



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

by  
Rebecca Webb

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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.

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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

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### List of Publications

Type of Publication	Number	Reference
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- [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 16<sup>th</sup> 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 41<sup>st</sup> International Meeting, Melbourne, November, 2003. (*Chapter 4*)

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## List of Abbreviations

3MAM	3-monoacetylmorphine
6MAM	6-monoacetylmorphine
7-NH <sub>2</sub> -CLO	7-aminoclonazepam
7-NH <sub>2</sub> -FLU	7-aminoflunitrazepam
7-NH <sub>2</sub> -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 <sub>L</sub>	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	<i>N</i> -diethylaminoethylene
DEL	delorazepam
DEM	demoxepam
DHC	dihydrocodeine
DHM	dihydromorphine
DIA	diazepam
DM $\beta$ CD	heptakis-(2,3-di- <i>O</i> -methyl)- $\beta$ -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 ( $\gamma$ )-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
$L_{\text{eff}}$	effective length
LLE	liquid-liquid extraction
LOD	limit of detection
LOP	loprazolam
LOQ	limit of quantification
LOR	lorazepam
LORM	lormetazepam
LPME	liquid-phase microextraction
$L_{\text{tot}}$	total length
M	molar
m	multiplet
$m/z$	mass to charge ratio
M3G	morphine-3-glucuronide
M6G	morphine-6-glucuronide
$\mu_{\text{app}}$	apparent mobility
Mec	meconium
MED	medazepam
$\mu_{\text{eff}}$	effective mobility
MEKC	micellar electrokinetic chromatography
$\mu_{\text{EOF}}$	electroosmotic flow mobility
MeOH	methanol
MEX	mexazoram
mg	milligram
$\mu\text{g}$	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 <sub>2</sub> -	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 <sub>s</sub>	product resolution
QSRR	quantitative structure-retention relationship
RBF	radial basis function
RIA	radioimmunoassay
R <sub>min</sub>	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 <sub>1/2</sub>	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
TM $\beta$ CD	heptakis-(2,3,6-tri- <i>O</i> -methyl)- $\beta$ -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 <sub>D</sub>	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  $\mu\text{g/mL}$ , 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.

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