush" that lasts only one or two minutes. This sensation is thought to be caused by the injected heroin crossing the blood-brain barrier (BBB) before being distributed by the bloodstream and converted into morphine. After the rush, the high lasts for four or five hours and is caused by the morphine diffusing from the bloodstream into the brain. Heroin and its biotransformation products exhibit their effects as agonists on the μ, κ and δ receptors in the CNS. Interactions with μ receptors cause central depression, consisting of supraspinal (μ_1) and spinal (μ_2) analgesia, respiratory depression, delayed gastrointestinal motility, miosis, euphoria and hypothermia [1]. Binding to κ receptors produces spinal analgesia, sedation, miosis, diuresis and mild respiratory depression. Most endogenous peptides interact with δ receptors, and it is these interactions that produce the effects of spinal analgesia, dysphoria, delusions, hallucinations, and respiratory stimulation [1] sometimes associated with heroin use. Opioids may also bind to σ receptors to produce central excitation, resulting in tachycardia, hypertension, tachypnea, mydriasis, and hallucinations [1].

Chronic administration of the drug may cause long-term effects, such as tolerance and physical dependence, to develop. Tolerance necessitates the consumption of larger quantities of the drug to achieve the desired effect. Opioid tolerance is initiated after the first dose, but is not usually significant until several weeks after chronic use

[1]. Physical dependence results in severe withdrawal symptoms, such as nausea, muscle spasms, cramps, anxiety, fever and diarrhoea if the drug is abruptly ceased. These withdrawal symptoms are most likely attributed to chemical imbalance, as neurons, which have become inhibited due to long-term drug use, begin producing neurotransmitters again. Other complications of chronic IV heroin usage include liver disease, pulmonary hypertension and peripheral nerve legions [3].

1.7.1.3 Metabolism and Pharmacokinetics

In trials involving the treatment of chronic pain, doses of 4 – 16 mg of heroin have been administered intravenously, however, addicted heroin users frequently consume single doses in the range 150 – 200 mg, and commonly require up to 500 mg or more per day [77]. Heroin has an analgesic potency two to three times that of morphine [78] and, due to the two acetyl groups, has better penetration across the BBB [1], crossing it within 15 – 20 seconds and achieving relatively high brain levels [78].

Following IV administration, heroin is rapidly metabolised via hydrolytic reactions to 6MAM, then further to morphine. Since morphine is relatively resistant to hydrolytic decomposition, further metabolism to the major metabolites M3G and M6G, as well as morphine-3-ethereal sulfate, occurs via synthetic reactions [5]. Minor metabolic pathways include formation of normorphine, morphine-3,6-diglucuronide and morphine *N*-oxide. Some of the metabolic pathways associated with heroin are illustrated in Figure 1-4. The extent of heroin metabolism is somewhat dependent on the route of administration, with routes avoiding first pass metabolism (e.g. IV, intramuscular, transdermal) producing fewer metabolites than other routes of administration (e.g. oral, sublingual) [79]. After administration of heroin in humans, 60 – 80% is excreted as morphine. 90% of this represents glucuronide conjugates [80], of which M3G is the most predominant. Extra-hepatic metabolism in the brain and kidneys, direct morphine elimination in urine, and excretion via other routes, such as faeces and perspiration, also occurs [44]. The prolonged effects of heroin are attributed to its active metabolites. The duration of action of the drug depends upon the distribution of the active metabolites throughout

the body, their half-lives. Heroin has a volume of distribution (V_D) of 25 L/kg and a short half-life ($t_{1/2}$) of 3 mins. 6MAM also has a short $t_{1/2}$ of around 6 – 25 mins. The V_D of morphine is around 3 – 5 L/kg, its plasma clearance is 15 – 20 mL/min/kg, and its $t_{1/2}$ is about 2 – 3 hrs [78].

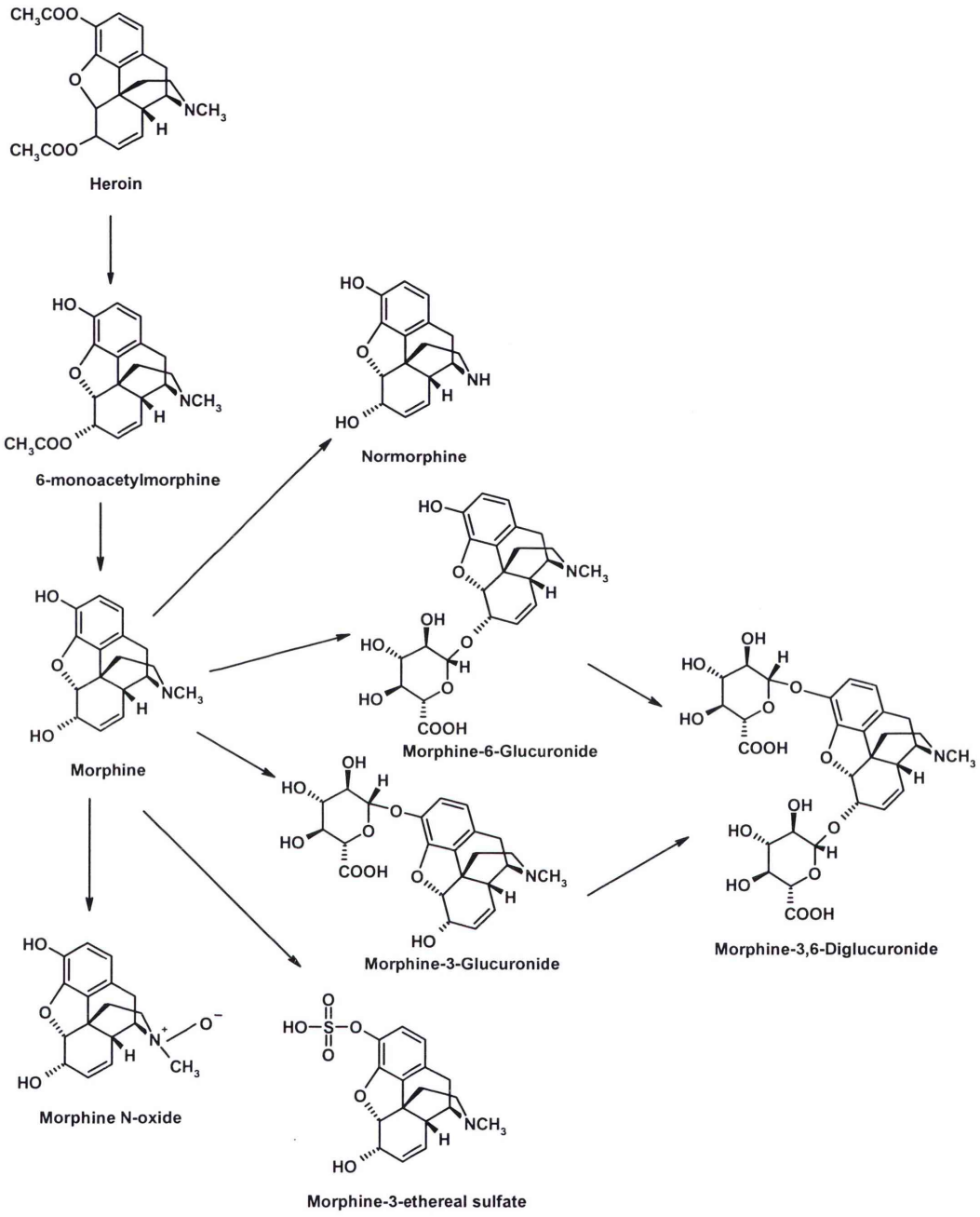


Figure 1-4 Some metabolic pathways of heroin

Glucuronidation of morphine involves UDPGT in the liver, however there is evidence to suggest that there may be more than one type of UDPGT to catalyse the this reaction, which explains the different proportions of the 3- and 6-glucuronide.

Since the 3-position represents a phenolic hydroxyl group, whilst the 6-position is an alcoholic hydroxyl group, it is possible that a different UDPGT catalyses the reaction at each position [81]. UDPGT isoenzymes are believed to be subject to genetic variation, which may account for the wide inter-patient variation in plasma concentrations of morphine, M3G and M6G in patients receiving morphine for pain [44], as well as subsequent decreased or increased analgesic effects [82].

Like morphine, M6G has a high binding affinity for the μ opioid receptor [29], with a number of studies suggesting that it has analgesic activity [50, 83, 84], and some even suggesting that it is a more powerful agonist than morphine [72, 84]. While its potency has been described as 4 – 200 times greater than that of morphine [49], some human clinical data suggest that it has a lesser respiratory depressant effect than an equipotent dose of morphine [29]. There is also evidence from other studies that suggest M6G has no analgesic activity [85, 86]. Whilst M3G shows a lack of affinity for μ, κ and σ opioid receptors [49, 51], it has been shown to antagonise some analgesic and respiratory depressive effects of morphine and M6G [44].

1.7.1.4 Analysis of Morphine and Metabolites in Biological Samples

The vast majority of published methods report the use of SPE for the extraction of morphine and its metabolites from biological samples, including whole blood, serum, plasma, cerebrospinal fluid, urine and vitreous humor. Less frequently used extraction methods include LLE [87-93], SPME [94] and immobilised antibodies [95]. In the past, M3G and M6G have suffered from poor recoveries with traditional LLEs from biological matrices, due to their pH-dependent nature and large difference in pK_a compared with the parent drug morphine. The main reason for the prevalence of SPE for the extraction of morphine and its metabolites is its ability to recover M6G and M3G. Mixed-mode SPE cartridges, such as Bond Elut Certify, have both reversed-phase and cation-exchange mechanisms in operation, enabling analytes with a range of polarities and pK_a's to be extracted simultaneously. Details

of the various types of SPE cartridge employed for the extraction of morphine and its metabolites are given in Table 1-2.

Table 1-2 SPE cartridges used for the extraction of heroin metabolites from biological samples

Cartridge Type	Brand	Reference
C_2	Baker	[96-98]
	Bond Elut	[99-101]
C_8	Bond Elut	[71, 101-105]
C_{18}	Bond Elut	[101, 106-112]
	Clean Up	[113]
	Nucleosil ODS-2	[114]
	Sep-Pak	[85, 115-123]
	Chromabond	[124]
Ion exchange	Bond Elut SCX	[101]
	Bond Elut CBA	[125]
	Oasis MCX	[126-128]
Mixed Mode	Bond Elut Certify	[36, 63, 98, 101, 129-140]
	Clean Screen DAU	[140, 141]
	Isolute Confirm HCX	[139]
	Chromabond Drug	[139]
	Bakerbond narc-2	[139]
Polymeric	Oasis HLB	[142, 143]

A wide variety of techniques have been employed for the analysis of opiates in biological samples. The most common non-chromatographic techniques for urine and blood/plasma analysis are immunoassay screening methods, such as enzyme immunoassay (EIA) [36, 144-148], radioimmunoassay (RIA) [149], enzyme linked immunosorbent assay (ELISA) [150-152], radioimmunoassay (RIA) [63, 153, 154], radioreceptor assay (RRA) [63], fluorescence polarisation immunoassay (FPIA) [147, 155], cloned-enzyme donor immunoassay (CEDIA) [147, 148, 156] and fluoroimmunoassay [157]. Although immunoassays have the advantage of low cost, high throughput and simplicity, the problem of cross-reactivity among morphine,

morphine metabolites and their analogues limits them to screening for drugs of abuse.

In terms of confirmatory techniques, the analysis of morphine and its metabolites in biological matrices is largely performed by HPLC with MS [96, 97, 99, 102, 103, 107, 108, 111, 115, 116, 126, 141, 158-161] or MS-MS [110, 113, 143, 162] detection due to its high levels of sensitivity and specificity. The applications and advantages of LC-MS for the determination of heroin and related opioids (including morphine, M3G and M6G) was published in a review by Pichini *et al.* in 1999 [69]. Other detection methods include UV [106, 114, 121, 138, 142], fluorescence [71, 85, 95, 100, 104, 105, 163, 164] and electrochemical detection [100, 117, 122, 139]. A handful of hyphenated detection methods (other than MS-MS) have been reported, and include ECD-F [112], F-UV [89, 125] and ECD-UV [120]. Published HPLC methods, including extraction and recovery, chromatographic conditions, run times and detection limits for the determination of morphine, selected metabolites and related analytes in biological specimens, are presented in Table 1-3.

While there have been reports on the use of GC for the analysis of morphine and its metabolites [36, 61, 87, 88, 90-94, 128, 136, 137, 140, 146, 148, 165-172], this technique is not used as routinely as HPLC. The main reason for this is that the analysis of polar drugs, such as the morphine glucuronides, requires a derivatisation step, which can cause the sample preparation process to become lengthy and tedious. CE is an emerging technique in toxicology and, as such, its application to the detection of morphine and its metabolites in biological samples has been reported [101, 129-135, 173-179]. However, as opposed to techniques such as HPLC and GC, CE is not used routinely in toxicological analyses due to its inferior sensitivity.

Table 1-3 Summary of published HPLC methods for heroin metabolites in biological samples

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[106]	B	MOR, C, MAM	SPE Bond Elut C_{18}	Spherisorb ODS 250x4.5mm, 5µm	0.05M sodium pentane sulfonic acid/ACN (70:30), pH 2.0	1.2	UV 220	0.2	>95	<10
[115]	U	MOR, M3G	SPE Sep-Pak C_{18}	ODS 150x4.6mm	50mM NH_4Ac/MeOH (86:14)	1.0	APCI-MS	1-3	n/a	n/a
[89]	B, Bi	MOR, C	LLE chloroform/IPA	Nova Pak phenyl 150x3.9mm, 5µm	ACN/phosphate buffer, pH 6.6 (10:90)	1.2	F220/370-UV 210	60-200	53-87	30
[118]	P, B, U	6MAM, MOR	SPE Sep-Pak C_{18}	Waters Bondapak phenyl 300x3.9mm, 10µm	25mM NH_4Ac/ACN (72:28), pH 6.5	1.0	ECD	1-4	92-99	n/a
[141]	U	MOR, C	SPE Clean Screen DAU	Zorbax RX C_8 250x4.6mm, 5µm	0.2% TFA, 0.1M NH_4Ac/MeOH (75:25)	1.25	TSP-MS	2	n/a	n/a
[117]	P, CSF	MOR, M3G, M6G	SPE Sep-Pak C_{18}	10µ Waters C_8 Resolve	MeOH/ACN/phosphate buffer, pH 7.5 (10:10:80)	n/a	EC	6	80-90	30
[104]	S	MOR, M6G, M3G	SPE Bond Elut C_8	Nucleosil C_{18} 250x4.8mm	1% ACN/TEAP buffer	1.0	F245/345	5	n/a	<10

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[120]	B	M3G, M6G, norMOR, MOR	SPE Sep-Pak C_{18}	Nova Pak C_{18} 150x3.9mm, 5µm	ACN/1mM SDS/phosphate buffer (24:76), pH 3	1.0	ECD-UV 210	10	70-80	<36
[163]	P, CSF	MOR, M3G, M6G	On-line SPE	Lichrospher 60RP select B 250x4mm, 5µm	ACN/200mM phosphate buffer, pH 3.0 (gradient)	0.8	F210/350	0.85-3.4	>95	10
[96]	S	MOR, M3G, M6G	SPE Baker C_2	Supelcosil ABZ 250x4.6mm, 5µm	H_2O/MeOH (85:15) gradient	0.8	ESI-MS (SIM)	10-100	70-95	10
[161]	U, B	6MAM, MOR, M3G, M6G, AC	SPE Bakerbond Diol; 0.22µm filters	Zorbax TMS 250x4.6mm, 5µm	15-50% MeOH/acetate buffer/TEA	1.2	TSP-MS	10-50	n/a	15
[139]	B, S	MOR, 6MAM, C	SPE Bond Elut Certify, Isolute Confirm HCX, Chromabond Drug, Bakerbond narc-2	C_{18} 250x4.6mm,	ACN/5mM citrate buffer/20mM lithium perchlorate, pH 5 (12:88)	0.8	ECD	n/a	30-73	<30

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[100]	P	MOR, M3G, M6G	SPE Bond Elut C_2	Nova Pak 75x3.9mm, 4µm (two in tandem)	MeOH/10mM phosphate buffer, pH 4.0 (10:90)	1.0	F210/335, EC	1-10	51-90	15
[71]	S	MOR, M3G, M6G	SPE Bond Elut C_8	Nucleosil C_{18}	TEAP	n/a	F220/340	7.5-25	64-77	n/a
[116]	S	MOR, M3G, M6G	SPE Sep-Pak C_{18}	ODS C_{18} 100x4.6mm	3mM formic acid/ACN gradient	1.0	APCI-MS	0.84-5	n/a	10
[95]	B	MOR, M3G, M6G	Immobilised antibodies	Lichrospher 60RP Select B 250x4mm, 5µm	10mM phosphate buffer/2mM heptane sulfonic acid/ACN, pH 3, gradient	1.0	F240/340	10	73-82	20
[109]	S, B, U, CSF, VH	MOR, M3G, M6G, C, C6G, 6MAM	SPE Bond Elut C_{18}	Superspher RP 18 125x3mm, 4µm	ACN/50mM ammonium formate buffer, pH 3.0 (5:95)	0.6-1.1	APCI-MS (SIM)	0.5-100	85-98	17
[108]	B, CSF, VH, U	MOR, M3G, M6G, 6MAM,	SPE Bond Elut C_{18}	Superspher Select B 125x3mm	50mM formate buffer, pH 3.0/ACN	0.6	APCI-MS	0.1-1	82-89	n/a

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[114]	P	HER, MOR, C, norMOR, M3G, M6G, C6G, DHM, 3MAM, 6MAM	SPE Nucleosil ODS-2 C_{18}	Spherisorb C_{18} 125x2mm, 3µm	Phosphoric acid/ACN/hexylamine, gradient	0.2	UV 210	25	>80	20
[122]	P	MOR, M3G, M6G, norMOR	SPE Sep-Pak C_{18}	Shodex-AR 150x4.6mm	10mM phosphate buffer, pH 2.1/1mM SDS/26% ACN	n/a	ECD	n/a	n/a	n/a
[85]	P	MOR, M6G	SPE Sep-Pak C_{18}	C_{18} AB 100x2mm	ACN/1.25mM SDS/10mM phosphate buffer (18:82)	0.4	F245/335	10	n/a	n/a
[121]	P	MOR, M3G, M6G	SPE Sep-Pak C_{18}	C_{18} 150x4.6mm	ACN/SDS/10mM phosphate buffer, pH 2.1 (26.5:73.5)	0.8	UV 210	n/a	n/a	n/a
[97]	S mice	HER, 6MAM, MOR, M3G, M6G, C	SPE Baker C_2	Supelcosil LC-Si 250x2.1mm, 5µm	H_2O/MeOH/ACN/ formic acid (59.8:5.2:34:65:0.35)	0.23	MS	0.5-4	44-99.8	<25

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[107]	B, S, U, CSF	MOR, M3G, M6G, MAM, C, C6G, DHC, DHM	SPE Bond Elut C_{18}	Superspher RP18 125x3mm, 4µm	ACN/50mM ammonium formate, pH 3.0 (10:90)	0.3-0.6	APCI-MS	0.1-100	73-98	35
[105]	P	MOR, M3G, M6G	SPE Bond Elut C_8	Alltima C_{18} 250x4.6mm, 5µm	ACN/phosphate buffer, pH 2.2 (27.5:72.5)	1.0	F210/340	5-50	90-102	n/a
[119]	P	MOR, M3G, M6G, HM	SPE Sep-Pak C_{18}	Waters C_8 Resolve Cartridge 100x8mm, 10µm	MeOH/ACN/12.5mM phosphate buffer, pH 7.2 (12:12:76)	n/a	ECD	6	91-93	20
[99]	P rat	MOR, M3G, M6G, norMOR	SPE Bond Elut C_2	Zorbax SB-phenyl 250x4.6mm, 5µm	0.1% formic acid/18% MeOH	0.7	ESI-MS	3.8-12	64-100	<20
[111]	S	MOR, M3G, M6G	SPE Bond Elut C_{18}	Lichrospher C_{18} 30x4mm	ACN/10mM formate buffer (6:94)	0.3	ESI-MS	1-5	97-104	<5
[112]	P	MOR, M3G, M6G	SPE Bond Elut C_{18}	Spherisorb 250x4.6mm	70mM phosphate buffer/0.5mM EDTA, pH 5.85/MeOH (92:8)	1.0	ECD-F280/335	<8	n/a	n/a

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[103]	S, B	MOR, M3G, M6G, C, 6MAM, C6G	SPE Bond Elut C_8	Inertsil ODS-3 150x3mm, 5µm	1mM ammonium formate, pH 3/ACN, gradient	0.4	ESI-MS	0.5-5	70-84	15
[110]	P	MOR, M3G, M6G	SPE Bond Elut C_{18}	Inertsil silica 50x3mm, 5µm	Formic acid/water/ACN (1:10:90)	1.0	TSP-MS-MS	0.5-1	70-93	3
[159]	S, U, CSF	MOR, M3G, M6G, norMOR	SPE C_2	Lichrospher RP18 125x3mm, 5µm	H_2O/ACN/THF/formic acid (100:1:1:0.1) gradient	1.0	ESI-MS	0.3-15	65-88	7
[113]	P	MOR, M6G, M3G	SPE Clean Up C_{18}	YMC ODS-AQ 150x2mm, 3µm	Water/ACN/formic acid (95:5:0.1)	0.2	ESI-MS-MS	0.25-0.5	n/a	7
[102]	B, P	MOR, M3G, M6G	SPE Bond Elut C_8	Hypersil ODS 60x4.6mm, 3µm	ACN/NH_4Ac gradient	0.8	MS	3.4-7.4	64-91	n/a
[126]	P (rat)	MOR, M3G, M6G	SPE Oasis MCX	Mightysil RP18 100x2mm,3µm	50mM NH_4Ac/ACN gradient	0.15	ESI-MS (SIM)	0.1	70-82	25
[142]	S	MOR, M3G, M6G	SPE Oasis HLB	Symmetry Shield RP8 150x4.6mm, 5µm	20mM phosphate buffer/ACN	1.0	UV 210	2.3-20	90-99	15

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[138]	P	MOR, HM, C, OC, HC	SPE Bond Elut Certify	Chromolith RP monolithic silica 100x4.6mm	Pentanesulfonic acid/phosphate buffer, pH 2.9/ACN, gradient	8.0	UV 208	8.7-38.6	95-104	2
[125]	B, U	Various (18)	SPE Bond Elut CBA	Hyposil phenyl 53x7mm, 3µm	Water/MeOH/TEAF, pH 4.5, gradient	2.0	UV 240-F280/335	1-9	>60	12
[162]	P	MOR, M3G, M6G	SPE C_{18}	Betasil silica 50x3mm, 5µm	ACN/water/TFA	0.7	MS-MS	0.5-10	43-72	3
[98]	Mec	6MAM, MOR, M3G, M6G, C	SPE Bond Elut Certify, Baker C_2	Zorbax Eclipse XDB-C_{18} 150x4.6mm	Acetic acid/ACN, gradient	1.0	MS	0.3-1.2	72-90	14
[158]	P	MOR, norMOR, M3G, M6G	ACN precipitation	Luna phenyl-hexyl 75x4.6mm, 3µm	0.1% formic acid/ACN gradient	1.0	ESI-MS (SIM)	$<10^{-5}$	70-91	<10
[127]	P	MOR, M3G, M6G	SPE Oasis MCX	Atlantis dC_{18} 150x2.1mm, 5µm	ACN/MeOH/10mM formate, pH 3 (2.5:2.5:95)	0.2	ESI-MS	0.5-5	62-90	<10

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[124]	U	MOR, M3G, M6G, 6MAM, C, C6G, AC, NOS, PAP	SPE Chromabond C_{18}	Phenomenex C_{18} Aqua 150x2mm, 4µm	H_2O/ACN/5mM NH_4Ac, gradient	0.2	ESI-MS-MS	0.1-7.4	45-98	19
[143]	P dog	MOR, M3G, M6G	SPE Oasis HLB	Symmetry C_{18} 150x2.1mm, 5µm	ACN/acetic acid (1:24); MeOH/formic acid (1:19)	0.1	MS-MS	0.5-25	n/a	n/a
[180]	P rat	MOR, OC, M3G, norOC	ACN precipitation	Agilent Zorbax SB-C_{18} 50x2.1mm, 5µm	Formic acid/MeOH/H_2O, gradient	0.15	ESI-MS-MS	3.8-5.0 (LOQ)	84-100	8

n/a – not available or not described, [a] references in chronological order, [b] flow rate in mL/min, [c] wavelengths in nm, [d] detection limits in ng/mL, [e] recoveries in per cent, [f] run times in min

Abbreviations: Specimens: bile (Bi), blood (B), cerebrospinal fluid (CSF), meconium (Mec), plasma (P), serum (S), urine (U), vitreous humor (VH); **Analytes:** acetylcodeine (AC), codeine (C), codeine-6-glucuronide (C6G), dihydrocodeine (DHC), dihydromorphine (DHM), heroin (HER), hydrocodone (HC), hydromorphone (HM), 3-monoacetylmorphine (3MAM), 6-monoacetylmorphine (6MAM), morphine (MOR), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), normorphine (norMOR), noscapine (NOS), oxycodone (OC), papaverine (PAP); **Method details:** acetonitrile (ACN), ammonium acetate (NH_4Ac), atmospheric pressure chemical ionisation (APCI), diode array detection (DAD), electrochemical detection (ECD), electrospray ionisation (ESI), isopropanol (IPA), fluorescence detection (F), liquid-liquid extraction (LLE), mass spectrometry (MS), methanol (MeOH), solid-phase extraction (SPE), tetrahydrofuran (THF), thermospray (TSP), triethylamine (TEA), triethylammonium phosphate (TEAP), trifluoroacetic acid (TFA), ultraviolet detection (UV).

1.7.1.5 Toxicological Data

There is often great variation in the blood morphine concentrations found in cases of fatal heroin overdose, with some fatalities possessing concentrations of as little as 10 ng/mL, and others with concentrations up to 4000 ng/mL [3, 35]. This large variation is most likely attributed to incomplete metabolism of morphine as a result of differing survival times after heroin injection, however it may also be due to the ambiguous reporting of free morphine or total morphine (the sum of morphine, M3G and M6G), which gives limited information. For comparative purposes, Table 1-4 illustrates the free and total morphine concentrations found in the plasma of fatal heroin overdoses, rapid fatal overdoses and non-fatal overdoses. Where available, the ratio of free morphine (MOR_{free}) to total morphine (MOR_{tot}), which may indicate the survival time after exposure to heroin, is also reported. Since the extent of metabolism is less following a rapid death compared to a delayed death, higher MOR_{free}/MOR_{tot} ratios may be indicative of rapid deaths following heroin administration.

The ratio between the molar concentration of M6G or M3G to morphine has also been suggested as a means of assessing the time elapsed since heroin or morphine administration [104, 108, 164], with lower ratios of M6G/MOR or M3G/MOR indicative of a shorter survival time after administration. High levels of M6G may be associated with chronic exposure to heroin, since M6G may accumulate with repeated administration of the drug [181]. Therefore, determination of the individual glucuronide metabolites may give a clearer picture as to the cause of heroin overdose, since it enables an estimation regarding survival time to be made, and it may indicate if death was a result of chronic or first-time administration of heroin. Table 1-5 summarises the few studies that have reported molar ratios and concentrations of morphine, M3G and M6G in heroin fatalities.

A number of studies have focused on the determination of morphine glucuronide concentrations in patients receiving morphine therapy in an attempt to account for

variations in analgesia, or to better understand the mechanism of tolerance. As seen in Table 1-6, morphine therapy patients can tolerate concentrations that would be fatal in some cases of heroin overdose. In addition, the concentrations of M3G and M6G are generally higher in these patients compared with heroin fatalities.

Table 1-4 Free and total morphine concentrations and ratios in fatal heroin overdose victims and survivors

Ref.	Fatal overdose			Rapid fatal overdose			Non-fatal overdose		
	MOR_{tot}[a]	MOR_{free}[a]	MOR_{free}/MOR_{tot}	MOR_{tot}[a]	MOR_{free}[a]	MOR_{free}/MOR_{tot}	MOR_{tot}[a]	MOR_{free}[a]	MOR_{tot}/MOR_{free}
[30]	2210 ± 4990 (110-36600)	420 ± 1580 (0-14500)	0.186 ± 0.225 (0-0.833)	923 ± 680 (310-2960)	260 ± 210 (0-870)	0.347 ± 0.246 (0-0.833)	n/a	n/a	n/a
[182]	980 (33-5000)	208 (0-2800)	0.293 ± 0.203	n/a	n/a	n/a	396 (10-2110)	30 (0-128)	0.135 ± 0.200
[32]	276.6 ± 148.3 (12-742)	88.1 ± 79.9 (2-513)	n/a	n/a	n/a	n/a	143.8 ± 142.7 (6-540)	54.4 ± 71.0 (3-291)	n/a
[20]	520 ± 530 (10-3400)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
[61]	n/a	220	n/a	n/a	360	n/a	n/a	n/a	n/a
[91]	534-1570	800-1350	0.684-0.860	n/a	n/a	n/a	n/a	n/a	n/a

Where available, data are presented as mean ± standard deviation (range)

[a] concentration ranges and means are in ng/mL

Table 1-5 Concentration ranges and molar ratios for MOR, M3G and M6G in heroin fatalities

Ref.	MOR conc. (ng/mL)	M3G conc. (ng/mL)	M6G conc. (ng/mL)	M3G/MOR molar ratio	M6G/MOR molar ratio	M3G/M6G molar ratio
[120]	380 (70-930)	500 (160-1040)	140 (90-290)	n/a	n/a	n/a
[71]	184 (117-312)	610 (478-775)	59 (50-104)	2.9 (2.02-3.50)	0.33 (0.12-0.46)	n/a
[70]	310 ± 220 (60-800)	610 ± 570 (130-2220)	130 ± 110 (20-500)	n/a	n/a	n/a
[108]	265 ± 323 (8-1539)	395 ± 226 (111-941)	139 ± 73 (32-332)	2.8 ± 4.0 (0.30-15.6)	1.6 ± 3.6 (0.06-16.78)	3.3 ± 1.7 (0.70-6.88)
[104]	665 (232-1700)	1862 (481-5800)	780 (316-2300)	2.52 (1.11-7.76)	1.2 (0.34-2.85)	0.49 (0.23-0.78)
[160]	127 (118-136)	126 (124-127)	30 (28-32)	0.62 (0.58-0.65)	0.15	n/a
[35]	273 ± 189 (60-894)	n/a	n/a	n/a	n/a	n/a

Where available, data are presented as mean ± standard deviation (range)

n/a – not available or not described

Table 1-6 Concentration ranges and molar ratios for MOR, M3G and M6G in morphine therapy patients

Ref.	MOR conc. (ng/mL)	M3G conc. (ng/mL)	M6G conc. (ng/mL)	M3G/MOR	M6G/MOR	M3G/M6G
[45]	36 ± 53 (3-320)	1075 ± 1352 (54-9640)	219 ± 325 (9-2287)	39.2 ± 24.4 (7.7-126.3)	7.2 ± 5.0 (0.7-30.8)	6.1 ± 2.7
[49]	79.8 ± 91.0 (8.5-356)	1625 ± 1495 (103-5472)	228 ± 182 (11.4-570)	34.2 ± 28.0 (4.7-120.1)	4.5 ± 4.0 (0.95-16.2)	8.2 ± 3.3 (3.0-17.0)
[46]	28 ± 17 (5-81)	681± 471 (283-2535)	90 ± 65 (32-350)	32.9 ± 22.3 (8.8-115.3)	4.3 ± 3.8 (0.9-18.1)	7.8 ± 2.0 (2.8-12.3)
[183]	25 (0.7-156)	896 (40-7771)	147 (7.5-1477)	n/a	n/a	n/a
[184]	112 ± 50	758 ± 182	280 ± 116	31.0 ± 6.0 (0.19-92)	3.4 ± 0.48 (0.03-8)	55.0 ± 47.0 (0.18-940)
[185]	51* (0.9-479)	1000* (48-18640)	118* (9-4263)	15* (4-111)	2.3* (1-23)	5.7* (3-15)
[186]	109 ± 102	3061 ± 3515	357 ± 261	33.7 ± 24.2	5 ± 2.7	n/a
[187]	17 (9-30)	694 (351-914)	110 (53-160)	37.4 (26.1-50.5)	6.1 (4.2-8.5)	6.3 (5.8-6.9)
[48]	22.5 ± 6.0	2167 ± 524	335 ± 82.7	150 ± 18 (15-481)	23 ± 4 (3-111)	6.5
[188]	n/a	479 (420-645)	78 (60-94)	n/a	n/a	n/a
[189]	24 (11-47)	700 (214-2072)	114 (30-329)	28.5 ± 17.0	4.9 ± 3.8	6.4 ± 1.5
[190]	n/a	n/a	n/a	12 ± 4.5 (0.25-116)	2.7 ± 0.5 (0.05-8.0)	n/a
[105]	(29-2051)	(360-32900)	(72-4040)	(15.5-32.3)	(2.2-4.5)	n/a

Where available, data are presented as mean ± standard deviation (range)

n/a – not available or not described

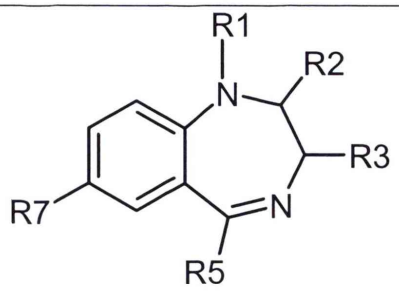
* median values reported

1.7.2 Benzodiazepines

1.7.2.1 Structural Features of Benzodiazepines

The classical benzodiazepines are based upon a 5-aryl-1,4-benzodiazepine structure, which is characterised by a benzene ring fused on the 10- and 11-positions of a 1,4-diazepine ring [191]. The structure of these 1,4-benzodiazepines may be modified by variation of the substituents at positions 1, 2, 3, 5 and 7, as depicted in Table 1-7. Other variations of the benzodiazepine structure include a 1,3-diazole (imidazole) or 1,2,4-triazole ring at the 1,2-position [191]. Table 1-7 illustrates the structures of some of these imidazo- and triazolo-benzodiazepines. For a benzodiazepine compound to possess activity, there should be an electron attracting substituent at position 7, and positions 6, 8 and 9 should be unsubstituted. Activity can be increased with smaller *N*-substituents at the 1-position, phenyl substituents at position 5, and 5-phenyl groups with substituents at the 2 -position. Since benzodiazepines are wide and varied in terms of their structure, it is of no surprise that their pK_a's show considerable variation as well. Many have low pK_a values, e.g. flunitrazepam pK_a = 1.8, alprazolam pK_a = 2.4; some have both a high and a low acid dissociation constant, e.g. clonazepam pK_a = 1.5, 10.5, lorazepam pK_a = 1.3, 11.5; and some have a dissociation constant close to neutral pH, e.g. midazolam pK_a = 6.2 [192].

Table 1-7 Structures of selected 1,4-benzodiazepines



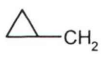
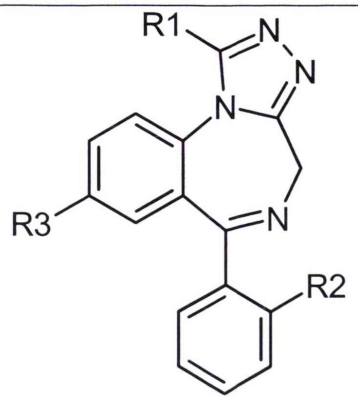
Compound	R_1	R_2	R_3	R_5	R_7
Bromazepam	H	=O	H	2 -pyridyl	Br
Clonazepam	H	=O	H	2-Cl-phenyl	NO_2
Delorazepam	H	=O	H	2-Cl-phenyl	Cl
Diazepam	CH_3	=O	H	Phenyl	Cl
Flunitrazepam	CH_3	=O	H	2-F-phenyl	NO_2
Flurazepam	DEAE	=O	H	2-F-phenyl	Cl
Halazepam	CF_3CH_2	=O	H	Phenyl	Cl
Lorazepam	H	=O	OH	2-Cl-phenyl	Cl
Lormetazepam	CH_3	=O	OH	2-Cl-phenyl	Cl
Medazepam	CH_3	*H*	H	Phenyl	Cl
Nordiazepam	H	=O	H	Phenyl	Cl
Nitrazepam	H	=O	H	Phenyl	NO_2
Oxazepam	H	=O	OH	Phenyl	Cl
Prazepam		=O	H	Phenyl	Cl
Temazepam	CH_3	=O	OH	Phenyl	Cl
Tetrazepam	CH_3	=O	H	1,2-dehydrocyclohexyl	Cl

Table 1-8 Structures of selected imidazo- and triazolo-benzodiazepines



Compound	R_1	R_2	R_3
Alprazolam	CH_3	H	Cl
Estazolam	H	H	Cl
Midazolam[a]	CH_3	F	Cl
Triazolam	CH_3	Cl	Cl

[a] diazole ring replaces triazole ring

1.7.2.2 Pharmacological Action and Effects

Benzodiazepines are among the most widely prescribed psychotropic drugs in the world, with almost 7 million prescriptions being dispensed through pharmacies in Australia alone during 2001 [193]. Alprazolam, clonazepam, diazepam and lorazepam are among the top 100 most commonly prescribed medications [194], and between 0.5% and 5.8% of the adult population use benzodiazepines on a long-term basis of one year or more [195]. Benzodiazepines were first synthesised in the mid 1950s, when chlordiazepoxide was discovered to have sedative, anticonvulsant and muscular relaxant effects [196]. It was later marketed in 1960 under the name Librium. Diazepam was introduced in 1963 and, since then, many analogues, such as clonazepam and lorazepam, have been developed and marketed for use in clinical practice.

Benzodiazepines are most commonly used to treat anxiety, insomnia and panic disorder, but may also be used in the management of agitation in acute alcohol

withdrawal, and in a number of psychiatric conditions including psychotic and agitated states, social phobia, obsessive-compulsive disorder, pain syndromes, seizures, drug withdrawal and side-effects generated by antidepressants and neuroleptics [193]. The classification of benzodiazepines into ultrashort-, short-, medium- and long-acting depends upon their rate of elimination from the blood [197]. Long-acting benzodiazepines, such as diazepam, are used to treat anxiety and have half-lives exceeding 24 hours due to long-acting pharmacologically active metabolites [198]. Short-acting benzodiazepines, such as triazoram, have a half-life of less than 14 hours [193] and are often used to treat insomnia. The advantage of short-acting benzodiazepines is less daytime residual sleepiness, however they are associated with rebound insomnia once treatment is discontinued [197]. The half-life of ultrashort-acting benzodiazepines, such as triazolam and midazolam, is less than 5 hours [198].

The effects of benzodiazepines, which include anticonvulsant, sedative, anaesthetic and skeletal muscle relaxant effects, are largely a result of their depressant effect on the CNS. Specifically, they interact with binding sites on $GABA_A$ receptors, which act via the production and inhibition of γ-aminobutyric acid (GABA), and comprise the brain's major inhibitory neurotransmitter system [197, 199]. $GABA_A$ receptors are divided into five subunits, and are classified into three major groups (α, β and γ) and several minor ones [197]. The receptor comprises of a central Cl^--selective ion channel, around which the five subunits are arranged pseudo-symmetrically [199]. $GABA_A$ agonists either bind directly to the ion channel of the $GABA_A$ receptor, or to allosteric sites that include benzodiazepine, neurosteroid and barbiturate sites [197]. Benzodiazepines do not have intrinsic activity, however they increase the frequency of channel opening and closing by enhancing the action of GABA, and increasing its affinity for the binding site [197]. Partial agonists (e.g. bretazenil) can maintain low-efficacy anticonvulsant and anxiolytic effects, without inducing the sedation, motor impairment, dependence and alcohol potentiation effects often produced by full agonists.

Withdrawal effects, as a result of both physiological and pharmacological dependence, may be experienced after discontinuation of normal therapeutic doses of benzodiazepines, and include anxiety, insomnia, restlessness, agitation, irritability and muscle tension [193]. Down-regulation of GABA, leading to CNS excitation, is the most likely cause of withdrawal symptoms [193, 194]. For short-acting benzodiazepines, withdrawal symptoms usually begin within 6 – 12 hours, peak within 2 – 4 days, and subside in 1 – 3 weeks [193]. For long-acting benzodiazepines, they typically appear within 24 – 48 hours, peak in 4 – 7 days, and subside in 2 – 4 weeks [193]. The incidence of dependence, and therefore the potential for withdrawal symptoms, tends to increase in proportion to the period of time the drug was taken. Tolerance to the various effects of benzodiazepines develops at different rates, with tolerance to the hypnotic and anticonvulsant effects developing rapidly, and tolerance to the anxiolytic effects developing more slowly [193, 194]. Benzodiazepine overdose can occur, and may cause impaired coordination, slurred speech, confusion, coma, diminished reflexes, hypertension, seizures and respiratory depression [193].

1.7.2.3 Metabolism and Pharmacokinetics

The majority of benzodiazepines are metabolised by phase I reactions, including dealkylation, hydroxylation, reduction and acetylation, to produce metabolites that have a degree of biological activity, which may be more or less than that of the parent compound [191]. Phase II, or conjugation reactions produce glucuronide metabolites, which generally have no significant biological activity [191].

The 1,4-benzodiazepines, including diazepam, nordiazepam, prazepam, halazepam, temazepam and oxazepam are all primarily metabolised through nordiazepam and/or temazepam to oxazepam and further to glucuronide conjugates, as depicted in Figure 1-5. Medazepam is also metabolised to oxazepam through diazepam and/or normedazepam. This makes oxazepam a common target metabolite for the detection of 1,4-benzodiazepines in a toxicological or post-mortem setting [191]. The triazolo-

benzodiazepines are metabolised slightly differently, with the 1,4-triazolo ring preventing the oxidative metabolism exhibited by the 1,4-benzodiazepines, thus avoiding the formation of active metabolites with long elimination half-lives [200]. The nitrobenzodiazepines, which are characterised by a NO_2 substituent at position-7, are metabolised to 7-amino metabolites via phase I reactions, involving the reduction of the nitro group to an amino group. These 7-amino metabolites are also common targets in urine and post-mortem blood specimens [191].

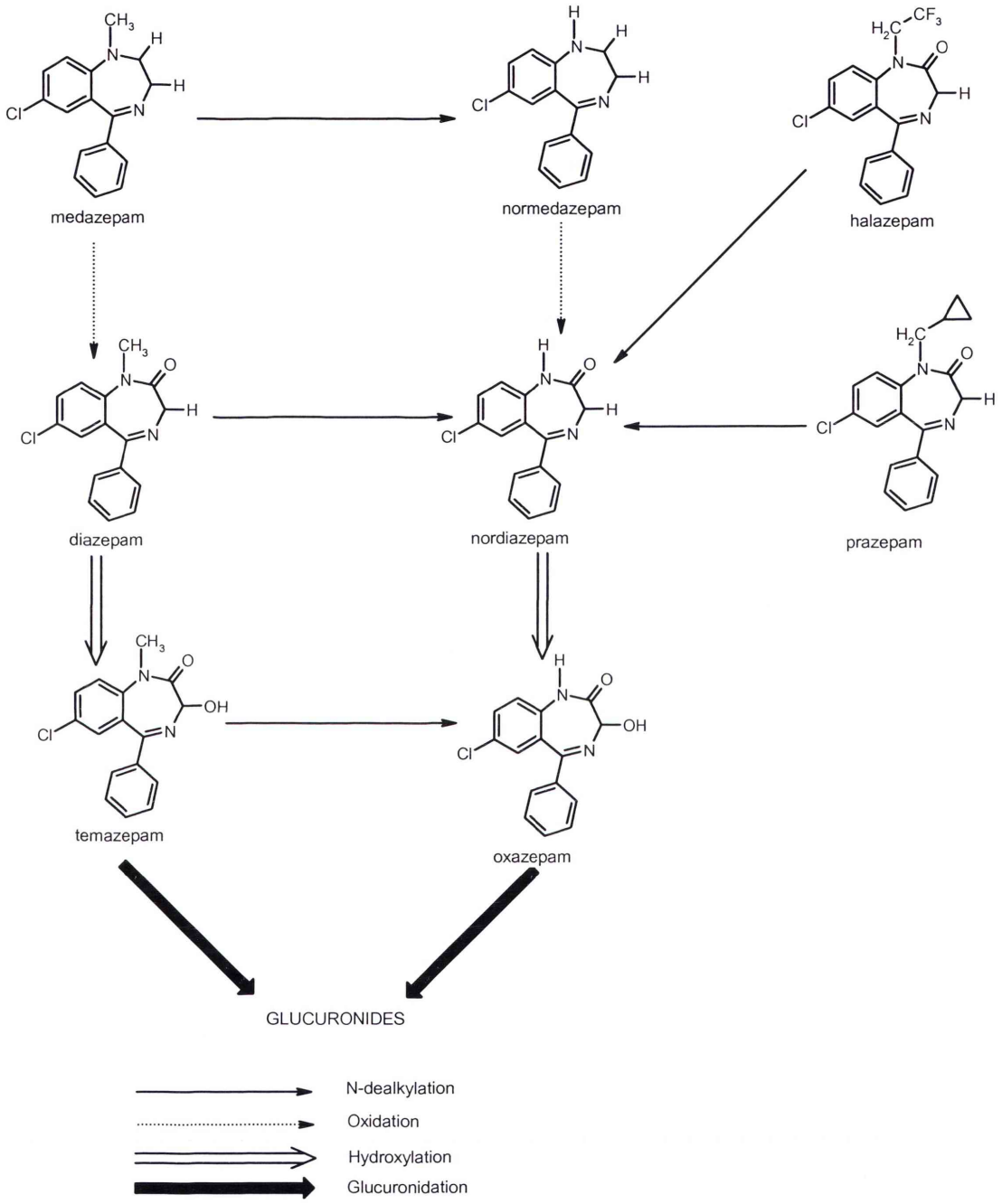


Figure 1-5 Metabolic pathways of some 1,4-benzodiazepines

Since benzodiazepines are classified as either short- or long-acting according to their rate of elimination, it is of no surprise that there is a large amount of variation in the pharmacokinetic characteristics of this class of drug. Onset of action for orally administered benzodiazepines depends on the rate of absorption from the GI tract [198, 201], with rapidly absorbed drugs reaching peak effect quicker than drugs that are absorbed more slowly. Absorption rates are determined by the physiochemical properties of the drug itself, as well as the pharmaceutical characteristics of the capsule or tablet preparation [202]. Circumstances of drug administration, such as the presence of absence of food in the stomach or the co-administration of other drugs, may also affect absorption rates. The distribution of benzodiazepines throughout the body, and hence their duration of action, depends on their lipophilicity [198, 201] and half-life [201]. Some pharmacokinetic characteristics of selected benzodiazepines are illustrated in Table 1-9 [196, 201, 203-205], however it is often difficult to assess the duration of action from these parameters alone. For example, elimination half-life does not necessarily predict clinical duration of action after a single dose, since it is distribution rather than rate of elimination that usually determines duration of action [202].

Table 1-9 Pharmacokinetic parameters of some benzodiazepines

Drug	t_{max}[a]	$t_{1/2}$[b]	V_D[c]	Protein binding[d]	Active metabolites
Alprazolam	1-2	6-16	0.72	80	Yes
Clonazepam	1-4	18-50	1.5-6	87	No
Diazepam	1-2	20-70	1.0	96-98	Yes
Lorazepam	1-5	8-25	1.1-1.5	85-94	No
Oxazepam	2-4	4-15	0.6-2	97	No
Midazolam	0.25-0.8	2	1.1	95	Yes
Chlordiazepoxide	1-4	8-28	0.3	89-94	Yes
Temazepam	1-3	5-8	1.3-1.5	76	Yes
Lormetazepam	2	8-12	n/a	>85	n/a
Flunitrazepam	1-2	9-25	3.7	80	Yes
Nitrazepam	0.5-2	16-40	2.4-4.8	85-88	No
Triazolam	1-2	3	1.1	90	Yes

[a] t_{max} in hours, [b] $t_{1/2}$ in hours, [c] V_D in L/kg, [d] protein binding in per cent

1.7.2.4 Analysis of Benzodiazepines in Biological Samples

A number of different methods have been reported for the LLE of benzodiazepines from biological samples, including whole blood, serum, plasma, cerebrospinal fluid, urine and vitreous humor. After pH adjustment to slightly alkaline conditions (pH 9-10) with the use of sodium carbonate, phosphate, borate or tetraborate buffers, common extraction solvents include diethyl ether [206-213], butyl chloride [214-218], ethyl acetate [219], dichloromethane [220-222], chloroform [223, 224], *n*-butyl acetate [225-227], toluene [228, 229], DMSO [230] and benzene [231]. Solvent systems comprising of a combination of solvents have also been reported, and include hexane/ethyl acetate [232], ammonium carbonate/chloroform [233], heptane/ethyl acetate/dichloromethane [234], diethyl ether/dichloromethane [235], diethyl ether/chloroform [236], heptane/dichloromethane [226], *n*-hexane/dichloromethane [237, 238], chloroform/IPA [52], diethyl ether/ethyl acetate [239], *n*-hexane/ethyl acetate [240], toluene/dichloromethane [93, 241-244], butyl chloride/dichloromethane [245], benzene/isoamyl alcohol [246], chloroform/ethyl

acetate [247], ether/IPA [248], *n*-butyl chloride/ethyl acetate [249], methyl isobutyl ether/chloroform [250], toluene/hexane/isoamyl alcohol [251], dichloromethane/acetone [252] and *n*-pentane/ethyl acetate [253].

Another commonly employed method for the extraction of benzodiazepines from biological matrices is SPE. Details of the various types of SPE cartridges, and authors who have utilised them, are presented in Table 1-10. Column-switching techniques [254-261], SPME [262-269], SFE [270], LPME [271], Soxhlet extraction [272], online dialysis [273, 274] and direct injection [275-278] have also been reported.

Table 1-10 SPE cartridges used for the extraction of benzodiazepines from biological samples

Cartridge Type	Brand	Reference
C_1	Pre-Sep	[279]
C_2	Bond Elut	[280-283]
C_8	Bond Elut	[283, 284]
C_{18}	Bond Elut	[52, 226, 283, 285-290]
	Clean Up	[291]
	Empore	[292]
	Extra-Sep	[293]
	Sep-Pak	[207, 231, 283, 294-296]
	UltraClean	[297]
	Superclean	[298]
Ion exchange	Oasis MCX	[128]
Mixed mode	Bond Elut Certify	[68, 137, 226, 299-309]
	Isolute Confirm HCX	[310, 311]
	Clean Screen DAU	[68, 312, 313]
	Bakerbond narc-2	[287]
Polymeric	Oasis HLB	[314]

A wide variety of techniques have been employed for the analysis of benzodiazepines in biological samples. The most common non-chromatographic techniques for urine and blood/plasma analysis are immunoassay screening methods,

such as enzyme immunoassay (EIA) [222, 300, 306, 311, 315-322], radio immunoassay (RIA) [317, 319, 321, 323], enzyme linked immunosorbent assay (ELISA) [150-152, 324-327], fluorescence polarisation immunoassay (FPIA) [213, 222, 306, 316-322, 328-332] and cloned enzyme donor immunoassay (CEDIA) [155, 315, 316, 333]. Radioreceptor assays (RRA) have also been reported in the literature [300, 333-337].

In terms of confirmatory techniques, GC, and/or GC-MS, MS, HPLC, LC-MS or LC-MS-MS, CE and CE-MS methods have been reported. In 1998, Drummer *et al.* published an excellent review of methods for the determination of benzodiazepines in biological matrices [191], which outlined the various extraction and analysis methods commonly employed in the measurement of this class of drugs. Due to its high level of sensitivity and specificity, especially when coupled with MS detection, HPLC is the most frequently employed confirmatory technique for the determination of benzodiazepines in biological matrices. Details of previously reported HPLC methods, including extraction, analysis, detection techniques and detection limits are given in Table 1-11.

The analysis of benzodiazepines in biological matrices by GC has been reported in a number of studies [93, 128, 218, 222, 225, 227-230, 238, 241-252, 262, 266, 268, 269, 271, 273, 282, 298, 306-309, 311, 313, 319, 338-343], however lengthy sample preparation involving derivatisation with BSTFA/TMCS (*N,O*-bis-(trimethylsilyl)trifluoroacetamine/trimethylchlorosilane) [137, 242, 244, 251, 307, 339, 342], HFBA (heptafluorobutyric anhydride) [93] or MTBSTFA (*N*-methyl-*N*-(tert-butyldimethylsilyl)-trifluoroacetamide) [309] is often required following extraction. For this reason, HPLC is generally a superior technique, since it enables the simultaneous analysis of a number of benzodiazepines, including their 7-amino metabolites, without derivatisation.

A handful of emerging techniques have been reported for the analysis of benzodiazepines in biological samples, including TOF-SIMS [344] and CE. CE is a

relatively new technique in the area of toxicology, and is rarely used routinely as it generally lacks the sensitivity necessary for toxicological analyses. However, coupled with MS detection, adequate detection limits can be achieved. In 1998, Smyth *et al.* published a review on the application of CE to the determination of benzodiazepines in formulations and biological samples [345] and, in the same year, a review was published by Tagliaro *et al.* on the determination of illicit and abused drugs (including benzodiazepines) by CE [346]. The details of the limited number of studies focusing on the CE analysis of benzodiazepines in biological samples are presented in Table 1-12.

Table 1-11 Summary of published HPLC methods for benzodiazepines in biological samples

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[240]	S	CLB, CLO, NIT	LLE *n*-hexane/ethyl acetate	Supelco RP C_8 150x4.6mm, 5µm	ACN/1.75mM HCl/50mM sodium acetate (36:10:54)	1.5	UV 220	0.5-1	>97	8
[232]	S	Numerous (14)	LLE hexane/ethyl acetate	Novapak C_{18} 150x3.9mm, 4µm	30mM phosphate buffer/ACN/MeOH (61:29.2:9.8), pH 6.4	1.8	UV 214	75-1200	>85	8
[209]	P	MID	LLE diethyl ether	Bondapak C_{18} 300x3.9mm	ACN/0.05M phosphate, pH 4.45 (30:70)	1.0	UV 210	<5	70-75	10
[294]	P, U	BROM, CHL, MED, MID, NIT, TEM, TRI, CLOT, PRA	SPE Sep-Pak C_{18}	Ultrabase C_{18} 30x4.6mm, 5µm	MeOH/water (various percentages)	1.0	UV 212	n/a	>85	n/a
[347]	U	7-NH_2-NIT, 7-NH_2-FLU, OXA, nor-DIA, nor-CHL, nor-FLU, DEM	Extrelut 20, Extrelut 3	Lichrospher RP8, 250x4mm, 5µm	0.01M phosphate, pH 3.5/0.02M MSA/ACN gradient	1.0	UV 234	100	86	n/a

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[291]	B, S	BROM, DIA, FLU, MID, nor-DIA, OXA, TRI, TET	SPE CleanUp C_{18}	Kontrosorb 10 RP18 250x4.6mm	ACN/phosphate, pH 2.3 (156:340, w/w)	1.3	DAD (various)	2	75-94	n/a
[257]	P	ALP, TRI	column-switching	Spheri-5 RP-8 100x4.6mm, 5µm	ACN/H_2O/phosphoric acid, pH 3.2	1.0	UV 230	1	86	<10
[274]	P	DIA, NIT, OXA	On-line dialysis	C_{18} RoSil 150x3.1mm, 5µm	MeOH/20mM acetate buffer, pH 5.0 (various compositions)	0.5	UV 254	20-25	37-50	n/a
[348]	B	BRO, CLO, nor-FLUD, DIA, FLU, KET, LOP, LOR, NIT, TRI	SPE Extrelut	Novapak C_{18} 150x3.9, 4µm	MeOH/50mM NH_4Ac (60:40)	0.4	MS-MS (SRM)	50	60-75	<20
[280]	U	OXA	SPE Bond-Elut C_2	Ultrabase C_{18} 35x4.6mm, 5µm	MeOH/H_2O (60:40)	0.5	F364/469	4	>95	<10
[283]	P, U	OXA, DIA, TEM, nor-DIA, BRO, MID, ADI	Sep-Pak C_{18}, Bond Elut C_2, C_8, C_{18}	Ultrabase C_{18} 35x4.6mm, 5µm	MeOH/H_2O (60:40)	Various	UV various	63-135	>90	n/a

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[235]	P, U	MID, FLUM, 1-OH-MID, 4-OH-MID	LLE DCM/diethyl ether	Novapak C_{18} 100x8mm, 4µm	ACN/0.04M phosphate/1% TEA, pH 7.2 (32:68)	1.5	UV 220	4-10	>80	40
[288]	P	DIA, nor-DIA	SPE Bond-Elut C_{18}	Bondapak C_{18} 150x3.9mm, 10µm	MeOH/30mM phosphate, pH 3.5 (55:45)	1.5	UV 229	5	>98	10
[281]	P	LOR, LOR-gluc	SPE Bond-Elut C_2	APEX octadecyl 250x4.6mm, 5µm	ACN/50mM phosphate, 20mM TEA pH 7.0 (33:67)	2.2	UV 230	0.5	73-75	10
[258]	P	CLB, DIA, nor-CLB, nor-DIA, FLU	Column-switching	Ultrasphere ODS 75x4.6mm, 3µm	ACN/65mM phosphate/1% TEA, pH 5.4 (33:67)	1.0	UV 230	20	>93	20
[210]	P	BROM	LLE diethyl ether	Novapak C_{18} 150x3.9mm, 5µm	ACN/H_2O/TEAP, pH 7.4 (700:300:4)	2.0	UV 240	50	>85	4
[208]	B	Numerous (15)	LLE diethyl ether + acid cleanup	Novapak phenyl 18 150x4.6mm, 5µm	15-28% ACN/40mM phosphate buffer, pH 3.8 gradient	0.8	UV 240	1-100	14-79	n/a

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[286]	S	OXA, TEM, TRI, ALP, DIA, nor-DIA, CHL	SPE Bond Elut C_{18}	Bakerbond C_8 250x4.6mm, 5µm	ACN/MeOH/H_2O/ perchloric acid (5:1:4.5:0.0015)	2.0	UV 230	10-110	67-114	11
[211]	P	FLU	LLE diethyl ether	Novapak C_{18} 150x3.9mm, 4µm	ACN/H_2O/TEA pH 7.5 (300:700:4)	2.0	UV 230	5	n/a	<8
[300]	U	Numerous (not given)	SPE Bond Elut Certify	Lichrospher RP18 250x4mm, 5µm	ACN/20mM phosphate pH 5.4 (40:60)	1.0	UV 252	6	n/a	n/a
[285]	P, U	TEM, OXA	SPE Bond Elut C_{18}	Lichrospher RP8 125x4mm, 5µm	ACN/0.01M phosphate buffer, pH 5.6	1.6	UV 254	5	60-99	n/a
[259]	P	DIA	Column-switching	Inertsil ODS-2 150x4.6mm, 5µm	ACN/H_2O (50:50)	1.0	UV 280	n/a	>90	15
[206]	S (rat)	ALP, OH-ALP	LLE diethyl ether	Ultrasphere C_{18} 150x2mm, 5µm	MeOH/ACN/43mM sodium acetate, pH 2.4 (45:8:47)	0.3	UV 230	5	79-85	10
[260]	P	MID, OH-MID, FLUR	Column-switching	Lichrospher RP-select B 250x4mm, 5µm	NaOH/phosphate buffer/ACN	1.0	UV 230	2	80-89	<25

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[279]	P	MID, 1-OH-MID, 4-OH-MID	SPE Presep C_1	Spherisorb C_8 100x4.6mm, 5µm	ACN/MeOH/0.02M phosphate, 0.2% TBABr, pH 4.1 (5:35:60)	1.5	UV 254	15	80-83	n/a
[293]	P	CLO	SPE Extra-Sep C_{18}	Velosep C_{18} 100x3.2mm, 3µm	ACN/0.5% acetic acid (32:68)	0.5	UV 306	5	90	<15
[221]	U	DIA, nor-DIA, OXA, TEM	LLE DCM	Lichrospher RP8 250x4mm, 5µm	H_2O/MeOH/TEA, pH 5.5 (70:30:0.1)	0.7	UV 240	2	>82	<20
[349]	B, L	ALP	SPE Bio-Rad	Bio-Rad C_8 100x2.1mm, 3µm	ACN/0.01M phosphate buffer, 0.01% *N,N*-dimethyloctylamine (30:70), pH 6.4	0.3	UV 242	18	84	<15
[212]	P	MID, OH-MID	LLE diethyl ether	Spherisorb CN 150x4.6mm, 5µm	MeOH/2-propanol with 0.015% perchloric acid (75:25)	1.5	UV 215	2	86-87	8
[217]	B	CLO, FLU, TEM, 7-NH_2-CLO, 7-NH_2-FLU, 7-NH_2-NIT	LLE butyl chloride	Novapak phenyl 150x3.9mm, 4µm	ACN/40mM phosphate, pH 3.75 (28:72)	0.8	UV 240	1-8	53-100	20

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[290]	P	ALP, CLO, NIT	SPE Bond Elut C_{18}	Novapak C_{18} 4µm	ACN/MeOH/10mM phosphate, pH 3.7 (30:2:100)	1.5	UV 240	1.5	94-100	12
[289]	P, S	CLB, nor-CLB	SPE Bond Elut C_{18}	Novapak C_{18} 150x3.9mm, 4µm	ACN/MeOH/10mM potassium phosphate, pH 3.7 (30:2:100)	1.5	UV 240; DAD 210-365	4.5	>97	<14
[236]	P	FLU, 3-OH-FLU, 7-NH_2-FLU, nor-FLU, 7-NH_2.nor-FLU, PRA	LLE diethyl ether/ chloroform	Rsil CN 300x3.9mm, 10µm	MeOH/30mM phosphate buffer, pH 4.0 (17:83)	1.6	UV 242	2.5-5	30-83	20
[213]	S, P	MID, OH-MID	LLE diethyl ether	Nucleosil 250x4.0mm, 5µm	MeOH/0.02M sodium acetate, pH 7.4 (55:45)	1.2	UV 215	1.5-3	58-78	20
[287]	S, U	DIA, nor-DIA, NIT, FLU, MED	SPE C_{18} Bond Elut, Bakerbond narc-2	Lichrospher 60-RP 100x2.0mm, 5µm	MeOH/H_2O/ACN (1:1:1), pH 6	0.1	ESI-MS-MS (SRM)	2	90-110	n/a

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[299]	B	BRO, CLO, nor-FLUD, DIA, FLU, KET, LOP, LOR, NIT, TRI	SPE Bond Elut Certify	Novapak C_{18} 150x3.9mm, 4µm	MeOH/50mM NH_4Ac (60:40)	0.4	TSP-MS-MS (SRM)	0.05-0.5	~50	<20
[226]	U	CHL, DIA, LOR, NIT, nor-DIA, OXA, TEM	LLE butyl acetate, DCM/heptane ; SPE Bond Elut C_{18}, Bond Elut Certify	HiChrom C_{18} 250x4mm, 5µm	H_2O/MeOH/ACN/ acetic acid (51:28:16:6)	1.0	UV 241	100-700	70-105	n/a
[350]	U	NIT, FLU, FLUD, DIA, CLOT, MED	SPME	Superiorex ODS 250x1.5mm	ACN/H_2O (35:65)	0.1	MS, UV 220	1-6	n/a	130
[234]	S	ALP, BROM, CLO, CHL, DIA, EST, FLU, FLUR, LOR, NIT, TRI, MID	LLE heptane/ EtOAc/DCM	ODS Hypersil 200x4.6mm, 5µm	Phosphate buffer/ACN (70:30)	1.0	UV 210	200		50

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[233]	U	OXA, CHL, CAR, MED, EST, nor-FLUD, TRI, DEL	LLE ammonium carbonate/ chloroform	SB-C_{18} Mac Mod Rapid Resolution 15x2.1mm, 3µm	3mM NH_4Ac, pH 3.3/ACN (67:33)	1.9	MS	>50	58-88	25
[207]	S	TRI, EST	LLE diethyl ether; SPE Sep-pak C_{18}	Capcell Pak C_{18} 150x4.6mm	0.1% acetic acid/40% ACN	1.0	UV 254	20-80	50-103	5
[223]	U	BROM, CAR, EST, nor-FLUD, ALP, TRI	LLE chloroform	Zorbax SB-C_{18} 15x2.1mm, 3µm	Formic acid/ACN/H_2O (0.01:33:67)	1.0	TSP-MS-MS (SRM)	n/a	n/a	<1
[215]	B	CLO, MID, FLU, OXA	LLE butyl chloride	Lichrospher C_8 125x3mm, 5µm	20mM phosphate buffer, pH 2.1/ACN (65:35)	0.3	UV 220	2-3.5	60-90	n/a
[255]	P, S	CLO, DIA, FLU, MID, OXA	Column-switching	Lichrospher C_8 125x3mm, 5µm	30-35% 20mM phosphate buffer, pH 2.1/ACN gradient	0.3-0.5	UV 254	15-24	82-109	<25
[216]	B, S	CLO, DIA, FLU, MID, OXA	LLE butyl chloride	Lichrospher C_8 125x3mm, 5µm	20mM phosphate buffer, pH 2.1/ACN (65:35)	0.3	UV 220	2-3.5	60-109	<25

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[312]	U, B	FLU, nor-FLU, 7-NH_2-FLU	SPE CleanScreen DAU	Altima C_{18} 150x2.1mm, 5µm	MeOH/H_2O with 0.03% NH_4OH (60:40)	0.3	MS	0.1-1	24-96	<10
[284]	S	BROM, CHL, OXA, NIT, LOR, ETI, CLO, FLU, DIA, CLOT	SPE Bond Elut C_8	Develosil ODS 150x0.3mm, 5µm	0.1M NH_4Ac buffer/ACN gradient	0.05	UV 240	2.5-5	78-98	35
[263]	U, S	DIA, nor-DIA, TEM, OXA, 7-NH_2-FLU, nor-FLUD, CLO	SPME	Supelco C_{18} 50x2.1mm, 3µm	MeOH/50mM NH_4Ac (60:40)	0.3	ESI-MS	0.02-2	40-107	12
[351]	U	OXA, DIA, TEM	Direct injection	Cyclobond 100x4.6mm	Phosphate buffer, pH 7/MeOH (75:25)	0.8	UV 240	50	97-99.8	n/a
[254]	B	CLO, DIA, FLU, MID, OXA	Column-switching	Licrospher C_8 125x3mm, 5µm	20mM phosphate buffer, pH 2.1/ACN linear gradient	0.3-0.5	UV 254	10-15	80-90	25

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[220]	S, U	CLB, nor-CLB	LLE DCM	Supelcosil LC-8-DB 250x4.6mm, 5µm	ACN/H_2O/0.5M phosphate buffer	1.5	UV 228	1-3000	100-103	12
[292]	P (dog)	DIA, OXA, TEM, nor-DIA	SPE Empore C_{18}	Discovery C_{18} 50x2.1mm, 5µm	30-100% ACN/NH_4Ac, pH 6.8, gradient	0.25	ESI-MS-MS (SRM)	0.3	44-87	5
[352]	S	Various (28)	SPE Isolute Confirm HCX	Symmetry C_{18} 150x2.0mm, 5µm	ACN/H_2O/MeOH, gradient	0.1-0.2	ESI-MS	0.1-8	n/a	n/a
[256]	U, P	CLO, OXA, TEM, nor-DIA, DIA	Column-switching	Supelcosil C_{18} 50x4.6mm, 5µm	H_2O/MeOH (54:46)	1.0	UV 230	>45	>95	20
[270]	VH, B	TEM, DIA, nor-DIA	SFE	Hyposil ODS 250x4.6mm, 5µm	Phosphate buffer/MeOH (30:70)	1.0	UV 254	n/a	n/a	n/a
[52]	U	ALP, OH-ALP, OXA, FLU, 7-NH_2-FLU, nor-DIA, LOR, DIA	LLE chloroform/ IPA, Bond Elut TCA C_{18}	Hypersil C_{18} 100x4.6mm, 3µm	40mM phosphate (pH 4.5)/0.4% octylamine/50% ACN	1.0	UV 240	8-20	51-83	<20

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[224]	B, P, U	BRO, OXA, LOR, NIT, CLO, nor-DIA, FLU, MID, DIA	LLE chloroform	Lichrospher RP8 150x4.6mm, 5µm	30mM acetate buffer, pH 4.6/ACN (55:45)	1.0	UV 250; ECD	2-14	82-92	<15
[219]	P	FLUM	LLE ethyl acetate	Spherisorb ODS 250x4.6mm, 5µm	10mM NH_4Ac/ACN (75:25), pH 5.4	1.0	UV 250	<2.5	92	20
[267]	U	DEL	SPME	Supelcosil LC-18-DB 250x4.6mm, 5µm	ACN/H_2O (65:35)	1.0	UV 230	5	n/a	10
[276]	S	BRO, DIA, FLU, HAL, MED, NIT, OXA, TET	Direct injection	Eclipse XDBC-8 150x4.6mm, 5µm; Kromasil C_{18} 120x4.6mm, 5µm	60mM SDS/5% butanol/10mM phosphate buffer, pH 7.0	1.0	UV 230	4-18	>95	20

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[301]	H	DIA, nor-DIA, OXA, ALP, OH-ALP, NIT, FLU, CLO, 7-NH_2-CLO, 7-NH_2-FLU, 7-NH_2-NIT	Proteinase K digestion, SPE Bond Elut Certify	Zorbax phenyl 50x2.1mm, 3µm	ACN/MeOH/20mM formate buffer (various gradients)	0.25	MS-MS	25-125	34-112	<10
[239]	P	FLU, 7-NH_2-FLU, nor-FLU	LLE diethyl ether/ethyl acetate	Superspher 60RP Select B 125x2mm	5mM ammonium formate, pH 3/ACN gradient	0.4-0.8	APCI-MS	<5	n/a	<10
[261]	U	Numerous (22)	Column-switching	Capcell Pak C_{18} 150x1.5mm, 5µm	10mM NH_4Ac/ACN, gradient	0.15	APCI-MS (SIM)	2-10	>90	30
[265]	S	OXA, TEM, nor-DIA, DIA, CLO	SPME	Lichrospher 100 RP-18e 150x4.0mm, 5µm	H_2O/MeOH (37:63) gradient	0.4	UV 230	22-29	>90	16
[310]	P, U	FLU, 7-NH_2-FLU, 3-OH-FLU, nor-FLU	Automated SPE Isolute HCX	Noca-Pak C_{18} 150x2.1mm, 4µm	H_2O/ACN/MeOH (49.5:5.5:45)	0.15	ESI-MS	0.02-0.2	81	10
[275]	P, U	ETI, BRO, LOR	Direct injection	Shodex Mspak 50x4.6mm, 6µm	20mM NH_4Ac/formic acid/ACN gradient	n/a	ESI-MS	2-5	>78	35

Ref.[a]	Specimen	Analytes	Extraction	Column	Mobile phase	Flow rate[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[278]	P	NIT, CLB, OXA, LOR	Direct injection	Hisep 250x4.6mm, 5µm	ACN/18mM NH_4Ac (15:85), pH 2.5	2.0	UV 254	160	>97	<15
[214]	B	Numerous (18)	LLE - butyl chloride	Zorbax XDB C_8 150x4.6mm, 5µm	MeOH/formate buffer, pH 9 gradient	0.7	MS-MS	n/a	>80	16
[237]	P, H (rat)	ALP, EST, MID, OH-ALP, OH-MID	DCM-MeOH-NH_4OH (H); LLE *n*-hexane-DCM	Mightysil RP18 100x2.0mm, 3µm	ACN/H_2O/1% acetic acid (various gradients)	0.15	ESI-MS (SIM)	n/a	n/a	n/a
[353]	B	Numerous (33)	SPE ChemElut	Xterra MS C_{18} 150x2.1mm, 3.5µm	MeOH/formic acid, pH 3, gradient	0.2	MS-MS	0.1-12.6	52-114	45
[314]	P	ALP, 4-OH-ALP, 1-OH-ALP	SPE Oasis HLB	Luna C_{18} 100x2.0mm, 3µm	Formic acid/ACN, gradient	0.3	MS	0.05	>82	10

n/a – not available or not described, [a] references in chronological order, [b] flow rate in mL/min, [c] wavelengths in nm, [d] detection limits in ng/mL, [e] recoveries in per cent, [f] run times in min

Abbreviations: Specimens: bile (Bi), blood (B), cerebrospinal fluid (CSF), hair (H), liver (L), meconium (Mec), plasma (P), serum (S), urine (U), vitreous humor (VH); **Analytes:** adinazolam (ADI), alprazolam (ALP), 7-aminoclonazepam (7-NH2-CLO), 7-aminoflunitrazepam (7-NH2-FLU), 7-aminonitrazepam (7-NH2-NIT), 7-aminonorflunitrazepam (7-NH2-nor-FLU), bromazepam (BROM), brotizolam (BRO), carbamazepine (CAR), chlordiazepoxide (CHL), clobazam (CLB), clonazepam (CLO), clotiazepam (CLOT), delorazepam (DEL), demoxepam (DEM), diazepam (DIA), estazolam (EST), etizolam (ETI), fludiazepam (FLUD), flumazenil (FLUM), flunitrazepam (FLU), flurazepam (FLUR), halazepam (HAL), 1-hydroxyalprazolam (1-OH-ALP), 4-hydroxyalprazolam (4-OH-ALP), 3-hydroxyflunitrazepam (3-OH-

FLU), 1-hydroxymidazolam (1-OH-MID), 4-hydroxymidazolam (4-OH-MID), ketazolam (KET), loprazolam (LOP), lorazepam (LOR), lorazepam-glucuronide (LOR-gluc), medazepam (MED), midazolam (MID), nitrazepam (NIT), norchlordiazepoxide (nor-CHL), norclobazam (nor-CLB), nordiazepam (nor-DIA), norfludiazepam (nor-FLUD), norflunitrazepam (nor-FLU), hydroxyalprazolam (OH-ALP), oxazepam (OXA), prazepam (PRA), temazepam (TEM), tetrazepam (TET), triazolam (TRI); **Method details:** acetonitrile (ACN), ammonium acetate (NH_4Ac), atmospheric pressure chemical ionisation (APCI), dichloromethane (DCM), diode array detection (DAD), electrochemical detection (ECD), electrospray ionisation (ESI), ethyl acetate (EtOAc), isopropanol (IPA), fluorescence detection (F), liquid-liquid extraction (LLE), mass spectrometry (MS), methanesulfonic acid (MSA), methanol (MeOH), selected-ion monitoring (SIM), selected-reaction monitoring (SRM), sodium dodecyl sulfate (SDS), solid-phase extraction (SPE), solid-phase microextraction (SPME), tetrahydrofuran (THF), thermospray (TSP), triethylamine (TEA), triethylammonium phosphate (TEAP), trifluoroacetic acid (TFA), ultraviolet detection (UV).

Table 1-12 Summary of published CE methods for benzodiazepines in biological samples

Ref.[a]	Specimen	Analytes	CE mode	Extraction	Capillary	Running buffer	Voltage[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[302, 303]	U	FLU, OXA, DIA	MEKC	SPE Bond Elut Certify	Fused-silica 75μm L_{tot} 90cm, L_{eff} 70cm	Borate-phosphate buffer, pH 9.1/ 75mM SDS	20	UV 195-320	100	80-90	<25
[296]	S	NIT, nor-CHL, CHL, OXA, nor-DIA, LOR, DIA	MEKC	SPE Sep-Pak C_{18}	Fused-silica 50μm L_{tot} 72cm, L_{eff} 50cm	30mM SDS/30mM borate, pH 9.3/10% ACN	30	UV 200	n/a	n/a	<16
[295]	U	NIT, 7-NH_2-NIT	MEKC	SPE Sep-Pak C_{18}	Fused-silica 50μm L_{tot} 72cm	60mM SDS/6mM phosphate-borate, pH 8.5-MeOH (85:15)	20	UV 220	0.1-0.2	78-100	<25
[304]	U	FLU, DIA, MID, CLO, BROM, TEM, OXA, LOR	MEKC	SPE Bond Elut Certify	Fused-silica 75μm L_{eff} 68cm	75mM SDS/borate-phosphate buffer, pH 9.2/5% IPA	30	UV 195-320	n/a	70-90	36

Ref.[a]	Specimen	Analytes	CE mode	Extraction	Capillary	Running buffer	Voltage[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[305]	U	FLU, TEM, nor-DIA, LOR, OXA	MEKC	SPE Bond Elut Certify	Fused-silica 75µm L_{tot} 110cm,L_{eff} 70cm	71.25mM SDS/borate-phosphate-IPA (95:5)	30	UV 200	n/a	n/a	35
[231]	S	NIT, DIA, EST, BRO, TRI, FLUR	MEKC	SPE Sep-Pak C_{18}; LLE benzene	Fused-silica 50µm L_{tot} 72cm, L_{eff} 50cm	5mM phosphate-borate, pH 8.5/50mM SDS/15% MeOH	25	UV 200	25-200	45-117	<25
[253]	S	NIT, CLO, CLB, DIA, nor-DIA, nor-CLB	MEKC	LLE CAPSO buffer, *n*-pentane/ EtOAc	Fused-silica 50µm L_{tot} 47cm, L_{eff} 40cm	50mM borate buffer, pH 9.5/ 18mM SDS/14% ACN	20	UV 214	10-20	90-96	<10
[272]	H	Numerous (15)	CZE	Soxhlet	Fused-silica 75µm L_{tot} 70cm, L_{eff} 58cm	0.02M citric acid, pH 2.50/15% MeOH	20	UV 230, ESI-MS	UV: 23-12000; MS: 4-50	n/a	<25

Ref.[a]	Specimen	Analytes	CE mode	Extraction	Capillary	Running buffer	Voltage[b]	Detection method[c]	LOD[d]	Recovery[e]	Run time[f]
[277]	S	LORM, LOR	MEKC	Direct injection	Fused-silica 75µm L_{tot} 57cm, L_{eff} 50cm	15mM borate-phosphate buffer, pH 8/30mM SDS/15% MeOH	25	UV DAD	500	n/a	20
[297]	U	DIA, BROM, FLU, OXA, TEM, LOR	CZE	SPE UltraClean C_{18}	CEofix coated fused-silica 75µm L_{eff} 50cm	100mM formic acid, pH 2.4/CEofix accelerator/TFA	18 (UV), 30 (MS)	UV 200, 240, MS, MS-MS	100	29-83	12

n/a – not available or not described, [a] references in chronological order, [b] voltages in kV, [c] wavelengths in nm, [d] limits of detection in ng/mL, [e] recoveries in per cent, [f] run times in min

Abbreviations: Specimens: hair (H), serum (S), urine (U); **Analytes:** 7-aminonitrazepam (7-NH2-NIT), brotizolam (BRO), bromazepam (BROM), chlordiazepoxide (CHL), clobazam (CLB), clonazepam (CLO), diazepam (DIA), estazolam (EST), flunitrazepam (FLU), flurazepam (FLUR), lorazepam (LOR), lormetazepam (LORM), midazolam (MID), nitrazepam (NIT), norchlordiazepoxide (nor-CHL), norclobazam (nor-CLB), nordiazepam (nor-DIA), oxazepam (OXA), temazepam (TEM), triazolam (TRI); **Method details:** capillary zone electrophoresis (CZE), effective length (L_{eff}), electrospray ionisation (ESI), isopropanol (IPA), liquid-liquid extraction (LLE), mass spectrometry (MS), methanol (MeOH), micellar electrokinetic capillary chromatography (MEKC), sodium dodecyl sulfate (SDS), solid-phase extraction (SPE), total length (L_{tot}), ultraviolet detection (UV).

1.7.2.5 Toxicological Data

Although many of the benzodiazepines are structurally similar, they possess wide and varied pharmacokinetic behaviour. Therapeutic dosing regimes therefore necessitate that doses be adjusted to consider the pharmacokinetic aspects of the particular benzodiazepine in question. A consequence of this is that therapeutic and toxic ranges vary from one benzodiazepine to the next. The therapeutic and toxic concentration ranges presented in Table 1-13 were first published in German by Uges *et al.* in 1990 [354], and then again in an English review by Schutz in 1997 [205]. In addition, Baselt and Cravey [3] noted the concentrations of several benzodiazepines found in fatal intoxications (Table 1-14). Fatalities involving benzodiazepines may either be a result of benzodiazepine intoxication alone, in which case the serum benzodiazepine concentration is likely to be in the toxic range; or due to the concomitant use of other CNS depressants, in which case the serum concentration of benzodiazepines could be within the therapeutic range.

Table 1-13 Therapeutic and toxic ranges of benzodiazepines in serum

Substance	Therapeutic Range (μg/mL)	Toxic Range (μg/mL)
Bromazepam	0.08 – 0.17	>0.25 – 0.5
Chlordiazepoxide	0.7 – 2.0	>3.5 – 10.0
Clonazepam	0.03 – 0.06	>0.10
Diazepam	0.124 – 1.5	>1.5 – 3.0
Flunitrazepam	0.005 – 0.015	>0.050
Lorazepam	0.02 – 0.25	>0.03 – 0.05
Lormetazepam	0.002 – 0.010	
Medazepam	0.01 – 0.15	>0.06
Midazolam	0.08 – 0.25	
Nitrazepam	0.03 – 0.12	>0.02 – 0.05
Nordiazepam	0.2 – 0.8	>2.0
Oxazepam	1.0 – 2.0	>3.0 – 5.0
Prazepam	0.05 – 0.20	>1.0
Temazepam	0.3 – 0.8	>1.0
Triazolam	0.002 – 0.020	

Table 1-14 Fatal concentration ranges of selected benzodiazepines in blood

Substance	Blood Concentration (ng/mL)
Alprazolam	0.12 – 0.39
Chlordiazepoxide	20.0 – 26.0
Diazepam	5.0 – 19.0
Nitrazepam	1.2 – 9.0
Temazepam	0.9 – 14.0

1.8 Project Objectives

The drugs of interest in this research were the metabolites of heroin and benzodiazepines. Research objectives were divided into these two components accordingly.

The analysis of benzodiazepines has been studied extensively, and has primarily focused on HPLC separations. However, novel approaches employing emerging analysis and optimisation techniques, such as capillary zone electrophoresis (CZE) and artificial neural networks (ANNs), are rarely explored. Further research into these areas could enable significant advantages in method development, optimisation and sample throughput in forensic toxicology to be realised. Therefore, the primary objectives of this component of research were to:

i) synthesise the 7-aminobenzodiazepine metabolites, 7-aminoclonazepam and 7-aminonitrazepam, from the parent drugs as a prelude to further studies involving the analysis of these metabolites;
ii) develop a fast CZE separation for the analysis of nine benzodiazepines, through the use of a dynamic doubly-coated capillary;
iii) evaluate the suitability of the CZE method for the determination of these analytes in forensic samples;
iv) explore the usefulness of ANNs in optimising an HPLC gradient elution separation of nine benzodiazepines;
v) assess the suitability of the optimised HPLC method for the analysis of these analytes in post-mortem blood samples.

The ambiguous reporting of either free or total morphine concentrations limits the interpretation of heroin-related fatalities. Simultaneous determination of morphine, M3G and M6G could enable the relationship between heroin concentration and effects to be better understood, and allow the possible contribution of M3G and M6G

to be explored in more detail. Therefore, the primary objectives of the morphine component of this research were to:

i) synthesise the major morphine metabolites, M3G and M6G, from morphine as a prelude to further studies involving the analysis of these metabolites;
ii) develop a rapid and simple HPLC method for the analysis of morphine and related analytes that may be applied to whole post-mortem blood;
iii) evaluate the suitability of the optimised HPLC method for the analysis of morphine, M3G and M6G in post-mortem blood samples;
iv) acquire some preliminary data on morphine glucuronide concentrations and ratios in heroin-related fatalities.

Chapter Two

Synthesis of Drug Metabolites

2 Synthesis of Standard Drug Metabolites

2.1 Summary

Synthesis of metabolites from parent drugs can be a relatively inexpensive means of overcoming the cost of purchasing metabolites commercially. In this work, standards of the two main morphine metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), and the benzodiazepine metabolites 7-aminonitrazepam and 7-aminoclonazepam, were synthesised based on previously reported methods as a prelude to further analyses of these metabolites in forensic samples. Yields of 48, 25, 74 and 70% were obtained for M3G, M6G, 7-aminonitrazepam and 7-aminoclonazepam respectively.

Keywords: synthesis; drug standards; morphine-6-glucuronide; morphine-3-glucuronide; 7-aminonitrazepam; 7-aminoclonazepam

2.2 Introduction

Drug and metabolite standards are essential in the development and validation of analytical methods for toxicological analyses. Whilst parent drugs are usually readily available from a number of commercial sources, it is often difficult to obtain samples of the corresponding metabolites. Many standards relevant to forensic toxicology are classified as S8 or S9 drugs according to the Standard for the Uniform Scheduling of Drugs as Poisons. In some cases, it may be difficult to source an Australian supplier of some of the more uncommon metabolites, such as M3G and M6G. The bureaucratic impediments to importing such drugs, including obtaining the relevant licences, can add months or years onto any project. Cost is another important issue to consider in forensic toxicology. At $A 101.20 and $A 182.90 for only 1 mL of M3G and M6G respectively (1 mg/mL) [355], the importance of performing the analysis of these metabolites must be weighed against the cost of purchasing the standards.

The synthesis of metabolites from parent drugs is a relatively inexpensive approach to overcome the inavailability and cost of purchasing metabolites commercially. Since the biological processes by which metabolites are formed *in vivo* are either phase I or phase II reactions, many can be synthesised by simple reduction, oxidation or dealkylation reactions of the parent drug. Numerous procedures for the most common metabolites have been published previously, however, if a particular procedure cannot be found, similar methods can be adapted to synthesise the metabolite of interest.

2.2.1 Methods for the Synthesis of M3G, M6G, 7-Aminonitrazepam and 7-Aminoclonazepam

Published methods for the synthesis of M3G have focused on a relatively simple condensation reaction between morphine and the bromo sugar, acetobromo-α-D-glucuronic acid methyl ester (Figure 2-1). The reaction occurs selectively at the 3-position since the phenolic group at the 3-position is more reactive than the alcoholic group at the 6-position. This method, first reported by Yoshimura *et al.* [356], involves the hydrolysis of morphine with sodium hydroxide-acetone. A yield of only 27% was obtained and a large amount of the bromo sugar was required to push the reaction. Subsequently, an improved method was developed by Berrang *et al.* [357], in which a lithium hydroxide-methanol mixture afforded a yield of 53%. Based on these findings in the literature, M3G was prepared according to the schematic depicted in Figure 2-2, utilising lithium hydroxide-methanol and the bromo sugar. The final product, M3G, was crystallised from the reaction mixture with acetic acid.

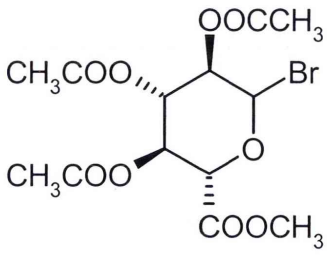


Figure 2-1 Structure of acetobromo-α-D-glucuronic acid methyl ester (II)

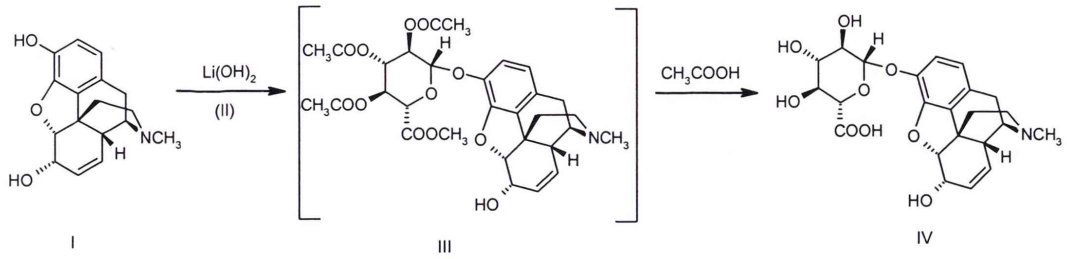


Figure 2-2 Synthesis of morphine-3-glucuronide (IV)

Previous methods involving the synthesis of M6G have utilised a Koenigs-Knorr coupling of a sugar moiety (commonly acetobromo-α-D-glucuronic acid methyl ester (Figure 2-1)) to morphine that has been protected at the 3-position (commonly 3-acetylmorphine) [356, 358-361]. This coupling procedure is based on the transfer of a glycoside group to a nucleophile by the use of glycosyl halides in the presence of heavy-metal salts (generally silver salts) [362]. The choice of catalyst has been shown to impact on the anomeric configuration of the glycosidic linkage. For example, the use of $ZnBr_2$ tends to produce mainly morphine-6α-D-glucuronide [358, 359], whilst Ag_2CO_3 affords the pharmacologically significant morphine-6β-D-glucuronide [356, 360, 361]. There are, however, a number of disadvantages associated with this method, including the expense and toxicity of heavy-metal salts and the sensitivity of glycosyl halides to hydrolysis [362].

Other synthetic routes to M6G have involved the imidate coupling method [363]. This acid catalysed reaction of *O*-nucleophiles with *O*-glycosyl trichloroacetimidates, usually with boron trifluoride in dichloromethane as a solvent, affords the formation of the β-product [362]. The synthesis of M6G from morphine-3,6-diglucuronide has also been reported [364]. This method involved the selective cleavage of the phenolic 3-glucuronide group using β-glucuronidase as a catalyst. Selective glucuronidation at the 6-position has also been achieved using human liver microsomes [365].

Based on the available literature, the Koenigs-Knorr coupling method using Ag_2CO_3 was selected for the synthesis of the 6-glucuronide. A schematic outlining the various steps in the synthesis of M6G is presented in Figure 2-3. To protect the 3-position and allow reaction at the 6-position, 3-monoacetylmorphine was first prepared by the reaction of morphine with acetic anhydride in the presence of sodium bicarbonate to neutralise the acetic acid formed. The coupling reaction was then performed with Ag_2CO_3 as a catalyst, and the resulting product was deprotected stepwise firstly with sodium methoxide, then with barium hydroxide and oxalic acid to produce the final product, M6G.

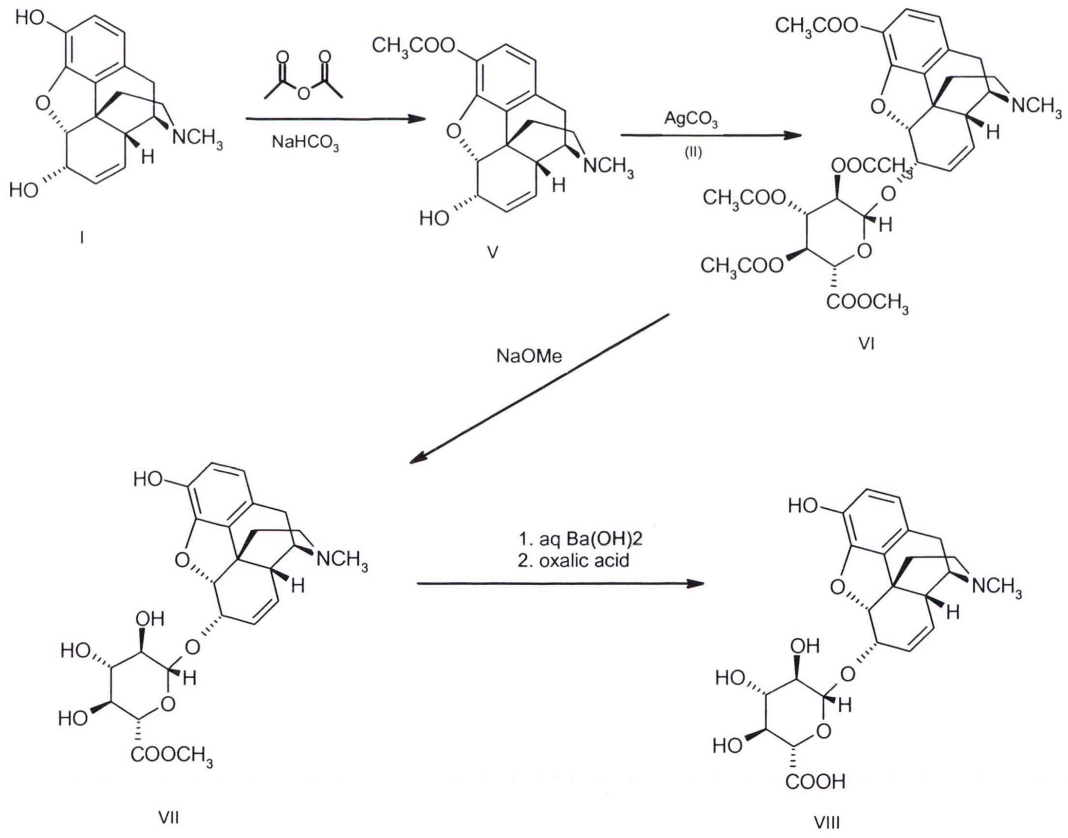


Figure 2-3 Synthesis of morphine-6-glucuronide (VIII)

Nitrobenzodiazepines, which are characterised by a NO_2 substituent at position-7, are metabolised *in vivo* to 7-amino metabolites via phase I reactions, involving the

reduction of the nitro group to an amino group. The synthesis of these 7-aminobenzodiazepines can therefore be performed with ease using a reducing agent. Harsh conditions, such as the use of acid and high temperatures, are not required. This method has been successfully employed by Zecca *et al.* [366], who used tin (II) chloride as the reducing agent to produce 7-aminoflunitrazepam in a yield of 78%. Feely *et al.* [367] also successfully employed this method to prepare the 7-amino metabolites of both nitrazepam and flunitrazepam. In this study, 7-aminonitrazepam and 7-aminoclonazepam were prepared based on these previously reported methods, as illustrated in Figure 2-4.

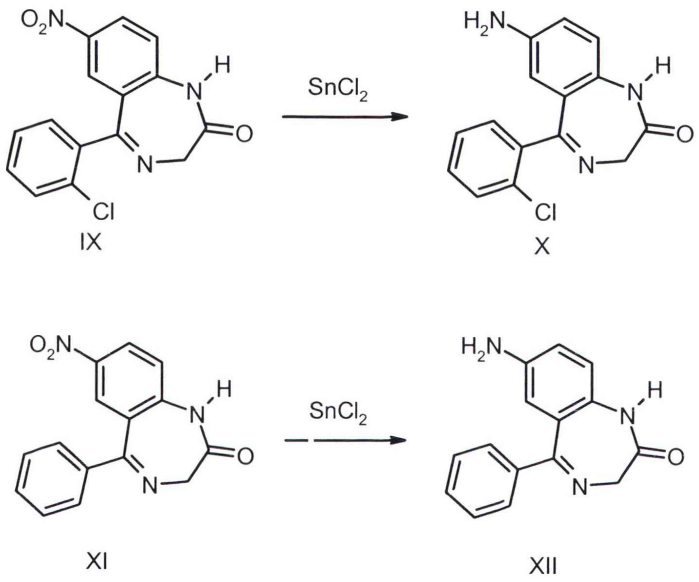


Figure 2-4 Synthesis of 7-aminoclonazepam (X) and 7-aminonitrazepam (XII)

2.2.2 Objectives

The objective of this study was to synthesise the two main morphine metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), as well as the 7-amino benzodiazepine metabolites 7-aminoclonazepam and 7-aminonitrazepam from the parent drugs. This was performed as a prelude to further studies involving the analysis of these metabolites in forensic samples.

2.3 Experimental

2.3.1 Chemicals

All reagents were of reagent grade, unless stated otherwise. Morphine hydrochloride (MOR) was obtained from Glaxo Australia Pty Ltd (Port Fairy, Victoria, Australia). Acetobromo-α-D-glucuronic acid methyl ester ($C_{13}H_{17}O_9Br$), lithium hydroxide monohydrate ($LiOH{\cdot}H_2O$), 6-monoacetylmorphine (6MAM), clonazepam (CLO) and nitrazepam (NIT) were obtained from Sigma-Aldrich (Sydney, New South Wales, Australia). Chloroform, dichloromethane, acetone, ethyl acetate (EtOAc), petroleum spirit, ethanol, sodium-dried benzene and sodium were from Crown Scientific (Sydney, New South Wales, Australia). Acetic acid (CH_3COOH) was from BDH HiPerSolv (Sydney, New South Wales, Australia). Acetic anhydride and hydrochloric acid (HCl) were from APS Chemicals (Sydney, New South Wales, Australia). Sodium bicarbonate, ($NaHCO_3$) anhydrous sodium sulfate (Na_2SO_4) barium hydroxide ($Ba(OH)_2$), oxalic acid ($H_2C_2O_4$), tin (II) chloride ($SnCl_2$), potassium carbonate (K_2CO_3), silver nitrate ($AgNO_3$) and sodium carbonate (Na_2CO_3) were purchased from Ajax Chemicals (Sydney, New South Wales, Australia). For HPLC analysis, formate buffer was prepared from formic acid (HCOOH, APS Chemicals, Sydney, New South Wales, Australia) and MilliQ water (18 $M\Omega cm^{-1}$). Ammonia solution (NH_4OH, APS Chemicals, Sydney, New South Wales, Australia) was used for the pH adjustment of the mobile phase, and the organic modifiers were methanol (MeOH, Merck, HPLC Grade) and acetonitrile (ACN, Merck, HPLC Grade). $CDCl_3$ and DMSO-d_6 (99.8% pure) for NMR analysis were obtained from Sigma-Aldrich (Sydney, New South Wales, Australia). Certified standards of morphine-3β-D-glucuronide (M3G), morphine-6β-D-glucuronide (M6G) from Lipomed (Arlesheim, Switzerland) and 7-aminoclonazepam and 7-aminonitrazepam from Novachem Pty Ltd (Melbourne, Victoria, Australia) were purchased to assist in mass spectra identification and peak allocation.

2.3.2 Instrumentation

GC-MS analysis was performed on a Hewlett-Packard 5890 Gas Chromatograph with a 5970 Series Mass Selective Detector. Products that could not be analysed by GC-MS were analysed by LC-MS-MS using a Perkin-Elmer SCIEX API 365 LC-MS-MS system, with an ElectroSpray Ionisation (ESI) source, a micro PE Series 200 LC pump and vacuum degasser. Multiple reaction monitoring (MRM) of the molecular ions of M3G and M6G and their predominant fragments in positive ion mode was employed. The selected MRM transition for M3G and M6G was *m/z* 462 → 286. For 7-aminonitrazepam and 7-aminoclonazepam, only full Q1 scans were performed.

The HPLC method used for the analysis of M3G and M6G was one previously validated for morphine-related analytes [368]. Briefly, it involved an Alltima Cyano 100 x 2.1 mm stationary phase (Alltech Associates, Sydney, New South Wales, Australia) with a mobile phase comprising of 10 mM HCOOH, pH 2.8:MeOH (98:2) at a flow rate of 0.2 mL/min. The HPLC method employed for the analysis of 7-aminonitrazepam and 7-aminoclonazepam is presented in Chapter 4. A 1.8 μm Zorbax SB-C18 50 x 4.6 mm stationary phase (Agilent Technologies, Sydney, Australia) was employed with a mobile phase consisting of 25 mM formate buffer (pH 2.8), 10% MeOH, ACN gradient 0 to 15 minutes, 6.5 to 48.5% (linear profile) at a flow rate of 1 mL/min and detection at 280 nm. Confirmation of the products was based on either the Q1 or product ion fragmentation in addition to the chromatographic retention time when compared with pure standards.

H^1 NMR spectra were obtained using a Bruker DRX NMR spectrometer operating at 300.13 MHz. Chemical shifts are quoted on the δ scale, followed by proton integration, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, bs = broad singlet, bt = broad triplet, dt = double triplet), coupling in Hertz and proton assignment.

2.3.3 Morphine-3-Glucuronide (M3G)

The procedure for the synthesis of M3G was based on the method previously reported by Berrang *et al.* [357]. To a solution of morphine hydrochloride (100 mg, 0.3 mmol) and $LiOH{\cdot}H_2O$ (12.5 mg) in methanol was added **II** (100 mg, 0.25 mmol) as a solid. The reaction mixture was allowed to stand at room temperature for 30 mins before a solution of $LiOH{\cdot}H_2O$ (35 mg) in water (0.5 mL) was added with stirring over 30 mins to produce the intermediate product **III**. After concentrating the mixture to a small volume, the pH was adjusted to 8 using acetic acid. At this point, unreacted morphine precipitated and was removed. The filtrate was concentrated to a syrup and methanol/acetone (50:50, 1mL) was added to aid the crystallisation of **IV**. Crystallisation was completed by freezing overnight. After drying, a yield of 69 mg (48%) of pure M3G (**IV**) was obtained as a white powder. LC, MS and NMR data are presented below. The spectra were very clean, indicating a highly pure product.

ESI-MS: calculated for $C_{23}H_{27}NO_9$ *m/z* 461.2, found: $[M^+{+}1]$ 462.2, 286.1; LC: single peak at t_r = 1.88; ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.70 (d *J* 8, H-2), 6.47 (d *J* 8, H-1), 5.57 (d *J* 10, H-7), 5.25 (m *J* 10, H-8), 5.02 (d *J* 6, H-1'), 4.74 (d *J* 7, H-5), 4.54 (s, H-6), 4.12 (s, H-9), 3.69 (d *J* 8, H-5'), 3.38 (dt *J* 8, H-4',3'), 3.30 (m *J* 8, H-2'), 3.18 (3H s, H-17), 3.16-2.58 (4H m, H_2-10, 16), 2.59 (t *J* 8, H-14).

2.3.4 Morphine-6-Glucuronide (M6G)

2.3.4.1 Protection (I → V)

V was prepared according to the method of Welsh *et al.* [369] with some modifications. A saturated solution of $NaHCO_3$ was prepared and added (40 mL) to a 100 mL separatory funnel. To this was added a solution of morphine hydrochloride (107 mg, 0.3 mmol) in methanol (0.5 mL). At this point, CO_2 gas was produced as HCl was removed from the molecule. The separatory funnel was shaken and the gas released for approximately 10 – 15 mins until gas formation had ceased. Acetic anhydride (0.5 mL) was added in three approximately equal portions and the mixture was allowed to stand for 30 minutes to ensure all effervescence had ceased. The

compound was then extracted three times with chloroform (45 mL) and the bottom organic layer collected. After drying with anhydrous Na_2SO_4, the reaction mixture was evaporated *in vacuo* to produce the viscous compound 3-monoacetylmorphine (**V**) in a yield of 102 mg (94%).

MS: calculated for $C_{19}H_{21}NO_4$ *m/z* 327.2, found: $[M^+]$ 327; ESI: found: $[M^++1]$ 328.2; GC: single peak at t_r = 12.22 mins. To verify acetylation occurred at the 3-position, and not the 6-position, GC-MS analysis of a mixture of the synthesised product and 6-monoacetylmorphine was performed: found: t_r = 12.22 (3MAM), 12.31 (6MAM).

2.3.4.2 Condensation (V → VI)

The procedure for the synthesis of M6G from 3-monoacetylmorphine was based on the method previously reported by Yoshimura *et al.* [356]. Silver carbonate was freshly prepared from sodium carbonate (100 mg, 0.94 mmol) and silver nitrate (300 mg, 1.8 mmol), and left under vacuum overnight to remove all traces of water. Since the reaction is moisture-sensitive, care was taken at each step to ensure all traces of moisture were removed from glassware. **V** (102 mg, 0.31 mmol) and sodium-dried benzene (13 mL) were added to a two-neck round-bottom flask. To one of the necks on the flask, a condenser with a dried tube was connected. A stopper was place in the other neck. The mixture was stirred and heated under reflux at 100°C for approximately 6 hours. During this time, **II** (180 mg, 0.45 mmol) in benzene (2 mL) and Ag_2CO_3 (180 mg, 0.65 mmol) were slowly added through the stoppered neck of the flask. The solution changed colour from colourless to dark brown as AgBr was formed. The reaction was monitored by TLC using a $CHCl_3$:MeOH (4:1) solvent system until it was shown to be complete. At this point, solid AgBr was filtered from the reaction mixture and washed with benzene. This was performed several times until the filtrate was reasonably clear. The filtrate was then extracted 3 times with cold 0.5% HCl (20 mL). And the aqueous bottom layer was collected. A solution of sodium bicarbonate was added to bring the pH of the solution to 8. After extracting 3 times with chloroform (20 mL), the extract was dried over anhydrous sodium sulfate

then filtered through filter paper. The extract was then evaporated *in vacuo* and recrystallised from ethanol to produce colourless prisms. The yield of **VI** was 150 mg (75%).

ESI-MS: calculated for $C_{32}H_{37}NO_{13}$ *m/z* 643.2, found: $[M^+ + 1]$ 644.2.

2.3.4.3 Deprotection (VI → VIII)

1% NaOMe was freshly prepared by dissolving sodium (100 mg) in methanol (10 mL). To a suspension of **VI** (150 mg, 0.23 mmol) in MeOH (1 mL) was added NaOMe (0.5 mL) and the mixture was allowed to stand overnight. The reaction mixture was then evaporated to dryness *in vacuo* before reconstitution in $Ba(OH)_2$ (5 mL, 0.43 N). The pH was adjusted to 6 with oxalic acid (2 N) and the precipitated barium oxalate was removed by filtration. After evaporating the filtrate to dryness *in vacuo* the residue was recrystallised from H_2O-ethanol to the final product M6G (**VIII**) in a yield of 27 mg (25%). Clean MS and NMR spectra indicate the high purity of the product. The MS fragmentation and NMR shifts are presented below.

ESI-MS: calculated for $C_{23}H_{27}NO_9$ *m/z* 461.2, found: $[M^+ + 1]$ 462.2, 286.1; LC: single peak at t_r = 2.29; ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.67 (d *J* 8, H-2), 6.46 (d, *J* 8 H-1), 5.75 (d *J* 10, H-8), 5.28 (d *J* 10, H-7), 5.09 (d *J* 7, H-5), 4.72 (d *J* 7, H-1'), 4.52 (bs, H-6), 4.10 (bs, H-9), 3.73 (d *J* 8, H-5'), 3.45 (dt *J* 8, H-4',3'), 3.38 (t *J* 8, H-2'), 3.22 (3H bs, H-17), 3.20-2.56 (4H m, H_2-10, 16), 2.60 (t *J* 8, H-14), 2.23 (2H bt, H-15).

2.3.5 7-Aminonitrazepam and 7-Aminoclonazepam

Clonazepam (**IX**) (25.7 mg, 0.0814 mmol) or nitrazepam (**XI**) (25.3 mg, 0.0899 mmol) was dissolved in methanol (4 mL) in a round-bottom flask. Anhydrous $SnCl_2$ (350 mg, 1.8 mmol), which had been dried overnight, was added to the reaction mixture and stirred for 6 hours. During this time, the mixture changed from yellow to brown, and the reaction was monitored by TLC using a solvent system comprising of EtOAc/Pet Spirit/EtOH (45:45:10). The reaction mixture was then evaporated *in*

vacuo and an aqueous solution of K_2CO_3 added until the pH was alkaline. After extracting three times with dichloromethane, the organic layer was dried over Na_2SO_4, filtered through fluted filter paper and evaporated *in vacuo*. The crude product was then dissolved in acetone before being subjected to preparative TLC with a solvent system comprised of EtOAc/Pet Spirit/EtOH (45:45:10). This afforded 16.3 mg (70%) of pure yellow solid 7-aminoclonazepam (**X**) and 16.8 mg (74%) of pure yellow solid 7-aminonitrazepam (**XII**). LC, MS and NMR data for both compounds are presented below.

ESI-MS: calculated for $C_{15}H_{12}ClN_3O$ (**X**) *m/z* 285.73, found: $[M^+ + 1]$ 286.7; LC: single peak at $t_r = 4.178$; ^1H NMR (300 MHz, $CDCl_3$) δ 3.65 (2H s, H-8), 4.30 (2H s, H-3), 6.55 (d *J* 2.6, H-7), 6.81-6.84 (dd *J* 2.6, H-9), 6.91 (d *J* 8.6, H-10), 7.34-7.44 (3H m, H-2', 3', 4'), 7.55-7.59 (2H m, H-1', 5'), 8.12 (s, H-1).

ESI-MS: calculated for $C_{15}H_{13}N_3O$ (**XII**) *m/z* 251.29, found: $[M^+ + 1]$ 252.3; LC: single peak at $t_r = 2.189$; ^1H NMR (300 MHz, $CDCl_3$) δ 3.61 (2H s, H-8), 4.35 (2H s, H-3), 6.32 (d *J* 2.6, H-7), 6.77-6.81 (dd *J* 2.6, H-9), 6.89 (d *J* 8.6, H-10), 7.26-7.37 (3H m, H-2', 3', 4'), 7.47-7.50 (m, H-5'), 8.07 (s, H-1).

2.4 Conclusion

M3G, M6G, 7-aminonitrazepam and 7-aminoclonazepam were successfully synthesised based on previously reported methods. Although the yields for M3G, 7-aminonitrazepam and 7-aminoclonazepam were quite good, that of M6G could be improved. Future work in this area could therefore focus on improving the yield of M6G through the use of different catalysts to assist the Koenigs-Knorr coupling reaction. Another approach may be investigation of the imidate coupling reaction, since there have been limited studies on the synthesis of M6G by this method. Minimal laboratory facilities were required to synthesise M6G, M3G, 7-aminonitrazepam and 7-aminoclonazepam, and while the synthesis of M6G required several steps, the remaining metabolites were prepared according to relatively simple procedures. The synthesis of drug metabolites therefore provides an alternative to

purchasing through expensive commercial sources and gives toxicology laboratories improved access to these necessary compounds.

Chapter Three

Development of a Rapid Method for the Analysis of Benzodiazepines by Capillary Zone Electrophoresis (CZE)

3 Development of a Rapid Method for the Analysis of Benzodiazepines by Capillary Zone Electrophoresis (CZE)

3.1 Summary

The number of studies focusing on the analysis of benzodiazepines by capillary zone electrophoresis (CZE) has been limited, mainly due to the lengthy analysis times that arise as a result of the reduction in the EOF at low pH. In this work, fast CZE separations of benzodiazepines at low pH were investigated through the use of doubly-coated capillaries and cyclodextrins. A rapid CZE-DAD method for the simultaneous determination of nine benzodiazepines was developed using a capillary coated with a polycation of poly(diallyldimethylammonium chloride) (PDDAC) and a polyanion of dextran sulfate (DS). The selected BGE conditions were 100 mM ammonium phosphate buffer, pH 2.5, which gave baseline resolution between each analyte and a run time of less than 6.5 minutes. This method offers improvements in both resolution and run time, compared to those attained under analogous conditions with an uncoated capillary. Cyclodextrins in conjunction with doubly-coated capillaries were not successful in further improving the run time or separation of the nine benzodiazepines. However, an uncoated capillary with a BGE of 10 mM β-cyclodextrin (βCD) in 100 mM phosphate buffer, pH 2.5 improved the resolution that could be attained on a bare fused-silica alone. The coated method was validated and was successfully applied to the analysis of benzodiazepines in spiked beverages.

Keywords: doubly-coated capillary; dynamic coating; benzodiazepines; capillary zone electrophoresis; cyclodextrins; drink spiking

3.2 Introduction

Capillary electrophoresis (CE) is an emerging technique in forensic toxicology and the pharmaceutical industry, and is considered to be complementary to HPLC, offering advantages such as low solvent and sample requirements, high efficiency and rapid analysis times. However, one of the limitations of CE is that it lacks a

stationary phase interaction as a means to modify separation selectivity [370]. Small selectivity changes can be brought about by varying the pH, ionic strength, or using an organic modifier in the background electrolyte (BGE), however analytes with very similar charge/size ratios are often difficult to separate. The use of additives, also referred to as pseudo-stationary phases, to interact with analytes and improve separation has been termed electrokinetic chromatography (EKC), since it combines the electrophoretic separation of CE with a chromatographic component resulting from the addition of the pseudo-stationary phase [371]. In capillary zone electrophoresis (CZE), the charged solutes are separated on the basis of their effective electrophoretic mobilities, whereas neutral solutes cannot be separated. EKC with the use of additives in the BGE overcomes the problem of separating neutral compounds by CE. When the additive is comprised of surfactant micelles (the most common being SDS), the approach is termed micellar electrokinetic chromatography (MEKC). In MEKC, surfactants above their critical micelle concentration (CMC) are added to the running buffer. Neutral solutes partition between the micellar and aqueous phases, and separate according to their partition coefficients [372]. Other pseudo-stationary phases may consist of cationic, anionic, zwitterionic or neutral species.

Flow through the capillary in CE separations is the result of an applied electric field and the electro-osmotic flow (EOF). The EOF is created by a double electric layer, which forms due to pairing between ionised silanol groups on the capillary wall and cationic species in the buffer. Bulk flow is created by the applied electric field, which causes the migration of buffer cations towards the cathode. Migration times of solutes in CE are dependent upon the charge/size ratio of the solute, in addition to the level of EOF in the capillary [373]. The size of the EOF depends on pH, since this governs the extent of silanol dissociation. It decreases rapidly below pH 6 and, at pH 2 – 3, the EOF is almost completely eliminated resulting in increased analysis times for cationic compounds and loss of the ability to analyse anionic compounds unless the polarity is reversed [374]. The coating of capillaries in CE is often performed to stabilise the EOF and eliminate associated peak tailing, poor efficiency [375] and

peak area variability [373] by reducing adsorption of buffer or sample components to the capillary surface. It is especially relevant for the analysis of basic analytes, which are positively charged and therefore attracted to the negatively charged capillary wall. Capillary coating may also be used to assist in changing the direction and/or speed of the EOF.

Capillary coatings can be classified as either static or dynamic. In static or permanent coating procedures, the coating material is permanently attached to the capillary wall by way of covalent bonds. One of the first static coating techniques utilised polyacrylamide as the coating material. This method was developed by Hjertén [376] and has since been used for the separation of proteins, DNA and some small molecules. In these capillaries, solute adsorption is reduced and the EOF is eliminated since the capillary wall is uncharged. This coating method has been successfully applied by Jinno *et al.* [372] for the analysis of toxic drugs including barbiturates and benzodiazepines. Variations on this method in which different polymers, such as poly(vinylpyrrolidone) [377], poly(ethylene glycol) and poly(vinyl alcohol) [378], are employed as coating materials have also been reported. The main drawback with this particular type of coated capillary is that elimination of the EOF can result in lengthy analysis times. In addition, the coating procedures are often lengthy and require multi-step chemical reactions, which can produce a large variance between capillaries.

In contrast to permanent coatings, dynamic coatings are relatively simple to prepare. The capillary is rinsed with a solution containing the coating reagent, which becomes adsorbed onto the capillary wall through electrostatic interactions with the silanol groups. The main drawback with dynamic coatings is the limited pH range in which the analyses can be performed. Dynamically coated capillaries also require occasional regeneration, and addition of the coating agent into the BGE may be necessary [379]. A number of papers detail the use of capillaries coated with a monolayer of a positively charged polymer [380-382]. In these cases, the EOF is reversed and cations migrate slower than the EOF, resulting in long analysis times.

Alternatively, a second anionic polymer layer can be electrostatically adsorbed onto the first cationic polymer layer to create a doubly-coated capillary. This results in a pH-independent EOF and allows for very rapid cationic separations. Doubly-coated capillaries were first introduced by Katayama *et al.*, who prepared dynamically coated capillaries by successive flushing with Polybrene and dextran sulfate (DS) to produce a stable coating with pH-independent EOF in the range pH 2 – 11. Since then, numerous doubly-coated capillaries have been prepared with the aid of different polymers, however they are essentially based on Katayama's initial coating procedure.

The commercially available CEofix® or CElixir® buffer systems comprise of an initiator buffer, which contains the polycation, and an accelerator containing the polyanion. The accelerator buffer is flushed through the capillary after the initiator buffer, and the polyanions adsorb to the first layer of polycations to form a double layer. The polyanion layer contains sulfate groups, which are relatively insensitive to changes in pH, resulting in a reproducible and large EOF [297, 383]. After each analysis, the coating is removed with NaOH and reapplied. This coating procedure has been successfully employed in the analysis of basic drugs [373, 383, 384], the major opium alkaloids [385] and basic impurities and adulterants in heroin [386]. Vanhoenacker *et al.* [297] also employed this coating procedure for the analysis of benzodiazepines.

Other non-commercial dynamic coating methods have been described, including the poly(diallyldimethylammonium chloride) (PDDAC)/DS coated capillary utilised by Zakaria *et al.* [371] for the analysis of alkaloids. This PDDAC/DS coating method was also successfully applied by Kelly *et al.* [387] for the chiral separation of methadone and its two major metabolites. Polybrene/poly(vinylsulfonate) doubly-coated capillaries for the analysis of peptides has been described by Catai *et al.* [388], and a similar coating procedure was reported by Graul and Schlenoff [389], whereby alternating layers of PDDAC and sodium poly(styrenesulfonate) enabled the efficient and reproducible separation of basic proteins.

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of D-glucose units bonded through α-1,4-linkages [390]. They are an example of chiral selectors [391] and are often used in enantiomeric separations. Due to the presence of primary and secondary hydroxyl groups, the outer surface of the cyclodextrin molecule is hydrophilic, while the inner cavity is hydrophobic. Only molecules that can be fitted into the cavity of the cyclodextrin can form stable inclusion complexes [390]. Separation is achieved as a result of differences in partitioning of the analytes between the aqueous BGE and the hydrophobic core of the cyclodextrin [371], in a similar manner to micellar separations. The hydrophobic character of the cyclodextrin cavity can be increased by replacing the hydroxyl groups on the rim of the cavity with methyl, hydroxyethyl, hydroxypropyl, sulfate and acetyl groups [390]. Cyclodextrins have been employed to improve separation in a variety of studies including the determination of aromatic bases [370], chiral [390, 391] and achiral [351] separations of benzodiazepines by HPLC, and the complementary use of cyclodextrins with coated capillaries [384].

3.2.1 Previous CE Methods for the Analysis of Benzodiazepines

Benzodiazepines are used in the clinical treatment of anxiety, insomnia and panic disorder [193]. Their potential for abuse is large, and they are often used by heroin users is to enhance the feeling of high from the opiates or to reduce opiate withdrawal symptoms [58]. Considering their abuse-potential, the analysis of benzodiazepines is of interest from a forensic and toxicological standpoint. As such, a number of reviews focusing on the analysis of benzodiazepines have been published [191, 392]. While HPLC remains the most common technique for their analysis, CE methods have recently been developed to accommodate both biological and aqueous media. Refer to Table 1-12 and Table 3-1 for details of these methods. Most analyses are performed with MEKC [192, 231, 253, 277, 296, 302-305, 372, 393-400], however CZE [272, 297, 397, 398, 401, 402] and CEC have also been reported [403-405]. A specific review regarding the application of CE to the analysis of benzodiazepines has been published by Smyth and McClean [345]. Note that the

effective length of the capillary (L_{eff}) refers to the length of the capillary between the sample inlet and the detector.

Table 3-1 Summary of published CE methods for the analysis of benzodiazepines

Ref.[a]	Analytes	CE type	Capillary	Running buffer	Voltage[b]	Detection method[c]	Run time[d]
[394]	19 analytes, inc. LOR, DIA	MEKC	Fused-silica 25-50μm L_{eff} 25-100cm	85mM SDS/8.5mM phosphate/8.5mM borate/15% ACN, pH 8.5	25-30	UV 210	40
[395]	BROM, NIT, CLO, CLB, TEM, OXA, BRO, LOR, nor-DIA, LORM	MEKC	Fused-silica 50μm L_{eff} 44cm	25mM SDS/75mM glycine/250mM TEA/20% MeOH	25	UV 235	12
[399]	BRO, FLU, CLB, NIT, CLO, CHL, TEM, LOR, FLUR, DIA, HAL, OXA, nor-DIA, LORM	MEKC	Fused-silica 75μm L_{tot} 87cm, L_{eff} 80cm	20mM borate-phosphate, pH 7/15mM SC/35mM SDC	20	UV 214, 254	25
[400]	Numerous (17)	MEKC	Fused-silica, C_8 and C_{18} coated 50μm L_{tot} 60cm, L_{eff} 40cm	100mM borate, pH 8.5/25mM SDS/5mM urea	30	UV 245	70
[396]	ALP, BRO, CHL, CLB, CLO, CLOR, OXA, PRA, TRI	MEKC	Fused-silica 75μm L_{eff} 47cm	50mM borate/50mM SDS/20mM γ-CD/2mM urea , pH 9.2/1% THF	15	UV 214	24

Ref.[a]	Analytes	CE type	Capillary	Running buffer	Voltage[b]	Detection method[c]	Run time[d]
[397]	17 analytes inc. FLU, DIA	MEKC and CZE	Fused-silica 50μm L_{tot} 55cm, L_{eff} 35cm	MEKC: 25mM borate, pH 9.24/20% MeOH/100mM SDS CZE: 50mM phosphate, pH 2.35	20	UV 200	~40
[398]	CHL, DIA, NIT, FLUR	MEKC and CZE	Fused-silica 50μm L_{tot} 70cm, L_{eff} 63cm	CZE: 20mM citric acid, pH 2.5/15% MeOH MEKC: 75mM SDS/phosphate-borate buffer/5% MeOH	20	UV 200	CZE: 8 MEKC: 21
[372]	EST, OXA, ALP, FLUD, HALO, DIA, ETI, CLOX, CLOT, FLUR	MEKC	Polyacrylamide-coated 50μm L_{tot} 60cm, L_{eff} 40cm	100mM borate, pH 8.5/5mM urea/10mM SDS	18	UV 254	10
[404]	DIA, NIT	CEC	Fused-silica 50-75μm 33-50cm packed with Hypersil ODS (3μm) and Apez ODS (3μm)	ACN/5mM NH_4Ac (gradient)	30	MS (SIM) and UV 254	25

Ref.[a]	Analytes	CE type	Capillary	Running buffer	Voltage[b]	Detection method[c]	Run time[d]
[403]	DIA, LOR, OXA, TEM, TOF	CEC	Electropak phenyl-bonded silica 75µm L_{tot} 47cm, L_{eff} 40cm	Tris HCl, pH 8/ACN (60:40)	20	UV 220	18
[401]	DIA, nor-DIA, OXA, TEM	CZE	Fused-silica 75µm L_{tot} 95cm L_{eff} 34cm	20mM acetic acid/15% MeOH, pH 2.50	20.5	ESI-MS	15
[405]	FLU, nor-FLU, 7-NH_2-FLU, Ac-FLU	CEC	Fused-silica 50µm L_{tot} 48cm, L_{eff} 40cm	70mM octane/800mM 1-butanol/80mM SDS/ 10mM borate buffer, pH 9	25	UV 220	6
[192]	FLU, CLO, CHL, ALP, LOR, TRI, MID, PRA	MEKC	Fused-silica 50µm L_{tot} 80cm, L_{eff} 72cm	20mM SDS/borate buffer/7% ACN	30	PDA	16
[393]	CLOX, CHL, NIT, FLU, BROM, OXA, EST, DIA, FLUD, MEX	MEKC	Fused-silica 75µm L_{tot} 57cm, L_{eff} 50cm	2% DS/20mM SDS/boric acid, pH 9.2	20	UV 254	<25

n/a – not available or not described, [a] references in chronological order, [b] voltages in kV, [c] wavelengths in nm, [d] run times in min

Abbreviations: Analytes: acetyl-flunitrazepam (Ac-FLU), 7-aminoflunitrazepam (7-NH_2-FLU), alprazolam (ALP), bromazepam (BROM), brotizolam (BRO), chlordiazepoxide (CHL), clobazam (CLB), clonazepam (CLO), clorazepate (CLOR), clotiazepam (CLOT), cloxazolam (CLOX), diazepam (DIA), estazolam (EST), etizolam (ETI), fludiazepam (FLUD), flunitrazepam (FLU), flurazepam (FLUR), halazepam (HAL), haloxazolam (HALO), lorazepam (LOR), lormetazepam

(LORM), mexazoram (MEX), midazolam (MID), nitrazepam (NIT), nordiazepam (nor-DIA), norflunitrazepam (nor-FLU), oxazepam (OXA), prazepam (PRA), temazepam (TEM), tofisopam (TOF), triazolam (TRI); **Method details:** acetonitrile (ACN), ammonium acetate (NH_4Ac), capillary electrochromatography (CEC), capillary zone electrophoresis (CZE), cyclodextrin (CD), dextran sulfate (DS), effective length (L_{eff}), electrospray ionisation (ESI), mass spectrometry (MS), methanol (MeOH), micellar electrokinetic capillary chromatography (MEKC), photodiode array (PDA), sodium cholate (SC), sodium deoxycholate (SDC), sodium dodecyl sulfate (SDS), tetrahydrofuran (THF), total length (L_{tot}), triethylamine (TEA), ultraviolet detection (UV).

Analyses of basic compounds are usually performed by CZE at low pH, however the low pK_a values of benzodiazepines makes them difficult to ionise, and therefore difficult to analyse by CZE. In addition, low pHs cause reduction of the EOF, which can result in long analysis times and low signal-to-noise ratios. For these reasons, the majority of benzodiazepine separations are performed by MEKC at high pH, as inspection of Table 1-12 and Table 3-1 will confirm. Despite this, CZE offers possible advantages over MEKC including better efficiency and precision, improved resolution, BGE simplicity and the possibility of coupling with MS.

In one of the few CZE studies of benzodiazepines, Tagliaro *et al.* [397] developed a CZE method for the analysis of drugs of forensic interest, including diazepam and flunitrazepam, on a bare fused-silica capillary at low pH. The method employed 50 mM phosphate buffer at pH 2.35 and gave good separation for most of the basic analytes. The authors compared this CZE method to a MEKC method (25 mM borate, pH 9.24/20% MeOH/100 mM SDS) and found intra-day RSDs of migration times to be far superior, ranging from 0.2 to 1.2% for CZE as compared to 2.0 to 8.6% for MEKC. The analytical sensitivity was also 3-4 times better than in MEKC due to higher efficiency.

The CZE analysis of four benzodiazepines at low pH was conducted in a study by McGrath *et al.* [398]. The BGE consisted of 20 mM citric acid + 15% methanol at pH 2.5, and the total analysis time was 8 minutes. This method was compared to a MEKC method (75 mM SDS/6 mM sodium tetraborate/12 mM disodium hydrogen phosphate + 5% MeOH) and the authors found that while CZE gave slightly inferior efficiencies to those obtained by MEKC, it was superior in terms of its fast analysis time. The same buffer conditions were also applied in two studies by McClean *et al.* [272, 401], both of which involved the analysis of 15 benzodiazepines in under 20 minutes by CE-MS.

A coated CZE method could allow the EOF to be introduced and permit a fast cationic separation, thereby overcoming the problem of long run times associated

with analysis at low pH. To date, the only method published for the analysis of benzodiazepines using a doubly-coated capillary was has been by Vanhoenacker *et al.* [297], who employed the CEofix coating system for the analysis of six benzodiazepines by CE-DAD, CE-MS and CE-MS-MS in 100 mM formic acid at pH 2.4. While run times of around 12 minutes were possible using MS-MS detection, significantly larger run times of 20 minutes were attained using DAD. MS-MS detection enables shorter analysis times to be obtained when the Multiple Reaction Monitoring (MRM) mode is utilised. This involves monitoring selected ion fragments of each analyte. As a result, analytes do not need to be baseline resolved to enable their accurate quantification. Less importance is therefore given to resolution, and analysis times can be reduced. Since the coupling of MS to CE remains an expensive method of detection, and is generally beyond the financial means of many analytical laboratories, it is of interest to develop a rapid CZE method using the more readily available DAD for the analysis of benzodiazepines.

3.2.2 Objectives

The main objective of this work was to develop a fast CZE separation for the analysis of nine benzodiazepines, primarily through the use of a dynamic doubly-coated capillary. The secondary objective of this study was to assess the effectiveness of cyclodextrins in enhancing resolution and reducing run time on both coated and bare fused-silica capillaries. The application of the method to the analysis of spiked beverages was also investigated.

3.3 Experimental

3.3.1 Chemicals

All reagents were of analytical grade unless stated otherwise. Nitrazepam, oxazepam, alprazolam, flunitrazepam, temazepam and diazepam were obtained from Sigma-Aldrich (Sydney, New South Wales, Australia). 7-aminoflunitrazepam, 7-aminonitrazepam and 7-aminoclonazepam were either synthesised in-house (Chapter 2) or purchased from Novachem Pty Ltd (Melbourne, Victoria, Australia). All buffers were prepared in MilliQ grade (18.2 $M\Omega cm^{-1}$) water by dilution of stock

solutions of phosphoric acid (H_3PO_4) (APS Chemicals, Sydney, New South Wales, Australia). Ammonia solution (NH_4OH, APS Chemicals, Sydney, New South Wales, Australia) was used for pH adjustment. 1N NaOH (HPCE grade) was obtained from Agilent Technologies (Sydney, New South Wales, Australia). Poly(diallydimethylammonium chloride) (PDDAC, 20 wt% in water) and dextran sulfate (DS) were obtained from Sigma-Aldrich (Sydney, New South Wales, Australia). β-cyclodextrin (βCD, 1135 g/mol), heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (DMβCD, 1331 g/mol) and heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TMβCD, 1430 g/mol) were purchased from Sigma-Aldrich (Sydney, New South Wales, Australia). Prior to use, all buffers were degassed by sonication and filtered using 0.45 μm Nylon syringe filters (Bonnett Equipment, Sydney, New South Wales, Australia).

3.3.2 Instrumentation

Separations were performed on an Agilent Chemstation Capillary Electrophoresis System (Agilent Technologies, USA), equipped with diode array detector (DAD). Uncoated 50μm I.D. fused-silica capillaries of 69 cm total length (60 cm effective length) were purchased from Polymicro Technologies (Phoenix, USA). Detection windows were made by burning the outer polyimide coating to expose the bare capillary. Aqueous standards of each analyte were prepared by dilution of stock solutions with MilliQ water. Duplicate injections for each standard were performed hydrodynamically at 50.0 mbar for two seconds. Separations were carried out at 25°C with an applied voltage of +30 kV, and detection at 200 nm. At the beginning of each day, the capillary was flushed with 0.1N NaOH for five minutes, MilliQ water for five minutes, and running buffer for ten minutes. Prior to each injection, the capillary was flushed with the running buffer for three minutes. The position of the EOF was verified for each condition by injection of an acetone solution (10% acetone in running buffer).

Coated capillaries were prepared by a method described previously [371, 387]. To summarise, the capillary was flushed with 1N NaOH for thirty minutes, followed by MilliQ water for fifteen minutes, and was then allowed to stand for thirty minutes. A solution of 1% v/v PDDAC was flushed through the capillary for fifteen minutes, and then left to stand for fifteen minutes. A two minute flush with MilliQ water preceded a fifteen minute flush with 1.5% w/v DS solution. After allowing the capillary to stand for thirty minutes, MilliQ water was flushed through for five minutes.

3.3.3 Calculations

The apparent mobility of each analyte was calculated according to Equation 3-1. The resulting value was then substituted into Equation 3-2 for the calculation of effective mobility.

Equation 3-1 Calculation of apparent mobility (μ_{app})

$$\mu_{app} = \frac{L_{eff} \cdot L_{tot}}{t_m \cdot V}$$

where L_{eff} and L_{tot} are the effective and total capillary lengths (in cm) respectively, t_m is the migration time (in sec), and V is the applied voltage (in V).

Equation 3-2 Calculation of effective mobility (μ_{eff})

$$\mu_{eff} = \mu_{app} - \mu_{EOF}$$

where μ_{app} is the apparent mobility and μ_{EOF} is the mobility of the EOF.

For each experimental condition, the resolution of each analyte pair was calculated using Equation 3-3. The product resolution was calculated by the multiplication of each of the individual resolution values, as shown in Equation 3-4.

Equation 3-3 Calculation of resolution (R_s)

$$R_s = \frac{(t_2 - t_1)}{0.5(w_1 + w_2)}$$

where t_1 and t_2 are the migration times for each peak pair, and w_1 and w_2 are the peak widths at baseline

Equation 3-4 Calculation of product resolution (PR_s)

$$PR_S = PP1 * PP2 * PP3 * \quad * PP9$$

where PP1, PP2, PP3, , PP9 are the resolutions between the 1st peak pair, 2nd peak pair, 3rd peak pair, ., 9th peak pair

3.3.4 Method Validation

The method was validated for aqueous standards and calibration curves were obtained by analysing working standard solutions, diluted to obtain final concentrations of 17.0, 22.7, 45.5, 68.2 and 90.9 μg/mL for diazepam, nitrazepam, flunitrazepam, temazepam and oxazepam, and 51.1, 68.2, 150.0, 204.5 and 272.7 μg/mL for alprazolam. Calibration standards were analysed each day, and standard curves were constructed using linear regression.

Accuracy and precision were calculated at high and low concentration for each drug, with five replicates performed at each concentration. Accuracy was expressed as the calculated concentration as a percentage of nominal concentration. Precision (%CV) was determined to be the standard deviation in peak area divided by the average of the three replicates, expressed as a percentage. The limit of detection (LOD) was defined as a signal to noise ratio (S/N) of 3:1 and the limit of quantification (LOQ) as a S/N of 10:1.

3.4 Results and Discussion

3.4.1 Preliminary Screening of BGE Parameters

Properties of the BGE, including buffer type, concentration and pH, can greatly influence the level of EOF and hence the analysis time in CE separations. If the pH of the BGE is too high, the analytes may be insufficiently ionised to achieve complete separation. If the pH is too low, the EOF is minimised, resulting in long analysis times and low signal-to-noise ratios. This is of particular importance in CZE separations of basic analytes. In addition to pH, the ionic strength of the BGE can influence the analysis time. High ionic strength BGEs can shield the effective charge

of the analytes, thereby reducing mobility and increasing migration times [406]. High ionic strength will also decrease the EOF.

With this in mind, a BGE of ammonium phosphate was selected for investigation in the range pH 1.9 – 2.5 (constant concentration 100 mM). In this acidic range, the positively charged basic analytes had an effective mobility greater than the EOF, causing them to migrate ahead of the EOF under the application of a positive potential. The effect of the ionic strength of this BGE on the separation was also investigated in the range 25 – 100 mM (constant pH 2.5). Figure 3-1 is an example of the separation attained in 100 mM phosphate buffer, pH 2.3 on a bare fused-silica capillary. As would be expected at low pH, 7-aminonitrazepam, which can be protonated on the $-NH_2$ group (pK_a 4.6) and the C=N group (pK_a 2.5), migrates through the capillary first. It is closely followed by 7-aminoflunitrazepam and 7-aminoclonazepam, which can also be protonated at these two positions. The remainder of the analytes were separated in line with their pK_a values of the monoprotonated species; that is diazepam (pK_a 3.3), nitrazepam (pK_a 3.2), alprazolam (pK_a 2.4), flunitrazepam (pK_a 1.8), oxazepam (pK_a 1.7) then temazepam (pK_a 1.6).

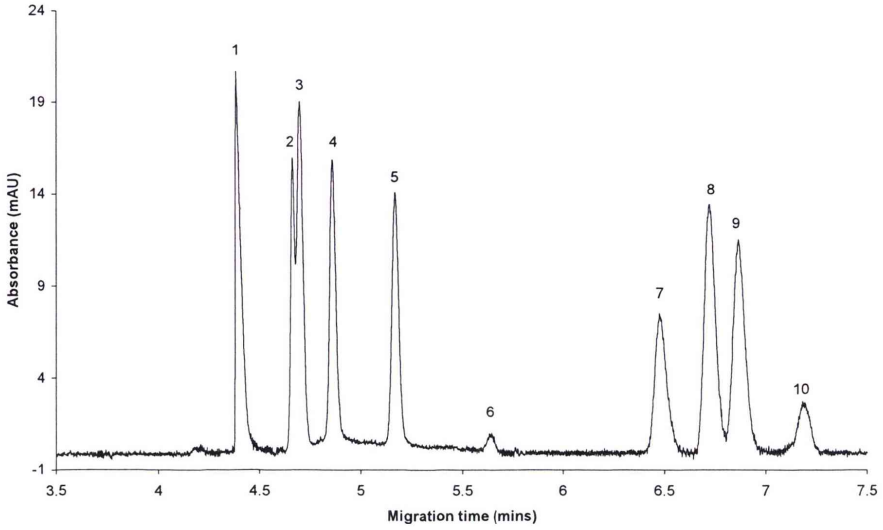


Figure 3-1 Example electropherogram of a standard mixture of nine benzodiazepines on an uncoated capillary

Conditions: 60 cm x 50 μm bare fused-silica capillary, 100 mM phosphate buffer, pH 2.3, applied voltage 28 kV, temperature 25 °C, injection 2s at 50 mbar, detection λ 200 nm; **Migration order:** 1. 7-NH_2-NIT (t_m 4.387), 2. 7-NH_2-FLU (t_m 4.662), 3. 7-NH_2-CLO (t_m 4.698), 4. DIA (t_m 4.858), 5. NIT (t_m 5.168), 6. ALP (t_m 5.672), 7. FLU (t_m 6.477), 8. OXA (t_m 6.722), 9. TEM (t_m 6.866), 10. EOF (t_m 7.183)

The average effective mobilities and resolutions for each analyte with varying phosphate buffer concentration (pH 2.5) are illustrated in Figure 3-2. Increasing ionic strength of the buffer had little effect on the effective mobilities of the six parent benzodiazepines, however a slight increase in effective mobility was observed for the 7-amino metabolites. This may be because the shielding effect of the BGE was less pronounced on the 7-amino metabolites, which have an effective +2 charge, as compared to the parent compounds, which only have a +1 charge. As a result, the migration times of the 7-aminobenzodiazepines were less affected by the increase in ionic strength. This caused their effective mobilities to increase relative to the migration time of the EOF and other analytes. Average resolutions between consecutive peaks and overall product resolutions (PR_s) are presented for each condition in Table 3-2. Co-migration of 7-aminoflunitrazepam and 7-

aminoclonazepam (PP2) at 75 and 100 mM rendered these concentrations ineffective for the complete separation of the nine analytes. A BGE concentration of 25 mM was also ineffective for the resolution of all analytes, due to co-migration of 7-aminonitrazepam and 7-aminoflunitrazepam (PP1). The only condition in which separation ($PR_s > 0$) of all nine analytes was possible in phosphate buffer (pH 2.5) occurred at a concentration of 50 mM. However, at this concentration, the resolution between 7-aminoflunitrazepam and 7-aminoclonazepam (PP2) was <1.5, indicating that complete baseline separation was not achieved.

Table 3-2 Average resolutions of consecutive peaks with varying phosphate buffer concentration on an uncoated capillary

Concentration (mM)	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8	PP9	PR_s (x 10^7)
25	0.000	0.824	0.477	3.352	14.050	17.430	4.321	2.615	3.144	0.000
50	5.510	1.289	3.944	16.277	17.464	19.576	4.688	2.798	3.541	0.724
75	7.043	0.000	3.061	8.321	11.489	14.688	1.963	0.853	2.079	0.000
100	7.613	0.000	3.286	8.938	13.435	18.155	2.053	0.812	2.185	0.000

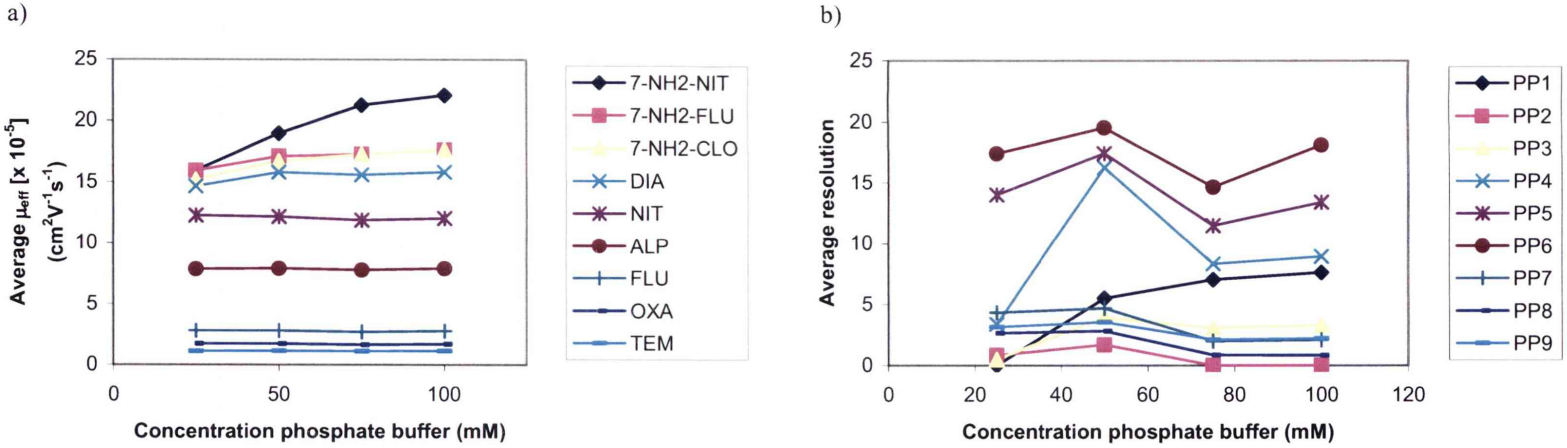


Figure 3-2 Influence of phosphate buffer concentration on a) average effective mobilities and b) average resolution for each analyte at pH 2.5 on an uncoated capillary

Table 3-3 Average resolutions of consecutive peaks with varying pH on an uncoated capillary

pH	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8	PP9	PRs (x 10^7)
2.1	2.234	0.553	3.193	5.064	10.195	14.406	4.968	3.273	7.771	0.03705
2.2	5.299	0.605	3.446	6.376	10.242	16.598	4.796	2.877	6.084	0.1005
2.3	6.343	0.626	3.273	7.394	10.574	16.561	3.487	1.923	4.586	0.05170
2.4	6.424	0.604	3.078	8.000	11.875	14.664	2.950	1.422	3.584	0.02502
2.5	4.975	0.695	2.275	8.682	8.613	11.134	1.933	0.835	2.278	0.002407

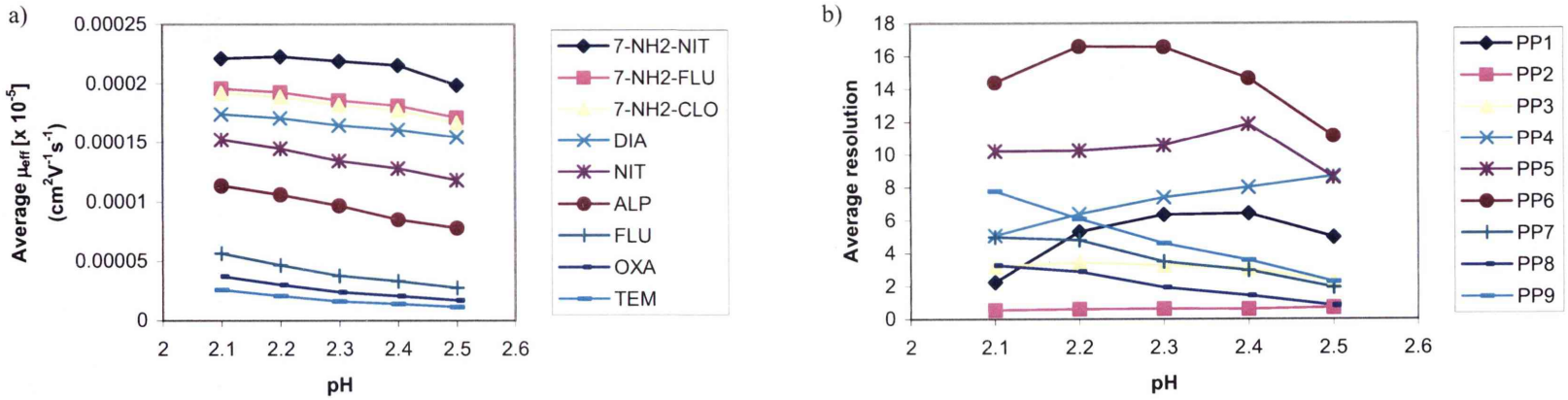


Figure 3-3 Influence of pH on a) average effective mobilities and b) average resolution for each analyte in 100 mM phosphate buffer on an uncoated capillary

Figure 3-3 highlights the effect of varying the pH of the BGE (100 mM phosphate buffer) on the average effective mobilities and resolutions for each analyte. Increasing pH tended to reduce the effective mobility, and hence increase the migration time of all analytes. This is because the pK_a of benzodiazepines is low, so that even at low pH, the species were hardly protonated. As the pH was increased, the positive charge on each analyte was reduced even further, causing them to become more neutral and migrate closer to the EOF. The effect of pH on resolution can also be explained in terms of this observation. The last three peaks, flunitrazepam, oxazepam and temazepam (PP7, PP8 and PP9), which have the lowest pK_as, experienced a reduction in resolution since they tended to merge with the EOF as the pH was increased. However, the effect on earlier migrating peaks was an increase in separation (PP1, PP5 and PP6) due to more interaction with the capillary wall. Table 3-3 illustrates the average resolution values for consecutive peaks, as well as the product resolution (PR_s) for each condition. At pHs 1.9 and 2.0, the peak shape of the 7-amino metabolites and alprazolam was poor and the signal-to-noise ratio was low. As a result, migration times and peak widths could not be accurately obtained for these analytes. For this reason, the resolution values obtained at these pH values were not included in Table 3-3. In the case of alprazolam, the poor peak shape may have been a result of ring-opening, which is known to occur in acidic medium [257]. For the remaining conditions, PR_s was >0 at all pHs investigated, indicating that no species co-migrated. However, the resolution between 7-aminoflunitrazepam and 7-aminoclonazepam was <1.5 for each condition, indicating that baseline separation could not be obtained under the conditions investigated.

3.4.2 Coated Capillary Method Development

In order to improve the separation, a dynamic doubly-coated capillary with a cationic layer of PDDAC and an anionic layer of DS, as previously described by Zakaria *et al.* [371], was investigated. An ammonium phosphate BGE was studied in the range pH 1.9 – 2.5 (constant concentration 100 mM) and ionic strengths of 25 – 100 mM (constant pH 2.5) for purposes of direct comparison with the results obtained on the uncoated capillary.

The average effective mobilities and resolutions of the nine benzodiazepines with varying phosphate buffer concentration (pH 2.5) on a coated capillary are illustrated in Figure 3-4. BGE ionic strength had little effect on the effective mobility of the six parent benzodiazepines, however, once again, the mobilities of the three 7-amino metabolites increased slightly due to the shielding effect of the BGE being less pronounced on these analytes. Table 3-4 presents average resolution values and product resolutions (PR_s) for the separations at each condition. The effect of BGE concentration on average resolution was much more significant. At 25 mM, 7-aminonitrazepam and 7-aminoflunitrazepam (PP1) were insufficiently resolved, however, since the effective mobility of 7-aminonitrazepam significantly increased with increasing BGE concentration, so too did the resolution of PP1. The peak pairs most affected by BGE concentration in terms of resolution were PP1 (7-aminonitrazepam and 7-aminoflunitrazepam) and PP4 (diazepam and nitrazepam). As the BGE concentration was increased, so too did the resolution between these peak pairs. Under the conditions studied, the highest PR_s occurred at a BGE concentration of 100 mM.

Table 3-4 Average resolutions of consecutive peaks with varying phosphate buffer concentration on a coated capillary

Concentration (mM)	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8	PP9	PR_s (x 10^7)
25	0.546	1.455	2.624	1.847	14.339	18.863	4.365	2.657	3.273	0.003951
50	4.117	2.392	2.715	16.836	19.373	22.589	5.033	2.870	3.866	1.100
75	13.372	2.538	6.420	17.811	19.453	22.210	4.871	2.839	4.192	9.722
100	15.621	2.743	7.542	16.580	17.731	21.391	4.821	2.773	4.587	12.46

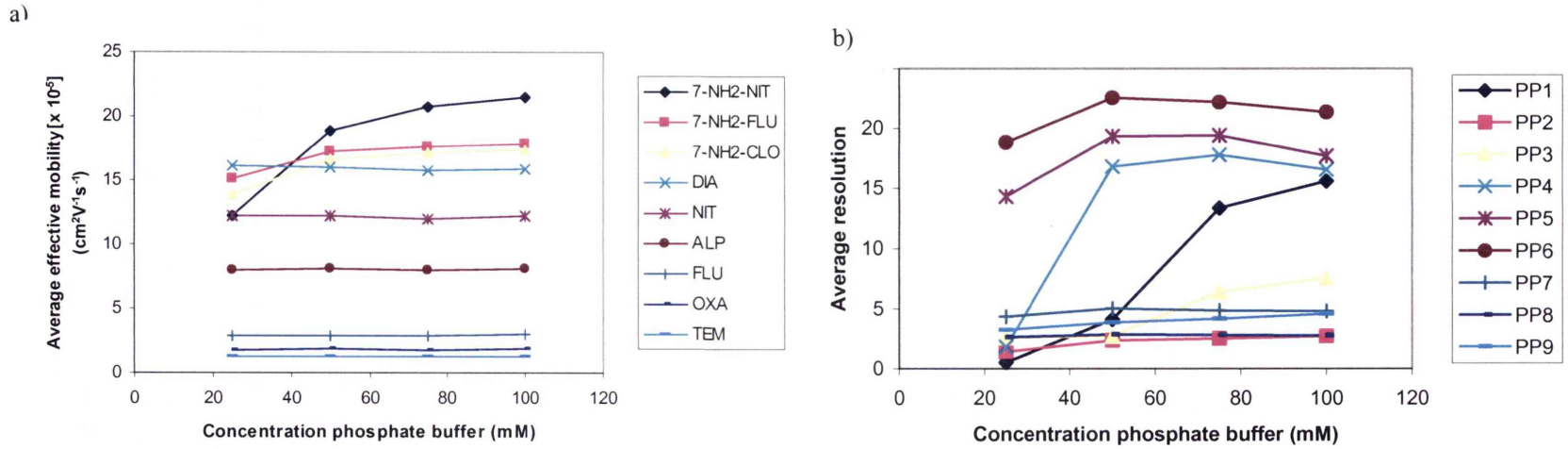


Figure 3-4 Influence of phosphate buffer concentration on a) average effective mobilities and b) average resolution for each analyte at pH 2.5 on a coated capillary

Table 3-5 Average resolutions of consecutive peaks with varying pH on a coated capillary

pH	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8	PP9	PRs (x 10^7)
2.0	7.564	6.883	0.706	0.000	4.000	8.769	10.000	6.884	16.279	0.0000
2.1	7.831	1.773	0.000	0.000	4.503	18.189	8.871	6.033	10.442	0.0000
2.2	1.746	0.000	0.000	2.536	15.037	21.549	7.076	4.771	7.348	0.0000
2.3	1.458	0.000	0.000	9.402	15.505	21.377	6.294	4.041	5.971	0.0000
2.4	0.825	1.256	0.587	7.846	13.959	19.154	5.126	3.057	7.799	0.01560
2.5	15.256	2.671	7.600	16.842	17.845	21.257	4.735	2.721	4.314	11.00

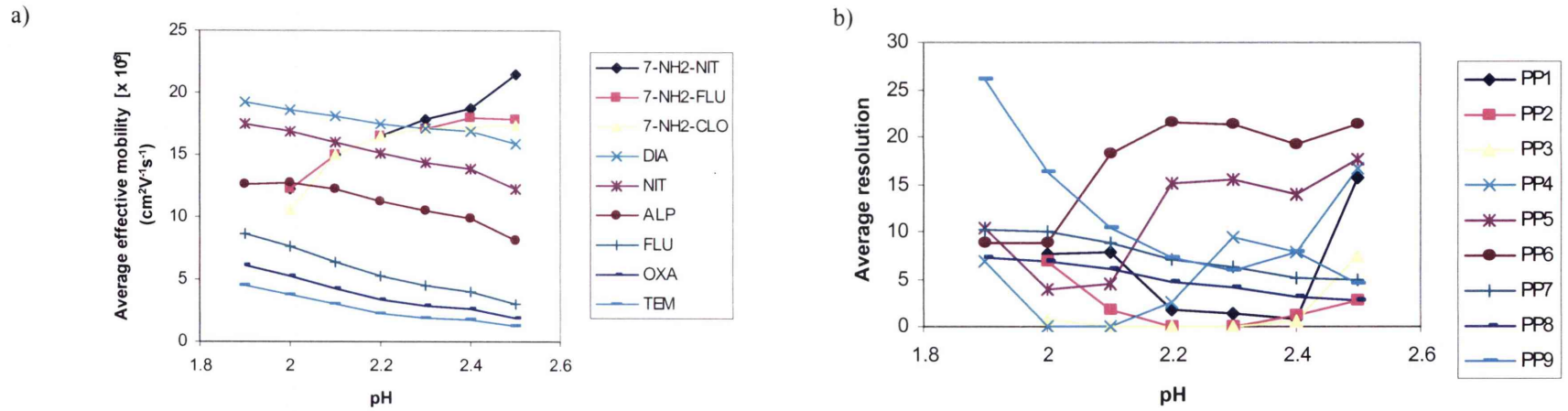


Figure 3-5 Influence of pH on a) average effective mobilities and b) average resolution for each analyte in 100 mM phosphate buffer on a coated capillary

Figure 3-5 illustrates the average resolutions and effective mobilities of the nine analytes with varying pH. In general, an increase in pH correlated to a reduction in effective mobility for the six parent benzodiazepines. Only the 7-aminobenzodiazepines showed an increase in effective mobility. This can be attributed to the low pK_a values of the parent compounds. Even at pH 1.9, these analytes were hardly protonated, resulting in long migration times. As the pH was increased, the effect was even more pronounced and they began to migrate with the EOF. Consequently, a decrease in effective mobility for the parent analytes was observed with increasing pH. Resolutions between consecutive peaks and product resolutions (PR_s) for the separation at each condition are presented in Table 3-5. Once again, poor peak shape was observed for the 7-aminobenzodiazepines and alprazolam at pH 1.9, making it difficult to accurately determine the migration times and peak widths, so this condition was omitted from the Table. The effect of pH on resolution was variable, with some peak pairs showing greater resolution as pH was increased, and others tending to display reduced resolution. This was largely a result of the effect of pH on effective mobility discussed above. At low pH, the 7-amino metabolites tended to co-migrate as one peak after diazepam, nitrazepam and alprazolam. However, as the pH was increased and the effective mobilities of the three metabolites increased relative to the six parent compounds, they began to migrate first. This change in migration order makes it difficult to assess trends in resolution. At pH 2.3 and below, $PR_s = 0$, due to co-migration of the 7-amino metabolites. As can be seen in Table 3-5, the maximum PR_s, and hence the best separation in 100 mM phosphate buffer occurred at pH 2.5.

Considering all the conditions evaluated on the coated capillary (BGE concentration 25 – 100 mM, pH 1.9 – 2.5), the best separation was found to occur in 100 mM phosphate buffer at pH 2.5. Under these conditions, basline separation of all nine analytes was possible in a run time of less than 6.5 minutes. An example of an electropherogram obtained under these conditions is illustrated in Figure 3-6.

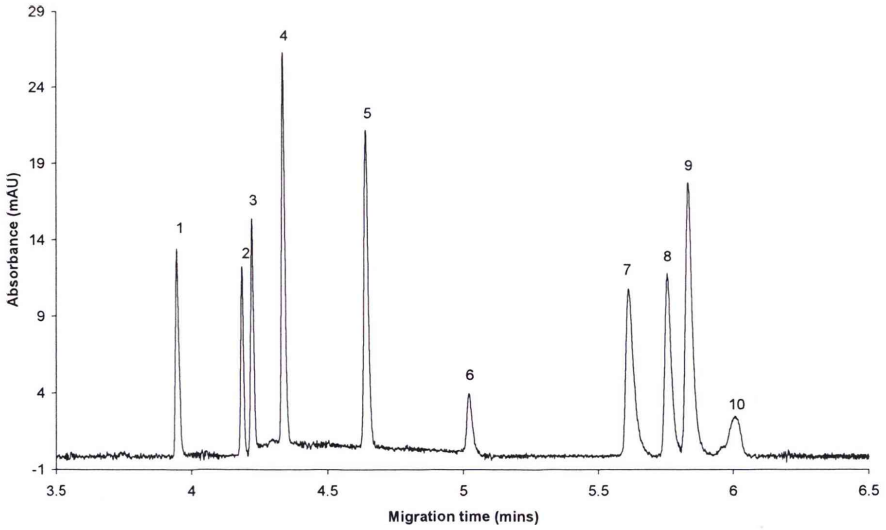


Figure 3-6 Example electropherogram of a standard mixture of nine benzodiazepines on a coated capillary

Conditions: 60 cm x 50 μm PDDAC/DS coated capillary, 100 mM phosphate buffer, pH 2.5, applied voltage 28 kV, temperature 25 °C, injection 2s at 50 mbar, detection λ 200 nm; **Migration order:** 1. 7-NH_2-NIT (t_m 3.948), 2. 7-NH_2-FLU (t_m 4.186), 3. 7-NH_2-CLO (t_m 4.223), 4. DIA (t_m 4.337), 5. NIT (t_m 4.641), 6. ALP (t_m 5.022), 7. FLU (t_m 5.614), 8. OXA (t_m 5.757), 9. TEM (t_m 5.834), 10. EOF (t_m 6.007)

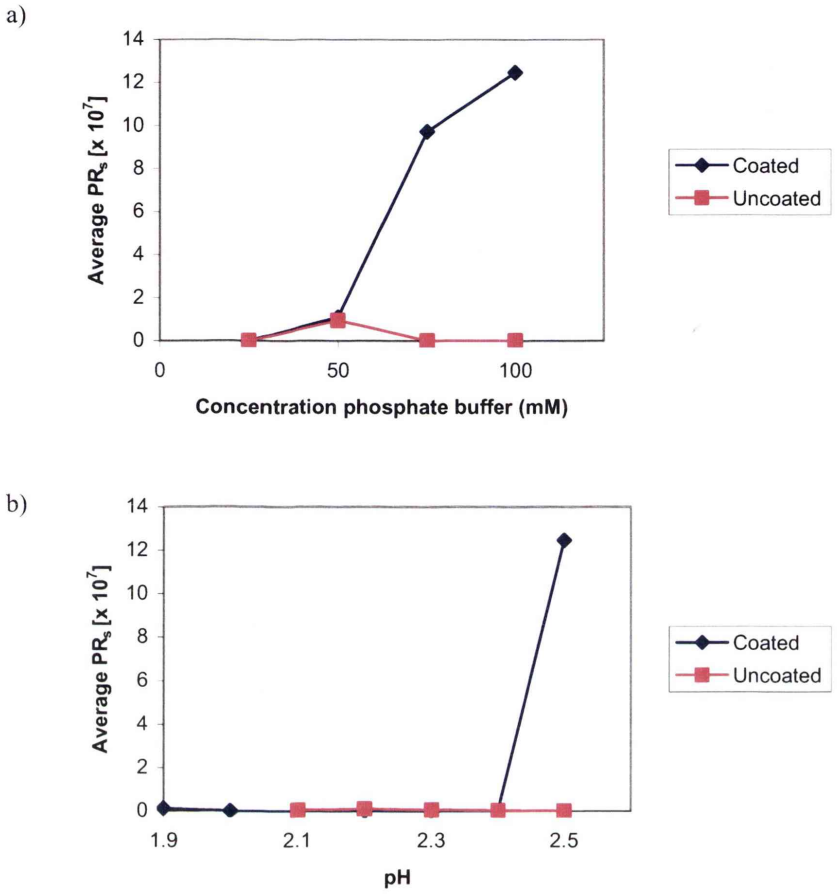
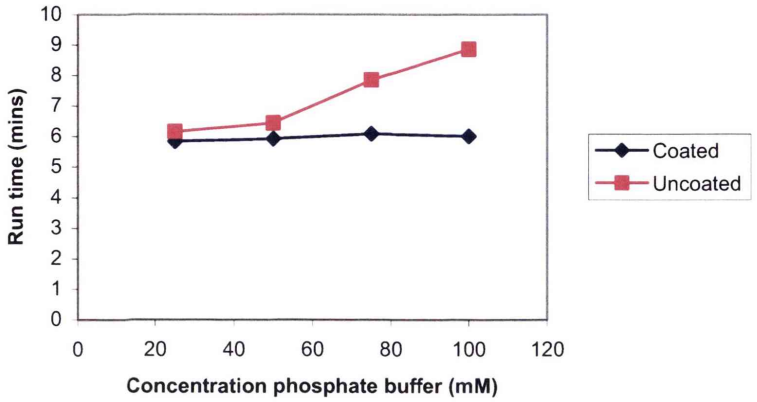


Figure 3-7 Influence of a) phosphate buffer concentration and b) pH on average product resolution for coated and uncoated capillaries

Compared with an uncoated bare fused-silica capillary, separations performed on the coated capillary were superior in terms of resolution, as shown in Figure 3-7. While comparable resolutions were obtained in 25 and 50 mM phosphate buffer, the coated capillary gave a significantly better separation at 75 and 100 mM. And although slightly better separations were obtained on an uncoated capillary below pH 2.4, there was a dramatic increase in product resolution at pH 2.5. Both these observations were attributable to the baseline separation of 7-aminoflunitrazepam and 7-aminoclonazepam on the coated capillary. This was not achievable on an uncoated capillary under any of the conditions.

a)



b)

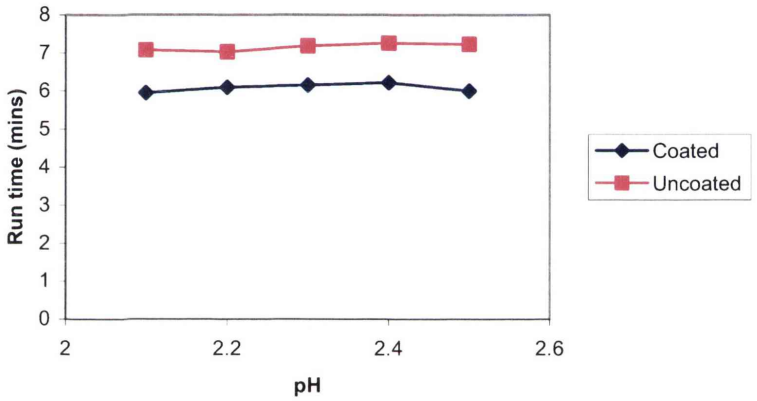


Figure 3-8 Influence of a) phosphate buffer concentration and b) pH on run time for coated and uncoated capillaries

In addition to improved resolution, the coated capillary gave a faster run time than the bare fused-silica capillary under all BGE concentrations and pHs. This is graphically depicted in Figure 3-8. The run time on the uncoated capillary was affected by the BGE concentration, showing a steady increase as the BGE concentration was increased. In contrast, the run time on the uncoated capillary remained relatively constant at around 6 minutes. pH had little effect on the run times of either the coated or uncoated capillaries, however the coated capillary improved the run time by about one minute, or around 17%. Whilst not a dramatic

improvement, it would become more significant during routine analysis when there is a high sample throughput.

This method compares favourably with the other CZE methods for benzodiazepine analysis. The CZE method developed by McGrath *et al.* [398] for the analysis of chlordiazepoxide, diazepam, flurazepam and nitrazepam had a total analysis time of 8 minutes for the four analytes when employing a 20 mM citric acid + 15% MeOH BGE. McClean *et al.* [272] described a CZE method in which fifteen benzodiazepines were analysed in 20 mM citric acid + 15% MeOH at pH 2.5. Under these conditions, only twelve analytes could be resolved within 20 minutes using DAD, and many of these did not have baseline resolution. Clearly, the current method, which is able to baseline resolve nine benzodiazepines, offers considerable advantages in terms of analysis time and resolution when compared with previous CZE methods. Furthermore, the present method has a shorter run time than many of the MEKC methods presented in Table 3-1 and Table 1-12, when consideration is given to the number of analytes studied. Compared to these MEKC methods, the CZE method presented here offers the advantage of BGE simplicity as well as a shorter analysis time.

The analysis of benzodiazepines by CE using doubly-coated capillaries has been previously reported [297]. The method, described by Vanhoenacker *et al.,* utilised the CEofix buffer system for the preparation of the coated capillaries, and MS detection to achieve the separation of six benzodiazepines in a run time of around 12 minutes. The method presented here for the separation of nine benzodiazepines on a PDDAC/DS coated capillary in 100 mM phosphate buffer at pH 2.5 permits the separation of a larger number of analytes in half the run time of the previously reported method. Whilst MS capabilities allow for better LODs compared with DAD, the coupling of CE with MS is not always financially viable, and it is of benefit to have a fast CE method that employs the more common DAD.

3.4.2.1 Stability of the EOF

In contrast to some previous findings [370, 371], the EOF of the doubly-coated PDDAC/DS capillary was not reproducible and tended to decrease as more injections were performed. This finding was in agreement with Kelly *et al.* [387], who also found a large amount of variation in the EOF. The likely reason for this seems to be the gradual removal of DS from the surface of the capillary wall by the running buffer during analyses. In an attempt to regenerate the DS coating, the capillary was periodically flushed with 1.5% DS solution. While this initially seemed successful in restoring the EOF, the improvement was only temporary, and subsequent analyses once again saw the EOF steadily decreasing. Kelly *et al.* suggested that running buffer containing a small amount of DS may reduce variation in the EOF by preventing the removal of the DS coating during analysis. This approach was investigated in the range 0 – 2% DS in 100 mM phosphate buffer at pH 2.5. EOF values were determined by an injection of acetone as a neutral marker, with ten replicates performed at each concentration, and the coefficient of variation (%CV) of the migration time was calculated. As indicated in Figure 3-9, the %CV declined as the percentage of DS in the running buffer was increased. A minimum %CV of 0.029% was obtained at 1.5% DS. At concentrations of DS above 1.5%, the %CV began to increase. Thus, it would appear that instability of the EOF on the PDDAC/DS coated capillary is largely due to removal of DS from the capillary surface, and that the addition of DS to the running buffer can help combat this problem.

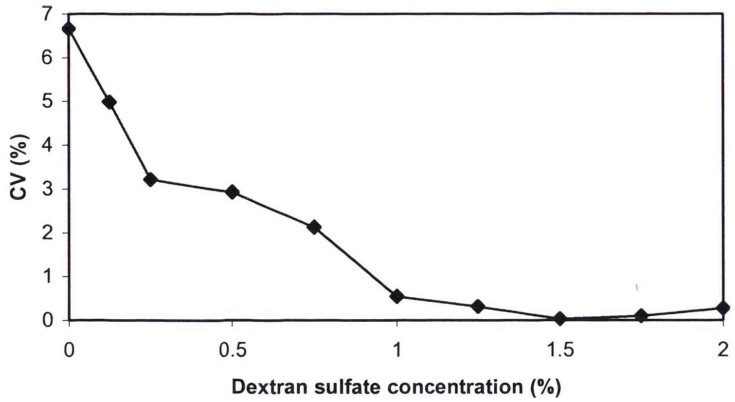


Figure 3-9 Effect of DS in running buffer on the coefficient of variation (%) of the EOF

The preliminary testing conditions were then applied to the separation of a standard mixture of the nine benzodiazepines. 1.5% DS was added to the running buffer (100 mM phosphate buffer, pH 2.5) and ten replicate injections were performed. Unfortunately, the addition of DS interfered with the separation mechanism, causing distorted peak shapes and co-migration of most of the analytes with the EOF. This is likely due to the positively charged benzodiazepines interacting with the negatively charged DS to form neutral species which, being uncharged, migrate with the EOF. As such, the best approach for maintaining a reproducible EOF when analysing positively charged species on the PDDAC/DS coated capillary seems to be periodic flushing with DS.

3.4.3 Investigation of Cyclodextrins

It has been found that in aqueous media, the cavity size of β-cyclodextrins most favourably accommodates the aromatic ring structures of benzodiazepine molecules [351]. With this in mind, three cyclodextrins, β-cyclodextrin (βCD), heptakis-(2,3-di-*O*-methyl)-β-cyclodextrin (DMβCD) and heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TMβCD), were added to the running buffer (100 mM phosphate buffer, pH 2.5) and investigated on both a PDDAC/DS coated capillary and a bare fused-silica capillary in the range 5 – 25 mM. The purpose was to determine if the

combination of a doubly-coated capillary with cyclodextrins offered any advantage in the analysis of benzodiazepines as compared with the use of a coated capillary alone, and/or if cyclodextrins applied with a bare-fused silica capillary offered any resolution or run time improvements as compared to either the coated or uncoated capillaries alone.

All cyclodextrins studied on the PDDAC/DS coated capillary had minimal effect on the average effective mobilities of each analyte and no effect on the migration order. The average resolution values for consecutive peaks at each condition on a coated capillary are presented in Table 3-6. Figure 3-10 illustrates the influence of cyclodextrin type and concentration on the product resolution (PR_s) of the separation. For both TMβCD and DMβCD, the maximum PR_s was obtained at a CD concentration of 5 mM. However, DMβCD at this concentration had limited success in resolving all nine analytes, as the separation between oxazepam and temazepam (PP8) was incomplete. Increasing the concentration of TMβCD and DMβCD above 5 mM only served to reduce the PR_s, and hence decrease separation. In both cases, the reduction in PR_s at higher concentrations of CD was a result of decreased resolution between oxazepam and temazepam (PP8). This is likely to be due to a greater interaction of oxazepam and temazepam with TMβCD and DMβCD occurring at higher cyclodextrin concentrations. 10 mM βCD gave the greatest separation on the PDDAC/DS coated capillary. A further increase in CD concentration to 25 mM however, saw a reduction in the PR_s due to decreased resolution between oxazepam and temazepam (PP8). βCD is less hydrophobic compared to the other cyclodextrins and has greater affinity for the moderately protonated analytes at pH 2.5, which may explain its effectives over TMβCD and DMβCD.

Table 3-6 Average resolutions for consecutive peaks with varying type and concentration of cyclodextrin on a coated capillary

	Conc (mM)	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8	PP9	PRs ($x10^7$)
TMβCD	5	7.267	1.882	5.374	16.781	16.559	18.110	3.426	1.570	3.867	0.7691
	10	8.429	1.600	4.465	17.689	17.627	18.756	3.275	1.300	3.390	0.5083
	25	9.089	1.717	5.056	17.729	16.477	16.471	3.248	1.133	3.240	0.4527
DMβCD	5	10.092	1.615	9.717	14.917	12.859	21.968	3.432	0.758	2.371	0.4116
	10	15.330	3.110	17.85	14.410	9.692	19.521	3.994	0.000	1.944	0.000
	25	18.452	3.505	30.11	14.400	5.728	16.143	2.450	0.000	0.063	0.000
βCD	5	12.454	2.575	5.549	13.558	14.811	19.892	3.736	1.492	2.907	1.152
	10	13.004	3.634	5.936	12.959	14.671	20.546	4.799	1.509	2.970	2.357
	25	12.902	3.946	7.322	14.169	12.260	18.122	5.443	0.544	2.403	0.8355

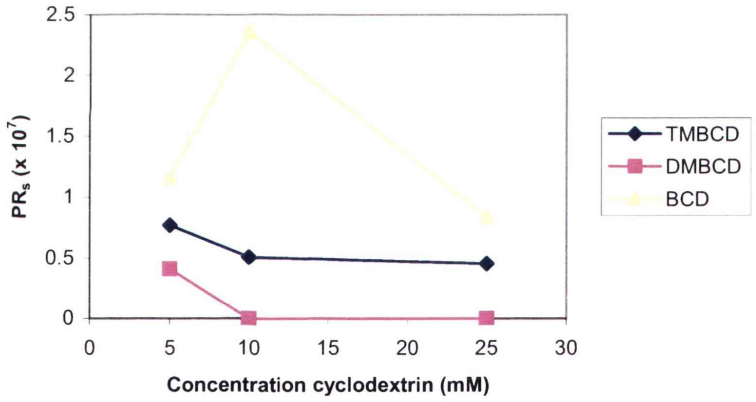


Figure 3-10 Influence of cyclodextrin type and concentration on product resolution (PR_s) for a coated capillary

Table 3-7 Average resolutions for consecutive peaks with varying type and concentration of cyclodextrin on an uncoated capillary

	Conc (mM)	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8	PP9	PRs ($x10^7$)
TMβCD	5	8.098	0.986	4.349	9.049	13.366	15.859	2.101	0.778	2.028	0.0221
	10	7.662	0.909	4.217	9.251	10.367	16.209	2.113	0.772	1.998	0.0149
	25	8.769	0.000	3.955	10.332	14.505	13.243	2.308	0.666	1.988	0.0000
DMβCD	5	8.696	3.546	14.22	18.808	17.979	25.232	3.068	0.571	2.606	1.709
	10	19.375	2.583	23.85	17.620	13.951	30.719	5.766	0.000	2.721	0.000
	25	23.463	5.200	28.07	10.346	1.614	19.687	2.267	0.000	1.573	0.000
βCD	5	8.794	1.043	5.708	9.821	13.720	21.507	6.630	3.345	6.971	2.347
	10	10.210	1.394	6.839	11.131	24.775	16.963	5.963	1.976	7.558	4.055
	25	6.279	2.937	4.836	10.617	11.030	17.115	3.705	0.497	2.905	0.0956

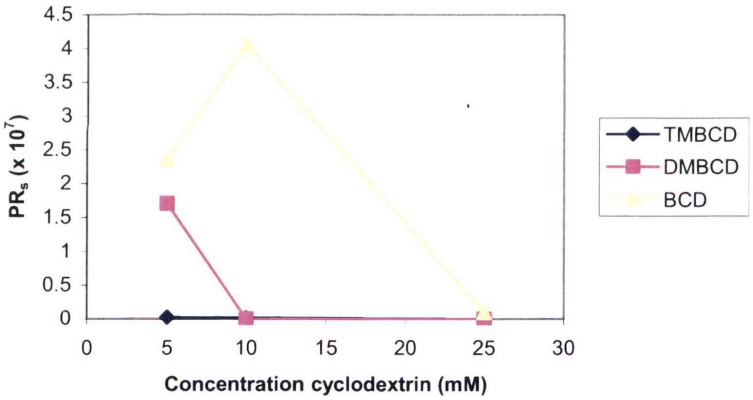


Figure 3-11 Influence of cyclodextrin type and concentration on product resolution (PR_s) for an uncoated capillary

The average resolution values between consecutive peaks on a bare fused-silica capillary with added cyclodextrins are presented in Table 3-7. As with the coated capillary, the addition of cyclodextrins to the running buffer had no effect on the migration order and minimal effect on the average effective mobilities of each analyte. Figure 3-11 highlights the effect of cyclodextrin type and concentration on the product resolution (PR_s) of the separation. TMβCD was unsuccessful in resolving

the nine benzodiazepines at any of the concentrations studied, due to insufficient separation between 7-aminoflunitrazepam and 7-aminoclonazepam (PP2), and oxazepam and temazepam (PP8). DMβCD had limited success at a concentration of 5 mM, however the resolution between oxazepam and temazepam (PP8) was <1, indicating incomplete resolution. βCD at a concentration of 10 mM was effective at separating all nine analytes, although the resolution between 7-aminoflunitrazepam and 7-aminoclonazepam (PP2) was <1.5, indicating the two analytes were not baseline resolved. While the resolution between these two analytes improved as the concentration of βCD was increased, the resolution between oxazepam and temazepam (PP8) decreased significantly, rendering higher concentrations inappropriate.

In order to compare the effectiveness of the investigated cyclodextrins in separating the nine benzodiazepines, the maximum product resolution obtained for each cyclodextrin on both coated and uncoated capillaries was compared, as illustrated in Figure 3-12. For TMβCD and DMβCD, this occurred at a concentration of 5 mM, while for βCD, the maximum PR_S was at 10 mM. Clearly, the PDDAC/DS coated capillary in the absence of any cyclodextrins gave the best separation, with none of the cyclodextrins offering any improvement in the resolution that had already been obtained. 10 mM βCD with an uncoated capillary gave the next best separation, followed by 10 mM βCD in conjunction with the PDDAC/DS coated capillary. Although not offering any advantage over the coated capillary alone, 10 mM βCD in the running buffer was more successful in resolving the nine analytes than the use of a bare fused-silica capillary alone.

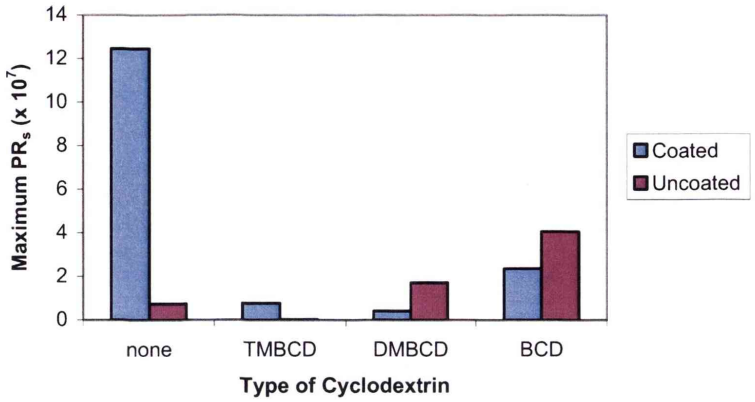


Figure 3-12 Maximum product resolution (PR_s) attained for each type of cyclodextrin on coated and uncoated capillaries

The run times obtained with and without cyclodextrins on both coated and uncoated capillaries are compared in Figure 3-13. The coated capillary without the use of cyclodextrins gave fastest run time. No advantage in run time was gained with the addition of cyclodextrins to the running buffer. The interaction of the benzodiazepines with the cyclodextrin cavity is no doubt responsible for the increase in analysis time. However, the effect is not great, since at pH 2.5 the analytes are mostly protonated and strong interactions with the cyclodextrins would not be expected.

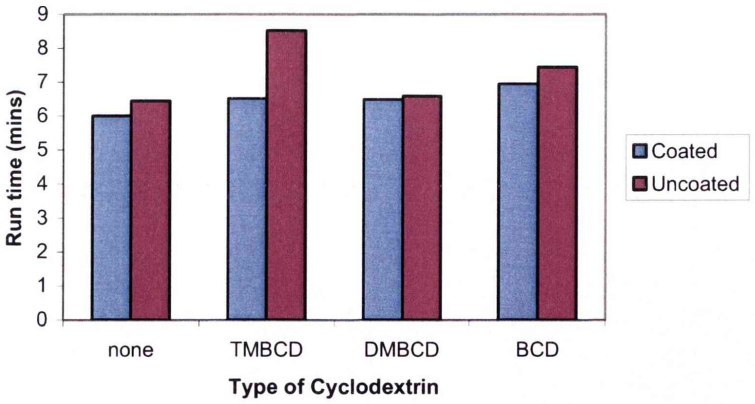


Figure 3-13 Run times for each type of cyclodextrin on coated and uncoated capillaries

The use of cyclodextrins in conjunction with doubly-coated capillaries for the analysis of benzodiazepines has not been reported previously, however Lurie *et al.* [384] detailed the use of a dynamically coated capillary with the addition of cyclodextrins in the run buffer at pH 2.5 for the analysis of moderately basic seized drugs. They found that the cyclodextrins not only allowed for chiral analysis, but that it also enhanced separation for achiral solutes. In another study [386], the same authors found that the combination of a dynamically coated capillary with cyclodextrin in the running buffer was well suited to the analysis of basic solutes in heroin, as it improved selectivity and precision, and reduced analysis time. While these studies demonstrate success in resolving basic analytes by using doubly-coated capillaries in combination with cyclodextrins, the findings of this study disagree with these results. Better separation was attained on the doubly-coated capillary alone, than when cyclodextrins were added to the running buffer. On the coated capillary, cyclodextrins only served to reduce to resolution between analytes. However, the addition of 10 mM βCD in the running buffer on an uncoated capillary improved the separation that had previously been obtained on this type of capillary.

One notable effect was observed with all cyclodextrins; that is, there was splitting of the alprazolam peak. Since ring-opening of alprazolam can occur in acidic medium [257], it is possible that alprazolam exists in two forms – an opened-ring and a closed-ring structure. These two forms are likely to interact differently with the added cyclodextrins, thus becoming partially separated and producing the peak-splitting effect. The partial separation of two chiral forms of alprazolam is another possible explanation for this peak-splitting effect. Obviously, this unwanted effect should be considered before implementing β-cyclodextrins in separations involving alprazolam in acidic medium. However, the addition of βCD to the running buffer still remains a useful alternative for improving the separation of benzodiazepines on uncoated capillaries.

3.4.4 Method Validation

Given that the best separation was attained on a PDDAC/DS coated capillary with 100 mM phosphate buffer at pH 2.5 as the running buffer, this method was validated for aqueous standards, and the calibration data for each analyte is presented in Table 3-8. Calibration curves were linear in the specified concentration ranges and correlation coefficients (r^2) ranging from 0.9891 – 0.9976 were established.

Accuracy and repeatability of peak areas and migration times were determined by analysis of five replicate samples at high and low concentration. As shown in Table 3-9, the peak area repeatability was in the order of 0.9 – 9.7% RSD (relative standard deviation) and the migration time repeatability was 0.3 – 0.8% RSD. Accuracies were greater than 95%.

Table 3-8 Calibration and LOD data

Analyte	Correlation coefficient (r^2)	Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
7-aminonitrazepam	0.9972	17.0 – 90.9	4.0	13.3
7-aminoflunitrazepam	0.9976	17.0 – 90.9	3.5	11.7
7-aminoclonazepam	0.9933	17.0 – 90.9	2.7	9.0
Diazepam	0.9976	17.0 – 90.9	3.0	10.1
Nitrazepam	0.9960	17.0 – 90.9	3.4	11.4
Alprazolam	0.9891	51.1 – 272.7	41.5	138.2
Flunitrazepam	0.9949	17.0 – 90.9	6.6	21.9
Oxazepam	0.9946	17.0 – 90.9	4.2	13.9
Temazepam	0.9938	17.0 – 90.9	4.8	16.1

Table 3-9 Accuracy and precision data

Analyte	Concentration (μg/mL)	Accuracy (%)	RSD peak areas (%)	RSD migration times (%)
7-aminonitrazepam	90.9	99	0.9	0.5
	17.0	97	2.6	0.5
7-aminoflunitrazepam	90.9	101	1.0	0.5
	17.0	110	3.2	0.4
7-aminoclonazepam	90.9	100	0.8	0.6
	17.0	111	6.1	0.4
Diazepam	90.9	101	0.9	0.6
	17.0	112	5.6	0.4
Nitrazepam	90.9	100	2.8	0.6
	17.0	108	1.3	0.3
Alprazolam	272.7	95	3.1	0.8
	150.0	102	3.9	0.3
Flunitrazepam	90.9	101	3.0	0.8
	22.7	105	9.7	0.4
Oxazepam	90.9	97	3.6	0.8
	17.0	115	6.7	0.5
Temazepam	90.9	103	2.0	0.8
	17.0	119	9.6	0.5

As shown in Table 3-8, the limit of detection (LOD) for each compound ranged from 2.7 μg/mL (7-aminoclonazepam) to 41.5 μg/mL (alprazolam), while the limit of quanitification (LOQ) was between 9.0 μg/mL (7-aminoclonazepam) and 138.2 μg/mL (alprazolam). Typical toxic benzodiazepine concentrations are in the order of 1 to 5 μg/mL, with some (e.g. flunitrazepam) 100-fold lower (Refer to Table 1-13). This method, and CE-DAD in general, lacks the sensitivity required for the analysis of authentic biological samples. This is mainly due to the small injection volumes and small optical path lengths for UV detection. While the method may useful in cases of large fatal overdoses where concentrations are in the order of 15 to 30 μg/mL, it would not be suitable for the analysis of benzodiazepines at therapeutic and toxic concentrations.

3.4.5 Application of the Method

Given that the LODs of the method were significantly greater than the concentrations one would expect to find in therapeutic or even toxic instances of benzodiazepine administration, the method was instead applied to spiked beverages.

The term 'drink spiking' refers to drugs or alcohol being added to a drink without the consent of the person consuming it [407]. The spiking substance, commonly benzodiazepines such as flunitrazepam, alprazolam, diazepam, temazepam and oxazepam [408, 409], can be added to both alcoholic and non-alcoholic drinks. The effects can range from vomiting to loss of consciousness, and can also include poor coordination and balance, slurred speech, muscle spasms, respiratory difficulties and loss of control. Often the victim will have lasting anterograde amnesia for events that occur under the influence of the drug [409]. For an incident to be defined as drink spiking, it need not involve further criminal victimisation, although such offences, including drug facilitated sexual assault and robbery, can occur [410]. A recent report by the Australian Institute of Criminology (AIC) [410] estimated that there were between 3000 and 4000 suspected incidents of drink spiking across Australia in the 2002/03 financial year, with approximately one third of incidents estimated to have been associated with sexual assault. Since the AICs report, there has been much

public attention in Australia surrounding the issue of drink spiking. Previously, there was no separate offence category in any Australian jurisdiction for the act of spiking someone's drink, however the AICs report has prompted the NSW Government to create a new summary offence of drink spiking, which will attract a maximum penalty of two years in jail. The Federal Government is also drafting national laws, which are expected to be announced in mid-2005, and are expected to outlaw drink spiking throughout the country.

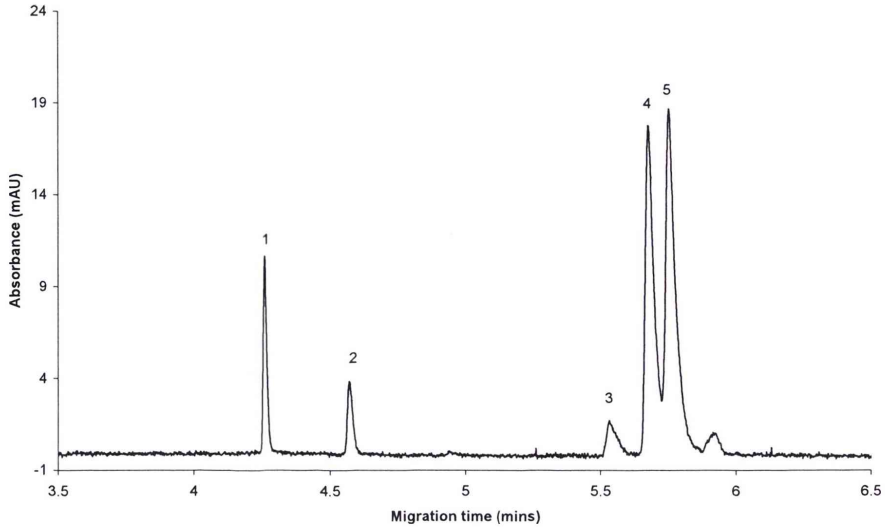
While there have been a number of studies focusing on the analysis of biological samples after the administration of spiked beverages, only a handful of studies have focused on the analysis of the spiked beverage itself. Bishop *et al.* [192] applied a MEKC method with an analysis time of less than 14 minutes for the analysis of benzodiazepines and γ-hydroxybutyric acid (GHB) in a number of beverages. The main disadvantage of this method is that it requires an ethyl acetate LLE, which is quite time consuming and messy. Rao *et al.* [411] employed a HPLC method for the determination of alprazolam, diazepam and chloralhydrate in the alcoholic beverage toddy. The analysis time was 15 minutes and the only sample pre-treatment required in this instance was filtration through 0.45 μm Nylon membranes.

Given the recent concern regarding drink spiking, the PDDAC/DS coated method was applied to the analysis of beverages spiked with diazepam, nitrazepam, alprazolam, flunitrazepam, temazepam and oxazepam. A variety of drinks frequently consumed at bars and parties, including Bacardi™ rum, Victorian Bitter™ beer, Jacob's Creek™ Semillon Sauvignon Blanc 2003 white wine, Coca-cola™, Jim Beam™ bourbon and Just Juice™ orange juice, were chosen for the analysis. Beverages were spiked with each drug at a concentration designed to simulate a prescription tablet. Information regarding tablet strengths was obtained from the manufacturers' websites and a standard drink was assumed to be 200 mL. Thus, the following final concentrations were obtained: diazepam 50 μg/mL, nitrazepam 25 μg/mL, alprazolam 10 μg/mL, flunitrazepam 25 μg/mL temazepam 150 μg/mL and

oxazepam 150 μg/mL. Beverages were analysed by direct injection, following filtration through 0.45 μm Nylon filters where necessary. Recovery was calculated for each analyte as the average peak area in the spiked samples as a percentage of the average peak area in aqueous standards. Example electropherograms of each beverage, both spiked and blank, are illustrated in Figure 3-14 to Figure 3-19.

In the case of spiked Bacardi (Figure 3-14), the sample matrix was very clean and there were no interfering peaks. This was also the case for bourbon (Figure 3-15) and Coca-cola (Figure 3-16), which only exhibited one peak corresponding to the EOF. In these beverages, there were no difficulties with peak assignment and each analyte could be quantified. However, the sample matrices for spiked wine (Figure 3-17), beer (Figure 3-18) and orange juice (Figure 3-19) were more complicated. In the case of wine, there was a small peak at t_m = 4.5 mins in the blank sample, which also corresponds to the migration time of nitrazepam. As a result, nitrazepam could not be accurately quantified in this sample. While the beer and orange juice also had complex electropherograms, there was no peak overlap between any of the matrix components and the analyte peaks in either of these cases, thus permitting the quantification of each analyte.

a)



b)

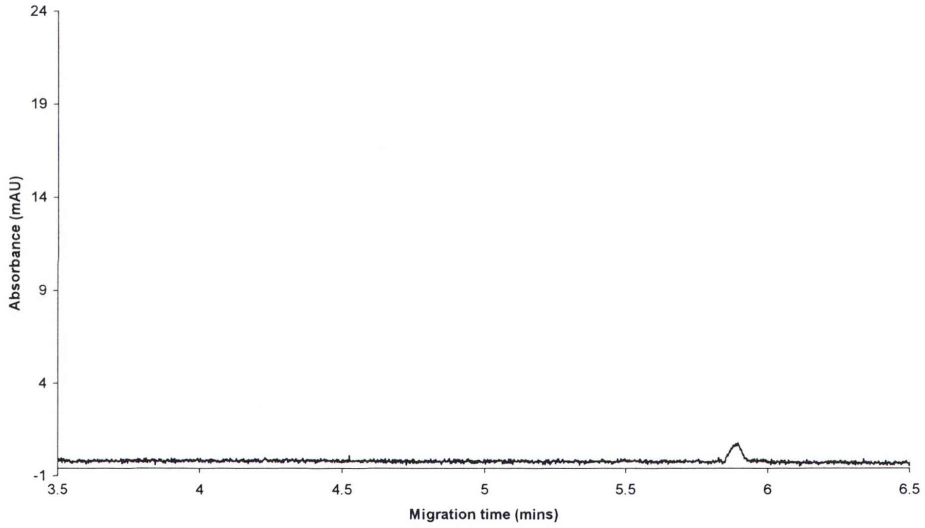


Figure 3-14 Example electropherograms of a) spiked Bacardi and b) blank Bacardi

Conditions: 60 cm x 50 μm PDDAC/DS coated capillary, 100 mM phosphate buffer, pH 2.5, applied voltage 28 kV, temperature 25 °C, injection 2s at 50 mbar, detection λ 200 nm; **Migration order:** 1. DIA, 2. NIT, 3. FLU, 4. OXA, 5. TEM.

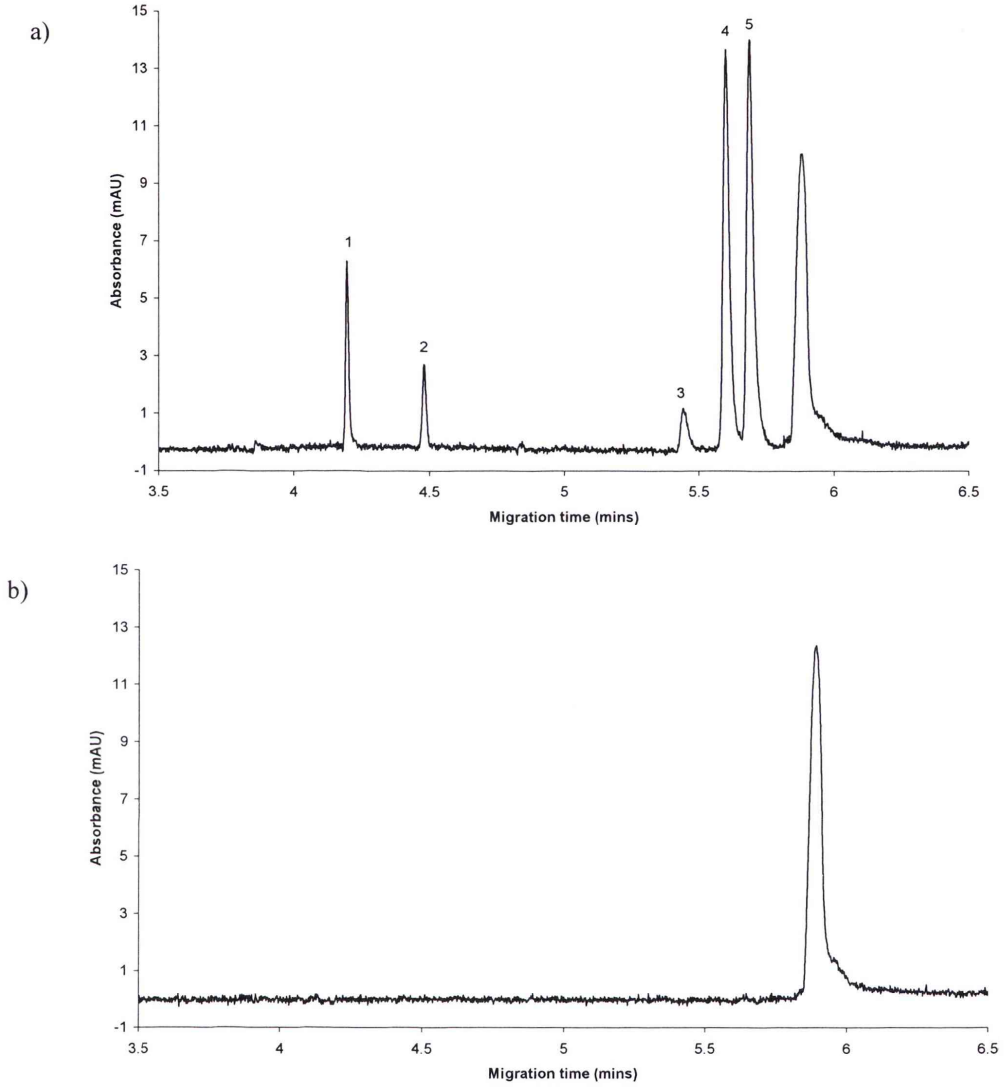


Figure 3-15 Example electropherograms of a) spiked bourbon and b) blank bourbon

Conditions: 60 cm x 50 μm PDDAC/DS coated capillary, 100 mM phosphate buffer, pH 2.5, applied voltage 28 kV, temperature 25 °C, injection 2s at 50 mbar, detection λ 200 nm; **Migration order:** 1. DIA, 2. NIT, 3. FLU, 4. OXA, 5. TEM.

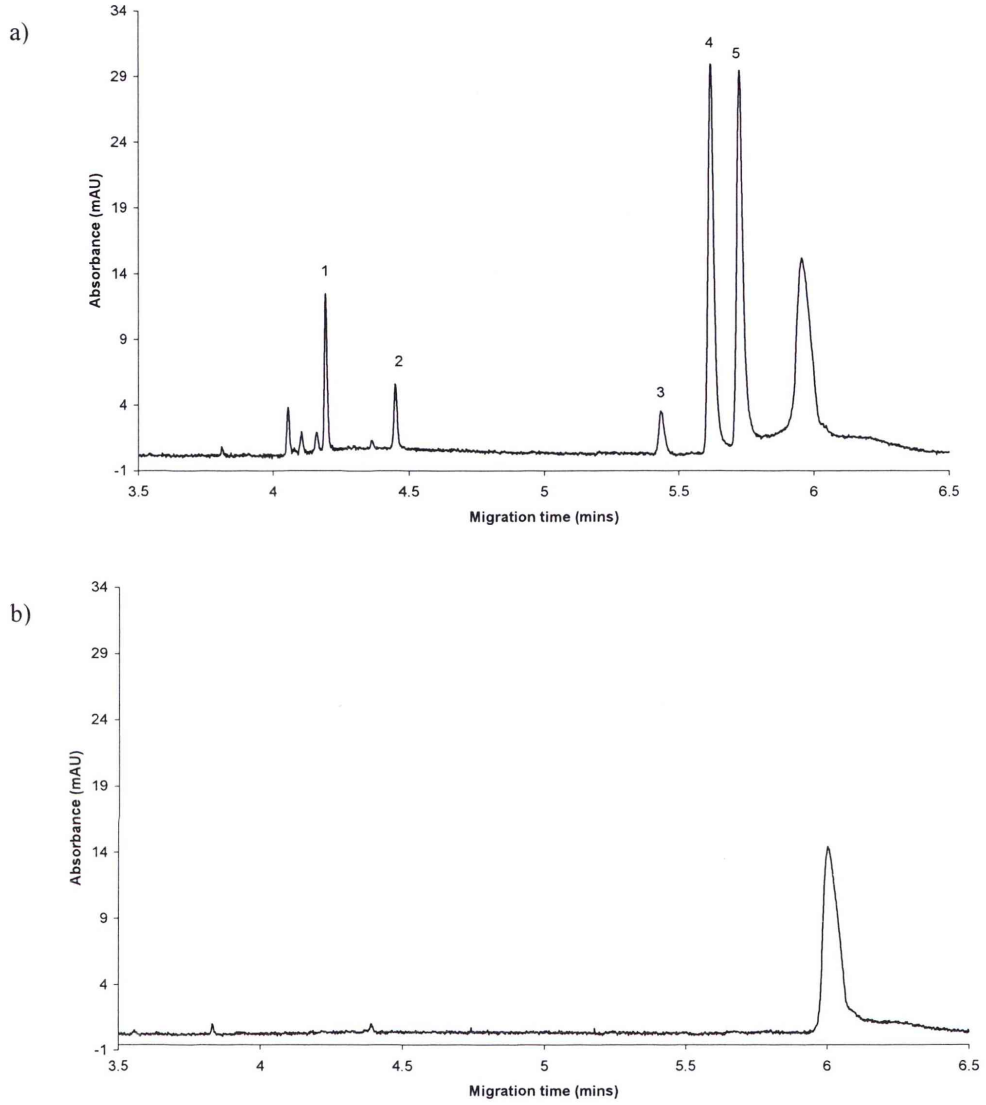
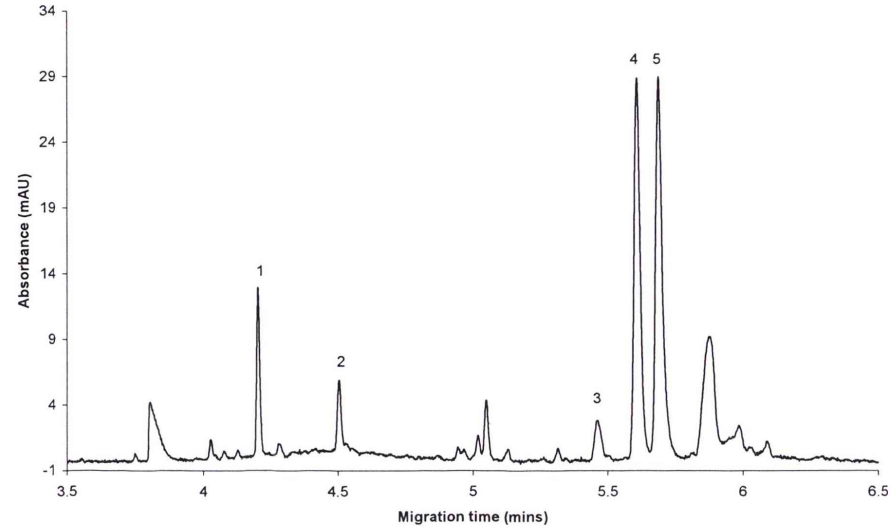


Figure 3-16 Example electropherograms of a) spiked Coca-cola and b) blank Coca-cola

Conditions: 60 cm x 50 μm PDDAC/DS coated capillary, 100 mM phosphate buffer, pH 2.5, applied voltage 28 kV, temperature 25 °C, injection 2s at 50 mbar, detection λ 200 nm; **Migration order:** 1. DIA, 2. NIT, 3. FLU, 4. OXA, 5. TEM.

a)



b)

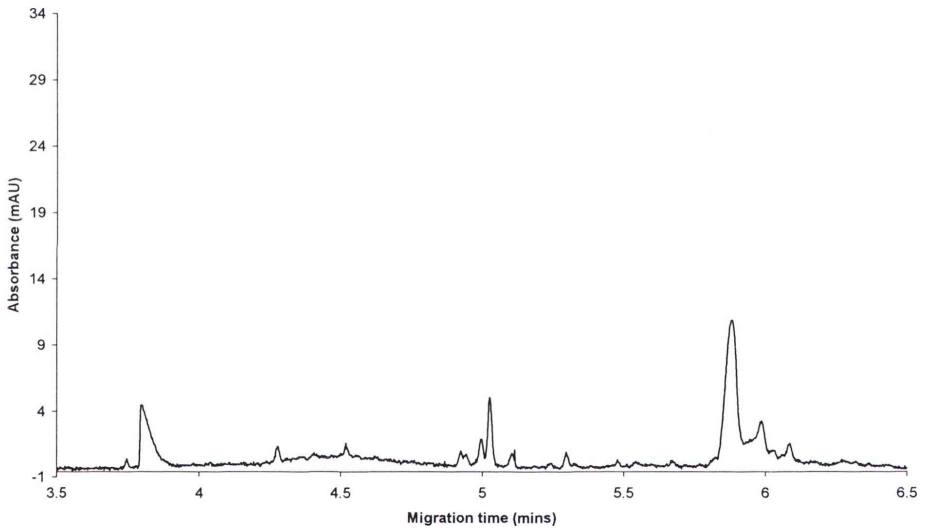
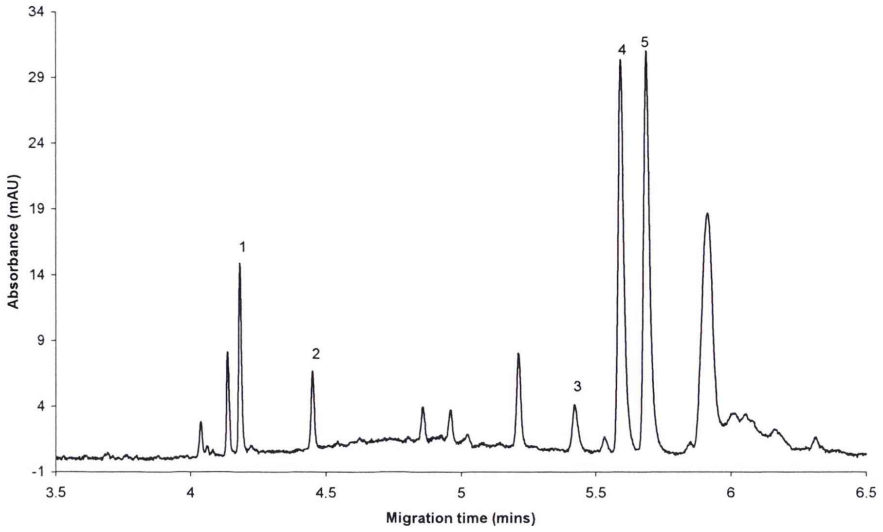


Figure 3-17 Example electropherograms of a) spiked wine and b) blank wine

Conditions: 60 cm x 50 μm PDDAC/DS coated capillary, 100 mM phosphate buffer, pH 2.5, applied voltage 28 kV, temperature 25 °C, injection 2s at 50 mbar, detection λ 200 nm; **Migration order:** 1. DIA, 2. NIT, 3. FLU, 4. OXA, 5. TEM.

a)



b)

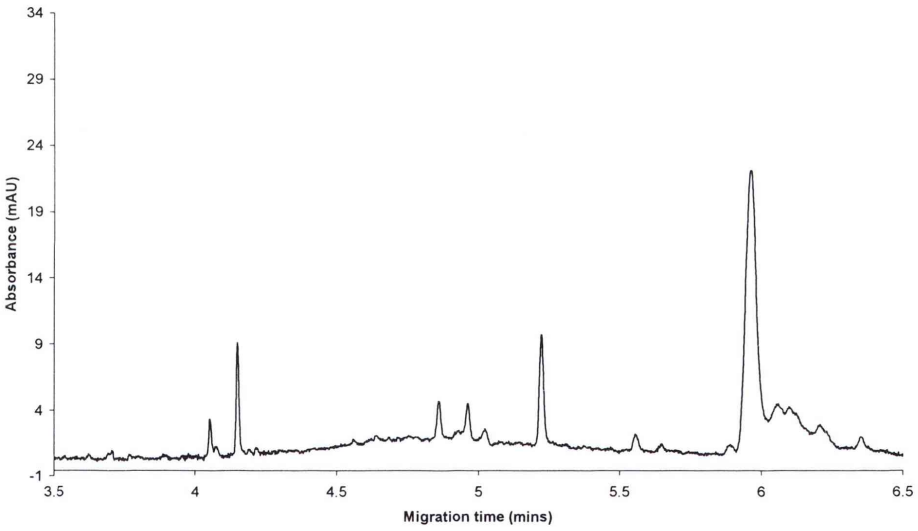


Figure 3-18 Example electropherograms of a) spiked beer and b) blank beer

Conditions: 60 cm x 50 μm PDDAC/DS coated capillary, 100 mM phosphate buffer, pH 2.5, applied voltage 28 kV, temperature 25 °C, injection 2s at 50 mbar, detection λ 200 nm; **Migration order:** 1. DIA, 2. NIT, 3. FLU, 4. OXA, 5. TEM.

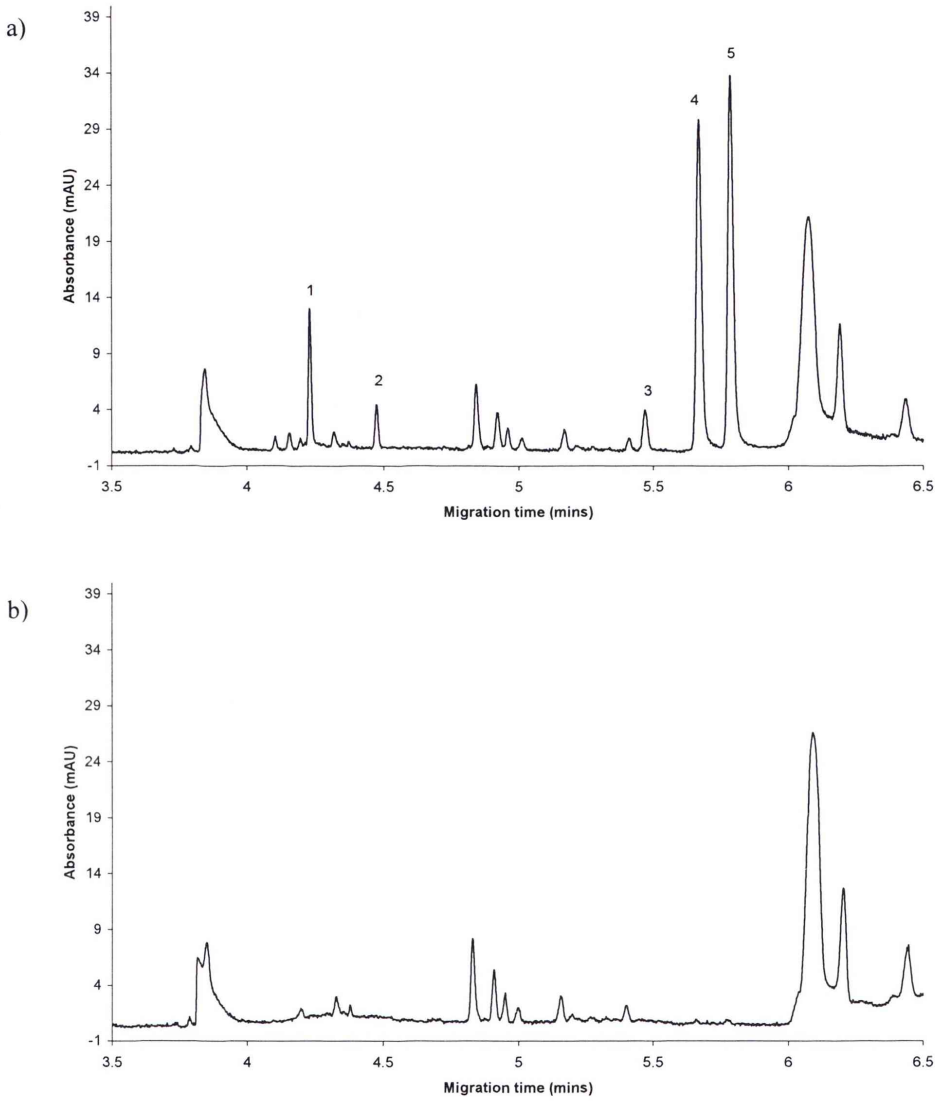


Figure 3-19 Example electropherograms of a) spiked orange juice and b) blank orange juice

Conditions: 60 cm x 50 μm PDDAC/DS coated capillary, 100 mM phosphate buffer, pH 2.5, applied voltage 28 kV, temperature 25 °C, injection 2s at 50 mbar, detection λ 200 nm; **Migration order:** 1. DIA, 2. NIT, 3. FLU, 4. OXA, 5. TEM.

The recovery data for each analyte is reported in Table 3-10. While the CZE-DAD method was capable of quantifying diazepam, nitrazepam, flunitrazepam, temazepam and oxazepam, it lacked the sensitivity required to detect alprazolam at a concentration designed to simulate a standard drink spiked with a prescription tablet of this particular drug.

Recoveries of 79.4 – 135.8% were obtained for Bacardi, 79.4 – 120% for white wine, 49.8 – 71.8% for bourbon, 75.9 – 110.4% for beer, 51.3 – 96.8% for orange juice and 65.2 – 101.7% for Coca-cola. The recoveries for bourbon and orange juice are somewhat lower than those obtained for the other beverages. In the case of orange juice, this may be due to the analytes adhering to particulate material in the juice, since the sample was filtered after spiking rather than before. In addition, the recovery of nitrazepam from spiked wine cannot be quoted with certainty, given the interfering component from the sample matrix. An extraction step could be performed to rectify these potential problems.

Table 3-10 Recovery of each drug from spiked beverages

Beverage	Compound	Concentration added (μg/mL)	Concentration found (μg/mL)	Recovery
Bacardi	Diazepam	50	46.9	93.8
	Nitrazepam	25	25.4	101.6
	Alprazolam	10	BLOD	n/a
	Flunitrazepam	25	34.0	135.8
	Temazepam	150	119.2	79.4
	Oxazepam	150	158.6	105.8
Wine	Diazepam	50	39.7	79.4
	Nitrazepam	25	20.7	82.7
	Alprazolam	10	BLOD	n/a
	Flunitrazepam	25	30.0	120.2
	Temazepam	150	130.7	87.1
	Oxazepam	150	152.3	101.5

Beverage	Compound	Concentration added (μg/mL)	Concentration found (μg/mL)	Recovery
Bourbon	Diazepam	50	28.6	57.2
	Nitrazepam	25	17.9	71.8
	Alprazolam	10	BLOD	n/a
	Flunitrazepam	25	13.3	53.3
	Temazepam	150	74.7	49.8
	Oxazepam	150	88.7	59.1
Beer	Diazepam	50	41.9	83.9
	Nitrazepam	25	19.0	75.9
	Alprazolam	10	BLOD	n/a
	Flunitrazepam	25	27.6	110.4
	Temazepam	150	131.0	87.3
	Oxazepam	150	149.4	99.6
Orange juice	Diazepam	50	35.4	70.7
	Nitrazepam	25	12.8	51.3
	Alprazolam	10	BLOD	n/a
	Flunitrazepam	25	21.9	87.6
	Temazepam	150	115.3	76.9
	Oxazepam	150	145.2	96.8
Coca-cola	Diazepam	50	34.0	68.1
	Nitrazepam	25	16.3	65.2
	Alprazolam	10	BLOD	n/a
	Flunitrazepam	25	25.4	101.7
	Temazepam	150	130.5	87.0
	Oxazepam	150	143.8	95.9

Abbreviations: below limit of detection (BLOD)

Compared to the other published methods for the analysis of spiked beverages [192, 411], the PDDAC/DS coated method offers considerably shorter run times. Another advantage of this method is that it does not require an extraction step. Direct injection was sufficient to detect and quantify five of the six benzodiazepines analysed, however the sensitivity was not adequate for the detection of alprazolam at 10 μg/mL. Given sufficient volume of a beverage, an extraction and concentration step may enable this drug to be detected and quantified.

3.5 Conclusions

Following investigation of BGE parameters on a PDDAC/DS dynamic doubly-coated capillary, a rapid CZE method for the simultaneous determination of nine benzodiazepines was developed. The selected BGE conditions were 100 mM ammonium phosphate buffer, pH 2.5, which gave baseline resolution between each analyte and a run time of less than 6.5 minutes. This method offers significant improvements both in terms of resolution and run time when compared with CZE analyses of benzodiazepines on bare fused-silica capillaries. It also represents a more rapid coated method for the analysis of benzodiazepines using DAD than has been previously reported. The validated method was successfully applied to beverages that had been spiked with benzodiazepines at concentrations simulating prescription tablets.

Cyclodextrins in conjunction with doubly-coated capillaries were not successful in improving the run time or separation of the nine benzodiazepines already obtained on the PDDAC/DS coated capillary. However, an uncoated capillary with a BGE of 10 mM βCD in 100 mM phosphate buffer, pH 2.5 was able to improve the resolution that had been obtained on a bare fused-silica capillary alone. While resolution values were still inferior to those obtained on the coated capillary, the addition of βCD to the running buffer represents a useful alternative to improve the separation of benzodiazepines.

3.6 Future Work

Due to small injection volumes and small optical path lengths for UV detection, CE-DAD lacks the sensitivity required for authentic biological samples. However, the method reported here has potential applications for the quantitation of benzodiazepines in biological matrices if sensitivity problems can be overcome. Future work should therefore centre on resolving this issue. One possibility for improving sensitivity may be the use of "bubble" or Z-shaped cells for detection. These cells effectively extend the light path of the detection window and can improve sensitivity 3- to 5-fold over standard capillaries by increasing the inner

diameter at the detection window. This permits the sensitivity of a wide inner diameter capillary and the low current generation of a narrow one [406], however it may also lead to band broadening. In these cases, the High Sensitivity Cell (Agilent Technologies) may be more appropriate since it is designed to minimise band broadening. Another possibility may be adaptation of the method to enable MS or MS-MS detection. In this case, the effects of dextran sulfate and cyclodextrin additives on both APCI and ESI interfaces demands further investigation.

It should be noted that the results obtained in the drink spiking study are specific to the particular brand of each beverage analysed. It is possible that there may be some variation in the matrices of beverages of different brands. Future work should be performed to determine if this is the case.

Chapter Four

Optimisation of Chromatographic Separations Using Artificial Neural Networks (ANNs) and a Novel Chromatographic Function: Application to the Gradient HPLC Separation of Benzodiazepines

4 Optimisation of Chromatographic Separations Using Artificial Neural Networks (ANNs) and a Novel Chromatographic Function: Application to the Gradient HPLC Separation of Benzodiazepines

4.1 Summary

Artificial neural networks (ANNs) were used in conjunction with experimental design to optimise a gradient HPLC separation of nine benzodiazepines. Using the best performing ANN, the optimum conditions predicted were 25 mM formate buffer (pH 2.8), 10% MeOH, ACN gradient 0 to 15 minutes, 6.5 to 48.5% (linear profile), which gave resolution values greater than 1.3 for each analyte pair and a run time of less than 13 minutes. The error associated with the prediction of retention times and peak widths under these conditions was less than 5% for six of the nine analytes. A novel chromatographic function, the Ideal Separation Function (ISF), was developed to incorporate run time, resolution and minimum retention into a single function designed to assess the quality of chromatographic separations. The ISF was found to offer significant advantages over other optimisation functions, which often fail to locate the optimum separation due to an inability to account for run time or detect poorly resolved peaks. The optimised method, with LODs in the range of 0.0057 – 0.023 μg/mL and recoveries between 58 – 92%, was successfully applied to authentic post-mortem samples. This method represents a more flexible and convenient means for optimising gradient elution separations using ANNs than has been previously reported.

Keywords: artificial neural networks (ANNs); gradient elution; optimisation; HPLC; benzodiazepines; optimisation functions

4.2 Introduction

As an analytical technique, high-performance liquid chromatography (HPLC) offers a number of advantages, including fast analysis times, high reproducibility and low

detection limits. However, the optimisation of HPLC separations in order to obtain satisfactory resolution, sensitivity and analysis times, can be a complicated and time-consuming process given the large number of parameters that may be varied, and the possibility of interactions between variables.

Chemometrics is concerned with optimising conditions in order to achieve the resolution of all analytes within a reasonable analysis time [412]. This can be achieved using "hard" models to predict retention behaviour, whereby knowledge of physico-chemical constants, such as pK_a values and pH stability data, is required [413]. Alternatively, a "soft" modelling or experimental approach can be employed, which does not require the formulation of mathematical models in order to achieve high levels of precision [414]. Experimental optimisation strategies can be classified as either univariate or multivariate approaches. The most common strategy involves a univariate approach in which each experimental parameter is varied in a selected range while keeping the other parameters constant. However, this approach has two main disadvantages. Firstly, although valuable information can be drawn from univariate investigation, the optimum conditions can only be attained by investigating all possible combinations of the factors, which can be time-consuming and costly. Secondly, interactions between two or more variables cannot be detected. If this is the case, the conditions selected may be far from the optimum ones, since the influence of any given variable depends on the magnitude of the other variables [412].

Experimental designs are methods for making a plan of experiments by providing the framework for changing all the relevant factors simultaneously within a small number of experimental runs [415]. They are multivariate approaches that can overcome the problems associated with univariate techniques by reducing the number of experiments required and providing information about the most relevant variables and the possible interactions that exist between them [412]. When defining the experimental space using experimental designs, the most common approach is to first select the variables thought to have the largest effect on the system by

conducting several preliminary univariate experiments. The range for each variable is then divided into a number of levels, usually low, medium and high. Experiments are then performed at the data points representing all possible combinations of these levels. Experimental design encompasses a number of different designs, including the full factorial, simplex, central composite (e.g. Box-Behnken), and fractional factorial (e.g. Plackett-Burman) designs. The type of design selected is usually based upon the complexity of the experiment at hand. Full factorial designs require a large number of experiments and are therefore usually used for studying a small number of factors at a small number of levels. Fractional factorial designs, used to determine the main effects while ignoring interaction effects [416], require fewer experiments to enable a larger number of variables to be studied. Central composite designs are the most commonly employed optimisation methods since they allow interaction effects to be studied with fewer experiments than a full factorial design.

The data obtained from experimental design methods can be used to create models which correlate experimental conditions with the measured responses. These response surfaces are three-dimensional plots, which aim to describe chromatographic behaviour in an experimental space by depicting the relationship between the response (often a measure of the separation quality e.g. resolution) and several input variables (e.g components of the mobile phase). The response is measured at a variety of experimental points, according to an experimental design, and the response surface is constructed by plotting the measured response against two of the variables, while any remaining variables are kept constant. As optimisation strategies, the most important feature of response surfaces is that they enable the global optimum, rather than a local optimum, to be located.

4.2.1 Artificial Neural Networks (ANNs)

An alternative multivariate approach for the optimisation of chromatographic separations can be found in artificial neural networks (ANNs). ANNs are predictive data-processing programs modelled on the human brain, which have the ability to model and solve non-linear problems and discover the approximate rules which

govern the optimal solution to these problems [417]. Unlike many other data-processing systems, which are programmed to arrive at the correct answer, ANNs are able to "learn" from a set of training examples that contain both the inputs and the desired outputs by plotting mapping functions. The relationships learnt using the mapping function from a particular data set can then be applied to new data, and predictions or generalisations can be made. The basic processing unit of the ANN is the neuron, or node. In the most common type of ANN, the multi-layer perceptron (MLP) feed-forward neural network, the network architecture is comprised of three layers of neurons; an input layer that receives the data, at least one hidden layer that processes the information received at the input, and an output layer, which is the observable response. The number of neurons in each layer is adjustable and governed by the complexity of the experiment at hand, with more variables requiring a greater number of neurons. Each neuron in the input layer is connected to each neuron in the hidden layer, and each one in the hidden layer is connected to each neuron in the output layer. The connections between the neurons are known as the weights. It is these weights that determine the behaviour of the ANN and how its behaviour changes over time during the learning process. An example of an ANN with three input nodes, four hidden nodes and two output nodes is shown in Figure 4-1.

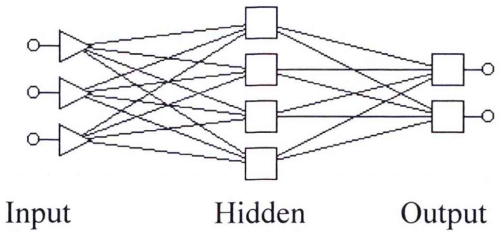


Figure 4-1 Example of ANN architecture (MLP 3:3-4-2:2)

The error of a network is defined as the squared difference between the outputs and the target output values, summed over the number of output nodes and training patterns. The goal of training the network is to adjust the weights between layers in a direction so as to minimise the error. The most common type of training algorithm used in analytical applications is the back propagation technique [418-421]. Backpropagation is a supervised learning method, meaning it requires the targets to the inputs to be known prior to training. The training weights are initially given arbitrary values and an iterative approach is taken to find their optimal values. Each iteration is known as an epoch. Output nodes are informed of the target values and, based on the difference between the calculated output and the target output, the node determines the direction and the amount by which the weight has to move to minimise the error. The output nodes then propagate the amount of their errors to the hidden nodes. This error is then used by the hidden nodes to determine in which direction, and by how much, their weights should change. The method used to calculate these weight changes is the gradient descent method. After an appropriate number of iterations, the network will arrive at a minimum error, and training is stopped. At this point, the calculated outputs should be as close as possible to the experimental output values. Backpropagation has a low memory requirement, achieves acceptable error quickly, and is most appropriate for training MLP networks. Other training algorithms include Quasi-Newton, Levenberg-Marquardt, Quick Propagation, Delta-bar-Delta and the conjugate gradient technique [422].

The biggest problem encountered when training ANNs is the tendency for over-learning or over-fitting, whereby the ANN does not describe the response adequately, despite the fact that the training data may fit well. Continued training causes over-fitting of the data, resulting in a network that has poor ability to generalise, or low predictive power. To prevent over-fitting, the data is split into a training set and a verification set. A good indication that over-fitting has occurred is when the verification error is significantly larger than the training error [420]. By watching the error on the validation set, training can be stopped before over-fitting occurs. Over-

fitting can also occur when the training data contains noise. If too few data are used, the ANN will model the noise instead of the underlying features [418].

Finding the optimal number of hidden nodes also presents a problem in ANN training. This is usually determined by trial and error, whereby the user increases the number of nodes in the hidden layer until the error starts to increase. At this point, the preceding architecture is deemed to have the best performance [420]. Another problem that may occur when training an ANN using the gradient descent method is that the local minimum of an error surface may be found, rather than the true global minimum. However, the probability of the existence of a local minimum goes down rapidly as the number of weights in the network is increased [423], so the addition of extra hidden nodes generally prevents this problem. Another means of preventing the system from getting stuck in a local minimum is to include a momentum term, μ. The momentum term takes into account the most recent weight corrections, and thus prevents sudden fluctuations in the direction of the weight change.

ANNs have been applied to a number of different areas of chemical information processing including classification [420, 424-427], calibration [428, 429] and optimisation [418, 430, 431]. They have been used to resolve or quantify overlapped peaks in chromatograms and electropherograms [415, 432-435], to model quantitative structure-retention relationships (QSRR) [436-440] and to estimate post-mortem intervals [441]. The applications of ANNs in the area of analytical chemistry are numerous, with studies of their implementation in CE [413, 414, 419, 429, 431-433, 435, 441-448], HPLC [418, 449-455], ion chromatography [430, 456-459] and GC [460] having been reported. Some have compared ANN results with those obtained using linear and non-linear regression methods, and found the predictive results to be better for ANNs [418, 419, 428, 432, 445, 451, 452, 461]. Many authors have found that the use of experimental design in conjunction with ANNs results in fewer experiments required to search for and find the optimum separation conditions [413-415, 431, 447, 455, 459, 462].

4.2.2 Previous Applications of Artificial Neural Networks to Gradient Elution Separations

Isocratic elution is the simplest elution method for separating analytes in HPLC, however it demands a compromise between the resolution of weakly retained analytes and the total analysis time [457]. When isocratic conditions fail to give optimal retentions, gradient elution is a useful technique because it can separate both weakly retained and strongly retained analytes within the same run [457] without compromising analysis time. The main disadvantage associated with gradient elution, as compared with isocratic elution, is that optimisation is more difficult to achieve. As a result, the vast majority of gradient elution separations are optimised by trial-and-error [463]. Despite this, a number of methods for optimising gradient elution conditions have been explored, including simplex procedures [464] and linear solvent strength models [439, 465]. More recently, ANNs have been employed to tackle the problem of optimising gradient elution separations, however the number of studies in this area has been limited.

Madden *et al.* [457] used an ANN to predict anion retention times in ion chromatography separations obtained with linear hydroxide gradients. The ANN was trained on the retention data obtained using three different gradient slopes. Each gradient had the same starting and finishing composition, with the only variable being time. This assumes the correct final concentration is selected to ensure that all components will elute within the gradient time. In addition, slow gradients can produce long analysis times, which render the optimisation process even more time-consuming. For example, if the initial and final compositions were selected as 10 and 70% respectively, a gradient with a slope of 3 would take place over 20 minutes, however a gradient with a slope of 1 would take place over a much longer time of 60 minutes. The three training gradients and four additional gradients were used as input variables to the selected ANN to predict the retention times for each analyte at the seven gradient conditions. The authors found the relative errors between the predicted and experimental data averaged 0.29%, indicating good predictive ability

of the ANN. However, while the ANN was shown to be capable of predicting retention times, the optimum separation conditions were selected from only seven possible gradient conditions. For the true optimum to be located, retention times should be predicted over all possible gradient conditions within an experimental space.

ANNs were also employed by Buciński *et al.* [454] for the prediction of gradient conditions for the HPLC separation of flavonoids. Four different gradient experiments were performed and the retention data from each gradient experiment comprised the input data for four separate ANNs. From this, the ANN and corresponding gradient condition that gave the best separation according to the relative error of predicted retention times was selected. So, rather than interpolating over a range of gradient conditions to locate the optimum, the method only enabled the optimum from a set of four gradient conditions to be established.

In 2003, Shan *et al.* [466] used an ANN to optimise mobile phase pH and acetonitrile gradient steepness in HPLC. Following in the steps of Madden *et al.*, the gradient was modelled using constant initial and final concentrations of organic modifier, with the only variable being the time over which the gradient was performed. The retention time of each analyte was modelled using an individual ANN comprised of two inputs (pH and gradient time), a hidden layer and one output (retention time of the analyte). So, for the separation of the seventeen amino acids in this study, an arduous seventeen individual ANNs were constructed. After the retention times of each solute under all possible pH and gradient conditions were predicted, resolution function values were calculated and a response surface was generated. This enabled the optimum pH and gradient conditions for the separation of all analytes to be found.

4.2.3 Defining the Optimum Separation

The response considered in optimisation processes can be one or more individual responses, such as resolution and analysis times, or based on multicriteria decision

making approaches. The latter is an effective alternative that considers all responses simultaneously. It combines individual responses, such as resolution and analysis time, into one objective function that measures the quality of the separation. This enables an optimum value from all of the responses to be achieved.

The product resolution and the normalised product resolution are two of the most commonly used optimisation functions. However, neither of these functions considers factors such as analysis time and asymmetrical peaks. Since both involve multiplication of individual peak pair resolution values, it comes as no surprise that the overall term will be greatest when the spread of peak is largest. More often than not, this will occur over a long run time, which is generally not ideal from the chromatographer's point of view. A number of authors have attempted to tackle the problem of assessing the optimal separation by defining their own optimisation functions, some of which are presented in Table 4-1. A review into the various functions developed to optimise chromatographic separations was published by Siouffi *et al.* in 2000 [467]. Some of the more popular or recent functions are discussed in detail below.

One of the earlier objective functions to be developed, the chromatographic response function (CRF) introduced by Berridge [464] considers the resolution between consecutive peak pairs, the maximum acceptable analysis time and the minimum retention time of the first peak to ensure all analytes are sufficiently retained on the column. The CRF optimises to a maximum and includes three weighting parameters, giving preference to one of resolution, run time or retention of all analytes on the column, which can be varied to suit the needs of the chromatographer. Since it sums the resolutions of all peak pairs, unresolved peaks have little effect on the CRF value compared to pairs with large resolution, which results in the quality of the chromatogram being determined by well resolved peak pairs. In reality, analytical problems tend to be caused by poorly resolved peaks.

The chromatographic resolution statistic (CRS) introduced by Schlabach and Excoffier [468] considers the average resolution in addition to the desired optimum resolution and the minimum acceptable resolution. It also contains a term which takes into account the total analysis time. The optimum separation conditions occur when the function value is minimised. The CRS function gives more weight to time over resolution, which means that chromatograms with shorter run times, but with unresolved peaks, are favoured over chromatograms with longer run times that have all peaks resolved.

The chromatographic exponential function (CEF), introduced by Morris *et al.* [469] considers the resolution between consecutive peak pairs, the desired resolution, the desired analysis time and the retention time of the final peak in the separation. The function optimises to a minimum and the adjustable parameter *a* permits a choice of emphasis on either resolution or run time.

Liu *et al.* [448] devised a novel criterion Q, which considers the resolution between consecutive peak pairs, and the resolution between the first and last peaks. In this way, it factors analysis time into the function.

Table 4-1 Optimisation functions for assessing chromatographic separations

Reference	Optimisation function	Definition	Comment
[464]	$\mathrm{CRF} = \sum_{i=1}^{L} R_i + L^{w_1} - w_2\lvert T_A - T_L\rvert - w_3(T_1 - T_0)$	Chromatographic Response Function R_i = ith and $(i+1)$th peak resolution L = no. peaks in chromatogram T_A = max. acceptable time T_L = final peak retention time T_1 = first peak retention time T_0 = min. retention time of first peak w_1, w_2, w_3 = weighting parameters	Optimises to a maximum
[468]	$\mathrm{CRS} = \left\{ \sum_{i=1}^{n-1} \left[\frac{(R_{i,i+1} - R_{opt})^2}{R_{i,i+1}(R_{i,i+1} - R_{min})^2} \right] + \sum \frac{(R_{i,i+1})^2}{(n-1)R_{av}^2} \right\} \frac{t_f}{n}$	Chromatographic Resolution Statistic $R_{i,i+1}$ = consecutive solute pairs resolution R_{av} = average resolution of all solute pairs R_{opt} = desired resolution R_{min} = min. resolution t_f = last solute retention time n = no. solutes	Optimises to a minimum

Reference	Optimisation function	Definition	Comment
[469]	$\mathrm{CEF} = \left[\left(\sum_{i=1}^{n-1}\left(1-e^{a(R_{opt}-R_s)}\right)^2\right)+1\right]\left[\frac{1+t_f}{t_{max}}\right]$	Chromatographic Exponential Function R_{opt} = desired resolution R_i = ith peak pair resolution t_{max} = max. acceptable time t_f = final peak retention time a = slope adjustment factor n = no. expected peaks	Optimises to a minimum
[448]	$\mathrm{Q} = \frac{R_{f\text{-}l}}{t_l}\prod_{i=1}^{n-1}\frac{R_i}{\frac{1}{n-1}\sum_{i=1}^{n-1}R_i}$	Q = quality of separation t_l = migration time of last peak $R_{f\text{-}l}$ = resolution between first and last peaks n = no. of peaks in chromatogram R_i = resolution of ith peak pair	Optimises to a maximum
[470]	$d_i = 0$; if $Y_i \leq Y^-$ $d_i = \left(\frac{Y_i - Y^-}{Y^+ - Y^-}\right)^r$; if $Y^- < Y_i < Y^+$ $d_i = 1$; if $Y_i \geq Y^+$	Derringer's desirability function Y_i = response Y^- = undesirable response value Y^+ = desirable response value	$d = 0$ for completely undesirable response $d = 1$ for fully desirable response
[471]	$\prod \mathrm{R_s} = \prod_{i=1}^{n-1}\frac{\mathrm{k}_{i+1} - \mathrm{k}_i}{\mathrm{k}_{i+1} + \mathrm{k}_i + 2}$	R_i = ith peak pair resolution n = no. peaks in chromatogram	Optimises to a maximum
[472]	$\mathrm{CRIT}_A(i,j) = \left[\frac{(t_j/t_i)_{predicted}}{(t_j/t_i)_{required}}\right] - 1$	$\mathrm{CRIT}_A(i,j)$ = function for a given pair of solutes (i,j) in a binary mobile phase (A)	Optimum when $\mathrm{CRIT_A}(i,j) > 0$

Reference	Optimisation function	Definition	Comment
[402]	$\xi = \text{Min}(R_s)$ if $\text{Min}(R_s) \leq 0$ $\xi = R_1 + \frac{1}{t_a}$ if not	$\text{Min}(R_s)$ = resolution of worst separated peak pairs R_1 = min. acceptable resolution t_a = analysis time	Optimises to a maximum
[473]	$\text{COF} = \sum_{i=1}^{n} A_i \ln\frac{R_i}{R_{id}} + B(t_m - t_n)$	Chromatographic Optimisation Function R_i = resolution for ith peak pair R_{id} = desired resolution for the ith peak pair t_m = desired max. run time t_n = retention time of last peak A_i, B = weighting factors	Optimises to zero
[474]	$\text{CR} = \frac{y}{\Delta y / \Delta x}$	y = response to be optimised Δy = change in response Δx = change in mobile phase composition	Optimum when $y \approx 1$ and $\Delta y / \Delta x \approx 0$
[475]	$S = \frac{V_B - V_A}{V_A + V_B}$	S = separation factor V_A = peak elution volume for peak A V_B = peak elution volume for peak B	Optimises to a maximum

4.2.4 Objectives

Development of HPLC methods by univariate approaches can be quite time consuming, particularly when optimisation of a number of parameters is required to obtain satisfactory resolution, sensitivity and analysis time. The main objective of this work was to explore the usefulness of ANNs in predicting resolution, retention times, and the subsequent optimal separation conditions in a gradient elution separation of nine benzodiazepines. A secondary objective of this study was to assess the effectiveness and limitations of several objective functions in determining the quality of a separation with respect to resolution and run time. Where inadequacies were found to occur, the goal was to devise a novel function that could overcome these limitations and describe the quality of separation effectively. The application of the optimised method to post-mortem blood samples was also investigated.

4.3 Experimental

4.3.1 Chemicals

All reagents were of analytical grade unless stated otherwise. Nitrazepam, oxazepam, alprazolam, flunitrazepam, temazepam and diazepam were obtained from Sigma-Aldrich (Sydney, New South Wales, Australia). 7-aminoflunitrazepam, 7-aminonitrazepam and 7-aminoclonazepam were either synthesised in-house (Chapter 2) or obtained from Novachem Pty Ltd (Melbourne, Victoria, Australia). Formic acid (HCOOH, APS Chemicals Ltd, Sydney, New South Wales, Australia) and ammonia solution (NH_4OH, APS Chemicals, Sydney, New South Wales, Australia) were used in the preparation of mobile phases. Methanol (MeOH, Merck HPLC grade), acetonitrile (ACN, Merck HPLC grade), ethyl acetate (EtOAc), butyl chloride and diethyl ether were purchased from Crown Scientific (Sydney, New South Wales, Australia). MilliQ grade water (18.2 $M\Omega cm^{-1}$) was used throughout the experiments. Buffers were prepared fresh each day and degassed by sonication prior to use. Whole sheep blood (Oxoid Australia Pty Ltd, Theberton, SA, Australia) was used for the preparation of spiked calibration standards. Prior to reconstitution, extracted samples

were evaporated to dryness using a Heto VR Maxi vacuum concentrator (Medos, Sydney, New South Wales, Australia).

4.3.2 Instrumentation

Experiments were performed on a Waters Alliance 2690 Separations Module, with Waters 996 Photodiode Array Detector (Waters, Sydney, Australia). All data manipulation was executed using Waters Millennium Software, Version 3.05. Separations were performed at 30 °C on a Zorbax SB-C18 column (50 x 4.6 mm, 1.8μm) (Agilent Technologies, Sydney, New South Wales, Australia). The mobile phase comprised of ammonium formate buffer (pH 2.80), ACN and MeOH at 1 mL/min. Duplicate 10 μL injections for each standard were performed and detection was at 280 nm.

ANN modelling of each experimental space was performed using Trajan Neural Network Simulator Version 6.0 (Trajan Software Ltd, Durham, UK). Three-dimensional response surfaces were generated using Minitab® Release 14 Statistical Software.

4.3.3 Calculations

For each experimental condition, the resolution of each analyte pair was calculated using Equation 4-1. The product resolution was calculated by the multiplication of each of the individual resolution values, as shown in Equation 4-2.

Equation 4-1 Calculation of resolution (R_s)

$$R_S = \frac{(t_2 - t_1)}{0.5(w_1 + w_2)}$$

where t_1 and t_2 are the retention times for each peak pair, and w_1 and w_2 are the peak widths at baseline

Equation 4-2 Calculation of product resolution (PR_s)

$$PR_S = PP1 * PP2 * PP3 * \quad * PP9$$

where PP1, PP2, PP3, , PP9 are the resolutions between the 1st peak pair, 2nd peak pair, 3rd peak pair, ., 9th peak pair

The relative error between experimental and predicted resolution values was calculated using Equation 4-3. Similarly, the relative error between measured and predicted retention times was calculated according to Equation 4-4.

Equation 4-3 Calculation of relative error ($\%e_i$) for resolution

$$\%e_i = \frac{\left| R_{s\ exp} - R_{s\ pred} \right|}{R_{s\ exp}} \text{ x } 100$$

where $R_{s\ exp}$ and $R_{s\ pred}$ are the experimental and predicted resolutions respectively for a particular peak pair, *i*.

Equation 4-4 Calculation of relative error ($\%e_i$) for retention times

$$\%e_i = \frac{\left| t_{r\ exp} - t_{r\ pred} \right|}{t_{r\ exp}} \text{ x } 100$$

where $t_{r\ exp}$ and $t_{r\ pred}$ are the experimental and predicted retention times respectively for a particular analyte, *i*.

4.3.4 Sample Preparation

Numerous methods have been published for the extraction of benzodiazepines from biological samples, with LLE being the most popular technique (refer to Table 1-11). Four LLEs, based on previously reported methods, were therefore evaluated with regards to the recovery of each benzodiazepine from spiked whole blood. The extraction details for each LLE technique are summarised in Table 4-2.

Table 4-2 Evaluated LLE techniques for benzodiazepine analytes

Extraction name	Details	Reference
Butyl chloride	25 µL 28% ammonia solution + 5 mL butyl chloride	[216]
Ethyl Acetate	1 mL 2M ammonia solution + 5 mL ethyl acetate	[219]
Diethyl ether/Ethyl acetate (1:1; v/v)	1 mL 2M ammonia solution + 5 mL diethyl ether/ethyl acetate (1:1, v/v)	[239]
Diethyl ether	100 µL 1M borate buffer (pH 9) + 1 mL diethyl ether	[206]

In each case, a 100 μL aliquot of a mixed aqueous standard at high (3.6 μg/mL 7-NH_2-CLO, 7-NH_2-NIT, 7-NH_2-FLU; 4.8 μg/mL NIT, OXA, TEM, DIA, ALP, FLU) and low (0.432 μg/mL 7-NH_2-CLO, 7-NH_2-NIT, 7-NH_2-FLU; 0.576 μg/mL NIT, OXA, TEM, DIA, ALP, FLU) concentrations was added to 0.5 mL of whole sheep blood. The two concentration levels were prepared and analysed in replicate. Following addition of the extraction solvent, each sample was vertically agitated for 2 minutes and centrifuged at 3000 rpm for 15 minutes. The organic layer was then transferred to a clean plastic tube, evaporated to dryness using a vacuum centrifuge and reconstituted in 100 μL MeOH prior to analysis.

4.3.5 Method Validation

Calibration curves were obtained by analysing drug-free whole blood, spiked with working standard solutions to obtain final concentrations of 0.432, 1.08, 1.44, 3.60 and 6.00 μg/mL for 7-aminonitrazepam, 7-aminoflunitrazepam and 7-aminoclonazepam, and 0.576, 1.44, 1.92, 4.80 and 8.00 μg/mL for oxazepam, nitrazepam, temazepam, flunitrazepam, diazepam and alprazolam. Calibration standards were analysed each day, and a standard curve constructed using linear regression.

Accuracy and precision were calculated at high and low concentration for each drug, with five replicates at each concentration. Accuracy was expressed as the calculated concentration as a percentage of nominal concentration. Precision (%CV) was determined to be the percentage of the average divided by the peak area ratio of the three replicates. Recovery was calculated as the average peak area of each analyte in the spiked samples as a percentage of the average peak area of each analyte in aqueous standards.

The limit of detection (LOD) was defined as a signal to noise ratio (S/N) of 3:1, and the limit of quantification (LOQ) was defined as a S/N of 10:1.

4.3.6 Application of the Method

The method was applied to post-mortem blood samples obtained from the Division of Analytical Laboratories (DAL) following coronial consent. All samples were preserved femoral blood taken as specimens at autopsy. Potential cases were selected based on independent quantitative results indicating the presence of benzodiazepines. Cases in which an insufficient volume of sample remained for analysis, or in which significant sample decomposition had occurred, were rejected. Recent cases, and cases requested by the Coroner to be kept beyond the maximum time of 12 months for the purposes of further legal proceedings, were also not analysed to preserve the integrity of the evidence.

4.4 Results and Discussion

4.4.1 Preliminary Screening of Mobile Phase Parameters

Resolution of ionogenic compounds in HPLC separations can be affected by a number of parameters, including solvent composition and pH. With this in mind, ammonium formate buffer was selected for investigation in the range pH 2.8 – 4.8 (constant concentration 25 mM), which is the useful buffering capacity of formate (pK_a 3.75). A low pH mobile phase was chosen to eliminate ion-exchange interactions and improve peak shape of the basic analytes. The StableBond column was selected for its stability in the range pH 2 – 4. The effect of buffer ionic strength was also investigated in the range 25 – 50 mM (constant pH 2.8). To avoid excessively long analysis times, all pH and concentration experiments were conducted with the addition of 30% ACN to the mobile phase.

The average retention times and resolutions for each analyte with varying pH (constant concentration 25 mM) are illustrated in Figure 4-2. pH had very little effect on the retention times of each analyte, with only diazepam exhibiting a slight increase in retention as pH was increased. The effect on resolution was accordingly minimal. Average resolutions between consecutive peaks and overall product resolutions (PR_S) are presented for each condition in Table 4-3. The resolution

between temazepam and diazepam (PP8) showed a slight increase as pH was increased, which was attributable to the increased retention time of diazepam that was observed. 7-aminonitrazepam and 7-aminoclonazepm (PP1), 7-aminoflunitrazepam and oxazepam (PP3) and nitrazepam and alprazolam (PP5) exhibited a general slight decrease in resolution as pH was increased. This is likely due to increased peak widths, which was observed with increasing pH. Since temazepam and flunitrazepam (PP7) co-eluted under all pH conditions investigated, PR_S was equal to zero in each case. However, PR_S^* was calculated in a similar manner to PR_S, omitting cases where $R_S = 0$ (i.e. PP7). From this, a general trend in product resolution could be observed while disregarding peaks that completely overlapped. The greatest PR_S^* was obtained at pH 3.8, however the resolution between alprazolam and flunitrazepam (PP6) was 1.271 at this pH, indicating that the analyte pair was not baseline resolved. Inspection of Table 4-3 reveals that pH 2.8 was closer to completely resolving all analytes (excluding PP7). This suggests that PR_S^* is not necessarily an adequate indicator of the best separation. The reason for this is that, in each instance, there are large gaps between the elution of 7-aminoflunitrazepam and oxazepam (PP3), nitrazepam and alprazolam (PP5), and temazepam and diazepam (PP8). These large resolution values of PP3, PP5 and PP8 tend to distort the value of PR_S, causing excessive emphasis to be placed on the well-resolved analytes, when it is the separation of the partially or incompletely resolved analytes of PP1, PP4 and PP6 that should govern the best separation.

Table 4-3 Average resolutions of consecutive peaks with varying pH

pH	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8	PRs	PR_s^*
2.8	2.612	2.811	17.50	1.483	9.671	1.415	0.000	10.02	0.000	22600
3.8	1.528	3.456	16.38	2.199	9.177	1.271	0.000	12.75	0.000	28300
4.8	0.9243	3.353	15.29	2.106	8.702	1.285	0.000	12.40	0.000	13800

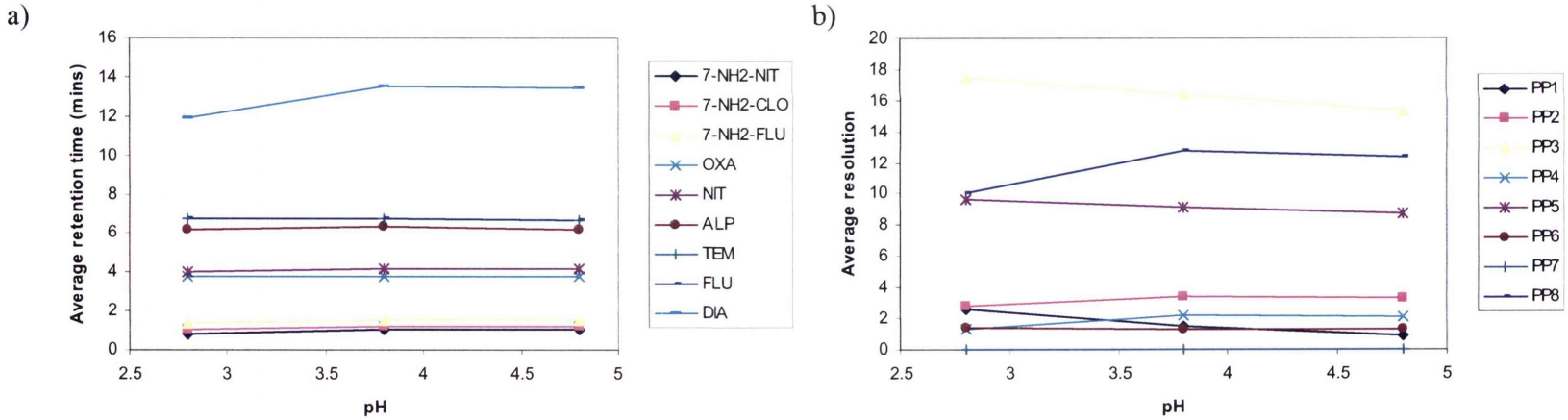


Figure 4-2 Influence of pH on a) average retention times and b) average resolution for each analyte

Table 4-4 Average resolutions of consecutive peaks with varying formate buffer concentration

Concentration (mM)	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8	PRs	PR_s^*
25	2.612	2.811	17.502	1.483	9.672	1.415	0.000	10.022	0.000	22600
50	2.680	2.977	17.176	1.404	9.364	1.245	0.000	9.972	0.000	22400
75	2.612	2.850	16.479	1.360	9.356	1.202	0.000	9.805	0.000	18400

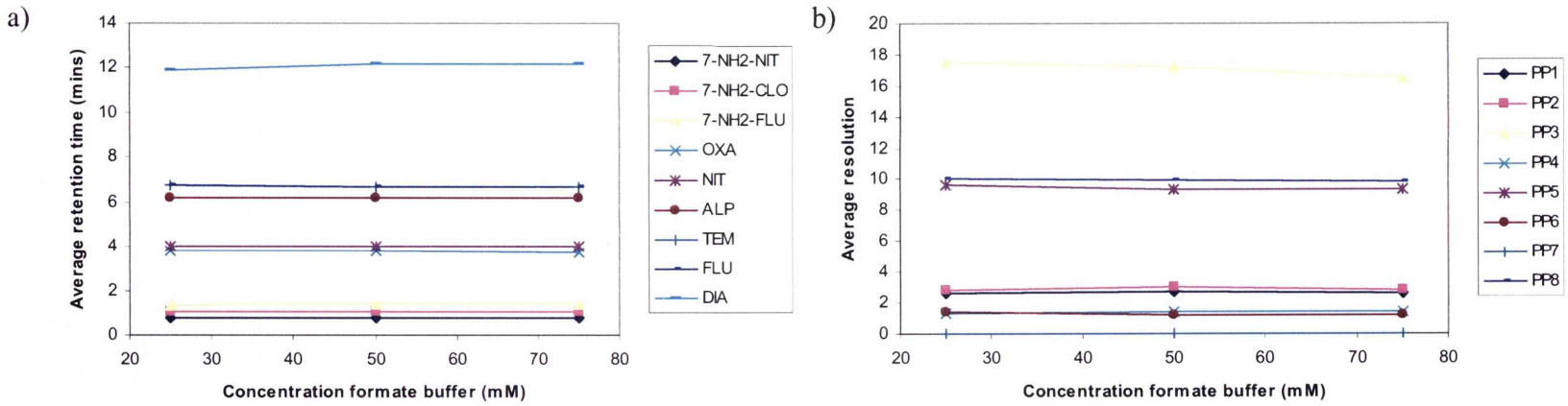


Figure 4-3 Influence of formate buffer concentration on a) average retention times and b) average resolutions for each analyte

The average retention times and resolutions for each analyte with varying formate buffer concentration (constant pH 2.8) are illustrated in Figure 4-3. Concentration had very little effect on the retention times of any of the analytes. Accordingly, the effect of increasing concentration on the resolution of each analyte pair was minimal. Average resolutions between consecutive peaks and overall product resolutions (PR_s) are presented for each condition in Table 4-4. Again, temazepam and flunitrazepam (PP7) co-eluted under all conditions investigated, so PR_s^* was calculated to give a more meaningful representation of the overall separation. A slight decrease in resolution between 7-aminoflunitrazepam and oxazepam (PP3) was observed with increasing concentration, mainly due to a slight increase in peak widths. This translated into an overall decrease in PR_s^* as concentration was increased, once again due to the excessive emphasis PR_s places on well-resolved peak pairs such as PP3.

The effect of organic modifier type on the separation was also investigated. Figure 4-4 illustrates the effect of varying %MeOH on the average retention times and resolution of each analyte. From these graphs, it can be seen that there is very poor resolution between a number of analytes in 60% MeOH. Reducing this to 50% MeOH improves the separation slightly, however some analytes remain unresolved. In 40% MeOH, the resolution between the early eluting 7-amino metabolites is still poor and, in addition, the run time is around 40 minutes. It is apparent from these graphs that an isocratic composition of MeOH would be incapable of separating all nine analytes within 40 minutes. The effect of varying an isocratic percentage of ACN was also investigated, as shown in Figure 4-5. From these graphs it can be seen that an isocratic percentage of ACN was also incapable of separating all nine analytes. Despite the fact that neither MeOH nor ACN could separate all nine analytes isocratically, it was interesting to note that the choice of organic modifier greatly affected selectivity, with some analytes displaying a greater affinity for one organic modifier over the other. For example, flunitrazepam eluted a lot sooner in MeOH than it did in ACN, enabling it to be completely resolved from temazepam in MeOH. ACN was ineffective at even partially resolving these analytes. This difference in elution order is illustrated in Figure 4-6.

Table 4-5 Average resolutions of consecutive peaks with varying methanol composition

%MeOH	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8
40	2.461	1.632	8.623	4.549	7.491	7.851	9.849	14.357
50	0.093	1.023	7.691	4.747	6.437	2.598	0.513	7.824
60	0.000	0.000	0.000	4.107	1.395	0.113	0.106	2.456

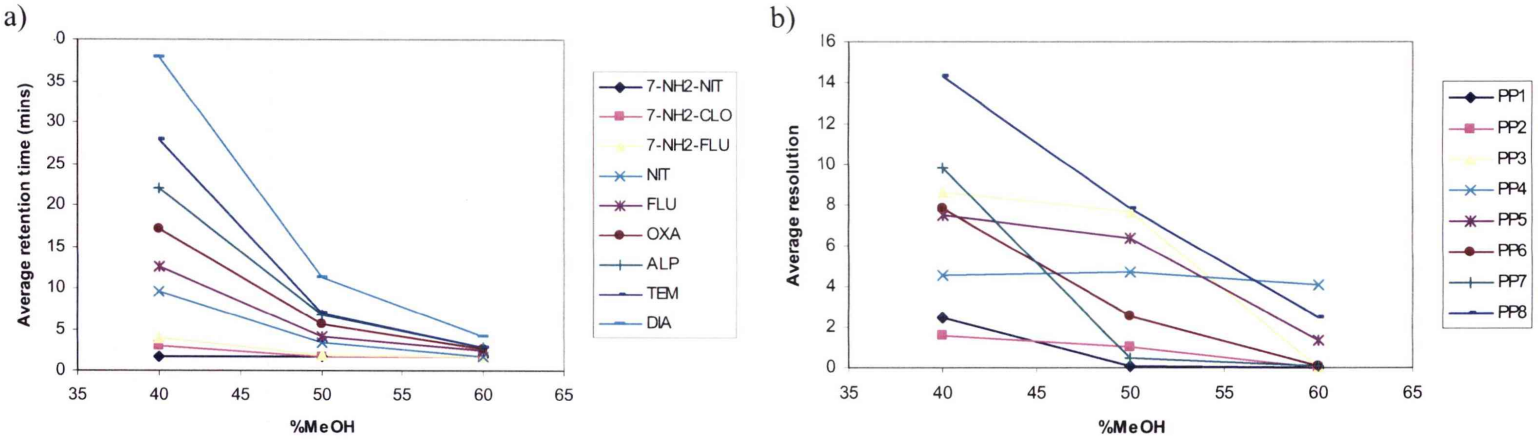


Figure 4-4 Influence of methanol composition on a) average retention times and b) average resolutions for each analyte

Table 4-6 Average resolutions of consecutive peaks with varying acetonitrile composition

%ACN	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8
30	2.641	2.801	17.082	1.267	9.704	1.425	0.000	10.050
35	0.000	2.414	6.120	2.316	0.795	3.701	0.283	6.508
40	0.000	0.000	3.541	0.788	1.248	3.089	0.884	5.284

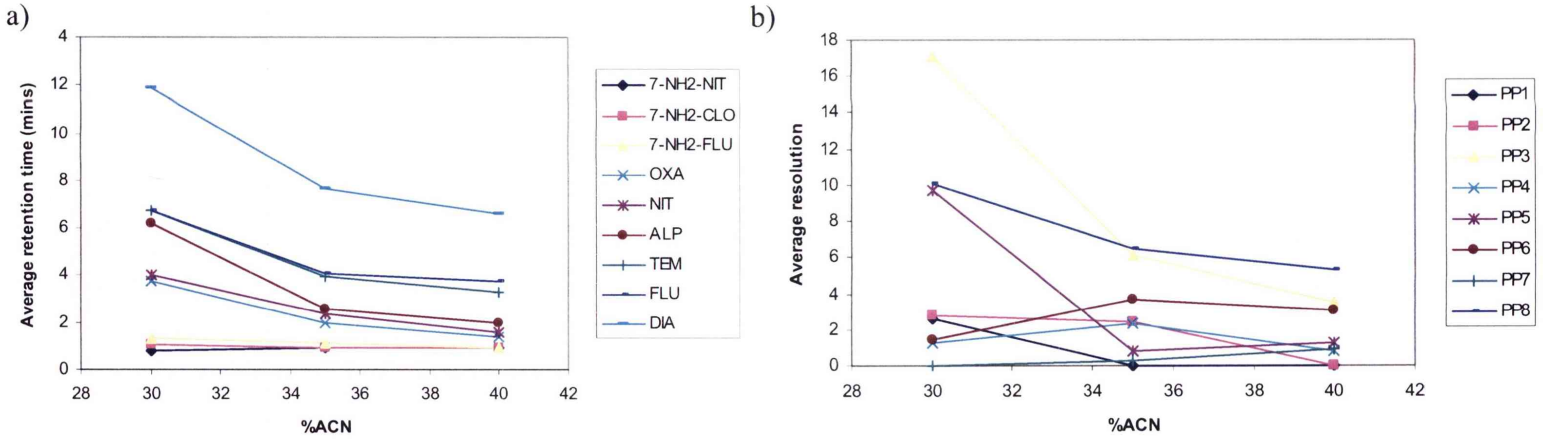
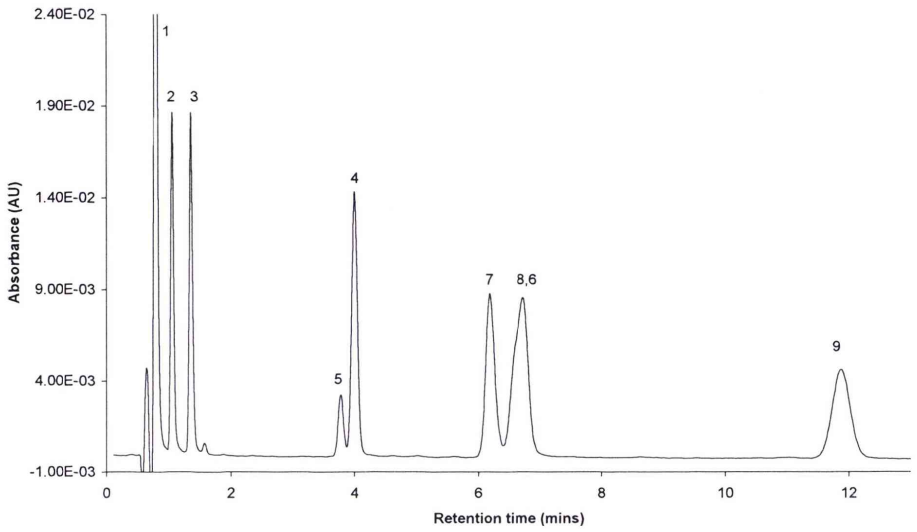


Figure 4-5 Influence of acetonitrile composition on a) average retention times and b) average resolutions for each analyte

a)



b)

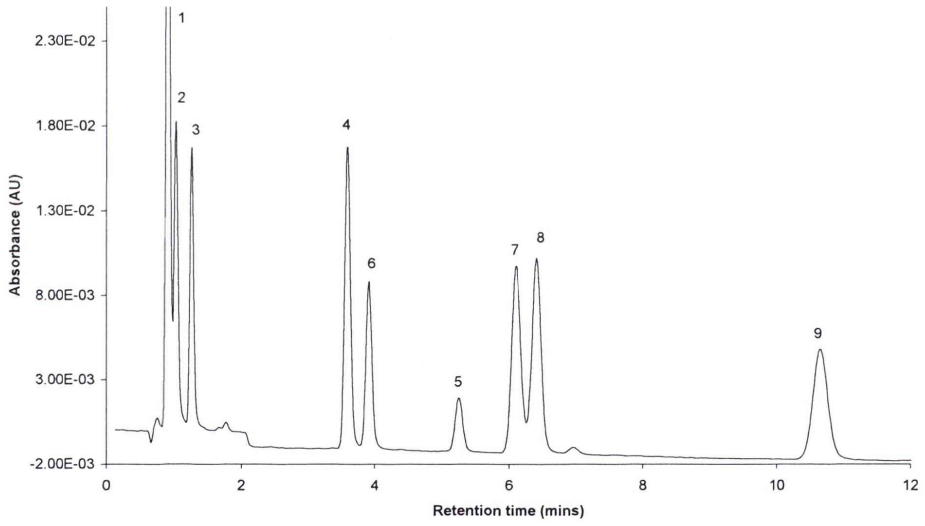


Figure 4-6 Effect on elution order of a) ACN and b) MeOH

a) Conditions: Zorbax SB-C18 column (50 x 4.6 mm, 1.8μm), 25 mM formate buffer:ACN (70:30), pH 2.8, temperature 30 °C, 10 μL injection, detection λ 280 nm; **b) Conditions:** Zorbax SB-C18 column (50 x 4.6 mm, 1.8μm), 25 mM formate buffer:MeOH (50:50), pH 2.8, temperature 30 °C, 10 μL injection, detection λ 280 nm; **Elution order:** 1. 7-NH_2-NIT, 2. 7-NH_2-CLO, 3. 7-NH_2-FLU, 4. NIT, 5. OXA, 6. FLU, 7. ALP, 8. TEM, 9. DIA.

4.4.2 ANN Modelling of the Experimental Space

One of the initial hurdles encountered when employing ANNs to optimise a chromatographic separation is defining the experimental space. The parameters and space in which the separation is modelled must be selected carefully. The optimum conditions must lie within the chosen experimental space, otherwise the optimal separation will not be found. Therefore, the chromatographer must attempt to gauge approximately where the optimum conditions lie by conducting preliminary experiments.

The preliminary investigation of mobile phase parameters in the previous section demonstrated that pH and concentration had little effect on the separation. In addition, it was shown that MeOH and ACN were incapable of separating all nine analytes under isocratic conditions, however they did affect selectivity. Therefore, gradient conditions using methanol alone and acetonitrile alone were investigated as preliminary experiments. This included varying the initial composition of the organic modifier, the slope of the gradient, and also a number of gradient profiles available using the Waters Millennium software. Unfortunately, none of these conditions were able to separate all nine analytes, so a ternary mobile phase system, involving buffer, ACN and MeOH was investigated. This was found to give improved separation, with the most promising results obtained using an isocratic percentage composition of MeOH and a gradient with respect to ACN. The reason for this improved separation is likely due to the differences in selectivity brought about by MeOH and ACN. While ternary systems can be difficult to optimise, especially when elution order is complicated due to selectivity changes, complex systems such as this are suited to optimisation by ANNs.

The experimental space for ANN training was therefore defined using the variables %MeOH and ACN gradient. %MeOH was defined as the isocratic percentage composition of methanol in the mobile phase. ACN gradient was defined as the percentage increase in acetonitrile composition divided by the duration of the linear

gradient. The increase in ACN over time was designed to replace the aqueous buffer only. This is shown graphically in Figure 4-7, which is an example depicting the change in mobile phase composition over 20 minutes given an initial 5% composition of ACN, an ACN gradient of 2%/min and an isocratic composition of 15% MeOH. In contrast to the other studies employing ANNs to model gradient separations, whereby the initial and final organic modifier concentrations were fixed, the initial composition of ACN (initial %ACN) was introduced as a third factor. This was done to give greater flexibility and to avoid the long analysis times required to generate slow gradients when initial and final compositions are fixed. Thus, the experimental space was as follows: 10 – 30% MeOH; 1 – 3%/min ACN gradient; 5 – 10% initial %ACN. Formate buffer concentration and pH were kept constant at 25 mM and pH 2.80 respectively.

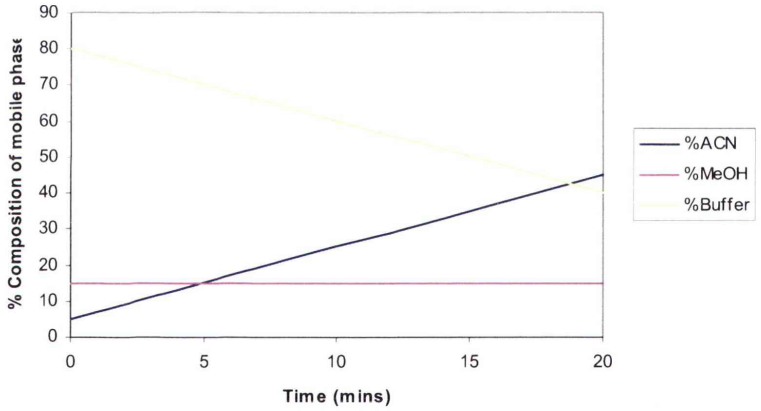


Figure 4-7 Example of percentage composition of each mobile phase component with varying time (15% MeOH, 2%/min ACN, 5% initial ACN)

Fifteen experiments based on a central composite design were performed, of which nine were assigned as training points and six as verification (select) points, as illustrated in Figure 4-8 and Table 4-7. Each experiment was performed in replicate with 10 µL injections of a mixed aqueous standard containing 100 µg/mL each of diazepam, nitrazepam, alprazolam, flunitrazepam, oxazepam, temazepam, 7-aminonitrazepam, 7-aminoflunitrazepam and 7-aminoclonazepam. ANN

architectures were constructed using an automated heuristic approach whereby the number of nodes in the hidden layer was varied. The most suitable ANNs were deemed to be the ones that had a sufficiently low training error with a corresponding verification error of the same magnitude, in order to minimise the likelihood of over-fitting. Data sheets containing a large number of possible points within the experimental space (10 – 30% MeOH at increments of 2%, 1 – 3%/min ACN gradient at increments of 0.1%/min, 5 – 10% initial ACN at increments of 0.5%) were created and the selected ANNs were applied to predict the output at each experimental point. In order to find the most appropriate ANN to model the chromatographic system, the effect of varying the output used as training data was investigated by comparing ANNs trained on resolution outputs to ANNs trained on retention time and peak width outputs. In addition, the effect of data quality and quantity was investigated by comparing ANNs trained on the data from each replicate to ANNs trained on the average data of both replicates.

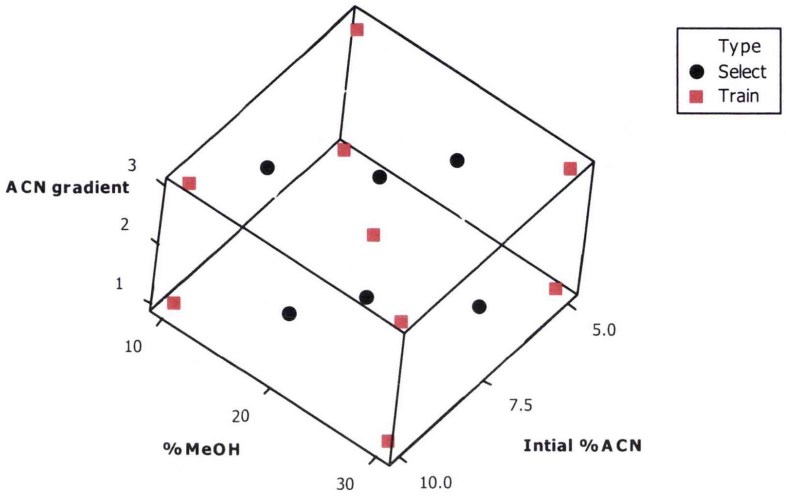


Figure 4-8 Schematic of experimental space

Table 4-7 Values for each experiment in three factor design

Experiment No.	ACN grad (%/min)	%MeOH (%)	Initial %ACN (%)
1	1	20	7.5
2	2	10	7.5
3	3	20	7.5
4	2	30	7.5
5	2	20	10
6	2	20	5
7	2	20	7.5
8	3	30	10
9	3	10	10
10	1	30	10
11	1	10	10
12	3	30	5
13	3	10	5
14	1	30	5
15	1	10	5

4.4.2.1 Resolutions as the Output

The resolution between various peak pairs is one of the more common outputs used when training an ANN to model chromatographic behaviour. The values of the three factors, and the corresponding replicate resolution data were used as the inputs and outputs respectively to train the ANN, giving a total of eighteen training points and twelve verification points.

The ANN selected for the prediction of resolutions had a MLP architecture and consisted of 3 input nodes, 12 hidden nodes and 8 output nodes (resolutions for each of the 8 peak pairs) (MLP 3:3-12-8:8). The training and verification errors for this network were 0.064 and 0.053 respectively. To determine the predictive ability of the ANN, the observed resolution values obtained from the fifteen experiments were plotted against the resolution values predicted by the ANN. As illustrated in Figure 4-9, good correlation was obtained between the experimentally gathered resolution values and predicted resolution values ($r^2 = 0.9978$). The correlation coefficients for each individual peak pair, seen in Table 4-8, ranged from 0.9637 for PP8 to 0.9932 for PP2.

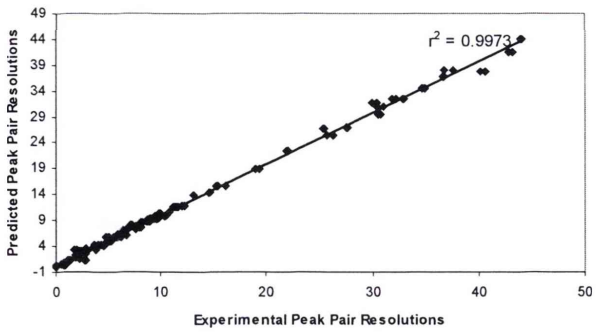


Figure 4-9 Overall correlation between experimental and predicted peak pair resolutions for a 3:3-12-8:8 MLP ANN trained on replicate data

Table 4-8 Correlation equations and correlation coefficients between experimental and predicted resolutions for each peak pair trained on replicate data

Peak Pair	Correlation Equation	Correlation Coefficient (r^2)
PP1	y = 1.002764x - 0.01869	0.9924
PP2	y = 0.937375x + 0.53108	0.9932
PP3	y = 0.928463x + 2.15322	0.9869
PP4	y = 0.939507x + 0.235102	0.9746
PP5	y = 0.953216x + 0.247804	0.9678
PP6	y = 0.978822x + 0.116831	0.9781
PP7	y = 1.001838x - 0.00445	0.9819
PP8	y = 0.925115x + 0.670236	0.9637

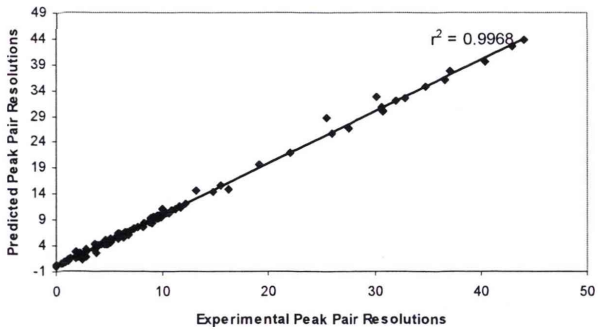


Figure 4-10 Overall correlation between experimental and predicted peak pair resolutions for a 3:3-12-8:8 MLP ANN trained on average data

Table 4-9 Correlation equations and correlation coefficients between experimental and predicted resolutions for each peak pair trained on average data

Peak Pair	Correlation Equation	Correlation Coefficient (r^2)
PP1	y = 1.043498x - 0.56158	0.9917
PP2	y = 0.972381x + 0.171401	0.9887
PP3	y = 0.935624x + 2.178615	0.9819
PP4	y = 0.920977x + 0.24634	0.9743
PP5	y = 0.960804x + 0.122996	0.9716
PP6	y = 1.000169x - 0.04357	0.9901
PP7	y = 0.970762x + 0.020807	0.9890
PP8	y = 0.943275x + 0.502977	0.9813

A second ANN was constructed using the average resolution values of each replicate. This halved the training and verification data, giving a total of 9 training points and 6 verification points. Based on this method, the ANN that gave the best performance for the prediction of resolution values had a MLP architecture comprising of 3 input nodes, 12 hidden nodes and 8 output nodes (MLP 3:3-12-8:8). The ANN had a training error of 0.057, which was smaller than that of the network trained on each replicate, however it had a similar verification error of 0.051. As before, the observed average resolution values obtained for each analyte in the fifteen experiments were plotted against the predicted values generated by the ANN. Good overall correlation ($r^2 = 0.9968$) was found to exist between average experimentally obtained resolutions and predicted resolutions, as shown in Figure 4-10. The correlation coefficients for each individual peak pair were between 0.9716 for PP5 and 0.9917 for PP1.

Each ANN was applied to predict the resolution values of each peak pair throughout the experimental space by inputting a grid pattern of %MeOH in the range 10 – 30% at increments of 2%, ACN gradient in the range 1 – 3%/min at increments of 0.2%/min, and initial %ACN in the range 5 – 10% at increments of 0.5%. From this predicted data, three experimental points were selected and run to determine if the predicted data was consistent with this new experimental data. Both the ANN trained on replicate data and the ANN trained on average data demonstrated good ability to predict and learn the training data by virtue of their correlation coefficients, which were greater than 0.99. However, the predictive ability of both networks diminished considerably when previously unseen data was presented. Table 4-10 illustrates the experimental resolution values and the predicted resolution values from the two separate ANNs for this unseen data. The relative errors of predicted resolution values to experimental resolution values are displayed in parentheses. The ANN trained on replicate data gave relative errors between 0.752% and 51.9%. The largest error occurred in the prediction of the resolution values for PP4 and PP5, which correspond to the nitrazepam-oxazepam and oxazepam-flunitrazepam peak pairs respectively. One reason for this large error may be due to the change in selectivity or 'peak-swapping' effect that takes place between these three analytes. In several of

the experiments used to train the ANNs, flunitrazepam and oxazepam swapped elution order. When the peak pair resolution values were calculated, the change in elution order was not considered. Consequently, under some conditions PP5 may represent the oxazepam-flunitrazepam peak pair, whilst under other conditions PP5 may represent the nitrazepam-flunitrazepam peak pair. This is likely to confound the ANN during training, since the network may not accurately recognise the change in elution order. As a result, the ANN cannot accurately predict the resolution values between these pairs of peaks. This finding is in disagreement with the results of Havel *et al.* [431] who employed an ANN to model the resolution between several metal complexes and found excellent agreement between experimental and predicted data, despite a change in selectivity for two of the analytes.

As indicated in Table 4-10, the ANN generated using average resolution values gave relative errors between 0.453% and 46.8% for the three experimental conditions tested. Again, the largest error occurred in the prediction of the resolution values for PP4 and PP5. As discussed above, this is likely due to the network's inability to predict the elution order of nitrazepam, flunitrazepam and oxazepam. While still generating a substantial error in its prediction of resolution values, its average error and standard deviation were less than that of the ANN trained on replicate data. A possible explanation for this is that taking the average resolution from two replicate injections may eliminate some noise from the data, thus enabling the ANN to train more effectively. The ANN trained on data from both replicates must attempt to learn two slightly different resolution values for the same input data point, which introduces noise into the training data and produces higher training and verification errors.

Table 4-10 Experimental and predicted resolution values for each ANN with corresponding relative errors

	ACN grad	%MeOH	Initial %ACN	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8
Exp. Data	2.4	10	9.5	14.07	9.64	30.16	1.66	5.86	2.03	1.27	10.10
	2.2	22	10	6.74	7.14	29.28	4.13	1.63	5.11	0.58	9.40
	2	12	5	17.72	11.74	33.84	2.68	5.17	3.06	1.10	10.15
ANN Trained on Replicate Data	2.4	10	9.5	13.12 (6.74%)	10.12 (4.97%)	34.91 (15.8%)	2.53 (51.9%)	6.10 (4.05%)	2.80 (37.9%)	1.19 (6.09%)	10.21 (1.12%)
	2.2	22	10	6.29 (6.55%)	7.20 (0.921%)	30.28 (3.40%)	4.09 (0.752%)	2.31 (41.1%)	5.08 (0.753%)	0.43 (26.8%)	9.09 (3.31%)
	2	12	5	16.64 (6.10%)	12.43 (5.85%)	38.69 (14.3%)	3.74 (39.4%)	5.40 (4.42%)	3.67 (19.8%)	0.86 (22.3%)	9.58 (5.70%)
	Average Relative Error (%)			13.8 ± 14.7							
ANN Trained on Average Data	2.4	10	9.5	12.81 (8.94%)	9.68 (0.453%)	34.00 (12.7%)	2.44 (46.8%)	5.60 (4.50%)	2.56 (26.2%)	1.20 (4.88%)	10.33 (2.31%)
	2.2	22	10	5.51 (18.2%)	6.32 (11.4%)	30.06 (2.65%)	4.52 (9.60%)	0.98 (40.0%)	4.99 (2.46%)	0.54 (7.98%)	9.24 (1.66%)
	2	12	5	16.12 (9.04%)	12.52 (6.62%)	38.39 (13.4%)	3.51 (30.8%)	5.39 (4.18%)	3.60 (17.5%)	0.77 (30.2%)	9.74 (4.06%)
	Average Relative Error (%)			13.2 ± 12.5							

Where applicable, data are presented as resolution value (relative error)

4.4.2.2 Retention Times as the Output

Another common method employed when training ANNs to model chromatographic separations is to use retention time data as the output variable. This enables the retention time of each analyte to be predicted at any point within the experimental space and, from this, it is often only a simple matter to convert the predicted retention times to resolution values in order to determine the best separation.

For the first ANN, the training data comprised of the values of the three factors as the inputs, and the replicate retention time data for each analyte as the outputs, giving a total of thirty experimental points, of which twelve were assigned as verification points. The network that gave the best performance had a MLP architecture consisting of 3 inputs nodes, 20 hidden nodes and 9 output nodes (retention times for each analyte) (MLP 3:3-20-9:9), with low training and verification errors of 0.021 and 0.029 respectively. To determine the predictive ability of the ANN, the observed retention data from each of the thirty experiments were plotted against the predicted retention data generated by the ANN, as shown in Figure 4-11. Overall, the correlation between the experimental and predicted retention times was good (r^2 = 0.9981) suggesting good predictive ability of the ANN. Correlation coefficients for each individual analyte, depicted in Table 4-11, ranged from 0.9862 for 7-aminoclonazepam to 0.9991 for nitrazepam. Although the correlation between experimental and predicted retention data for 7-aminoclonazepam was slightly less than desirable, this analyte was well-resolved under all conditions within the experimental space, so any errors in its prediction were of little concern.

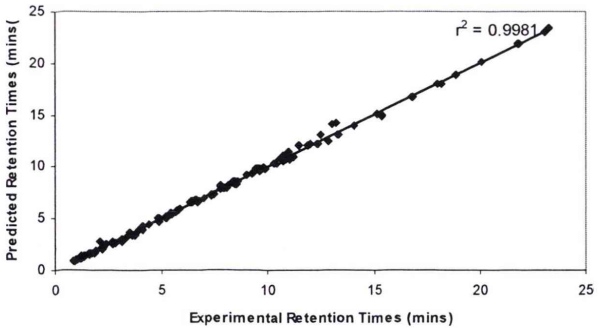


Figure 4-11 Overall correlation between experimental and predicted retention times using a 3:3-20-9:9 MLP ANN trained on replicate data

Table 4-11 Correlation equations and correlation coefficients between experimental and predicted retention times for each analyte trained on replicate data

Analyte	Correlation Equation	Correlation Coefficient (r^2)
7-aminonitrazepam	y = 1.010977x - 0.00796	0.9948
7-aminoclonazepam	y = 0.970867x + 0.043127	0.9862
7-aminoflunitrazepam	y = 1.000594x - 0.02537	0.9974
nitrazepam	y = 0.998949x + 0.022088	0.9991
oxazepam	y = 0.991519x + 0.098903	0.9982
flunitrazepam	y = 0.995733x + 0.080019	0.9987
alprazolam	y = 0.99003x + 0.192517	0.9968
temazepam	y = 0.984992x + 0.257944	0.9971
diazepam	y = 0.979522x + 0.40367	0.9951

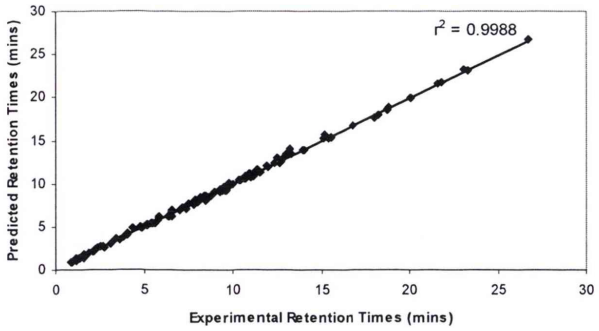


Figure 4-12 Overall correlation between experimental and predicted retention times using a 3:3-12-9:9 MLP ANN trained on average data

Table 4-12 Correlation equations and correlation coefficients between experimental and predicted retention times for each analyte trained on average data

Analyte	Correlation Equation	Correlation Coefficient (r^2)
7-aminonitrazepam	y = 0.999635x + 0.002161	0.9987
7-aminoclonazepam	y = 0.990025x + 0.19223	0.9941
7-aminoflunitrazepam	y = 0.990246x + 0.04997	0.9977
nitrazepam	y = 0.990533 + 0.021159	0.9981
oxazepam	y = 0.997337x + 0.029419	0.9986
flunitrazepam	y = 0.99673x + 0.070527	0.9990
alprazolam	y = 0.997974x + 0.00615	0.9990
temazepam	y = 0.991526x + 0.059858	0.9978
diazepam	y = 1.000917x - 0.07015	0.9991

A second ANN was trained using the same experimental data, however the average retention times for each replicate were taken and assigned as outputs for the training and verification data, as opposed to considering each replicate individually in the manner described previously. This gave a total of fifteen experimental data points, including six verification points. The best performing network had low training and verification errors of 0.017 and 0.015 respectively, and comprised of 3 input nodes, 12 hidden nodes and 9 output nodes arranged in a MLP architecture (MLP 3:3-12-9:9). As before, the predictive ability of the ANN was assessed by plotting the observed retention data from the fifteen experiments against the predicted retention data. From Figure 4-12, good overall correlation ($r^2 = 0.9988$) between experimental and predicted data can be observed. As seen in Table 4-12, the correlation coefficients for each individual analyte were all greater than 0.99, indicating strong ability of the selected ANN to predict retention times.

By inputting a grid pattern of %MeOH in the range 10 – 30% at increments of 2%, ACN gradient in the range 1 – 3%/min at increments of 0.2%/min, and initial %ACN in the range 5 – 10% at increments of 0.5%, both ANNs were applied to predict the retention times of each analyte throughout the experimental space. Three experimental points were then selected and run to determine if the predicted data was consistent with this new experimental data. By virtue of their correlation coefficients, which were greater than 0.99, both the ANN trained on replicate data and the ANN trained on average data demonstrated good ability to predict and learn the training data. However, each ANN performed differently when this previously unseen data was presented. Table 4-13 illustrates the experimental and predicted retention times for each analyte generated by both networks for this new data, with relative errors given in parentheses. The ANN trained on both replicates gave relative errors in the range 0.121% to 27.3% when tested on the new data. The largest error occurred in the prediction of the retention time of 7-aminoclonazepam, although large errors were also found in the prediction of the remaining two 7-amino metabolites. Despite this, the selected ANN was able to predict the retention times of the remaining benzodiazepines with consistently small errors. Since the three 7-amino metabolites

were well-resolved under all experimental conditions, the error associated with the prediction of their retention times was of little bearing. The accurate prediction of the retention times of nitrazepam, oxazepam, flunitrazepam, alprazolam and temazepam was of more importance, given the difficulties associated with their separation.

The ANN trained on average retention data gave relative errors between 0.787% and 25.6%. Again, the largest error was associated with the prediction of 7-aminoclonazepam, and the 7-amino metabolites in general. Inspection of the relative errors for the remaining analytes in Table 4-13 reveals that they are greater than the relative errors obtained for the ANN trained on replicate data. This is contrary to the previous finding, whereby the ANN trained on average resolution data was associated with the smaller average relative error. The reason for this may lie in the quality and quantity of data in the training set. Generally, the greater the number of cases available for training, the better the predictive ability of the ANN. However, if the training data contains noise, this relationship does not hold true. The ANN trained on both retention replicates had a training data set comprised of thirty cases, while the ANN trained on the average of both replicates had only fifteen training points. Since the replicate retention time data was consistent, the training data contained very little noise. Consequently, the network trained on this data was better able to learn, and hence predict, due to the greater number of training cases available.

Table 4-13 Experimental and predicted retention times for each ANN with corresponding relative errors

	ACN grad	%MeOH	Initial %ACN	7-NH_2-NIT	7-NH_2-CLO	7-NH_2-FLU	NIT	OXA	FLU	ALP	TEM	DIA
Exp. Data	2.4	10	9.5	1.896	3.729	5.094	9.746	10.014	10.987	11.331	11.544	13.365
	2.2	22	10	1.277	2.061	2.941	7.051	7.681	7.935	8.848	8.963	10.736
	2	12	5	2.392	4.865	6.674	12.448	12.927	13.872	14.445	14.652	16.706
ANN Trained on Replicate Data	2.4	10	9.5	1.404 (26.0%)	2.712 (27.3%)	4.084 (19.8%)	9.304 (4.53%)	9.737 (2.77%)	10.700 (2.61%)	11.450 (1.05%)	11.530 (0.121%)	13.320 (0.337%)
	2.2	22	10	0.939 (26.5%)	1.531 (25.7%)	2.259 (23.2%)	6.602 (6.37%)	7.287 (5.13%)	7.545 (4.92%)	8.801 (0.531%)	8.893 (0.781%)	10.710 (0.242%)
	2	12	5	1.968 (17.7%)	4.252 (12.6%)	5.981 (10.4%)	11.970 (3.84%)	12.680 (1.91%)	13.840 (0.231%)	14.620 (1.21%)	14.740 (0.601%)	16.630 (0.455%)
	Average Relative Error (%)			8.4 ± 9.7								
ANN Trained on Average Data	2.4	10	9.5	1.419 (25.1%)	2.773 (25.6%)	4.192 (17.7%)	9.101 (6.62%)	9.546 (4.67%)	10.520 (4.25%)	10.790 (4.78%)	11.310 (2.03%)	12.840 (3.93%)
	2.2	22	10	0.989 (22.6%)	1.642 (20.3%)	2.420 (17.7%)	6.398 (9.26%)	7.155 (6.85%)	7.492 (5.58%)	8.326 (5.90%)	8.353 (6.81%)	10.190 (5.09%)
	2	12	5	1.962 (18.0%)	4.144 (14.8%)	5.749 (13.9%)	12.350 (0.787%)	13.070 (1.11%)	13.750 (0.879%)	14.920 (3.29%)	15.000 (2.38%)	17.290 (3.50%)
	Average Relative Error (%)			9.3 ± 7.7								

Where applicable, data are presented as retention time (relative errors)

Given the various outputs (resolution vs. retention times) and types of training data (replicates vs. averages) that were considered, the best performing ANN was found to be the one trained on replicate retention time data. This network gave excellent overall correlation between experimental and predicted retention times for the training data set and, more importantly, it gave the lowest relative errors when presented with new data. Unfortunately, knowledge of analyte retention times does not directly indicate the best separation, and resolutions between peak pairs must first be determined. Further manipulation of the predicted retention data was therefore required in order to ascertain the optimum separation conditions.

In many HPLC gradient separations, peak widths remain more or less constant and calculation of resolution becomes a simple matter of calculating the difference in retention times between consecutive peaks. However, in this case, the peak widths were found to vary considerably with the percentage of organic modifier in the mobile phase, with lower percentages associated with increased peak widths. Since resolution values could not be calculated simply as the difference in retention time between consecutive peaks, Equation 4-1 was instead applied. Consequently, an additional ANN was constructed in order to predict the peak widths for each analyte throughout the experimental space. For this ANN, the values of each factor were again entered as inputs, while the average peak widths for each analyte comprised the nine outputs. Average data was used to eliminate the noise produced by slight variations in peak width, giving a total of nine training points and six verification points.

The best performing ANN had a MLP architecture consisting of 3 input nodes, 12 hidden nodes and 9 output nodes (peak widths for nine analytes) (MLP 3:3-12-9:9), with a training error of 0.087 and a verification error of 0.085. The average peak widths obtained at each of the fifteen experiments were plotted against the predicted peak widths generated by the ANN for the same fifteen experiments. Overall correlation between experimental and predicted peak widths, as illustrated in Figure 4-13, was satisfactory (r^2 = 0.9838). As seen in Table 4-14, the correlation

coefficients obtained for each individual analyte showed more variation, ranging between 0.9023 for 7-aminonitrazepam and 0.9951 for diazepam, which may account for the lower than desired overall correlation coefficient. Given 7-aminonitrazepam had the least retention on the column and, as a result, the smallest peak width, the low correlation coefficient for 7-aminonitrazepam is to be expected. Even slight differences between predicted and experimental peak widths for this analyte become magnified and cause the data to appear comparatively non-linear. In any case, the peak width of 7-aminonitrazepam remained constant throughout the entire experimental space, so the low predictive power associated with this analyte was not of great concern. More important was the ANNs strong ability to predict the peak width of diazepam which, being the strongest retained analyte, varied considerably throughout the experimental space according to the percentage of organic modifier in the mobile phase.

After considering the ability of the ANN to predict peak widths for the experimental data set it was trained on, a grid pattern was again generated and the ANN applied to predict the peak widths of each analyte throughout the experimental space. Three experimental points were then selected and run to determine if the predicted data was consistent with this new experimental data. Table 4-15 contains the experimental peak widths for the new data set, the predicted peak widths generated by the ANN and the corresponding relative error associated with the prediction. Relative errors ranged from 0.282% to 19.1%, with the largest error associated with the prediction of the peak width of alprazolam.

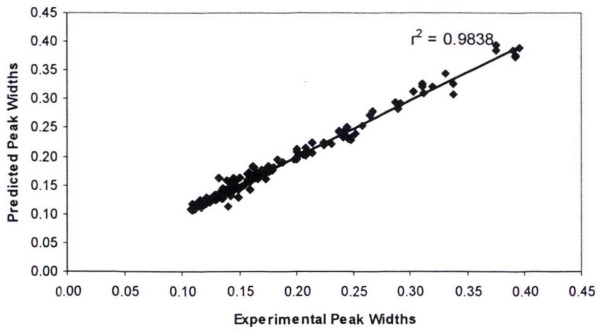


Figure 4-13 Overall correlation between experimental and predicted peaks widths for a 3:3-12-9:9 MLP ANN

Table 4-14 Correlation equations and correlation coefficients between experimental and predicted peak widths for each analyte

Analyte	Correlation Equation	Correlation Coefficient (r^2)
7-aminonitrazepam	y = 0.924151x + 0.007697	0.9023
7-aminoclonazepam	y = 1.026312x - 0.00341	0.9706
7-aminoflunitrazepam	y = 0.990677x + 0.001907	0.9759
nitrazepam	y = 0.936984x + 0.011939	0.9940
oxazepam	y = 0.989787x - 0.00122	0.9509
flunitrazepam	y = 0.996042 - 0.00221	0.9738
alprazolam	y = 1.008963x - 0.00067	0.9917
temazepam	y = 1.024757x - 0.00517	0.9878
diazepam	y = 0.974807x - 0.005016	0.9951

Table 4-15 Experimental and predicted peak widths with corresponding relative errors

	ACN grad	%MeOH	Initial %ACN	7-NH_2-NIT	7-NH_2-CLO	7-NH_2-FLU	NIT	OXA	FLU	ALP	TEM	DIA
Exp. Data	2.4	10	9.5	0.12	0.14	0.15	0.16	0.16	0.17	0.17	0.17	0.19
	2.2	22	10	0.11	0.12	0.13	0.15	0.15	0.16	0.20	0.20	0.18
	2	12	5	0.13	0.15	0.16	0.18	0.18	0.19	0.18	0.19	0.21
Predicted Data	2.4	10	9.5	0.11 (8.08%)	0.12 (10.3%)	0.13 (11.8%)	0.15 (6.40%)	0.14 (9.09%)	0.16 (6.53%)	0.15 (11.0%)	0.15 (11.6%)	0.17 (8.17%)
	2.2	22	10	0.11 (3.60%)	0.12 (4.03%)	0.12 (6.61%)	0.15 (4.06%)	0.17 (13.4%)	0.17 (6.24%)	0.21 (5.60%)	0.20 (1.20%)	0.18 (0.681%)
	2	12	5	0.13 (0.282%)	0.14 (7.12%)	0.14 (8.94%)	0.18 (2.96%)	0.16 (11.1%)	0.17 (11.0%)	0.22 (19.1%)	0.22 (13.2%)	0.20 (4.96%)
	Average Relative Error (%)			7.7 ± 4.3								

Where applicable, data are presented as peak widths (relative errors)

The predicted retention times and peak widths were used to determine the resolution between each analyte according to Equation 4-1, and the optimum separation conditions were determined according to the product resolution (PR_s), which was calculated by multiplying peak pair resolutions according to Equation 4-2. The data generated was then sorted in order of descending PR_s to identify the conditions for which PR_s was a maximum. To observe the overall trend, three-dimensional resolution response surfaces were generated by plotting PR_s against two of the variables. The third variable remained constant; its value chosen to include the region of maximum PR_s.

Figure 4-14 illustrates the effect of varying the initial %ACN and ACN gradient on PR_s. From this response surface, it can be seen that the best separation according to PR_s occurred at low ACN gradient and low initial %ACN. PR_s tended to decrease slowly as ACN gradient and initial %ACN were increased. The influence of initial %ACN and %MeOH on PR_s is highlighted in Figure 4-15, which shows that the maximum PR_s was at low %MeOH and low initial %ACN. Since the maximum occurred at a specific point rather than over a broad region, PR_s tended to decrease sharply as %MeOH and initial %ACN were increased beyond this point. Finally, the influence of %MeOH and ACN gradient on PR_s is depicted in Figure 4-16. As is evident from this response surface, the maximum PR_s occurred at low %MeOH and low ACN gradient. Again, PR_s decreased sharply as %MeOH and ACN gradient were increased beyond this maximum. To summarise, PR_s response surfaces predicted the optimum separation to be at low %MeOH, low ACN gradient and low initial %ACN.

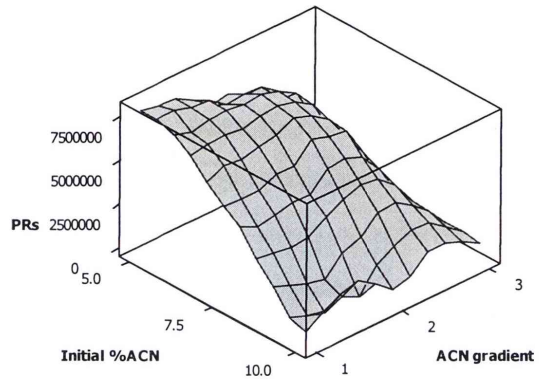


Figure 4-14 Influence of initial %ACN and ACN gradient product resolution (at 10% MeOH)

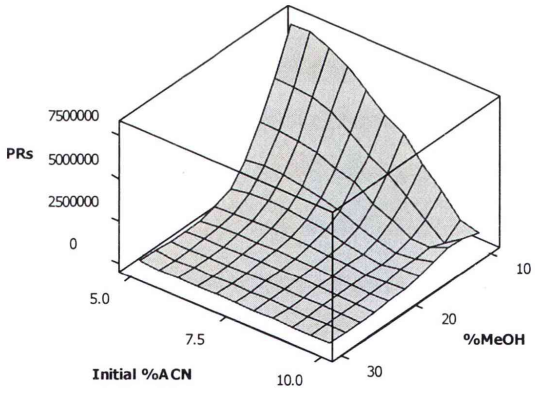


Figure 4-15 Influence of %MeOH and initial %ACN product resolution (at ACN gradient 1)

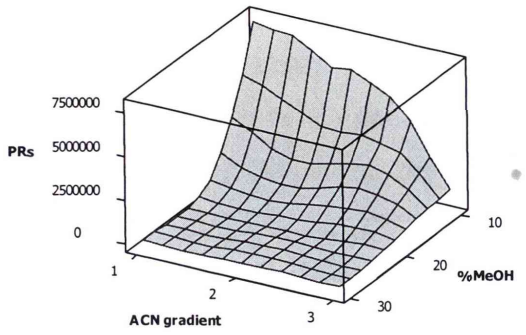


Figure 4-16 Influence of ACN gradient and %MeOH on product resolution (at 5% initial ACN)

Table 4-16 Resolutions between consecutive peaks at the optimum predicted by PR_s

	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8
Experimental	20.241	13.474	36.937	3.117	5.551	3.281	0.785	9.881
Predicted	17.812	15.267	43.232	4.293	6.177	3.413	0.738	10.036

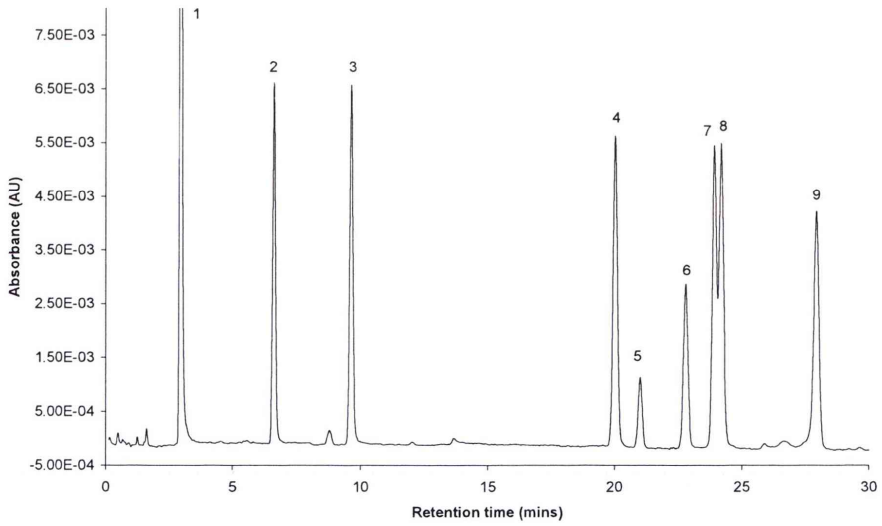


Figure 4-17 Example chromatogram of the separation optimised using product resolution

Conditions: Zorbax SB-C18 column (50 x 4.6 mm, 1.8μm), temperature 30 °C, 10 μL injection, detection λ 280 nm, 25 mM formate buffer, pH 2.8, 10% MeOH, ACN gradient 0 to 30 minutes, 5.5 to 35.5%; **Elution order:** 1. 7-NH_2-NIT, 2. 7-NH_2-CLO, 3. 7-NH_2-FLU, 4. NIT, 5. OXA, 6. FLU, 7. ALP, 8. TEM, 9. DIA.

Inspection of the response surfaces and data revealed the optimum conditions according to PR_s to be 10% MeOH, 5.5% initial composition ACN and a gradient of 1 (0 to 30 mins, 5.5 to 35.5%). To ascertain that these conditions were indeed the optimum, they were verified experimentally. An example of the separation attained under these conditions is illustrated in Figure 4-17 and the corresponding resolutions between consecutive peaks are presented in Table 4-16. Clearly, the optimum conditions according to PR_s were not ideal, since they produced a run time of more than 25 minutes. In addition, the resolution between alprazolam and temazepam (PP7) was 0.785, indicating that these analytes were barely resolved. The reason for this is that PR_s determines the optimum separation to be the one which has the greatest spread of peaks. This generally occurs over a long period of time, which equates to a long run time. Also, the impact of poorly resolved analytes is insignificant compared with the resolution of analytes that are separated by a number of minutes, which means that the optimum separation according to PR_s may not have all peaks resolved. In order to find the optimum separation conditions in this case, other optimisation functions need to be considered to ensure that run time and the resolution of poorly resolved peaks are taken in account.

4.4.3 Development of a Novel Function

The response values given by many optimisation functions (e.g. product resolution, PR_s) do not adequately reflect the quality of a chromatogram. In the case of PR_s, the maximum value represents the largest spread of peaks. However, its value is largely influenced by the resolution of the well-resolved peaks, when realistically it is the incompletely resolved peaks that present problems in analytical separations. In addition, PR_s is more likely to favour longer analysis times, since the greatest spread of peaks occurs over a longer period of time. In practise, one may be willing to sacrifice a small amount of resolution for faster analysis times.

Before the optimum separation conditions can be determined, the optimum separation must first be defined. For the purposes of the benzodiazepine analysis

under investigation, the optimum separation was defined as one in which all peaks were baseline resolved (R_s > 1.5) and all analytes eluted within a run time of 10 minutes. However, since it is of more importance to have acceptable resolution between analytes, slightly longer analysis times would be accepted if the resolution criterion was close to being satisfied. Given the tendency for 7-aminonitrazepam to elute at the void under conditions of high organic modifier composition, the optimum separation should also have the first peak sufficiently retained on the column so as not elute at the void.

As illustrated in Table 4-1, there are a number of available functions for assessing chromatographic separations. To determine if any of these functions were capable of accurately assessing separations according to the definition of the optimum separation specified above, several of the more common or recent functions were tested with regards to the separation of benzodiazepines. Rather than considering all fifteen chromatograms from the three factor analysis, the sample size was reduced to seven chromatograms, as shown in Table 4-17. These chromatograms were evaluated according to the product resolution (PR_s), normalised product resolution (NPR_s), chromatographic resolution statistic (CRS) [468], chromatographic response factor (CRF) [464], chromatographic exponential function (CEF) [469] and quality of separation function (Q) [448].

Table 4-17 Experiments selected for the comparison of optimisation functions

Experiment No.	%MeOH	ACN gradient
1	10	1
2	10	3
3	30	1
4	30	3
5	20	2
6	15	2
7	25	2

The function values calculated for each of the seven separations are presented in Table 4-18. Resolution and product resolution were calculated according to Equation 4-1 and Equation 4-2 respectively. The remaining functions were calculated according to the equations listed below. Given that some functions optimise to a maximum (PR_s, NPR_s, CRF and Q) while others optimise to a minimum (CRS and CEF), the values cannot be compared directly. Each value was therefore converted into a chromatogram ranking, whereby 1 represented the optimum separation according to the function value, and 7 represented the worst separation. The chromatogram rankings for each function were then compared.

Equation 4-5 Calculation of normalised product resolution

$$NPR_s = \Pi \frac{R_s}{(n-1)^{-1} \Sigma R_s}$$

where R_s = resolution of consecutive solute pairs, n = no. solutes

Equation 4-6 Calculation of CRS

$$CRS = \left\{ \sum_{i=1}^{n-1} \left[\frac{(R_{i,i+1} - R_{opt})^2}{R_{i,i+1}(R_{i,i+1} - R_{min})^2} \right] + \Sigma \frac{(R_{i,i+1})^2}{(n-1)R_{av}^2} \right\} \frac{t_f}{n}$$

where $R_{i,i+1}$ = consecutive solute pairs resolution, R_{av} = average resolution of all solute pairs, R_{opt} = desired resolution, R_{min} = min. resolution, t_f = last solute retention time, n = no. solutes [468]

Equation 4-7 Calculation of CRF

$$CRF = \sum_{i=1}^{L} R_i + L^{w_1} - w_2 \left| T_A - T_L \right| - w_3(T_1 - T_0)$$

where R_i = ith and (i+1)th peak resolution, L = no. peaks in chromatogram, T_A = max. acceptable time, T_L = final peak retention time, T_1 = first peak retention time, T_0 = min. retention time of first peak, w_1, w_2, w_3 = weighting parameters [464]

Equation 4-8 Calculation of CEF

$$CEF = \left[\left(\sum_{i=1}^{n-1} \left(1 - e^{a(R_{opt} - R_s)}\right)^2 \right) + 1 \right] \left[\frac{1 + t_f}{t_{max}} \right]$$

where R_{opt} = desired resolution, R_i = ith peak pair resolution, t_{max} = max. acceptable time, t_f = final, peak retention time, a = slope adjustment factor, n = no. expected peaks [469]

Equation 4-9 Calculation of Q

$$Q = \frac{R_{f\text{-}l}}{t_l} \prod_{i=1}^{n-1} \frac{R_i}{\frac{1}{n-1} \sum_{i=1}^{n-1} R_i}$$

where t_l = migration time of last peak, $R_{f\text{-}l}$ = resolution between first and last peaks, n = no. of peaks in chromatogram, R_i = resolution of ith peak pair [448]

Table 4-18 Comparison of selected optimisation functions for evaluating separations

Exp.	Resolution								t_{first} (mins)	t_{final} (mins)	PR_s	NPR_s	CRS	CRF	CEF	Q
	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8								
1	8.952	8.382	40.182	5.259	5.765	3.173	1.456	7.667	1.041	20.304	3238000	0.02979	7.084	78.29	24.05	0.1176
2	9.060	7.320	30.534	2.595	5.595	1.347	1.582	7.510	1.047	10.502	470700	0.02318	2.644	72.79	14.23	0.1421
3	2.259	2.574	21.879	5.273	1.073	5.981	0.806	8.452	0.741	10.477	29340	0.01662	57.57	55.88	35.28	0.07751
4	2.222	2.583	20.055	4.750	0.000	5.586	0.577	6.108	0.739	6.813	0	0	N.D	43.76	670.7	0
5	4.643	4.950	30.144	4.999	2.284	4.747	1.159	8.192	0.841	10.933	356500	0.03072	7.542	68.14	17.94	0.1774
6	6.523	6.331	32.386	4.272	3.943	3.431	1.376	7.964	0.919	12.173	847300	0.03841	3.410	71.93	17.77	0.2137
7	3.266	3.631	25.118	5.344	0.705	5.582	0.930	7.961	0.785	9.488	10410	0.005629	86.39	56.08	177.4	0.03230

N.D – function not defined at this value

Exp.	Chromatogram ranking					
	PR_s	norm PR_s	CRS	CRF	CEF	Q
1	1	3	3	1	4	4
2	3	4	1	2	1	3
3	5	5	5	6	5	5
4	7	7	N.D	7	7	7
5	4	2	4	4	3	2
6	2	1	2	3	2	1
7	6	6	6	5	6	6

As can be seen in Table 4-18, all the evaluated functions were able to accurately distinguish chromatograms 3, 4 and 7 as being undesirable due to the poor resolution of PP5 and PP7 in chromatograms 3 and 4, and PP7 in chromatogram 5. However, each differed in their ability to rank the remaining four chromatograms in accordance with the optimum separation definition.

As observed in Table 4-18, PR_s ranked chromatogram 1 as the optimum separation, largely due to the emphasis it places on well-resolved peak pairs. While close to baseline resolution is achieved for each analyte in this chromatogram, the separation is far from optimal given the extremely long run time. The next best separation according to PR_s is attained in chromatogram 6, where the minimum resolution is close to 1.4 and the run time is significantly shorter. Since a small amount of resolution can be sacrificed for a shorter analysis time, chromatogram 6 is closer to fitting the definition of the optimum separation and should be given preference over chromatogram 1. PR_s determines the best separation to be the greatest spread of peaks, thus it cannot evaluate chromatograms in accordance with the definition of the optimum separation specified here, as it gives preference to long run times.

Chromatogram 6 is ranked highest by the normalised product resolution, NPR_s, which, as discussed above, has good resolution and run time. However, the next best separation according to NPR_s is chromatogram 5, which has a pair of analytes (PP7) with $R_s < 1.2$, which is considerably less resolution than desired. The separation ranked third was that of chromatogram 1. In this case, the minimum resolution is close to 1.5, but the run time of 20 minutes is longer than desired. In reality, neither chromatogram 5 nor chromatogram 1 are particularly desirable, however for the purposes of assessing the ability of each optimisation function in ranking chromatograms, a choice between the two must be made. According to the definition outlined previously, a small amount of resolution may be sacrificed for a shorter run time. Since an R_s value of 1.2 translates into a substantial loss of resolution, preference should be given to chromatogram 1, which is closer to resolving all analytes, despite the long run time. Therefore, NPR_s cannot determine the best

separation in accordance with the optimum separation definition, since it places too much emphasis on run time.

CRS [468] defines chromatograms 2 and 6 as the best separations. In both these cases, the resolutions and run times are close to the desired values. Importantly, unlike NPR_s, CRS ranks chromatogram 1 above chromatogram 5, meaning it can sacrifice a small amount of resolution for a shorter run time. The main drawback with the CRS function is that it is not useful in separations where two analytes co-elute ($R_s = 0$), as seen in chromatogram 4. In such cases, the value of CRS is undefined since it renders the denominator of the first term in Equation 4-6 equal to zero. So although the CRS can accurately detect optimal separations, it is not useful for plotting response surfaces, since there may be regions that are undefined in cases where $R_s = 0$.

Like PR_s, CRF [464] favours long analysis times and ranks chromatogram 1 as the optimum. Chromatograms 2 and 6, which have acceptable resolution and are more ideal due to shorter analysis times, are ranked next. Clearly, the CRF does not have the ability to accurately assess the quality of chromatograms within the bounds of the criteria specified for the optimum separation, since it is largely influenced by well-resolved peak pairs.

Chromatograms 2 and 6 are ranked highest by the CEF [469], which is in agreement with the optimum separation definition. However, rather than favouring longer analysis times in which the minimum resolution is achieved, the CEF favours short analysis times. As a result, chromatogram 5, which has $R_s < 1.2$ for PP7, is ranked higher than chromatogram 1. Since more weight is given to short analysis times than resolution, the CEF is not ideal for assessing chromatographic separations in accordance with the stipulated definition.

The Q [448] function ranked chromatogram 6 as optimal, which is justified given the reasonable resolution and run time. Ranked second was chromatogram 5, followed

by chromatogram 2. As discussed above, chromatogram 5 has one peak pair with R_s < 1.2, whereas chromatogram 2 is closer to satisfying both the resolution and run time criteria. Consequently, Q is not an appropriate function for determining the optimum separation since it does not give enough weight to resolution.

Since none of the available optimisation functions could satisfy the criteria and appropriately assess the seven chromatograms, a novel objective function was developed. To ensure that the function could assess chromatographic separations in accordance with the stipulated criteria for the optimum separation, it was given a number of properties. First was its ability to place more weight on unresolved peak pairs as opposed to well-resolved peaks, thereby ensuring that resolutions far greater than optimal (R_s = 1.5) had minimal effect on the function. The second attribute was its capacity to assign more weight to resolution over run time, thereby preventing chromatograms with shorter run times and unresolved peaks from being favoured. And finally, the function was given the capability to prevent chromatograms with short run times, but with the first peak insufficiently retained on the column, from being favoured.

The Ideal Separation Function (ISF), as shown in Equation 4-10, was developed to incorporate both resolution and run time, and achieve a maximum when both these conditions are ideal. It was also designed to recognise separations where the first peak elutes at the void as undesirable. Its design was based on a combination of previously developed functions, including the CEF and CRS.

Equation 4-10 The Ideal Separation Function (ISF)

$$\mathrm{ISF} = \frac{1}{n-1}\left[\sum_{i=1}^{n-1}\left(1-e^{2(R_{min}-R_s)}\right)\right] + \frac{1}{2}\left(\frac{t_{max}}{t_{final}}\right) + \left(1-e^{t_{min}-t_{first}}\right)$$

where n is the number of peaks in a chromatogram, R_s is the resolution between consecutive peaks, R_{min} is the minimum acceptable resolution for consecutive peaks, t_{max} is the maximum acceptable run time, t_{final} is the retention time of the final peak, t_{first} is the retention time of the first peak and t_{min} is the minimum acceptable retention time for the first peak

The first term in the ISF considers the resolution of consecutive peaks R_s, and the minimum desired resolution for the separation R_{min}, which can be varied to suit the requirements of the chromatographer. An R_{min} of 1.5 is chosen when baseline resolution of all components is desired. When the resolution between any peak pair is less than R_{min}, the term will be negative, and when the resolution is greater than R_{min}, the term will be positive. The exponential function, also employed by Morris *et al.* [469] in the development of the CEF, prevents excessively large R_s values between one pair of analytes from significantly affecting the overall ISF value. This is important because it is the incompletely resolved analytes that should govern the best separation, rather than peak pairs which exhibit large resolution values. As indicated in Figure 4-18, the term approaches 1 as R_s increases, ensuring that no additional weight is given to very large resolution values in any separation. The coefficient of R_s in the equation is 2 so that the function becomes increasingly negative if the minimum resolution of any peak pair is not achieved. The term is summed over each peak pair, with any negative values in the separation decreasing its final value. The term is then averaged by dividing by the number of peak pairs in the chromatogram. If a sufficient number of poorly resolved peaks are present in any separation, the value of this first term may be negative.

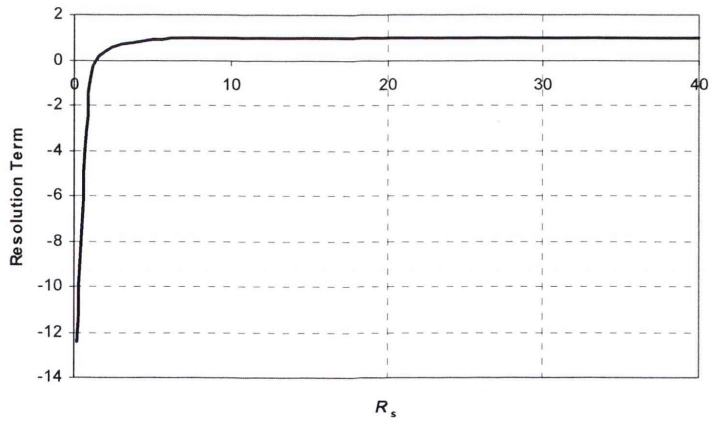


Figure 4-18 Range and domain for the resolution term of the ISF

Conditions: $y = 1-e^{2(R_{min}-R_s)}$, $R_{min} = 1.5$

The second term in the ISF relates to the overall run time. The variable t_{max} is the maximum acceptable analysis time, and can be given any value, depending on the requirements of the separation. If the final peak t_{final} elutes after the maximum analysis time, the term will be less than one. If t_{final} elutes within the specified analysis time, then the term will be greater than one. This can be seen graphically in Figure 4-19. The term is halved to place less weight on run time and comparatively more on resolution.

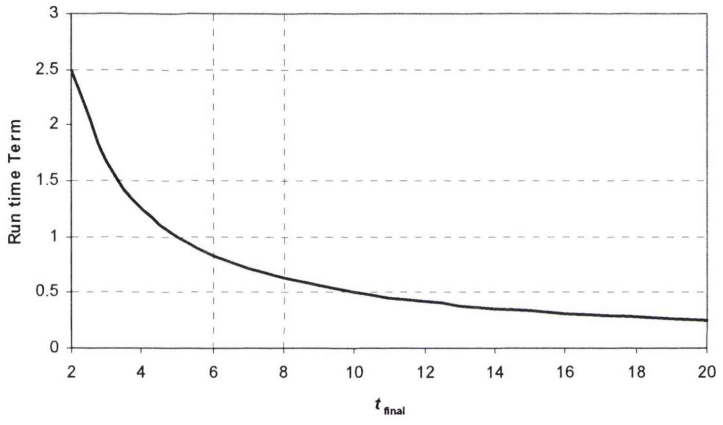


Figure 4-19 Range and domain for the run time term of the ISF

Conditions: $y = t_{max} / 2t_{final}$, $t_{max} = 10$

Unlike many of the other optimisation functions, the ISF includes a term designed to reduce the value of the function in chromatograms where peaks elute at the void. The variable t_{min} is the minimum desired retention time of the first peak, and it should be given a value slightly greater than that of the void if there are no early-eluting endogenous peaks. Since it is of no more benefit to have the first peak, t_{first}, elute long after the void, an exponential function was again utilised. When t_{first} elutes after t_{min}, the value of the function is positive and approaches 1. If t_{first} elutes before t_{min}, then the term is negative and reduces the overall function value. This can be seen graphically in Figure 4-20.

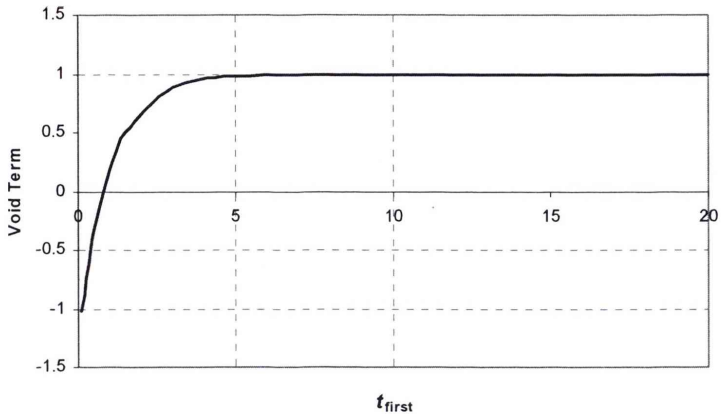


Figure 4-20 Range and domain for the void term of the ISF

Conditions: $y = 1-e^{t_{min}-t_{first}}$, $t_{min} = 0.8$

Finally, rather than multiplying all three terms together, which could result in an overall positive value if two of the terms are negative, all three terms are summed so that the ISF optimises to a maximum. The ISF's performance was assessed by calculating its value for each of the seven chromatograms, in the manner described previously. To allow direct comparison with the other functions, each ISF value was again converted into a chromatogram ranking, as shown in Table 4-19.

Table 4-19 Assessment of separations according to the ISF

Exp.	ISF	Ranking
1	1.319	3
2	1.405	1
3	0.580	5
4	-1.679	7
5	1.224	4
6	1.359	2
7	-0.128	6

Like CRS and CEF, ISF ranks chromatogram 2 as the optimal, followed closely by chromatogram 6. In both these chromatograms, the resolution is $\approx$ 1.4, which is acceptable given that both have close to the maximum specified run time of 10 minutes. Chromatogram 1 is ranked third, which has a similar resolution to that of chromatograms 2 and 6, although the run time is double that of the maximum specified run time. However, this ranking is justified given that longer analysis times are acceptable if the minimum resolution is achieved. The next highest ranked chromatogram is 5, which has a good run time of around 10 minutes, however the resolution of PP7 is < 1.2. ISF easily distinguishes chromatograms 3, 7 and 4 as being unacceptable due to early elution of the first peak and poor resolution values. And, unlike CRS, ISF is defined when $R_s = 0$ so a response surface can be plotted for all values of R_s, even when two analytes co-elute.

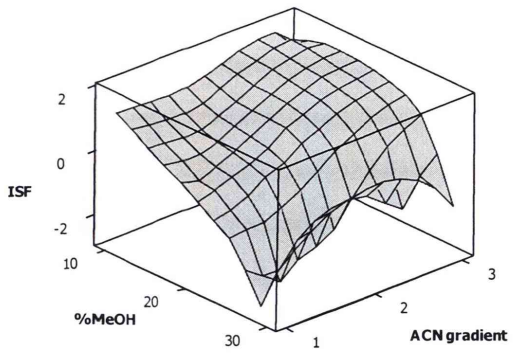
Considering the various optimisation functions that were assessed in their ability to identify the optimum separation, the ISF performed the best. It was able to rank the seven chromatograms in accordance with the specified definition of the optimum separation by giving appropriate weight to the parameters of resolution, run time and minimum retention.

4.4.4 Optimisation of the Separation Using ISF

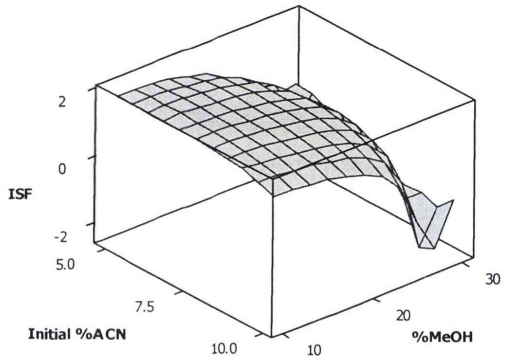
Given that the ISF performed better than any of the other optimisation functions tested, it was applied to the retention time and peak width data predicted by the ANNs. The variables R_{min} and t_{max} were assigned the values 1.5 and 10 respectively. The void volume was determined to be 0.7 minutes, so t_{min} was assigned the value 0.9. From this data, three resolution response surfaces were generated by plotting ISF against two of the variables while the third variable was kept constant, as illustrated in Figure 4-21. According to these response surfaces, the maximum ISF and hence the best separation could be found at low %MeOH, high ACN gradient and low initial %ACN. Sorting the data generated in order of descending ISF value gave the maximum value of ISF at 10% MeOH, 6.5% initial ACN composition and a gradient of 2.8 (0 to 15 mins, 6.5 to 48.5%). The ANN data predicted that these conditions

would give resolution values > 1.5 and a run time of less than 13 minutes. Although this run time was slightly longer than the preferred run time of 10 minutes, it was deemed acceptable given that the resolution criterion of $R_s > 1.5$ was satisfied. The conditions producing run times of less than 13 minutes were considered inappropriate, since they would have produced unacceptably low resolution values.

a)



b)



c)

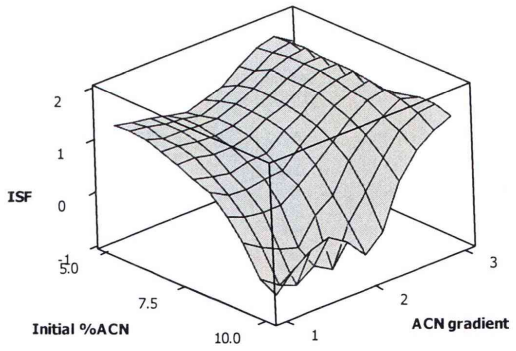


Figure 4-21 ISF response surfaces for varying a) %MeOH and ACN gradient, b) initial %ACN and ACN gradient and c) initial %ACN and %MeOH

The optimum conditions according to the ISF were run and an example chromatogram of the optimised separation in 25 mM formate buffer (pH 2.80), 10% MeOH with a gradient of 0 to 15 minutes, 6.5 to 48.5% ACN is illustrated in Figure 4-22. The predicted and experimental retention times and peak widths, with associated relative errors under these conditions are illustrated in Table 4-20. The errors concerning the prediction of retention times at the optimum ranged from 0.039 – 14.39%, with an average of 4.78%. The largest error was associated with the prediction of the retention time of 7-aminonitrazepam. Even though the absolute difference between the experimental and predicted retention times for this analyte was only 0.315 minutes, this equated to a large percentage error since 7-aminonitrazepam had the least retention on the column. The errors relating to the prediction of peak widths at the optimum ranged from 1.41 – 7.12%, with an average of 3.57%. Again, the largest error was associated with the prediction of 7-aminonitrazepam. According to Zakaria *et al.* [476], predictive errors using ANNs, or indeed any other model, should be less than 5% for optimisation purposes. The results for six of the nine analytes are in agreement with this. While higher than desired, the errors associated with the prediction of the 7-amino metabolites had no bearing on the final outcome since these analytes were well-resolved under all conditions, including the optimum.

Table 4-21 illustrates the predicted and experimental resolution values, calculated using retention times and peak widths obtained from predicted and experimental data respectively. The error relating to the prediction of resolution values ranged from 0.771 – 20.4%. This large error results from the addition of small errors in retention and peak width data, which must be inputted into the resolution equation. Unfortunately, the largest error was associated with the prediction of the resolution between alprazolam and temazepam (PP7), which was the most difficult peak pair to separate. So while baseline resolution was predicted for PP7 (R_s = 1.63), this was not observed when the conditions were run. Experimentally, a resolution value of R_s = 1.35 was obtained for this peak pair.

Table 4-20 Predicted and experimental retention times and peak widths with corresponding errors for the optimum separation conditions

Analyte	Predicted t_r (mins)	Experimental t_r (min)	Error (%)	Predicted width	Experimental width	Error (%)
7-NH_2-NIT	1.874	2.189	14.39	0.122	0.131	7.12
7-NH_2-CLO	3.716	4.178	11.06	0.125	0.133	6.22
7-NH_2-FLU	5.094	5.515	7.628	0.131	0.138	5.25
NIT	9.404	9.669	2.741	0.142	0.149	4.52
OXA	9.618	9.908	2.927	0.128	0.130	1.89
FLU	10.560	10.757	1.831	0.142	0.145	1.89
ALP	10.890	11.044	1.394	0.140	0.143	2.31
TEM	11.130	11.247	1.040	0.155	0.157	1.41
DIA	12.850	12.845	0.039	0.153	0.155	1.49

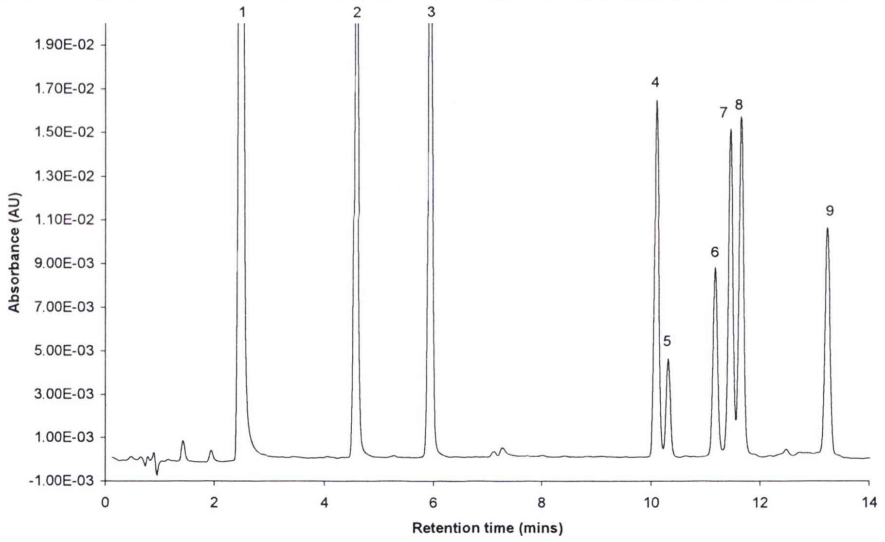


Figure 4-22 Example chromatogram of the optimised separation of nine benzodiazepines

Conditions: 10% MeOH, ACN gradient 0 to 15 minutes, 6.5 to 48.5%, for remaining conditions and elution order refer to Figure 4-17.

Table 4-21 Predicted and experimental resolution values for the optimum separation conditions

	PP1	PP2	PP3	PP4	PP5	PP6	PP7	PP8
Predicted	14.9	10.8	31.6	1.59	6.98	2.34	1.63	11.2
Experimental	15.1	9.87	29.0	1.71	6.17	1.99	1.35	10.2
Error (%)	0.771	9.35	9.06	7.41	13.1	17.5	20.4	9.22

Despite the fact that baseline resolution was not achieved between alprazolam and temazepam (PP7), and the run time exceeded the stipulated 10 minutes, the ISF was still more successful than PR_s in locating the best possible separation. The optimum conditions predicted using PR_s gave the greatest spread of peaks, and the resolution of PP7 did not have a significant effect on its value. As a result, the predicted resolution of PP7 was only 0.738 (experimental $R_s = 0.785$) at the optimum and the run time was more than 25 minutes. Based on the variables of a maximum run time of 10 minutes, a minimum retention time of 0.9 minutes and a desired resolution of 1.5, the ISF was able to locate the separation conditions that gave a predicted resolution of $R_s = 1.6$ for PP7 within a much shorter run time.

The optimisation of gradient elution separations using ANNs has been previously reported, however the method presented here offers a number of advantages. Firstly, unlike the methods presented by Madden *et al.* [457] and Shan *et al.* [466], the initial and final compositions of the organic modifier do not need to be constrained. Instead, the initial composition of organic modifier and the slope of the gradient can be used as input variables for an ANN, and the final composition can be calculated from the gradient slope after substituting in an appropriate time. Not only does this allow for greater flexibility because it does not rely on fixed starting and finishing compositions, but it also effectively opens up the experimental space and increases the possibility of finding the optimum separation conditions. In addition, having flexible starting and final concentrations avoids the use of long analysis times that are required to implement slow gradients when concentrations are fixed.

In contrast to the work presented here, the majority of studies employing ANNs to optimise gradient elution separations do not represent true optimisation strategies. Rather than defining an experimental space and modelling the entire region, these studies model only a handful of gradient conditions, and the optimum is selected from this limited study. For example, Madden *et al.* [457] employed three gradients to train an ANN, which was then used to predict the retention times at the three training gradients plus an additional four. The optimum separation conditions were

then selected as one of these seven gradients. A similar method was employed by Buciński *et al.* [454] who only established the optimum from a set of four gradients, rather than interpolating over a range of gradient conditions to locate the optimum. The major fault with these methods is that by limiting the input data, they risk missing the optimum completely. Clearly, the method presented here is a more useful gradient optimisation method, since it enables retention times to be predicted over a wide range of gradients within the experimental space, rather than for just a select few conditions. It also offers the possibility of generating response surfaces to observe overall trends in resolution, thus enabling the optimum to be easily located.

Finally, in contrast to the study by Shan *et al.* [466], who modelled the retention of seventeen amino acids using seventeen individual ANNs, the current method only requires two ANNs (one for retention times and one for peak widths) to determine the optimum separation. The only possible disadvantage is that the substitution of predicted retention times and peak widths into the resolution equation could cause an accumulation of error. However, this risk must be taken in gradient separations whereby peak widths can vary from one condition to the next. Using the data generated from only two ANNs to model a complex separation, which undergoes changes in selectivity as well as peak widths, is a major advantage and a simplification of Shan's method.

4.4.5 Sample Preparation

Given that the HPLC separation of nine benzodiazepines was successfully optimised using ANNs, the suitability of the method for the analysis of the analytes in post-mortem blood was assessed. Prior to this however, an appropriate extraction technique had to be developed. Four LLE techniques, including butyl chloride, ethyl acetate, diethyl ether and diethyl ether/ethyl acetate (1:1, v/v), were evaluated with regards to the recovery of each benzodiazepine from spiked whole blood. Average analyte recoveries for these extraction techniques are presented in Figure 4-23.

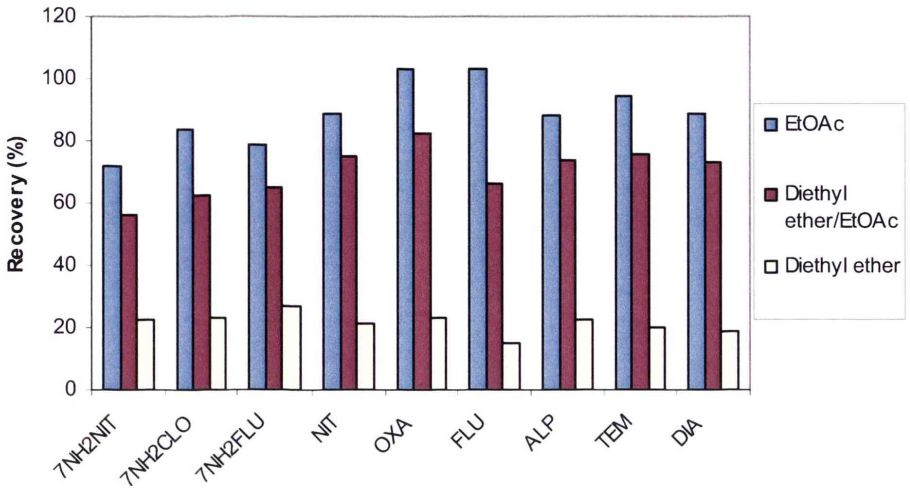


Figure 4-23 Comparison of average recovery obtained for ethyl acetate, diethyl ether/ethyl acetate and diethyl ether LLEs

Butyl chloride was the least efficient extraction solvent since, in addition to the analytes of interest, it extracted a large number of endogenous blood components that produced many interfering peaks in the chromatogram. As such, the recovery data for the butyl chloride LLE could not be accurately calculated and was not included in Figure 4-23. As a result, butyl chloride was excluded from further consideration as a potential LLE solvent.

From the remaining solvents in Figure 4-23, diethyl ether was the least effective extraction technique, giving recoveries of only 15 – 30% for each analyte. Despite the low recoveries, the diethyl ether extract was very clean, with only one matrix peak present at 2.5 mins which did not co-elute with any of the analytes.

Ethyl acetate gave the greatest recoveries, which ranged from 70 – 103%, however this solvent also extracted some endogenous blood components, and the resulting extract contained several small interfering peaks. This is the likely reason for the extraction recoveries of oxazepam and flunitrazepam exceeding 100%.

Diethyl ether/ethyl acetate successfully removed most matrix components, giving a very clean extract with only one small matrix peak at 2.5 mins, as shown in Figure 4-24. In addition, good recoveries in the range of 56 – 83% were obtained using this solvent system. Even though diethyl ether/ethyl acetate did not give the highest recoveries, it was deemed to be the most appropriate LLE technique. Unlike ethyl acetate alone, it gave clean extracts, resulting in a higher degree of accuracy for the method and, unlike diethyl ether alone, it did not compromise recovery to a great extent. Diethyl ether/ethyl acetate LLE was therefore employed for the remaining method validation and application.

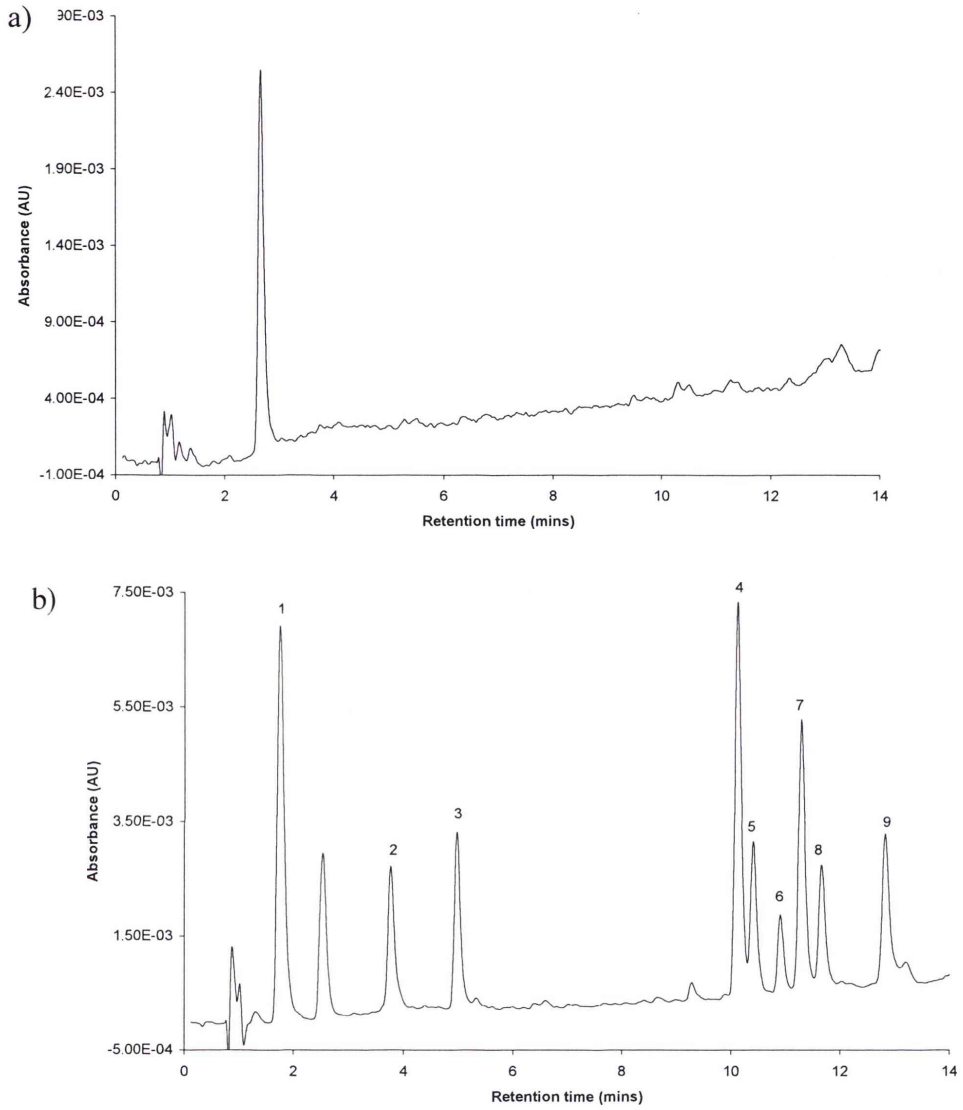


Figure 4-24 Example chromatograms of a) blank and b) spiked whole blood extracted with diethyl ether/ethyl acetate (1:1)

Conditions: Zorbax SB-C18 column (50 x 4.6 mm, 1.8μm), temperature 30 °C, 10 μL injection, detection λ 280 nm, 25 mM formate buffer, pH 2.8, 10% MeOH, ACN gradient 0 to 15 minutes, 6.5 to 48.5%; **Elution order:** 1. 7-NH_2-NIT, 2. 7-NH_2-CLO, 3. 7-NH_2-FLU, 4. NIT, 5. OXA, 6. FLU, 7. ALP, 8. TEM, 9. DIA.

4.4.6 Method Validation

The optimised method was validated for spiked whole blood samples, and calibration data for each analyte is presented in Table 4-22. Calibration curves were linear in the

specified concentration ranges and correlation coefficients (r^2) ranging from 0.9973 – 0.9993 were established in these ranges. The limits of detection (LOD) were between 0.0057 μg/mL (nitrazepam) and 0.023 μg/mL (flunitrazepam).

Intra-assay precision and accuracy were determined by analysis of five replicate samples at high and low concentration within the same validation batch. Inter-assay precision and accuracy were assessed by analysing five replicate samples at high and low concentrations over three validation batches. As shown in Table 4-23, intra-assay precision was between 0.18 and 3.0%, while inter-assay precision was between 0.15 and 13%. Accuracies were greater than 97%.

Table 4-22 Calibration and LOD data for benzodiazepine analytes in blood

Analyte	Correlation coefficient (r^2)	Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
7-aminonitrazepam	0.9985	0.43 – 6.0	0.0063	0.021
7-aminoclonazepam	0.9984	0.43 – 6.0	0.014	0.046
7-aminoflunitrazepam	0.9987	0.43 – 6.0	0.012	0.039
Nitrazepam	0.9983	0.58 – 8.0	0.0057	0.019
Oxazepam	0.9973	0.58 – 8.0	0.018	0.049
Flunitrazepam	0.9993	0.58 – 8.0	0.023	0.076
Alprazolam	0.9988	0.58 – 8.0	0.0084	0.028
Temazepam	0.9981	0.58 – 8.0	0.020	0.065
Diazepam	0.9980	0.58 – 8.0	0.017	0.055

Table 4-23 Intra- and inter-assay precision and accuracy data for benzodiazepine analytes in blood

Analyte	Concentration (μg/mL)	Intra-assay precision (%CV)	Intra-assay accuracy (%)	Inter-assay precision (%CV)	Inter-assay accuracy (%)	Recovery (%, mean±SD)
7-aminonitrazepam	6.0	0.18	98	0.15	98	58 ± 5
	0.43	0.81	115	13	115	
7-aminoclonazeapm	6.0	0.19	98	8.6	98	64 ± 1
	0.43	1.5	114	8.8	116	
7-aminoflunitrazepam	6.0	0.19	98	2.8	98	61 ± 4
	0.43	1.6	118	12	116	
Nitrazepam	8.0	0.59	98	4.3	97	77 ± 8
	0.58	1.9	109	1.3	111	
Oxazepam	8.0	1.7	98	12	97	86 ± 11
	0.58	3.0	113	6.0	114	
Flunitrazepam	8.0	0.24	99	4.8	98	92 ± 20
	0.58	1.8	117	19	113	
Alprazolam	8.0	0.21	99	8.7	98	75 ± 6
	0.58	0.52	111	3.1	112	
Temazepam	8.0	0.49	98	9.2	97	75 ± 6
	0.58	2.0	112	0.62	112	
Diazepam	8.0	0.26	98	3.8	98	75 ± 13
	0.58	1.7	118	0.43	117	

4.4.7 Method Application

Following compilation of a list of potential post-mortem cases for analysis (n = 13), five were rejected on the basis that they were recent cases, or cases requested by the Coroner to be kept pending further legal proceedings. A further three cases were rejected due to insufficient sample volume or decomposition. The remaining five samples were analysed and the benzodiazepine concentrations found in each sample are reported in Table 4-24. Example HPLC-DAD chromatograms of two post-mortem samples are illustrated in Figure 4-25.

Table 4-24 Blood concentrations of benzodiazepines in post-mortem blood samples

Case no.	Benzodiazepine detected	Concentration (μg/mL)	Additional drugs detected[a]
2402494	DIA	1.2	
	TEM	4.0	
	OXA	BLOQ	
2500058	DIA	1.4	CAR (2.8)
	TEM	0.12	PAR (<3)
2401190	OXA	4.6	
2401509	OXA	1.8	
2402756	DIA	0.3	ALC (0.164)

[a] drugs detected in addition to the nine benzodiazepines, as determined by independent analysis at DAL. Where appropriate, concentration values in parentheses are given in μg/mL, except ALC in g/100mL.

Abbreviations: alcohol (ALC), below limit of quantification (BLOQ), carbamazepine (CAR), diazepam (DIA), oxazepam (OXA), paracetamol (PAR), temazepam (TEM).

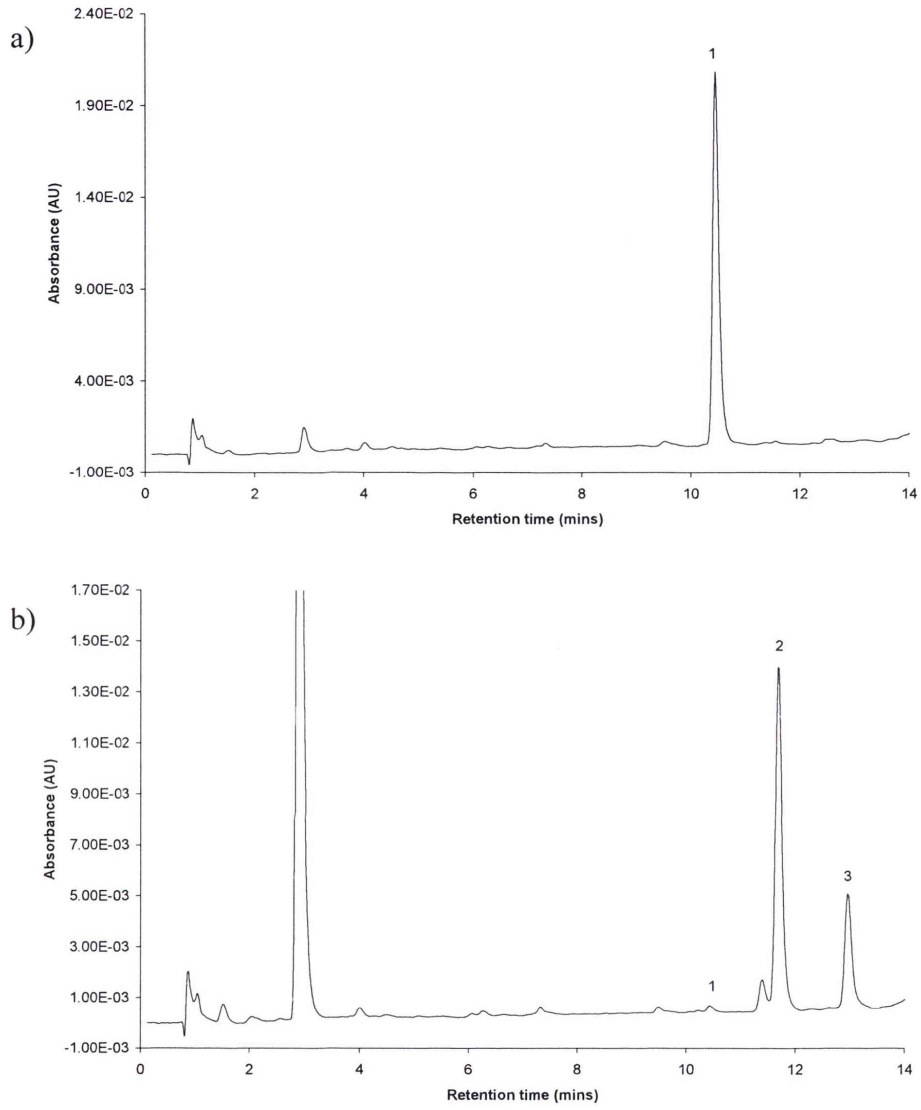


Figure 4-25 Example chromatograms for post-mortem blood samples testing positive for benzodiazepines a) case 2401190 b) case 2402494

Conditions: Zorbax SB-C18 column (50 x 4.6 mm, 1.8μm), temperature 30 °C, 10 μL injection, detection λ 280 nm, 25 mM formate buffer, pH 2.8, 10% MeOH, ACN gradient 0 to 15 minutes, 6.5 to 48.5%; **Elution order:** 1. OXA, 2. TEM, 3. DIA.

In case 2402494, diazepam and temazepam were found at concentrations of 1.2 μg/mL and 4.0 μg/mL respectively. It should be noted that the presence of temazepam in blood could have arisen from its administration as a parent drug, or via

the metabolism of diazepam. In any case, the concentration of temazepam was found to be within the fatal range of 4 – 9 μg/mL [78], while diazepam was found to be within the therapeutic range of 0.05 – 2 μg/mL [78]. Oxazepam was also detected, however its concentration was below the LOQ. Given the sub-therapeutic concentration found, its presence could be due to partial metabolism of temazepam.

In case 2500058, diazepam was found at a concentration of 1.4 μg/mL and temazepam at a concentration of 0.12 μg/mL. Considering the small concentration of temazepam, its presence is likely to be due to metabolism of diazepam, rather than consumption of the drug in its parent form. Whilst diazepam can be toxic at concentrations above 1.5 μg/mL [78], its fatal range is usually in the order of 5 μg/mL. When considered in isolation, the diazepam concentration found in this case would not be considered fatal, and the presence of other drugs must be taken into account. Paracetamol at a concentration of <3 μg/mL, is well within its therapeutic ranges and is unlikely to have contributed towards death. Although the concentration of carbamazepine was also within its therapeutic range, it is a CNS depressant and, as such, its depressive effects are additive to those of diazepam and temazepam. The concomitant use of diazepam and carbamazepine may therefore have lowered the concentration of both drugs required to produce a toxic or even fatal effect.

4.6 μg/mL of oxazepam was found in case 2401190, which falls within the toxic range (>2 μg/mL [78]). No other drugs were detected and, at this concentration, benzodiazepine-induced CNS depression resulting in death would be likely. Oxazepam was also the only drug found in case 2401509, yet its concentration was at the upper limit of the therapeutic range. At a concentration of 1.8 μg/mL, and in the absence of any other CNS depressants, toxicity and death would not be expected. The presence of systemic disease, which would lower the toxic concentration range, may have been a contributing factor in this instance. Another possibility is that death was delayed and metabolism of a larger dose of oxazepam to its glucuronide conjugates had occurred.

Diazepam was found to be present in case 2402756 at a concentration of 0.3 μg/mL, which is within the therapeutic range. However, a high blood alcohol concentration of 0.164 g/100mL was found in addition to the benzodiazepine. Since the CNS depressant effects of diazepam and alcohol are additive, the combination of these two drugs may have produced sufficient respiratory depression to result in death.

Compared to other studies employing UV detection for the determination of benzodiazepines (Refer to Table 1-11), the method presented here offers comparable run times and detection limits when consideration is given to the number of analytes involved. Whilst MS capabilities offer the possibility for lower LODs and shorter run times, the coupling of HPLC to DAD is still more widespread, and it is therefore beneficial to have a rapid and sensitive HPLC-DAD method capable of quantifying benzodiazepines in post-mortem samples. The HPLC-DAD method described here was sensitive enough to enable quantification of benzodiazepines found at fatal, toxic and even therapeutic concentrations in post-mortem samples. In one case, the concentration of oxazepam was BLOQ, however the method was still sensitive enough to enable its detection.

4.5 Conclusions

A combination of a three factor experimental design and ANNs was applied to the optimisation of a gradient elution HPLC separation of nine benzodiazepines. Following investigation of a number of ANNs, the optimum conditions predicted were 25 mM formate buffer (pH 2.8), 10% MeOH, ACN gradient 0 to 15 minutes, 6.5 to 48.5%, which gave resolution values greater than 1.3 for each analyte pair and a run time of less than 13 minutes. The error associated with the prediction of retention times and peak widths under these conditions was less than 5% for six of the nine analytes studied. This method represents a more flexible and convenient means for optimising gradient separations than has been previously reported. The optimised method was validated for blood and successfully applied to authentic post-

mortem samples. The limits of detection of the method ranged from 0.0057 – 0.023 μg/mL, and recoveries were in the order of 58 – 92%.

A novel chromatographic function, ISF, was developed to incorporate run time, resolution and minimum retention into a single function designed to assess the quality of chromatographic separations. ISF was found to offer significant advantages over other optimisation functions, such as the product resolution, which often fail to locate the optimum separation due to an inability to account for run time or detect poorly resolved peaks.

4.6 Future Work

This work has demonstrated the applicability of ANNs to modelling linear gradient separations, however their ability to model non-linear gradient profiles should be investigated further. One possibility may be to derive the equation for each profile and apply appropriate variables that give a unique descriptor of the non-linear profile, such as the y-intercept or second derivative, as training inputs. This would enable a wide variety of gradients to be investigated and perhaps enable a more appropriate optimum to be found.

The optimised method for the analysis of nine benzodiazepines has proven to be useful for the quantification of these drugs in authentic post-mortem samples. However, considering the low concentrations at which some of these drugs may be encountered, particularly when used in conjunction with other CNS depressants or when the therapeutic index is low (e.g. flunitrazepam), future work could focus on improving the LOQs of this method. Given that the optimised mobile phase is volatile, coupling of the HPLC with MS or MS-MS should accomplish this with ease.

Chapter Five

Simultaneous Quantification of Morphine, Morphine-3-Glucuronide, Morphine-6-Glucuronide and Codeine in Post-Mortem Blood Samples

5 Simultaneous Quantification of Morphine, Morphine-3-Glucuronide, Morphine-6-Glucuronide and Codeine in Post-Mortem Blood Samples

5.1 Summary

A simple and rapid HPLC method for the simultaneous determination of morphine, M3G, M6G and codeine in post-mortem blood was developed based on fluorescence detection. After SPE using C_{18} cartridges, the analytes were separated by RP-HPLC in a mobile phase consisting of 25 mM ammonium acetate buffer (pH 4.8) and an ACN gradient of 0 to 36%, 0 to 6 mins at 1 mL/min, and determined at an excitation wavelength of 280 nm and an emission wavelength of 335 nm. This gave baseline resolution between each analyte and a run time of less than 5 minutes. The analytes were determined in twelve post-mortem blood samples taken from heroin-related deaths; six of which involved the concomitant use of benzodiazepines. Cases involving the use of benzodiazepines in conjunction with heroin were found to have lower ratios of M6G/MOR and M3G/MOR, suggesting that death occurred more rapidly in this sample set. This sample set also contained fewer samples with quantifiable levels of morphine, which reflects the additive effect of heroin and benzodiazepines. Higher average concentrations of M6G and higher M6G/M3G ratios were found in cases involving the use of heroin only, which may suggest chronic heroin exposure in this sample set. High ratios of M6G/M3G coupled with low M6G/MOR ratios may indicate the possible contribution of M6G towards heroin overdose, however more cases need to be acquired to verify this.

Keywords: morphine; morphine-3-glucuronide (M3G); morphine-6-glucuronide (M6G); heroin overdose; HPLC; fluorescence detection.

5.2 Introduction

Heroin (diacetylmorphine) use may be regarded as a significant illicit drug problem in Australia. Heroin users are at a greater risk of premature mortality than their non-

heroin-using peers, and although there are a number of factors that may contribute towards this excess mortality, overdose remains the leading cause. While there has been a decline in the number of heroin fatalities in Australia since 1999, in part due to a reduction in the availability of heroin over this period, official reports suggest that since 2002, heroin consumption has again been on the rise [15]. Increased heroin production in Afghanistan, and the corresponding increase in heroin consumption in Australia [9], have sparked fears that the overdose rate will again rise.

Heroin overdoses are often described as being the result of a quantity or quality (purity) of heroin in excess of the person's current tolerance to the drug [30, 31]. This being the case, overdose deaths resulting from large or highly pure intakes of heroin should correspond to high post-mortem blood morphine concentrations. However, in a significant number of fatal overdose cases, the morphine concentrations found are no greater than many non-fatal overdoses [32] and, in some cases, may even be below toxic levels [30, 31]. In addition, a number of studies have reported finding large variation in the blood morphine concentrations found in fatal heroin overdoses, with some possessing concentrations of only 10 ng/mL, and others of up to 4000 ng/mL [3, 35]. This large variation could be attributed to differences in tolerance or the incomplete metabolism of morphine as a result of differing survival times after heroin injection. It could also be due to the co-administration of other depressant drugs, such as benzodiazepines, which can increase the likelihood of a fatal overdose due to potentiation of the respiratory depressant effects of heroin [30]. In combination with benzodiazepines, heroin can cause respiration to slow or even cease, and a smaller than usual dose may be required to exert a fatal effect [63].

The variation in blood morphine concentrations found in heroin-related fatalities may also be due to ambiguous reporting of the morphine concentration data. In some instances, the morphine concentration reported represents the concentration of free morphine only, and in other instances it corresponds to the concentration of free morphine plus morphine conjugates (total morphine). This particularly confounds interpretation in instances where death occurs some time after heroin administration.

If unconjugated free morphine is reported in such cases, below toxic concentrations of morphine may be found due to extensive metabolism.

Another possible explanation for the variable blood morphine concentrations found in fatal heroin overdoses may relate to the potential for inter-individual differences in drug effect due to genetic variations in the amount of active and inactive metabolites produced. Following administration, heroin is first metabolised to the pharmacologically active metabolites, 6-monoacetylmorphine (6MAM) and morphine, before uridine diphosphate-glucuronosyltransferase (UDPGT) enzymes metabolise it further to the main metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). Like morphine, M6G has a high binding affinity for the μ opioid receptor [29] and is pharmacologically active [50, 83, 84]. There is even some evidence to suggest that M6G is a more powerful agonist than morphine [72, 84]. On the other hand, the most prevalent metabolite, M3G, shows a lack of affinity for μ, κ and σ opioid receptors [49, 51] and may even antagonise the respiratory depressant and analgesic effects of M6G and morphine [44, 50, 51]. UDPGT isoenzymes are believed to be subject to genetic variation, meaning that the potential exists for inter-individual differences in the extent of metabolism to M3G and M6G. Since the primary mechanism involved in heroin overdose is opioid-induced depression of respiration with resulting hypoxia and death [29], high concentrations of active M6G may be associated with increased CNS depression and a greater risk of respiratory failure. This idea is supported by some clinical studies, which attribute inter-patient variations in analgesia to wide differences in plasma concentrations of morphine and M6G in patients receiving morphine for pain [44-49].

Determining morphine and its glucuronide metabolites simultaneously in post-mortem samples could enable the contribution of M3G and M6G towards heroin overdose to be explored in greater detail. However, many laboratories do not routinely perform this analysis, and report only free or total morphine. Since M3G is the main metabolite, the total morphine concentration is often only indicative of the

pharmacologically inactive M3G concentration, which does not contribute towards respiratory depression, and therefore gives little information regarding the nature of the overdose. Reporting of free morphine does not consider the contribution of either M3G or M6G.

Measurement of the individual glucuronide concentrations may also give a good indication of the post-mortem interval. The ratio between the molar concentration of M6G or M3G to morphine has been suggested as a means of assessing the time elapsed since heroin administration [104, 108, 164]. The negligible metabolism that occurs when death rapidly follows heroin intake may result in low M6G/MOR or M3G/MOR ratios, thus suggesting a short survival time. Conversely, high ratios of M6G/MOR or M3G/MOR may indicate a delayed death. In addition, determination of the individual glucuronide metabolites may indicate if death was a result of chronic or first-time administration of heroin. M6G has been shown to accumulate with repeated administration of the drug, so high levels of M6G may be associated with chronic exposure to heroin [181].

5.2.1 Previous Investigations of M3G and M6G Concentrations and Ratios in Heroin Fatalities

A number of reviews on the determination of opiates in biological samples have been published [65, 69, 477], and many HPLC methods have been reported in the literature for the determination of morphine and metabolites in biological samples, including whole blood [89, 95, 102, 103, 106-109, 118, 120, 125, 139, 161, 164], plasma [85, 99, 100, 102, 105, 110, 112-114, 117-119, 121, 122, 124, 126, 127, 138, 143, 158, 162-164], serum [71, 96, 97, 103, 104, 107, 109, 111, 116, 138, 139, 142, 143, 159], urine [107-109, 115, 118, 125, 141, 159, 161], bile [89], CSF [107-109, 117, 159, 163], vitreous humor [108, 109] and meconium [98] (Refer to Table 1-3 for details). Despite the large quantity of literature relating to the analysis of morphine and its glucuronides, only a limited number of studies have focused on their analysis in post-mortem samples. No studies have compared the concentrations

of morphine, M3G and M6G in fatalities involving the concomitant use of heroin and benzodiazepines to fatalities involving heroin only.

In 1995, Gerostamoulos *et al.* [120] developed a method for the analysis of M3G, M6G, morphine and normorphine in post-mortem blood using a SPE technique followed by HPLC with electrochemical and UV detection. Nine post-mortem blood samples were analysed and the mean concentrations of M3G, M6G and morphine were reported to be 0.5 ± 0.33 μg/mL, 0.14 ± 0.08 μg/mL and 0.38 ± 0.28 μg/mL respectively. The method, which had a run time of more than 30 minutes, was applied by the same authors in a second study [70] involving the analysis of morphine, M3G and M6G in 40 heroin-related deaths. While valuable data regarding the post-mortem redistribution of morphine was obtained, the run time was exceedingly long and it would be of considerable benefit to have a more rapid method for the determination of morphine, M3G and M6G in post-mortem samples.

A fast HPLC method for the determination of morphine, M6G and M3G was developed by Aderjan *et al.* [104] to determine the concentrations of these analytes in the serum of twenty heroin consumers and in ten heroin-related deaths. The method had a run time of less than 10 minutes, and concentrations of 0.45 ± 0.47 μg/mL (MOR), 0.68 ± 0.56 μg/mL (M6G), and 1.7 ± 1.9 μg/mL (M3G) were reported in the fatalities. The main disadvantage of this method, and another similar method reported by the same authors [71], was that it could only be applied to the analysis of serum, rather than whole blood. This is a considerable disadvantage in post-mortem toxicology, where sample volumes are often limited and direct analysis of the sample is preferred.

A few studies have determined M6G/MOR and M3G/MOR ratios in order to determine the time elapsed since heroin administration. Previous findings have suggested that low ratios of M3G/MOR and M6G/MOR are indicative of short survival times following drug intake [95, 104, 108]. In the study by Beike *et al.* [95],

all seven post-mortem samples had ratios of M6G/MOR < 1. These deaths were interpreted as occurring within an hour of intake. Bogusz *et al.* [108] found the average M6G/MOR ratio (n = 21) to be 1.6 ± 3.6, however, a slightly higher M3G/MOR ratio of 2.8 ± 4.0 was observed. Higher ratios again were observed in the study by Aderjan *et al.* [104], who found the average M6G/MOR ratio in ten overdose cases to be 2.6 ± 2.2, while the average M3G/MOR ratio was 5.0 ± 6.5. In contrast, Skopp *et al.* [71] measured the ratios of M3G/MOR and M6G/MOR in four cases of heroin overdose, and found that a definite relationship could not be established.

While some of the studies mentioned above have calculated M6G/M3G in addition to M3G/MOR and M6G/MOR, few have attempted to draw conclusions from this data. Antonilli *et al.* [181] conducted a study in which higher ratios of M6G/M3G were found in a group comprising of current injecting heroin users as compared with a group on non-heroin users treated with morphine. They found the ratio of M6G/M3G was > 1 in both the blood and urine of chronic heroin users, and reasoned that there was a reduction of M3G in favour of M6G in chronic injecting heroin users. They postulated that the significant amounts of cadmium, which can be found in heroin, could selectively inhibit the synthesis of M3G. Considering the limited data that is available regarding the ratios of M3G/MOR, M6G/MOR and especially M6G/M3G, it would be of interest to gather some additional cases to further aid the interpretation of heroin toxicity.

5.2.2 Objectives

The ambiguous reporting of either free or total morphine concentrations limits the interpretation of heroin-related fatalities. Simultaneous determination of morphine, M3G and M6G could enable the relationship between heroin concentration and effects to be better understood. The first objective of this work was to develop a fast and simple method for the analysis of morphine and its two glucuronide metabolites that could be applied to whole blood. Since no studies have compared the concentrations of morphine, M3G and M6G in heroin fatalities to those involving the

concomitant use of heroin and benzodiazepines, the second objective of this study was to acquire some preliminary data on morphine glucuronide concentrations and ratios in these two types of heroin-related fatalities.

5.3 Experimental

5.3.1 Chemicals

All reagents were of analytical grade, unless stated otherwise. Morphine hydrochloride (MOR) and codeine (COD) were obtained from Glaxo Australia Pty Ltd (Port Fairy, Victoria, Australia). Morphine-3β-D-glucuronide (M3G), morphine-6β-D-glucuronide (M6G) and 6-monoacetylmorphine (6MAM) were either synthesised in-house (Chapter 2) or purchased from Novachem (Melbourne, Victoria, Australia). Formic acid (HCOOH), acetic acid (CH_3COOH), potassium dihydrogen phosphate (KH_2PO_4), ammonium carbonate ($NH_4HCO_3.NH_2COONH_4$) and ammonia solution (all from APS Chemicals Ltd, Sydney, New South Wales, Australia) were used in the preparation of mobile phases and buffers. Methanol (MeOH, Merck HPLC grade), acetonitrile (ACN, Merck HPLC grade), ethyl acetate (EtOAc), butyl chloride, chloroform and diethyl ether were purchased from Crown Scientific (Sydney, New South Wales, Australia). MilliQ grade water (18.2 $M\Omega cm^{-1}$) was used throughout the experiments. Buffers were prepared fresh each day and degassed by sonication prior to use. Duplicate 5 μL injections for each standard and sample were performed.

Whole sheep blood (Oxoid Australia Pty Ltd, Theberton, SA, Australia) was used for the preparation of spiked calibration standards. For solid-phase extraction, Bond Elut C_{18} SPE cartridges (500 mg, 6 mL) were purchased from Varian (Melbourne, Victoria, Australia) and Xtract SPE cartridges (200 mg, 3 mL) were from PM Separations (Brisbane, Queensland, Australia). Prior to reconstitution, extracted samples were evaporated to dryness using a Heto VR Maxi vacuum concentrator (Medos, Sydney, New South Wales, Australia).

5.3.2 Instrumentation

Experiments were performed on a Waters Alliance 2690 Separations Module, with Waters 996 Photodiode Array Detector and 470 Scanning Fluorescence Detector (Waters, Sydney, New South Wales, Australia). All data manipulation was executed using Waters Millennium Software, Version 3.05. Separations were performed on either Zorbax SB-C18 (50 x 4.6 mm, 1.8μm) (Agilent Technologies, Sydney, New South Wales, Australia) or Atlantis dC_{18} (50 x 4.6 mm, 3 μm) (Waters, Sydney, New South Wales, Australia) columns.

5.3.3 Calculations

Where applicable, the resolution of each analyte pair was calculated using Equation 5-1.

Equation 5-1 Calculation of resolution (R_s)

$$R_s = \frac{(t_2 - t_1)}{0.5(w_1 + w_2)}$$

where t_1 and t_2 are the migration times for each peak pair, and w_1 and w_2 are the peak widths at baseline

5.3.4 HPLC Method Development

Simplicity was a key requirement for the development of the HPLC method, so UV and fluorescence detection methods, which are easily accessible and available to most laboratories, were investigated. To ascertain the optimum detection wavelength for fluorescence detection, the signal/noise (S/N) ratio of an injection of M6G was measured at four different excitation and emission wavelengths. S/N ratios were then compared for UV and fluorescence detection to determine the most appropriate detection method. The effect of organic modifier type was studied by comparing the separation attained using a gradient run of 0 – 15 minutes, 0 – 30% ACN with that obtained using a gradient of 0 – 15 minutes, 0 – 30% MeOH (in 25 mM acetate buffer, pH 4.7). To investigate the influence of pH on the separation of the five analytes, ammonium formate, ammonium acetate and ammonium phosphate buffers were chosen to cover a wide pH range. Experiments were conducted at a constant

concentration of 25 mM in the range pH 2.75 – 4.75 for formate (pK_a 3.75), pH 3.8 – 5.4 for acetate (pK_a 4.7) and pH 6.2 – 7.2 for phosphate (pK_a 7.2). Buffer concentration was investigated in acetate buffer in the range 25 – 50 mM (constant pH 3.8) and the effect of temperature on the separation was also studied in the range 25 – 50 °C (25 mM acetate buffer, pH 4.7). To avoid excessively long analysis times, all initial pH, concentration and temperature experiments were conducted with an ACN gradient of 0 – 15 minutes, 0 – 15%. Initial experiments were performed on a Zorbax SB-C18 column (50 x 4.6 mm, 1.8μm), however pH, concentration and temperature experiments were repeated on an Atlantis dC_{18} column (50 x 4.6 mm, 3 μm) to evaluate the most suitable stationary phase for the separation. It should be noted that ANNs were not used in this Chapter, since preliminary experiments suggested that the optimum could be found with relative ease.

5.3.5 Sample Preparation

Numerous methods have been published for the extraction of morphine and related analytes from biological samples (Refer to Table 1-3). SPE is the most commonly employed technique, however LLE and protein precipitation methods have also been reported. To determine the best extraction technique, four LLEs (including two protein precipitation methods) and two SPEs were evaluated with regards to the recovery of each analyte from spiked whole blood.

The extraction details for each LLE technique are summarised in Table 5-1. While butyl chloride/chloroform has previously been reported for the extraction of morphine from plasma [90], diethyl ether/ethyl acetate has not been reported, and was selected based on its successes as an elution solvent in SPE [137] and in extracting benzodiazepines (Chapter 4). Protein precipitation using ACN has been employed for the extraction of morphine and its glucuronides from plasma [158] and serum [180], and recoveries of more than 70% have been reported. MeOH is a less commonly used solvent for precipitating plasma proteins, however it has been reported for the extraction of morphine and its glucuronides from whole blood [161].

Table 5-1 Evaluated LLE techniques for the extraction of morphine and related analytes

Extraction name	Details	Reference
Butyl chloride/chloroform	200 µL 4 M carbonate buffer (pH 9) + 2 mL butyl chloride/chloroform (5:1)	[90]
Diethyl ether/ethyl acetate	1 mL 2 M ammonia solution + 2 mL diethyl ether/ethyl acetate (1:1)	[219]
ACN precipitation	2 mL ACN	[158]
MeOH precipitation	2 mL MeOH	[161]

In each case, a 100 µL aliquot of a mixed aqueous standard at high (4.0 µg/mL M3G, M6G, MOR, COD, 6MAM) and low (0.5 µg/mL M3G, M6G, MOR, COD, 6MAM) concentrations was added to 0.5 mL of whole sheep blood. The two concentration levels were prepared and analysed in replicate. Following addition of the extraction solvent, each sample was vertically agitated for 2 minutes and centrifuged at 3000 rpm for 15 minutes. The organic layer was then transferred to a clean plastic tube, evaporated to dryness using a vacuum centrifuge and reconstituted in 100 µL mobile phase prior to analysis.

Two SPE cartridge types were investigated with regards to their ability to extract morphine and its metabolites from whole blood. Numerous studies have reported the use of C_{18} cartridges for the extraction of morphine, M3G and M6G (refer to Table 1-2) with recoveries greater than 70% in most cases. Mixed-mode SPE cartridges have both reversed-phase and cation-exchange mechanisms in operation, enabling analytes with a range of polarities and pK_a's to be extracted simultaneously. Although they are often used for the extraction of morphine, mixed-mode cartridges have not been reported for the extraction of the glucuronides. Given the widely varying pK_a's morphine (pK_a = 9) and its glucuronides (pK_a = 2.8), a mixed-mode cartridge was also considered in the evaluation.

All SPE procedures follow a similar pattern of conditioning the cartridge, loading the sample, rinsing to remove matrix components, and finally eluting the analytes of

interest with an appropriate solvent. Variation generally occurs in buffer pH and elution solvent selection. Table 5-2 summarises the SPE procedures employed for the extraction from blood. The procedure selected for the C_{18} SPE cartridge was based on the method of Dienes-Nagy *et al.* [103]. A variation on this basic procedure was also employed, whereby the elution solvent comprised of MeOH/CH_3COOH, as reported by Bogusz *et al.* [109]. For the mixed-mode cartridge, the procedure employed was based on that reported by Chen *et al.* [68] with some modifications.

Table 5-2 Evaluated SPE methods for the extraction of morphine and related analytes

Extraction name	Details	Reference
Xtract Mixed-mode	Condition: 1.5 mL MeOH, 1.5 mL acetate buffer (pH 6) Load sample: 0.5 mL blood + 1 mL acetate buffer Rinse: 2 x 1.5 mL acetate buffer, 0.5 mL CH_3COOH, 2 x 1.5 mL MeOH Elute: 2 mL DCM/IPA (9:1) with 2% ammonia solution	[68]
Bond Elut C_{18}	Condition: 4 mL MeOH, 4 mL H_2O, 3 mL 10 mM ammonium carbonate buffer (pH 9.3) Load sample: 0.5 mL blood + 3 mL carbonate buffer Rinse: 3 x 2 mL carbonate buffer Elute: 2 mL MeOH	[103]
	Condition: 4 mL MeOH, 4 mL H_2O, 3 mL 10 mM ammonium carbonate buffer (pH 9.3) Load sample: 0.5 mL blood + 3 mL carbonate buffer Rinse: 3 x 2 mL carbonate buffer Elute: 2 mL MeOH/CH_3COOH (9:1, v/v)	[109]

In each case, a 100 µL aliquot of a mixed aqueous standard at high (4.0 µg/mL M3G, M6G, MOR, COD, 6MAM) and low (0.5 µg/mL M3G, M6G, MOR, COD, 6MAM) concentrations was added to 0.5 mL of whole sheep blood. The two concentration levels were prepared and analysed in replicate. Following addition of buffer, each sample was vertically agitated for 2 minutes and centrifuged at 3000 rpm for 15 minutes. The supernatant was then loaded onto the pre-conditioned SPE cartridge

and, following the elution step, each sample was evaporated to dryness using a vacuum centrifuge before reconstitution in 100 μL mobile phase prior to analysis.

Samples containing benzodiazepines in addition to opiates were prepared and analysed according to the method described in Chapter 4. Briefly, it involved the addition of 1 mL 2 M ammonia solution and 5 mL diethyl ether/ethyl acetate (1:1) to 0.5 mL blood, followed by vertical agitation for 2 minutes and centrifugation at 3000 rpm for 15 minutes. The organic layer was then transferred to a clean plastic tube, evaporated to dryness using a vacuum centrifuge and reconstituted in 100 μL MeOH prior to analysis by HPLC-DAD on a Zorbax SB-C_{18} column (50 x 4.6 mm, 1.8 μm) at 280 nm. The mobile phase comprised of 25 mM formate buffer (pH 2.8), 10% MeOH, ACN gradient 0 to 15 minutes, 6.5 to 48.5% at a flow rate of 1 mL/min.

5.3.6 Method Validation

Calibration curves were obtained by analysing drug-free whole blood, spiked with working standard solutions to obtain final concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 μg/mL for M3G, M6G, morphine, codeine and 6MAM. Calibration standards were analysed each day, and a standard curve constructed using linear regression.

Accuracy and precision were calculated at high and low concentration for each drug, with five replicates at each concentration. Accuracy was expressed as the calculated concentration as a percentage of nominal concentration. Precision (%CV) was determined to be the percentage of the average divided by the peak area ratio of the three replicates. Recovery was calculated as the average peak area of each analyte in the spiked samples as a percentage of the average peak area of each analyte in aqueous standards. To study matrix effects, samples of blood without any additions were analysed as blanks.

The limit of detection (LOD) was defined as a signal to noise ratio (S/N) of 3:1, and the limit of quantification (LOQ) was defined as a S/N of 10:1.

5.3.7 Method Application

The method was applied to post-mortem blood samples obtained from the Division of Analytical Laboratories (DAL) following coronial consent. All samples were preserved femoral blood taken as specimens at autopsy. Heroin use was confirmed by the mention of heroin use in the case circumstances. Cases in which it was known that death was entirely drug-related were selected based on independent quantitative results indicating the presence of opiates arising from suspected heroin use, both with and without the presence of benzodiazepines. Cases in which an insufficient volume of sample remained for analysis, or in which significant sample decomposition had occurred, were rejected. Recent cases, and cases requested by the Coroner to be kept beyond the maximum time of 12 months to allow for retesting were rejected to preserve the volume of sample.

5.4 Results and Discussion

5.4.1 HPLC Method Development

To ascertain the optimum detection wavelength for fluorescence detection, S/N ratios were measured at four different excitation and emission wavelengths. Since M6G was associated with the weakest signal, an injection of 25 μg/mL M6G was performed at the four detection wavelengths of 280/335, 210/335, 280/355 and 210/355 nm, and the S/N ratio measured. The various excitation/emission wavelengths and corresponding S/N ratios are presented in Figure 5-1. From this graph, it is evident that the best S/N ratio for an injection of M6G was obtained with detection at 280/335 nm, followed by detection at 280/355 nm. Very poor S/N ratios were obtained at excitation/emission wavelengths of 210/335 and 210/355 nm.

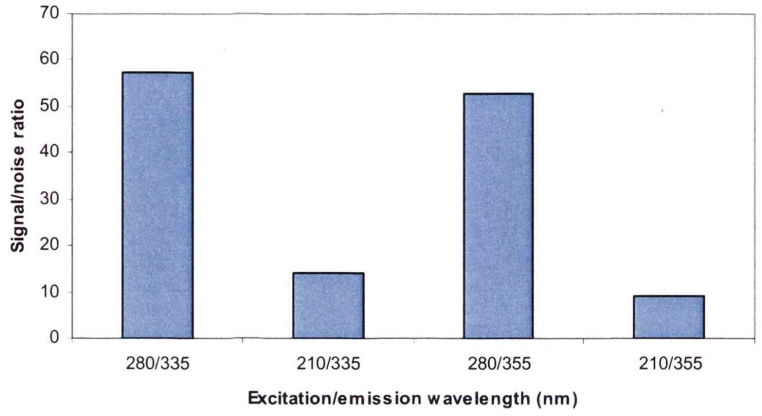


Figure 5-1 Effect of various excitation and emission wavelengths on the signal/noise ratio of M6G

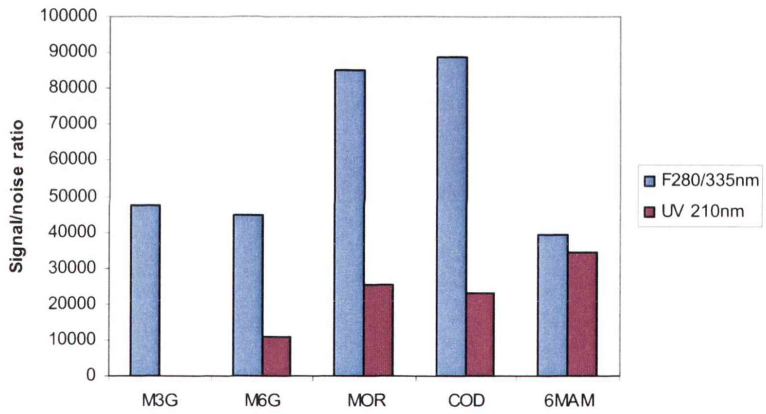


Figure 5-2 Comparison of signal/noise ratios obtained for each analyte using UV and fluorescence detection

Signal/noise (S/N) ratios were compared for UV and fluorescence detection to determine the most appropriate detection method. The wavelengths selected for comparison were 210 nm for UV detection and 280/335 nm for fluorescence detection. A number of UV wavelengths were investigated, however all gave comparable responses. UV 210 nm was selected for comparison with fluorescence detection because it was representative of the best wavelengths investigated. Figure 5-2 shows that fluorescence detection gave superior signal/noise (S/N) ratios for all

analytes. UV detection was virtually incapable of detecting M3G due to an unstable baseline and high levels of noise present at the time corresponding to its retention time. Fluorescence detection at 280/335 nm was shown to be the superior mode of detection for the analysis, and was therefore the detection method of choice for the remaining work.

The influence of organic modifier type on the separation of the five analytes was studied by comparing a gradient run of ACN (0 – 30% ACN, 0 – 15 mins) with a similar gradient run of MeOH (0 – 30% MeOH, 0 – 15 mins). For this investigation, the pH and concentration of acetate buffer were kept constant at 4.7 and 25 mM respectively. Figure 5-3 illustrates the effect of ACN and MeOH gradients on the retention times of each analyte. Compared with MeOH, ACN tended to reduce the retention time of all analytes. The effect was more pronounced on the later-eluting analytes where the percentage of organic modifier was highest. It can be seen that the overall run time was reduced by 3 minutes when ACN was used instead of MeOH. This result is to be expected since ACN is a stronger organic solvent than MeOH. No changes in selectivity were found to occur between organic modifier types, however a large amount of peak-tailing was observed on the codeine peak in MeOH. ACN was therefore deemed to be the most appropriate organic modifier since it reduced the overall analysis time and improved the peak shape of codeine.

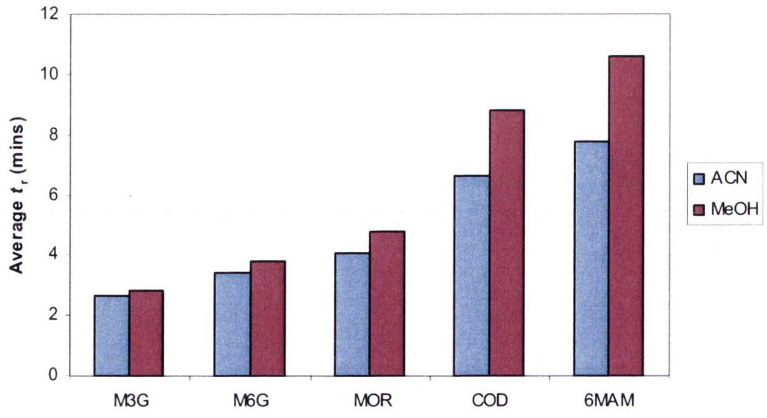


Figure 5-3 Effect of organic modifier type on retention times of each analyte

To study the effect of pH on the separation, formate buffer was investigated in the range pH 2.7 – 4.7. The average resolutions for each analyte pair with varying formate buffer pH (constant concentration 25 mM) are presented in Table 5-3, and the effect of varying pH on retention times and resolution is graphically depicted in Figure 5-4. From the graph, it can be seen that increasing the pH of formate buffer tended to increase the retention times of morphine, codeine and 6MAM by the same degree, whilst the retention times of M3G and M6G tended to decrease. The analyte pair resolutions over this pH range showed little change for PP1 (M3G and M6G), PP3 (morphine and codeine) and PP4 (codeine and 6MAM), however a dramatic increase in the resolution of PP2 (M6G and morphine) was observed. While M6G and morphine co-eluted at pH 2.65, the analytes were significantly more resolved at pH 4.65. This is attributable to the increasing retention time of morphine and the corresponding decrease in retention time for M6G that was observed over this pH range. It should also be noted that the peak shapes of M3G and M6G were poor at all formate buffer pHs investigated as a result of peak-splitting, which tended improve as the pH was increased. A large amount of peak-fronting was also found to occur on the M3G peak under these conditions. Given that the pK_a's of M3G and M6G are both 2.8 [478], these analytes would likely exist simultaneously in a protonated and unprotonated form when the pH of the mobile phase is at or around pH 2.8. The effect of peak-splitting may be caused by the partial resolution of these two forms. As the pH is increased above the pK_a of M3G and M6G, the analytes tend to exist in only one ionised form, and the peak-splitting effect is less pronounced.

Table 5-4 illustrates the average resolution of each analyte pair with varying acetate buffer pH. The effect of varying acetate buffer pH on average retention times and resolution is also graphically presented in Figure 5-5. From the graphs, it can be seen that increasing acetate buffer pH caused a slight increase in the retention times of morphine, codeine and 6MAM, whilst the retention times of M3G and M6G remained relatively constant. A slight general decrease in resolution with increasing pH was observed for PP1 (M3G and M6G) and PP4 (codeine and 6MAM), due to

slight increases in retention times of the earlier eluting analyte in each of these pairs. Accordingly, the resolutions between PP2 (M6G and morphine) and PP3 (morphine and codeine) showed a slight general increase over this pH range. Once again, splitting of the M3G and M6G peaks was observed, although the effect was minimal above pH 4.8. Fronting of the M3G peak again occurred at all acetate buffer pHs.

Ammonium phosphate buffer was also investigated at pH 6.2 and 7.2. The peak shapes of M3G and M6G improved significantly in this buffer, however, the system backpressure increased to more than 3500 psi. Compared to the backpressure obtained using formate and acetate buffers, this amounted to an increase of around 1000 psi. Since phosphate buffers tend to be less soluble than formate or acetate buffers, it is possible that the formation of a fine precipitate within the column may have caused the increase in backpressure. Alternatively, the higher pH, which is approaching the upper limit of the column's tolerance, may have caused the column packing material to deteriorate, thereby producing fine particulate matter that could block the column. Given the high backpressure that was obtained in this buffer, no further testing was performed and it was excluded as a potential mobile phase candidate in order to conserve column life. Ammonium acetate was selected as the mobile phase for all further method development on this column, since improved peak shapes for M3G and M6G were obtained in this buffer.

Table 5-3 Average resolution of each analyte pair with varying pH of formate buffer on the Zorbax SB-C_{18} stationary phase

pH	PP1	PP2	PP3	PP4
2.7	4.020	0.000	20.05	10.87
3.7	4.760	3.300	20.80	11.02
4.7	5.195	4.945	21.43	10.67

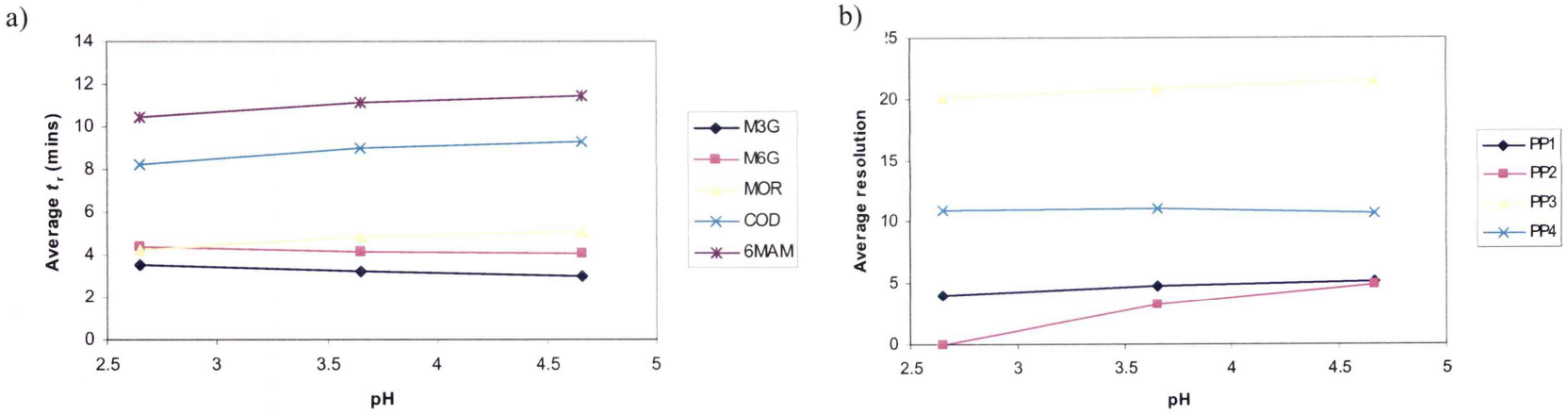


Figure 5-4 Effect of varying formate buffer pH on a) average retention times and b) average resolution for each analyte on the Zorbax SB-C_{18} stationary phase

Table 5-4 Average resolution of each analyte pair with varying pH of acetate buffer on the Zorbax SB-C_{18} stationary phase

pH	PP1	PP2	PP3	PP4
3.8	4.950	3.380	21.49	10.94
4.8	5.390	5.550	21.92	10.40
5.4	5.210	7.200	23.01	9.255

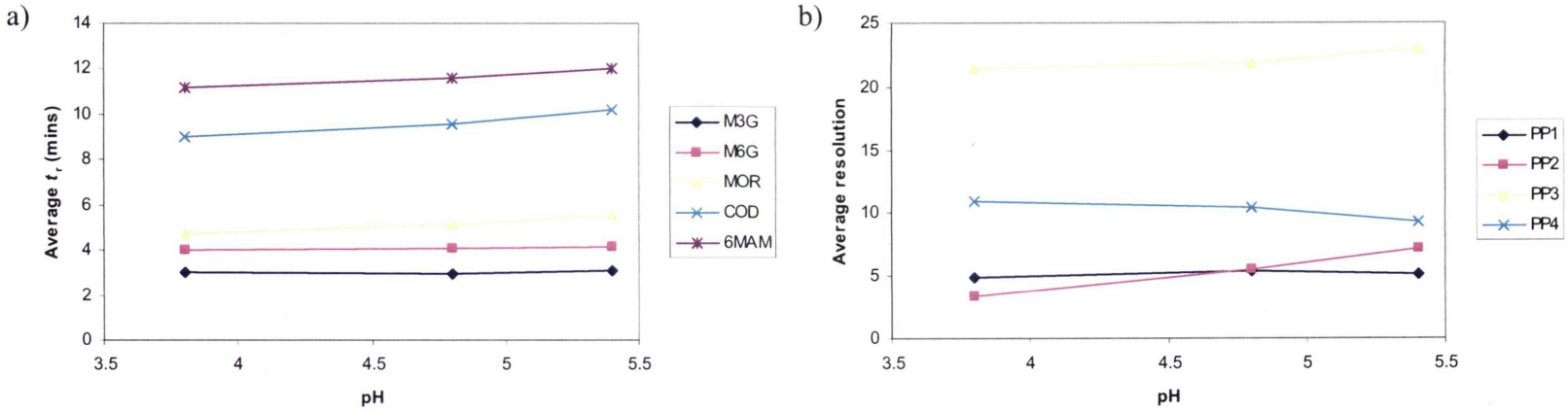


Figure 5-5 Effect of varying acetate buffer pH on a) average retention times and b) average resolution for each analyte on the Zorbax SB-C_{18} stationary phase

The effect of varying buffer concentration on analyte retention times and resolutions was investigated in acetate buffer in the range 25 – 50 mM (pH 3.8). The results of this investigation are illustrated in Figure 5-6, and the corresponding resolution data is presented in Table 5-5. It can be seen that concentration did not have a great effect on retention times, with only a very slight decrease observed for each analyte over the concentration range investigated. The effect of concentration on resolution was accordingly minimal. The peak shape of M3G and M6G was affected by concentration, with peak-splitting becoming more significant as the concentration was increased, possibly due to increased ion-exchange interactions between one of the ionised forms of the glucuronides and the residual silanol groups on the column. Given that the peak-splitting of M3G and M6G worsened as the buffer concentration was increased, all further experiments were performed at a concentration of 25 mM.

Temperature was investigated in 25 mM acetate buffer (pH 4.7) in the range 25 – 50 °C. The average resolution of each analyte pair with varying temperature is presented in Table 5-6 and illustrated graphically in Figure 5-7. The effect of varying temperature on the retention times of each analyte can also be seen in Figure 5-7. From the graphs, increasing temperature tended to decrease the retention time of each analyte. This is expected; since higher temperatures increase the interaction rate between each analyte and the stationary phase, which in turn causes the retention time for most analytes to decrease. As a result of the reduced retention times at higher temperatures, there was a general decrease in resolution for most analyte pairs. Splitting of the M3G and M6G peaks was once again evident; this time as the temperature was increased. This may have been due to increased interactions between the stationary phase and one ionised form of the glucuronides at higher temperatures. Peak-fronting of M3G was observed at all temperatures, although the effect was minimised as the temperature was increased.

Table 5-5 Average resolution of each analyte pair with varying buffer concentration on the Zorbax SB-C_{18} stationary phase

Concentration (mM)	PP1	PP2	PP3	PP4
25	5.175	3.780	21.75	11.04
35	4.665	3.490	20.79	10.62
50	4.725	3.665	20.58	10.55

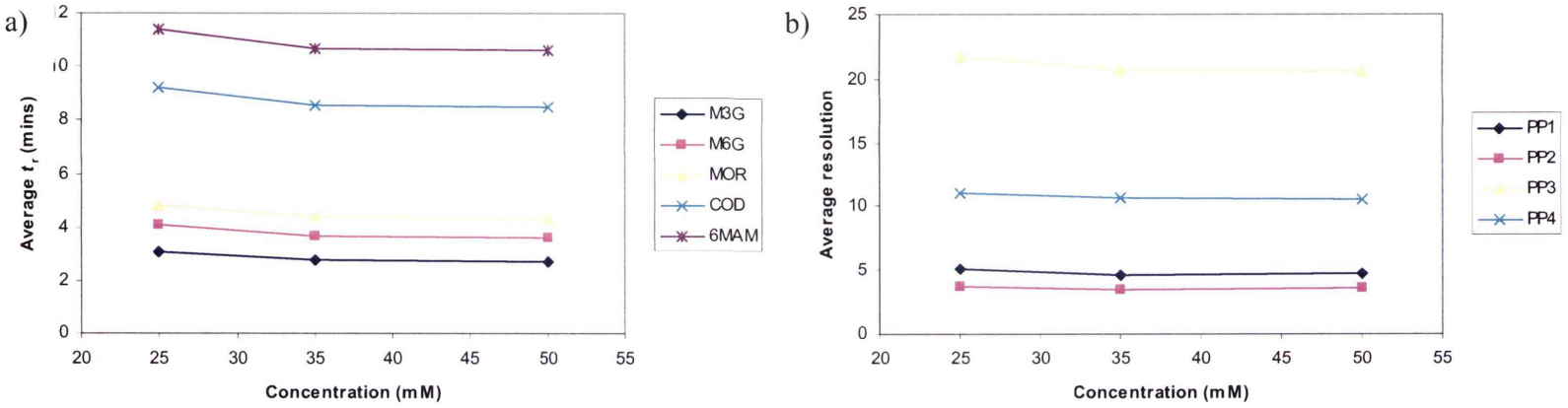


Figure 5-6 Effect of varying buffer concentration on a) average retention times and b) average resolution of each analyte on the Zorbax SB-C_{18} stationary phase

Table 5-6 Average resolution of each analyte pair with varying temperature on the Zorbax SB-C_{18} stationary phase

Temperature (°C)	PP1	PP2	PP3	PP4
25	7.117	5.450	20.27	9.916
30	3.352	3.758	16.01	7.781
40	4.572	3.817	15.89	8.638
50	1.220	0.9356	11.73	5.357

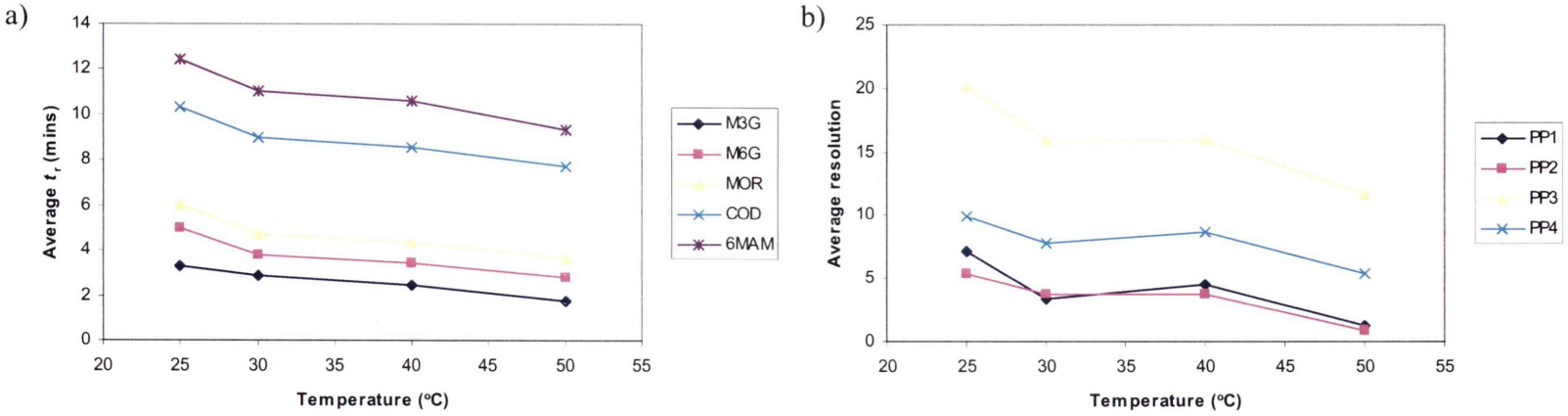


Figure 5-7 Effect of varying temperature on a) average retention times and b) average resolution of each analyte on the Zorbax SB-C_{18} stationary phase

Since the peak-fronting of M3G could not be resolved under any of the mobile phase and temperature conditions investigated, the Atlantis dC_{18} column was evaluated as an alternative stationary phase. For all pH, concentration and temperature experiments on this column, it was found that an ACN gradient of 0 – 36% ACN, 0 – 6 minutes offered a considerable reduction in run time, while still ensuring satisfactory resolution between all analytes. All further method development was therefore conducted with this gradient.

The resolutions of each analyte pair with varying formate buffer pH (constant concentration 25 mM) on the Atlantis dC_{18} stationary phase are depicted in Table 5-7. The influence of pH on retention time and resolution can be seen graphically in Figure 5-8. From these graphs, it is evident that increasing pH tended to increase the retention times of morphine, codeine and 6MAM, while the retention times of M3G and M6G tended to decrease. This had the effect of increasing the resolution of PP2 (M6G and morphine) which, at pH 2.8 was completely unresolved, and also PP1 (M3G and M6G) and PP3 (morphine and codeine). The resolution of PP4 (codeine and 6MAM) remained consistent over the entire pH range since the retention times of codeine and 6MAM increased by an approximately equal amount. While peak-splitting of the M3G and M6G peaks was again present at low pH, fronting of the M3G was not observed.

Acetate buffer pH was varied in the range pH 3.8 – 5.8 (constant concentration 25 mM) on the Atlantis stationary phase and the resulting resolutions of each analyte pair are presented in Table 5-8. Figure 5-9 graphically illustrates the influence of acetate buffer pH on the retention times and resolutions of each analyte. It can be seen that increasing the pH tended to increase the retention times of all analytes. This had the effect of increasing the resolution between PP1 (M3G and M6G), PP2 (M6G and morphine) and PP3 (morphine and codeine), however there was a reduction in resolution between PP4 (codeine and 6MAM) due to a marked increase in the peak width of 6MAM. Once again, the M3G peak did not front, however peak-splitting of M3G and M6G occurred below pH 4.8. Acetate buffer at pH 4.8 was therefore

chosen for the remaining method development on this column, since peak-splitting was eliminated in this mobile phase.

Table 5-7 Average resolution of each analyte pair with varying formate buffer pH on the Atlantis dC_{18} stationary phase

pH	PP1	PP2	PP3	PP4
2.8	3.396	0.000	11.27	5.705
3.8	4.859	2.832	12.20	5.722
4.8	5.064	3.868	12.14	5.626

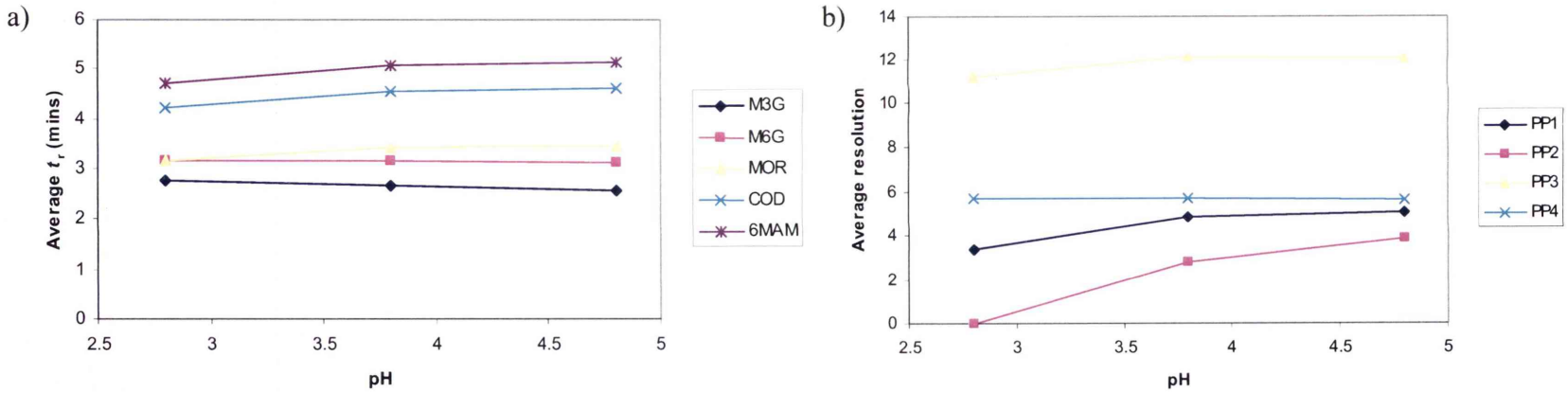


Figure 5-8 Effect of varying formate buffer pH on a) average retention times and b) average resolution for each analyte on the Atlantis dC_{18} stationary phase

Table 5-8 Average resolution of each analyte pair with varying acetate buffer pH on the Atlantis dC_{18} stationary phase

pH	PP1	PP2	PP3	PP4
3.8	4.530	1.790	11.75	5.656
4.8	5.149	3.918	12.21	5.562
5.8	6.439	5.453	12.44	4.496

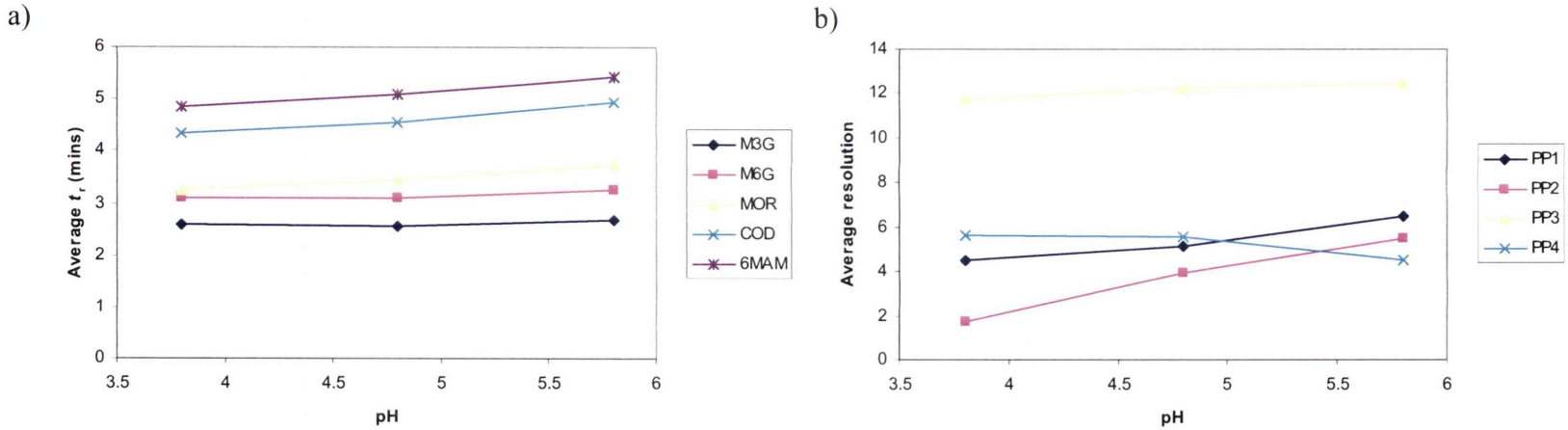
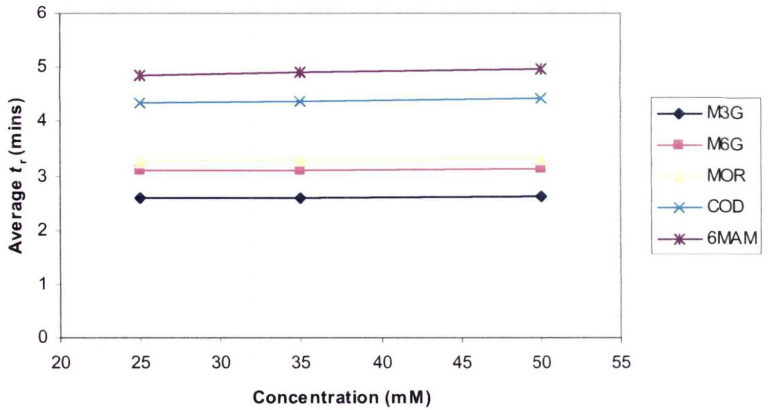


Figure 5-9 Effect of varying acetate buffer pH on a) average retention times and b) average resolution for each analyte on the Atlantis dC_{18} stationary phase

Table 5-9 Average resolution of each analyte pair with varying concentration on the Atlantis dC_{18} stationary phase

Concentration (mM)	PP1	PP2	PP4	PP4
25	4.530	1.790	11.75	5.656
35	4.487	1.930	11.81	5.688
50	4.202	2.216	11.84	5.593

a)



b)

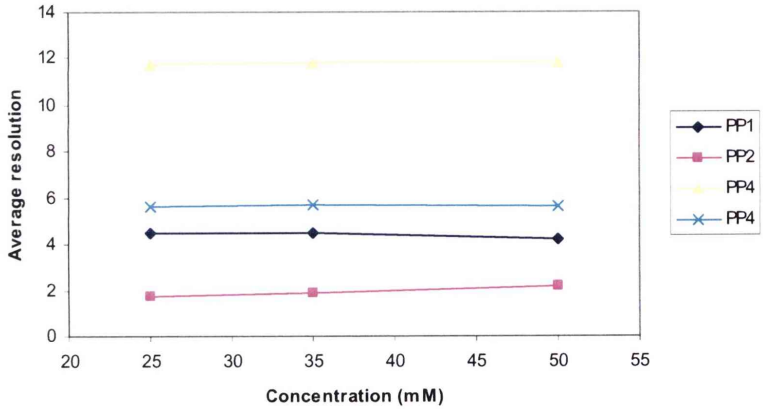


Figure 5-10 Effect of varying concentration on a) average retention times and b) average resolution for each analyte on the Atlantis dC_{18} stationary phase

Table 5-10 Average resolution of each analyte pair with varying temperature on the Atlantis dC_{18} stationary phase

Temperature (°C)	PP1	PP2	PP3	PP4
25	4.530	1.790	11.75	5.656
30	5.105	0.8227	11.14	5.410
35	4.875	0.8449	10.77	5.224
40	4.599	0.9217	10.88	5.079

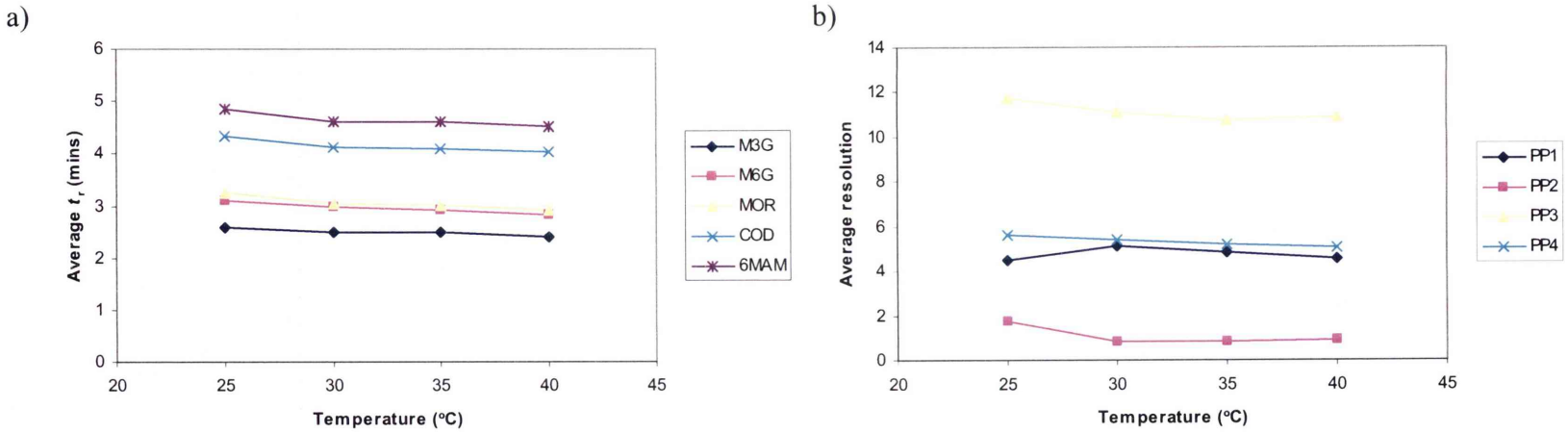


Figure 5-11 Effect of varying temperature on a) average retention times and b) average resolution of each analyte on the Atlantis dC_{18} stationary phase

Figure 5-10 illustrates the effect of varying the concentration of acetate buffer (pH 4.8) on the retention times and resolution of each analyte. Average resolutions for each analyte pair at each of the concentrations studied are presented in Table 5-9. From this data and the accompanying graphs, it can be seen that varying the buffer concentration had little effect on the retention times of the five analytes. The effect on resolution was accordingly very small. However, once again high buffer concentrations were shown to cause peak-splitting of the M3G and M6G peaks. The optimum buffer concentration was therefore determined to be 25 mM.

The influence of temperature on the separation of morphine, M3G, M6G, 6MAM and codeine was investigated in the range 25 – 40 °C. Resolution data for each analyte at the various temperatures are presented in Table 5-10, while Figure 5-11 is a graphical representation of the effect of temperature on analyte retention times and resolution. The data shows that analyte retention times tended to decrease with increasing temperature. At temperatures above 30 °C, analyte pair resolutions showed very little change with increasing temperature, with only PP1 and PP4 exhibiting a slight decrease. The greatest effect on resolution occurred between 25 and 30 °C for most of the analyte pairs and was characterised by a decrease in resolution between PP2 (M6G and morphine), PP3 (morphine and codeine) and PP4 (codeine and 6MAM), while the resolution between PP1 (M3G and M6G) increased. The reduction in resolution can be reasoned in terms of the increase in the peak widths of M6G, morphine, codeine and 6MAM that was found to occur as the temperature was increased. However, increasing temperature had the effect of reducing the peak width of M3G initially, causing a corresponding increase in resolution. As the temperature was further increased, the peak width increased, thus causing the reduction in resolution. Higher temperatures caused peak-splitting of the M6G and M3G peaks, again possibly due to higher temperatures increasing interactions between the stationary phase of the column and an ionised form of both M3G and M6G. This may have contributed to the increased peak width of M3G at

higher temperatures. Thus, to eliminate peak-splitting, the optimum temperature was determined to be 25 °C.

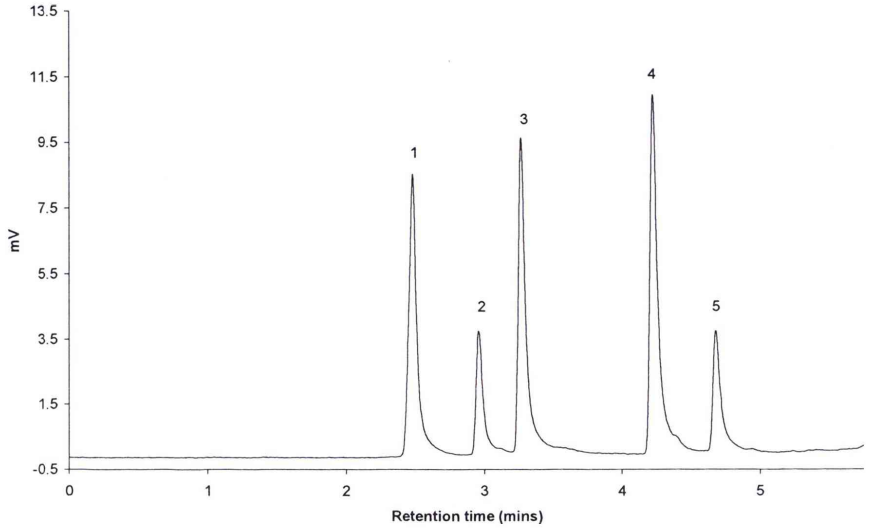


Figure 5-12 Example chromatogram of the separation of M3G, M6G, morphine, codeine and 6MAM

Conditions: Atlantis dC_{18} column (50 x 4.6 mm, 3 μm), 5 μL injection, temperature 25 °C, detection λ 280/335 nm, 25 mM acetate buffer, pH 4.8, ACN gradient 0 to 6 minutes, 0 to 36%, flow rate 1 mL/min; **Elution order:** 1. M3G, 2. M6G, 3. MOR, 4. COD, 5. 6MAM

After consideration of the results of the mobile phase and stationary phase investigation, the optimum separation conditions were selected. From the results of the stationary phase evaluation, the Atlantis dC_{18} was chosen in favour of the Zorbax SB-C_{18}, since it improved the peak shape of M3G by eliminating peak-fronting. On this stationary phase, acetate buffer at pH 4.8 was shown to reduce splitting of the M3G and M6G peaks, which occurred in formate buffer at low pH, while still giving short analysis times. Since high buffer concentration was found to have a detrimental effect on the peak shape of M3G and M6G, the optimum concentration was found to be 25 mM. High temperatures were also found to have an adverse effect on peak shape, so an optimum temperature of 25 °C was chosen. An ACN gradient of 0 – 36% ACN, 0 – 6 minutes at a flow rate of 1 mL/min was found to reduce the run time to 5 minutes, while still permitting baseline resolution between all analytes.

Fluorescence detection at 280/335 nm offered the best S/N ratios when compared with UV detection at 210 nm. An example of the separation attained under these optimum separation conditions is illustrated in Figure 5-12.

5.4.2 Sample Preparation

The results of the evaluation of four LLEs are presented in Table 5-11 and Figure 5-13. From the graph, it can be seen that varying degrees of success were had with each extraction method, however no method was able to recover all five analytes.

In terms of the protein precipitation methods, ACN precipitation gave recoveries ranging from 16 – 44% for M6G, morphine and codeine, however M3G and 6MAM were unable to be recovered. MeOH proved to be a much more efficient extraction method, with recoveries between 39 – 70% for four of the five analytes. Compared to ACN, MeOH gave comparable recoveries for codeine and three- to four-times greater recoveries for M6G and morphine. In addition, MeOH was successful in recovering M3G, however once again 6MAM could not be recovered.

The LLEs using butyl chloride/chloroform and diethyl ether/EtOAc were even less successful than the protein precipitation methods. While butyl chloride/chloroform gave good recoveries for codeine and was the only solvent to recover 6MAM, it gave the lowest recovery for morphine and could not recover either of the glucuronide metabolites. Diethyl ether/ethyl acetate gave low recoveries of around 20% for morphine and codeine, and was additionally ineffective at extracting either the glucuronide metabolites or 6MAM. The inability of either butyl chloride/chloroform or diethyl ether/ethyl acetate to extract M3G and M6G can be explained in terms of the buffer pH and the pK_a's of the glucuronide metabolites. Both extractions were performed at basic pH where morphine, codeine and, to a lesser extent, 6MAM are neutral and can therefore be extracted into the organic layer. On the other hand, the glucuronide metabolites, with pK_a's of 2.8, are salts at this basic pH. This causes M3G and M6G to remain in the aqueous layer, rather than be extracted into the organic layer. LLEs at pH 2 were attempted in order to improve the recovery of M3G

and M6G. However, this tended to extract acidic blood components, which caused the accumulation particulate matter in the sample. Filtration through 0.22 μm Nylon syringe filters was attempted, but the samples were still insufficiently clean for analysis. Additionally, even if the samples could have been adequately cleaned up, it is unlikely that morphine, codeine and 6MAM would have been recovered since these analytes would have been ionised at pH 2.

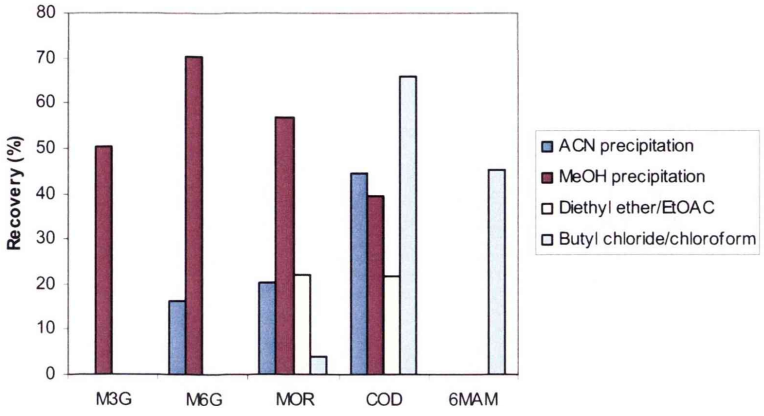


Figure 5-13 Comparison of recoveries of each analyte using precipitation and LLE techniques

Table 5-11 Recoveries of each analyte using precipitation and LLE techniques

Extraction Method	Analyte	Recovery (%)
ACN precipitation	M3G	0.00
	M6G	16.3
	MOR	20.4
	COD	44.4
	6MAM	0.00
MeOH precipitation	M3G	50.2
	M6G	70.1
	MOR	56.7
	COD	39.3
	6MAM	0.00
Diethyl ether/ethyl acetate	M3G	0.00
	M6G	0.00
	MOR	22.2
	COD	21.9
	6MAM	0.00
Butyl chloride/chloroform	M3G	0.00
	M6G	0.00
	MOR	4.16
	COD	66.0
	6MAM	45.1

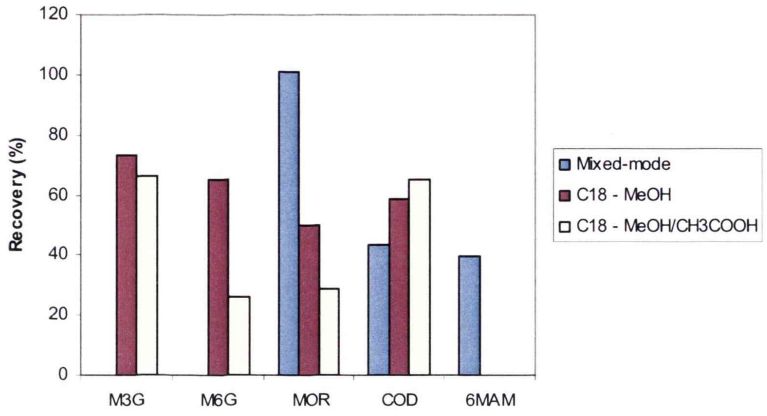


Figure 5-14 Comparison of recoveries of each analyte using SPE techniques

Table 5-12 Recoveries of each analyte using SPE techniques

Extraction cartridge	Analyte	Recovery (%)
Xtract Mixed-mode	M3G	0.00
	M6G	0.00
	MOR	101
	COD	43.2
	6MAM	39.8
Bond Elut C_{18} – MeOH elution	M3G	73.1
	M6G	65.2
	MOR	49.8
	COD	58.6
	6MAM	0.00
Bond Elut C_{18} – MeOH/CH_3COOH elution	M3G	66.2
	M6G	26.2
	MOR	28.6
	COD	65.3
	6MAM	0.00

The results of the SPE evaluation are presented in Figure 5-14 and Table 5-12. The Xtract mixed-mode cartridge gave relatively clean extracts and good extraction

recoveries for morphine, codeine and 6MAM. However, the main analytes of interest, M3G and M6G, were not recovered in the DCM/IPA fraction. The CH_3COOH/MeOH wash step, where acidic drugs can be recovered on mixed-mode cartridges, was also analysed and did not yield the glucuronide metabolites. One possible explanation for this is that the buffer added to the sample initially was at pH 6 and, given the low pK_a's of M3G and M6G, the analytes would be negatively charged and would not adhere to the SPE sorbent. As a result, they would pass through the cartridge in the rinse step. Even the addition of CH_3COOH, which acidifies the sample and causes acidic drugs to elute from the cartridge, would be too late to recover the glucuronides. An attempt was made to recover glucuronides by adjusting the buffer to pH 2 with ammonium phosphate. It was thought that this pH would be sufficient to protonate all analytes of interest, including the glucuronides, and cause them to adhere to the SPE packing material. The same procedure was followed, using phosphate buffer (pH 2) instead of acetate buffer (pH 6). The acidic fraction was collected after the addition of CH_3COOH and the basic fraction after the addition of DCM/IPA. Upon analysis of the two fractions, it was found that neither fraction contained any of the analytes of interest. Possibly, the extreme pH of the buffer had a detrimental effect on the solid-phase packing material, preventing any interaction with the analytes, and causing them to pass straight through the cartridge.

The Bond Elut C_{18} SPE gave good recoveries for four of the five analytes. Again, 6MAM was not extracted, possibly due to greater retention on the cartridge. A stronger elution solvent, such as ACN, could be investigated in order to recover this analyte. The recoveries obtained using MeOH/CH_3COOH as the elution solvent were less than those obtained using MeOH alone. On the other hand, MeOH/CH_3COOH gave a cleaner chromatogram and removed or reduced a number of matrix peaks. In this case, a decision regarding the importance of recovery versus extract purity was made and, based on the results of the sample preparation method development, SPE with the Bond Elut C_{18} cartridge and MeOH elution solvent was selected for all further sample preparation. Since the focus of this study was on the analysis of M3G and M6G, the inability to extract 6MAM was deemed to be of little importance. Its

selection was based on the fact that it gave the optimum recoveries of M3G and M6G, which was deemed to be the most important factor for consideration. An example chromatogram of spiked whole blood that was extracted by SPE with the Bond Elut C_{18} cartridge and MeOH elution solvent is presented in Figure 5-15.

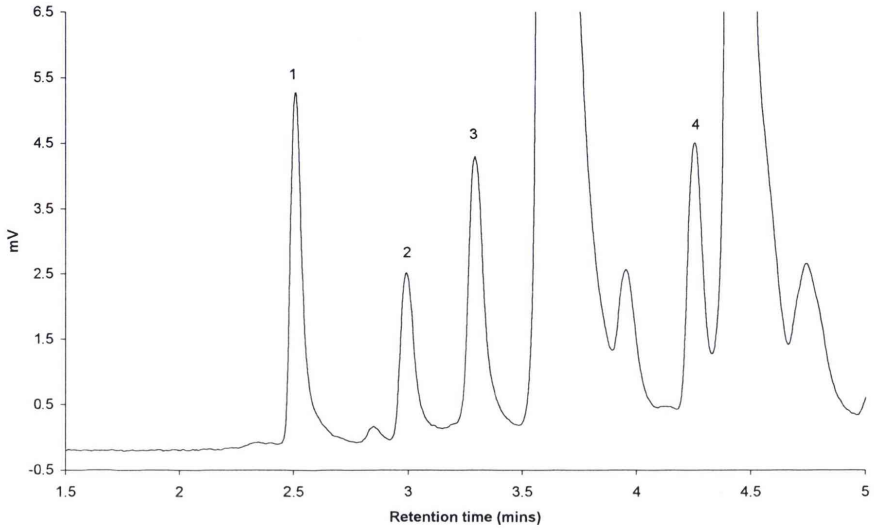


Figure 5-15 Example chromatogram of spiked whole blood extracted using Bond Elut C_{18} SPE

Conditions: Atlantis dC_{18} column (50 x 4.6 mm, 3 μm), 5 μL injection, temperature 25 °C, detection λ 280/335 nm, 25 mM acetate buffer, pH 4.8, ACN gradient 0 to 6 minutes, 0 to 36%; **Elution order:** 1. M3G, 2. M6G, 3. MOR, 4. COD.

5.4.3 Method Validation

The optimised method was validated for spiked whole blood samples, and calibration data for each analyte is presented in Table 5-13. Calibration curves were linear in the specified concentration ranges, and correlation coefficients (r^2) ranging from 0.9929 – 0.9997 were established.

Intra-assay precision and accuracy were determined by analysis of five replicate samples at high and low concentration within the same validation batch. Inter-assay precision and accuracy were assessed by analysing five replicate samples at high and low concentrations over three validation batches. The intra- and inter-assay precision, accuracy and recovery data for morphine, M3G, M6G and codeine in

blood are given in Table 5-14. The limits of detection (LOD) were between 0.015 μg/mL (morphine) and 0.045 μg/mL (M6G).

The method validation results show inter- and intra-assay accuracy to be satisfactory for all four analytes. Intra-assay precision was satisfactory for three of the analytes, however it was outside generally accepted guidelines (%CV $\leq$ 20% [479]) for codeine. Inter-assay precision was also slightly outside the accepted guidelines for codeine and M6G. The recovery data for M3G, M6G and morphine was acceptable, however the recovery of codeine was slightly low. Furthermore, the error associated with the recovery of codeine was quite high. SPE has traditionally suffered from variable recoveries between batches of cartridges, and even within the same batch. This may explain the source of error in the recovery of codeine, as well as the imprecision in the inter- and intra-assay precision data. However, further investigation is required to ascertain if this is indeed the cause.

Table 5-13 Calibration and LOD data for morphine and related analytes in blood

Analyte	Correlation coefficient (r^2)	Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Morphine-3-glucuronide	0.9929	0.25 – 4.0	0.021	0.068
Morphine-6-glucuronide	0.9997	0.25 – 4.0	0.045	0.15
Morphine	0.9986	0.25 – 4.0	0.015	0.048
Codeine	0.9955	0.25 – 4.0	0.023	0.078

Table 5-14 Intra- and inter-assay precision and accuracy data for morphine and related analytes in blood

Analyte	Concentration (μg/mL)	Intra-assay precision (%CV)	Intra-assay accuracy (%)	Inter-assay precision (%CV)	Inter-assay accuracy (%)	Recovery (%, mean±SD)
Morphine-3-glucuronide	4.0	2.2	96	18.1	95	62 ± 9
	0.50	5.5	103	14.4	109	
Morphine-6-glucuronide	4.0	5.4	100	22.2	97	51 ± 11
	0.50	4.2	95	23.9	107	
Morphine	4.0	2.2	100	9.6	100	50 ± 11
	0.50	8.2	94	7.4	102	
Codeine	4.0	11.9	99	20.4	97	39 ± 14
	0.50	25.5	84	30.2	102	

5.4.4 Method Application

A list of fifteen potential post-mortem cases involving heroin only was compiled, however seven were rejected on the basis that they were recent cases, or cases requested by the Coroner to be kept for the possibility of retesting. A further two cases were rejected due to insufficient sample volume or decomposition. The remaining six samples were analysed and the concentrations of morphine, M3G, M6G and codeine found in each sample are reported in Table 5-15. An initial list of twenty post-mortem cases involving the combined use of heroin with benzodiazepines was compiled. Unfortunately, only six cases could be analysed after consideration was given to the cases that had to be kept, were decomposed or contained insufficient sample volume for analysis. The concentrations of morphine, M3G, M6G and codeine found in these cases are presented in Table 5-16. Also shown are the concentrations of benzodiazepines found, as determined by the method presented in Chapter 4.

In each Table, ratios of M3G/MOR, M6G/MOR and M6G/M3G are calculated where possible. Where the concentration found was below the LOQ but above the LOD, the LOD value was assigned. A concentration of 0 μg/mL was assigned to analytes whose concentrations were below the LOD. Therefore, ratios in which the numerator was below the LOD were equated to zero. Ratios in which the denominator was below the LOD were undefined.

Table 5-15 Molar ratios and blood concentrations of morphine, M3G, M6G and codeine in heroin fatalities

Case no.	M3G[a]	M6G[a]	MOR[a]	COD[a]	M3G/MOR[b]	M6G/MOR[b]	M6G/M3G[b]
2402273	0.26	0.18	BLOQ (>0.015)	BLOQ (>0.023)	14.3	9.0	0.6
2402603	0.11	0.15	0.087	BLOQ (>0.023)	1.1	1.3	1.2
2400980	BLOD	BLOD	0.410	0.100	0.0	0.0	n/a
2400776	0.15	0.23	BLOD	BLOQ (>0.023)	n/a	n/a	1.4
2400547	0.29	0.37	BLOQ (>0.015)	BLOQ (>0.023)	16.0	18.6	1.2
2400535	BLOQ (>0.021)	BLOQ (>0.045)	0.081	BLOQ (>0.023)	0.2	0.4	2.0
Mean	0.14 ± 0.1	0.16 ± 0.1	0.10 ± 0.1				
Range	0 – 0.29	0 – 0.37	0 – 0.41				
Median	0.13	0.17	0.051				

n/a – cannot be determined, [a] all concentrations in µg/mL, [b] where concentrations are >LOD but <LOQ, ratios are calculated using the LOD

Abbreviations: below limit of detection (BLOD), below limit of quantification (BLOQ), codeine (COD), limit of quantification (LOQ), morphine (MOR), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G).

Table 5-16 Molar ratios and blood concentrations of morphine, M3G, M6G, codeine and benzodiazepines in heroin/benzodiazepine fatalities

Case no.	M3G[a]	M6G[a]	MOR[a]	COD[a]	M3G/MOR[b]	M6G/MOR[b]	M6G/M3G[b]	Benzodiazepine detected[a]
2402272	0.40	BLOD	0.50	BLOQ (>0.023)	0.7	0.0	0.0	DIA (0.072)
2402251	BLOD	BLOD	0.091	BLOD	0.0	0.0	n/a	DIA (0.080)
2402552	BLOD	0.17	BLOD	BLOD	n/a	n/a	n/a	DIA (0.58) OXA (BLOQ)
2402519	0.185	BLOD	BLOD	BLOD	n/a	n/a	0.0	OXA (0.41)
2402384	0.134	BLOQ (>0.045)	BLOQ (>0.015)	BLOQ (>0.023)	7.4	2.3	0.3	7-NH_2-CLO (0.11)
2402642	BLOD	BLOD	BLOQ (>0.015)	BLOD	0.0	0.0	n/a	ALP (0.3)
Mean	0.12 ± 0.1	0.036 ± 0.06	0.10 ± 0.2					
Range	0 – 0.40	0 – 0.17	0 – 0.50					
Median	0.067	0	0.015					

n/a – cannot be determined, [a] all concentrations in µg/mL, [b] where concentrations are >LOD but <LOQ, ratios are calculated using the LOD

Abbreviations: alprazolam (ALP), 7-aminoclonazepam (7-NH_2-CLO), below limit of detection (BLOD), below limit of quantification (BLOQ), codeine (COD), diazepam (DIA), morphine (MOR), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), oxazepam (OXA), temazepam (TEM).

From Table 5-15, it can be seen that morphine was detected in five of the six cases involving heroin only, however two of these cases had concentrations below the LOQ. For the purposes of estimating the morphine concentrations and ratios in these cases, the morphine concentration was assigned the value of the LOD (0.015 μg/mL). M3G and M6G were quantified in four of the six opiate cases. One of the six cases had concentrations of M3G and M6G below the LOQ, yet greater than the LOD (0.021 μg/mL for M3G and 0.045 μg/mL for M6G). In this case, the LOD values for M3G and M6G are reported as estimates and to assist in the calculation of ratios. In only one case were the concentrations of M3G and M6G below the LOD. Codeine was below the LOQ in all but one of the heroin cases. Codeine in the urine or blood of heroin users is usually attributable to the metabolism of small amounts of acetylcodeine that sometimes exists as an impurity in heroin [477], so only small amounts would be expected.

The concentrations of morphine, M3G, M6G, codeine and benzodiazepines found in the heroin/benzodiazepine cases are presented in Table 5-16. Morphine could only be quantified in two of these cases and was not detected in another two samples. A further two cases had morphine concentrations above the LOD (0.015 μg/mL) but below the LOQ. The lower frequency of cases containing quantifiable concentrations of morphine in this sample set, as compared with the sample set containing only heroin cases, may reflect the lower levels of morphine that are required to exert a fatal effect when heroin is used in conjunction with other CNS depressants such as benzodiazepines. M6G could only be detected in two of the heroin/benzodiazepine cases, and quantified in only one of these. Slightly better results were obtained for M3G, which could be quantified in three cases. This may indicate higher levels of M3G relative to M6G in these cases, or it may be a result of the lower LOD for M3G. The concentrations of benzodiazepines in this sample set were found to be within therapeutic ranges (DIA 0.05 – 2 μg/mL, OXA 0.5 – 2 μg/mL, 7-NH_2-CLO 0.03 – 0.15 μg/mL [78]) for all but one of the samples. In this case, alprazolam was found at a concentration of 0.3 μg/mL, which is within its toxic range of 0.2 – 0.4

μg/mL [78]. Less heroin would have been required to exert a fatal effect in this case, which may account for the lower levels of morphine found.

Mean, range and median concentrations for M3G, M6G and morphine in heroin and heroin/benzodiazepine cases are presented at the bottom of Table 5-15 and Table 5-16 respectively. The range of morphine concentrations in the two sample sets was similar, however, since the highest level of M6G was found in a case of intoxication by heroin only, a greater range of M6G concentrations was found for the heroin cases. In contrast, a greater range of M3G concentrations was found for the heroin/benzodiazepine cases, since the highest level of M3G was found in a case involving both heroin and benzodiazepines. The median concentrations of all three analytes were higher in the heroin cases than in the heroin/benzodiazepine cases. The greater frequency of heroin cases with analyte concentrations above the LOD, as compared with the heroin/benzodiazepine cases, is the likely reason for this.

As is evident from the large standard deviation and range for each analyte, the concentrations in both sample sets were scattered. This finding is concordant with other studies [61, 104, 108, 120], and can reflect different survival times, individual susceptibility to heroin, co-administration of other CNS depressants or the presence of systemic disease. The large standard deviation is further accentuated by the small data set and the number of cases that had analyte concentrations below the LOD. While the two sample sets had similar mean concentrations of M3G and morphine, the heroin cases had a much higher mean concentration of M6G, compared with that of the heroin/benzodiazepine cases. One explanation for this is that these cases may have been chronic heroin users, since M6G may accumulate with repeated administration of the drug [181]. Another possible explanation may be that the majority of the heroin cases were delayed deaths, while the heroin/benzodiazepine cases occurred more rapidly. Since glucuronidation of morphine proceeds rapidly, and levels of M3G and M6G may exceed that of free morphine 30 mins after IV injection of heroin [480], high levels of M6G would suggest a delayed death.

However, an accompanying higher average M3G level would also be expected, and this is not the case.

Examination of the ratios of M3G/MOR and M6G/MOR, as opposed to individual M3G or M6G concentrations, can give a clearer indication as to whether a death occurred rapidly or some time after heroin administration. The molar ratio between the concentration of M6G or M3G to morphine may assist in determining the time elapsed since heroin administration [104, 108, 164]. Low M6G/MOR or M3G/MOR ratios indicate negligible metabolism of morphine to its glucuronides, thus suggesting a short survival time. Conversely, high ratios of M6G/MOR or M3G/MOR may indicate a delayed death. M3G/MOR and M6G/MOR ratios for heroin and heroin/benzodiazepine cases are presented in Table 5-15 and Table 5-16 respectively. It can be seen that the highest M6G/MOR and M3G/MOR ratios were associated with heroin use only, while heroin use in conjunction with benzodiazepines tended to produce lower average ratios of M6G/MOR and M3G/MOR. The comparatively lower ratios of M3G/MOR and M6G/MOR for the heroin/benzodiazepine group suggest more rapid deaths. This finding is supported by the concomitant use of benzodiazepines and heroin which can potentiate the respiratory depressant effects and induce them more rapidly. The possibility of a rapid death could also be confirmed by the absence of morphine in urine and/or the presence of 6MAM.

Figure 5-16a) and Figure 5-16b) illustrate the relationship between morphine concentration and the M3G/MOR and M6G/MOR ratios in both sample sets. From Figure 5-16a), it can be seen that the two cases with very high morphine concentrations (Cases 2400980 and 2402272) had M6G/MOR ratios less than 2, indicating incomplete distribution of heroin and likely rapid deaths. At the other end of the scale, cases with very low levels of morphine (e.g. Cases 2402384, 2400547 and 2402273) had high ratios of M6G/MOR; a likely indication of a delayed death. A similar pattern was observed for the M3G/MOR ratio in Figure 5-16b).

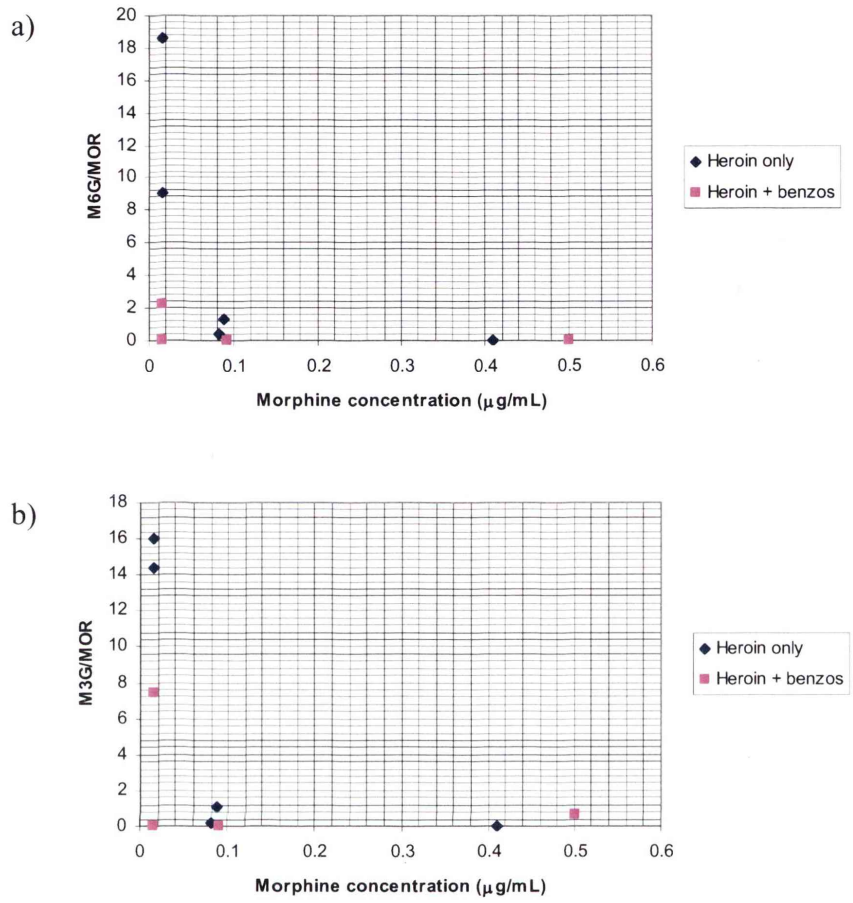


Figure 5-16 Relationship between the free morphine concentration and the ratio of a) M6G/MOR b) M3G/MOR in heroin and heroin/benzodiazepine fatalities

Since M6G may be a more powerful agonist than morphine [72, 84], while M3G may antagonise the respiratory depressant and analgesic effects of M6G and morphine [44, 50, 51], the ratio of M6G/M3G may indicate the potential for respiratory depression. Higher ratios suggest comparatively greater concentrations of the agonist and the possibility of greater respiratory depression. Lower ratios would suggest lower concentrations of the agonist, comparatively higher concentrations of the antagonist, and a lower potential for respiratory depression. High ratios of M6G/M3G may be particularly significant in cases where the M6G/MOR or M3G/MOR ratio is low. This is because the considerable levels of unmetabolised

morphine in such cases would add to the respiratory depressive effect of M6G, while low levels of M3G would have little antagonistic effect. M6G/M3G ratios for heroin and heroin/benzodiazepine cases are presented in Table 5-15 and Table 5-16 respectively. Figure 5-17 illustrates the relationship between M6G/MOR and M6G/M3G in heroin and heroin/benzodiazepine fatalities. Unfortunately, the M6G/M3G ratio could not be calculated for three of the heroin/benzodiazepine cases and one of the heroin cases, since M3G was below the LOD. More cases, particularly heroin/benzodiazepine cases, should be acquired to add to the value of these results. However, from the data, two distinct separate regions can be seen on the graph, with the heroin only cases lying in the upper region of the graph, and the heroin/benzodiazepine cases lying in the lower region. In all heroin/benzodiazepine cases for which ratios could be calculated, the ratio of M6G/M3G was less than 0.5, indicating considerably higher concentrations of M3G as compared with M6G. This is generally expected, since M3G is the major metabolite. In contrast, all of the heroin only cases had ratios of M6G/M3G greater than 0.5, and three of these cases had a M6G/M3G ratio greater than 1, indicating comparatively higher concentrations of M6G relative to M3G. The reason for this is unclear, however it is possible that M6G forms preferentially over M3G in cases of chronic heroin use [181].

In two cases with high M6G/M3G ratios (Cases 2402603 and 2400535), low ratios of M6G/MOR were also found, indicating that death occurred rapidly following heroin administration, and that relatively high levels of morphine were still present. In these cases, a more potent respiratory depressive effect might be expected, due to the higher levels of M6G relative to M3G, coupled with the high levels of morphine. While this may indicate the potential of M6G to contribute towards heroin overdose, given the limited data set, more cases should be acquired before definitive conclusions are made.

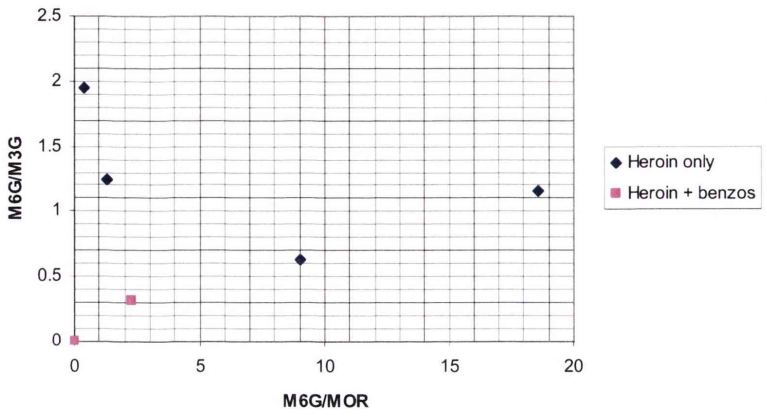


Figure 5-17 Relationship between M6G/MOR and M6G/M3G in heroin and heroin/benzodiazepine fatalities

The analysis of morphine and its glucuronide metabolites in post-mortem samples by HPLC with fluorescence detection has been previously reported [71, 95, 104], however it should be noted that, unlike some of the other studies [71, 95], interference from endogenous peaks in the extracts of post-mortem blood was found to occur in some cases, as seen in Figure 5-18. This may in part be due to inefficiency of the extraction method which, despite giving good recoveries, extracted endogenous blood components and did not selectively extract the analytes of interest. It may also be a result of the low specificity of fluorescence detection. The use of other detection techniques, such as MS or MS-MS, particularly in selected ion monitoring (SIM) mode, would overcome the issue of specificity.

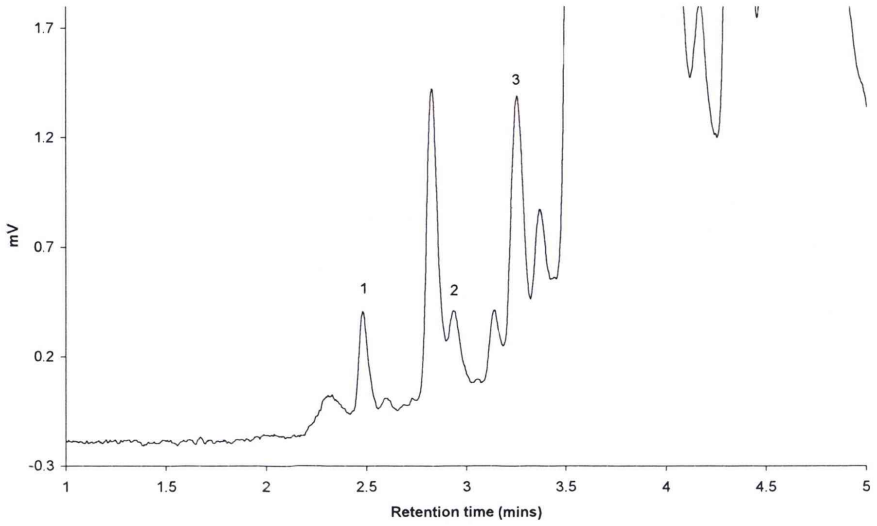


Figure 5-18 Example chromatogram of a post-mortem blood sample from a heroin-related death (Case 2402603)

Conditions: Atlantis dC_{18} column (50 x 4.6 mm, 3 μm), 5 μL injection, temperature 25 °C, detection λ 280/335 nm, 25 mM acetate buffer, pH 4.8, ACN gradient 0 to 6 minutes, 0 to 36%; **Elution order:** 1. M3G, 2. M6G, 3. MOR.

Despite this, the method presented here offers a number of advantages. Firstly, unlike the methods presented by Aderjan *et al.* [104] and Skopp *et al.* [71], this method can be applied to the analysis of whole blood, rather than serum only. This is of considerable advantage in post-mortem toxicology, where sample volumes are often limited and direct analysis of the sample is preferred. Compared with other HPLC methods employing fluorescence detection, the method presented here is also advantageous in terms of run time, which is less than those of Aderjan *et al.* (6.5 mins) [104], Beike *et al.* (10 mins) [95], Huwyler *et al.* (10 mins) [163] and Rotshteyn *et al.* (15 mins) [100], and includes two additional analytes. It is also more rapid than many of the HPLC methods employing MS detection, as is evident by inspection of Table 1-3. Unfortunately, the sensitivity of the method was not as high as many of the previously reported methods. This is to be expected when more sophisticated and sensitive detection techniques are employed, such as MS and MS-MS, however the sensitivity was approximately 10-fold lower than other

fluorescence detection methods, which have LODs in the range of 0.005 – 0.01 μg/mL [104].

There is limited available data concerning the concentrations of morphine, M3G and M6G in heroin fatalities, and no studies concerning these concentrations in the presence of benzodiazepines. Compared to the study by Gerostamoulos *et al.* [120], who found concentrations of M3G, M6G and morphine in nine heroin fatalities to be 0.5 ± 0.33 μg/mL, 0.14 ± 0.08 μg/mL and 0.38 ± 0.28 μg/mL respectively, similar concentrations of M6G, but lower concentrations of M3G and morphine were found. Similarly, compared with the concentrations found by Dienes-Nagy *et al.* [103] of 0.50 μg/mL (M3G), 0.094 μg/mL (M6G) and 0.32 μg/mL (MOR), lower concentrations of M3G and morphine, but higher concentrations of M6G were found here. Aderjan *et al.* [104] found all three analytes at even higher concentrations of 0.45 ± 0.47 μg/mL (MOR), 0.68 ± 0.56 μg/mL (M6G), and 1.7 ± 1.9 μg/mL (M3G).

A few studies have determined ratios of M3G/MOR and M6G/MOR to determine the time elapsed since heroin administration. The results of this study are in agreement with previous findings that suggest low ratios of M3G/MOR and M6G/MOR are indicative of short survival times following drug intake [95, 104, 108]. In the study by Beike *et al.* [95], all seven samples had M6G/MOR < 1. The findings of the present study are in agreement with this, since six of the nine cases had M3G/MOR and M6G/MOR < 2. A similar finding was observed in the study by Bogusz *et al.* [108], who found the average M6G/MOR ratio (n = 21) to be 1.6 ± 3.6. However, a slightly higher M3G/MOR ratio of 2.8 ± 4.0 was observed. Higher ratios again were observed in the study by Aderjan *et al.* [104], who found the average M6G/MOR ratio in ten overdose cases to be 2.6 ± 2.2, while the average M3G/MOR ratio was 5.0 ± 6.5.

In a study by Antonilli *et al.* [181], a group comprising of current injecting heroin users had higher ratios of M6G/M3G, as compared with a group of non-heroin users

treated with morphine. They found the ratio of M6G/M3G was > 1 in both the blood and urine of chronic heroin users, and reasoned that there was a reduction of M3G in favour of M6G in chronic injecting heroin users. Aderjan *et al.* [104] found the concentration of M6G to be higher in cases of death than in living addicts. However, the average ratio of M6G/M3G in this study was < 1 at 0.76 ± 0.71, while Bogusz *et al.* [108] found M6G/M3G ratio to be 0.303 ± 0.16. In a similar vein to Antonilli's study, the present investigation found the average M6G/M3G ratio to be > 1 for cases involving heroin only. In the cases involving the concomitant use of heroin and benzodiazepines, the ratio was < 1. This may suggest that the deaths involving heroin only were all chronic heroin users. It is unclear why chronic heroin exposure alters morphine glucuronidation, however it has been postulated that heroin contains significant amounts of cadmium, which could selectively inhibit the synthesis of M3G [181]. The current study was a preliminary study, performed on a small sample of subjects. More data should be acquired before definitive conclusions are made.

5.5 Conclusions

A simple and rapid HPLC method for the simultaneous determination of morphine, M3G, M6G, 6MAM and codeine was developed based on fluorescence detection. The analytes were isolated from 0.5 mL of whole post-mortem blood using C_{18} SPE cartridges. While satisfactory recoveries were obtained for morphine, M3G, M6G and codeine, 6MAM could not be recovered. After separation employing a mobile phase of 25 mM ammonium acetate buffer (pH 4.8) and an ACN gradient of 0 to 36%, 0 to 6 mins at 1 mL/min, the analytes were determined at an excitation wavelength of 280 nm and an emission wavelength of 335 nm. Under these conditions, baseline resolution was obtained between each analyte pair and the run time was less than 5 minutes.

The analytes were determined in twelve post-mortem blood samples taken from heroin-related deaths. Six of these cases involved the concomitant use of benzodiazepines, which were quantified according to a HPLC-DAD method. High concentrations of free morphine were associated with low ratios of M3G/MOR and

M6G/MOR, and were indicative of rapid deaths related to the incomplete distribution of heroin. Cases involving the use of benzodiazepines in conjunction with heroin were found to have lower ratios of M6G/MOR and M3G/MOR, suggesting that death occurred more rapidly in this sample set. This sample set also contained fewer samples with quantifiable levels of morphine, which reflects the additive effect of heroin and benzodiazepines. Higher average concentrations of M6G and higher M6G/M3G ratios were found in cases involving the use of heroin only, which may suggest chronic heroin exposure in this sample set. High ratios of M6G/M3G coupled with low M6G/MOR ratios may indicate the possible contribution of M6G towards heroin overdose, or a protective effect due to antagonism by M3G, however more cases need to be acquired to verify this.

5.6 Future Work

Due to the low sensitivity of fluorescence detection, the quantification of all analytes was not possible in some of the post-mortem samples analysed in this work. The low specificity of fluorescence detection also presented problems in the form of endogenous matrix peaks attributed to the post-mortem nature of samples. To improve the sensitivity and specificity of the method, MS or MS-MS detection should replace fluorescence detection. Given the volatile nature of the mobile phase described in this work, direct transfer of the HPLC method to another detector should not present any problems. An improved extraction method that selectively removes the analytes of interest from the sample matrix would also help eliminate the problem of interfering matrix peaks. The application of an additional wash step, to remove matrix components from the SPE cartridge without severely impacting on analyte recoveries, should be investigated.

The concentrations and ratios of M3G, M6G and morphine in heroin-related deaths were investigated in this work, however more samples should be acquired to enhance the value of the results and enable more meaningful conclusions to be made regarding the contribution of M3G and M6G towards heroin overdose. Future work should also consider the analysis of M3G, M6G and morphine in cases of non-fatal

heroin overdose. This would serve as a comparative tool to enable any differences in the glucuronide levels or ratios between fatal and non-fatal cases to be determined. The addition of two control groups comprising of patients undergoing short- and long-term treatment with morphine may help ascertain the role of M3G and M6G in the formation of tolerance.

Chapter Six

Overall Conclusions

6 Overall Conclusions

This research has focused on the development of analytical methods for the quantitation of benzodiazepines and opiates in forensic samples. Both these classes of drugs are regularly encountered in forensic toxicology, especially in cases of fatal heroin overdose, and thus quantitative methods for their analysis are particularly relevant.

Drug and metabolite standards are essential in the development and validation of analytical methods for toxicological analyses. Whilst parent drugs are usually readily available from a number of commercial sources, it is often difficult or expensive to obtain standards of the corresponding metabolites. M3G, M6G, 7-aminonitrazepam and 7-aminoclonazepam were therefore synthesised based on previously reported methods in yields of 48, 25, 74 and 70% respectively. Minimal laboratory facilities were required, and while the synthesis of M6G required several steps, the remaining metabolites were prepared with relative ease. This work demonstrated that the synthesis of drug metabolites can be a viable alternative to purchasing through expensive commercial sources.

The number of studies focusing on the analysis of benzodiazepines by capillary zone electrophoresis (CZE) has been limited, mainly due to the lengthy analysis times that arise as a result of the reduction in the EOF at low pH. Fast CZE separations of benzodiazepines at low pH were therefore investigated, and a rapid CZE-DAD method for the simultaneous determination of nine benzodiazepines was developed using a capillary coated with a polycation of poly(diallyldimethylammonium chloride) (PDDAC) and a polyanion of dextran sulfate (DS). Under the selected BGE conditions of 100 mM ammonium phosphate buffer (pH 2.5), baseline resolution was obtained betweeb each analyte within a run time of 6.5 minutes. When compared with CZE analyses of benzodiazepines on bare fused-silica capillaries, the method was found to offer significant improvements in terms of resolution and run time. The

run time was also significantly shorter than previously reported coated methods for the analysis of benzodiazepines by CZE with DAD detection. While the inferior detection limits of CZE-DAD did not enable the method to be applied to biological samples, it was successfully applied to the analysis of some beverages that had been spiked with benzodiazepines at concentrations simulating prescription tablets.

Since the CZE-DAD method did not have sufficiently low detection limits for the quantification of benzodiazepines in post-mortem samples, attention was turned to the development of a HPLC method. While ANNs have been employed to optimise isocratic HPLC separations, their usefulness in optimising gradient elution separations has not been studied extensively. Thus, a three factor experimental design and ANNs were applied to the optimisation of a gradient elution HPLC separation of nine benzodiazepines. The best performing ANN was found to be the one trained on replicate retention time data. The optimum conditions were selected by way of a novel chromatographic function, ISF, which was developed to incorporate run time, resolution and minimum retention into a single function designed to assess the quality of chromatographic separations. ISF was found to offer significant advantages over other optimisation functions, such as the product resolution, which often fail to locate the optimum separation due to an inability to account for run time or detect poorly resolved peaks. Under the optimum conditions of 25 mM formate buffer (pH 2.8), 10% MeOH, ACN gradient 0 to 15 minutes, 6.5 to 48.5%, the error associated with the prediction of retention times and peak widths was less than 5% for six of the nine analytes studied. The method was shown to be a more flexible and convenient means for optimising gradient separations than has been previously reported. Detection limits of the method were sufficient to enable its application to authentic post-mortem samples.

The ambiguous reporting of either free or total morphine concentrations limits the interpretation of heroin-related fatalities. Therefore, a simple and rapid HPLC method for the simultaneous determination of morphine, M3G, M6G, 6MAM and codeine was developed. Baseline resolution and a run time of under 5 minutes was

possible in a mobile phase of 25 mM ammonium acetate buffer (pH 4.8) and an ACN gradient of 0 to 36%, 0 to 6 mins, with fluorescence detection at 280/335. The analytes were determined in twelve post-mortem blood samples taken from heroin-related deaths; six of which involved the concomitant use of benzodiazepines. Unfortunately, the low sensitivity of the method did not enable the quantification of all analytes in the samples. However, preliminary data concerning the concentrations and ratios of morphine, M3G and M6G in these two groups of heroin-related fatalities was gathered. High concentrations of free morphine were associated with low ratios of M3G/MOR and M6G/MOR, and were indicative of rapid deaths related to the incomplete distribution of heroin. Cases involving the use of benzodiazepines in conjunction with heroin were found to have lower ratios of M6G/MOR and M3G/MOR, suggesting that death occurred more rapidly in this sample set. This sample set also contained fewer samples with quantifiable levels of morphine, which reflects the additive effect of heroin and benzodiazepines. Higher average concentrations of M6G and higher M6G/M3G ratios were found in cases involving the use of heroin only, which may suggest chronic heroin exposure in this sample set. High ratios of M6G/M3G coupled with low M6G/MOR ratios may indicate the possible contribution of M6G towards heroin overdose, however more cases need to be acquired to verify this.

Chapter Seven

References

7 References

[1] B. Levine, Principles of forensic toxicology, American Association for Clinical Chemistry, Inc., USA, 1999.

[2] M. Hesse, Alkaloids - nature's curse or blessing?, Wiley-VCH, Zurich, 2002.

[3] R.C. Baselt, R.H. Cravey, Disposition of toxic drugs and chemicals in man, Year Book Medical Publishers, Chicago, 1989.

[4] K.A. Sporer, Acute heroin overdose, *Ann. Intern. Med.*, 130 (1999) 584

[5] U. Boerner, The metabolism of morphine and heroin in man, *Drug Metab. Rev.*, 4 (1975) 39

[6] National Health and Medical Research Council, *Standard for the Uniform Scheduling of Drugs and Poisons No. 8*, Australian Government Publishing Service, 1993.

[7] J. Lonie, A Social History of Drug Control in Australia, Research Paper 8, Royal Commission into the Non-Medical Use of Drugs South Australia, 1979 (The Sackville Commission).

[8] L. Maher, W. Swift, M. Dawson, Heroin purity and composition in Sydney, Australia, *Drug Alc. Rev.*, 20 (2001) 439

[9] JIAFT-West, Special Counterdrug Summary - Drug Trends in Oceania, (2003)

[10] Australian Crime Commission, *Australian Illicit Drug Report 2001-2002 - Heroin*, www.crimecommission.gov.au/content/publications/aidr_2002/03_Heroin.pdf (2002). Accessed March, 2004.

[11] W.D. Hall, J.E. Ross, M.T. Lynskey, M.G. Law, L.J. Degenhardt, How many dependent heroin users are there in Australia?, *MJA*, 173 (2000) 528

[12] S. Darke, D. Zador, Fatal heroin 'overdose': a review, *Addiction*, 91 (1996) 1765

[13] M. Warner-Smith, S. Darke, M. Lynskey, W. Hall, Heroin overdose: causes and consequences, *Addiction*, 96 (2001) 1113

[14] P. Dietze, J. Fitzgerald, Interpreting changes in heroin supply in Melbourne: droughts, gluts or cycles?, *Drug Alc. Rev.*, 21 (2002) 295

[15] NDARC, Australian drug trends 2002: findings of the Illicit Drug Reporting System (IDRS). Monograph no. 50, (2002)

[16] S. Darke, R.P. Mattick, L. Degenhardt, The ratio of non-fatal to fatal heroin overdose, *Addiction*, 98 (2003) 1169

[17] J. Kimber, M. MacDonald, I. van Beek, J. Kaldor, D. Weatherburn, H. Lapsley, R.P. Mattick, The Sydney Medically Supervised Injecting Centre: client characteristics and predictors of frequent attendance during the first 12 months of operation, *Journal of Drug Issues*, 33 (2003) 639

[18] D.J. Collins, H.M. Lapsley, The Social Costs of Drug Abuse in Australia in 1988 and 1992. National Drug Strategy Monograph Series No. 30, Australian Government Publishing Service, Canberra, Australia, 1996.

[19] P. Dietze, C. Fry, S. Sunjic, G. Bammer, D. Zador, D. Jolley, G. Rumbold, Using ambulance attendances to recruit people who have experienced non-fatal heroin overdose, *Drug Alcohol Depend.*, 67 (2002) 99

[20] J. Gerostamoulos, V. Staikos, O.H. Drummer, Heroin-related deaths in Victoria: a review of cases for 1997 and 1998, *Drug Alcohol Depend.*, 61 (2001) 123

[21] J. Wallington, J. Gerostamoulos, O.H. Drummer, Heroin deaths in Victoria: 2001. Report No. 5 - February 2002, Victorian Institute of Forensic Medicine & Department of Forensic Medicine - Monash University.

[22] NDARC, Costs of illegal drug use. The consequences of alcohol and drug use: Implications for policy, Monograph no. 15, (1992)

[23] P. Mendes, Drug wars down under: the ill-fated struggle for safe injecting facilities in Victoria, Australia, *Int. J. Soc. Welf.*, 11 (2002) 140

[24] D. Weatherburn, B. Lind, The impact of law enforcement activity on a heroin market, *Addiction*, 92 (1997) 557

[25] G. Rumbold, C. Fry, The heroin market place project: examining the short term impact of the Port Macquarie heroin seizure on the characteristics of the

retail heroin market in Melbourne, Turning Point Alcohol and Drug Centre Inc., Melbourne, 1999.

[26] D. Weatherburn, C. Jones, K. Freeman, T. Makkai, Supply control and harm reduction: lessons from the Australian heroin 'drought', *Addiction*, 98 (2003) 83

[27] I. van Beek, The Sydney Medically Supervised Injecting Centre: a clinical model, *Journal of Drug Issues*, 33 (2003) 625

[28] K.A. Sporer, Strategies for preventing heroin overdose, *BMJ*, 326 (2003) 442

[29] J.M. White, R.J. Irvine, Mechanisms of fatal opioid overdose, *Addiction*, 94 (1999) 961

[30] M.J. Burt, J. Kloss, F.S. Apple, Postmortem blood free and total morphine concentrations in medical examiner cases, *J. Forensic Sci.*, 46 (2001) 1138

[31] S. Darke, W.D. Hall, D. Weatherburn, B. Lind, Fluctuations in heroin purity and the incidence of fatal heroin overdose, *Drug Alcohol Depend.*, 54 (1999) 155

[32] J. Gutiérrez-Cebollada, R. de la Torre, J. Ortuño, J.M. Garcés, J. Camí, Psychotropic drug consumption and other factors associated with heroin overdose, *Drug Alcohol Depend.*, 35 (1994) 169

[33] S. Darke, L. Topp, S. Kaye, W. Hall, Heroin use in New South Wales, Australia, 1996-2000: 5 year monitoring of trends in price, purity, availability and use from the Illicit Drug Reporting System (IDRS), *Addiction*, 97 (2002) 197

[34] D. Zador, Heroin overdose: new directions for research, *Addiction*, 94 (1999) 975

[35] F. Tagliaro, Z. De Battisti, F. Smith, M. Marigo, Death from heroin overdose: findings from hair analysis, *Lancet*, 351 (1998) 1923

[36] R. Kronstrand, R. Grundin, J. Jonsson, Incidence of opiates, amphetamines, and cocaine in hair and blood in fatal cases of heroin overdose, *Forensic Sci. Int.*, 92 (1998) 29

[37] D. Westerling, J. Säwe, G. Eklundh, Near fatal intoxication with controlled-release morphine tablets in a depressed woman, *Acta Anaesthesiol. Scan.*, 42 (1998) 586

[38] S. Siegel, Pavlovian conditioning and drug overdose: when tolerance fails, *Addict. Res. Theory*, 9 (2001) 503

[39] S. Siegel, Drug anticipation and drug addiction. The 1998 H. David Archibald Lecture, *Addiction*, 94 (1999) 1113

[40] S. Siegel, L.G. Allan, Learning and homeostasis: drug addiction and the McCollough effect, *Psychol. Bull.*, 124 (1998) 230

[41] S.J. Larson, S. Siegel, Learning and tolerance to the ataxic effect of ethanol, *Pharmacol., Biochem. Behav.*, 61 (1998) 131

[42] S. Siegel, S.J. Larson, Disruption of tolerance to the ataxic effect of ethanol by an extraneous stimulus, *Pharmacol., Biochem. Behav.*, 55 (1996) 125

[43] M.C.A. Buster, G.H.A. van Brussel, W. van den Brink, An increase in overdose mortality during the first 2 weeks after entering or re-entering methadone treatment in Amsterdam, *Addiction*, 97 (2002) 993

[44] S. Mercadante, The role of morphine glucuronides in pain, *Palliative Med.*, 13 (1999) 95

[45] M. Holthe, P. Klepstad, K. Zahlsen, P.C. Borchgrevink, L. Hagen, O. Dale, S. Kaasa, H.E. Krokan, F. Skorpen, Morphine glucuronide-to-morphine plasma ratios are unaffected by the *UGT2B7* H268Y and *UGT1A1*28* polymorphisms in cancer patients on chronic morphine therapy, *Eur. J. Clin. Pharmacol.*, 58 (2002) 353

[46] P. Klepstad, S. Kaasa, P.C. Borchgrevink, Start of oral morphine to cancer patients: effective serum morphine concentrations and contribution from morphine-6-glucuronide to the analgesia produced by morphine, *Eur. J. Clin. Pharmacol.*, 55 (2000) 713

[47] M. Tateishi, K. Ohashi, K. Kobayashi, T. Hashimoto, J. Yamaguchi, H. Fujioka, K. Izawa, M. Masada, Interindividual variation in the ratio between plasma morphine and its metabolites in cancer patients, *Int. J. Clin. Pharm. Res.*, 23 (2003) 75

[48] T. Wolff, H. Samuelsson, T. Hedner, Morphine and morphine metabolite concentrations in cerebrospinal fluid and plasma in cancer pain patients after slow-release oral morphine administration, *Pain*, 62 (1995) 147

[49] M. Ashby, B. Fleming, M. Wood, A. Somogyi, Plasma morphine and glucuronide (M3G and MG6) concentrations in hospice inpatients, *J. Pain Symptom Manag.*, 14 (1997) 157

[50] R.T. Penson, S.P. Joel, K. Bakhshi, S.J. Clark, R.M. Langford, M.L. Slevin, Randomized placebo-controlled trial of the activity of the morphine glucuronides, *Clin. Pharmacol. Ther.*, 68 (2000) 667

[51] L.L. Christrup, Morphine metabolites, *Acta Anaesthesiol. Scan.*, 41 (1997) 116

[52] M. Segura, J. Barbosa, M. Torrens, M. Farré, C. Castillo, J. Segura, R. de la Torre, Analytical methodology for the detection of benzodiazepine consumption in opioid-dependent subjects, *J. Anal. Toxicol.*, 25 (2001) 130

[53] S.G. Darke, J.E. Ross, W.D. Hall, Benzodiazepine use among injecting heroin users, *MJA*, 162 (1995) 645

[54] C.L. Fry, R.B. Bruno, Recent trends in benzodiazepine use by injecting drug users in Victoria and Tasmania, *Drug Alc. Rev.*, 21 (2002) 363

[55] D. Zador, S. Sunjic, S. Darke, Heroin-related deaths in New South Wales, 1992: toxicological findings and circumstances, *MJA*, 164 (1996) 204

[56] M. Gossop, D. Stewart, S. Treacy, J. Marsden, A prospective study of mortality among drug misusers during a 4-year period after seeking treatment, *Addiction*, 97 (2002) 39

[57] S. Darke, W. Hall, S. Kaye, J. Ross, J. Duflou, Hair morphine concentrations of fatal heroin overdose cases and living heroin users, *Addiction*, 97 (2002) 977

[58] V. Navaratnam, K. Foong, Opiate dependence - the role of benzodiazepines, *Curr. Med. Res. Opin.*, 11 (1990) 620

[59] S. Darke, J. Ross, M. Teesson, M. Lynskey, Health service utilization and benzodiazepine use among heroin users: findings from the Australian Treatment Outcome Study (ATOS), *Addiction*, 98 (2003) 1129

[60] J. Ross, S. Darke, The nature of benzodiazepine dependence among heroin users in Sydney, Australia, *Addiction*, 95 (2000) 1785

[61] B.A. Goldberger, E.J. Cone, T.M. Grant, Y.H. Caplan, B.S. Levine, J.E. Smialek, Disposition of heroin and its metabolites in heroin-related deaths, *J. Anal. Toxicol.*, 18 (1994) 22

[62] T.M. Garrick, D. Sheedy, J. Abernethy, A.E. Hodda, C.G. Harper, Heroin-related deaths in Sydney, Australia. How common are they?, *Am. J. Addiction*, 9 (2000) 172

[63] A. Steentoft, K. Worm, C.B. Pedersen, M. Sprehn, T. Mogensen, M.B. Sørenson, E. Nielsen, Drugs in blood samples from unconscious drug addicts after the intake of an overdose, *Int. J. Legal Med.*, 108 (1996) 248

[64] W.E. Lambert, J.F. Van Bocxlaer, A.P. De Leenheer, Potential of high-performance liquid chromatography with photodiode array detection in forensic toxicology, *J. Chromatogr. B*, 689 (1997) 45

[65] O.H. Drummer, Postmortem drug analysis: analytical and toxicological aspects, *Ther. Drug Monit.*, 24 (2002) 199

[66] J. Blanchard, Evaluation of the relative efficacy of various techniques for deproteinizing plasma samples prior to high-performance liquid chromatographic analysis, *J. Chromatogr.*, 226 (1981) 455

[67] X.-H. Chen, J.-P. Franke, R.A. Zeeuw, Solid-phase extraction for systematic toxicological analysis, *Forensic Sci. Rev.*, 4 (1992) 147

[68] X.-H. Chen, J.-P. Franke, J. Wijsbeek, R.A. de Zeeuw, Isolation of acidic, neutral, and basic drugs from whole blood using a single mixed-mode solid-phase extraction column, *J. Anal. Toxicol.*, 16 (1992)

[69] S. Pichini, I. Altieri, M. Pellegrini, P. Zuccaro, R. Pacifici, The role of liquid chromatography-mass spectrometry in the determination of heroin and related opioids in biological fluids, *Mass Spectrom. Rev.*, 18 (1999) 119

[70] J. Gerostamoulos, O.H. Drummer, Postmortem redistribution of morphine and its metabolites, *J. Forensic Sci.*, 45 (2000) 843

[71] G. Skopp, R. Lutz, B. Ganßmann, R. Mattern, R. Aderjan, Postmortem distribution pattern of morphine and morphine glucuronides in heroin overdose, *Int. J. Legal Med.*, 109 (1996) 118

[72] C. Ulens, L. Baker, A. Ratka, D. Waumans, J. Tytgat, Morphine-6β-glucuronide and morphine-3-glucuronide, opioid receptor agonists with different potencies, *Biochem. Pharmacol.*, 62 (2001) 1273

[73] C. Mignat, U. Wille, A. Ziegler, Affinity profiles of morphine, codeine, dihydrocodeine and their glucuronides at opioid receptor subtypes, *Life Sci.*, 56 (1995) 793

[74] K. Oguri, I. Yamada-Mori, J. Shigezane, T. Hirano, H. Yoshimura, Enhanced binding of morphine and nalorphine to opioid delta receptor by glucuronate and sulfate conjugations at the 6-position, *Life Sci.*, 41 (1987) 1457

[75] Z.R. Chen, R.J. Irvine, A.A. Somogyi, F. Bochner, Mu receptor binding of some commonly used opioids and their metabolites, *Life Sci.*, 48 (1991) 2165

[76] A.H. Beckett, A.F. Casy, N.J. Harper, Analgesics and their antagonists: some steric and chemical considerations. Part III - The influence of the basic group on biological response, *J. Pharm. Pharmacol.*, 8 (1956) 874

[77] K.M. Rentsch, Arterial and venous pharmacokinetics of intravenous heroin in subjects who are addicted to narcotics, *Clin. Pharmacol. Ther.*, 70 (2001) 237

[78] A.C. Moffat, M.D. Osselton, B. Widdop, Clarke's analysis of drugs and poisons: in pharmaceuticals, body fluids and postmortem material, Pharmaceutical Press, UK, 2004.

[79] C.C. Faura, S.L. Collins, A. Moore, H.J. McQuay, Systematic review of factors affecting the ratios of morphine and its major metabolites, *Pain*, 74 (1998) 43

[80] R.E. Aderjan, G. Skopp, Formation and clearance of active and inactive metabolites of opiates in humans, *Ther. Drug Monit.*, 20 (1998) 561

[81] M.W.H. Coughtrie, B. Ask, A. Rane, B. Burchell, R. Hume, The enantioselective glucuronidation of morphine in rats and humans. Evidence for the involvement of more than one UDPGT isoenzyme, *Biochem. Pharmacol.*, 38 (1989) 3273

[82] M.F. Fromm, K. Eckhardt, S. Li, G. Schanzle, U. Hofmann, G. Mikus, M. Eichelbaum, Loss of analgesic effect of morphine due to coadministration of rifampin, *Pain*, 72 (1997) 261

[83] T.M. Buetler, O.H.G. Wilder-Smith, C.H. Wilder-Smith, S. Aebi, T. Cerny, R. Brenneisen, Analgesic action of I.V. morphine-6-glucuronide in healthy volunteers, *Br. J. Anaesth.*, 84 (2000) 97

[84] P.I. Thompson, S.P. Joel, L. John, J.A. Wedzicha, M. Maclean, M.L. Slevin, Respiratory depression following morphine and morphine-6-glucuronide in normal subjects, *Br. J. Clin. Pharmacol.*, 40 (1995) 145

[85] J. Lötsch, G. Kobal, A. Stockmann, K. Brune, G. Geisslinger, Lack of analgesic activity of morphine-6-glucuronide after short-term intravenous administration in healthy volunteers, *Anesthesiology*, 87 (1997) 1348

[86] J. Lötsch, G. Kobal, G. Geisslinger, No contribution of morphine-6-glucuronide to clinical morphine effects after short-term administration, *Clin. Neuropharmacol.*, 21 (1998) 351

[87] T.A. Jennison, B.S. Finkle, D.M. Chinn, D.J. Crouch, The quantitative analysis of 1-α-acetylmethadol and its principal metabolites in biological specimens by gas chromatography-chemical ionization-multiple ion monitoring mass spectrometry, *J. Chromatogr. Sci.*, 17 (1979) 64

[88] H.-M. Lee, C.-W. Lee, Determination of morphine and codeine in blood and bile by gas chromatography with a derivitization procedure, *J. Anal. Toxicol.*, 15 (1991) 182

[89] K.L. Crump, I.M. McIntyre, O.H. Drummer, Simultaneous determination of morphine and codeine in blood and bile using dual ultraviolet and fluorescence high-performance liquid chromatography, *J. Anal. Toxicol.*, 18 (1994) 208

[90] B. Fryirs, M. Dawson, L.E. Mather, Highly sensitive gas chromatographic-mass spectrometric method for morphine determination in plasma that is suitable for pharmacokinetic studies, *J. Chromatogr. B*, 693 (1997) 51

[91] F. Moriya, Y. Hashimoto, Distribution of free and conjugated morphine in body fluids and tissues in a fatal heroin overdose: is conjugated morphine stable in postmortem specimens?, *J. Forensic Sci.*, 42 (1997) 736

[92] H.J. Leis, G. Fauler, G. Raspotnig, W. Windischhofer, Quantitative analysis of morphine in human plasma by gas chromatography-negative ion chemical ionization mass spectrometry, *J. Chromatogr. B*, 744 (2000) 113

[93] P. Lillsunde, L. Michelson, T. Forsstrom, T. Korte, E. Schultz, K. Ariniemi, M. Portman, M.-L. Sihvonen, T. Seppala, Comprehensive drug screening in blood for detecting abused drugs or drugs potentially hazardous for traffic safety, *Forensic Sci. Int.*, 77 (1996) 191

[94] U. Staerk, W.R. Külpmann, High-temperature solid-phase microextraction procedure for the detection of drugs by gas-chromatography-mass spectrometry, *J. Chromatogr. B*, 745 (2000) 399

[95] J. Beike, H. Kohler, G. Blaschke, Antibody-mediated clean-up of blood for simultaneous HPLC determination of morphine and morphine glucuronides, *Int. J. Legal Med.*, 110 (1997) 226

[96] R. Pacifici, S. Pichini, I. Altieri, A. Caronna, A.R. Passa, P. Zuccaro, High-performance liquid chromatography-electrospray mass spectrometric determination of morphine and its 3- and 6-glucuronides: application to pharmacokinetic studies, *J. Chromatogr. B*, 664 (1995) 329

[97] P. Zuccaro, R. Ricciarello, S. Pichini, R. Pacifici, I. Altieri, M. Pellegrini, G. D'Ascenzo, Simultaneous determination of heroin, 6-monoacetylmorphine, morphine, and its glucuronides by liquid chromatography-atmospheric pressure ionspray-mass spectrometry, *J. Anal. Toxicol.*, 21 (1997) 268

[98] S. Pichini, R. Pacifici, M. Pellegrini, E. Marchei, E. Perez-Alarcon, C. Puig, O. Vall, O. Garcia-Algar, Development and validation of a liquid chromatography-mass spectrometry assay for the determination of opiates and cocaine in meconium, *J. Chromatogr. A*, 794 (2003) 281

[99] M. Zheng, K.M. McErlane, M.C. Ong, High-performance liquid chromatography-mass spectrometry analysis of morphine and morphine

metabolites and its application to a pharmacokinetic study in male Sprague-Dawley rats, *J. Pharm. Biomed. Anal.*, 16 (1998) 971

[100] Y. Rotshteyn, B. Weingarten, A highly sensitive assay for the simultaneous determination of morphine, morphine-3-glucuronide, and morphine-6-glucuronide in human plasma by high-performance liquid chromatography with electrochemical and fluorescence detection, *Ther. Drug Monit.*, 18 (1996) 179

[101] M.R. Taylor, S.A. Westwood, D. Perrett, Determination of phase II metabolites in equine urine by micellar electrokinetic capillary chromatography, *J. Chromatogr. A*, 745 (1996) 155

[102] G. Skopp, L. Pötsch, A. Klingmann, R. Mattern, Stability of morphine, morphine-3-glucuronide, and morphine-6-glucuronide in fresh blood and plasma and postmortem blood samples, *J. Anal. Toxicol.*, 25 (2001) 2

[103] A. Dienes-Nagy, L. Rivier, C. Giroud, M. Augsburger, P. Mangin, Method for quantification of morphine and its 3- and 6-glucuronides, codeine, codeine glucuronide and 6-monoacetylmorphine in human blood by liquid chromatography-electrospray mass spectrometry for routine analysis in forensic toxicology, *J. Chromatogr. A*, 854 (1999) 109

[104] R. Aderjan, S. Hofmann, G. Schmitt, G. Skopp, Morphine and morphine glucuronides in serum of heroin consumers and in heroin-related deaths determined by HPLC with native fluorescence detection, *J. Anal. Toxicol.*, 19 (1995) 163

[105] A. Vermeire, J.P. Remon, M.T. Rosseel, F. Belpaire, J. Devulder, M.G. Bogaert, Variability of morphine disposition during long-term subcutaneous infusion in terminally ill cancer patients, *Eur. J. Clin. Pharmacol.*, 53 (1998) 325

[106] I.R. Tebbett, Rapid extraction of codeine and morphine in whole blood for HPLC analysis, *Chromatographia*, 23 (1987) 377

[107] M.J. Bogusz, R.-D. Maier, K.-D. Krüger, U. Kohls, Determination of common drugs of abuse in body fluids using one isolation procedure and

liquid-chromatography-atmospheric-pressure chemical-ionization mass spectrometry, *J. Anal. Toxicol.*, 22 (1998) 549

[108] M.J. Bogusz, R.-D. Maier, S. Driessen, Morphine, morphine-3-glucuronide, morphine-6-glucuronide, and 6-monoacetylmorphine determined by means of atmospheric pressure chemical ionization-mass spectrometry-liquid chromatography in body fluids of heroin victims, *J. Anal. Toxicol.*, 21 (1997) 346

[109] M.J. Bogusz, R.-D. Maier, M. Erkens, S. Driessen, Determination of morphine and its 3- and 6-glucuronides, codeine-glucuronide and 6-monoacetylmorphine in body fluids by liquid chromatography atmospheric pressure chemical ionization mass spectrometry, *J. Chromatogr. B*, 703 (1997) 115

[110] W. Naidong, J.W. Lee, X. Jiang, M. Wehling, J.D. Hulse, P.P. Lin, Simultaneous assay of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human plasma using normal-phase liquid chromatography-tandem mass spectrometry with a silica column and an aqueous organic mobile phase, *J. Chromatogr. B*, 735 (1999) 255

[111] M. Blanchet, G. Bru, M. Guerret, M. Bromet-Petit, N. Bromet, Routine determination of morphine, morphine 3-β-Þ-glucuronide and morphine 6-β-Þ-glucuronide in human serum by liquid chromatography coupled to electrospray mass spectrometry, *J. Chromatogr. A*, 854 (1999) 93

[112] L.L. Christrup, P. Sjøgren, N.-H. Jensen, A.-M. Banning, K. Elbaek, A.K. Ersbøll, Steady-state kinetics and dynamics of morphine in cancer patients: Is sedation related to the absorption rate of morphine?, *J. Pain Symptom Manag.*, 18 (1999) 164

[113] M.H. Slawson, D.J. Crouch, D.M. Andrenyak, D.E. Rollins, J.K. Lu, P.L. Bailey, Determination of morphine, morphine-3-glucuronide, and morphine-6-glucuronide in plasma after intravenous and intrathecal morphine administration using HPLC with electrospray ionization and tandem mass spectrometry, *J. Anal. Toxicol.*, 23 (1999) 468

[114] D. Bourquin, T. Lehmann, R. Hammig, M. Buhrer, R. Brenneisen, High-performance liquid chromatographic monitoring of intravenously administered diacetylmorphine and morphine and their metabolites in human plasma, *J. Chromatogr. B*, 694 (1997) 233

[115] M. Nishikawa, K. Nakajima, K. Igarashi, F. Kasuya, M. Fukui, H. Tsuchihashi, Determination of morphine-3-glucuronide in human urine by LC/APCI-MS, *Eisei Kagaku*, 38 (1992) 121

[116] N. Tyrefors, B. Hyllbrant, L. Ekman, M. Johansson, B. Långström, Determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human serum by solid-phase extraction and liquid chromatography-mass spectrometry with electrospray ionisation, *J. Chromatogr. A*, 729 (1996) 279

[117] A.W.E. Wright, J.A. Watt, M. Kennedy, T. Cramond, M. Smith, Quantitation of morphine, morphine-3-glucuronide and morphine-6-glucuronide in plasma and cerebrospinal fluid using solid-phase extraction and high-performance liquid chromatography with electrochemical detection, *Ther. Drug Monit.*, 16 (1994) 200

[118] P.P. Rop, F. Grimaldi, J. Burle, M.N. De Saint Leger, A. Viala, Determination of 6-monoacetylmorphine and morphine in plasma, whole blood and urine using high-performance liquid chromatography with electrochemical detection, *J. Chromatogr. B*, 661 (1994) 245

[119] A.W.E. Wright, M.T. Smith, Improved one-step solid-phase extraction method for morphine, morphine-3-glucuronide, and morphine-6-glucuronide from plasma and quantitation using high-performance liquid chromatography with electrochemical detection, *Ther. Drug Monit.*, 20 (1998) 215

[120] J. Gerostamoulos, O.H. Drummer, Solid phase extraction of morphine and its metabolites from postmortem blood, *Forensic Sci. Int.*, 77 (1995) 53

[121] D. Wu, Y.-S. Kang, U. Bickel, W.M. Pardridge, Blood-brain barrier permeability to morphine-6-glucuronide is markedly reduced compared with morphine, *Drug Metab. Dispos.*, 25 (1997) 768

[122] M. Hoffman, J.-C. Xu, C. Smith, C. Fanelli, V. Pascal, C. Degaetano, G. Meenan, M. Lehrer, M. Lesser, M. Citron, A pharmacodynamic study of morphine and its glucuronide metabolites after single morphine dosing in cancer patients with pain, *Cancer Invest.*, 15 (1997) 542

[123] S.E. Bartlett, M.T. Smith, The apparent affinity of morphine-3-glucuronide at μ_1-opioid receptors results from morphine contamination: demonstration using HPLC and radioligand binding, *Life Sci.*, 57 (1995) 609

[124] F. Musshoff, J. Trafkowski, B. Madea, Validated assay for the determination of markers of illicit heroin in urine samples for the control of patients in a heroin prescription program, *J. Chromatogr. B*, 811 (2004) 47

[125] R. Dams, T. Benijits, W.E. Lambert, A.P. De Leenheer, Simultaneous determination of in total 17 opium alkaloids and opioids in blood and urine by fast liquid-chromatography-diode-array detection-fluorescence detection, after solid-phase extraction, *J. Chromatogr. B*, 773 (2002) 53

[126] T. Toyo'oka, M. Yano, M. Kato, Y. Nakahara, Simultaneous determination of morphine and its glucuronides in rat hair and rat plasma by reversed-phase liquid chromatography with electrospray ionization mass spectrometry, *Analyst*, 126 (2001) 1339

[127] D. Whittington, E.D. Kharasch, Determination of morphine and morphine glucuronides in human plasma by 96-well plate solid-phase extraction and liquid chromatography-electrospray ionization mass spectrometry, *J. Chromatogr. B*, 796 (2003) 95

[128] J. Yawney, S. Treacy, K.W. Hindmarsh, F.J. Burczynski, A general screening method for acidic, neutral, and basic drugs in whole blood using the Oasis MCX® column, *J. Anal. Toxicol.*, 26 (2002) 325

[129] A.B. Wey, J. Caslavska, W. Thormann, Analysis of codeine, dihydrocodeine and their glucuronides in human urine by capillary immunoassays and capillary electrophoresis-ion trap mass spectrometry, *J. Chromatogr. A*, 895 (2000) 133

[130] A.B. Wey, W. Thormann, Head-column field-amplified sample stacking in presence of siphoning - Application to capillary electrophoresis-electrospray

ionization mass spectrometry of opioids in urine, *J. Chromatogr. A*, 924 (2001) 507

[131] A.B. Wey, W. Thormann, Capillary electrophoresis-electrospray ionization ion trap mass spectrometry for analysis and confirmation testing of morphine and related compounds in urine, *J. Chromatogr. A*, 916 (2001) 225

[132] E. Hufschmid, R. Theurillat, U. Martin, W. Thormann, Exploration of the metabolism of dihydrocodeine via determination of its metabolites in human urine using micellar electrokinetic capillary chromatography, *J. Chromatogr. B*, 668 (1995) 159

[133] E. Hufschmid, R. Theurillat, C.H. Wilder-Smith, W. Thormann, Characterization of the genetic polymorphism of dihydrocodeine O-demethylation in man via analysis of urinary dihydrocodeine and dihydromorphine by micellar electrokinetic capillary chromatography, *J. Chromatogr. B*, 678 (1996) 43

[134] R.B. Taylor, A.S. Low, R.G. Reid, Determination of opiates in urine by capillary electrophoresis, *J. Chromatogr. B*, 675 (1996) 213

[135] A.B. Wey, C.-X. Zhang, W. Thormann, Head-column field-amplified samples stacking in binary system capillary electrophoresis. Preparation of extracts for determination of opioids in microliter amounts of body fluids, *J. Chromatogr. A*, 853 (1999) 95

[136] A. Solans, M. Carnicero, R. de la Torre, J. Segura, Comprehensive screening procedure for detection of stimulants, narcotics, adrenergic drugs, and their metabolites in human urine, *J. Anal. Toxicol.*, 19 (1995) 104

[137] P.G.M. Zweipfenning, A.H.C.M. Wilderink, P. Horsthuis, J.-P. Franke, R.A. de Zeeuw, Toxicological analysis of whole blood samples by means of Bond-Elut Certify columns and gas chromatography with nitrogen-phosphorus detection, *J. Chromatogr. A*, 674 (1994) 87

[138] W.V. Caulfield, J.T. Stewart, Rapid determination of selected drugs of abuse in human plasma using a monolithic silica HPLC column and solid phase extraction, *J. Liq. Chromatogr. and Rel. Technol.*, 25 (2002) 2977

[139] M.J. Bogusz, R.-D. Maier, K.-H. Schiwy-Bochat, U. Kohls, Applicability of various brands of mixed-phase extraction columns for opiate extraction from blood and serum, *J. Chromatogr. B*, 683 (1996) 177

[140] M.Y. Salem, S.A. Ross, T.P. Murphy, M.A. ElSohly, GC-MS determination of heroin metabolites in meconium: evaluation of four solid-phase extraction cartridges, *J. Anal. Toxicol.*, 25 (2001) 93

[141] S.S.C. Tai, R.G. Christensen, R.C. Paule, L.C. Sander, M.J. Welch, The certification of morphine and codeine in a human urine standard reference material, *J. Anal. Toxicol.*, 18 (1994) 7

[142] E. Brandsteterova, E. Blahova, J. Netriova, Simple generic SPE assay for HPLC analysis of morphine and its glucuronides in serum samples, *J. Liq. Chromatogr. and Rel. Technol.*, 25 (2002) 2521

[143] N. Mabuchi, S. Takatsuka, M. Matsuoka, K. Tagawa, Determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide in monkey and dog plasma by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry, *J. Pharm. Biomed. Anal.*, 35 (2004) 563

[144] R.S. Niedbala, K.W. Kardos, J. Waga, D. Fritch, L. Yeager, S. Doddamane, Laboratory analysis of remotely collected oral fluid specimens for opiated by immunoassay, *J. Anal. Toxicol.*, 25 (2001) 310

[145] A.D. Fraser, D. Worth, Experience with a urine opiate screening and confirmation cutoff of 2000 ng/mL, *J. Anal. Toxicol.*, 23 (1999) 549

[146] L. Presley, M. Lehrer, W. Seiter, D. Hahn, B. Rowland, M. Smith, K.W. Kardos, D. Fritch, S. Salamone, R.S. Niedbala, E.J. Cone, High prevalence of 6-acetylmorphine in morphine-positive oral fluid specimens, *Forensic Sci. Int.*, 133 (2003) 22

[147] L.R. Baden, G. Horowitz, H. Jacoby, G.M. Eliopoulos, Quinolones and false-positive urine screening for opiates by immunoassay technology, *JAMA*, 286 (2001) 3115

[148] G. Ceder, A.W. Jones, Concentrations of unconjugated morphine, codeine and 6-acetylmorphine in urine specimens from suspected drugged drivers, *J. Forensic Sci.*, 47 (2002) 366

[149] N.P. Lemos, R.A. Anderson, R. Valentini, F. Tagliaro, R.T.A. Scott, Analysis of morphine by RIA and HPLC in fingernail clipping obtained from heroin users, *J. Forensic Sci.*, 45 (2000) 407

[150] K.A. Moore, C. Werner, R.M. Zannelli, B. Levine, M.L. Smith, Screening postmortem blood and tissues for nine cases of drugs of abuse using automated microplate immunoassay, *Forensic Sci. Int.*, 106 (1999) 93

[151] P. Kemp, G. Sneed, T. Kupiec, V. Spiehler, Validation of a microtiter plate ELISA for screening of postmortem blood for opiates and benzodiazepines, *J. Anal. Toxicol.*, 26 (2002) 504

[152] S. Kerrigan, W.H. Phillips, Comparison of ELISAs for opiates, methamphetamine, cocaine metabolite, benzodiazepines, phencyclidine, and cannabinoids in whole blood and urine, *Clin. Chem.*, 47 (2001) 540

[153] I.B. Collison, V. Spiehler, S. Guluzian, P.R. Sedgwick, Setting cutoff concentrations for immunoassay screening of postmortem blood, *J. Forensic Sci.*, 43 (1998) 390

[154] A.J. Ruttenber, K. H.D., P. Santinga, The role of ethanol abuse in the etiology of heroin-related death, *J. Forensic Sci.*, 35 (1990) 891

[155] T. Keller, A. Schneider, R. Dirnhofer, R. Jungo, W. Meyer, Fluorescence polarization immunoassay for the detection of drugs of abuse in human whole blood, *Med. Sci. Law*, 40 (2000) 258

[156] E.M. Hattab, B.A. Goldberger, L.M. Johannsen, P.W. Kindland, F. Ticino, C.W. Chronister, R.L. Bertholf, Modification of screening immunoassays to detect sub-threshold concentrations of cocaine, cannabinoids, and opiates in urine: use for detecting maternal and neonatal drug exposures, *Annals of Clinical & Laboratory Science*, 30 (2000) 85

[157] M.E. Eldefrawi, N.L. Azer, N. Nath, N.A. Anis, M.S. Bangalore, K.P. O'Connell, R.P. Schwartz, J. Wright, A sensitive solid-phase

fluoroimmunoassay for detection of opiates in urine, *Appl. Biochem. Biotechnol.*, 87 (2000) 25

[158] D. Projean, T. Minh Tu, J. Ducharme, Rapid and simple method to determine morphine and its metabolites in rat plasma by liquid chromatography-mass spectrometry, *J. Chromatogr. B*, 787 (2003) 243

[159] G. Schanzle, S. Li, G. Mikus, U. Hofmann, Rapid, highly sensitive method for the determination of morphine and its metabolites in body fluids by liquid chromatography-mass spectrometry, *J. Chromatogr. B*, 721 (1999) 55

[160] M.J. Bogusz, G. Skopp, Postmortem distribution pattern of morphine glucuronides in heroin overdose [letter], *Int. J. Legal Med.*, 110 (1997) 114

[161] A. Polettini, A. Groppi, M. Montagna, Application of thermospray liquid chromatography-tandem mass spectrometry (HPLC/TSP/MS/MS) to the confirmatory analysis of heroin metabolites in biological fluids, *Adv Forensic Sci Pro Meet Int Assoc Forensic Sci*, 5 (1995) 197

[162] W.Z. Shou, M. Pelzer, T. Addison, X. Jiang, W. Naidong, An automated 96-well solid phase extraction and liquid chromatography-tandem mass spectrometry method for the analysis of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human plasma, *J. Pharm. Biomed. Anal.*, 27 (2002) 143

[163] J. Huwyler, S. Rufer, E. Kusters, J. Drewe, Rapid and highly automated determination of morphine and morphine glucuronides in plasma by on-line solid-phase extraction and column liquid chromatography, *J. Chromatogr. B*, 674 (1995) 57

[164] G. Skopp, L. Pötsch, B. Ganßmann, R. Aderjan, R. Mattern, A preliminary study on the distribution of morphine and its glucuronides in the subcompartments of blood, *J. Anal. Toxicol.*, 22 (1998) 261

[165] G. Ceder, A.W. Jones, Concentration ratios of morphine to codeine in blood of impaired drivers as evidence of heroin use and not medication with codeine, *Clin. Chem.*, 47 (2001) 1980

[166] H. Schutz, J.C. Gotta, F. Erdmann, M. Rißе, G. Weiler, Simultaneous screening and detection of drugs in small blood samples and bloodstains, *Forensic Sci. Int.*, 126 (2002) 191

[167] C. Meadway, S. George, R. Braithwaite, Interpretation of GC-MS opiate results in the presence of pholcodine, *Forensic Sci. Int.*, 127 (2002) 131

[168] W. Nowatzke, J. Zeng, A. Saunders, A. Bohrer, J. Koenig, J. Turk, Distinction among eight opiate drugs in urine by gas chromatography-mass spectrometry, *J. Pharm. Biomed. Anal.*, 20 (1999) 815

[169] C.L. O'Neal, A. Poklis, The detection of acetylcodeine and 6-acetylmorphine in opiate positive urines, *Forensic Sci. Int.*, 95 (1998) 1

[170] A. Geier, D. Bergemann, L. von Meyer, Evaluation of a solid-phase extraction procedure for the simultaneous determination of morphine, 6-monoacetylmorphine, codeine and dihydrocodeine in plasma and whole blood by GC/MS, *Int. J. Legal Med.*, 109 (1996) 80

[171] K.A. Hadidi, J.S. Oliver, Stability of morphine and buprenorphine in whole blood, *Int. J. Legal Med.*, 111 (1998) 165

[172] W. Weinmann, M. Renz, S. Vogt, S. Pollack, Automated solid-phase extraction and two-step derivatisation for simultaneous analysis of basic illicit drugs in serum by GC/MS, *Int. J. Legal Med.*, 113 (2000) 229

[173] H. Keski-Hynnilä, K. Raanaa, J. Taskinen, R. Kostiainen, Direct analysis of nitrocatechol-type glucuronides in urine by capillary electrophoresis-electrospray ionisation mass spectrometry and tandem mass spectrometry, *J. Chromatogr. B*, 749 (2000) 253

[174] F. Tagliaro, C. Poiesi, R. Aiello, R. Dorizzi, S. Ghielmi, M. Marigo, Capillary electrophoresis for the investigation of illicit drugs in hair: determination of cocaine and morphine, *J. Chromatogr.*, 638 (1993) 303

[175] F. Tagliaro, R. Valentini, G. Manetto, F. Crivellente, G. Carli, M. Marigo, Hair analysis by using radioimmunoassay, high-performance liquid chromatography and capillary electrophoresis to investigate chronic exposure to heroin, cocaine and/or ecstasy in applicants for driving licences, *Forensic Sci. Int.*, 107 (2000) 121

[176] P. Wernly, W. Thormann, D. Bourquin, R. Brenneisen, Determination of morphine-3-glucuronide in human urine by capillary zone electrophoresis and micellar electrokinetic capillary chromatography, *J. Chromatogr.*, 616 (1993) 305

[177] N.W. Barnett, B.J. Hindson, S.W. Lewis, Determination of morphine, oripavine and pseudomorphine using capillary electrophoresis with acidic potassium permanganate chemiluminescence detection, *Analyst*, 125 (2000) 91

[178] W.-S. Wu, J.-L. Tsai, Analysis of morphine and morphine-3β-D glucuronide in human urine by capillary zone electrophoresis with minimal sample pretreatment, *Biomed. Chromatogr.*, 13 (1999) 216

[179] J.L. Tsai, W.-S. Wu, H.-H. Lee, Qualitative determination of urinary morphine by capillary zone electrophoresis and ion trap mass spectrometry, *Electrophoresis*, 21 (2000) 1580

[180] S.R. Edwards, M.T. Smith, Simultaneous determination of morphine, oxycodone, morphine-3-glucuornide, and noroxycodone concentrations in rat serum by high performance liquid chromatography-electrospray ionization-tandem mass spectrometry, *J. Chromatogr. B*, 814 (2005) 241

[181] L. Antonilli, F. Semeraro, C. Suriano, L. Signore, P. Nencini, High levels of morphine-6-glucuronide in street heroin addicts, *Psychopharmacology*, 170 (2003) 200

[182] C. Meissner, S. Recker, A. Reiter, H.J. Friedrich, M. Oehmichen, Fatal versus non-fatal heroin "overdose": blood morphine concentrations with fatal outcome in comparison to those of intoxicated drivers, *Forensic Sci. Int.*, 130 (2002) 49

[183] P. Klepstad, O. Dale, S. Kaasa, K. Zahlsen, T. Aamo, P. Fayers, P.C. Borchgrevink, Influences on serum concentrations of morphine, M6G and M3G during routine clinical drug monitoring: a prospective survey in 300 adult cancer patients, *Acta Anaesthesiol. Scan.*, 47 (2003) 725

[184] T. Wolff, H. Samuelsson, T. Hedner, Concentrations of morphine and morphine metabolites in CSF and plasma during continuous subcutaneous morphine administration in cancer pain patients, *Pain*, 68 (1996) 209

[185] P. Klepstad, P.C. Borchgrevink, O. Dale, K. Zahlsen, T. Aamo, P. Fayers, B. Fougner, Routine drug monitoring of serum concentrations of morphine, morphine-3-glucuronide and morphine-6-glucuronide do not predict clinical observations in cancer patients, *Palliative Med.*, 17 (2003) 679

[186] C.C. Faura, A. Moore, J.F. Horga, C.W. Hand, H.J. McQuay, Morphine and morphine-6-glucuronide plasma concentrations and effect in cancer pain, *J. Pain Symptom Manag.*, 11 (1996) 95

[187] C. Quigley, S. Joel, N. Patel, A. NBaksh, M. Slevin, Plasma concentrations of morphine, morphine-6-glucuronide and morphine-3-glucuronide and their relationship with analgesia and side effects in patients with caner-related pain, *Palliative Med.*, 17 (2003) 185

[188] D. Bigler, C.B. Christensen, J. Eriksen, N.-H. Jensen, Morphine, morphine-6-glucuronide and morphine-3-glucuronide concentrations in plasma and cerebrospinal fluid during long-term high-dose intrathecal morphine administration, *Pain*, 41 (1990) 15

[189] A.A. Somogyi, R.L. Nation, C. Olweny, P. Tsirgiotis, J. van Crugten, R.W. Milne, J.F. Cleary, C. Danz, F. Bochner, Plasma concentrations and renal clearance of morphine, morphine-3-glucuronide and morphine-6-glucuronide in cancer patients receiving morphine, *Clin. Pharmacokinet.*, 24 (1993) 413

[190] H. Samuelsson, T. Hedner, R. Venn, A. Michalkiewicz, CSF and plasma concentrations of morphine and morphine glucuronides in cancer patients receiving epidural morphine, *Pain*, 52 (1993) 179

[191] O.H. Drummer, Methods for the measurement of benzodiazepines in biological samples [review], *J. Chromatogr. B*, 713 (1998) 201

[192] S.C. Bishop, M. Lerch, B.R. McCord, Micellar electrokinetic chromatographic screening method for common sexual assault drugs administered in beverages, *Forensic Sci. Int.*, 141 (2004) 7

[193] M.J. Barker, M. Jackson, K.M. Greenwood, S.F. Crowe, Cognitive effects of benzodiazepine use: a review, *Aust. Psychol.*, 38 (2003) 202

[194] L.P. Longo, B. Johnson, Addiction: Part 1. Benzodiazepines - side effects, abuse risk and alternatives, *American Family Physician*, 61 (2000) 2121

[195] M.J. Barker, K.M. Greenwood, M. Jackson, S.F. Crowe, Cognitive effects of long-term benzodiazepine use - a meta-analysis, *CNS Drugs*, 18 (2004) 37

[196] C. McGrath, G.D. Burrows, T.R. Norman, The benzodiazepines: a brief review of pharmacology and therapeutics, *Anxiolytics*, (2000) 1

[197] T.A.D. Smith, Type A γ-aminobutyric acid ($GABA_A$) receptor subunits and benzodiazepine binding: significance to clinical syndromes and their treatment, *British Journal of Biomedical Science*, 58 (2001) 111

[198] D.J. Greenblatt, R.I. Shader, M. Divoll, J.S. Harmatz, Benzodiazepines: a summary of pharmacokinetic properties, *Br. J. Clin. Pharmac.*, 11 (1981) 11S

[199] D. Berezhnoy, Y. Nyfeler, A. Gonthier, H. Schwob, M. Goeldner, E. Sigel, On the benzodiazepine binding pocket in $GABA_A$ receptors, *J. Biol. Chem.*, 279 (2004) 3160

[200] P.D. Garzone, P.D. Kroboth, Pharmacokinetics of the newer benzodiazepines, *Clinical Pharmacokinetics*, 16 (1989) 337

[201] C.C. Crone, G.M. Gabriel, Treatment of anxiety and depression in transplant patients - pharmacokinetic considerations, *Clin. Pharmacokinet.*, 43 (2004) 361

[202] D.J. Greenblatt, M. Divoll, D.R. Abernethy, H.R. Ochs, R.I. Shader, in Burrows/Norman/Davies (Editor), Antianxiety agents, 1984.

[203] R. Yuan, D.A. Flockhart, J.D. Balian, Pharmacokinetic and pharmacodynamic consequences of metabolism-based drug interactions with alprazolam, midazolam, and triazolam, *J. Clin. Pharmacol.*, 39 (1999) 1109

[204] J.-P. Cano, P. Coassolo, Notion de demi-vie et metabolisme des benzodiazepines, *L'Encephale*, 9 (1983) 75B

[205] H. Schutz, Analytical Toxicology for Clinical, Forensic and Pharmaceutical Chemists, 1997, p. 369.

[206] L. Jin, C.E. Lau, Determination of alprazolam and its major metabolites in serum microsamples by high-performance liquid chromatography and its application to pharmacokinetics in rats, *J. Chromatogr. B*, 654 (1994) 77

[207] M. Yoshida, A. Akane, Subzero-temperature liquid-liquid extraction of benzodiazepines for high-performance liquid chromatography, *Anal. Chem.*, 71 (1999) 1918

[208] I.M. McIntyre, M.L. Syrjanen, K. Crump, S. Horomidis, A.W. Peace, O.H. Drummer, Simultaneous HPLC gradient analysis of 15 benzodiazepines and selected metabolites in postmortem blood, *J. Anal. Toxicol.*, 17 (1993) 202

[209] H.A. Adams, B. Weber, B. Bachmann-M, M. Guerin, G. Hempelmann, Die simultane Bestimmung von Ketamin und Midazolam mittels Hochdruck-Flussigkeits-chromatographie und UV-Detektion (HPLC/UV), *Anaesthesist*, 41 (1992) 619

[210] H. Le Solleu, F. Demotes-Mainard, G. Vincon, B. Bannwarth, The determination of bromazepam in plasma by reversed-phase high-performance liquid chromatography, *J. Pharm. Biomed. Anal.*, 11 (1993) 771

[211] F. Benhamou-Batut, F. Demotes-Mainard, L. Labat, G. Vincon, B. Bannwarth, Determination of flunitrazepam in plasma by liquid chromatography, *J. Pharm. Biomed. Anal.*, 12 (1994) 931

[212] B. Lehmann, R. Boulieu, Determination of midazolam and it unconjugated 1-hydroxy metabolite in human plasma by high-performance liquid chromatography, *J. Chromatogr. B*, 674 (1995) 138

[213] P. Bourget, V. Bouton, A. Lesne-Hulin, P. Amstutz, M. Benayed, D. Benhamou, P.L. Dufieux, G. Goursot, S. Grosbuis, J.P. Haberer, F. Jardin, P. Kirstetter, J. Marty, A. Mercatello, B. Page, J.L. Pourriat, T. Vassal, Comparison of high-performance liquid chromatography and polyclonal fluorescence polarization immunoassay for the monitoring of midazolam in the plasma of intensive care unit patients, *Ther. Drug Monit.*, 18 (1996) 610

[214] H.M. Rivera, G.S. Walker, D.N. Sims, P.C. Stockham, Application of liquid chromatography-tandem mass spectrometry to the analysis of benzodiazepines in blood, *Eur. J. Mass Spectrom.*, 9 (2003) 599

[215] A. El Mahjoub, C. Staub, Stability of benzodiazepines in whole blood samples stored at varying temperatures, *J. Pharm. Biomed. Anal.*, 23 (2000) 1057

[216] A. El Mahjoub, C. Staub, Simultaneous determination of benzodiazepines in whole blood or serum by HPLC/DAD with a semi-micro column, *J. Pharm. Biomed. Anal.*, 23 (2000) 447

[217] M.D. Robertson, O.H. Drummer, High-performance liquid chromatographic procedure for the measurement of nitrobenzodiazepines and their 7-amino metabolites in blood, *J. Chromatogr. B*, 667 (1995) 179

[218] O.H. Drummer, S. Horomidis, S. Kourtis, M.L. Syrjanen, P. Tippett, Capillary gas chromatographic drug screen for use in forensic toxicology, *J. Anal. Toxicol.*, 18 (1994) 134

[219] L. Zedkova, G.A. Rauw, G.B. Baker, N.J. Coupland, A rapid high-pressure liquid chromatographic procedure for determination of flumazenil in plasma, *Journal of Pharmacological and Toxicological Methods*, 46 (2001) 57

[220] P.K. Kunicki, Simple and sensitive high-performance liquid chromatographic method for the determination of 1,5-benzodiazepine clobazam and its active metabolite *N*-desmethylclobazam in human serum and urine with application to 1,4-benzodiazepine analysis, *J. Chromatogr. B*, 750 (2001) 41

[221] K. Chiba, H. Horii, T. Chiba, Y. Kato, T. Hirano, T. Ishizaki, Development and preliminary application of high-performance liquid chromatographic assay of urinary metabolites of diazepam in humans, *J. Chromatogr. B*, 668 (1995) 77

[222] A.D. Fraser, W. Bryan, A.F. Isner, Urinary screening for α-OH triazolam by FPIA and EIA with confirmation by GC/MS, *J. Anal. Toxicol.*, 16 (1992) 347

[223] J. Zweigenbaum, K. Heinig, S. Steinborner, T. Wachs, J. Henion, High-throughput bioanalytical LC/MS/MS determination of benzodiazepines in human urine: 1000 samples per 12 hours, *Anal. Chem.*, 71 (1999) 2294

[224] M. Wilhelm, H.-J. Battista, D. Obendorf, HPLC with simultaneous UV and reductive electrochemical detection at the hanging mercury drop electrode: a

highly sensitive and selective tool for the determination of benzodiazepines in forensic samples, *J. Anal. Toxicol.*, 25 (2001) 250

[225] H. Gjerde, E. Dahlin, A.S. Christophersen, Simultaneous determination of common benzodiazepines in blood using capillary gas chromatography, *J. Pharm. Biomed. Anal.*, 10 (1992) 317

[226] K. Wolff, D. Garretty, A.W.H. Hay, Micro-extraction of commonly abused benzodiazepines for urinary screening by liquid chromatography, *Ann. Clin. Biochem.*, 34 (1997) 61

[227] B. Aebi, R. Sturny-Jungo, W. Bernhard, R. Blanke, R. Hirsch, Quantitation using GC-TOF-MS: example of bromazepam, *Forensic Sci. Int.*, 128 (2002) 84

[228] R. Nishioka, Determination of triazolam in serum by deactivated metal capillary gas chromatography with electron-capture detection, *J. Chromatogr. B*, 681 (1996) 401

[229] D.F. LeGatt, D.P. McIntosh, Clobazam and norclobazam quantitation in serum by capillary gas chromatography with electron-capture detection, *Clin. Biochem.*, 26 (1993) 159

[230] W.A. Joern, Confirmation of low concentrations of urinary benzodiazepines, including alprazolam and triazolam, by GC/MS: An extractive alkylation procedure, *J. Anal. Toxicol.*, 16 (1992) 363

[231] M. Tomita, T. Okuyama, Application of capillary electrophoresis to the simultaneous screening and quantitation of benzodiazepines, *J. Chromatogr. B*, 678 (1996) 331

[232] P.R. Puopolo, M.E. Pothier, S.A. Volpicelli, J.A. Flood, Single procedure for detection, confirmation, and quantification of benzodiazepines in serum by liquid chromatography with photodiode-array detection, *Clin. Chem.*, 37 (1991) 701

[233] K. Heinig, J. Henion, Fast liquid-chromatographic-mass spectrometric determination of pharmaceutical compounds, *J. Chromatogr. B*, 732 (1999) 445

[234] T.-L. Chang, K.-W. Chen, Y.-D. Lee, K. Fan, Determination of benzodiazepines in clinical serum samples: comparative evaluation of REMEDi system, aca Analyzer, and conventional HPLC performance, *J. Clin. Lab. Anal.*, 13 (1999) 106

[235] K. Chan, R.D.M. Jones, Simultaneous determination of flumazenil, midazolam and metabolites in human biological fluids by liquid chromatography, *J. Chromatogr.*, 619 (1993) 154

[236] F. Berthault, P. Kintz, P. Mangin, Simultaneous high-performance liquid chromatographic analysis of flunitrazepam and four metabolites in serum, *J. Chromatogr. B*, 685 (1996) 383

[237] T. Toyo'oka, Y. Kumaki, M. Kanbori, M. Kato, Y. Nakahara, Determination of hypnotic benzodiazepines (alprazolam, estazolam, and midazolam) and their metabolites in rat hair and plasma by reversed-phase liquid-chromatography with electrospray ionization mass spectrometry, *J. Pharm. Biomed. Anal.*, 30 (2003) 1773

[238] T. Edeki, D.W. Robin, C. Prakash, I.A. Blair, A.J.J. Wood, Sensitive assay for triazolam in plasma following low oral doses, *J. Chromatogr.*, 577 (1992) 190

[239] H.H. Maurer, T. Kraemer, C. Kratzsch, F.T. Peters, A.A. Weber, Negative ion chemical ionization gas chromatography-mass spectrometry and atmospheric pressure chemical ionization liquid chromatography-mass spectrometry of low-dosed and/or polar drugs in plasma, *Ther. Drug Monit.*, 24 (2002) 117

[240] M.A. Zilli, G. Nisi, Simple and sensitive method for the determination of clobazam, clonazepam and nitrazepam in human serum by high-performance liquid chromatography, *J. Chromatogr.*, 378 (1986) 492

[241] D.M. Song, V. Khaykis, K. Kohlhof, Determination of flumazenil in plasma by gas chromatography-negative ion chemical ionization mass spectrometry, *J. Chromatogr. B*, 663 (1995) 263

[242] D.M. Song, S. Zhang, K. Kohlhof, Gas chromatographic-mass spectrometric method for the determination of flurazepam and its major metabolites in mouse and rat plasma, *J. Chromatogr. B*, 658 (1994) 142

[243] D.M. Song, S. Zhang, K. Kohlhof, Determination of chlordiazepoxide in mouse plasma by gas chromatography-negative-ion chemical ionization mass spectrometry, *J. Chromatogr. B*, 660 (1994) 95

[244] D.M. Song, S. Zhang, K. Kohlhof, Quantitative determination of clonazepam in plasma by gas chromatography-negative ion chemical ionization mass spectrometry, *J. Chromatogr. B*, 686 (1996) 199

[245] B.-I. Podkowik, S. Masur, Gas chromatographic determination of midazolam in low-volume plasma samples, *J. Chromatogr. B*, 681 (1996) 405

[246] J.F. Kelly, D.J. Greenblatt, Rapid and sensitive gas chromatographic determination of estazolam, *J. Chromatogr.*, 621 (1993) 102

[247] L.E. Fisher, S. Perch, M.F. Bonfiglio, S.M. Geers, Simultaneous determination of midazolam and flumazenil concentrations in human plasma by gas chromatography, *J. Chromatogr. B*, 665 (1995) 217

[248] T.V. Beischlag, T. Inaba, Determination of nonderivatized para-hydroxylated metabolites of diazepam in biological fluids with a GC megabore column system, *J. Anal. Toxicol.*, 16 (1992) 236

[249] P.H. Dickson, W. Markus, J. McKernan, H.C. Nipper, Urinalysis of α-hydroxyalprazolam, α-hydroxytriazolam, and other benzodiazepine compounds by GC/EIMS, *J. Anal. Toxicol.*, 16 (1992) 67

[250] R. Meatherall, GC-MS confirmation of urinary benzodiazepine metabolites, *J. Anal. Toxicol.*, 18 (1994) 369

[251] R.L. Fitzgerald, D.A. Rexin, D.A. Herold, Benzodiazepine analysis by negative chemical ionization gas chromatography/mass spectrometry, *J. Anal. Toxicol.*, 17 (1993) 342

[252] K.E. Brooks, N.B. Smith, Efficient extraction of basic, neutral, and weakly acidic drugs from plasma for analysis by gas chromatography-mass spectrometry, *Clin. Chem.*, 37 (1991) 1975

[253] M. Imazawa, Y. Hatanaka, Micellar electrokinetic capillary chromatography of benzodiazepine antiepileptics and their desmethyl metabolites in blood, *J. Pharm. Biomed. Anal.*, 15 (1997) 1503

[254] A. El Mahjoub, C. Staub, Semiautomated high-performance liquid chromatographic method for the determination of benzodiazepines in whole blood, *J. Anal. Toxicol.*, 25 (2001) 209

[255] A. El Mahjoub, C. Staub, High-performance liquid chromatographic method for the determination of benzodiazepines in plasma or serum using the column-switching technique, *J. Chromatogr. B*, 742 (2000) 381

[256] W.M. Mullett, J. Pawliszyn, Direct LC analysis of five benzodiazepines in human urine and plasma using an ADS restricted access extraction column, *J. Pharm. Biomed. Anal.*, 26 (2001) 899

[257] W. Rieck, D. Platt, High-performance liquid chromatographic method for the determination of alprazolam in plasma using the column-switching technique, *J. Chromatogr. B*, 578 (1992) 259

[258] C. Lacroix, F. Wojciechowski, P. Danger, Monitoring of benzodiazepines (clobazam, diazepam and their main active metabolites) in human plasma by column-switching high-performance liquid chromatography, *J. Chromatogr.*, 617 (1993) 285

[259] H. Iwase, K. Gondo, T. Koike, I. Ono, Novel precolumn deproteinization method using a hydroxyapatite cartridge for the determination of theophylline and diazepam in human plasma by high-performance liquid chromatography with ultraviolet detection, *J. Chromatogr. B*, 655 (1994) 73

[260] R. Lauber, M. Mosimann, M. Buhrer, A.M. Zbinden, Automated determination of midazolam in human plasma by high-performance liquid chromatography using column switching, *J. Chromatogr. B*, 654 (1994) 69

[261] A. Miki, M. Tatsuno, M. Katagi, M. Nishikawa, H. Tsuchihashi, Simultaneous determination of eleven benzodiazepine hypnotics and eleven relevant metabolites in urine by column-switching liquid chromatography-mass spectrometry, *J. Anal. Toxicol.*, 26 (2002) 87

[262] M. Krogh, H. Grefslie, K.E. Rasmussen, Solvent-modified solid-phase microextraction for the determination of diazepam in human plasma samples by capillary gas chromatography, *J. Chromatogr. B*, 689 (1997) 357

[263] H. Yuan, Z. Mester, H. Lord, J. Pawliszyn, Automated in-tube solid-phase microextraction coupled with liquid chromatography for the determination of selected benzodiazepines, *J. Anal. Toxicol.*, 24 (2000) 718

[264] K. Jinno, Y. Han, Drug analysis by capillary electrophoretic methods, *Biomed. Chromatogr.*, 12 (1998) 126

[265] W.M. Mullett, K. Levsen, D. Lubda, J. Pawliszyn, Bio-compatible in-tube solid-phase microextraction capillary for the direct extraction and high-performance liquid chromatographic determination of drugs in human serum, *J. Chromatogr. A*, 963 (2002) 325

[266] G. Frison, L. Tedeschi, S. Maietti, S.D. Ferrara, Determination of midazolam in human plasma by solid-phase microextraction and gas chromatography/mass spectrometry, *Rapid Commun. Mass Spectrom.*, 15 (2001) 2497

[267] A. Aresta, L. Monaci, C.G. Zambonin, Determination of delorazepam in urine by solid-phase microextraction coupled to high performance liquid chromatography, *J. Pharm. Biomed. Anal.*, 28 (2002) 965

[268] F. Guan, H. Seno, A. Ishii, K. Watanabe, T. Kumazawa, H. Hattori, O. Suzuki, Solid-phase microextraction and GC-ECD of benzophenones for detection of benzodiazepines in urine, *J. Anal. Toxicol.*, 23 (1999) 54

[269] K.J. Reubsaet, H.R. Norli, P. Hemmersbach, K.E. Rasmussen, Determination of benzodiazepines in human urine and plasma with solvent modified solid phase micro extraction and gas chromatography; rationalisation of method development using experimental design strategies, *J. Pharm. Biomed. Anal.*, 18 (1998) 667

[270] K.S. Scott, J.S. Oliver, The use of vitreous humor as an alternative to whole blood for the analysis of benzodiazepines, *J. Forensic Sci.*, 46 (2001) 694

[271] H.G. Ugland, M. Krogh, K.E. Rasmussen, Liquid-phase microextraction as a sample preparation technique prior to capillary gas chromatographic-

determination of benzodiazepines in biological matrices, *J. Chromatogr. B*, 749 (2000) 85

[272] S. McClean, E. O'Kane, J. Hillis, W.F. Smyth, Determination of 1,4-benzodiazepines and their metabolites by capillary electrophoresis and high-performance liquid chromatography using ultraviolet and electrospray ionisation mass spectrometry, *J. Chromatogr. A*, 838 (1999) 273

[273] R. Herraez-Hernandez, A.J.H. Louter, N.C. Van der Merbel, Automated on-line dialysis for sample preparation for gas chromatography: determination of benzodiazepines in human plasma, *J. Pharm. Biomed. Anal.*, 14 (1996) 1077

[274] N.C. Van de Merbel, J.M. Teule, H. Lingemann, U.A.T. Brinkman, Dialysis as an on-line sample-pretreatment technique for column liquid chromatography: influence of experimental variables upon the determination of benzodiazepines in human plasma, *J. Pharm. Biomed. Anal.*, 10 (1992) 225

[275] X.-P. Lee, T. Kumazawa, J. Sato, Y. Shoji, C. Hasegawa, C. Karibe, T. Arinobu, H. Seno, K. Sato, Simple method for the determination of benzodiazepines in human body fluids by high-performance liquid chromatography-mass spectrometry, *Anal. Chim. Acta*, 492 (2003) 223

[276] M.-E. Capella-Peiro, D. Bose, A. Martinavarro-Dominguez, M. Gil-Agusti, J. Esteve-Romero, Direct injection micellar liquid chromatographic determination of benzodiazepines in serum, *J. Chromatogr. B*, 780 (2002) 241

[277] J.J.B. Nevado, G.C. Penalvo, M.J.P. Calderon, Determination of lormetazepam and its main metabolite in serum using micellar electrokinetic capillary chromatography with direct injection and ultraviolet absorbance detection, *J. Chromatogr. B*, 773 (2002) 151

[278] C. Pistos, J.T. Stewart, Direct injection HPLC method for the determination of selected benzodiazepines in plasma using a Hisep column, *J. Pharm. Biomed. Anal.*, 33 (2003) 1135

[279] V. Mastey, A.-C. Panneton, F. Donati, F. Varin, Determination of midazolam and two of its metabolites in human plasma by high-performance liquid chromatography, *J. Chromatogr. B*, 655 (1994) 305

[280] L.A. Berrueta, B. Gallo, F. Vicente, Analysis of oxazepam in urine using solid-phase extraction and high-performance liquid chromatography with fluorescence detection by post-column derivitization, *J. Chromatogr.*, 616 (1993) 344

[281] T. Kondo, D.C. Buss, P.A. Routledge, A method for rapid determination of lorazepam by high-performance liquid chromatography, *Ther. Drug Monit.*, 15 (1993) 35

[282] Z. Lin, O. Beck, Procedure for verification of flunitrazepam and nitrazepam intake by gas chromatographic-mass spectrometric analysis of urine, *J. Pharm. Biomed. Anal.*, 13 (1995) 719

[283] M. Casas, L.A. Berrueta, B. Gallo, F. Vicente, Solid-phase extraction of 1,4-benzodiazepines from biological fluids, *J. Pharm. Biomed. Anal.*, 11 (1993) 277

[284] T. Taguchi, K. Doge, K. Tsunoda, N. Furuta, S. Watanabe, K. Sato, Capillary high-performance liquid chromatography for nine benzodiazepines in human serum, *Jpn. J. Forensic Toxicol.*, 18 (2000) 221

[285] J. Chopineau, F. Rivault, V. Sautou, M.F. Sommier, Determination of temazepam and its active metabolite, oxazepam in plasma, urine and dialysate using solid-phase extraction followed by high performance liquid chromatography, *J. Liq. Chromatogr.*, 17 (1994) 373

[286] H.-L. Tai, R.J. Osiewicz, D.P. Bofinger, A salt-free isocratic high-pressure liquid chromatographic method for the quantitation of benzodiazepines in serum, *Clin. Biochem.*, 26 (1993) 165

[287] M. Kleinschnitz, M. Herderich, P. Schreier, Determination of 1,4-benzodiazepines by high-performance liquid chromatography-electrospray tandem mass spectrometry, *J. Chromatogr. B*, 676 (1996) 61

[288] F. Kamali, Determination of plasma diazepam and desmethyldiazepam by solid-phase extraction and reversed-phase high-performance liquid chromatography, *J. Pharm. Biomed. Anal.*, 11 (1993) 625

[289] K.K. Akerman, Analysis of clobazam and its active metabolite norclobazam in plasma and serum using HPLC/DAD, *Scand. J. Clin. Lab. Invest*, 56 (1996) 609

[290] K.K. Akerman, J. Jolkkonen, M. Parviainen, I. Penttila, Analysis of low-dose benzodiazepines by HPLC with automated solid-phase extraction, *Clin. Chem.*, 42 (1996) 1412

[291] F. Musshoff, T. Daldrup, A rapid solid-phase extraction and HPLC/DAD procedure for the simultaneous determination and quantification of different benzodiazepines in serum, blood and post-mortem blood, *Int. J. Legal Med.*, 105 (1992) 105

[292] Z. Liu, J. Short, A. Rose, S. Ren, N. Contel, S. Grossman, S. Unger, The simultaneous determination of diazepam and its three metabolites in dog plasma by high-performance liquid chromatography with mass spectrometry, *J. Pharm. Biomed. Anal.*, 26 (2001) 321

[293] B.C. Sallustio, C. Kassapidis, R.G. Morris, High-performance liquid chromatography determination of clonazepam in plasma using solid-phase extraction, *Ther. Drug Monit.*, 16 (1994) 174

[294] M. Casas, L.A. Berrueta, B. Gallo, F. Vicente, Solid phase extraction conditions for the selective isolation of drugs from biological fluids predicted using liquid chromatography, *Chromatographia*, 34 (1992) 79

[295] M. Tomita, T. Okuyama, Simultaneous determination of nitrazepam and its metabolites in urine by micellar electrokinetic capillary chromatography, *J. Chromatogr.*, 621 (1993) 249

[296] M.A. Evenson, J.E. Wiktorowicz, Automated capillary electrophoresis applied to therapeutic drug monitoring, *Clin. Chem.*, 38 (1992) 1847

[297] G. Vanhoenacker, F. de l'Escaille, D. De Keukeleire, P. Sandra, Analysis of benzodiazepines in dynamically coated capillaries by CE-DAD, CE-MS and CE-MS^2, *J. Pharm. Biomed. Anal.*, 34 (2004) 595

[298] A.J.H. Louter, E. Bosma, J.C.A. Schipperen, J.J. Vreuls, U.A.T. Brinkman, Automated on-line solid-phase extraction-gas chromatography with nitrogen-phosphorus detection: determination of benzodiazepines in human plasma, *J. Chromatogr. B*, 689 (1997) 35

[299] A.M.A. Verweij, M.L. Hordijk, P.J.L. Lipman, Liquid chromatographic-thermospray tandem mass spectrometric quantitative analysis of some drugs with hypnotic, sedative and tranquillising properties in whole blood, *J. Chromatogr. B*, 686 (1996) 27

[300] B. Borggaard, I. Joergensen, Urinary screening for benzodiazepines with radioreceptor assay: comparison with EMIT d.a.u. benzodiazepine assay and high-performance liquid chromatography, *J. Anal. Toxicol.*, 18 (1994) 243

[301] R. Kronstrand, I. Nystrom, M. Josefsson, S. Hodgins, Segmental ion spray LC-MS-MS analysis of benzodiazepines in hair of psychiatric patients, *J. Anal. Toxicol.*, 26 (2002) 479

[302] P. Wernly, W. Thormann, Analysis of illicit drugs in human urine by micellar electrokinetic capillary chromatography with on-column fast scanning polychrome absorption detection, *Anal. Chem.*, 63 (1991) 2878

[303] P. Wernly, W. Thormann, Drug of abuse confirmation in human urine using stepwise solid-phase extraction and micellar electrokinetic capillary chromatography, *Anal. Chem.*, 64 (1992) 2155

[304] M. Schafroth, W. Thormann, D. Allemann, Micellar electrokinetic capillary chromatography of benzodiazepines in human urine, *Electrophoresis*, 15 (1994) 72

[305] L. Steinmann, W. Thormann, Toxicological drug screening and confirmation by electrokinetic capillary techniques: concept of an automated system, *J. Capillary Electrop.*, 2 (1995) 81

[306] T.R. Koch, R.L. Raglin, S. Kirk, J.F. Bruni, Improved screening for benzodiazepine metabolites in urine using the Triage™ panel for drugs of abuse, *J. Anal. Toxicol.*, 18 (1994) 168

[307] D.A. Black, G.D. Clark, V.M. Haver, J.A. Garbin, A.J. Saxon, Analysis of urinary benzodiazepines using solid-phase extraction and gas chromatography-mass spectrometry, *J. Anal. Toxicol.*, 18 (1994) 185

[308] Z.-P. Huang, X.-H. Chen, J. Wijsbeek, J.-P. Franke, R.A. de Zeeuw, An enzymic digestion and solid-phase extraction procedure for the screening for acidic, neutral, and basic drugs in liver using gas chromatography for analysis, *J. Anal. Toxicol.*, 20 (1996) 248

[309] R.E. West, D.P. Ritz, GC/MS analysis of five common benzodiazepine metabolites in urine as tert-butyl-dimethylsilyl derivatives, *J. Anal. Toxicol.*, 17 (1993) 114

[310] N. Jourdil, J. Bessard, F. Vincent, H. Eysseric, G. Bessard, Automated solid-phase extraction and liquid chromatography-electrospray ionization-mass spectrometry for the determination of flunitrazepam and its metabolites in human urine and plasma samples, *J. Chromatogr. B*, 788 (2003) 207

[311] A. Negrusz, C.M. Moore, T.L. Stockham, K.R. Poiser, J.L. Kern, R. Palaparthy, N.L.T. Le, P.G. Janicak, N.A. Levy, Elimination of 7-aminoflunitrazepam and flunitrazepam in urine after a single dose of Rohypnol®, *J. Forensic Sci.*, 45 (2000) 1031

[312] M.A. LeBeau, M.A. Montgomery, J.R. Wagner, M.L. Miller, Analysis of biofluids for flunitrazepam and metabolites by electrospray liquid chromatography/mass spectrometry, *J. Forensic Sci.*, 45 (2000) 1133

[313] C. Moore, G. Long, M. Marr, Confirmation of benzodiazepines in urine as trimethylsilyl derivatives using gas chromatography-mass spectrometry, *J. Chromatogr. B*, 655 (1994) 132

[314] A. Allqvist, A. Wennerholm, J.-O. Svensson, R.A. Mirghani, Simultaneous quantification of alprazolam, 4- and α-hydroxyalprazolam in plasma samples using liquid chromatography mass spectrometry, *J. Chromatogr. B*, 814 (2005) 127

[315] R.L. Fitzgerald, D.A. Herold, Improved CEDIA benzodiazepine assay eliminates sertraline crossreactivity, *J. Anal. Toxicol.*, 21 (1997) 32

[316] A.D. Fraser, R. Meatherall, Comparative evaluation of five immunoassays for the analysis of alprazolam and triazolam metabolites in urine: effect of lowering the screening and GC-MS cut-off values, *J. Anal. Toxicol.*, 20 (1996) 217

[317] W. Huang, D.E. Moody, Immunoassay detection of benzodiazepines and benzodiazepine metabolites in blood, *J. Anal. Toxicol.*, 19 (1995) 333

[318] R. Meatherall, Benzodiazepine screening using EMIT II and TDx: urine hydrolysis pretreatment required, *J. Anal. Toxicol.*, 18 (1994) 385

[319] R.L. Fitzgerald, D.A. Rexin, D.A. Herold, Detecting benzodiazepines: immunoassays compared with negative chemical ionization gas chromatography/mass spectrometry, *Clin. Chem.*, 40 (1994) 373

[320] A.D. Fraser, A.F. Isner, W. Bryan, Urinary screening for adinazolam and its major metabolites by the Emit d.a.u. and FPIA benzodiazepine assays with confirmation by HPLC, *J. Anal. Toxicol.*, 17 (1993) 427

[321] W. Huang, D.E. Moody, D.M. Andrenyak, D.E. Rollins, Immunoassay detection of nordiazepam, triazolam, lorazepam, and alprazolam in blood, *J. Anal. Toxicol.*, 17 (1993) 365

[322] O. Beck, P. Lafolie, P. Hjemdahl, S. Borg, G. Odelius, P. Wirbing, Detection of benzodiazepine intake in therapeutic doses by immunoanalysis of urine: two techniques evaluated and modified for improved performance, *Clin. Chem.*, 38 (1992) 271

[323] A. West, H. Kohler-Schmidt, S. Baudner, G. Blaschke, A specific immunoassay for the detection of flunitrazepam, *Int. J. Legal Med.*, 108 (1995) 105

[324] K. Walshe, A.M. Barrett, P.V. Kavanagh, S.M. McNamara, C. Moran, A.G. Shattock, A sensitive immunoassay for flunitrazepam and metabolites, *J. Anal. Toxicol.*, 24 (2000) 296

[325] L.Y. Gorczynski, F.J. Melbye, Detection of benzodiazepines in different tissues, including bone, using a quantitative ELISA assay, *J. Forensic Sci.*, 46 (2001) 916

[326] D. Laurie, A.J. Mason, N.H. Piggot, F.J. Rowell, J. Saviour, D. Strachan, J.D. Tyson, *Analyst*, 121 (1996) 951

[327] A.A. Elian, ELISA detection of clonazepam and 7-aminoclonazepam in whole blood and urine, *Forensic Sci. Int.*, 134 (2003) 54

[328] M.J. Janssen, K. Ensing, R.A. de Zeeuw, A fluorescent receptor assay for benzodiazepines using coumarin-labeled desethylflumazenil as ligand, *Anal. Chem.*, 73 (2001) 3168

[329] J. Becker, A. Correll, W. Koepf, C. Rittner, Comparative studies on the detection of benzodiazepines in serum by means of immunoassays (FPIA), *J. Anal. Toxicol.*, 17 (1993) 103

[330] P. Simonsson, A. Liden, S. Lindberg, Effect of β-glucuronidase on urinary benzodiazepine concentrations determined by fluorescence polarization immunoassay, *Clin. Chem.*, 41 (1995) 920

[331] A.D. Fraser, W. Bryan, Evaluation of the Abbott TDx serum benzodiazepine immunoassay for the analysis of lorazepam, adinazolam, and *N*-desmethyladinazolam, *J. Anal. Toxicol.*, 19 (1995) 281

[332] P.G. Agbuya, L. Li, M.V. Miles, A.L. Zaritsky, A.D. Morris, Development of a fluorescence polarization immunoassay for lorazepam quantification, *Ther. Drug Monit.*, 18 (1996) 194

[333] T. Nishikawa, H. Ohtani, D.A. Herold, R.L. Fitzgerald, *American Journal of Clinical Pathology*, 102 (1997) 605

[334] J. Bruhwyler, A. Hassoun, Potential use of a radioreceptor assay for the determination of benzodiazepine compounds in serum, *J. Anal. Toxicol.*, 17 (1993) 403

[335] T. Nishikawa, S. Suzuki, H. Ohtani, N.W. Eizawa, T. Sugiyama, T. Kawaguchi, S. Miura, Benzodiazepine concentrations in sera determined by radioreceptor assay for therapeutic-dose recipients, *American Journal of Clinical Pathology*, 102 (1994) 605

[336] K. Ensing, I.J. Bosman, A.C.G. Egberts, J.-P. Franke, R.A. de Zeeuw, *J. Pharm. Biomed. Anal.*, 12 (1994) 53

[337] K. Ensing, I.J. Bosman, A.C.G. Egberts, J.-P. Franke, R.A. de Zeeuw, *J. Pharm. Biomed. Anal.*, 12 (1994) 59

[338] D. Borrey, E. Meyer, W. Lambert, S. Van Calenbergh, C. Van Peteghem, A.P. De Leenheer, Sensitive gas chromatographic-mass spectrometric screening of acetylated benzodiazepines, *J. Chromatogr. A*, 910 (2001) 105

[339] S. Pirnay, I. Ricordel, D. Libong, S. Bouchonnet, Sensitive method for the detection of 22 benzodiazepines by gas chromatography-ion trap tandem mass spectrometry, *J. Chromatogr. A*, 954 (2002) 235

[340] L.E. Edinboro, A. Poklis, Detection of benzodiazepines and tribenzazolams by TRIAGE^{TM}: Confirmation by solid-phase extraction utilizing SPEC^{R}.3ML.MP3 microcolumns and GC-MS, *J. Anal. Toxicol.*, 18 (1994) 312

[341] J.L. Valentine, R. Middleton, C. Sparks, Identification of urinary benzodiazepines and their metabolites: comparison of automated HPLC and GC-MS after immunoassay screening of clinical specimens, *J. Anal. Toxicol.*, 20 (1996) 416

[342] S. Pirnay, S. Bouchonnet, F. Herve, D. Libong, N. Milan, P. d'Athis, F. Baud, I. Ricordel, Development and validation of a gas chromatography-mass spectrometry method for the simultaneous determination of buprenorphine, flunitrazepam and their metabolites in rat plasma: application to the pharmacokinetic study, *J. Chromatogr. B*, 807 (2004) 335

[343] H. Inoue, Y. Maeno, M. Iwasa, R. Matoba, M. Nagao, Screening and determination of benzodiazepines in whole blood using solid-phase extraction and gas chromatography/mass spectrometry, *Forensic Sci. Int.*, 113 (2000) 367

[344] J.-N. Audinot, M. Yegles, A. Labarthe, D. Ruch, R. Wennig, H.-N. Migeon, Detection and quantification of benzodiazepines in hair by ToF-SIMS: preliminary results, *Appl. Surf. Sci.*, 203-204 (2003) 718

[345] W.F. Smyth, S. McClean, A critical evaluation of the application of capillary electrophoresis to the detection and determination of 1,4-benzodiazepine

tranquilizers in formulations and body materials, *Electrophoresis*, 19 (1998) 2870

[346] F. Tagliaro, S. Turrina, P. Pisi, F.P. Smith, M. Marigo, Determination of illicit and/or abused drugs and compounds of forensic interest by capillary electrophoretic/electrokinetic methods, *J. Chromatogr. B*, 713 (1998) 27

[347] S.D. Ferrara, L. Tedeschi, G. Frison, F. Castagna, Solid-phase extraction and HPLC-UV confirmation of drugs of abuse in urine, *J. Anal. Toxicol.*, 16 (1992) 217

[348] A.M.A. Verweij, P.J.L. Lipman, P.G.M. Zweipfenning, Quantitative liquid chromatography, thermospray/tandem mass spectrometry (LC/TSP/MS/MS) analysis of some thermolabile benzodiazepines in whole blood, *Forensic Sci. Int.*, 54 (1992) 67

[349] M.A. Hall, C.A. Robinson, R.M. Brissie, High-performance liquid chromatography of alprazolam in postmortem blood using solid-phase extraction, *J. Anal. Toxicol.*, 19 (1995) 511

[350] K. Jinno, M. Taniguchi, M. Hayashida, Solid phase micro extraction coupled with semi-microcolumn high performance liquid chromatography for the analysis of benzodiazepines in human urine, *J. Pharm. Biomed. Anal.*, 17 (1998) 1081

[351] S.M. El-Gizawy, Simultaneous determination of diazepam, oxazepam and temazepam in spiked urine by HPLC, *Anal. Lett.*, 33 (2000) 629

[352] M. Rittner, F. Pragsst, W.-R. Bork, J. Neumann, Screening method for seventy psychoactive drugs or drug metabolites in serum based on high-performance liquid chromatography-electrospray ionization mass spectrometry, *J. Anal. Toxicol.*, 25 (2001) 115

[353] B.E. Smink, J.E. Brandsma, A. Dijkhuizen, K.J. Lusthof, J.J. de Gier, A.C.G. Egberts, D.R.A. Uges, Quantitative analysis of 33 benzodiazepines, metabolites and benzodiazepine-like substances in whole blood by liquid chromatography-(tandem) mass spectrometry, *J. Chromatogr. B*, 811 (2004) 13

[354] D.R.A. Uges, M. von Clarmann, M. Geldmacher-von Mallinckrodt, A.N.P. van Heijst, K. Ibe, M. Oellerich, H. Schutz, D. Stamm, F. Wunsch, Orientierende Angaben zu therapeutischen und toxischen Konzentrarionen von Arzneimittein und Giften in Blut, Serum oder Urin, Mitteilung XV der Senatskommission der Deutschen Forschungsgemeinschaft fur Klinisch-toxikologische Analytik, Weinheim, 1990.

[355] Sigma-Aldrich, Biochemicals & Reagents for Life Science Research,,, 2004-2005.

[356] H. Yoshimura, K. Oguri, H. Tsukamoto, The synthesis of codeine and morphine glucuronides, *Tetrahedron Lett.*, 4 (1968) 483

[357] B. Berrang, C.E. Twine, G.L. Hennessee, F.I. Carroll, Synthesis of morphine-3-glucuronide, *Synth. Commun.*, 5 (1975) 231

[358] I. Rukhman, A.L. Gutman, Synthesis of morphine 6-α-D-glucuronide, *Tetrahedron Lett.*, 41 (2000) 6889

[359] I. Rukhman, L. Yudovich, G. Nisnevich, A.L. Gutman, Selective synthesis of both isomers of morphine-6-β-glucuronide and their analogs, *Tetrahedron*, 57 (2001) 1083

[360] H. Yoshimura, K. Oguri, H. Tsukamoto, Metabolism of drugs. LX. The synthesis of codeine and morphine glucuronides, *Chem. Pharm. Bull.*, 16 (1968) 2114

[361] C. Lacy, M. Sainsbury, A synthesis of morphine-6-glucuronide, *Tetrahedron Lett.*, 36 (1995) 3949

[362] R.R. Schmidt, New methods for the synthesis of glycosides and oligosaccharides - are there alternatives to the Koenigs-Knorr method?, *Angewandte Chemie International Edition in English*, 25 (1986) 212

[363] R.R. Schmidt, *Angew. Chem., Int. Ed. Engl.*, 25 (1986) 212

[364] R.T. Brown, N.E. Carter, F. Scheinmann, N.J. Turner, Synthesis of morphine-6-glucuronide via a highly selective enzyme catalysed hydrolysis reaction, *Tetrahedron Lett.*, 36 (1995) 1117

[365] G.N. Jenkins, A.V. Stachulski, F. Schienmann, N.J. Turner, The enzymatic glucuronidation of 3-*O*-protected morphine - a new route to 7,8-dihydromorphine-6-glucuronide, *Tetrahedron: Asymmetry*, 11 (2000) 413

[366] L. Zecca, P. Ferrario, Synthesis and biodistribution of an ^{123}I labelled flunitrazepam derivative: a potential *in vivo* tracer for benzodiazepine receptors, *Appl. Radiat. Isot.*, 39 (1988) 353

[367] J. Feely, P.V. Kavanagh, S.M. McNamara, J.E. O'Brien, Simple preparation of the major urinary metabolites of flunitrazepam and nitrazepam, *Irish J. Med. Sci.*, 168 (1999) 8

[368] T. Kelly, *Separations of Morphine and Methadone Related Analytes in Human Hair by Liquid Chromatography-Mass Spectrometry and Capillary Electrophoresis*, PhD Thesis, University of Technology, Sydney, 2004.

[369] L.H. Welsh, O^3-Monoacetylmorphine, *J. Org. Chem.*, 19 (1954) 1409

[370] P. Zakaria, M. Macka, P.R. Haddad, Modelling, optimisation and control of selectivity in the separation of aromatic bases by electrokinetic chromatography using a natural cyclodextrin as a pseudostationary phase, *Electrophoresis*, 23 (2002) 1844

[371] P. Zakaria, M. Macka, P.R. Haddad, Separation of opiate alkaloids by electrokinetic chromatography with sulfated-cyclodextrin as a pseudo-stationary phase, *J. Chromatogr. A*, 985 (2003) 493

[372] K. Jinno, Y. Han, H. Sawada, Analysis of toxic drugs by capillary electrophoresis using polyacrylamide-coated columns, *Electrophoresis*, 18 (1997) 284

[373] K.D. Altria, Enhanced pharmaceutical analysis by CE using dynamic surface coating system, *J. Pharm. Biomed. Anal.*, 31 (2003) 447

[374] L. Bendahl, S.H. Hansen, B. Gammelgaard, Capillaries modified by noncovalent anionic polymer adsorption for capillary zone electrophoresis, micellar electrokinetic capillary chromatography and capillary electrophoresis mass spectrometry, *Electrophoresis*, 22 (2001) 2565

[375] R.S. Shah, Q. Wang, M.L. Lee, Cycloaliphatic epoxy resin coating for capillary electrophoresis, *J. Chromatogr. A*, 952 (2002) 267

[376] S. Hjertén, High-performance electrophoresis: elimination of electroendosmosis and solute adsorption, *J. Chromatogr.*, 347 (1985) 191

[377] H. Tian, L.C. Brody, D. Mao, J.P. Landers, Effective capillary electrophoresis-based heteroduplex analysis through optimization of surface coating and polymer networks, *Anal. Chem.*, 72 (2000) 5483

[378] D. Belder, K. Elke, H. Husmann, Influence of pH-value of methanolic electrolytes on electroosmotic flow in hydrophilic coated capillaries, *J. Chromatogr. A*, 868 (2000) 63

[379] J. Horvath, V. Dolník, Polymer wall coatings for capillary electrophoresis [review], *Electrophoresis*, 22 (2001) 644

[380] E. Córdova, J. Gao, G.M. Whitesides, Noncovalent polycationic coatings for capillaries in capillary electrophoresis of proteins, *Anal. Chem.*, 69 (1997) 1370

[381] F.B. Erim, A. Cifuentes, H. Poppe, J.C. Kraak, Performance of a physically adsorbed high-molecular-mass polyethyleneimine layer as coating for the separation of basic proteins and peptides by capillary electrophoresis, *J. Chromatogr. A*, 708 (1995) 356

[382] Y. Wang, P.L. Dubin, Capillary modification by noncovalent polycation adsorption: effects of polymer molecular weight and adsorption ionic strength, *Anal. Chem.*, 71 (1999) 3463

[383] G. Vanhoenacker, F. de l'Escaille, D. De Keukeleire, P. Sandra, Dynamic coating for fast and reproducible determination of basic drugs by capillary electrophoresis with diode-array detection and mass spectrometry, *J. Chromatogr. B*, 799 (2004) 323

[384] I.S. Lurie, P.A. Hays, K. Parker, Capillary electrophoresis analysis of a wide variety of seized drugs using the same capillary with dynamic coatings, *Electrophoresis*, 25 (2004) 1580

[385] I.S. Lurie, S. Panicker, P.A. Hays, A.D. Garcia, B.L. Geer, Use of dynamically coated capillaries with added cyclodextrins for the analysis of opium using capillary electrophoresis, *J. Chromatogr. A*, 984 (2003) 109

[386] I.S. Lurie, P.A. Hays, A.E. Garcia, S. Panicker, Use of dynamically coated capillaries for the determination of heroin, basic impurities and adulterants with capillary electrophoresis, *J. Chromatogr. A*, 1034 (2004) 227

[387] T. Kelly, P. Doble, M. Dawson, Chiral separation of methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP) by capillary electrophoresis using cyclodextrin derivatives, *Electrophoresis*, 24 (2003) 2106

[388] J.R. Catai, G.W. Somsen, G.J. de Jong, Efficient and reproducible analysis of peptides by capillary electrophoresis using noncovalently bilayer-coated capillaries, *Electrophoresis*, 25 (2004) 817

[389] T.W. Graul, J.B. Schlenoff, Capillaries modified by polyelectrolyte multilayers for electrophoretic separations, *Anal. Chem.*, 71 (1999) 4007

[390] E. Ameyibor, J.T. Stewart, Enantiomeric HPLC separation of selected chiral drugs using native and derivitized β-cyclodextrins as chiral mobile phase additives, *J. Liq. Chromatogr. and Rel. Technol.*, 20 (1997) 855

[391] A. Walhagen, L.-E. Edholm, Chiral separation on achiral stationary phases with different functionalities using β-cyclodextrin in the mobile phase and application to bioanalysis and coupled columns, *Chromatographia*, 32 (1991) 215

[392] H.H. Maurer, Liquid chromatography-mass spectrometry in forensic and clinical toxicology [review], *J. Chromatogr. B*, 713 (1998) 3

[393] Y. Suzuki, H. Arakawa, M. Maeda, The capillary electrophoresis separation of benzodiazepine drug using dextran sulfate and SDS as running buffer, *Biomed. Chromatogr.*, 18 (2004) 150

[394] R. Weinberger, I.S. Lurie, Micellar electrokinetic capillary chromatography of illicit drug substances, *Anal. Chem.*, 63 (1991) 823

[395] I. Bechet, M. Fillet, P. Hubert, J. Crommen, Determination of benzodiazepines by micellar electrokinetic chromatography, *Electrophoresis*, 15 (1994) 1316

[396] M.F. Renou-Gonnord, K. David, Optimized micellar electrokinetic chromatographic separation of benzodiazepines, *J. Chromatogr. A*, 735 (1996) 249

[397] F. Tagliaro, F.P. Smith, S. Turrina, V. Equisetto, M. Marigo, Complementary use of capillary zone electrophoresis and micellar electrokinetic capillary chromatography for mutual confirmation of results in forensic drug analysis, *J. Chromatogr. A*, 735 (1996) 227

[398] G. McGrath, S. McClean, E. O'Kane, W.F. Smyth, F. Tagliaro, Study of the capillary zone electrophoretic behavior of selected drugs, and its comparison with their analytical techniques for their formulation assay, *J. Chromatogr. A*, 735 (1996) 237

[399] S. Boonkerd, M.R. Detaevernier, J. Vindevogel, Y. Michotte, Migration behaviour of benzodiazepines in micellar electrokinetic chromatography, *J. Chromatogr. A*, 756 (1996) 279

[400] K. Jinno, Y. Han, M. Nakamura, Analysis of anxiolytic drugs by capillary electrophoresis with bare and coated capillaries, *J. Capillary Electrop.*, 3 (1996) 139

[401] S. McClean, E. O'Kane, W.F. Smyth, The identification and determination of selected 1,4-benzodiazepines by an optimised capillary electrophoresis-electrospray mass spectrometric method, *Electrophoresis*, 21 (2000) 1381

[402] E. Peyrin, Y.C. Guillaume, Chemometric approach to the treatment of benzodiazepine separation and peak broadening in capillary electrophoresis, *J. Chromatogr. A*, 849 (1999) 563

[403] X. Cahours, P. Morin, M. Dreux, Influence of ionic strength and organic modifier on performance in capillary electrochromatography on phenyl silica stationary phase, *J. Chromatogr. A*, 845 (1999) 203

[404] M.R. Taylor, p. Teale, Gradient capillary electrochromatography of drug mixtures with UV and electrospray ionisation mass spectrometric detection, *J. Chromatogr. A*, 768 (1997) 89

[405] X. Cahours, S. Cherkaoui, G. Rozing, J.-L. Veuthey, Microemulsion electrokinetic chromatography versus capillary electrochromatography-UV-

mass spectrometry for the analysis of flunitrazepam and its major metabolites, *Electrophoresis*, 23 (2002) 2320

[406] Agilent Technologies, Chiral analysis with the Agilent capillary electrophoresis system, *Agilent Technologies Application Note*, (2000).

[407] Drink spiking - for workers. Australian Drug Foundation: Drug Info Clearinghouse <http://druginfo.adf.org.au/article.asp?id=4911>: Australian Drug Foundation; 2002. Accessed November, 2004.

[408] A. Negrusz, R.E. Gaensslen, Analytical developments in toxicological investigation of drug-facilitated sexual assault, *Anal Bioannal Chem*, 376 (2003) 1192

[409] R.H. Schwartz, R. Milteer, M.A. LeBeau, Drug-facilitated sexual assault ('date rape'), *South .Med. J.*, 93 (2000) 558

[410] N. Taylor, J. Prichard, K. Charlton. National Project on Drink Spiking: Investigating the nature and extent of drink spiking in Australia: Australian Institute of Criminology; 2004 Nov.

[411] R.N. Rao, P. Parimala, S. Khalid, S.N. Alvi, Detection of the adulteration of traditional alcoholic beverages by the separation and determination of alprazolam, chloralhydrate and diazepam using reversed-phase high-performance liquid chromatography, *Anal. Sci.*, 20 (2004) 383

[412] S. Sentellas, J. Saurina, Chemometrics in capillary electrophoresis. Part A: methods for optimization, *J. Sep. Sci.*, 26 (2003) 875

[413] V. Dohnal, M. Farkova, J. Havel, Prediction of chiral separations using a combination of experimental designs and artificial neural networks, *Chirality*, 11 (1999) 616

[414] Q. Li, Y. Zhou, H. Wang, H. Zhang, S. Liu, X. Chen, Z. Hu, Application of artificial neural networks in multifactor optimization of selectivity in capillary electrophoresis, *Anal. Lett.*, 33 (2000) 2333

[415] G. Bocaz-Beneventi, R. Latorre, M. Farkova, J. Havel, Artificial neural networks for quantification in unresolved capillary electrophoresis peaks, *Anal. Chim. Acta*, 452 (2002) 47

[416] D.K. Bempong, I.L. Honigberg, Multivariate analysis of capillary electrophoresis separation conditions for *Z-E* isomers of clomiphene, *J. Pharm. Biomed. Anal.*, 15 (1996) 233

[417] M. Buscema, A brief overview and introduction to artificial neural networks, *Subst. Use Misuse*, 37 (2002) 1093

[418] H.J. Metting, P.M. Coenegracht, Neural networks in high-performance liquid chromatography optimization: response surface modeling, *J. Chromatogr. A*, 728 (1996) 47

[419] M. Jalali-Heravi, Z. Garkani-Nejad, Prediction of electrophoretic mobilities of sulfonamides in capillary zone electrophoresis using artificial neural networks, *J. Chromatogr. A*, 927 (2001) 211

[420] P. Doble, M. Sandercock, E. Du Pasquier, P. Petocz, C. Roux, M. Dawson, Classification of premium and regular gasoline by gas chromatography/mass spectrometry, principal component analysis and artificial neural networks, *Forensic Sci. Int.*, 132 (2003) 26

[421] J. Zupan, J. Gasteiger, Neural networks in chemistry and drug design, Wiley-VCH, 1999.

[422] Trajan Software Ltd, *Trajan 6.0 Professional Neural Network Simulator*, 2001.

[423] M. Smith, Neural networks for statistical modeling, International Thomson Computer Press, 1993.

[424] S. Dreiseitl, L. Ohno-Machado, Logistic regression and artificial neural network classification models: a methodology review, *J. Biomed. Inform.*, 35 (2002) 352

[425] S.R. Johnson, J.M. Sutter, H.L. Engelhart, P.C. Jurs, J. White, J.S. Kauer, T.A. Dickinson, D.R. Walt, Identification of multiple analytes using an optical sensor array and pattern recognition neural networks, *Anal. Chem.*, 69 (1997) 4641

[426] P.K. Hopke, X.-H. Song, Classification of single particles by neural networks based on the computer-controlled scanning electron microscopy data, *Anal. Chim. Acta*, 348 (1997) 375

[427] H.-L. Liu, X.W. Cao, R.J. Xu, N.Y. Chen, Independent neural network modeling of class analogy for classification pattern recognition and optimization, *Anal. Chim. Acta*, 342 (1997) 223

[428] H.C. Goicoechea, M.S. Collado, M.L. Satuf, A.C. Olivieri, Complementary use of partial least-squares and artificial neural networks for the non-linear spectrophotometric analysis of pharmaceutical samples, *Anal. Bioanal. Chem.*, 374 (2002) 460

[429] P. Polaskova, G. Bocaz, H. Li, J. Havel, Evaluation of calibration data in capillary electrophoresis using artificial neural networks to increase precision of analysis, *J. Chromatogr. A*, 979 (2002) 59

[430] G. Srecnik, Z. Debeljak, S. Cerjan-Stefanovic, M. Novic, T. Bolancab, Optimization of artificial neural networks used for retention modelling in ion chromatography, *J. Chromatogr. A*, 973 (2001) 47

[431] J. Havel, M. Breadmore, M. Macka, P.R. Haddad, Artificial neural networks for computer-aided modelling and optimisation in micellar electrokinetic chromatography, *J. Chromatogr. A*, 850 (1999) 345

[432] R.M. Latorre, S. Hernandez-Cassou, J. Saurina, Artificial neural networks for quantification in unresolved capillary electrophoresis peaks, *J. Sep. Sci.*, 24 (2001) 427

[433] V. Dohnal, F. Zhang, H. Li, J. Havel, Quantitative chiral analysis in capillary electrophoresis from unresolved peaks using derivative electropherograms, experimental designs, and artificial neural networks, *Electrophoresis*, 24 (2003) 2462

[434] H. Miao, M. Yu, S. Hu, Artificial neural networks aided deconvolving overlapped peaks in chromatograms, *J. Chromatogr. A*, 749 (1996) 5

[435] Y. Zhang, H. Li, A. Hou, J. Havel, Artificial neural networks based on genetic input selection for quantification in overlapped capillary electrophoresis peaks, *Talanta*, 65 (2005) 118

[436] J.R.M. Smits, W.J. Melssen, G.J. Daalmans, G. Kateman, Using molecular representations in combination with neural networks. A case study: prediction of the HPLC retention index, *Comput. Chem.*, 18 (1994) 157

[437] Y.L. Loukas, Artificial neural networks in liquid chromatography: efficient and improved quantitative structure-retention relationship models, *J. Chromatogr. A*, 904 (2000) 119

[438] J.V. Turner, D.J. Cutler, I. Spence, D.J. Maddalena, Selective descriptor pruning for QSAR/QSPR studies using artificial neural networks, *J. Comput. Chem.*, 24 (2003) 891

[439] R. Kaliszan, T. Baczek, B. Bucinski, M. Sztupecka, Prediction of gradient retention from the linear solvent strength (LSS) model, quantitative structure-relationships (QSRR), and artificial neural networks (ANN), *J. Sep. Sci.*, 26 (2003) 271

[440] D.J. Maddalena, G.A.R. Johnston, Prediction of receptor properties and binding affinity of ligands to benzodiazepine/$GABA_A$ receptors using artificial neural networks, *J. Med. Chem.*, 38 (1995) 715

[441] G. Bocaz-Beneventi, F. Tagliaro, F. Bortolotti, G. Manetto, J. Havel, Capillary zone electrophoresis and artificial neural networks for estimation of the post-mortem interval (PMI) using electrolytes measurements in human vitreous humour, *Int. J. Legal Med.*, 116 (2002) 5

[442] S. Frias-Garcia, M.J. Sanchez, M.A. Rodriguez-Delgado, Optimization of a solid-phase microextraction procedure for the determination of herbicides by micellar electrokinetic chromatography, *J. Sep. Sci.*, 27 (2004) 660

[443] L. Huitao, W. Ketai, X. Hongping, C. Xingguo, H. Zhide, Application of experimental design and artificial neural networks to separation and determination of active components in traditional Chinese medicinal preparations by capillary electrophoresis, *Chromatographia*, 55 (2002) 579

[444] S. Casamento, B. Kwok, C. Roux, M. Dawson, P. Doble, Optimization of the separation of organic explosives by capillary electrophoresis with artificial neural networks, *J. Forensic Sci.*, 48 (2003) 1075

[445] M. Jalali-Heravi, Z. Garkani-Nejad, Prediction of electrophoretic mobilities of alkyl- and alkenylpyridines in capillary electrophoresis using artificial neural networks, *J. Chromatogr. A*, 971 (2002) 207

[446] M. Farkova, E.M. Pena-Mendez, J. Havel, Use of artificial neural networks in capillary zone electrophoresis, *J. Chromatogr. A*, 848 (1999) 365

[447] S. Malovana, S. Frias-Garcia, J. Havel, Artificial neural networks for modeling electrophoretic mobilities of inorganic cations and organic cationic oximes used as antidote contra nerve paralytic chemical weapons, *Electrophoresis*, 23 (2002) 1815

[448] B.F. Liu, J.F. Zhang, Y.T. Lu, Predicting and evaluating separation quality of micellar electrokinetic capillary chromatography by artificial neural networks, *Electrophoresis*, 23 (2002) 1279

[449] E. Marengo, V. Gianotti, S. Angioi, M.C. Gennaro, Optimization by experimental design and artificial neural networks of the ion-interaction reversed-phase liquid chromatographic separation of twenty cosmetic preservatives, *J. Chromatogr. A*, 1029 (2004) 57

[450] H. Wang, W. Liu, Optimization of a high-performance liquid chromatography system by artificial neural networks for separation and determination of antioxidants, *J. Sep. Sci.*, 27 (2004) 1189

[451] T.D. Booth, K. Azzaoui, I.W. Wainer, Prediction of chiral chromatographic separations using combined multivariate regression and neural networks, *Anal. Chem.*, 69 (1997) 3879

[452] S. Agatonovic-Kustrin, M. Zecevic, L. Zivanovic, I.G. Tucker, Application of neural networks for response surface modeling in HPLC optimization, *Anal. Chim. Acta*, 364 (1998) 265

[453] P.M.J. Coenegracht, H.J. Metting, E.M. van Loo, G.J. Snoeijer, D.A. Doornbos, Peak tracking with a neural network for spectral recognition, *J. Chromatogr.*, 631 (1993) 145

[454] A. Bucinski, T. Baczek, Optimization of HPLC separations of flavonoids with the use of artificial neural networks, *Polish Journal of Food and Nutritional Sciences*, 11 (2002) 47

[455] J. Havlis, J.E. Madden, A.L. Revilla, J. Havel, High-performance liquid chromatographic determination of deoxycytidine monophosphate and methyldeoxycytidine monophosphate for DNA demethylation monitoring:

experimental design and artificial neural network optimisation, *J. Chromatogr. B*, 755 (2001) 185

[456] J. Havel, J.E. Madden, P.R. Haddad, Prediction of retention times for anions in ion chromatography using artificial neural networks, *Chromatographia*, 49 (1999) 481

[457] J.E. Madden, N. Avdalovic, P.R. Haddad, J. Havel, Prediction of retention times for anions in linear gradient elution ion chromatography with hydroxide eluents using artificial neural networks, *J. Chromatogr. A*, 910 (2001) 173

[458] G. Sacchero, M.C. Bruzzoniti, C. Sarzanini, E. Mentasti, H.J. Metting, P.M.J. Coenegracht, Comparison of prediction power between theoretical and neural-network models in ion-interaction chromatography, *J. Chromatogr. A*, 799 (1998) 35

[459] E. Marengo, M.C. Gennaro, S. Angelino, Neural network and experimental design to investigate the effect of five factors in ion-interaction high-performance liquid chromatography, *J. Chromatogr. A*, 799 (1998) 47

[460] M. Jalali-Heravi, Z. Garkani-Nejad, Use of self-training artificial neural networks in modeling of gas chromatographic relative retention times of a variety of organic compounds, *J. Chromatogr. A*, 945 (2002) 173

[461] M. Jalali-Heravi, Z. Garkani-Nejad, Prediction of relative response factors for flame ionization and photoionization detection using self-training artificial neural networks, *J. Chromatogr. A*, 950 (2002) 183

[462] J. Havel, E.M. Pena-Mendez, A. Rojas-Hernandez, J.-P. Doucet, A. Panaye, Neural networks for optimization of high-performance capillary zone electrophoresis methods. A new method using a combination of experimental design and artificial neural networks, *J. Chromatogr. A*, 793 (1998) 317

[463] S. Heinisch, J.-L. Rocca, M. Kolosky, Computerized optimization of the mobile phase composition in gradient elution reversed-phase HPLC, *Chromatographia*, 29 (1990) 482

[464] J.C. Berridge, Unattended optimisation of reversed-phase high-performance liquid chromatographic separations using the modified simplex algorithm, *J. Chromatogr.*, 244 (1982) 1

[465] L.R. Snyder, J. Saunders, *J. Chromatogr. Sci.*, 7 (1969) 195

[466] Y. Shan, R. Zhao, Y. Zhang, W. Zhang, Y. Tian, Retention modeling and simultaneous optimization of pH value and gradient steepness in RP-HPLC using feed-forward neural networks, *J. Sep. Sci.*, 26 (2003) 1541

[467] A.M. Siouffi, R. Phan-Tan-Luu, Optimization methods in chromatography and capillary electrophoresis, *J. Chromatogr. A*, 892 (2000) 75

[468] T.D. Schlabach, J.L. Excoffier, Multi-variate ranking function for optimizing separations, *J. Chromatogr.*, 439 (1988) 173

[469] V.M. Morris, J.G. Hughes, P.J. Marriott, Examination of a new chromatographic function, based on an exponential resolution term, for use in optimization strategies: application to capillary gas chromatography separation of phenols, *J. Chromatogr. A*, 755 (1996) 235

[470] G. Derringer, R. Suich, Simultaneous optimization of several response variables, *J. Qual. Technol.*, 12 (1980) 214

[471] P.J. Schoenmakers, A.C.J.H. Drouen, H.A.H. Billiet, L. de Galan, A simple procedure for the rapid optimization of reversed-phase separations with ternary mobile phase mixtures, *Chromatographia*, 15 (1982) 688

[472] S. Heinisch, E. Lesellier, C. Podevin, J.-L. Rocca, A. Tchapla, Computerized optimization of RP-HPLC separation with nonaqueous or partially aqueous mobile phases, *Chromatographia*, 44 (1997) 529

[473] J.L. Glajch, J.J. Kirkland, K.M. Squire, J.M. Minor, Optimization of solvent strength and selectivity for reversed-phase liquid chromatography using an interactive mixture-design statistical technique, *J. Chromatogr.*, 199 (1980) 57

[474] P.F. De Aguiar, Y. Vander Heyden, D.L. Massart, Study of different criteria for the selection of a rugged optimum in high-performance liquid chromatography optimisation, *Anal. Chim. Acta*, 348 (1997) 223

[475] P. Jones, C.A. Wellington, Optimisation in chromatography: theory and application to the separation of aromatic acids in reversed-phase liquid chromatography, *J. Chromatogr.*, 213 (1981) 357

[476] P. Zakaria, M. Macka, P.R. Haddad, Mixed-mode electrokinetic chromatography of aromatic bases with two pseudo-stationary phases and pH control, *J. Chromatogr. A*, 997 (2003) 207

[477] O.H. Drummer, Postmortem toxicology of drugs of abuse, *Forensic Sci. Int.*, 142 (2004) 101

[478] Advanced Chemistry Development (ACD/Labs) Software Solaris V4.67: 1994-2005.

[479] F. Bressolle, M. Bromet-Petit, M. Audran, Validation of liquid chromatographic and gas chromatographic methods: Applications to pharmacokinetics, *J. Chromatogr. B*, 686 (1996) 3

[480] R. Osborne, S. Joel, D. Trew, M. Slevin, Morphine and metabolite behavior after different routes of morphine administration: Demonstration of the importance of the active metabolite morphine-6-glucuronide, *Clin. Pharmacol. Ther.*, 47 (1990) 12