FUNDAMENTALS OF ANALYTICAL TOXICOLOGY

FUNDAMENTALS OF ANALYTICAL TOXICOLOGY

Clinical and Forensic

Second Edition

Robert J. Flanagan
King's College Hospital NHS Foundation Trust
UK

Eva Cuypers
Maastricht Multimodal Molecular Imaging Institute
Mastricht University
The Netherlands

Hans H. Maurer
Department of Experimental and Clinical Toxicology
Saarland University
Germany

Robin Whelpton
Formerly School of Biological and Chemical Sciences
Queen Mary University of London
UK

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Preface

The analytical toxicologist may be required to detect, identify, and in many cases measure a wide variety of compounds in samples from almost any component of the body or in related materials such as residues in syringes or in soil. Many difficulties may be encountered. The analytes may include gases such as carbon monoxide, drugs, solvents, pesticides, metal salts, and naturally-occurring toxins. Some poisons may be individual chemicals and others complex mixtures. New drugs, pesticides, and other substances continually present novel challenges in analysis and in the interpretation of the results of the analysis. The analyte might be an endogenous compound such as acetone, or an exogenous compound such as a drug and/or metabolite(s) of the drug, whilst the sample matrix may range from urine to bone.

Many biological samples contain muscle, connective tissue, and so forth, which may have to be separated or degraded prior to an analysis, as well as a multitude of small and large molecular weight compounds. The concentration of the analyte to be measured can range from g L^{-1} (parts per thousand) in the case of blood ethanol to μg L^{-1} (parts per thousand million) in the case of plasma digoxin, and even ng L^{-1} (parts per million million) in the case of the potent opioid carfentanil. The stability of the analytes in biological samples also varies considerably, ranging from a few minutes for protease sensitive peptides and esters such as aspirin and diamorphine, to several years for some other drugs and pesticides.

This book aims to give principles and practical information on the analysis of drugs, poisons and other relevant analytes in biological and related specimens, particularly clinical and forensic specimens, i.e. it is a 'toolkit' in modern parlance. As such, this volume extends the scope of the World Health Organization (WHO) basic analytical toxicology manual[1] and builds on the success of the first edition of this work that appeared in 2007.[2] Moreover, it is intended to complement Dr Randall Baselt's *Disposition of Toxic Drugs and Chemicals in Man* (Edition 12. Seal Beach: Biomedical Publications, 2020), which remains the seminal reference work as regards the interpretation of analytical toxicology data.

A major difficulty in writing any textbook is deciding on the order of presentation. Having taken account not only of reviewer comments on the first edition, but also of the advances in analytical methods on the one hand, and the range of analyses that may now be required on the other, the material has been updated, expanded and presented in a new order. However, much of the discussion of the historical development of analytical toxicology present in the first edition has been removed to save space. On the other hand, some discussion of more traditional methods such as thin-layer chromatography has been retained for the simple reason that such methods are still used in many parts of the world.

After providing some background information, Section A outlines basic laboratory operations (aspects of sample collection, transport, storage, and disposal, use of internal standards, method implementation/validation, quality control and quality assessment, staff training, laboratory

[1] Flanagan RJ, Braithwaite R, Brown SS, Widdop B, De Wolff FA. *Basic Analytical Toxicology*. Geneva: WHO, 1995; available at www.who.int/ipcs/publications/training_poisons/analytical_toxicology.pdf

[2] Flanagan RJ, Taylor A, Watson ID, Whelpton R. *Fundamentals of Analytical Toxicology*. Chichester: Wiley, 2007

accreditation, etc.) and basic methodology ranging from simple colour tests through spectrophotometry to immunoassay and enzyme-based assays. Section B discusses separation science in detail (chromatography and electrophoresis, mass spectrometry, and ion mobility spectrometry). Section C reviews xenobiotic absorption, distribution and metabolism, and pharmacokinetics. Section D aims to unify this material and discusses point-of-contact testing, laboratory-based substance misuse and general toxicology screening, therapeutic drug monitoring, and trace elements and toxic metals analysis. The section concludes with a general discussion on the interpretation of analytical toxicology results.

Health and Safety

This book is intended for use by scientists trained appropriately in laboratory work. Care should be taken to ensure the safe handling of all chemical and biological materials, and particular attention should be given to the possible occurrence of allergy, infection, fire, explosion, or poisoning (including transdermal absorption or inhalation of toxic vapours). Readers are expected to consult current local health and safety regulations and to adhere to them.

Nomenclature, Symbols, and Conventions

We have followed IUPAC nomenclature for chemical names except when Chemical Abstracts nomenclature or trivial names are more readily understood. With regard to symbols, we have adopted the convention that variables and constants are italicized, but labels and mathematical operators are not. Thus, for example, the acid dissociation constant is written K_a, K being the variable, a being a label to denote that it is an acid dissociation constant. The notation for the negative logarithm of K_a is pK_a – p is a mathematical symbol and is not italicized. Where the subscript is a variable then it is italicized, so the concentration at time t, is C_t, but the concentration at time 0 is C_0. Note especially that relative molecular mass (molecular weight, relative molar mass), the ratio of the mass of an atom or molecule to the unified atomic mass unit (u), is referred to throughout as M_r. The unified atomic mass unit, sometimes referred to as the dalton (Da), is defined as one twelfth of the mass of one atom of ^{12}C. The symbol amu for atomic mass unit can sometimes be found, particularly in older works. The unified atomic mass unit is not a Système International (SI) unit of mass, although it is (only by that name, and only with the symbol u) accepted for use with SI.

As to drugs and pesticides, we have used recommended International Non-proprietary Name (rINN) or proposed International Non-proprietary Name (pINN) whenever possible. For misused drugs, the most common chemical names or abbreviations have been used. It is worth noting that for rINNs and chemical nomenclature, it is now general policy to use 'f' for 'ph' (e.g. sulfate not sulphate), 't' for 'th' (e.g. chlortalidone not chlorthalidone) and 'i' for 'y' (mesilate not mesylate for methanesulfonate, for example). However, so many subtle changes have been introduced that it is difficult to ensure compliance with all such changes. Names that may be encountered include the British Approved Name (BAN), the British Pharmacopoeia (BP) name, the United States Adopted Name (USAN), the United States National Formulary (USNF) name, and the United States Pharmacopoeia (USP) name. Where the rINN is markedly different from common US usage, for example acetaminophen rather than paracetamol, meperidine instead of pethidine, the alternative is given in parentheses at first use and in the index.

Isotopically-labelled compounds are indicated using the usual convention of square brackets to denote the substituted atoms, and site of substitution where known. For example, [$^2H_3$-*N*-methyl]-hyoscine indicates that the hydrogen atoms in the *N*-methyl group have been substituted by deuterium – this should not be confused with *N*-methylhyoscine (methscopolamine).

A useful source of information on drug and poison nomenclature is the *Merck Index Online* (www.rsc.org/Merck-Index/). Chemical Abstracts Service (CAS) Registry Numbers (RN) provide a unique identifier for individual compounds, but it is important to note that salts, hydrates, racemates, etc., each have their own RNs. Similarly, when discussing dosages we have tried to be clear when referring to salts, and when to free acids, bases, or quaternary ammonium compounds.

The oxidation number of metal ions is given by, for example, iron(II), but older terminology such as ferrous and ferric iron for Fe^{2+} and Fe^{3+}, respectively, will be encountered in the literature.

We emphasize that cross-referral to an appropriate local or national formulary is mandatory before any patient treatment is initiated or altered. Proprietary names must be approached with caution – the same name is sometimes used for different products in different countries.

Uniform Resource Locators

Uniform resource locators (URLs, web addresses) were correct at the time of printing. If the cited links are broken, readers should use an appropriate search engine or other resource to find the current URL unless directed otherwise.

Amount Concentration and Mass Concentration

In parts of Europe some laboratories report analytical toxicology data in 'amount concentration' using what have become known as SI molar units (μmol L^{-1}, etc.), whilst others, especially in the US, continue to use mass concentration [so-called 'traditional' units (mg L^{-1}, etc. or even mg dL^{-1})]. Most published analytical toxicology and pharmacokinetic data are presented in SI mass units per millilitre or per litre of the appropriate fluid [the preferred unit of volume is the litre (L)], or units that are numerically equivalent in the case of aqueous solutions:

$$[\text{parts per million}] = \mu g\ g^{-1} = \mu g\ cm^{-3} = \mu g\ mL^{-1} = mg\ L^{-1} = mg\ dm^{-3} = g\ m^{-3}$$

When preparing written statements for a court of law or other purpose outside the normal reporting channels it is advisable to write out the whole unit of measurement in full (milligrams per litre, for example).

An exception to the above is carboxyhaemoglobin saturation (COHb), which is usually reported as a percentage of the total haemoglobin present in the sample (% COHb) – the SI convention is that fractions of one should be used rather than percentages, but this is generally ignored.

We have followed the recommendations of the UK NPIS/ACB (National Poisons Information Service/Association for Clinical Biochemistry and Laboratory Medicine) for reporting analytical toxicology results and have used the litre as the unit of volume and SI mass units except for lithium (and sometimes toxic metals/trace elements), methotrexate, and thyroxine (NPIS/ACB. Laboratory analyses for poisoned patients: Joint position paper. *Ann Clin Biochem* 2002; 39: 328–39). More information on SI is available (Flanagan RJ. SI units – Common sense not dogma is needed. *Br J Clin Pharmacol* 1995; 39: 589–94).

Conversion from mass concentration (ρ) to amount concentration (c) ('molar units') and *vice versa* is simple if the molar mass (M) of the compound of interest is known. Thus, if a solution contains 302.4 g L^{-1} of a compound of M_r 151.2 g mol^{-1}

$$c = \rho / M \text{ For example: } (302.4\ g\ L^{-1})/(151.2\ g\ mol^{-1}) = 2\ mol\ L^{-1}$$

$$\rho = c\ M \text{ For example: } (2\ mol\ L^{-1}) \times (151.2\ g\ mol^{-1}) = 302.4\ g\ L^{-1}$$

However, such conversions always carry a risk of error. Especial care is needed in choosing the correct M_r if the drug is supplied as a salt, hydrate, etc. This can cause great discrepancies especially if the contribution of the accompanying anion or cation is high. Most analytical measurements are reported in terms of the free acid or base, and not the salt.

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List of Abbreviations

AA	atomic absorption
AACC	American Association for Clinical Chemistry
AAFS	American Academy of Forensic Sciences
AAG	α_1-acid glycoprotein (also AGP)
AAS	atomic absorption spectrometry
ABC	ATP-binding cassette (transporter)
ABPR	automated back-pressure regulator
ABTS	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AC	alternating current
ACB	Association for Clinical Biochemistry and Laboratory Medicine (UK)
ACCT	American Academy of Clinical Toxicology
ACEI	angiotensinogen-converting enzyme inhibitor
ACGIH	American Conference of Governmental Industrial Hygienists
AChE	acetylcholinesterase
ACOT	acyl-CoA-thioesterase
ACT	low-range activated coagulation time
ADH	alcohol dehydrogenase
ADHD	attention deficit hyperactivity disorder
ADME	absorption, distribution, metabolism, and excretion
AED	atomic emission detector/detection
AES	atomic emission spectroscopy
AFID	alkali-flame ionization detector/detection
AFS	atomic fluorescence
AHFS	American Hospital Formulary Service
2-AI	2-aminoindane
AIC	Akaike information criterion
AIDS	acquired immunodeficiency syndrome
ALDH	aldehyde dehydrogenase
6-AM	6-acetylmorphine
AMC	Analytical Methods Committee
AMDIS	Automated Mass Spectral Deconvolution and Identification System
AMS	ambient mass spectrometry
ANOVA	analysis of variance
AOX	aldehyde oxidase
APCI	atmospheric pressure chemical ionization
APDC	ammonium pyrrolidine dithiocarbamate
API	atmospheric pressure ionization
APPI	atmospheric pressure photoionization
aps	average particle size
AR	analytical reagent
ASE	accelerated solvent extraction

ASV	anodic stripping voltammetry
ATCA	2-aminothiazoline-4-carboxylic acid
ATP	adenosine triphosphate
ATR	attenuated total reflection
AUC	area under plasma concentration–time curve
AV	assigned value
BAC	blood alcohol concentration
BAN	British Approved Name
BBB	blood brain barrier
BC	background correction
BCRP	breast cancer resistance protein
BCSFB	blood–CSF barrier
1,4-BD	1,4-butanediol
BDZ	benzodiazepine
BE	benzoylecgonine
BEH	bridged ethylsiloxane/silica hybrid
BEI	biological exposure index
BGE	background electrolyte
BHO	butane hash (honey) oil
BLT	Toxicological Society of Belgium and Luxembourg
BMDMCS	bromomethyldimethyldichlorosilane
BP	British Pharmacopoeia
BPR	back-pressure regulator
BrAC	breath alcohol concentration
BRB	blood–retinal barrier
BSA	bovine serum albumin
BSTFA	*N,O*-bis(trimethylsilyltrifluoro)acetamide
BZP	benzylpiperazine
CAD	charged aerosol detector/detection
CAS	Chemical Abstracts Service
2C-B	2,5-dimethoxy-4-bromophenethylamine
CBD	cannabidiol
CBQCA	3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde
CCD	charge coupled device
CCS	collision cross-section
CD	circular dichroism
CD	cyclodextrin
CDER	Center for Drug Evaluation and Research
CDM	concentration dependant model
CDT	carbohydrate deficient transferrin
CE	capillary electrophoresis
CEA	chiral eluent additive
CEC	capillary electrochromatography
CECF	1-chloroethyl chloroformate
CEDIA	cloned enzyme donor immunoassay
CER	charge exchange reaction
CGE	capillary gel electrophoresis

ChE	cholinesterase
4-CHFB	4-carboxyhexafluorobutyryl
2C-I	2,5-dimethoxy-4-iodophenethylamine
CI	confidence interval
CID	collision-induced dissociation
CIEF	capillary isoelectric focusing
CIP	Cahn–Ingold–Prelog
CITP	capillary isotachophoresis
CJD	Creutzfeldt–Jakob Disease
CL	plasma (whole body) clearance
CLEC	chiral ligand exchange chromatography
CLD	chemiluminescence detector/detection
CLIA	chemiluminescent immunoassay
CLND	chemiluminescent nitrogen detector/detection
CLSI	Clinical and Laboratory Standards Institute
CMC	critical micellar concentration
CMDMCS	chloromethyldimethyldichlorosilane
CMIA	chemiluminescent magnetic immunoassay
CML	chronic myeloid leukaemia
CNLSD	condensation nucleation light scattering detector/detection
CNS	central nervous system
COHb	carboxyhaemoglobin
COMT	catechol *O*-methyl transferase
CoR	coefficient of repeatability
2C-P	2,5-dimethoxy-4-propylphenethylamine
CPD	continuing professional development
CPHP	4-(4-chlorophenyl)-4-hydroxypiperidine
CPP	chlorophenylpiperazine
CPR	chlorophenol red
CR	cross reactivity
CSF	cerebrospinal fluid
CSL	Commonwealth Serum Laboratories
CSP	chiral stationary phase
CSV	cathodic stripping voltammetry
CT	computerized tomography/tomographic
CV	coefficient of variation (relative standard deviation, RSD)
2C-X	2,5-dimethoxy substituted phenylethylamines
CYP	cytochrome
2,4-D	2,4-dichlorophenoxyacetic acid
DAD	diode-array detector/detection
DART	direct analysis in real time
2,4-DB	4-(2,4-dichlorophenoxy)butyric acid
DBS	dried blood spot
DC	direct current
DCM	dichloromethane
DCPP	2-(2,4-dichlorophenoxy)propionic acid
DEA	US Drug Enforcement Administration
DESI	desorption electrospray ionization

DDA	data dependent acquisition
DFC	drug facilitated crime
DFG	Deutsche Forschungsgemeinschaft
DFO	deferoxamine (desferrioxamine)
DFSA	drug facilitated sexual assault
DHT	dihydrotestosterone
DIA	data independent acquisition
DIOS	direct ionization on silicon
DLIS	digoxin-like immunoreactive substance(s)
DLLME	dispersive liquid–liquid microextraction
DMAA	dimethylamylamine
p-DMAB	*p*-dimethylaminobenzaldehyde
DMCS	dimethylchlorosilane
DMS	differential mobility spectrometry
DNOC	dinitro-*o*-cresol (4,6-dinitro-2-methylphenol)
DNP	dinitrophenol
DNSCl	dansyl chloride
DoA	drugs of abuse
DOAC	direct-acting oral anticoagulant
DOM	2,5-dimethoxy-4-methylamfetamine
DOPA	3-(3,4-dihydroxyphenyl)alanine
DP	discriminating power
DPP	differential pulse polarography
DPX	disposable pipette extraction
DTAB	dodecyltrimethylammonium bromide
DTAF	5-(4,6-dichlorotriazinyl)amino fluorescein
DTI	direct thrombin inhibition
DTIMS	drift time ion mobility spectrometry
DTTO	Drug Treatment and Testing Order
DUI	driving under the influence
DUS	dried urine spot
EA	enzyme acceptor
EAPCCT	European Association of Poisons Centres and Clinical Toxicologists
EASI	easy ambient sonic spray ionization
EBC	exhaled breath condensate
EC	electrochemical
ECD	electron capture detection
ECF	extracellular fluid
ECPLA	*N*-ethyl-*N*-cyclopropyl lysergamide
ED	electrochemical detector/detection
ED	enzyme donor (biochemistry)
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine
EDTA	ethylenediaminetetra-acetic acid (or sodium salt)
EDXRF	energy dispersive X-ray fluorescence
EHC	enterohepatic cycling
EI	electron ionization
EIA	enzyme immunoassay

ELISA	enzyme-linked immunosorbent assay
ELSD	evaporative light scattering detector/detection
EM	extensive metabolizer
EMA	European Medicines Agency
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EME	ecgonine methyl ester
EMIT	enzyme-multiplied immunoassay technique
ENFSI	European Network of Forensic Science Institutes
EOF	electro-osmotic flow
EPA	Environmental Protection Agency (US)
EPN	ethyl *p*-nitrophenyl thionobenzene phosphonate
EPTIS	European Proficiency Testing Information System
EQA	external quality assessment
ESI	electrospray ionization
ESR	electron spin resonance spectroscopy
ETAAS	electrothermal atomization atomic absorption spectroscopy
EtG	ethyl glucuronide
EtS	ethyl sulfate
EWDTS	European Workplace Drug Testing Society
FA	fluoroamfetamine
FAAS	flame atomic absorption spectroscopy
FAB	fast atom bombardment
FAEE	fatty acid ethyl ester
FAIMS	high field asymmetric waveform ion mobility spectroscopy
FDA	US Food and Drug Administration
FEP	flame emission photometry
FFAP	free fatty acid phase
FIA	flow-injection analysis
FID	flame ionization detector/detection
FITC	fluorescein isothiocyanate
FMO	flavin-containing monooxygenase
FMD	formaldehyde dehydrogenase
FPD	flame photometric detector/detection
FPN	ferric / perchloric / nitric (acids)
FPIA	fluorescence polarization immunoassay
FPP	fully porous particles
FQ	3-(2-furoyl)quinoline-2-carboxaldehyde
FSCE	free solution capillary electrophoresis
FT	Fourier transform
FTIR	Fourier transform infra-red
FTICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
5-FU	5-fluorouracil
FWHM	full width of peak at half maximum height
GBL	γ-butyrolactone
GC	gas chromatography
GC-C-IRMS	GC-combustion-isotope ratio mass spectrometry
GFC	gel filtration chromatography
GFR	glomerular filtration rate

GHB	γ-hydroxybutyrate
GI	gastrointestinal
GIST	gastrointestinal stromal tumours
GLC	gas–liquid chromatography
GPC	gel permeation chromatography
G-6-P	glucose-6-phosphate
GPC	gel permeation chromatography
G-6-PDH	glucose-6-phosphate dehydrogenase
GPR	general purpose reagent
GSC	gas–solid chromatography
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione *S*-transferase
GTFCh	Society of Toxicological and Forensic Chemistry
GTN	glyceryl nitrate (trinitrin)
H	height equivalent to a theoretical plate (HETP)
HbA1c	glycated haemoglobin
HCD	high energy collision dissociation
HCG	human chorionic gonadotropin
HEMA	2-hydroxyethyl mercapturic acid (*N*-acetyl-*S*-(2-hydroxyethyl)-L-cysteine)
He-PDPID	pulsed discharge helium photoionization
HETP	height equivalent to a theoretical plate
HFB	heptafluorobutyryl
HFBA	heptafluorobutanoic acid
HFBA	heptafluorobutyryl anhydride
HHb	deoxygenated haemoglobin
5-HIAA	5-hydroxyindoleacetic acid
HILIC	hydrophilic interaction liquid chromatography
HIV	human immunodeficiency virus
HLoQ	higher limit of quantification
HMDS	hexamethyldisilazane
HPLC	high-performance liquid chromatography
HPT	hybrid particle technology
HPTLC	high-performance thin-layer chromatography
hR_f	retention (retardation) factor $\times$ 100
HRMS	high resolution mass spectrometry
HRT	hormone replacement therapy
HS	headspace
HSA	human serum albumin
HSCT	haematopoietic stem cell transplantation
5-IAI	5-iodo-2-aminoindane
IC	ion chromatography
ICE	immobilized coating extraction
ICL	isotopic internal calibration
ICP(-MS)	inductively coupled plasma (mass spectrometry)
i.d.	internal diameter
IDE	insulin-degrading enzyme
I-EC	ion exchange chromatography

IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IFMA	immunofluorometric assay
IgG	immunoglobulin G
IgM	immunoglobulin M
ILAC	International Laboratory Accreditation Cooperation
i.m.	intramuscular
IM	intermediary metabolizer
IMS	ion mobility spectrometry
INR	international normalized ratio
i.p.	intraperitoneal
IP	ionization potential
IPED	image and performance enhancing drug
IQC	internal quality control
IR	infra-red
IRI	immunoreactive insulin
IRMA	immunoradiometric assay
ISCC	Inter-Society Color Council
ISE	ion selective electrodes
ISF	interstitial fluid
ISI	ion spray ionization
ISO	International Organization for Standardization
ISRP	Internal surface reverse-phase
ISTD	internal standard
ITSP	instrument top sample preparation
i.v.	intravenous
IUPAC	International Union of Pure and Applied Chemistry
LA	laser ablation
LADESI	laser ablation/desorption electrospray ionization
LAMPA	*N*,*N*-methylpropyl lysergamide
LAT	latex agglutination test
LAT1	large amino acid transporter type 1
LBA	ligand binding assay
LBW	lean body weight
LC	liquid chromatography
LD	loading dose
LDI	laser desorption/ionization
LDTD™	laser diode thermal desorption
LDV	low dead volume
LIF	laser-inducted fluorescence
LLE	liquid–liquid extraction
LLoD	lower limit of detection
LLoQ	lower limit of quantitation
LoD	limit of detection
LoQ	limit of quantification
LPG	liquified petroleum gas
LPME	liquid phase microextraction
LSD	lysergic acid diethylamide

LTG	London Toxicology Group
MA	methoxyamfetamine
MAbs	monoclonal antibodies
MALDI	matrix-assisted laser desorption ionization
6-MAM	6-monoacetylmorphine (6-AM)
MAOI	monoamine oxidase inhibitor
MATE	multidrug and toxin exclusion protein
MCPP	2-(4-chloro-2-methylphenoxy)propionic acid
MDA	3,4-methylenedioxyamfetamine
MDAI	5,6-methylenedioxy-2-aminoindane
MDBP	3,4-methylenedioxybenzylpiperazine
MDEA	3,4-methylenedioxyethylamfetamine
MD-GC	multidimensional gas chromatography
MDMA	3,4-methylenedioxymetamfetamine
MDMAI	5,6-methylenedioxy-*N*-methyl-2-aminoindane
MDQ	minimum detectable quantity
MDR	multi-drug resistance
MEIA	microparticle enzyme immunoassay
MEKC	micellar electrokinetic (capillary) chromatography
3-MeO-PCE	3-methoxyeticyclidine
3-MeO-PCP	3-methoxyphencyclidine
4-MeO-PCP	4-methoxyphencyclidine
MeOPP	4-methoxyphenylpiperazine
MEPS	microextraction in packed syringe
MetHb	methaemoglobin
MF	mass fragmentography
MFO	mixed function oxidase
MIBK	methyl isobutyl ketone (4-methyl-2-pentanone)
MIC	minimum inhibitory concentration
MIP	molecularly imprinted polymer
MML	mean list length
M–M	Michaelis–Menten
4-MMC	mephedrone (4-methylmethcathinone)
6-MP	6-mercaptopurine
MPA	mycophenolic acid
MPD	microwave plasma detector/detection
M_r	relative molecular (atomic) mass
MRM	multiple reaction monitoring
MRP	multidrug resistance-associated protein
MS	mass spectrometry/spectrometric
MSI	mass spectrometry imaging
MSTFA	*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide
MTBE	methyl *tert*-butyl ether
MTPA	(*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid

MU	measurement uncertainty
MXE	methoxetamine
NAA	neutron activation analysis
NAC	*N*-acetyl-L-cysteine
NAD	nicotine adenine dinucleotide
NADP	nicotine adenine dinucleotide phosphate
NAPA	*N*-acetylprocainamide
NAPQI	*N*-acetyl-*p*-benzoquinoneimine
NAT2	*N*-acetyltransferase type 2
NBCS	newborn calf serum
NBD	7-nitrobenz-2-oxa-1,3-diazole
NBOMe	*N*-benzyl substituted phenethylamine
NBS	National Bureau of Standards (US)
NCI	negative ion chemical ionization
NDA	2,3-naphthalenedialdehyde
NIH	National Institutes of Health (US)
NIOSH	National Institute for Occupational Safety and Health (US)
NIST	National Institute for Standards and Technology (US)
NLM	National Library of Medicine (US)
NMR	nuclear magnetic resonance
NOAC	non-vitamin K antagonist oral anticoagulant
NPD	nitrogen–phosphorus detector/detection
NPIS	National Poisons Information Service (UK)
NPS	novel (new) pharmaceutical substance
NPT	near patient testing
NSAID	non-steroidal anti-inflammatory drug
NSD	nitrogen-selective detector/detection
OAT	organic anion transporter
OATP	organic anion-transporting polypeptide
OCT	organic cation transporter
ODS	octadecylsilyl
OE	optical emission
OES	optical emission spectroscopy
O_2Hb	oxygenated haemoglobin
OP	organophosphorus/organophosphate
OPA	*o*-phthaldialdehyde (1,2-phthalic dicarboxaldehyde)
OPLC	overpressured-layer chromatography
OPLC	optimum performance laminar chromatography
OR	optical rotation
OT	Orbitrap mass analyzer
OTC	over-the-counter
PAN	1-(2-pyridylazo)-2-naphthol
PAPS	3′-phosphoadenosine-5′-phosphosulfate
PBA	phenylboronic acid
PBS	phosphate buffered saline
PCE	eticyclidine
PCI	positive ion chemical ionization
PCP	phencyclidine

PCR	polymerase chain reaction
PCT	proximal convoluted tubule
PDD	pulsed discharge detector/detection
PDE5	phosphodiesterase type 5
PDECD	pulsed discharge electron capture detector/detection
PDED	pulsed discharge helium ionization emission detector/detection
PDMS	polydimethylsiloxane
PDVB	polydivinylbenzene
PEEK	polyetheretherketone
PEG	polyethylene glycol
PEths	phosphatidylethanol species
PETIA	particle enhanced turbidimetric immunoassay
PETINIA	particle enhanced turbidimetric inhibition immunoassay
PFK	perfluorokerosene
PFTBA	perfluorotributylamine
PGC	porous graphitic carbon
P-gp	P-glycoprotein
PID	photoionization detector/detection
pINN	proposed International Non-proprietary Name
PK	pharmacokinetic/s
PKI	protein kinase inhibitor
PLE	pressurized liquid extraction
PLOT	porous layer open tubular
1P-LSD	1-propionyl-lysergic acid diethylamide
PM	poor metabolizer
POCT	point of contact testing
POC(L)T	point of care (laboratory) testing
PSA	potentiometric stripping analysis
PSA	polar surface area
PSDVB	polystyrene-divinylbenzene
PSI	paperspray ionization
PT	proficiency testing
PTFE	polytetrafluoroethylene
PVC	polyvinylchloride
QA	quality assessment
QC	quality control
QED	quantitative ethanol detector/detection
QIT	quadrupole ion trap
QTOF	quadrupole time of flight
RA	radioactivity
RAM	restricted access media
RCF	relative centrifugal force
RD	relative density
RDX	1,3,5-trinitro-1,3,5-triazine
RF	radiofrequency
R_f	retention (retardation) factor
rGO	reduced graphene oxide
RI	refractive index

RI	retention index
RIA	radioimmunoassay
rINN	recommended International Non-proprietary Name
RIP	reactant ion peak
RLU	relative chemiluminescent unit
RMS	root mean square (error)
RN	(Chemical Abstracts) registry number
ROFT	rapid oral fluid testing
RPC	rotational planar chromatography
RPM/rpm	revolutions per minute
RSD	relative standard deviation
RTIC	reconstructed total ion chromatogram
RTL	retention time locking
SAM	*S*-adenosylmethionine
SAMHSA	Substance Abuse and Mental Health Services Administration (US)
SAX	strong anion exchange
s.c.	subcutaneous
SCOT	support coated open tubular
SCX	strong cation-exchange
SD	standard deviation
SDME	single-drop microextraction
SDPA	standard deviation of proficiency assessment
SDS	sodium dodecylsulfate
SE	sorbent extraction
SEC	size exclusion chromatography (gel permeation chromatography, GPC)
SELDI	surface enhanced laser desorption ionization
s.e.m.	standard error of the mean
SER	smooth endoplasmic reticulum
SERS	surface-enhanced Raman spectroscopy
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
SFTA	French Society of Analytical Toxicology
SG	specific gravity (relative density)
SHP	shield hydrophobic phase
SI	Système International
SIFT	selected ion flow tube
SIL	stable isotope labelled
SIM	selected ion monitoring
SIMS	secondary ion mass spectrometry
SLC	solute carrier
SLE	supported liquid extraction
S/N	signal-to-noise
SNRI	serotonin–norepinephrine reuptake inhibitor
SOFT	Society of Forensic Toxicologists (US)
SoHT	Society of Hair Testing
SOP	standard operating procedure
SPE	solid phase extraction
SPFM	spectrophotofluorimetry

SPME	solid phase microextraction
SPP	superficially porous particles
SR	sustained release
SRM	selected reaction monitoring
SS	sum of squares
SSRI	selective serotonin reuptake inhibitor
STA	systematic toxicological analysis
SWO	stomach wash-out
TBDMSIM	*tert*-butyldimethylsilylimidazole
TBW	total body water
TBM	total body mass
TCA	tricyclic antidepressant
TCD	thermal conductivity detector (katharometer)
TCP	tenocyclidine
TCPO	bis(2,4,6-trichlorophenyl)oxalate
TDM	therapeutic drug monitoring
TFMPP	1-(3-trifluoromethylphenyl)piperazine
THC	Δ^9-tetrahydrocannabinol
THC-COOH	11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol
THF	tetrahydrofuran
TIA	turbidimetric immunoassay
TIAFT	The International Association of Forensic Toxicologists
TIMS	trapped ion mobility spectrometry
TKI	tyrosine kinase inhibitor
TLC	thin-layer chromatography
TLV	threshold limit value
TMAH	tetramethylammonium hydroxide
TMB	3,3',5,5'-tetramethylbenzidine
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
TOF	time of flight
2,4,5-TP	2-(2,4,5-trichlorophenoxy)propionic acid
TPMT	thiopurine methyltransferase
TSIM	*N*-trimethylsilylimidazole
TWIMS	travelling wave ion mobility spectrometry
TXRF	total reflection X-ray fluorescence
UDP	uridine diphosphate
UDPGA	uridine diphosphate glucuronic acid
UGT	UDP-glucuronosyltransferase
UHPLC	ultra-high performance chromatography
UKIAFT	United Kingdom and Ireland Association of Forensic Toxicologists
ULoQ	upper limit of quantitation
UM	ultra-rapid metabolizer
UNODC	United Nations Office on Drugs and Crime
UPLC	Ultra-Performance Liquid Chromatography™
USAN	United States Adopted Name
USNF	United States National Formulary
USP	United States Pharmacopoeia

UV	ultraviolet
UV/Vis	ultraviolet/visible
V	volume of distribution
VAMS	volumetric absorptive microsampling
VKA	vitamin K antagonist
VOC	volatile organic compound
VSA	volatile substance abuse (misuse)
VUV	vacuum ultraviolet
WAX	weak anion exchange
WCOT	wall coated open tubular
WCX	weak cation exchange
WDXRF	wavelength dispersive X-ray fluorescence
WHO	World Health Organization
XRF	X-ray fluorescence

Section A
The Basics

1 Analytical Toxicology: Overview

1.1 Introduction

Analytical toxicology is concerned with the detection, identification, and measurement of drugs and other foreign compounds (xenobiotics) and their metabolites, and in some cases endogenous compounds, in biological and related specimens. The analytical toxicologist can play a useful role in the diagnosis, management, and indeed the prevention of poisoning, but to do so a basic knowledge of clinical and forensic toxicology is essential. Moreover, the analyst must be able to communicate effectively with clinicians, pathologists, coroners, police, members of the legal profession, and a range of other people. In addition, a good understanding of analytical chemistry, clinical chemistry, pathology, clinical pharmacology, pharmacokinetics, and occupational and environmental health is essential.

The use of physicochemical techniques in the detection, identification and measurement of drugs and other poisons in body fluids and tissues has its origins in the development of forensic toxicology. Important contributions came later from work to improve food safety and from occupational toxicology. Major advances in analytical methodology followed the introduction and application of refined physicochemical techniques such as spectrophotometry and chromatography in the late 1940s. In particular, ultraviolet (UV) and infra-red (IR) spectrophotometry, together with visible spectrophotometry (colorimetry), and paper and ion-exchange column chromatography were widely used. In the 1960s paper chromatography was largely superseded by thin-layer chromatography (TLC) as this latter technique offered advantages of speed of analysis and lower detection limits.

Improved instrumentation for UV/visible spectrophotometry (UV/Vis), spectrophotofluorimetry, atomic absorption spectrophotometry (AAS), electrochemistry, X-ray diffraction, nuclear magnetic resonance (NMR), and neutron activation analysis led to these techniques being applied to particular problems. However, whilst some more traditional methods still have their uses, gas and liquid chromatography (GC and LC, respectively), often linked to mass spectrometry (MS) in its various modes, on the one hand and immuno- and enzyme-based assays on the other, are the techniques that are used most widely today (Table 1.1).

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

Table 1.1 Methods for the analysis of drugs and other organic poisons in biological samples

Principle	Technique
Chemical	Colour test
Electrochemical	Biosensors Differential pulse polarography (DPP)
Spectrometric	High resolution mass spectrometry (HRMS)
	Mass spectrometry (MS)
	Nuclear magnetic resonance (NMR)
	Spectrophotofluorimetry (SPFM)
	Ultraviolet/visible absorption spectrophotometry (UV/Vis)
Kinetic	Flow-injection analysis (FIA)
Chromatographic	Gas chromatography (GC), includes gas–solid chromatography (GSC) and gas–liquid chromatography (GLC)
	(High performance) liquid chromatography [(HP)LC]
	(High performance) thin-layer chromatography [(HP)TLC]
	Ion-exchange chromatography (I-EC)
	Supercritical fluid chromatography [SFC]
Electrophoretic	Capillary (zone) electrophoresis [C(Z)E]
	Capillary electro-chromatography (CEC)
	Ion mobility spectrometry (IMS)
	Micellar electrokinetic (capillary) chromatography (MEKC)
Ligand immunoassay	Cloned enzyme donor immunoassay (CEDIA)
	Enzyme-linked immunosorbent assay (ELISA)
	Enzyme-multiplied immunoassay technique (EMIT)
	Fluorescence polarization immunoassay (FPIA)
	Latex agglutination test (LAT)
	Microparticle enzyme immunoassay (MEIA)
	Radioimmunoassay (RIA)
Enzyme-based assay	Alcohol dehydrogenase – ethanol
	Aryl acylamide amidohydrolase – paracetamol

1.2 Modern analytical toxicology

Recent years have seen many advances in methods for detecting, identifying, and measuring drugs and other poisons in biological fluids with consequent improvement in the scope and reliability of analytical results. The value of certain emergency assays and their contribution to therapeutic intervention has been clarified. Some such assays are performed for clinical purposes, but have overt medico-legal implications and require a high degree of analytical reliability. Examples include ‘brain death’ and child abuse screening (Flanagan, 2019), and instances of suspected iatrogenic poisoning. In addition, demand for the measurement of plasma

drug and sometimes metabolite concentrations to aid treatment (therapeutic drug monitoring, TDM), for substance misuse screening, and for laboratory analyses to monitor occupational exposure to certain chemicals has increased. There has also been increased demand for 'on-site' testing, be it either in the clinic, or at the roadside.

Despite analytical advances, it remains impossible to look for all poisons in all samples at the sensitivity required to diagnose poisoning. It is vital therefore that the reason for any analysis is kept clearly in mind. Although the underlying principles remain the same in the different branches of analytical toxicology, the nature and the amount of specimen available can vary widely, as may the time scale over which the result is required and the purpose for which the result is to be used. All these factors may in turn influence the choice of method(s) for a particular analysis. Over the last 10 years the dramatic expansion in the range of compounds that may be misused to achieve intoxication has further complicated the role of the analytical toxicologist (Pasin *et al*., 2017), as has the use of such unusual substances as ^{210}Po and the nerve agent 'Novichok' as murder weapons (Harrison *et al*., 2017; Vale *et al*., 2018).

1.2.1 Analytical methods

Immunoassays have found wide application in analytical toxicology. A range of techniques, for example enzyme-multiplied immunoassay technique (EMIT) and cloned enzyme donor immunoassay (CEDIA), are available and are often highly sensitive. Enzyme-based assays, such as that for paracetamol (acetaminophen), have also been described. However, all of these assays have the disadvantage that antibodies, enzymes, or specific binding proteins have to be prepared for each analyte or group of analytes before an analysis is possible. On the other hand, these and similar assays may often be used directly in small volumes of aqueous media ('homogenous assay'), in contrast to chromatographic methods, which often require some form of sample preparation procedure, for example liquid–liquid extraction (LLE), otherwise known as solvent extraction, prior to the analysis. Moreover, although immunoassays can be very sensitive, some may be poorly selective, the antibody recognizing several structurally similar molecules. Sometimes this cross-reactivity can be exploited, as in screening for classes of misused drugs such as benzodiazepines and opiates (Box 1.1).

There are practical considerations. Immunoassay is a batch process and therefore readily amenable to automation, whilst GC and LC involve sequential assays on the same instrument. Whilst automated sample preparation and shorter columns/faster analysis times can help, this remains a limiting factor. However, the infinitely greater selectivity of chromatographic methods even without MS detection means that such methods are often a prerequisite if the results are to withstand scrutiny in a court of law.

LC has achieved wide application in analytical toxicology since the 1970s. Gases and very volatile solvents excepted, many analytes are amenable to analysis by LC or a variant of the basic procedure. GC, on the other hand, is restricted to the analysis of either compounds or derivatives that are both stable and volatile at temperatures up to approximately 350 °C. This being said, GC with modern bonded-phase capillary columns, temperature programming, nitrogen–phosphorus detection (NPD), and MS detection either when used alone, or linked in series (MS/MS), has tremendous sensitivity and selectivity especially when combined with a selective sample preparation procedure.

The use of LC in the qualitative analysis of drugs and other poisons has been limited by the lack of a sensitive universal detector analogous to the flame ionization detector (FID) in GC and the poor performance of gradient elution systems. However, a range of sensitive MS detectors is now available for use with LC and together with modern narrow-bore packed columns and

Box 1.1 Opiates, opioids, and opium

- Opium is the dried residue obtained from the white, viscous fluid that exudes from the unripe seed head of the opium poppy (*Papaver somniferum*) when it has been cut
- The opium poppy is the major source of the drug morphine, but also contains the closely related alkaloids codeine and thebaine. Opium also contains meconin, noscapine, papaverine, and reticuline, amongst other compounds, which are not structurally related to morphine and are not analgesic
- Purified morphine and thebaine are used as starting materials for semi-synthetic drugs such as dihydrocodeine and oxycodone
- Acetylating morphine produces diamorphine, which was introduced in Germany in 1895 as an antitussive under the name *Heroin*. The addictive properties of diamorphine quickly became apparent and the importation, production, and use of diamorphine was banned in the US in 1924, for example
- The term 'heroin' is now generally used to refer to the impure diamorphine obtained by treating semi-purified opium with an acetylating agent, usually acetic anhydride. Acetylcodeine is a common contaminant of heroin
- Strictly, the term 'opiates' refers to substances obtained from opium, not all of which have morphine-like properties, whereas 'opioids' are materials with morphine-like properties
- The term 'opioid' can thus apply to not only naturally occurring and semi-synthetic compounds such as morphine and oxycodone, but also synthetic drugs such as methadone and dextropropoxyphene, as well as naturally occurring transmitters, for example met-enkephalin and leu-enkephalin
- The term 'narcotic' originally referred to any psychoactive compound with sleep-inducing properties. In the US it has since become associated with opiates and opioids, commonly morphine and heroin, as well as stimulants such as cocaine, but is clearly non-specific and is best avoided

eluent gradient programming, major advances have been made. The advent of accurate mass detection systems, i.e. detectors that can measure mass-to-charge ratio (*m/z*) to four decimal places, together with other parameters that can be measured to increase the certainty of peak assignment, has been yet another milestone (Grapp *et al.,* 2018). However, the high purchase and maintenance costs of such systems remains a major barrier to their widespread use.

An important consideration is that the MS detector is a reaction detector, i.e. the signal obtained is dependent on chemical reaction(s) occurring in the ionization source. Therefore, the signal obtained is dependent not only on the analyte, but also on the possible presence of co-eluting compounds that may affect the ionization of the analyte. This is not normally a problem in GC-MS because the carrier-gas (usually helium) has little influence and the selectivity of the system is such that the analyte is usually fairly pure when it arrives at the ionization source. Not so with LC-MS, where either the suppression, or enhancement of the analyte signal due to the presence of co-eluting compounds can be significant.

1.2.2 Systematic toxicological analysis

The problem in systematic toxicological analysis (STA, poisons screening, drug screening, unknown screening) is to detect reliably as wide a range of compounds as possible in as

little sample (plasma/serum/whole blood, urine, vitreous humour, stomach contents or vomit, or tissues) as possible at high sensitivity, but with no false positives (Maurer, 1999). Ideally some sample should be left to permit confirmation of the results using another technique and if indicated quantitation of any poison(s) present to aid clinical interpretation of the results.

In poisons screening it is important to adopt a systematic approach in order to eliminate possible contenders and to 'home in' on any compound(s) of interest present. STA can be divided into three key stages (Figure 1.1). The aim of the sample preparation step is to retain all the toxicologically important substances whilst removing potentially interfering sample matrix components. Thus, a wide range of analytes of interest, including lipophilic and polar, acidic, basic, and neutral species, should be isolated. To increase the yield of analyte(s) when analyzing urine, for example, the sample may be treated with β-glucuronidase/arylsulfatase to hydrolyze conjugated metabolites, but this may not be necessary if the conjugates themselves can be detected.

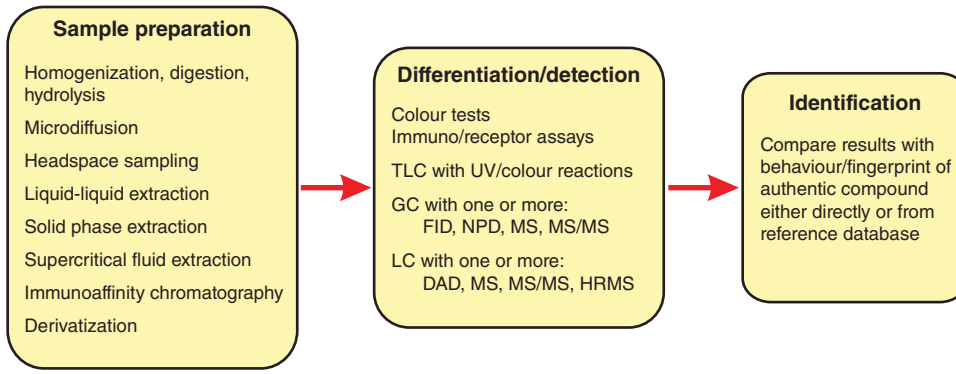

Figure 1.1 The three key steps in systematic toxicological analysis

The aim of the differentiation/detection step is to identify all relevant compounds in the minimum amount of time. This requires a combination of relatively non-specific ('universal') assays and highly selective methods. Immunoassays, particularly if the antibody has wide cross-reactivity, are useful for identifying classes of drugs. TLC has the advantage that all the non-volatile materials in the extract remain on the plate, whereas with GC and LC there is always the possibility that compounds have not been eluted from the analytical column. Obviously, one analytical technique cannot separate and identify all the possible compounds of interest. Only a finite number of compounds can be resolved on a single TLC plate, for example.

The greater the number and range of techniques that are available, the greater the probability that unknown substance(s) will be identified correctly. Investigation of the responses of various analytes to different detectors can provide valuable information about the nature of a compound. LC with diode array detection (LC-DAD) can provide spectral information to aid peak assignment, but is limited by relatively poor sensitivity and selectivity (Section 10.3.1). In contrast, hyphenated techniques such as GC-MS can provide robust analyte identification, particularly when combined with computerized libraries of electron ionization (EI) fragmentation data that can be searched rapidly to confirm compound identity (Grapp *et al.*, 2016). In addition, positive ion chemical ionization (PCI) MS can be used to give an indication of the molar mass of a substance (Section 13.3.1.2).

Analytes may be chemically modified to improve their chromatographic properties or 'detectability', but derivatization can also give useful qualitative information. One classic example is the so called 'acetone-shift' (reaction of acetone with a primary amine to give the corresponding Schiff's base). Amfetamine, for example, reacts with acetone to form *N*-(1-methyl-2-phenylethyl)propanimine (Figure 1.2). If the product does not chromatograph as expected, then the unknown substance was not amfetamine.

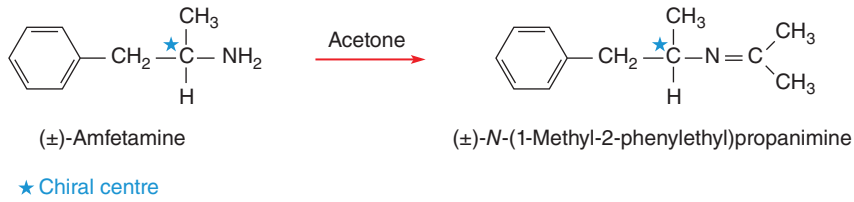

Figure 1.2 Reaction of amfetamine with acetone

The third step in STA is to compare the observed data with validated database information. Clearly, databases used in compound identification need to be regularly updated, and must include information on not only parent compounds, but also metabolites, common interferences, and possible contaminants. It is important that the analytical techniques used in establishing such databases are reproducible, both within and between laboratories.

1.2.3 Ethanol and other volatile substances

Enzymatic methods for plasma ethanol using alcohol dehydrogenase with spectrophotometric measurement of a coenzyme, for example, are available in kit form for clinical chemistry analyzers. GC analysis of ethanol either by direct injection of blood or urine diluted with deionized water, or by static headspace sampling (HS-GC), is also widely used, particularly in forensic work. GC-FID is advantageous because methanol, 2-propanol, and acetone may be separated and measured simultaneously. Methanol poisoning from ingestion of synthetic alcoholic drinks is one of the few causes of acute poisoning 'epidemics' and measurement of blood methanol is important in confirming the diagnosis and in monitoring treatment (Section 22.4.1.2). Detection of high concentrations of acetone, itself a metabolite of 2-propanol, and *vice versa*, may aid the diagnosis of ketoacidosis (Belsey & Flanagan, 2016).

Many more volatile compounds may be encountered in acute poisoning arising, for example, from deliberate inhalation of vapour in order to become intoxicated [volatile substance misuse, 'glue sniffing', solvent abuse, inhalant abuse, volatile substance abuse (VSA)]. Some of these volatile compounds have metabolites, which may be measured in urine in order to assess exposure, notably hippuric and methylhippuric (toluric) acids (from toluene and the xylenes, respectively) and trichloroacetic acid (from trichloroethylene). However, most volatile substances are excreted unchanged in exhaled air and therefore whole blood is the best sample for detecting and identifying these compounds (Section 22.4.23).

1.2.4 Trace elements and toxic metals

In order to help diagnose either deficiencies or chronic poisoning with these substances, where alterations from target ranges of only a few μg L^{-1} (parts per billion, ppb, i.e. parts per thousand

million) of blood or serum can be important, good accuracy and reproducibility are essential (Wang *et al*., 2014). Sample contamination during collection (for example from sample tubes, or even from syringe needles in the case of chromium and manganese) and within the laboratory itself can be a serious source of error. This applies especially to widespread elements such as lead and aluminium.

Modern methods for measuring toxic metals in biological materials (Table 1.2) vary enormously in terms of complexity, cost, accuracy, and sensitivity. In the case of serum iron, however, reliable kits based on the formation of a coloured complex remain widely used in clinical chemistry. Some techniques (isotope dilution mass spectrometry, neutron activation analysis), on the other hand, are in reality reference methods. Atomic absorption spectrophotometry (AAS) with either flame or electrothermal atomization using a graphite furnace has been employed widely, but is being superseded by inductively coupled plasma-mass spectrometry (ICP-MS).

Table 1.2 Methods for the analysis of toxic metals in biological materials

Technique	Mode	Variant
Electrochemical	Potentiometric	Ion selective electrodes
	Coulometric	(Differential pulse) polarography
		Anodic/cathodic stripping voltammetry (A/CSV)[a]
Spectrophotometric	Optical Emission (OES)	Flame emission photometry (FEP)[b]
		Direct-current plasma
		Inductively coupled plasma (ICP)
	Atomic Absorption (AAS)	Flame
		Hydride generation
		Electrothermal
		Cold vapour
	X-Ray	Fluorescence
	Nuclear	Neutron activation
		Proton activation
Mass spectrometric		Inductively coupled plasma (ICP-MS)

[a] Also known as potentiometric stripping analysis (PSA)
[b] Normally refers to the use of filters to select the emission wavelength – used mainly for potassium, lithium, and sodium assay

ICP-MS is a multi-element technique that can detect and measure isotopes with detection limits of μg L^{-1} to ng L^{-1}. Different isotopes of an element can also be measured. For some elements, the relative abundance of the isotopes depends upon the source of the metal. Therefore, by measuring the isotope ratios of an element such as lead in a sample from a chronically poisoned patient with those found in material present in the patient's immediate environment it may be possible to localize the source of exposure (Gulson, 2008).

Although ICP-MS measures (isotopes of) elements, it cannot differentiate between toxic and non-toxic arsenic species, for example, without a prior separation step (Hsu *et al*., 2011).

Even then, phenomena such as adduct formation and/or spectral interference from isotopes of different elements can confuse an analysis unless special precautions are taken (Balcaen *et al.*, 2015; Section 21.4.4.3).

1.3 Provision of analytical toxicology services

If poisoning is suspected and appropriate biological samples have been obtained, the stages in processing analytical work can be divided into pre-analytical, analytical, and post-analytical phases (Table 1.3).

Table 1.3 Steps in undertaking an analytical toxicological investigation

Pre-analytical	Obtain details of the current (suspected) poisoning episode, including any circumstantial evidence of poisoning, and the results of biochemical and haematological investigations, if any. Also obtain the patient's medical and occupational history, if available. Decide the priorities for the analysis
Analytical	Perform the agreed analysis
Post-analytical	Interpret the results in discussion with the physician looking after the patient or the pathologist. Perform additional analyses, if indicated, using either the original samples, or further samples from the patient. Save any unused or residual samples for possible future use

1.3.1 Samples and sampling

In analytical toxicology, clinical chemistry, and related fields, the words 'sample' and 'specimen' are used to denote a portion of a body fluid, tissue, incubation medium, etc. obtained under defined conditions. The samples encountered may range from relatively pure solutions of a drug to a piece of putrefying tissue. Liquids, such as blood, oral fluid (principally saliva), urine, and cerebrospinal fluid (CSF), are generally easier to sample and to analyze than solids and semi-solids, which require homogenization or digestion prior to analysis.

Blood plasma or serum is used in clinical work if quantitative measurements are needed in order to assess dosage or monitor treatment as in TDM. Urine is commonly used in qualitative work such as substance misuse screening because collection is non-invasive and the concentrations of many drugs and their metabolites tend to be higher than in blood, thereby facilitating analyte detection. Further aspects related to samples and sampling are discussed in Chapter 2.

1.3.2 Choice of analytical method

In responding to a given analytical problem, many factors must be considered. It may seem self-evident that the method used should be appropriate for the intended analysis. In practice, the choice of method depends on several factors. These include (i) the circumstances under which an analysis is requested (i.e. the question being asked), (ii) the speed with which the result is required, (iii) the sample to be analyzed, (iv) the nature of the analyte (if known), (v) the expected concentration of any analyte(s), (vi) the time available for the analysis, (vii) the apparatus available, (viii) the existence of a validated method in the laboratory, and (ix) the training and experience of the analyst.

The nature of the sample and the expected concentration of any analyte(s) are obvious influences on the choice of method. It may be possible to measure the concentration of a known substance in a relatively pure solution directly using a simple technique such as UV spectrophotometry. However, if the sample is a piece of post-mortem tissue such as liver then a wholly different approach will be required. Typically, a representative portion of the tissue will have to be homogenized and the analyte obtained in a relatively pure form by LLE, for example, of the homogenate at an appropriate pH. Further purification or extract concentration steps may be needed prior to instrumental analysis. In the case of organic poisons, this will usually be by a chromatographic method such as GC or LC because both qualitative and quantitative information can be obtained during the course of the analysis. The choice of instrument may influence the choice of sample preparation procedure, although this is not always the case.

For optically active (chiral) drugs (Table 1.4), the desired clinical activity resides predominantly in one enantiomer, the eutomer. The other enantiomer (the distomer) may be either pharmacologically inactive, or have different properties from its enantiomer, so administration of a racemate (a 50:50 mixture of enantiomers, Table 1.5) is the same as giving different compounds as far as the body is concerned. The supply of optically active compounds as pure enantiomers is sometimes indicated by the name used (dexamfetamine, dextropropoxyphene, escitalopram, levorphanol), but this does not always apply (hyoscine, morphine, physostigmine). Moreover, it is thought that some 50 % of currently used drugs are chiral, of which some 88 % are supplied as racemates, usually without any indication of the fact (Nguyen *et al*., 2006). Atropine is the approved name for (±)-hyoscyamine, for example.

With the exception of amfetamine (Section 22.4.21.1) and other misused drugs where the enantiomers have different actions such as dextromethorphan/levomethorphan (levorphanol) and perhaps escitalopram (Section 22.4.3), there are few clear indications for providing chiral methodology for routine analytical toxicology at present. In part this is because chiral analysis at the sensitivity required for the analysis of biological samples is difficult. It should be noted that not only is MS achiral, but also that positional isomers cannot be differentiated by MS unless either resolved chromatographically, or by differing fragmentation patterns. Nevertheless, chromatographic methods have made a major contribution to the development of pharmacology and therapeutics by providing methods to separate enantiomers on a preparative scale and on occasions in biological samples (Fortuna *et al*., 2014).

1.3.3 Method validation and implementation

Whatever analytical method is used it must be validated, i.e. it must be shown to be ‘fit for purpose’. Method validation is important not only when developing a method (Peters *et al*., 2007), but also when implementing a method for routine use (Wille *et al*., 2017). A number of terms important to understanding method validation are given in Table 1.6. A fundamental starting point in any assay is obtaining certified pure reference material, or at least the best approximation to such material that can be sourced. When preparing primary standards, particular attention should be paid to the M_r of salts and their degree of hydration (water of crystallization). Analytical results are normally reported in terms of free acid or base and not of a salt (Section 3.1.1).

A method must possess adequate sensitivity for the task in hand. The limit of sensitivity is a term often used to describe the limit of accurate measurement, but this is better defined as the lower limit of quantification (LLoQ). The limit of detection (detection limit) is a better term for limit of sensitivity. Whatever terminology is used, assessing the presence or absence of a

Table 1.4 Summary of chiral nomenclature

Number of chiral centres	If n = number of optical centres there will be $\mathbf{2^n}$ **isomers.** Molecules with two optical centres can exist as four molecules: two diasteromers (diastereoisomers), each consisting of two enantiomers, i.e. there are *two pairs of enantiomers*. The exception to this is if two molecules have a *plane of symmetry* (a plane that divides a molecule into two parts, each a mirror image of the other) and therefore cancel out their net optical rotation. In such cases they are known as **meso** forms
Nomenclature	Enantiomers possess a unique property in that they rotate plane-polarized light to the same extent, but in opposite directions. This is the basis of the (+)/(–) or d/l notation, the former being preferred as it avoids confusion with D/L, however it does not unequivocally distinguish between enantiomers because some molecules may change rotation on forming salts. The notation tells nothing about the absolute configuration, i.e. the spatial arrangement, of the atoms
Rotation of plane polarized light	Rotates to the right – dextrorotatory (+) Rotates to the left – levorotatory (–)
Fischer	The absolute stereochemistry in the Fischer notation gives the absolute spatial arrangement by reference to D-glyceraldehyde. The letters D or L are used (not to be confused with lower case d or l). The Fischer convention is still used for carbohydrates and amino acids. The original choice of D-glyceraldehyde was arbitrary, but was proved correct by X-ray crystallography
Cahn–Ingold–Prelog (CIP)	The CIP system is the definitive method of assigning absolute configuration. The letters *R* and *S* indicate spatial arrangements as follows: 1. Assign values to the substituent groups by highest atomic number[a] 2. Point the lowest value away 3. If the remainder go from high to low clockwise then *R* (rectus) 4. If the remainder go from high to low anticlockwise then *S* (sinister) 5. In case of a tie go to the next atoms along The Fischer convention cannot be simply converted to the CIP system, i.e. *R* does not always equate to D. All naturally occurring α-amino acids in mammalian proteins are L-. Using the CIP system, cysteine and cystine are *S*- the others, without sulfur, are *R*-

[a]The rules are in fact more detailed: Highest atomic number > highest atomic mass > *cis*- prior to *trans*- > like pairs (*RR*) or (*SS*) prior to unlike > lone pairs are considered an atom of atomic number 0

compound at the limit of assay sensitivity is always difficult and ideally reporting a positive finding in such circumstances requires corroboration from other evidence.

Quantitative methods must have good precision (reproducibility) and accuracy (the results must reflect the true concentration of the analyte). Selectivity (freedom from interference, specificity) is important when a single species is to be measured, but broad specificity may be useful when screening for the presence of a particular class of compounds as discussed above. The recovery of the analyte, i.e. the proportion of the compound of interest that is recovered

Table 1.5 Some terms used in stereochemistry

Absolute stereochemistry	The absolute spatial configuration of atoms of a molecule
Chiral	Hand-like, i.e. left- and right-handed mirror images
Enantiomer	One mirror image from of a pair of non-superimposable optically active compounds
Epimers	Optically active molecules with more than two chiral centres differing at only one chiral centre
Epimerization	Partial racemization of one chiral centre in a molecule with two or more chiral centres
Diastereomers	Stereoisomers that are not mirror images of each other
Inversion	Conversion of one enantiomer to the other
Meso	Optically inactive isomer in which the activity of chiral centres are balanced
Racemate	Equimolar mixture of both enantiomers of an optically active compound
Racemization	Conversion of a single enantiomer to a racemate

from the sample matrix during an extraction, for example, is important if sensitivity is limiting, but need not be an issue if the LLoD, accuracy, and precision of the assay are acceptable.

Ideally, whatever the methodology employed, quantitative assay calibration should be by analysis of standard solutions of each analyte (normally 6–8 concentrations across the calibration range) prepared in the same matrix and analyzed as a batch along with the test samples. A graph of response against analyte concentration should be prepared and used to calculate the analyte concentration in the sample (so-called 'external standard' method). Use of a full calibration sequence may not be possible in emergency toxicology, for example, and in such circumstances single-point calibration is often acceptable if properly validated (Section 3.2.4.10).

Any quantitative analysis is a measurement and, in common with all measurements, has associated errors, both random and systematic. In chromatographic and other separation methods the 'internal standard' method is often used to reduce the impact of systematic errors such as variations in injection volume or evaporation of extraction solvent during the analysis. Thus, a known amount of a second compound that behaves similarly to the analyte during the analysis, but elutes at a different place on the chromatogram or is otherwise detected independently of the analyte (the internal standard, ISTD) is added at an appropriate stage in the analysis. Subsequently, the detector response of the analyte relative to the response of the ISTD is plotted against analyte concentration when constructing a calibration graph (Section 3.3). In the case of MS methods, stable isotope-labelled analogues of the analyte should be used if available. The use of a marketed medicament should be avoided otherwise a significant bias may result if the patient has taken this drug.

1.3.4 Quality control and quality assessment

Once an analytical method has been validated and implemented it is important to be able to show that the method continues to perform as intended. In qualitative work, known positive and negative specimens should normally be analyzed in the same analytical sequence as the test sample. A negative control ('blank') helps to ensure that false positives (owing to, for example,

Table 1.6 Terms used when reporting method validation

Term	Notes
Accuracy	The difference between the measured value and the accepted ('true') value
Calibration range	The range of concentrations between the highest and lowest calibration standards. This should encompass the range of concentrations found in the test samples
Carry over	Signal from the last sample analysis enhancing response in the next analysis
Carry under	Signal from the last sample analysis reducing response in the next analysis
Coefficient of variation (CV)	An alternative term for relative standard deviation (RSD)
'Cut-off'	An analyte concentration above or below which positive results are not reported (see Section 18.1.2)
Higher or upper limit of quantification (HLoQ/ULoQ)	The highest concentration that can be quantified. Not always quoted, but important in assays with a clear upper limit to a calibration graph, for example immunoassays and fluorescence assays
Internal quality control (IQC)	A sample of known analyte concentration that is analyzed as if it were a real sample
Internal standard (ISTD)	A second compound, not the analyte, added at an appropriate stage in the assay to correct for systematic errors in the analysis
Lower limit of detection/limit of detection (LLoD/LoD)	The smallest amount of analyte that can be detected. Usually defined as some multiple (5 for example) of the baseline noise (signal to noise ratio = 5) or a multiple of the standard deviation (SD) of the blank signal
Linearity	A definable and reproducible relationship between a physicochemical measurement (e.g. UV absorption) and the concentration of the analyte. Not necessarily a straight line
Lower limit of quantification/limit of quantification (LLoQ/LoQ)	The lowest concentration that can be measured within defined limits. Usually a concentration for which the precision and accuracy have been set arbitrarily, e.g. RSD <20 %
Measurement uncertainty (MU)	The 95 % confidence interval of the sum of the IQC RSD values given by an assay over a defined period
Precision	The scatter of measured values about a mean value. Usually quoted as RSD – within-assay and between-assay precision is commonly given
Relative standard deviation (RSD)	The standard deviation of replicate measurements expressed as a percentage of the mean value: RSD = SD/Mean x 100 % Useful when comparing precision at different concentrations
Selectivity	The ability to distinguish between the analyte and some other compound
Signal to noise (S/N) ratio	Strictly, the response to the analyte divided by the amplitude of random electronic noise of the detection system. In practice, the background signal due to interfering compounds is often greater than the electronic noise

contaminated reagents or glassware, or carry-over from a previous analysis) are not obtained. Equally, inclusion of a true positive serves to check that any reagents have been prepared properly and have remained stable.

In quantitative work, assay performance is monitored by the systematic analysis of internal quality control (IQC) samples, independently prepared standard solutions of known composition prepared in the same matrix as the samples and not used in assay calibration. Plotting the results for the IQC samples on a chart allows the day-to day performance of the assay to be monitored and gives warning of any actual or impending problems. When new batches of calibration and IQC samples are prepared it is prudent to ensure comparability of the results obtained with those given by an earlier batch, or with the results obtained using external QC material.

An important parameter in the routine use of a method is measurement uncertainty (MU). This is expressed as ±1.96 CV %, in other words the 95 % confidence interval of the sum of the IQC CV values given by an assay over a defined period (Section 3.2.3.4). An especial issue concerns accuracy at a limit defined in legislation such as drink- and drugs-driving legislation. Here it is good practice to allow 3 SDs error (99.9 % of measurements will be either at, or above the limit) before reporting a positive result as a 'not less than' concentration.

Participation in appropriate external quality assessment (EQA)/proficiency testing (PT) schemes is also important. In such schemes, (sometimes lyophilized) plasma, serum, whole blood, urine, or hair specimens are sent to a number of participating laboratories. After reconstitution in deionized water if appropriate, the specimens are analyzed as if they were real samples and the results are reported before the true or target concentrations are made known (Section 3.6.2).

1.4 Applications of analytical toxicology

Requests for toxicological analyses include (i) emergency and general hospital toxicology, including 'poisons screening', and (ii) more specialized categories such as screening for substance misuse, forensic toxicology, TDM, and occupational/environmental toxicology. However, there is considerable overlap between all of these areas (Smith & Bluth, 2016).

1.4.1 Clinical toxicology

The specialized nature of analytical toxicological investigations and the expense of modern equipment dictate that facilities are concentrated in centres that are often remote from the patient. Frequently, routine clinical chemical tests will be performed at one site, whilst more complex toxicological analysis will be performed by a different department, possibly at a different location. The toxicology laboratory will usually undertake a range of analyses in addition to emergency toxicology.

Despite physical separation, the importance of direct liaison between the physician treating the patient and the analytical toxicologist cannot be over-emphasized (Flanagan *et al*., 2013; Thompson *et al*., 2014). Ideally, this liaison should commence before any specimens are collected because some analytes, toxic metals for example, require special precautions in specimen collection (Section 21.2). At the other extreme, residues of samples held in a clinical chemistry laboratory or by other departments, for example in the emergency department refrigerator or in the histology department, can be invaluable if the possibility of poisoning is raised in retrospect (Vuori *et al*., 2013).

Toxicology screening is normally performed using immunoassays and/or temperature programmed capillary GC-MS or gradient elution LC-MS. Proponents of STA sometimes overstate the case for absolute reproducibility of retention data. In practice many factors (clinical and

circumstantial evidence, availability of a particular poison, past medical history, occupation, number of peaks present on the chromatogram, selective detector responses, MS fragmentation patterns, and so on) are considered before reporting results. For example, Grapp *et al*. (2018) positively identified drugs by accurate mass measurement (±5 ppm for $[M+H]^+$; ±10 ppm for $[M-H]^-$), LC retention time (±0.35 min), isotopic pattern match (less than 10 *m/z* root mean square error [RMS, ppm]), isotope match intensity (less than 20 % RMS) and the presence of at least two fragment ions.

The range of analyses that can be offered by specialized laboratories, sometimes on an emergency basis, usually encompasses several hundred poisons. 'Poisons screens' must use reasonable amounts of commonly available samples (20–30 mL urine, 2–5 mL plasma). If any tests are to influence immediate patient management, the (preliminary) results should be available within 2–3 h of receiving the specimens (ideally 1 h in the case of paracetamol). In some cases, the presence of more than one poison may complicate the analysis and examination of further specimens may be required.

A quantitative analysis carried out on whole blood or plasma is usually needed to confirm poisoning unequivocally, but this may not be possible if laboratory facilities are limited, or if the compound is particularly difficult to measure. It is important to discuss the scope and limitations of the tests performed with the clinician concerned and to maintain high standards of laboratory practice, especially when performing tests on an emergency basis. It may be better to offer no result rather than misleading data based on unreliable tests. Clinicians often treat poisoned patients on the basis of suspicion and history rather than await the results of a laboratory test, but may change their approach once they have the result. The treatment of paracetamol poisoning is an example.

Circumstantial evidence of the compound(s) involved in a poisoning episode is often ambiguous and thus, on the rare occasions when an analysis for 'poisons' is indicated, it is advisable to perform a 'poisons screen' routinely in all but the simplest cases. Similarly, the analysis should not end after the first positive finding because additional, hitherto unsuspected compounds may be present. An exception is sub-lethal carbon monoxide poisoning, which can be difficult to diagnose even if carboxyhaemoglobin measurements are available – circumstantial evidence of poisoning may prove invaluable in such cases. Of course, a positive result on a 'poisons screen' does not of itself confirm poisoning because such a result may arise from incidental or occupational exposure to the poison in question, or the use of drugs in treatment.

Blood is often the easiest specimen to obtain from an unconscious patient and is needed for any quantitative measurements. Urine is also a valuable specimen not only because relatively large volumes are often available, but also because it is by far the easiest specimen to obtain, especially from patients likely to have damaged veins. Moreover, some compounds may be detected in urine after they have been cleared from blood. In addition, human urine presents less of a hazard than blood to laboratory staff. However, some compounds such as many benzodiazepines are extensively metabolized prior to excretion and then blood plasma is the specimen of choice for detecting the parent compound. Quantitative measurements in urine are generally of little use in emergency toxicology.

All poisons screens have limitations (Table 1.7). Thus, of the drugs commonly used to treat depression, lithium has to be looked for specifically, whilst those monoamine oxidase inhibitors (MAOIs) that act irreversibly, such as phenelzine, have a prolonged action in the body even though plasma concentrations are very low after overdosage. Any drug that is not bound to the enzyme may be excreted rapidly and may be difficult to detect except in a urine specimen obtained soon after the event. Tricyclic antidepressants are very lipophilic hence urinary concentrations, even after fatal poisoning, may be below the LLoD of the analytical

Table 1.7 Some analytes not normally included in analytical toxicology screens

Anabolic steroids	Heavy/toxic metal ions (antimony, arsenic, cadmium, lead, mercury)
Anticoagulants (apixaban, dabigatran, rivaroxaban, warfarin)	γ-Hydroxybutyrate (GHB)
Baclofen	Hypoglycaemic agents (gliclazide, insulin, metformin)
Carboxyhaemoglobin	Iron salts
Chloral hydrate	Laxatives (phenolphthalein)
Digoxin and other *Digitalis* glycosides	Lithium ion
2,4-Dinitrophenol (DNP)	Organophosphorus and other pesticides
Diuretics (furosemide, chlortalidone)	Thallium ion
Ethylene glycol (1,2-ethanediol)	Volatile compounds (butane, nitrous oxide)
Fungal/plant toxins (α-amanitin)	

method if death has occurred relatively soon after the ingestion. Poisoning with endogenous substances such as sodium and potassium is likewise often very difficult to diagnose (Belsey & Flanagan, 2016).

1.4.2 Forensic toxicology

Toxicological investigations of deaths (including deaths in road traffic accidents) are often undertaken if there is a possibility that drugs or other poisons may have been involved. These include instances where deliberate poisoning, including self-poisoning, is a possibility, especially if death has occurred in children or whilst in police custody, or when decomposition has taken place to such an extent that it is difficult to glean much information as to the cause of death from a conventional post-mortem examination. The value of giving as full a clinical, occupational, or circumstantial history as possible, together with a copy of the post-mortem report, if available, when submitting samples for analysis cannot be overemphasized. Not only might this help target the analysis to likely poisons, but also the interpretation of any analytical results may be greatly simplified.

Non-fatal incidents where toxicological investigations may be useful include collapse whilst in custody, alleged offences under road traffic legislation involving ethanol or other drugs, allegations of poisoning of relatives or pets, doping in sex offences, and other cases of assault. It may also be important to analyze samples from a suspect for the presence of drugs such as ethanol, which may have altered his or her perception or behaviour during the course of a crime.

The specimens available may range from fresh blood to decomposing tissues recovered from a partial skeleton, while the quantity available may range from a kilogram of liver to a dried blood stain. Difficult areas still include screening for a wide range of compounds which could affect driving performance in, say, 2 mL of whole blood while leaving sufficient sample for a quantitative measurement, and detecting drugs used in sexual or other assaults (drug facilitated crime, DFC). Detailed guidelines as to sample collection and the scope of the analyses required in this latter case are available (UNODC, 2011).

An important role of the Coroner in England and Wales, of the Procurator Fiscal in Scotland, and of the Medical Examiner or equivalent in other jurisdictions is to establish how the deceased came by their death. The data derived from such investigations may be invaluable in monitoring the incidence of fatal poisoning. The importance of adequately documenting all acute poisoning incidents both in the hospital notes and in the laboratory records becomes clear when it is remembered that even an apparently trivial case may eventually be reviewed in detail in a coroner's or other court. Required documentation includes correctly recorded patient and sample details, the date and time of collection of samples, details of physical examination, nature and timing of treatment, particularly drug treatment, results of investigations (including units), and conversations with poisons information services and the laboratory. The laboratory should fully document all analyses and keep copies of all the reports issued. Residues of samples should be kept, appropriately stored, e.g. at –20 °C, until the conclusion of the investigation.

In assessing the evidence of the analytical toxicologist, courts of law are concerned especially with the experience of the analyst, the origin and condition of the samples, and the analytical methods used. The ability to prove continuous and proper custody of the specimen is important. It used to be argued that evidence from a minimum of two unrelated analytical methods should be employed before a tentative identification is accepted, but with the advent of GC-MS and LC-MS methods this is often no longer the case, the MS data being regarded as orthogonal to the chromatographic data. The results should be presented together with sufficient information to ensure accurate interpretation of the findings by a coroner, magistrate, judge, and/or jury. There is always the possibility of an independent examination by a further expert instructed by another party in the case.

1.4.3 Testing for substance misuse

The value of blood, breath, or urinary measurements in the diagnosis of ethanol misuse and in monitoring abstinence is clear. Screening for substance misuse in urine is also valuable in monitoring illicit drug taking in dependent patients and guards against prescribing controlled drugs for patients who are not themselves drug dependent. Some substances disappear from biological samples very rapidly and, depending on the time between administration and sample collection, the parent compound may not be detectable. Sometimes, however, metabolite identification can be used to demonstrate systemic exposure to a particular drug. 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) is monitored to demonstrate systemic exposure to methadone, for example. Other samples, such as oral fluid, exhaled air, sweat, and meconium can also provide useful samples for specific purposes (Pleil, 2016; Chapter 18).

Analysis of hair, long advocated as a way to assess exposure to toxic metals, can also provide a history of exposure to illicit drugs and other organic poisons, but care is needed in the interpretation of results. Analytes including metabolites can arise in or on hair in many ways, and indeed attempts to remove external contamination may move analytes from the hair surface into the medulla, giving the impression that the analyte was present in the body when the hair was formed in the hair follicle (Cuypers & Flanagan, 2018).

Testing for substance misuse may also be valuable in the psychiatric assessment of patients presenting with no overt history of drug misuse. In addition, the diagnosis of maternal drug use, either during pregnancy or post-partum, can be important in the management of the neonate. The need for substance misuse screening of personnel in sensitive positions (armed forces, security services, pilots, drivers) or those applying for such positions ('employment' and 'pre-employment' screening, respectively), has become accepted in recent years. The detection of illicit or performance-enhancing drug use in sport has also assumed importance. In animal

sports the definition of an illicit compound is much easier than in man and can include any substance not normally derived from feedstuffs.

The illicit drugs encountered most commonly in the UK are opioids, mainly heroin (Box 1.1), cocaine, amfetamines including amfetamine, metamfetamine and methylenedioxymetamfetamine (MDMA, 'ecstasy'), and cannabis. In the US, misuse of cocaine either as the hydrochloride, or as the free base ('crack') is relatively common, and a range of additional compounds may also be encountered, including dextropropoxyphene (propoxyphene), fentanyl and its analogues, and phencyclidine (PCP, 'angel dust').

Worldwide, whilst traditional drugs such as alcohol, cocaine, heroin, and metamfetamine, continue to dominate, the last few years have seen the emergence of so-called 'legal highs' (new or novel pharmaceutical substances, NPS), which can be divided broadly into novel stimulants, synthetic cannabinoids, and analogues of fentanyl and other synthetic opioids such as carfentanil. Increasingly these compounds are mentioned on death certificates in England and Wales. The synthetic opioids and cannabinoids are particularly potent and thus dangerous (Handley *et al*., 2018).

The purity of 'street' drugs varies widely – heroin may be between 2 and 95 % pure, for example. Overdosage, either with excessively pure 'street' drug, or with drug 'cut' with a particularly toxic compound such as strychnine, is a further cause of acute poisoning 'epidemics'. Compounds such as atropine, barbiturates, chloroquine, ephedrine, levamisole, lidocaine, methaqualone, phenacetin, phentermine, quinine, and strychnine may be used to 'cut' street drugs. Serious acute poisoning may occur if tolerance to heroin or methadone has been reduced through abstinence. Methadone is widely used in the management opioid addiction, although buprenorphine is also employed in this role. Misuse of other opioids such as codeine, dihydrocodeine, oxycodone, and pethidine (meperidine) may also be encountered.

The availability of a variety of immunoassay kits has proved invaluable, especially in employment and pre-employment screening when large numbers of negative results are to be expected and high sensitivity is required. However, confirmation of positive results with MS linked either to capillary GC or LC is essential. In clinical samples TLC can be used to resolve drugs such as morphine from compounds such as codeine and pholcodine that are available in over-the-counter preparations. TLC requires a minimum of apparatus and is generally cost effective. It is also amenable to batch sample processing, but is labour intensive, analyte capacity is low, and interpretation of results can be anything but straightforward. Capillary GC, GC-MS, or LC-MS is used to detect and identify amfetamines, and increasingly to confirm immunoassay results.

Ingestion of diuretics and/or laxatives either to produce weight loss, or to provoke covert alterations in body biochemistry (Munchausen syndrome, factitious illness) is uncommon and can be difficult to diagnose. Collection of serial urine samples over several days is advisable (Section 22.4.12). Detection of the misuse of osmotic laxatives such as lactulose and bulk-formers such as bran is not possible analytically. The covert ingestion or administration of anticoagulants and antidiabetic drugs is also well documented, but can be difficult to diagnose.

1.4.4 Therapeutic drug monitoring

The measurement of plasma concentrations of drugs given in therapy is useful in assessing adherence and for compounds for which pharmacological effects cannot be assessed easily and for which the margin between adequate dosage and overdosage is small, as discussed in Chapter 20. The availability of a variety of immunoassay and other kits means that many TDM assays can be performed more conveniently by such means than by chromatographic methods. However, chromatographic assays are still important in the case of antiepileptic,

antimicrobial, antipsychotic, and immunosuppressant drugs, for amiodarone, where it has proved impossible to produce an antibody that does not cross-react significantly with thyroxine and tri-iodothyronine, and in general where active metabolites need to be measured as well as the parent compound. Examples include carbamazepine/carbamazepine-10,11-epoxide, clozapine/norclozapine, procainamide/*N*-acetylprocainamide, and amitriptyline/nortriptyline.

1.4.5 Occupational and environmental toxicology

The monitoring of occupational or environmental exposure to toxic substances is an important area. Metal ions such as lead and also some organochlorine pesticides such as chlordane and dieldrin have long half-lives in the body and thus accumulation can occur with prolonged exposure to relatively low concentrations. The manufacture of drugs can also present a hazard to those involved via either dermal, or inhalational absorption. The misuse of alcohol and of controlled drugs is of concern in occupational medicine, especially as regards screening for substance misuse amongst potential employees and amongst, for example, operators of heavy machinery and pilots, as discussed above.

Control of occupational exposure to toxic metals, volatile solvents, and of some other substances is an integral part of industrial hygiene and has been achieved, in part, by monitoring ambient air concentrations of the compound(s) under investigation. However, an individual's work pattern and attention to safety procedures may greatly influence exposure and 'biological effect' monitoring, where clinical chemical parameters such as blood zinc protoporphyrin are measured as an indicator of lead exposure, is required practice in certain occupations.

Not all poisons are amenable to effect monitoring and so 'biological' monitoring is performed widely. This involves measuring blood, urinary, or breath concentrations of a compound, and possibly of its metabolites. The ACGIH (2018) recommends Threshold Limit Values (TLVs) for more than 700 chemical substances and physical agents. There are also more than 50 Biological Exposure Indices (BEIs) that cover more than 80 chemical substances. The DFG (2018) 'MAK List' (maximum allowable concentration in workplace air) classifies more than 1000 compounds according to their toxicological profile and gives threshold values for their use.

The investigation of the accidental release of chemicals into the workplace or into the environment (so-called chemical incidents) is an important area. Examples include the Bhopal disaster in India when methyl isocyanate was released into the atmosphere and the Camelford incident in the UK, in which aluminium sulfate was accidentally added to the local drinking water supply. Toxicological analyses can be valuable not only in providing evidence of the nature and magnitude of an exposure, but also in demonstrating that no significant exposure has occurred, thereby allaying public apprehension. Clearly, the early collection of appropriate biological samples is essential.

In the absence of information to the contrary it is wise to collect 10 mL whole blood (2 x 5 mL EDTA anticoagulant) and at least 25–50 mL urine (no preservative) from exposed or possibly exposed individuals. The time and date of sampling and the patient's full names should be recorded on the samples and also on a separate record sheet. The samples should be stored at either 4 °C or –20 °C until the appropriate analyses can be arranged. If the incident is investigated in retrospect then samples may exist in a local hospital laboratory, for example.

The increasing use of herbal or other 'natural' remedies is an area of especial concern. Whilst most, but by no means all, herbal preparations are innocuous (Belsey & Karch, 2015; Byeon *et al*., 2019), adulteration or contamination with dust, pollens, insects, rodents, parasites, microbes, fungi, mould, toxins, pesticides, toxic metals, and/or prescription drugs, is not uncommon (Byard, 2010; Posadzki *et al*., 2013; Steyn *et al*., 2018). This adds to the difficulty

of diagnosing poisoning with these agents, each of which many contain dozens if not hundreds of different compounds, all at relatively low concentrations.

One area that has been neglected somewhat is that of food-derived poisons. Botulinum toxin and other toxins of microbiological origin are usually considered together with food poisoning. Poisoning from other naturally occurring substances, which include atropine from *Atropa belladonna*, solanine from potatoes, and cyanide from *Cassava* and from apple pips, also occurs, sometimes with homicidal intent (Bonnici *et al*., 2010). Here analysis of the foodstuff rather than biological samples can be more helpful in establishing the diagnosis in individual patients. Acute pesticide poisoning sometimes occurs after ingestion of contaminated produce and again analysis of the foodstuff can be helpful (Nasreddine & Parent-Massin, 2002).

1.5 Summary

It is impossible to divorce the study of the analytical methods used in performing toxicological analysis on biological and related samples from the study of toxicology itself, especially clinical and forensic toxicology. By the same token, the laboratory can do nothing to help in the diagnostic process unless someone, be it a clinician, pathologist, or some other person, first suspects poisoning and ensures that specimens are collected and sent for analysis. However, appropriate sample collection and handling is not always straightforward and indeed is a subject in its own right.

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2 Sample Collection, Transport, and Storage

2.1 Introduction

In analytical toxicology, no matter how complex the equipment and careful the analysis, the results may be rendered worthless if sample collection, transport, and storage have not been performed with the analysis in mind. Thus, it is important to be familiar with the nature and stability of the analyte(s), the nature of the sample matrix, and the circumstances under which the analyses are to be performed. Proper documentation of the history of the sample (origin, mode of collection, transport, storage, chain-of-custody documentation, and the like) is essential.

The analyte concentration in the specimen is generally assumed to be representative of the concentration in the particular fluid or tissue sampled. Anticoagulated whole blood, plasma (the fluid obtained on centrifugation of anticoagulated blood), or serum (the fluid remaining when blood has clotted) are widely used in clinical work. This is because not only is blood relatively easy to collect, but also a quantitative analysis can give useful information as to the magnitude of exposure and hence the severity of poisoning. Excretions (exhaled air, urine) or secretions (saliva, sweat) are often less useful as regards interpretation of quantitative data, but can be useful in qualitative work.

Variations in bioanalytical measurements may be subject-dependent and reflect normal physiological changes, whilst others may reflect sample collection and handling procedures (Table 2.1). Post-mortem specimens are a special problem because, generally, information on the analyte concentration in blood *at the time of death* is required. Post-mortem blood concentrations may not accurately reflect peri-mortem blood concentrations for several reasons. Haemolysis is common, for example, whilst haemostasis may lead to changes in the cellular composition of the 'blood' being sampled. There is also the possibility of contamination during collection, e.g. with stomach contents, and of leakage of analyte from adjacent tissues into blood. The leakage of intra-cellular potassium into plasma, which begins soon after death, is such an example.

2.2 Clinical samples and sampling

2.2.1 Health and safety

Biological samples may contain infective agents and must be handled with care, especially if originating from substance misusers, and must always be treated as if they are infective. The major common risks are associated with tuberculosis, hepatitis B and C, and human

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Table 2.1 Types of variables affecting clinical samples

Variable	Example(s)
(i) Physiological	
Age	Markers of bone turnover such as plasma alkaline phosphatase activity are increased in childhood
Sex	Sex hormones
Body weight	Urinary creatinine excretion increases with muscle mass
Recent food intake	Plasma glucose, triglycerides, etc. increase after a normal meal. May delay and/or reduce absorption of some drugs, but increase absorption of others
Diet	Malnutrition or fasting will reduce serum albumin, urea, and phosphate, amongst other parameters
Circadian variation	Some analytes (e.g. cortisol, iron) show diurnal changes in plasma concentration
Menstrual cycle	Plasma concentrations of luteinizing hormone, follicle stimulating hormone, oestradiol, and progesterone vary with the cycle
Seasonal	Vitamin D metabolites higher in summer
Pregnancy	Plasma concentrations of human chorionic gonadotropin, oestradiol, and other biochemicals vary throughout pregnancy
Psychological changes	Venepuncture or hospitalization may increase plasma concentrations of stress-related compounds such as catecholamines, cortisol, and prolactin
Physiological changes	Posture may affect measurements such as plasma aldosterone and albumin. Exercise can change the blood concentrations of compounds such as lactate
Drugs	Drug treatment may alter concentrations of some plasma constituents even in apparently healthy subjects (e.g. trimethoprim increases serum creatinine)
(ii) Sample/analyte variations	
Incorrect specimen	Value differences between plasma and serum, venous and arterial blood, random and 24 h urine samples (e.g. potassium is on average higher in serum than in plasma)
Incorrect collection	Contamination with ethanol as a skin disinfectant prior to venepuncture may invalidate ethanol assay; use of 2-propanol or other alcohols may also invalidate ethanol assay if used as an internal standard
Incorrect sample tube	The absence of an appropriate enzyme inhibitor may allow continued enzyme action such as catabolism of glucose or neuropeptides. Failure to acidify urine will decrease urinary catecholamines. Collection into EDTA will decrease plasma calcium. Use of Li heparin tube will invalidate plasma Li assay
Haemolysis	Red cell lysis may lead to changes in plasma constituents, particularly potassium, phosphate, and some enzymes, and may interfere with the analytical method
Cellular contamination	The presence of platelets following incorrect centrifugation will apparently raise plasma serotonin
Incorrect/excessive storage	Some compounds may oxidize even when frozen [e.g. formation of adrenochrome from 5-hydroxyindoleacetic acid (5-HIAA)], or be subject to bacterial degradation (e.g. amino acids in acidified urine). Loss of water when frozen (freeze-drying effect), but may attract water if tube open when contents thawed
Collection during an infusion	Collection near to an infusion site or using a needle used to give an infusion will give misleading concentrations of the compound being infused, or dilute other blood constituents
Drug treatment	Other drugs or metabolites may interfere in the assay

immunodeficiency virus (HIV). Urine is least likely to be infective. It is thought very likely that following solvent extraction or other robust sample preparation procedure, infective agents will be inactivated, except for variant Creutzfeldt-Jakob Disease (CJD), but in homogenous assays such as immunoassays samples may continue to be infective after incubation, even though diluted. Indeed, incubation may increase the titre of the infective agent.

Staff in regular contact with potentially infective materials must be properly trained in the safe handling and disposal of biological samples. Such staff should be vaccinated against hepatitis B, polio, tuberculosis, and tetanus, and possibly other diseases in specific countries. Sample handling should be performed with due attention to preventing droplets splashing into the eyes and minimizing aerosol formation (wear eye protection, perform mixing and other procedures in a microbiological safety cabinet, always use either sealable centrifuge tubes or a centrifuge with sealable rotors). Screw-capped sample tubes are preferable to those with push in stoppers because there is less risk of aerosol formation when opening the tube.

2.2.2 Clinical sample types

Clinical samples can be divided into (i) blood and related fluids, (ii) body fluids other than blood, (iii) excretory fluids/residues, and (iv) other clinical specimens (Table 2.2). A range of additional specimens may be collected for toxicological purposes. Special precautions will be needed with unstable analytes. Most compounds measured in urine can be considered stable for at least a few hours at room temperature because the urine may already have been held at body temperature for some time before it was voided. Nevertheless, stability testing should be included in any method validation work.

2.2.3 Blood and blood fractions

2.2.3.1 Arterial blood

Arterial blood is normally collected by an experienced medical practitioner – it is a relatively dangerous procedure – for the measurement of blood gases and is rarely submitted for toxicological analysis. Capillary blood, which closely approximates to arterial blood, can be obtained by pricking the heel, finger or ear lobe; this procedure is most often performed on small children. Applying pressure to encourage blood flow will dilute the sample with tissue fluid.

2.2.3.2 Venous blood

Venous blood is obtained by venepuncture of (usually) the median cubital vein of an arm remote from any infusion site. Either a hypodermic needle and syringe (1–50 mL) or a commercial vacuum-sampling system such as a Vacutainer™ may be employed. Care should be taken to ensure that 2-propanol or other alcohols are not introduced into the sample when using swabs to clean/disinfect the skin. A tourniquet can be used to distend the vein prior to venepuncture, but should be released immediately prior to sampling.

For repeated sampling, a small cannula may be inserted into, for example, a vein in an arm or hand that allows venous access via a rubber septum. However, maintaining patency may be a problem, and there may be risks of (i) inducing haemolysis and (ii) of specimen contamination due to use of anticoagulant or local anaesthetic solutions with such devices. The use of heparin anticoagulant solutions that contain phenolic preservatives should be avoided.

Following venepuncture, blood should be transferred into an appropriate container as soon as practicable. Some basic analytes and quaternary ammonium compounds, e.g. tricyclic antidepressants (TCAs) and paraquat, and aluminium bind to glass. Plastic tubes are thus preferred and are also less likely to shatter than glass, especially if frozen. On the other hand, if volatile

Table 2.2 Some clinical sample types

Blood & related fluids	**Blood** ('whole blood') is the fluid that circulates through the arteries, capillaries, and veins. The adult human body contains some 5–6 litres of blood. It is composed of plasma and blood cells. Normally venous blood is obtained (Section 2.2.3.2). If whole blood is to be analyzed, then the sample should be collected into an appropriate anticoagulant, mixed, and then frozen in order to lyse the cells before the analysis. **Dried blood spots (DBS)** are the residues remaining when whole blood has been allowed to dry on filter paper or another appropriate medium. **Post-mortem blood** is blood obtained after death, usually at autopsy. The site of sampling should always be recorded (Section 2.3.2.1). [N.B. Occult blood is altered blood found only in trace amounts particularly in faeces. It is not used as an analytical sample.]
	Blood cells include red cells (erythrocytes) and white cells (lymphocytes, leucocytes, platelets, etc.). All may be harvested from freshly collected blood with appropriate procedures (Section 2.2.3.5)
	Cerebrospinal fluid (CSF) is a plasma ultrafiltrate (i.e. its composition is that of plasma except that high M_r proteins are absent) that surrounds the elements of the central nervous system (CNS). It is obtained by lumbar puncture (needle aspiration from the spinal cord) and is usually collected into sterile tubes. Samples contaminated with blood should be discarded if microbiological assessment is required
	Cord blood is blood obtained from the umbilical cord at parturition. Normally venous cord blood is obtained in order to reflect neonatal, as opposed to placental, blood. It may be possible to obtain plasma or serum depending on the volume available
	Plasma is the liquid portion of blood (Section 2.2.3.4). It is obtained by centrifugation of anticoagulated whole blood
	Serum is the pale yellow fluid remaining when whole blood has clotted. Its composition is generally the same as plasma except that fibrinogen and factors associated with the clotting process are absent (Section 2.2.3.3). It is obtained by centrifugation of whole blood that has been allowed to clot in the sample tube
Body fluids other than blood	**Amniotic fluid** is the fluid that surrounds the foetus in the amniotic sac
	Aqueous humour is the watery fluid occupying the space between the cornea and the iris of the eye
	Bone marrow aspirate is a sample of the spongy tissue found within bones that produces blood cells
	Breast milk is the protein and fat-rich fluid produced by nursing mothers. The first expression of breast milk (colostrum) is especially rich in protein
	Gastric aspirate is an acidic aqueous fluid containing digestive enzymes, food residues, etc. obtained by aspiration from the stomach
	Intraosseous fluid is fluid derived from blood and interstitial fluid residing within bones
	Interstitial fluid is the fluid between blood vessels and cells
	Lymph is a yellowish fluid in the lymph channel secreted from the lymph glands
	Pericardial fluid is fluid present between the heart and the sac surrounding the heart known as the pericardium
	Peritoneal fluid is the fluid that accumulates in the peritoneum

Table 2.2 (*Continued*)

	Saliva is the viscous, clear secretion of the mucous glands in the mouth. It is related in composition to plasma, but also contains some digestive enzymes. **Oral fluid** is the preferred term for the mixture of saliva, gingival crevicular fluid (fluid from the tooth/gum margin), cellular debris, blood, mucus, food particles, and other material collected from the mouth
	Semen is produced by the testes and the prostate gland, and consists of seminal fluid, which may be obtained from semen by centrifugation, and spermatozoa
	Synovial fluid is the clear, viscous, lubricating liquid that fills the synovium (the membrane that surrounds a joint and creates a protective sac)
	Tears are the clear watery secretion of the tear ducts of the eye
	Vaginal fluid is the viscous secretion of the vagina
	Vitreous humour is the transparent, viscous fluid contained behind the lens in the eye
Excretory fluids/residues	**Bile** is the thick yellow-green fluid secreted by the liver via the gall bladder into the intestine
	Exhaled (expired) air generally contains less oxygen and more carbon dioxide and water vapour than ambient air, but may contain other volatile and non-volatile compounds
	Faeces are the brown, semi-solid residues of the digestive process (Section 2.2.6)
	Sweat is the aqueous fluid excreted by the pores of the skin
	Meconium is the dark green substance forming the first faeces of a newborn infant
	Urine is the yellow/yellow-green fluid produced by the kidney. It consists mainly of water, salts, urea, creatinine, and other metabolic products (Section 2.2.4)
Other samples	'**Cavity blood**' is the fluid remaining in the body cavity once the organs have been removed
	Bezoars are stones or concretions found in the alimentary tract of animals
	Bronchoalveolar lavage is obtained by washing the bronchi/alveoli with an appropriate solution and aspirating the resulting fluid
	Calculi ('stones') are hard crystalline deposits formed in various body cavities such as the kidney
	Dialysis fluid (extracorporeal or peritoneal) is the fluid remaining or recovered after dialysis has been performed
	Gastric lavage (stomach wash-out, SWO) is a specimen obtained by washing the stomach with an appropriate solution and aspirating the resulting fluid
	Hair (head, axilliary, or pubic) is sometimes used to assess exposure to poisons such as drugs or toxic metals
	Nails or nail clippings (finger or toe) are sometimes used to assess exposure to drugs or heavy metals
	Nasal swabs are fluid collected onto cotton swabs from inside the nose
	Stomach contents may be (i) gastric aspirate, (ii) gastric lavage, (iii) vomit, or (iv) the residue in the stomach at autopsy (Section 2.2.5)
	Tissue specimens are obtained either surgically, or at post-mortem. Tissue obtained from an aborted foetus and/or placenta may sometimes be presented for analysis. A biopsy sample is a small sample of a tissue obtained by a specialist sampling technique
	Vomit reflects the composition of gastric aspirate

compounds such as solvents or anaesthetic gases are to be analyzed, glass containers are preferred because of the risk of loss of analyte if plastic tubes are used (Section 2.3).

If blood has been collected into a syringe, it is essential that the syringe needle is removed and the blood allowed to flow gently into the collection tube in order to prevent haemolysis. This should be followed by gentle mixing to ensure contact with the anticoagulant if one is being used. Even mild haemolysis will invalidate a serum iron or potassium assay, and may invalidate plasma or serum assays for other analytes concentrated in red cells such as chlortalidone.

Provided the analyte is stable, anticoagulated whole blood can be kept at room temperature or refrigerated (2–8 °C) for 2 days or so before harvesting plasma. However, leaving plasma or serum in contact with red cells can either cause changes as a result of enzymatic activity, or redistribution of an analyte between cells and plasma. Thus, in general, plasma or serum should be separated from the blood cells as soon possible. If necessary, whole blood can be stored at –20 °C or below, but freezing will lyse most cell types.

A range of anticoagulants is available for *in vitro* use (Table 2.3). Sodium citrate tubes may contain 0.5 or 1 mL of an aqueous solution of anticoagulant and are unsuitable for quantitative work. Furthermore, citrate has strong buffering capacity and dilution of the sample may reduce the degree of plasma protein binding and consequently the plasma:red cell distribution of an analyte. It should be ensured that lithium heparin anticoagulant is not used if plasma lithium is to be measured (Arslan *et al*., 2016). Heparin too has been known to interfere in drug analysis.

Table 2.3 Anticoagulants for *in vitro* use

Anticoagulant	Concentration (mL^{-1} blood)	Comment
Lithium heparin	10–20 units	General biochemistry
Sodium heparin	10–20 units	General biochemistry
Sodium fluoride (with either EDTA or oxalate)	1–2 mg 6–10 mg	Glucose (inhibits glycolysis) General anticoagulant[a]
Sodium citrate	3 mg	Clotting studies – not recommended for other purposes because an aqueous solution dilutes the specimen
EDTA	2 mg	Haematology (stabilizes readily oxidized compounds)

[a] *ca*. 2 % w/v fluoride is recommended to attempt to stabilize analytes such as cocaine (Section 2.3) and to prevent fermentation of glucose to ethanol, for example

2.2.3.3 Serum

When whole blood is allowed to stand (15 min, room temperature) in a plain tube (no anticoagulant) a clot normally forms that will retract sufficiently to allow serum to be collected after centrifugation. For many analyses, serum is preferred to plasma because it produces less precipitate (of fibrin) on freezing and thawing.

2.2.3.4 Plasma

Separation of plasma from anticoagulated whole blood normally requires centrifugation, as do many of the phase separation procedures discussed in Chapter 4. The inter-relationship of

centrifuge rotor diameter, speed of centrifugation, and relative centrifugal force (g-force) is set out below (Box 2.1). Swing-out rotors are preferred for separating liquid phases.

Box 2.1 Calculating relative centrifugal force

- The relative centrifugal force (RCF, g) depends upon of the speed of the centrifuge in revolutions per minute (RPM) and the effective radius of rotation, r
- The radius of rotation varies along the length of the centrifuge tube
- RCF may be quoted as maximum, minimum, or average
- Conversion tables and nomograms for each rotor are normally supplied by the manufacturer of the centrifuge
- Modern centrifuges have the facility to set the RCF directly
- The RCF will be maximal at the bottom of the tube
- RPM for a required RCF can be calculated from:

$$\mathrm{RPM} = \sqrt{\frac{\mathrm{RCF}}{(1.118 \times 10^{-6})r}}$$

where r is in mm

- RCF from RPM is given by:

$$\mathrm{RCF} = (1.118 \times 10^{-6})r(\mathrm{RPM})^2$$

On centrifugation, anticoagulated whole blood (approximately 2000 g, 10 min; 2–8 °C if necessary) will separate into three layers: the bottom layer (normally 45 % or thereabouts by volume) consists of red cells. A thin intermediate layer of white cells and platelets called the 'buffy coat' is the next layer; and the upper, aqueous, straw-coloured layer is the plasma (about 50 % v/v). More plasma than serum can be separated from whole blood.

Some commercial tubes contain agents such as plastic beads or a gel that sits at the interface between the cells and the plasma to aid plasma collection. Gel separators have caused problems with some drug analyses (Berk *et al*., 2006), and although reformulated gels have been shown to have little effect on clozapine and norclozapine measurement (Handley *et al*., 2018), tubes containing gel separators are best avoided (Schrapp *et al.,* 2019). Modern blood-collection tubes may contain a range of additives including surfactants, which may interfere in immunoassays, for example (Bowen & Remaley, 2014). The use of such tubes will also invalidate many trace elements analyses (Chapter 21) and may impair analyses for solvents and other volatiles.

2.2.3.5 Blood cells

To collect erythrocytes, heparinized blood should be centrifuged (2000 g, 10 min), the plasma, buffy coat, and top 10 % of erythrocytes (mainly reticulocytes) removed, and the remaining erythrocytes carefully washed with isotonic phosphate-buffered saline (PBS), pH 7.4, to remove trapped plasma. The cells may be used directly or stored at –20 °C, either to cause haemolysis, or for storage. Platelets are usually isolated by the slow centrifugation (e.g. 300 g, 15 min) of anticoagulated whole blood to yield platelet-rich plasma, which is recentrifuged (2000 g, 10 min) to harvest the platelets. Other white blood cells are most commonly obtained either by centrifugation through media of appropriate density (according to manufacturers' instructions), or isolated by solid-phase antibody techniques.

Erythrocyte:plasma distribution

It is easier to measure whole blood:plasma analyte distribution than to attempt to measure the erythrocyte:plasma distribution, a measurement that requires the preparation of washed erythrocytes. Analyte is added to a portion of analyte-free heparinized whole blood and, after allowing time for equilibration and controlling the pH (the pH of blood tends to fall *in vitro*), one portion of the blood is centrifuged to obtain plasma and a second portion is frozen and thawed to give haemolyzed whole blood. After measuring the analyte concentration in the plasma (C_P) and in the haemolyzed whole blood (C_B), the analyte erythrocyte concentration (C_E) can be calculated if the haematocrit, H, (the proportion of erythrocytes in the blood) is known:

$$C_E = \frac{C_B - C_P(1 - H)}{H} \tag{2.1}$$

2.2.3.6 Dried blood spots

DBS are produced by the deposition of either haemolyzed whole blood, or capillary blood onto either filter paper, or dedicated paper cards. As compared with either whole blood or plasma, sample transport and storage are easier because refrigeration is not needed provided that the analyte is stable and can be recovered from the spot quantitatively (Wagner *et al*., 2016; Freeman *et al*., 2018). Problems are that (i) capillary blood is not venous blood, and (ii) volumetric blood collection directly from the patient onto the medium on which the blood is to be dried is extremely difficult (Sulochana *et al*., 2019).

Thus, for qualitative work such as screening for inborn errors of metabolism, or for detection of substance misuse, DBS are acceptable provided that the analyte is stable in the spot, but reliable quantitative work means measuring a known volume of venous blood onto the storage medium before allowing it to dry (Section 20.3.1.1). This being said, in forensic work, measurement of the haemoglobin content of a blood spot found at a scene, for example, may serve to give a surrogate measure of the amount of blood deposited initially.

2.2.3.7 Volumetric microsampling

Volumetric microsampling devices (Figure 2.1) aim to collect a fixed volume of capillary blood, which can then either be made available for analysis in its entirety or can be used to provide

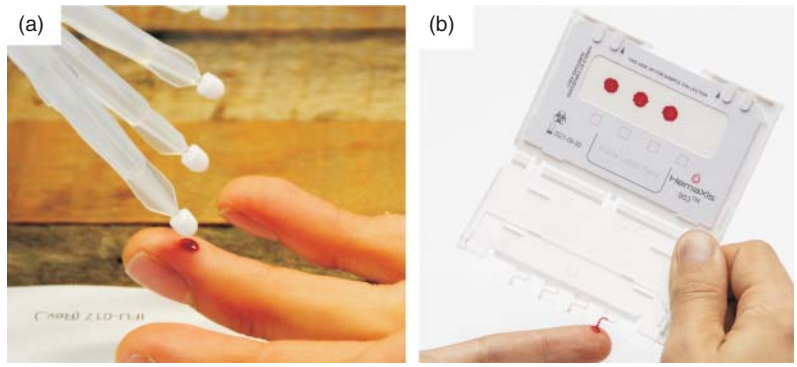

Figure 2.1 Volumetric blood microsampling devices. (a) Mitra (Neoteryx) uses an absorptive pad to collect a fixed volume of blood; (b) HemaXis DB uses a fixed volume capillary to transfer blood to a standard DBS card

a fixed volume of plasma for processing. These devices promise advantages over DBS related to sampling volume accuracy, simplified sample pre-treatment, and ease of automation (John *et al*., 2016).

Volumetric absorptive microsampling (VAMS®) aims to absorb a fixed volume of blood or other fluid (10 or 20 µL) using the Mitra® (Neoteryx) device (Spooner *et al*., 2015; Protti *et al*., 2019). Although some aspects still need to be investigated in depth (Kip *et al*., 2017), VAMS® may be a viable alternative to DBS. It simplifies sample collection and storage, and may allow sample collection in a patient's home (Kok & Fillet, 2018). The HemaXis™ DB 10 (DBS System SA) uses microfluidics to provide 10 µL whole blood from a reservoir onto a DBS card (Leuthold *et al*., 2015; Verplaetse & Henion, 2016). The HemaXis™ DX promises to produce plasma or serum without the use of a centrifuge, filtration membrane, or pump and with no haemolysis. The resulting sample can be stored in dried or liquid format. The Noviplex™ Plasma Prep Card gives a fixed volume of plasma from a reservoir of whole blood (Heussner *et al*., 2017).

2.2.4 Urine

Different urine specimens, e.g. random, early morning, end-of-shift, 24-hour (the total urine voided over a day), may be collected in the course of metabolic or other studies. In metabolic studies, it is important to note the time of the beginning and end of the collection period so that the rate of urine production can be calculated. A random urine sample is usually a midstream specimen. A preservative, for example 2 mol L^{-1} hydrochloric acid to prevent microbial growth and to stabilize phenols, may be added. Fresh urine is yellow/yellow-green in colour, but on storage in acidic solution the colour changes to yellow/brown and even to dark brown because of the oxidation of urobilinogen to urobilin. Crystals, particularly of uric acid and calcium oxalate, may form causing turbidity.

When random, early morning, or end-of-shift specimens are collected it is common practice to relate certain analytical results to a 'fixed' urinary constituent such as creatinine, which is considered to be excreted at a relatively constant rate in normal subjects. However, because creatinine is derived from creatine, there are situations such as muscle wasting, excessive exercise, or in bodybuilders dosing with creatine, when this is not strictly true. Note that many clinical chemistry laboratories report creatinine in mmol L^{-1} (1 mmol L^{-1} = 113 mg L^{-1}).

The concentrations of many drugs and metabolites, and of some endogenous constituents, will remain the same in acidified urine for over a week at room temperature, and for up to a month at 2–8 °C. Unacidified urine undergoes microbiological attack and many changes occur, including the complete loss of amino acids. For long term storage acidified urine can be stored at –20 °C, but it may be necessary to centrifuge the sample to remove any precipitate formed during storage prior to any analysis. Dried urine spots may also be produced in an analogous manner to dried blood spots.

2.2.5 Stomach contents

This specimen encompasses vomit, gastric aspirate, and gastric lavage fluid as well as the contents of the stomach at post-mortem. The nature of this sample can be very variable and additional procedures such as homogenization followed by filtration and/or centrifugation may be required to produce a sample amenable to analysis. Sometimes tablet residues may be apparent, study of which which might help identify the drug ingested.

2.2.6 Faeces

The analysis of faeces is rarely performed in clinical chemistry, but sometimes drug and possibly metabolite analysis may be required in pharmacokinetic and metabolism studies. Analyses may also be requested if, for example, drug leakage from ingested packets of drug is suspected. Unlike plasma, urine, and other fluid samples, faeces are not homogeneous, and thus it is often necessary to analyze the whole sample or homogenize the whole sample and prove that the fraction taken for analysis is representative of the whole. It may take more than a day after dosage before a drug or a drug metabolite appears in faeces. The stability of some drugs in meconium has been assessed (Wu *et al*., 2017).

2.2.7 Tissues

Histology specimens are usually collected into a preservative such as formalin (37 % w/v) aqueous formaldehyde solution). Such pre-treatment must be borne in mind if toxicological analyses are requested subsequently. Samples of tissue obtained post-mortem are normally kept at 2–8 °C prior to analysis.

2.3 Guidelines for sample collection for analytical toxicology

If poisoning is suspected, a 10 mL blood sample (lithium heparin or EDTA tube) should be taken from an adult (proportionally less from a young child) as soon as possible. In addition, 2 mL of blood should be collected in a fluoride/oxalate tube if ethanol is suspected. Note that tubes of this type for clinical use contain only *ca*. 0.1 % w/v fluoride (Table 2.3), whereas *ca*. 2 % w/v fluoride (40 mg sodium fluoride per 2 mL blood) is needed to inhibit fully microbial action in such specimens. Addition of fluoride may also help protect other labile drugs such as clonazepam, cocaine, and nitrazepam from degradation. If possible, the retention of an unpreserved blood sample is also advisable.

Table 2.4 Sample requirements: general analytical toxicology

Sample	Notes[a]
Whole blood	10 mL (lithium heparin or EDTA tube – use fluoride/oxalate if ethanol suspected; plastic tube if paraquat suspected; glass or plastic tube with minimal headspace if carbon monoxide or other volatiles suspected)
or Plasma/serum	5 mL (send whole blood if volatiles, metals, and some other compounds suspected – see above)
Urine[b]	20–50 mL (plain bottle, no preservative[c])
Gastric contents[d]	25–50 mL (plain bottle, no preservative)
Scene residues[e]	As appropriate

[a] Smaller volumes may often be acceptable, for example in the case of young children
[b] Normally the only sample that is required for substance misuse screening
[c] Sodium fluoride (2 % w/v) should be added if ethanol is suspected and blood is not available
[d] Includes vomit, gastric lavage (SWO, first sample), etc.
[e] Tablet bottles, drinks containers, aerosol canisters, etc. – pack entirely separately from biological samples, especially if poisoning with volatiles is a possibility

Collection of urine, stomach contents, and 'scene residues', i.e. material such as tablet bottles or drinks containers found at the scene of an incident may be helpful (Table 2.4). Samples of other appropriate fluids and tissues should also be collected as detailed below, especially when investigating deaths (Table 2.5), but may not be required for analysis unless either special investigations are required or decomposition is advanced (Dinis-Oliveira *et al*., 2010; Dinis-Oliveira *et al*., 2017; Belsey & Flanagan, 2016). However, such samples should be retained (2–8 or –20 °C) in case they are needed. The advantages and disadvantages of various specimens are detailed in Table 2.6. There are special considerations in sample collection and storage for metals/trace elements analysis (Section 21.2).

Information recorded on the sample container at the time the sample is collected should include the name of the patient (first and family or last name) and date of birth, patient/subject/animal number, the date and time of collection, and the sample type. Many laboratories will have a bespoke analysis request form to accompany the sample(s) on which this and any

Table 2.5 Sample requirements: post-mortem biochemistry and toxicology

Sample	Notes[a]
Heart whole blood (right ventricle)	20 mL unpreserved (normally qualitative toxicology only)
Jugular vein whole blood	20 mL unpreserved (normally qualitative toxicology only)
Peripheral whole blood	20 mL from femoral or other peripheral site ensuring no contamination from urine or from central or cavity blood. Collect one portion into 2 % w/v sodium fluoride and another into a plain tube
Urine	20–50 mL if available (plain tube, no preservative unless a portion is required for ethanol measurement)
Gastric contents[b]	25–50 mL (plain bottle, no preservative; record the total weight or volume)
Vitreous humour	Maximum available, plain tube, separate specimens from each eye if feasible. Avoid excessive suction to minimize the risk of aspirating retinal fragments. Collect one portion into 2 % w/v sodium fluoride if for ethanol measurement
Cerebrospinal fluid	5–10 mL, plain tube
Pericardial fluid	Maximum available, plain tube
Synovial fluid[c]	Maximum available, plain tube
Intra-osseous fluid	Maximum available, plain tube
Bile	Maximum available, plain tube
Liver and other tissues	Liver 10 g (deep inside right lobe), other tissues 10 g as appropriate[d]
Scene residues[e]	As appropriate

[a] Smaller volumes may often be acceptable, for example in the case of young children
[b] Includes vomit, gastric lavage (SWO, first sample), etc.
[c] Alternative if vitreous humour not available
[d] Because there is little information on drug distribution within solid tissues in man, collection of approximately 10 g specimens from several sites from organs such as the brain is recommended if the whole organ is available
[e] Tablet bottles, drink containers, aerosol canisters, etc. should be packed entirely separately from biological samples, especially if poisoning with volatiles is a possibility

Table 2.6 Advantages and disadvantages of different sample types in analytical toxicology

Specimen	Advantage	Disadvantage	Comment
Blood (plasma/serum or whole blood)	Detect parent compound. Interpretation of quantitative data	Limited volume. Low concentrations of many basic drugs and some other poisons	Interpretation of quantitative results from post-mortem blood may be difficult
Dried blood spot on filter paper	If known volume of venous blood added, then easy to store and transport (room temperature)	Almost impossible to get accurate volume of blood without use of a pipette or special device. Need analyte to be stable on the paper	Advocated for collecting capillary blood, but capillary blood not venous blood
Urine	Often large volume. High concentrations of many poisons, but sometimes only metabolites detectable	Not always available. Quantitative data not often useful	Standard sample for substance misuse screening
Gastric aspirate (stomach contents, SWO, vomit, etc.)	May contain large amounts of poison, particularly if ingested	If available, variable sample. Limited use if exposure is by inhalation or injection	Ensure no cross-contamination of other specimens during transport/storage/analysis
Oral fluid	Non-invasive. Qualitative information on exposure to many drugs	Variable sample hence little use for quantitative work. Low concentrations of many analytes	Interpretation of quantitative results may be difficult
Hair/nails or nail clippings	Usually available even if decomposition advanced	High sensitivity needed. May only give exposure data for the weeks/months before death. Susceptible to external contamination	Easy to store (room temperature)
Exhaled air	Non-invasive. Large volume available	Need live patient. Analyte must be volatile or present as an aerosol	Mainly used to assess ethanol ingestion, carbon monoxide exposure, and monitor volatile anaesthetics
Scene residues (tablet bottles, aerosol cans, etc. near patient)	May contain large amounts of poison	May not have been the poison taken	Ensure no cross-contamination of other specimens during transport/storage/analysis

Table 2.6 (*Continued*)

Specimen	Advantage	Disadvantage	Comment
Vitreous humour	May be used instead of urine if latter not available	Limited volume but normally two specimens	Analysis may be valuable to help interpret post-mortem blood data for ethanol and for some other compounds
Additional tissues (liver, brain, lung, kidney, etc.)	May contain large amounts of poison. If available then large quantity	Interference in analysis. Difficult to prepare calibrators/QCs, assess recovery, etc. Quantitative data not always easy to interpret	Analysis may be valuable to help interpret post-mortem blood data

Box 2.2 Information that should accompany a request for general toxicological analysis

- Name, sex, date of birth, date and time of death if appropriate, details of the sample(s) sent for analysis
- Name, address, and telephone number of clinician/pathologist and/or coroner's/police officer requesting the analysis, and address to which the report and invoice are to be sent. A post-mortem (reference) number may also be appropriate
- Circumstances of incident (including copy of sudden death report if available)
- Past medical history, including current or recent prescription medication, and details of whether the patient suffered from any serious potentially infectious disease such as hepatitis, tuberculosis, or HIV
- Information on the likely cause, and estimated time, of ingestion/death and the nature and quantity of any substance(s) implicated
- If the patient has been treated in hospital, a summary of the relevant hospital notes should be supplied to include details of emergency treatment and drugs given, including drugs given incidentally during investigative procedures
- Note of occupation/hobbies
- A copy of any preliminary pathology report, if available

other appropriate information such as a note of any preservative added to the sample, site of collection of blood, etc. should be recorded (Box 2.2). The date and time of receipt of all specimens by the laboratory should be recorded and a unique identifying number assigned in each case.

2.3.1 Sample collection and preservation

In general biological specimens should ideally be stored at 2–8 °C before transport to the laboratory. Exceptions to this include hair, nail, and filter-paper adsorbed DBS. Dried blood stains and other dried forensic specimens may of course be handled similarly (Schütz *et al*., 2002).

Each specimen bottle should be securely sealed to prevent leakage, and individually packaged in separate plastic bags. Particular attention should be paid to the packaging of samples to be transported by either post, or courier in order to comply with current health and safety regulations. Sample volumes or amounts smaller than those indicated in Tables 2.4 and 2.5 are often sufficient to complete the analyses required. Submission of very small samples may, however, result in reduced sensitivity and scope of the analyses undertaken, but nevertheless such samples should always be forwarded to the laboratory. Any residual specimen should be kept at –20 °C or below until investigation of the incident has been concluded.

In post-mortem work, the use of disposable hard plastic (polystyrene) tubes is recommended. If these are not available, then containers with secure closures appropriate to the specimen volumes should be used. Some laboratories provide specimen containers for collecting post-mortem blood and urine specimens. It may be important to note if urine was obtained by use of a catheter. Suitable packaging for sending specimens by post may also be supplied. When death has occurred in hospital and poisoning is suspected, any residual ante-mortem specimens should be obtained as a matter of urgency from the hospital pathology laboratory (not only chemical pathology and haematology, but also immunology, transfusion medicine, and virology departments may be a source of such specimens) and submitted for toxicological analysis in addition to post-mortem specimens. Recording the date and time of collection of such samples is important. Note that the availability of ante- or peri-mortem specimens does NOT negate the need to collect post-mortem specimens.

All organ and tissue samples, and any tablet bottles or scene residues, should be placed in separate containers to avoid any chance of cross-contamination. Sampling through tissues containing high concentrations of an analyte may lead to contamination of the sample. On the other hand, toxicological analysis of samples from an embalmed body, formalin-fixed specimens, or even on tissue from microscope slides may be requested. Clearly, concentrations of any drugs or metabolites that survive in such specimens are likely to have been altered by the embalming or fixation process. Formaldehyde is highly reactive, and the identification and the evaluation of possible analyte degradation products and/or formaldehyde derivatives may be helpful (Nikolaou *et al*., 2013; Takayasu, 2013; Ameline *et al*., 2019).

Sample integrity is of prime concern if there are medico-legal implications because evidence may have to be produced in court. Precautions to ensure sample integrity include: (i) proper sample labelling, (ii) use of tamper-proof containers, (iii) collection of samples such as hair, nail, and femoral blood before opening the body and documenting the fact, and (iv) proper accompanying documentation (chain-of-custody documents). Samples collected for clinical purposes (or even for the coroner) are often not of ‘evidential’ quality, but such samples may be all that is available. DNA testing may be used to establish the origin of certain samples if there is concern over sample integrity.

2.3.2 Blood

In analytical toxicology, plasma or serum is normally used for quantitative assays, but some poisons such as carbon monoxide, cyanide and many other volatile organic compounds, lead and other toxic metals, and some drugs such as chlortalidone, are found primarily in or associated with erythrocytes. Thus, haemolyzed whole blood should be used for such measurements. The space above the blood in the tube (‘headspace’) should be minimized if carbon monoxide, solvents, or other volatiles are suspected.

Provided that the samples have been collected and stored correctly, there are usually no significant differences in the concentrations of poisons between plasma and serum. However, if

a compound is not present to any extent within erythrocytes then using lysed whole blood will result in an approximately two-fold dilution of the specimen. A heparinized or EDTA whole blood sample will give either whole blood, or plasma as appropriate. The immunosuppressive drugs ciclosporin, everolimus, sirolimus, and tacrolimus are special cases because redistribution between plasma and erythrocytes begins once the sample has been collected and so the use of haemolyzed whole blood is indicated for the measurement of these compounds.

In addition to the obvious alteration in sample volume, careful interpretation of results is needed if solutions containing ascorbic acid (risk of loss of acid labile compounds, reduction of metabolites), sodium metabisulfite (reduction of metabolites), or sodium azide (interference in immunoassays) are added to specimens.

2.3.2.1 Collection of blood post-mortem

In order to maximize the reliability of measurements performed in post-mortem blood, it is recommended that (i) the interval between death and the post-mortem examination is minimized, (ii) the body/samples are stored at 2–8 °C before the examination/after collection, (iii) that blood is collected from two distinct peripheral site(s), preferably the femoral vein(s), after tying off the vein proximally to the site of sampling (Lemaire *et al*., 2016), and (iv) a preservative (approximately 2 % w/v, fluoride) is added to a portion of the blood sample/the sample from one vein. The exact site of blood sampling should be recorded, as should the time of sampling and (approximate) time of death if known.

If sufficient sample is obtained, this should be divided between unpreserved and preserved (fluoride) tubes, otherwise the entire sample should be preserved unless there is a possibility of poisoning with fluoride or compounds giving rise to fluoride *in vivo* such as fluoroacetate. Added fluoride may also enhance decomposition of organophosphorus compounds (Moriya *et al*., 1999). If only heart or cavity blood is available this should be clearly stated.

Post-mortem blood (*ca*. 20 mL) for qualitative analysis only should be taken from the heart (preferably right atrium), inferior vena cava, or another convenient large vessel. The precise sampling site must be recorded on the sample tube. The blood should be free-flowing.

2.3.3 Urine

The presence of metabolites may sometimes assist identification of a poison if chromatographic techniques are used, but not all poisons appear in urine as either parent compound, or metabolites. A 50 mL specimen from an adult, collected in a sealed, sterile container, is sufficient for most purposes. No preservative should be added. The sample should be obtained as soon as poisoning is suspected, ideally before any drug therapy has been initiated. However, some drugs, such as the tricyclic antidepressants (amitriptyline, imipramine, etc.), cause urinary retention, and a very early specimen may contain insignificant amounts of drug. Conversely, little poison may remain in specimens taken many hours or days after exposure even though the patient may be very ill, for example as in acute paracetamol poisoning.

High concentrations of some drugs or metabolites can impart characteristic colours to urine (Table 2.7). Strong smelling poisons such as camphor, ethchlorvynol, and methylsalicylate can sometimes be recognized in urine because they are excreted in part unchanged. Acetone, an endogenous metabolite, may arise also from metabolism of 2-propanol (Section 1.2.3). Chronic therapy with sulfa-drugs such as a sulfonamide may give rise to yellow or green/brown crystals in neutral or alkaline urine. Phenytoin, primidone, and sultiame may give rise to crystals in urine following overdosage. Surgical manipulation of paralytic ileus may promote absorption

Table 2.7 Some possible causes of coloured urine

Colour	Possible cause
Yellow/brown	Bilirubin, haemoglobin, myoglobin, porphyrins, urobilin
	Anthrone derivatives (e.g. from aloin, aloe, cascara, senna, rhubarb, etc.),[a] bromsulfthalein,[a] carotenes, chloroquine, congo red,[a] cresol, flavins (yellow/green fluorescence), fluorescein, mepacrine, methocarbamol (on standing), methyldopa (on standing), nitrobenzene, nitrofurantoin, pamaquine, phenolphthalein,[a] primaquine, quinine, santonin[a]
Red/brown	Bilirubin, haemoglobin, myoglobin, porphyrins, urobilin
	Aminophenazone, anisindione,[a] anthrone derivatives,[a] bromsulfthalein,[a] cinchophen, congo red,[a] cresol, deferoxamine,[b] ethoxazene, furazolidone, furazolium, levodopa (black on standing), methocarbamol, methyldopa, niridazole, nitrobenzene, nitrofurantoin, phenacetin, phenazopyridine, phenindione,[a] phenolphthalein,[a] phenothiazines, phensuximide, phenytoin, pyrogallol, rifampicin, salazosulfapyridine, santonin,[a] sulfamethoxazole, warfarin
Blue/green	Bile, biliverdin, indican (on standing)
	Acriflavine (green fluorescence), amitriptyline, azuresin, copper salts, ingido carmine, indometacin, methylene blue,[b] nitrofural, phenylsalicylate, resorcinol, toluidine blue,[b] triamterene (blue fluorescence), flunitrazepam (blue fluorescence)
	Methadone linctus adulteration
Black[c]	Blood (on standing), homogentisic acid, indican (on standing), porphobilin
	Cascara (on standing), levodopa (on standing), phenols including propofol, pyrogallol, resorcinol, thymol

[a] pH dependent
[b] Sometimes given i.v. to treat poisoning
[c] Some urinary bacteria possess an enzyme able to convert a tryptophan metabolite into a substance that interacts with the plastic of urine collection bags to produce indirubin (red) and indigo (blue) giving an intense purple/black colour. Although dramatic, purple urine bag syndrome is harmless and disappears after treatment of the infection (Kumar *et al*., 2018)

of orally administered methylene blue (Morell-Garcia *et al*., 2016). Characteristic colourless crystals of calcium oxalate may form at neutral pH after ingestion of ethylene glycol, oxalic acid, or water-soluble oxalates (Hanouneh & Chen, 2017; Section 22.4.1.2). Urine fluorescence may be due to fluorescein added to car antifreeze (often contains ethylene glycol and/or methanol) and possibly other products to aid leak detection.

For post-mortem work, if possible, 2 x 25 mL urine samples should be collected in sterile plastic container(s), one with preservative (2 % w/v fluoride). Ethanol may be produced (Foley, 2018), and also degraded by microbial action, unless appropriate precautions are taken. If only a small amount of urine is available, all should be preserved with fluoride (but see note on fluoride poisoning above) in a plain 5 mL plastic or glass tube. Boric acid or thiomersal [thimerosal; sodium 2-(ethylmercuriothio)benzoate] containers should NOT be used because of sample contamination with borates and mercury, respectively. Urine specimens collected post-mortem are valuable in screening for drugs or poisons, particularly illicit drugs, and are often used for quantitative ethanol or GHB analysis to corroborate the results of a blood analysis (Sections 22.4.1.1 and 22.4.11, respectively).

2.3.4 Stomach contents

Gastric lavage is rarely performed nowadays in treating acute poisoning. However, if a sample of stomach contents is obtained soon after a poisoning episode, large amounts of poison may be present while metabolites may be absent. When investigating possible poisoning, it is important to obtain the *first* sample of any lavage fluid because later samples may be very dilute. A representative portion (*ca*. 50 mL) without preservative should be taken for analysis. However, all stomach contents should be retained and the volume noted, and this information passed to the laboratory. If the blood concentration is difficult to interpret, most notably in post-mortem work, it can be helpful to measure the amount of poison present in the stomach.

Stomach contents are especially useful if poison(s) which are not easy to measure reliably in blood such as cyanide have been taken orally. However, great care is needed if cyanide salts or phosphides, for example aluminium phosphide, are thought to have been ingested, particularly on an empty stomach, because highly toxic hydrogen cyanide or phosphine gas may be released because of reaction with stomach acid. Additionally, the presence of these and other volatile materials can lead to cross-contamination of other biological specimens unless due precautions are taken.

With stomach contents (and also scene residues – Section 2.3.20), characteristic colours or smells (Table 2.8) may indicate a variety of substances. Many other compounds (for example, ethchlorvynol, methylsalicylate, paraldehyde, phenelzine) also have distinctive smells. Very low or high pH values may indicate ingestion of acids or alkali, while a green/blue colour suggests the presence of iron or copper salts.

Table 2.8 Smells associated with particular poisons.[a]

Smell	Possible cause
Almonds	Cyanide
Cloves	Oil of cloves
Fruity	Alcohols (including ethanol), esters
Garlic	Arsenic, phosphine
Mothballs	Camphor or naphthalene
Nail-polish remover	Acetone, butanone
Pears	Chloral
Petrol	Petroleum distillates (may be vehicle in pesticide formulation)
Phenolic (carbolic soap)	Disinfectants, cresols, phenols
Shoe polish	Nitrobenzene
Stale tobacco	Nicotine
Sweet	Chloroform and other halogenated hydrocarbons

[a]CARE – specimens containing cyanides may give off hydrogen cyanide gas, especially if acidified – stomach contents are often acidic. Genetic variation means that not everyone can detect hydrogen cyanide by smell. Similarly, sulfides evolve hydrogen sulfide and phosphides evolve phosphine – the ability to smell hydrogen sulfide (rotten egg smell) is lost at higher concentrations

Examination using a polarizing microscope may reveal the presence of tablet or capsule debris. Starch granules used as 'filler' in some tablets and capsules may be identified by microscopy using crossed polarizing filters when they appear as bright grains marked with a dark Maltese cross. If distinct tablets or capsules are observed, these should be placed in individual containers. Such items and any plant remains or specimens of plants thought to have been ingested should be examined separately.

The local poisons information service or pharmacy will normally have access to publications or other aids to the identification of legitimate and sometimes illicit tablets/capsules by weight, markings, colour, shape, and possibly other physical characteristics. Identification of such material by reference to a computerized product database may be possible.

2.3.5 *Oral fluid*

Whilst not normally considered in either emergency clinical or post-mortem work, there is much interest in the collection of oral fluid from individuals because collection is non-invasive and reflects to an extent recent drug or alcohol usage. Moreover, oral fluid collection for forensic purposes, drug-impaired driving for example, has the advantage that sampling can be carried out by suitably trained staff while the donor is under observation, hence it is more difficult to adulterate or substitute the specimen (Section 18.3.1.1). There is also interest in oral fluid collection for TDM purposes (Section 20.3.3).

2.3.6 *Sweat*

Sweat production is not uniform in either amount, or composition. 'Insensible sweat' is moisture that is lost from the body through the skin and does not form droplets. 'Sensible sweat', i.e. sweat that can be seen as liquid, arises from two types of gland, apocrine and eccrine. The former, which tend to be located in the axillae, pubic, and mammary areas, are larger and secrete a thicker substance. The surface of the skin is also covered with sebaceous secretions, chiefly lipids, higher concentrations being found on the scalp and forehead. Thus, the fluid collected for analysis by patches applied to the skin is generally a mixture of secretions and can only give an indication of drug exposure over the period when the device was attached to the skin.

Collection of sweat has been suggested as a means of testing for misused drugs. Surface contamination by exposure to drug use by others (e.g. smoking either cocaine, or cannabis) is a potential problem when analysing sweat. There are two methods of collecting and testing sweat. One is the Drugwipe, which may also be used with oral fluid, and has found use for roadside testing (Section 17.3.2.1). The other is a tamper-proof 'sticking-plaster' that may be used to collect sweat over several days and is generally used in detoxification clinics and US prisons. The patches are not amenable to point-of-contact testing (POCT).

2.3.7 *Exhaled air*

Measurement of concentrations of volatile substances in exhaled (expired) air by infra-red or other device is of course essential in roadside testing for ethanol and valuable in assessing exposure to poisons such as carbon monoxide (Chapter 17). Direct MS of exhaled air can also detect many compounds, including not only inhalational, but also i.v. anaesthetics such as propofol (Dong *et al*., 2017). However, the use of these techniques is limited by the need to take breath directly from live subjects. Similarly, collection of exhaled air into an impervious plastic (Tedlar™ or PTFE) bag can facilitate the analysis of a number of substances and metabolites

via subsequent GC or GC-MS analysis (Castellanos *et al*., 2016). Misused drugs can also be detected in exhaled air (Section 18.6).

2.3.8 Cerebrospinal fluid

CSF collected via needle aspiration is sometimes used to assess exposure to centrally acting drugs (Shen *et al*., 2004), and may be submitted for analysis if a possible drug administration error is under investigation. As with vitreous humour and synovial fluid, CSF is normally within a relatively protected environment, and thus may also provide a valuable sample for corroborative ethanol measurement, for example, in the event that other samples are not available (Tominaga *et al*., 2015).

2.3.9 Vitreous humour

Vitreous humour can sometimes be obtained even if a corpse has been extensively burnt or damaged, if putrefaction is beginning to occur, or if samples such as urine are not available (Arora *et al.,* 2016; Metushi *et al*., 2016). This specimen may be especially useful when investigating diabetes-related deaths and assessing other parameters such as renal function (Belsey & Flanagan 2016). It can also be valuable for the analysis of alcohols, digoxin, lithium, and some other compounds such as insulin analogues (Ojanperä *et al*., 2013). Vitreous humour glucose falls rapidly after death and potassium increases even with careful sample collection and such measurements must be interpreted with due caution (Belsey & Flanagan, 2016).

Vitreous humour is essentially a salt solution with very little protein, and thus any poisons or metabolites present can often be extracted as though they were in solution in buffer. Samples should be collected from each eye separately if possible, and sodium fluoride preservative (2 % w/v) added to one sample. Care must be taken during sampling because use of excessive suction can cause a significant change in the concentration of certain analytes and there is always the possibility of post-mortem change (Dinis-Oliviera *et al*., 2017). The presence of concurrent vitreous disease may also be a factor (Parsons *et al*., 2003).

2.3.10 Synovial fluid

Synovial fluid collected via needle aspiration has been used, for example, to assess the uptake of non-steroidal inflammatory drugs into their likely site of action (Day *et al*., 1999). As with CSF and vitreous humour, collection of synovial fluid may also be helpful in the event of traumatic death or extensive decomposition because it is located in a relatively protected environment.

2.3.11 Pericardial fluid

As with synovial fluid, pericardial fluid is normally within a relatively protected environment, and thus may also provide a valuable sample for drug screening or for corroborative ethanol measurement, for example, in the event that other samples are not available (Maeda *et al*., 2006; Tominaga *et al*., 2013; 2015).

2.3.12 Intraosseous fluid

Intraosseous fluid has also been suggested as a further specimen that may be useful if there has been extensive trauma, for example (Rodda *et al*., 2018).

2.3.13 Liver

Liver is easily collected post-mortem and readily homogenized. It may contain large amounts of drugs and metabolites, and may be the primary specimen submitted for analysis if blood is not available. A portion (10–20 g) of unpreserved wet tissue should be collected. The sample should be taken from the right lobe, if possible, to reduce the risk of contamination with bile and because any diffusion of poison from the stomach is less likely than in the left lobe. An analysis may, in some cases, help to establish whether either acute, or chronic exposure has occurred, but sometimes the analysis can do little more than establish exposure in the absence of reliable information to aid in the interpretation of quantitative results (Section 22.3.2).

2.3.14 Bile

Bile tends to contain higher concentrations of drugs and metabolites than other body fluids and thus can be a useful specimen if blood and urine are not available, or if ingestion of a very potent poison is suspected (Bévalot *et al*., 2016; Tominaga *et al*., 2016). Blood and bile concentrations are generally poorly correlated (Ferner & Aronson, 2018). At autopsy, bile can usually be collected by syringe aspiration from the gallbladder, or by incision-compression if the bile is too viscous. In the event of cholecystectomy, sampling can be performed directly in the common bile duct. The site of sampling should be recorded. Care should be taken to avoid contamination of the specimen by stomach or intestinal contents.

2.3.15 Other tissues

Other tissue samples may be useful when investigating deaths where volatile substances such as solvents or gases are implicated. Brain, subcutaneous fat, lung (apex), spleen, and kidney are the most useful; 10–20 g wet weight of unpreserved tissue should be collected into separate containers. The specimen should be placed in a specimen jar or nylon bag (VSA- or anaesthetic-related deaths), taking care not to overfill sample containers, and stored at –20 °C or below prior to and during transport to the laboratory.

Measurement of brain concentrations of certain poisons may be useful in specific instances, for example such measurements are said to be helpful when investigating possible cocaine-related deaths. Spleen is rich in erythrocytes and hence may provide a valuable alternative specimen in which to measure carboxyhaemoglobin saturation if blood is not available (Vreman *et al.,* 2006).

2.3.16 Insect larvae

Analysis of blowfly (*Calliphora vicina*) and other insect larvae that feed on rotting flesh may facilitate detection of many drugs originally present in human or animal tissues, although quantitative extrapolations are unreliable (Introna *et al*., 2001; Tracqui *et al*., 2004; da Silva *et al.,* 2017). Pupae may be preserved for years, but drug and metabolite concentrations in post-feeding and pupating larvae are much lower than in feeding larvae, suggesting that the larvae metabolize and eliminate drugs during development. Metabolism of nordazepam to oxazepam by blowfly larvae has been observed (Pien *et al*., 2004).

2.3.17 *Keratinaceous tissues (hair and nail)*

Hair and nail may be useful for detecting exposure to poisons that may have been eliminated from other commonly sampled fluids and tissues before the hair sample was collected (Section 18.5). Hair may also survive longer after burial than other tissues. If a single exposure to a poison is suspected, as in drug-facilitated sexual assault (DFSA, date rape), but the suspected agent is not detected in blood or urine, waiting for 1–2 months for head hair to grow and then performing segmental analysis may reveal the presence of the drug (head hair on average grows at the rate of 1 cm per month).

Head hair (a bunch of hairs the thickness of a pencil, *ca*. 100–200 hairs) should be either plucked in the case of a deceased person, or tied at the root end with cotton thread and then cut *ca*. 2 mm from the scalp at the vertex posterior (crown) of the head (Box 2.3; Figure 2.2), making sure the scissors are level with the scalp and not at an angle. Tying with thread helps preserve the alignment necessary for segmental analysis. The sample should be laid aligned in aluminium foil, with the proximal end clearly identified. Pubic or axillary hair may be substituted if no head hair remains, or if the head hair has been excessively bleached or permed, which tends to destroy sequestered drug, but the ability to perform segmental analysis is lost.

Box 2.3 Protocol for collection of head hair for testing for drug exposure

- The ideal sample is collected from the vertex (the crown) of the head by cutting approximately 2 mm from the scalp
- Take a sample of hair about the thickness of a pencil (100–200 hairs)
- Pinch the hair tightly with the fingers and tie with cotton thread at the root end before cutting
- Cut the sample as close as possible to the scalp, making sure the scissors are level with the scalp
- Still holding the sample tightly, align the cut root ends of the sample and carefully place flat on a piece of aluminium foil with the cut root ends projecting about 15 mm beyond the end of the foil
- Mark the root end of the foil and fold the foil around the hair and pinch tightly to keep in place
- Fold the foil again in half lengthwise
- Place the sample in a tamper-proof envelope. Complete and sign the request form, making sure that the donor also signs if necessary. If there are special instructions that do not appear on the form, but are felt relevant, make a note on a separate sheet and enclose with the sample

Nail clippings may be used to monitor uptake of antifungal drugs such as itraconazole and terbinafine (Leyden, 1998). In post-mortem work, whole nails should be lifted from the fingers or toes. This provides an even longer potential window for detecting exposure than hair. However, relatively little is known about the mechanisms of uptake and retention or drugs and metabolites in nail. In addition, the slower growth rate of nail, especially toe nail, as compared to hair

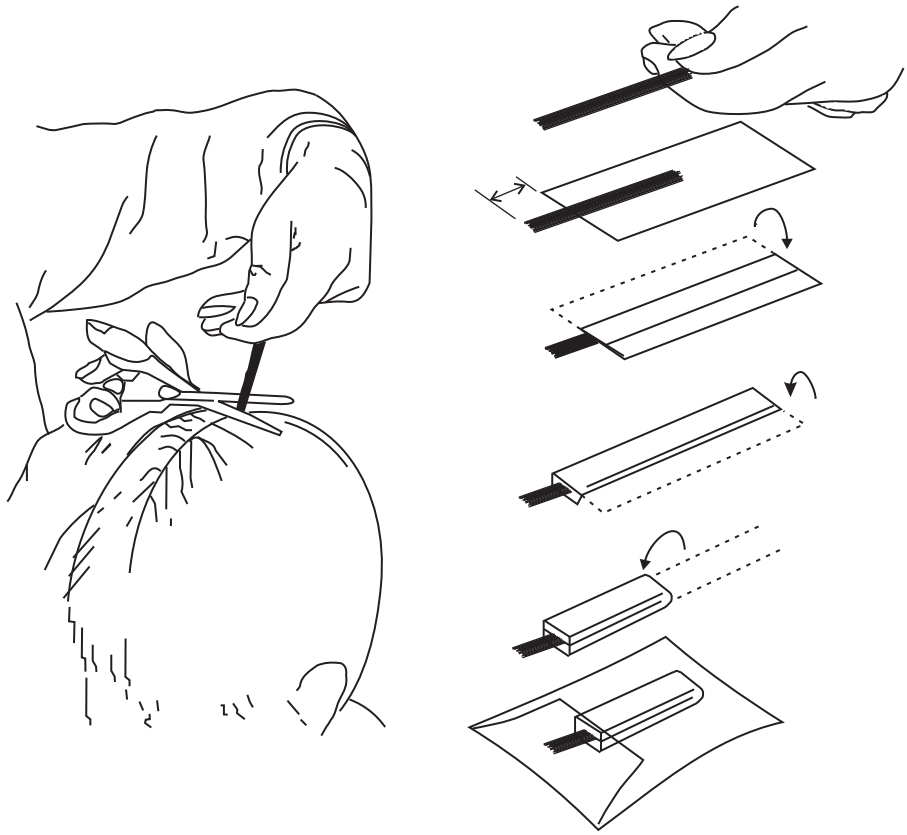

Figure 2.2 Schematic of head hair collection

makes segmental analysis, and hence interpretation of quantitative results, virtually impossible (Krumbiegel *et al*., 2016; Solimini *et al*., 2017).

2.3.18 Bone

Bone marrow aspirate may be useful in the identification of certain poisons in exhumations, for example, where all soft tissue has degenerated (Orfanidis *et al*., 2018). As with synovial fluid and pericardial fluid, bone marrow is normally within a relatively protected environment, and thus may also provide a valuable sample for drug screening or for corroborative ethanol measurement, for example, in the event that other samples are not available (Maeda *et al*., 2006; Cartiser *et al.,* 2011; Tominaga *et al*., 2013). Analysis of bone itself may be useful if chronic poisoning by arsenic or lead, for example, is suspected.

2.3.19 Injection sites

Possible injection sites should be excised, packaged individually and labelled with the site of origin. Appropriate ‘control’ material (i.e. from a site thought to not be an injection site) of similar composition should be supplied separately.

2.3.20 Scene residues

Such material, which may include tablets, powders, syringes, infusion fluids, infusion lines, bandages, foodstuffs, drinks, and so forth, may give valuable information as to the poison(s) involved in an incident, and should be packaged separately from any biological samples. This is especially important if volatile compounds are involved. If police attend a scene these materials may find their way to a forensic laboratory rather than accompanying the patient.

All items should be labelled and packed with care. Scene residues may be particularly valuable when investigating deaths that may involve medical, dental, veterinary, or nursing personnel, who may have access to agents that are difficult to detect once they have entered the body, and in deaths that have occurred in hospital. Investigation of deaths occurring during or shortly after anaesthesia should include the analysis of the anaesthetic(s) used, including inhalational anaesthetics, in order to exclude an administration error. Needles must be packaged within a suitable shield to minimize the risk of injury to laboratory and other staff.

2.4 Sample transport, storage, and disposal

It is usually advisable to contact the laboratory by telephone in advance to discuss urgent or complicated cases. Most specimens, particularly blood and urine, may be sent by post if securely packaged in compliance with current regulations. However, if legal action is likely to be taken on the basis of the results, it is important to be able to guarantee the identity and integrity of the specimen from when it was collected through to the reporting of the results. Thus, such samples should be protected during transport by the use of tamper-evident seals and should, ideally, be submitted in person to the laboratory by the coroner's officer or other investigating personnel. *Chain of custody* is a term used to refer to the process used to maintain and document the history of the specimen (Box 2.4).

Box 2.4 Chain of custody documents

- Name of the individual collecting the specimen(s)
- Name of each person or entity subsequently having custody of it, and details of how it has been stored
- Date(s) the specimen(s) collected or transferred
- Specimen or post-mortem number(s)
- Name of the subject or deceased
- Brief description of the specimen(s)
- Record of the condition of tamper-evident seals

Fully validated assays must include data on the stability of the analyte under specified sample collection and storage conditions (van de Merbel *et al*., 2014; Reed, 2016). In the absence of other information, biological specimens should be stored at 2–8 °C prior to analysis, if possible, and ideally any specimen remaining after the analysis should be kept at 2–8 °C for 3–4 weeks in case further analyses are required. In view of the medico-legal implications of some poison cases (for example, if it is not clear either how the poison was administered, or if the patient dies) then any specimen remaining should be kept (preferably at –20 °C) until investigation of the incident is concluded. Unfortunately, there are few stability data for whole blood specimens, let alone for post-mortem specimens.

Table 2.9 Some drugs, metabolites, and other poisons unstable in whole blood or plasma (data from Peters, 2007 and from Mata, 2016)

Volatile agents	Aerosol propellants, anaesthetic gases, carbon monoxide, cyanide, ethanol, ethchlorvynol, mercury, methanol, nicotine, organic solvents, paraldehyde, volatile nitrites (amyl nitrite, etc.)
Non-volatile substances	Acyl (ester) glucuronides, amiodarone, aspirin, bupropion, carbamate esters (e.g. physostigmine, pyridostigmine), ciclosporin,[a] cyanide, esters (e.g. 6-AM, benzocaine, cocaine, diltiazem, diamorphine, GHB, pethidine, methylphenidate, procaine, succinylcholine), *N*-glucuronides (e.g. olanzapine *N*-glucuronide), insulins, insulin C-peptide, LSD, nitrobenzodiazepines (clonazepam, flunitrazepam, loprazolam, nitrazepam and their 7-amino metabolites), glyceryl trinitrate and other nitrates and nitrites, nitrophenylpyridines (e.g. nifedipine, nisoldipine), olanzapine, *N*-oxide metabolites, *S*-oxide metabolites, paracetamol, peroxides and other strong oxidizing agents, phenelzine, phenothiazines,[b] piperazines, proinsulin, quinol metabolites (e.g. 4-hydroxypropranolol), rifampicin, sirolimus,[a] *N*-sulfate metabolites (e.g. minoxidil *N*-sulfate), tacrolimus,[a] thalidomide, thiol-containing drugs (e.g. captopril), thiopental, zopiclone

[a]Redistributes between plasma and erythrocytes on standing (use whole blood)
[b]Particularly those without an electronegative substituent at the 2-position

With regard to drugs, some compounds such as clonazepam, cocaine, nifedipine, nitrazepam, thiol drugs, and many phenothiazines and their metabolites are unstable in biological samples at room temperature (Table 2.9). Synthetic piperazines, for example, are generally unstable in whole blood, for example (Lau *et al*., 2018). Exposure to sunlight can cause up to 99 % loss of clonazepam in serum after 1 h at room temperature. Covering the outside of the sample tube in aluminium foil is a simple precaution in such cases. *N*-Glucuronides such as olanzapine *N*-glucuronide are unstable and may be present in plasma at high concentration; on decomposition the parent compound is reformed. Some compounds, notably olanzapine, appear relatively stable in plasma, but are dramatically unstable in haemolyzed blood.

Solid sodium fluoride may be added to at least partially inhibit microbial and some other degradative enzymes as discussed above (Section 2.3). Esters (including carbamates and organophosphates) may be rapidly hydrolyzed by plasma esterases, including cholinesterase. If physostigmine or cocaine are to be measured accurately the blood should be drawn into tubes containing an excess of neostigmine. Storage at –20 °C or below is recommended if the analysis cannot be performed immediately and if the stability of the analyte is unknown. However, even this may not be ideal because *N*- and *S*-oxides may be reduced to the parent compounds. Quinols such as 4-hydroxypropranolol, on the other hand, are readily oxidized.

Stabilization by addition of a reducing agent such as ascorbate or sodium metabisulfite has been advocated in some cases (Sørensen & Hasselstrøm, 2019), but is not performed routinely and in the case of olanzapine, for example, there is the possibility of reducing the *N*-oxide by such an addition. The monographs in the compendium by Baselt (2020) detail such stability data as there are for many compounds.

Storage at −5 to −70 °C should be accompanied by basic precautions to preserve sample integrity (Box 2.5). The requirements of the local ethics committee on the retention, storage, and safe disposal of clinical samples must be complied with.

Box 2.5 Guidance on freezer storage of samples

- Do not freeze whole blood if plasma or serum is to be analyzed
- Ensure that labelling is waterproof
- Ensure tubes are tightly sealed and well filled, but do not overfill tubes, especially glass tubes
- Do not keep too long to minimize freeze-drying effects
- Keep a record of freezer contents and freezer cleaning
- Keep a continuous record of freezer temperature via an electronic device
- Use a spark-proof freezer if flammable materials may be stored
- Fit an alarm in case of freezer failure
- Implement a defined sample disposal policy in collaboration with stakeholders

2.5 Common interferences

Plasticizers, particularly phthalates, may originate from plastic bags used to store transfusion blood, infusion tubing, and from soft plastic closures for blood tubes. Such compounds are often retained on reverse-phase LC systems (Section 10.5.3) and show good absorption at 254 nm and below. Polyvinylchloride (PVC), for example, can contain up to 40 % (w/w) di-2-ethylhexylphthalate and concentrations of this latter compound of up to *ca*. 0.5 g L^{-1} have been reported after storage of plasma in PVC bags for 14 days. Phospholipids are notorious for supressing ionization in LC-MS assays (Fabresse *et al*., 2017). A further consideration is that post-mortem specimens may contain putrefactive bases such as phenylethylamines and indole(s) that may interfere in the analysis of amfetamines and other stimulants. Hexanal may arise from breakdown of fatty acids. GC retention data and mass spectra of a number of plasticizers, pollutants, and other substances that may be encountered in toxicological analyses have been reported (Maurer *et al*., 2016).

Drugs may arise from unexpected sources including food and over-the-counter (OTC) medicines. Quinine may originate from ingestion of tonic water or malaria prophylaxis, for example, caffeine from caffeinated beverages (tea, coffee, cola) and some proprietary stimulants, chloroquine, and related compounds from malaria prophylaxis, and pholcodine and other opiate analogues from cough and cold cures. Morphine is a constituent of some antidiarrhoeal preparations and is present in poppy seeds. A caffeine metabolite, paraxanthine, may be a problem in some LC theophylline assays.

Lidocaine-containing gel is commonly used as a lubricant during procedures such as bladder catheterization or bronchoscopy, and measurable plasma concentrations of lidocaine and some metabolites may be attained. Lidocaine and propofol are commonly used in association with emergency procedures, but these compounds may be misused together. Ketamine and morphine may also be given as part of emergency procedures. The alkaloids emetine and cephaeline, and their metabolites, have been detected in stomach contents, plasma, and in urine after syrup of ipecacuanha (ipecac) was given to induce vomiting, especially in children, although this form of treatment is no longer recommended practice.

Sedatives such as pethidine (meperidine) may be given prior to computerized tomographic (CT) scans, lumbar puncture, or other investigations. Neuromuscular blocking agents such

as atracurium, which is metabolized to laudanosine, and vecuronium, may be encountered in samples from patients undergoing mechanical ventilation. 1,3-Propanediol (propylene glycol) is used as a vehicle in some i.v. infusions. Benzoic acid, which is metabolized to hippuric acid, is used as a preservative in some drugs and foods.

The antibiotic metronidazole is often encountered in samples from hospitalized patients. Medical imaging media may interfere in a range of assays (Lippi *et al*., 2014). Alcohols may originate from skin cleansing swabs. Such compounds and also drugs given in emergencies, amiodarone, anticonvulsants such as diazepam, and propofol, for example, may not be recorded on record sheets. Some compounds or their metabolites may have very long plasma half-lives. Chlorpromazine metabolites, for example, have been reported in urine many months after stopping therapy. Primary amines and even morphine, for example, can be acetylated in gastric contents if aspirin has been co-ingested (Naso-Kaspar *et al*., 2013).

Contamination with trace elements is a particularly difficult area (Chapter 21). Contamination with volatiles, such as solvents used in the laboratory, must be guarded against if one of the solvents in question is to be tested for in a biological or related sample. Glassware and other items must be kept clean and tested regularly for contamination via IQC procedures.

2.6 Summary

Although not in the immediate control of the laboratory, every effort must be made to ensure appropriate priority is given to sample collection and handling because if this is not done properly all subsequent effort is wasted. Care in sample collection is especially important in post-mortem and overt medico-legal work, but even in clinical work effort in providing advance information to clinicians and pathologists on sample requirements (site of collection, volume, addition of sodium fluoride, etc.) and feedback on the problems that will arise when mistakes are made can only prove beneficial.

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3 Basic Laboratory Operations

3.1 Introduction

Appropriate procedures to ensure that all stages of an analysis (sample integrity, the certification, traceability, and preparation of reference materials, assay validation, quality control, quality assurance, reporting of results, staff training, etc.) must be in place if reliable results are to be obtained and reported. The laboratory must be able to demonstrate to regulators that it has robust practices in place by implementing and validating appropriate quality management procedures. Laboratory accreditation, that is inspection and independent certification of laboratories to ensure as far as possible the quality and reliability of the work produced, is becoming increasingly important. This chapter discusses issues surrounding accreditation and quality, especially as regards quantitative analysis.

Written procedures, usually known as either standard operating procedures (SOPs), or laboratory procedures, should describe all aspects of laboratory operation, including laboratory management. The International Laboratory Accreditation Cooperation website (https://ilac.org) gives details of laboratory accreditation procedures. ILAC module G19 provides specific details on requirements for forensic toxicological investigations. The International Organization for Standardization (ISO) gives details of quality management systems (ISO 9000 family) that can be used to describe all types of operations including laboratory operation (www.iso.org/iso-9001-quality-management.html).

ISO Standards 15189 (www.iso.org/obp/ui/#iso:std:iso:15189:ed-3:v2:en) and 17025 (www.iso.org/obp/ui/#iso:std:iso-iec:17025:ed-3:v1:en) define standards for the operation of medical laboratories and of testing and calibration laboratories, respectively, and both are consistent with ISO 9001. The Society of Forensic Toxicologists (SOFT) and American Academy of Forensic Sciences (AAFS) have published detailed guidelines for the operation of forensic toxicology laboratories, much of which is applicable to clinical toxicology laboratories (SOFT/AAFS, 2006). The United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT) (Cooper *et al*., 2010) and the Society of Toxicological and Forensic Chemistry (GTFCh, 2009) have produced similar guidelines.

As discussed in Chapter 1, laboratory operations can be divided into pre-analytical, analytical, and post-analytical phases (Box 3.1). The actual analytical methods used will depend on local circumstances. It is not essential that uniform methodology is employed, only that the method used gives accurate, reliable, reproducible results when used for its designated purpose (Wille *et al*., 2011). Assay validation should conform as far as possible with the US Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER) guidance for bioanalytical method validation (FDA/CDER, 2018) or that of the European Medicines Agency (EMA, 2019). Data for within-day (repeatability), between-day, and total precision should be

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calculated according to the protocol proposed by the Clinical and Laboratory Standards Institute (CLSI, 2014).

Box 3.1 Stages in analytical toxicology laboratory operation

- **Pre-analytical**
  Procedures must be in place to advise on appropriate sample collection (including sample tubes and minimal sample requirements for routine assays), to ensure the safe transport, receipt, and storage of biological samples once in the laboratory, and to assign the appropriate priority for the analysis
- **Analytical**
  Validated (i.e. tried and tested) procedures must be used to perform the requested or appropriate analyses to the required degree of accuracy and reliability in an appropriate time scale
- **Post-analytical**
  A mechanism for reporting results and maintaining confidentiality by telephone, fax, or other electronic means and in writing must be in place. Proper interpretation of results, especially for less common analytes, must be provided. Full records of the analysis must be kept for at least 5 years (10 or more years if the case has medico-legal implications). Residues of samples must be stored appropriately until disposed of safely in an agreed time-frame

3.1.1 Reagents and standard solutions

Chemicals obtained from a reputable supplier are normally graded as to purity [analytical reagent grade (AR or AnalaR), general purpose reagent (GPR), or laboratory reagent grade, etc.]. In general, material of the highest available purity should be used in analytical work. For highly sensitive techniques, especially those using LC and LC-MS, specific LC-grade solvents and eluent additives are available (Section 10.2.4). Often the maximum limits of well-known or important impurities will be stated on the label together with recommended storage conditions.

Some chemicals readily absorb water vapour and either remain solid (hygroscopic, for example the sodium salt of phenytoin) or enter solution (deliquescent, for example trichloroacetic acid), and thus should be stored in a desiccator. Others (for example sodium hydroxide) readily absorb atmospheric carbon dioxide either when solid, or in solution. Other alkaline solutions readily absorb carbon dioxide with a subsequent decrease in pH. Bacteria, often visible as a cloudy precipitate, will grow in most aqueous solutions, especially phosphate buffers. Such growth will not only change the composition of the solution, but also may be a source of contamination, particularly if biological molecules (amino acids, prostaglandins) are to be assayed.

The concentrations of buffer solutions are frequently prepared as mol L^{-1} (older literature may use the symbol, M, for 'molar', i.e. mol L^{-1}). The relative molecular mass of the salt (M_r, sometimes referred to as formula weight, FWt) is usually to be found on the bottle label, or in the supplier's catalogue. To prepare a 1 mol L^{-1} solution of disodium hydrogen orthophosphate, for example, the appropriate M_r (in grams) of the salt available should be weighed and dissolved

in less than one litre of purified water and made up to volume with water. If the protocol requires a specified weight of salt to be taken, then it is important to either use the specified salt, or make the appropriate correction should that not be available. The other crucial information that is required is the number molecules of water of hydration per molecule of salt.

Preparing standard solutions of drugs and poisons can be even more complex. For drugs that are weak electrolytes it is usual to report the result of an assay in terms of the concentration of 'free' acid or base. However, many compounds are supplied as salts, and there may be several alternatives available. One supplier offers codeine as either the base (CAS 76-57-3; M_r 299.36), or as the sulfate (CAS 1420-53-7; M_r 696.81). Morphine may be available as the anhydrous sulfate, the hydrochloride, the sulfate pentahydrate (CAS 6211-15-0; M_r 758.83), or the tartrate (CAS 302-31-3). Although tartaric acid is tribasic, the salt is actually bimorphine hydrogen tartrate (Box 3.2).

Box 3.2 Example of weights of morphine salts required to make 1 litre of a 100 µmol L^{-1} solution of morphine

Compound	CAS number	M_r	Weight (mg) containing 10 mg free base	Weight (mg) for 100 µmol L^{-1}
Morphine	57-27-2	285.34	10.00	28.5
Morphine hydrochloride	52-26-6	321.80	11.28	32.1
Morphine sulfate	64-31-3	668.76	11.72	33.4
Morphine sulphate pentahydrate	6211-15-0	758.83	14.32	37.9
Morphine hydrogen tartrate trihydrate	6032-59-3	774.82	14.58	38.7

Explanations:

Morphine hydrochloride

Every 321.80 g of morphine hydrochloride contains 285.34 g morphine base, therefore correction is:

$$321.80 \div 285.34 = 1.128$$

Morphine sulfate

Every 668.76 g of morphine sulfate contains (285.34 × 2) g morphine base, therefore correction is:

$$668.76 \div (285.34 \times 2) = 1.172$$

Morphine sulfate pentahydrate

Every 758.83 g of morphine sulfate contains (285.34 × 2) g morphine base, therefore correction is:

$$758.83 \div (285.34 \times 2) = 1.332$$

Morphine hydrogen tartrate trihydrate

Every 774.82 g of morphine tartrate contains (285.34 × 2) g morphine base, therefore correction is:

$$774.82 \div (285.34 \times 2) = 1.358$$

Note that unless specifically defined otherwise, the use of the terms 'free' and 'total' drug in reports should be confined to non-protein bound drug measurements (Section 4.3) and to measurements performed after hydrolysis of conjugated metabolites (Section 4.4), respectively.

3.1.2 Reference compounds

The supplier, batch or lot number, purity, expiry date, and any other relevant information supplied with a compound should be recorded. Unless a certified, weighed amount (with uncertainty) of compound is supplied, it cannot be assumed that the quantity purchased is actually the amount supplied. For reference compounds supplied as certified solutions, the uncertainty of the assigned concentration should be available (e.g. 1.000 ± 0.005 g L^{-1}).

Compounds should be stored in the dark under conditions recommended by the supplier. To ensure good accuracy, the highest quality (purest) material available should be used to prepare calibration solutions. Every effort should be made to obtain a certificate of analysis or other appropriate documentation when a new supply of a compound is obtained, but supply of such documentation may be at the discretion of the manufacturer. It cannot be assumed that a simple statement such as 'purity >98 %' is accurate unless a certificate of analysis, ideally with accompanying evidence of the analysis, is supplied.

Reference materials, and indeed reagents, can be divided into portions when first received and stored in air-tight containers with minimal headspace. This may be appropriate for deliquescent and hydroscopic materials and those that are easily oxidized because the reference material is not exposed to the atmosphere so frequently. However, this may not be appropriate if only very small quantities of material are available, or storage space is limited. Reference compounds, etc. that have been stored in a refrigerator or freezer should be allowed to come to ambient temperature before the containers are opened to minimize the risk of water condensing on the material. The material must have been stored appropriately, and dried before use if necessary.

Obtaining reference samples of rarely encountered compounds and of metabolites can prove very difficult. In addition to chemical synthesis using a precursor or analogue as starting material, a further approach that has been suggested for metabolite synthesis is incubation of parent drug with fission yeast (*Schizosaccharomyces pombe*) genetically modified to express human CYP2D6 followed by metabolite isolation (Drăgan *et al*., 2011). Chiron AS (www.chiron.no/en/about-us/) and LGC Standards (www.lgcstandards.com/GB/en) are but two reputable suppliers that specialize in rarely encountered compounds and also stable-isotope labelled compounds for use in MS work.

In any event if primary standards, such as drugs, have been obtained from ill-defined sources, it is important to have some idea of the purity of each sample. Visual inspection may show inconsistencies in colour, which may indicate decomposition. Useful information can often be obtained by carrying out a qualitative chromatographic analysis (TLC, LC) or spectroscopic investigation (UV, IR, NMR). Drying a sample to constant weight will indicate if it contains moisture. A UV spectrum may be valuable. It is also possible to measure the absorbance of a solution of the drug and compare the result with literature values for specific absorbance, A^1_1 (Section 5.4.1).

It should be noted that wherever possible A^1_1 values are best measured at the λ_{max} so that errors in the wavelength calibration of the instrument are minimized. Also, the literature values may either be in error, or not sufficiently accurate for the purpose, but a large discrepancy between the quoted and observed value probably indicates a gross error. It is good practice to store the UV spectra for future reference.

Reference compounds withdrawn from use should be disposed of safely according to local protocols unless return to the supplier is requested.

3.1.3 Preparation and storage of calibration solutions

Assay calibration is normally by analysis of standard solutions containing each analyte over an appropriate range of concentrations prepared in the analyte-free matrix for which the calibration is performed (e.g. plasma, whole blood, urine, or other appropriate fluid). The chosen medium should be analyzed prior to the addition of the analyte(s) to ensure the absence of interferences.

Balances for weighing reagents or calibration standards, and automatic and semi-automatic pipettes, must be kept clean and checked for accuracy on a regular basis. Weighing of certified balance check weights should be recorded. Pipette accuracy can be documented by dispensing purified water and recording the weight dispensed. Weighing of reference compounds should normally be performed by one analyst and witnessed by a second analyst, as should other steps in preparing calibration standards and IQC solutions such as calculating and performing dilutions.

Unless the reference material is in short supply, then an 'appropriate' amount should be weighed. It is difficult to give an exact figure because this will depend on the accuracy of the balance used. If a well-maintained four-place balance, accurate to ±0.1 mg is used then 100 mg can be weighed to ±0.1 %. This may be sufficiently accurate, particularly if the accuracies of subsequent volumetric steps are not better than this. *Ideally*, a portion of standard reference compound should be removed (tipped) into a clean container and samples of this taken for weighing. Any excess should be disposed of, not returned to the stock container. This approach also prevents dirty spatulas contaminating the stock reference material.

To minimize operator bias, an approximate amount should be weighed accurately rather than attempting to weigh to a pre-determined value. For example, ideally a weight of 98.4 mg should be accepted and recorded rather than attempting to weigh exactly 100.0 mg. Although this can lead to complex calculation of calibration standard concentrations, the ready availability of calculators and spreadsheets means that it is not the problem it used to be when calibration graphs were drawn by hand. To avoid errors caused by transfer losses, samples can be weighed directly into volumetric flasks. This is particularly useful when only a small amount of material is available, which is often the case in analytical toxicology. It is of course important to consider the solubility of the material in the chosen solvent when choosing the appropriate size of flask.

Automated gravimetric dosing systems are available that provide full traceability for preparing reference solutions. Reference materials are weighed accurately using a four-place balance, and solutions accurately prepared using a liquid dispenser at the desired concentration, whilst accounting for the diluent density and temperature.

The volumetric part of preparing calibration curves is usually less accurate than the gravimetric (weighing) part. Good quality volumetric glassware and pipettes are required and must be used and cleaned correctly – volumetric glassware *should not* be dried by heating in an oven. Glass (bulb) pipettes may be appropriate, but biological fluids (plasma & blood) are more viscous than water and take longer to drain.

Semi-automatic pipettes are normally calibrated to measure aqueous fluids with specific gravities (SGs) of approximately 1, and thus should not be used for organic solvents or other solutions with SGs or viscosities greatly different from that of water. Positive displacement pipettes should be used for very viscous fluids such as whole blood. Using the same pipettor

with clean, properly fitted tips will ensure that any systematic error in the volume delivered will be cancelled out. Similarly, it is logical to use the same pipettor for delivery of sample (unknown) and standard solutions.

The most accurate dilutions are obtained by diluting 1+1. However, this is often impractical especially if the final dilution is into plasma or whole blood when a minimal amount of water should be added otherwise the blood will be markedly diluted. To avoid diluting the matrix, primary standards can be prepared in a suitable solvent such as methanol and the required volume of solution pipetted into a volumetric flask. The solvent is evaporated under a stream of air or nitrogen and the dry residue taken up in the matrix. This can be very successful with blood and plasma when protein binding helps to dissolve the analyte. However, different compounds can take different amounts of time to dissolve so it is important to test that dissolution is complete. The solvent should not interact with the analyte. Acetone would react with primary amines and use of methanol, although usually suitable, may result in transesterification of esters.

The range of the calibration solutions (calibrators) should cover the range of concentrations expected in the samples. The calibration curve should not be extrapolated beyond the lowest or highest calibrator. If the concentration of the analyte in some samples is below the lowest calibrator then the assay should be repeated with the inclusion of lower concentration calibrators, if that is possible, or the result reported as less than the lowest calibrator. High concentration samples should be diluted with appropriate 'blank' (analyte-free) matrix to fit within the calibration range provided that the validity of so doing has been demonstrated during method development, assuming sufficient sample is available. Dilutions should be made with the same (e.g. blank plasma from the subject), or very similar, matrix as the sample (e.g. that used for the calibration solutions).

Newborn calf serum (NBCS) is often used in the preparation of calibration solutions for plasma or serum assays. Freshly prepared calibration solutions should be validated before use by comparison either with existing calibration solutions, or with IQC solutions prepared in human plasma or serum, and the results recorded. If for any reason NBCS proves unsuitable, human plasma or serum from an appropriate source should be used.

Blood-bank whole blood (transfusion blood) may not be suitable for calibration standards. Such blood, and plasma derived from it, will (i) usually be diluted with citrate solution, which has a high buffering capacity, (ii) contain lidocaine and sometimes lidocaine metabolites, and (iii) may well contain plasticizers and other contaminants, which may interfere in chromatographic and possibly other assays, and may alter the distribution of drugs between red cells and plasma by altering protein binding. Commercially available equine or bovine blood may suffer from some of these same problems.

If freshly prepared calibration standards and the IQCs meet the criteria for acceptance, they should be transferred to labelled 3 mL plastic tubes and stored (−20 °C) until required. To meet health and safety requirements, cleaning records and lists of contents should be posted on all refrigerators and freezers, which should be fitted with failure alarms and temperature monitoring devices so that temperature records can be kept. Computerized and web-based temperature monitoring devices are available (Section 2.4).

Care must be taken when choosing a matrix for preparation of calibrators for endogenous analytes. Surrogate matrices, such as charcoal-stripped media, may be useful to avoid the need for standard addition calibration (Section 3.2.4.7), but should be carefully validated because the charcoal-stripping process is non-selective and is likely to have removed not only the analyte of interest, but also other compounds present in samples that may interfere. For analysis of endogenous proteins and peptides, especially when using MS, non-human matrices can be valuable because the amino acid sequence is often different to that of man.

3.2 Aspects of quantitative analysis

3.2.1 Analytical error

Replicate analyses of a sample usually do not produce identical values because of errors that are associated with the analysis. These errors may be either *random*, or *systematic*. Systematic errors (e.g. an incorrectly set pipetting device) should be investigated, and either eliminated, or an appropriate correction made. The impact of some systematic errors can be minimized by the way the work is performed such as use of the same pipette for calibration solutions and for samples. *Gross* errors are those such as dropping a sample or adding the wrong reagent – these may be either obvious to the eye, or revealed by a very poor recovery of ISTD, for example.

Any measurement is an estimate of the 'true' value. Thus, there is an error associated with that measurement – the difference between the true and the measured value. Consequently, when repeated measurements are made the results are a range of values. The result could be reported as the average value of the measurements ± the range of values obtained (largest to smallest value). However the values at the extremes will not give a good representation of the distribution of the results about the mean value. If a sufficiently large number of replicates are used, then the distribution can be seen from a frequency diagram. A set of all possible measurements (an infinite number) is known as the *population* and the histogram of results (Figure 3.1) represents a *sample* from that population.

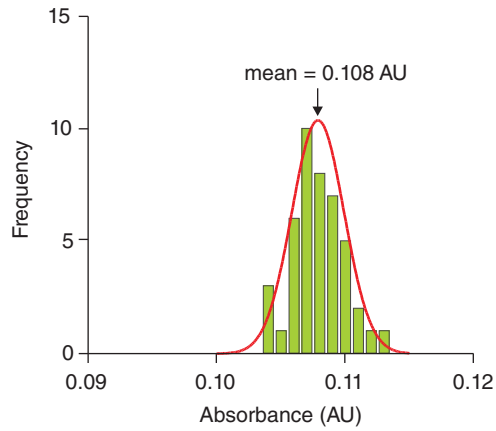


Figure 3.1 Histogram of replicate absorbance measurements (n = 44) for plasma sulfadimidine measured using a Bratton–Marshall colorimetric method (Whelpton *et al*., 1981). The solid line is a Gaussian curve fitted to the data (mean = 0.108 AU, SD = 0.002)

Population distributions for analytical measurements are frequently a normal or Gaussian distribution and, in the absence of any systematic errors, the population mean (μ) is the true value. Statistics based on such distributions are referred to as *parametric*. The spread of the curve can be described in terms of the population standard deviation (SD, σ): the greater the SD, the wider the curve will be. Furthermore, for a normal distribution, *approximately* 68 % of the results lie within $\pm 1\sigma$ of the mean, ~95 % are within $\pm 2\sigma$, and ~99.7 % are within $\pm 3\sigma$ of the mean. If values of μ and σ are known, then it is possible to calculate the exact proportion of

values that lie within any interval. Firstly, the standardized normal variable is obtained:

$$z = \frac{(x - \mu)}{\sigma} \tag{3.1}$$

and then tables of z-values can be used to determine the proportion of values lying outside the range or the NORMSDIST(z) function in Microsoft Excel can be used, which calculates:

$$f(z) = \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{z^2}{2}\right) \tag{3.2}$$

Using values of –1.96 and 1.96, returns 0.025 and 0.975, respectively. Thus 95 % of the results lie within $\pm 1.96\sigma$. Similarly, the formula can be used to show that 99 % of results lie within $\pm 2.58\sigma$ of the mean, and 99.9 % within $\pm 3.29\sigma$.

Clearly, μ and σ cannot be measured directly, but have to be estimated from sample values. The sample mean is:

$$\overline{x} = \frac{\sum x}{n} \tag{3.3}$$

and the sample SD, s is:

$$s = \sqrt{\frac{\sum (\overline{x} - x)^2}{n - 1}} \tag{3.4}$$

and the sample variance is s^2. In this case $(n - 1)$ is the number of degrees of freedom (ν) and so variance is the sum of squares divided by the degree of freedom. Note that population values take Greek characters, whereas sample values are distinguished by Roman characters.

The more measurements that are included in calculating the sample mean, the closer it will become to the population mean. The error in the sample mean is known as the standard error of the mean (*s.e.m*):

$$s.e.m. = \frac{s}{\sqrt{n}} \tag{3.5}$$

and this gives a measure of the variability of $\overline{x}$. However, it is often more informative to describe the SD as a proportion or percentage of the mean. This is the relative standard deviation, RSD:

$$\text{RSD} = \frac{s}{\overline{x}} \times 100\,\% \tag{3.6}$$

3.2.1.1 Confidence intervals

When n is large (>200), s is considered to be a reliable estimate of σ, and the general formula for the confidence interval (CI) is given by:

$$\overline{x} \pm \frac{zs}{\sqrt{n}} \tag{3.7}$$

The appropriate value of z is selected for the level of confidence that is required: 1.96 for 95 %, 2.326 for 99.0 %, etc. For smaller sampler sizes, the normal distribution does not hold true and s is a less reliable estimate of σ. Hence an alternative distribution, the t-distribution (Student's distribution) is used. The confidence intervals are:

$$\overline{x} \pm \frac{t_\nu s}{\sqrt{n}} \tag{3.8}$$

where $\nu = n - 1$. Values of t_ν are obtained from statistical tables for the required level of confidence.

A much-debated topic is the need for replicate analyses (n) because the RSD is reduced by the square root of n (Table 3.1). If the variability is large, triplicates or even quadruplicates may be needed, but in practice, with chromatographic methods especially and 'one-off' clinical/forensic samples, duplicates usually give a reasonable compromise between reagent and other costs, time, sample volume availability, and the need for accuracy. Furthermore, should one duplicate be lost during the analysis, one at least remains.

Table 3.1 Effect of replicate analyses (n) on RSD (arbitrary assay RSD of 10 %)

n	Square root	RSD (%)	n	Square root	RSD (%)
1	1.00	10.00	6	2.45	4.08
2	1.41	7.07	7	2.65	3.78
3	1.73	5.77	8	2.83	3.54
4	2.00	5.00	9	3.00	3.33
5	2.24	4.47	10	3.16	3.16

If insufficient sample to permit a duplicate analysis is available then use of a smaller sample volume in one or both replicates may be acceptable, provided that the volume is made up using 'blank' plasma/serum and such dilutions have been validated for the assay. A marked difference between duplicates indicates gross error and the need for the test to be repeated, if possible.

If *some* samples are assayed in duplicate for quality assessment (QA) purposes, the mean data should not be reported along with the results of the remaining samples because the errors differ from those of the singleton analyses. *One* of the results from each duplicate can be included, which one of the pair (e.g. the first) having been decided in advance.

3.2.2 Minimizing random errors

Each time a measurement is made there will be an associated error and the total error will be a function of the total number of measurements and how they relate to each other. When measured values are added or subtracted, the variance of the result is the sum of the variances for each measurement. For example, when weighing a sample, if the mass of the weighing boat is $M_1 \pm s_1$ and the mass with added sample is $M_2 \pm s_2$ then the combined SD, s_{diff}, is:

$$s_{\text{diff}} = \sqrt{s_2^2 + s_1^2} \tag{3.9}$$

The same relationship is used for the combined SD when values are added.

The relationship for multiplication and division is more complex, but the approximate values can be obtained using the RSDs. Thus, for a ratio of measurements:

$$\text{RSD}_{\text{ratio}} \approx \sqrt{\text{RSD}_1^2 + \text{RSD}_2^2} \tag{3.10}$$

3.2.2.1 Preparation of a solution of known concentration

As an example, 10 mg of analyte are weighed into a 10 mL flask and solvent is added to volume. Assuming that the weight of the flask is 12 g and that the weighings have a SD of 0.2 mg, then the SD for weighing, s_{w}, is:

$$s_{\text{w}} = \sqrt{0.2^2 + 0.2^2} = 0.283 \text{ mg} \tag{3.11}$$

The estimate of the weight of drug is 10 ± 0.283 mg, so the RSD_w is $\pm 0.283/10 \times 100 = 2.83$ %. If the volume of solvent added to the flask is 10 ± 0.1 mL (RSD = 1 %), then the RSD for the analyte concentration (RSD_{concn}) is:

$$RSD_{concn} = \sqrt{2.83^2 + 1^2} = \sqrt{9.01} = 3\% \tag{3.12}$$

and the concentration should be reported as 1 ± 0.03 g L^{-1}.

If the RSD of 3 % is deemed to be too high, then inspection of Equation (3.12) and the data of Table 3.2 show that a larger RSD is associated with a lower weight of analyte. Weighing 25 mg reduces the RSD to 1.5 %, but on the other hand increasing the weight to 50 mg gives only a small improvement. Weighing 100 mg would be a waste of compound because for this weight most of error in the concentration is due to the error in measuring the volume. This illustrates the important point that if the accuracy of an assay is to be improved, then the steps with the largest errors need to be identified and appropriate measures taken.

Table 3.2 Effect of amount of drug weighed on overall assay error

Weight of drug (mg)	RSD_w (%)	$(RSD_w)^2$	RSD_{concn} (%)
5	4.76	22.7	4.9
10	2.83	8.0	3.0
25	1.13	1.28	1.5
50	0.57	0.32	1.15
100	0.28	0.078	1.04

3.2.3 Accuracy and precision

When a sample is analyzed, the closer the measured concentration is to the actual concentration then the more *accurate* is the result. If repeat analyses are performed, then the less the variation between the results (i.e. the smaller the scatter about the mean value) then the greater is the *precision*. Thus, precision is an estimate of the size of random errors, whereas *bias* is an estimate of the systematic errors, and accuracy defines how close a measured value (or mean of values) is to the 'true' value and contains both types of error. The 'true' value can never be ascertained because there must always be some error in measuring it. A working definition is that accuracy is the closeness of agreement between a test result and an accepted reference value, for example, the concentration in a certified standard solution. The terms imprecision and inaccuracy may be encountered. The former is synonymous with precision whereas, for example, an inaccuracy of 10 % equates to an accuracy of 90 %.

Repeatability is used to describe within batch precision (i.e. when the same standard solutions and apparatus are used by a single analyst) whereas *reproducibility* refers to between-batch precision, when, usually, different standard solutions, apparatus, operators, and possibly laboratories are being compared.

3.2.3.1 Assessing precision and accuracy

To assess precision, replicate assays (e.g. $n = 8$) are performed, usually at several concentrations, and the means and RSDs calculated at each concentration. It is usual to quote the results as RSD

because the errors are generally proportional to the concentrations being measured. Indeed, most assays are designed and validated to ensure that the RSD does not exceed an arbitrary value, say 15 % over the concentration range. Intra-batch (within-day) precisions are measured from a single analytical sequence and inter-batch (between-day) values from the comparison of values from several analytical sequences. It is generally assumed that inter-batch RSDs will be greater than corresponding intra-batch values, but this may not always be the case.

Accuracy is more difficult to assess because a sample of known concentration is needed with which to compare results. EQA schemes provide one method of assessing accuracy (Section 3.6.2). However, such schemes are not available for all analytes. In the event that a scheme is unavailable, or is not sufficiently frequent, efforts should be made to assess accuracy using standard solutions either prepared in-house by an independent analyst, or by exchanging either calibrators or analyzed samples with other laboratories performing the same analysis.

3.2.3.2 Detecting systematic error (fixed bias)

If n replicate measurements have a mean concentration of $\overline{x}$ with SD, s then the result can be tested to see if it is significantly different from the target mean concentration, μ, using a t-test:

$$t_{(n-1)} = \frac{(\bar{x} - \mu)s}{\sqrt{n}} \tag{3.13}$$

If $t_{(n-1)}$ is greater than the critical value (obtained from statistical tables) then $\overline{x}$ is significantly different from μ. Thus, if the target value was for an EQA sample then the estimate contains a systematic error, also referred to as bias. In a similar way, the results from two analysts can be compared to assess if the differences in the mean values were due to random errors, in which case they should not be significantly different. If the samples have equal size variances, then the appropriate t-test is:

$$t_\nu = \frac{\overline{x}_1 - \overline{x}_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \tag{3.14}$$

where $\nu = n_1 + n_2 - 2$ and s is given by:

$$s = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{\nu}} \tag{3.15}$$

The F-test is used to ascertain whether or not the variances are significantly different:

$$F = \frac{s_1^2}{s_2^2} \tag{3.16}$$

Note that the variance ratio is always written such that $s_1 > s_2$ to ensure $F \geq 1$. Critical values of F are tabulated with ν_1 [$= (n_1 - 1)$] along one axis, and ν_2 [$= (n_2 - 1)$] along the other. A two-tailed test should be used. When the sample variances are not equal, the equation is more complex, but the principle of the test is the same.

3.2.3.3 Identifying sources of variation: analysis of variance

There are situations when more than two means need to be compared, for example when comparing results from several laboratories. There will be within-laboratory variation, but there may also be additional variation due to between-laboratory variation. This can be tested for using analysis of variance (ANOVA). As an example, data from an experiment testing the effects

of storage conditions (Table 3.3) give rise to mean values for replicate analyses of the freshly prepared and stored samples. The question is 'Do all the means come from the same population?' With each treatment having n replicates:

$$\text{Total sum of squares } (TSS) = \sum (\text{Measured concentration} - \text{Overall mean})^2$$

$$\text{Within-treatment sum of squares } (WSS) = \sum (\text{Measured concentration} - \text{Treatment mean})^2$$

$$\text{Between-treatment sum of squares } (BSS) = \sum n\,(\text{Treatment mean} - \text{Overall mean})^2$$

Table 3.3 Data from a typical stability experiment

Treatment	Measured concentration					Mean	s	s^2
Freshly prepared	104	100	98	95	101	99.6	3.36	11.3
Stored –20 °C (18 h)	100	97	95	103	98	98.6	3.05	9.3
Stored 4 °C (18 h)	98	94	89	100	95	95.2	4.21	17.7
Room temperature (4 h)	99	89	93	85	88	90.8	5.04	29.2
Overall mean						96.05	4.01	16.88

In the example, the within-treatment variance, σ_w^2, has 16 degrees of freedom as each replicate of n samples has $(n - 1)$ degrees of freedom and they are 4 sets of them, and so:

$$\sigma_w^2 = MS_w = \frac{WSS}{m(n-1)} \tag{3.17}$$

where m is the number of treatments. It is also referred to as the within-treatment *mean square* (*MS*) and is the mean within-treatment variance (Table 3.3). The between-treatment variance has (number of treatments – 1) degrees of freedom, represented by the between-treatment mean square MS_b, hence:

$$MS_b = \frac{BSS}{m-1} \tag{3.18}$$

If there was no additional variation due to different storage conditions, then MS_b should equal MS_w and

$$F = \frac{MS_b}{MS_w} \tag{3.19}$$

would not be significantly different from 1.

In the example, $MS_b = 78.98$, and $MS_w = 16.88$, so $F = 4.68$. The critical value ($P = 0.05$) of F for 3 and 16 degrees of freedom obtained from statistical tables is 3.239. Note that one-tailed tables are always used for one-way ANOVA. Because $4.48 > 3.239$, the storage conditions do have a significant effect on the mean concentrations measured.

ANOVA is included in the statistics available with Microsoft Excel. The output includes *BSS*, *WSS*, *TSS*, the degrees of freedom, MS_b, MS_w, F, and the level of significance, P (Figure 3.2).

Having shown that there is a statistically significant effect between storage conditions, further testing can be performed to test for differences between the treatments. Statistical programs usually include this option, so that the tests are automatically applied if F is significant. Bonferroni's t-test is used for multiple comparisons, whereas Dunnett's test compares treatment

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	5	498	99.6	11.3
Row 2	5	493	98.6	9.3
Row 3	5	476	95.2	17.7
Row 4	5	454	90.8	29.2

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	236.95	3	78.98333	4.680494	0.015666	3.238867
Within Groups	270	16	16.875			
Total	506.95	19				

Figure 3.2 Excel table of results for one-way ANOVA of the data of Table 3.3

means to a control mean (the freshly prepared solution in this example). Both tests showed that there was a significant difference between the mean value given by the freshly prepared solution and that given by a sample stored at room temperature. Testing for a linear trend is useful when the effect of time, for example, on a parameter is being examined.

3.2.3.4 Measurement uncertainty

Measurement uncertainty (MU) is a value associated with the result of a measurement that defines the range of values that could reasonably be attributed to the measured quantity. ISO 15189 states that MU may be calculated using values obtained by the measurement of IQC materials under 'intermediate precision' conditions that include as many routine changes as reasonably possible in the standard operation of a measurement procedure. Such changes might include use of different reagent and calibrator batches, different operators, and planned instrument maintenance.

MU is expressed as ±1.96 CV %, in other words the 95 % confidence interval of the sum of the IQC CV values given by an assay over a defined period. For blood alcohol, for example, MU could be calculated from the differences between the laboratory's EQA results and the mean values reported by the participants in the scheme (Wallace, 2010).

3.2.4 Calibration graphs

Normally a calibration graph of analyte response versus concentration in the calibration solutions is constructed. In chromatographic assays, FIA, etc., the response may be peak height or area, or peak height or area ratios to those of an ISTD (Section 3.4). The relationship may be a straight line or a curve.

Before ready access to curve-fitting programs, calibration graphs were plotted on graph paper and the concentrations interpolated manually. Having a straight line made the task easier and analysts tried to work within a 'linear' region of the curve even for techniques such as fluorescence that are not inherently linear. Least-squares fitting of calibration data can now be

performed with electronic calculators and has the advantage that statistical fitting allows an assessment of the quality of the results in terms of CI or SD.

3.2.4.1 Linear regression

Least-squares linear regression is a statistical method to obtain the best fit of a straight line ($y = a + bx$) to experimental data. The formula minimizes the sum of the squares of the residuals, $(\hat{y}_i - y_i)$ where $\hat{y}_i$ is the y-value calculated from the regression line at x_i (Figure 3.3). The sum of *squares* of the residuals is used because the sum of the residuals will be zero. Estimates of a and b can be obtained either by statistical fitting (e.g. using Microsoft Excel), or from a manually plotted graph. The regression line goes through the mean value of x and y (the centroid), a fact that can be exploited when plotting a graph manually.

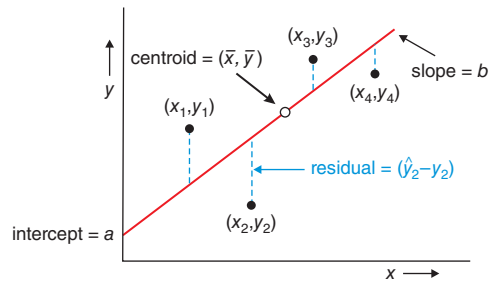


Figure 3.3 The principle of least-squares regression

Linear regression programs usually provide the correlation coefficient, r, which gives an indication of how y varies as a function of x. The explained variance is r^2. For example, if $r = 0.95$, $r^2 = 0.903$ so the explained variance or *percentage fit* is 90.3%, which indicates that ~10% of the change in y cannot be ascribed to the change in x.

Unless the data are a perfect fit to the regression line, there must be errors associated with the estimates of a and b. The standard errors, s_a and s_b, respectively, are used to calculate the CI values for the intercept and slope, respectively:

$$a \pm t_{(n-2)} s_a \text{ and } b \pm t_{(n-2)} s_b$$

Note that for linear regression the number of degrees of freedom is $n - 2$, because a straight line will pass through *any* two points and so a fit cannot be derived statistically from only 2 points. Having obtained values for a and b, concentrations in the test samples can be calculated from:

$$\text{concentration} = \frac{\text{response} - a}{b} \tag{3.20}$$

The equation to calculate the CIs for the interpolated concentrations is complex because the error will arise from errors in the estimate of the slope and the intercept and, of course, the response will have random errors. Provided that b and the sum of squares of x are relatively large, which they are in a well-constructed calibration graph, then an approximation may be used to calculate the CI:

$$\text{CI} = \pm \frac{t_{(n-2)} s_{x/y}}{b} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(\text{response} - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2}} \tag{3.21}$$

where m is the number of replicates used to obtain a value for the response of the unknown sample.

Features of Equation (3.21) are (i) that CI is smallest for signal responses that equal the mean of the responses in the calibration curve, (ii) replicate measurements can have a marked effect on reducing the CI, and (iii) increasing the number of calibration points reduces CI (Figure 3.4). Note how the CIs are symmetrically curved about the regression line and the effect that the number of calibration points has on the CI values.

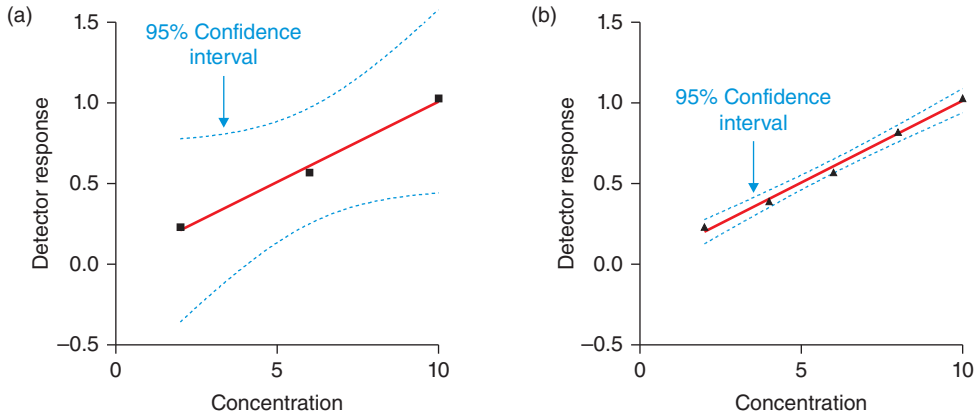


Figure 3.4 The effect of the number of data points on the 95 % confidence interval: (a) three points, (b) five points

Not surprisingly, the curve with only 3 points has very large confidence intervals; there is only one degree of freedom ($n - 2$) and t_ν is 12.71. With two additional standards, $\nu = 3$ and t_ν is 3.18. For a curve with only 3 points [Figure 3.4(a)], in this example the correlation coefficient was not significant, i.e. the slope was not significantly different from zero. The significance of r can be tested using:

$$t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}} \tag{3.22}$$

3.2.4.2 Testing for linearity

It should not be assumed that a detector response increases linearly with increasing concentration. Calibration data should be plotted or displayed on a computer screen and scrutinized [Figure 3.5(a)].

Fitting the data of Figure 3.5(a) to a straight line gave a value of $r = 0.998$. However, on close inspection the graph is obviously curved, i.e. the commonly held belief that a high value of r is proof that the calibration graph is linear is not true. It is the case that if the calibration graph were a perfect straight line, r would be unity, but a correlation coefficient approaching unity does not of itself prove that the calibration line is linear (AMC, 2005). Inspection of the points about the line shows two points below the line, then a 'run' of points above, and a point below. This pattern is seen more clearly if the y-residuals are plotted against concentration [Figure 3.5(b)]. If the calibration data did fit a straight line then the residuals, whose sum must equal zero, would be normally distributed about zero. The U-shape distribution in Figure 3.5(b) is typical of data that should be fitted to a curve.

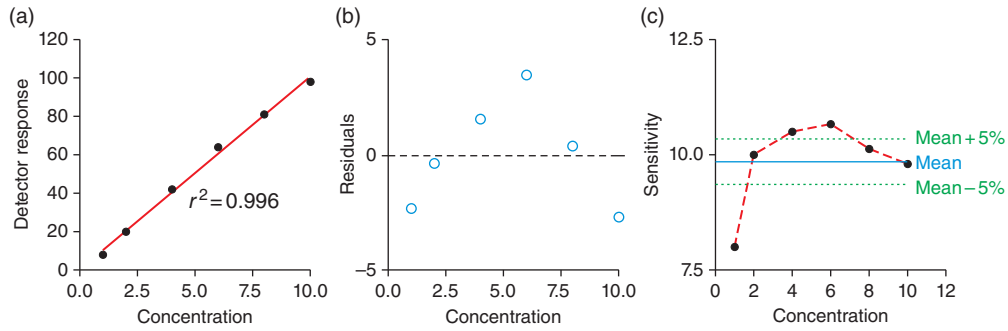


Figure 3.5 Testing calibration curves for linearity: (a) data fitted to a straight line (b) residual plot (c) plot of sensitivity (detector response/concentration) versus concentration

A further way to prove that a curve is non-linear is to plot *sensitivity* against concentration (or log concentration in the case of a calibration curve that spans a wide range of concentrations). Sensitivity has been defined as detector response divided by concentration, basically the slope of the calibration graph (http://www.iupac.org/goldbook/S05606.pdf).

If the calibration is perfectly linear then sensitivity will be the same at every concentration. However, if sensitivity varies in a systematic manner then fitting a straight line would be incorrect. For a calibration graph to be considered linear the sensitivity should not vary by more than ±5 % of the mean value [Figure 3.5(c)]. Clearly the data from the example have not been derived from linear data. An advantage of examining sensitivity as a function of concentration is that such plots can be used to define a working linear range. This is particularly useful for curvilinear plots often seen with fluorescence and MS detection and has been applied to defining the linear working ranges of detectors.

3.2.4.3 Weighted linear regression

Simple linear regression as described above assumes equal variance at all concentrations, i.e. the data are said to be homoscedastic. However, for calibration data the variance generally increases with concentration – indeed by ensuring that the RSD values cover a limited range, especially if chromatographic methods are used, and never exceed a predefined limit, the errors are going to be approximately proportional to the concentration. Thus, the errors associated with high concentrations are higher and these may unduly affect the slope of the calibration line. However, unweighted regression treats all points equally which in effect means that lower concentration points are more likely not to be fitted to the line, which can be a problem, particularly if the calibration curve extends over several orders of magnitude. The answer is to weight the data and to minimize the sum of weighted squares:

$$\text{Weighted sum of residual squares} = \Sigma[(\text{weight})(\text{residual})2] \tag{3.23}$$

The size of the random error in the measured response is given by the variance (s^2) and thus the data should be weighted by $1/s^2$, or the reciprocal of some value that is directly proportional to s, such as concentration or response. Equation (3.23) can be written:

$$\text{Weighted sum of residual squares} = \Sigma[1/s^2(\text{residual})^2] \tag{3.24}$$

Therefore, the points with the lowest errors assume more importance than those with the largest errors. If the RSD is constant over the concentration range used for the calibration

graph then the size of s is proportional to the size of the signal and, for a linear relationship, proportional to the concentration of the sample. Under these conditions the data can be weighted by 1/(response)^2 [or by 1/(concentration)^2, if the calibration is linear].

Many commercially available statistical programs allow the option of weighting data by $1/y^2$ or by $1/x^2$. The weighted calibration line passes through the weighted centroid and the confidence intervals are smallest at this point, but unlike the homoscedastic case are not symmetrical about the line, but increase disproportionately as the concentration increases.

Weighting data is not some method of manipulating the result to make it appear more acceptable, *it is the correct statistical treatment for heteroscedastic data*. However, this being said, when the errors are small and the concentration range limited, then simple linear regression may be shown to be fit for purpose.

3.2.4.4 Non-linear calibration

There is no reason why a calibration line should be a straight line over the entire calibration range. Indeed, some detectors, such as fluorescence detectors, are known to have a limited 'linear' range, and even the Beer–Lambert law (Section 5.4.1) does not hold at high analyte concentration. MS coupled to either GC or LC, and some immunoassays, such as EMIT (Section 6.4.1), may give a curvilinear response with changes in concentration. If the concentration–response relationship has been shown to be non-linear then a decision as which type of curve should be fitted has to be made.

The data of Figure 3.5(a), which are clearly not linear, can be fitted to a quadratic equation [Figure 3.6(a)]. Not only does the quadratic equation give a better fit to the data, but also the correlation coefficient is greater ($r = 0.9995$), confirming that r-values approaching 1.0 cannot be taken as being indicative of linearity. Inspection of the residual plot [Figure 3.6(b)], which are close to and randomly distributed about zero, confirm that the quadratic model is a better choice than the linear one.

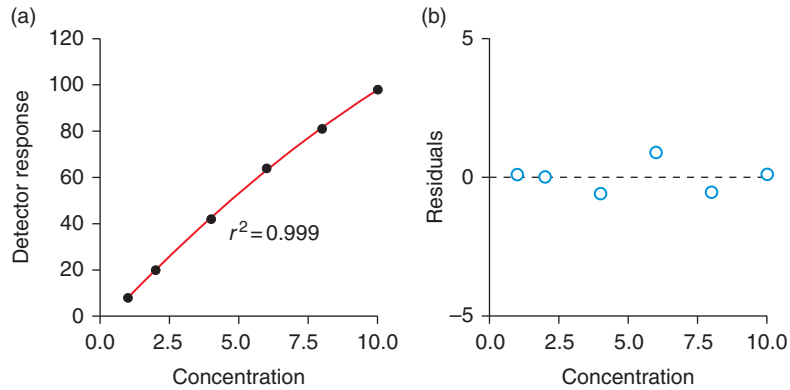


Figure 3.6 The data of Figure 3.5(a) fitted (a) to a quadratic function and (b) the resulting residuals

Sometimes a model for the relationship between detector response and concentration can be proposed. Calibration data for medazepam measured by GC-ECD in DC mode (Section 9.2.2.4) are shown in Figure 3.7. As the electrons are captured the standing current falls and so the curve might be expected to be defined by a hyperbola [Figure 3.7(a)]. The hyperbola passes though the points and the origin and asymptotes to a maximum value (the point where there is no longer

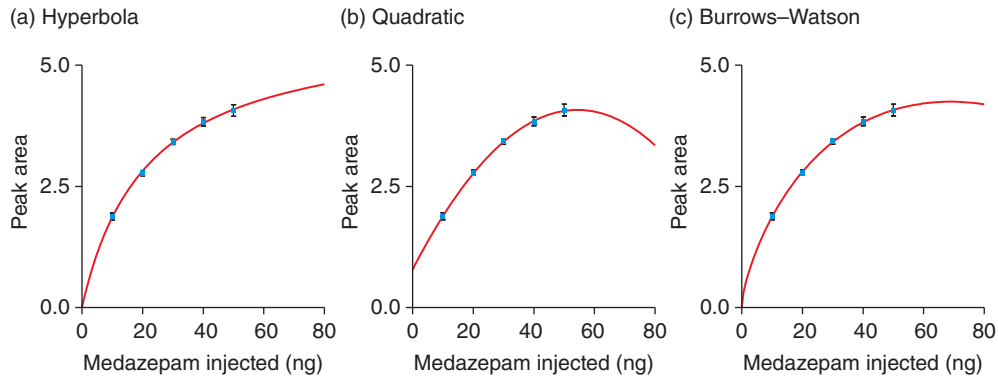


Figure 3.7 Calibration data for GC-ECD of medazepam fitted to (a) a hyperbola, (b) a quadratic function, and (c) Burrows–Watson equation

any standing current). However, the quadratic fit [Figure 3.7(b)] is clearly a suitable alternative over the working range of the calibration data.

With non-linear curves it is unusual to have a model for the relationship between the concentration and response and there may be several components leading to non-linearity. Thus, the choice of an equation is empirical and a quadratic or cubic expression may be suitable. For curvilinear plots of the type frequently seen in MS assays, Burrows & Watson (1994) recommend [Figure 3.7(c)]:

$$y = a + bx + cx \ln x \tag{3.25}$$

3.2.4.5 Residuals and standardized residuals

Inspection and plotting the residuals can be very useful in demonstrating that the most suitable equation and method of weighting have been applied to the calibration data, and may indicate the presence of outliers. Ideally, the residuals should be randomly distributed about zero [Figure 3.6(b)]. Curves such as that of Figure 3.5(b) indicate that an incorrect equation has been chosen. If the residual versus concentration plot shows an approximately constant value of residual over the concentration range then no weighting is required [Figure 3.8(a)]. However, the sizes of the residuals are often proportional to analyte concentration [Figure 3.8(b) and (c)]. Indeed, this is almost invariably the case as methods are developed to have more or less constant RSDs over the calibration range.

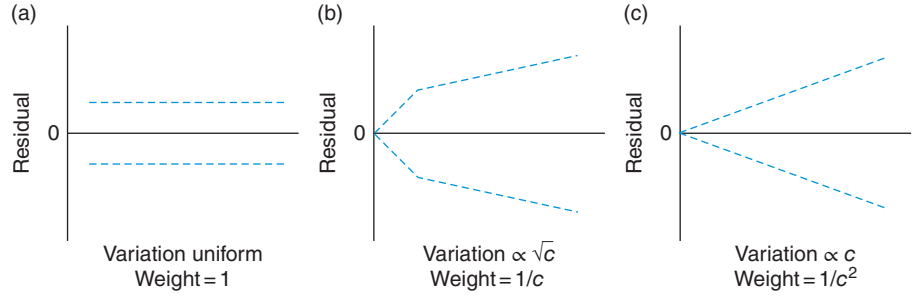


Figure 3.8 Use of residual plots to ascertain the most appropriate method to weight calibration data

3.2.4.6 *Blank samples and the intercept*

Blank samples, usually the drug-free matrix, should be prepared and analyzed along with the calibrators and unknown samples both with and without addition of an ISTD. The blank is an important part of QC as it should be free of interfering substances. In a chromatographic assay, for example, there should be no interfering peaks in the region of the analyte(s) or ISTD(s). The response of the blank should not be so high as to limit the working range of the assay.

The blank signal *should not* be subtracted from the responses of the calibrators as this alters the error model (Section 3.5.2). Similarly, *quantitative* photometric assays should not be performed against blank samples placed in the reference cell of a double beam spectrometer. Note, this is different from the *qualitative* investigations described in Chapter 5.

The blank is not a zero-concentration calibrator, and must not be included in the calibration data. Sample analyte values below the lowest calibrator should not be quantified. Replicate analyses of 'blank' signal are necessary to define the limit of detection (LoD, Section 3.2.4.8) and so it is important when using electronic data capture to ensure the software is not forcing the calibration curve though zero.

In practice, calibration graphs rarely pass through the origin (0,0), although the origin may be within the 95 % confidence intervals of the intercept. A positive intercept is most likely due to a contribution from background signals and would be expected to be close to the value given by the blank sample (this is a useful QC check). The intercept is the average contribution that the blank signal makes to calibrator signals. Negative intercepts are often a result of adsorptive losses, which are proportionality greater at lower analyte concentrations. Although statistical methods can be used to 'force' a calibration though the origin, this is not necessary as it is simple to calculate the concentrations in the unknown samples, for example from Equation (3.20).

3.2.4.7 *Method of standard additions*

In the preparation of calibration graphs described above, it has been assumed that the analyte-free plasma, urine, etc. are representative of the matrix to be analyzed. However, there may be situations when there are wide variations in the matrix (e.g. post-mortem blood) or when it is impossible to obtain suitable analyte-free matrix, as when measuring endogenous compounds, for example.

In the method of standard additions, a calibration graph is prepared using the sample to be analyzed. The calibration graph is drawn or calculated as usual and the unknown concentration obtained from the intercept on the concentration axis (Figure 3.9). This has two obvious drawbacks: (i) the volume of the unknown sample must be large enough to prepare the required

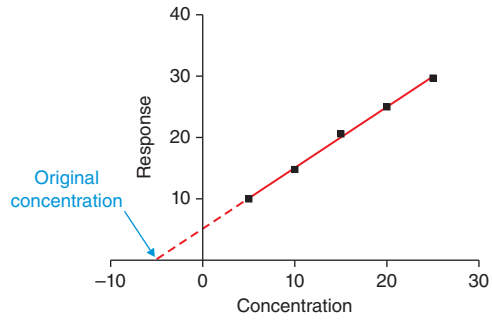


Figure 3.9 Calibration curve for the method of standard additions

number of calibrators, and (ii) it is time consuming because each unknown sample requires its own calibration curve. Furthermore, there is neither reproducibility data between one graph and the next, nor IQC data. The unknown sample could be divided and a known amount of analyte added to one portion, but this single-point calibration approach assumes a perfectly linear relationship between concentration and response.

3.2.4.8 Limits of detection and quantitation

An important criterion for an analytical method is the LoD, i.e. the minimum concentration (amount) of analyte that can be detected reliably and differentiated from any background signals measured in analyte-free samples. Defining the LoD is difficult because there are random errors associated with blank samples and with samples at or near the LoD. For chromatographic assays the LoD might be quoted as some arbitrary multiple of the signal to noise ratio (S/N) such as 3, 5, or 10 times. This, of course, presupposes that the S/N can be measured and with biological samples, the limiting factor is rarely instrument noise, but rather signals that are usually because of endogenous interferences.

Although there is no universally agreed definition of LoD, the IUPAC formula is frequently used:

$$\text{LoD} = \bar{y}_B + k\sigma_B \tag{3.26}$$

where $\overline{y}_B$ and σ_B are the mean and the SD of the blank signal, respectively. If the mean signal for the blank is close to zero, then some responses will be negative and it is important that these are measured. Using a value of 3 in Equation (3.26) (sometimes referred to as the 3σ convention) there is ~7 % probability that the analyte will be deemed to be present in the sample when in fact it is not. To reduce the error to the 5 % level requires the factor to be increased to 3.28 (Miller & Miller, 2018). Having derived an estimate for LoD, results below this can be reported as < LoD, but the best practice in analytical toxicology is to report them as 'not detected' because this does not imply that the analyte is present when in fact it may not be. Similar considerations apply to assessing LoD in MS assays (Section 13.11).

For quantitative analyses it is usual to quote the lowest concentration or amount that can be measured with defined values of precision and accuracy, the LLoQ. Again, the criteria are arbitrary, and the RSDs for precision and accuracy typically range from 10–15 %, depending on the requirement of the assay. For trace analyses and pharmacokinetic experiments it may be necessary to accept values as high as 20 %, although the RSDs for concentrations above the LLoQ would normally be considerably lower. The concentration of the lowest calibrator may be set to the LLoQ, although for some applications it may not be necessary to measure such low concentrations. In any event, *concentrations below the lowest standard must not be reported*. An alternative approach, using a value of $k = 10$ in Equation (3.26), is not widely used.

Reports do not always quote a value for ULoQ particularly when the assay has a wide linear range. However, for some techniques, such as immunoassay and fluorescence detection in LC, the working range of the assay should be defined by both LLoQ and ULoQ.

3.2.4.9 Curve fitting and choice of equation

How the calibration data are used to derive analyte concentrations depends on a number of factors. These include the reason for performing the assay, the type of assay, and whether the assay is a routine one involving large numbers of samples, or a single measurement of an analyte that is required rarely. In some instances, it may not be necessary to 'draw' a calibration graph, for example when using cut-off values in immunoassays (Section 6.6.2). The data station or

integrator may have the calibration routine built-in and the results expressed as concentrations (or amounts) directly.

When there are only a few samples to be analyzed, drawing a calibration on graph paper and interpolating the unknown concentrations by eye may be adequate, but for larger numbers this is tedious, time-consuming and prone to error. Furthermore, it is not amenable to statistical analysis. Many inexpensive hand-held calculators will perform linear regression, and once the slope (b) and intercept (a) of the calibration line are known it is simple to calculate the concentrations in the test samples using Equation (3.20).

For more complex calibration relationships, computer programs may be needed. These can include commercially available statistical and curve drawing packages, which are often included in GC-MS and LC-MS instrument control packages. Linear and quadratic equations have discrete solutions, and calibration curves can be generated using a spreadsheet such as Microsoft Excel.

The choice of calibration equation (model) should be dictated by the data. Statistical fitting allows various equations, for example quadratic and cubic, to be compared (Desharnais *et al*., 2017a,b). Simply choosing the equation that gives the lowest residual sum of squares (SS) is unhelpful because this will be the equation with the largest number of parameters. Consequently, most statistical packages compute 'goodness of fit' parameters, which take into account the number of parameters in the equation. A useful one is the Akaike information criterion (AIC):

$$\mathrm{AIC} = n \ln(SS) + 2m \tag{3.27}$$

where n is the number of data points and m is the number of parameters. The equation with the lowest AIC is statistically the most appropriate.

3.2.4.10 Single-point calibration

Multi-point calibration as described above is well suited to batch analyses. However, on occasions, for example emergency requests for rarely performed assays, single-point calibration may be appropriate. Single-point calibration methods should be validated, and the results compared with those from multi-point calibration (Peters *et al*., 2005). It is important to show that 'blank' samples from a variety of sources are free from interferences, to define the linear range of the assay, which should encompass the lowest and highest expected concentrations, and assess the size of the intercept. The concentration of the single-point calibrator should be chosen so that the 99 % confidence intervals for mean values are within 50 % of the target value.

With commercially produced immunoassays, manufacturers often develop an equation apparently enabling straight line calibration to be used. This can be 'checked' routinely with single-point calibration and use of a zero intercept. However, it is important to use multiple calibrators to confirm continued performance if, for example, a different batch of reagent is used because assay calibrators may be 'adjusted' to be reagent lot specific. Failure to perform multiple-point calibration may result in calibration drift.

3.2.5 Batch analyses

Whatever instrumentation is employed, quantitative batch assay calibration should normally be by analysis of standard solutions containing each analyte. The FDA/CDER (2018) and EMEA (EMA, 2019) guidelines specify that the LLoQ should be the lowest calibrator, and that for a linear calibration model, there should be a minimum of six equally spaced calibrators across the calibration range (Wille *et al*., 2011). For quadratic (second-order polynomial) models, at

least eight points should be used. The solutions should be matrix matched as far as possible and prepared in, for example, analyte-free NBCS or human serum. IQC procedures should be instituted. This involves the analysis in each analytical sequence of independently prepared standard solutions of known composition that are not used in assay calibration. Normally low, medium, and high concentrations of each analyte are prepared analyte-free matrix. If NBCS has been used to prepare the calibrators, human plasma or serum should be used to prepare the IQCs.

'Equally spaced' is open to interpretation as either linear (5, 10, 15, 20, 25, 30, 35), or exponential (5, 10, 20, 40, 80, 160, 320). Given that in analytical toxicology a wide concentration range is often encountered, but with most samples containing analyte towards the lower end of the calibration range, many authors chose this latter interpretation (Wille *et al*., 2011). Whichever method is chosen, the calibration should be tested for linearity (Section 3.2.4.2) and the data weighted as appropriate to ensure that the residuals are normally distributed about zero (Section 3.2.4.5).

Calibration standards are normally assayed in duplicate, once at the beginning and once at the end of the batch. IQC samples are analyzed at the beginning and end of the batch, and also after every 5–10 patient samples, as appropriate. EQA samples are analyzed to conform to the requirements of particular EQA schemes. During development and validation of a method, an important parameter to assess is maximum batch size. This accounts for stability of assay calibration (i.e. any 'drift') over time, but also the stability of prepared samples in an autosampler.

The performance of batch analyses, the analysis of a number of samples in the same analytical sequence, and analysis acceptance criteria should be as set out in method validation guidance (FDA/CDER, 2018). Typical assay acceptance criteria are (i) chromatography (reproducible peak shape and retention time, stable baseline), (ii) calibration graph ($r \geq 0.98$, intercept not markedly different from zero), and mean IQC results within acceptable limits (generally within 10 % of nominal value). Acceptance criteria for patient samples are (i) 'clean' chromatogram, i.e. absence as far as can be ascertained of interferences, (ii) duplicate values (peak height or area ratio to the ISTD) within 10 % except when approaching the LoD of the assay when duplicates within 20 % may be acceptable, and (iii) results within the calibration range. Clinical sample analyses falling outside acceptance limits may be repeated if sufficient sample is available.

If a result above the calibration range is obtained then, if possible, a portion of the sample should be diluted with 'blank' plasma/serum and reanalyzed. If a sample is from a suspected overdose patient, then sample dilutions (1+1, 1+3, 1+9) should be made using 'blank' matrix at the time of the initial analysis (if the available sample volume permits) and analyzed at the same time as the normal sample analysis. Post-mortem whole blood samples from suspected overdose cases should be diluted 1+3 and 1+9 with 'blank' human serum prior to analysis using standard methods. If the information available suggests a massive overdose, further dilutions may be made as appropriate prior to the analysis.

3.2.6 Random access analysis

Whilst immunoassays may be performed as batch analyses, they are more often subjected to random access analysis. Chromatographic and MS assays are more commonly performed using batch analysis with bracketed calibrators, although random access assays are sometimes employed for high-throughput quantitative assays. It is especially important for such assays to consider calibration frequency, and to ensure the same ISTD batch is used for calibrators and subsequent samples. Use of isotopic internal calibration using stable isotope labelled (SIL) analogues to facilitate random access MS assays is discussed in Section 13.10.3.

IQC in random access analysis is best performed at timed intervals at low, medium, and high concentration. Some immunoassays are used as screens for the presence of drugs or metabolites, and 'cut-off' concentration values are used to define positives. It is particularly important to ensure that the analytical performance at this cut-off point is properly investigated (i.e. IQCs at ± 25 % of cut-off value) to minimize the risk of false positives and negatives arising from poor assay performance.

3.3 Use of internal standards

An ISTD is a compound added in known amount at some stage in an assay with the aim of demonstrating method integrity and correcting for systematic errors (Section 1.3.3). Obviously, an ISTD is used mainly in chromatographic, electrophoretic, or MS assays (Chapters 9–13) where the resolving power of the analytical system permits independent measurement of the added compound. However, use of an ISTD may increase the analysis time, and thus it is worth ensuring that a suitable compound is employed. It should be shown that the use of internal standardization is beneficial, or at least is not deleterious, to assay performance.

Whenever possible the ISTD should be added to the biological sample in (preferably aqueous) solution. If there are extraction step(s), the ISTD should show similar behaviour to the analyte(s), i.e. the partition coefficients should be similar. It is self-evident that the ISTD should show a similar detector response to and be resolved from the analyte(s) and any other compounds likely to be present on the chromatogram or ions in the mass spectrum. In particular, the ISTD should be neither a drug that is likely to be present in the samples to be analyzed, nor a known or possible metabolite or decomposition product of the analyte(s) (Box 3.3).

For MS assays, SIL ISTDs are often employed. However, these can influence the result produced (Berg *et al*., 2014), depending on the isotope (^{2}H, ^{13}C, ^{15}N, etc.) and numbers of atoms substituted (Section 13.10). This should be considered during method development.

A constant amount of an ISTD must be added with a high degree of precision to each sample, with thorough mixing. Absolute accuracy in the amount added is generally less important, and within limits, an ISTD does not need to be as pure as the analyte. Depending on the reasons for its inclusion, the ISTD may be added either at the beginning, or at a later stage of a procedure.

3.3.1 Advantages of internal standardization

3.3.1.1 Reproducibility of injection volume

Manual injection of small volumes of an extract in GC especially is not very precise. Provided that the detector shows a linear response to the ISTD, the signal given by the ISTD should be directly proportional to the volume of sample injected. Thus, if all the samples contain the same concentration of internal standard a calibration curve of:

$$\frac{\text{analyte response}}{\text{internal standard response}}$$

against analyte concentration can be constructed. If the sample analyses are treated in the same way, response ratios can be used to calculate the analyte concentration in the sample, due allowance for differences in injection volume having been made. The same reasoning applies to situations where a volatile injection solvent is used. In this case the drug and ISTD concentrations will rise as the solvent evaporates, the response given by the ISTD being inversely proportional to the volume of solution remaining. Use of internal standardization is important in headspace analysis (Section 9.4).

Box 3.3 Some requirements for an internal standard

- Be completely resolved from potential interferences
- Elute near to (preferably just after the last) peak(s) of interest unless MS detection
- If SIL ISTD, should co-elute with the unlabelled analyte
- Have a similar detector response (peak height or area) to the analyte(s)
- Have similar chemical and physical properties to the analyte(s)
- Undergo any derivatization reaction in the same way as the analyte(s)
- Be chemically and physically stable on storage in solution and during the analysis
- Be easily available with adequate purity
- Not be a marketed drug

3.3.1.2 Instability of the detection system

Some detection systems are less stable than others. An electrochemical detectors (ED) in LC shows reduced response if contaminated, for example. A suitably chosen ISTD may compensate for such changes. However, the analyte:ISTD signal ratio *must* remain constant for a given mixture. This can be tested by perturbing the system, e.g. changing the working voltage of an ED slightly and observing the result (Whelpton, 2019).

3.3.1.3 Pipetting errors and evaporation of extraction solvent

Once an ISTD has been added, losses on extract transfer between vessels or extract concentration due to evaporation of a volatile solvent during the procedure should be reflected in the recovery of the ISTD. It can be argued, therefore, that inclusion of an ISTD obviates the need for quantitative transfers during the procedure, at least in part, and so cheaper glassware, e.g. Pasteur pipettes, or quicker methods, such as pouring, may be used when separating phases, for example. If the transfer volumes are measured, then good precision can usually be obtained with external standardization. Be this as it may, obtaining quantitative phase transfer and use of relatively non-volatile extraction solvents is to be recommended because the ISTD response should be relatively constant during an analytical sequence and any major change in response should alert the analyst to a potential problem with the assay (White *et al*., 2014).

3.3.1.4 Extraction efficiency

For an ISTD to compensate for extraction losses it must be as chemically similar to the analyte as possible. Therefore, in MS a SIL ISTD is most appropriate (Section 13.10), but if such compounds are unsuitable, as in non-MS methods, or unavailable, then the closest appropriate homologue should be selected. This is usually more successful than using an analogue or compound that is less similar chemically to the ISTD (Figure 3.10).

A problem arises when several compounds, e.g. a drug and its metabolites, are to be measured because the extraction characteristics of each compound are likely to differ. Some studies may require the use of more than one ISTD, e.g. if two different detectors are used, or if there is a long chromatographic sequence, such as in LC-MS/MS screening for misused drugs. If the extraction efficiency of the analyte is high (95 % or so), then inclusion of an ISTD may give only marginal improvement in assay precision. The same is true of situations where the extraction efficiency is lower, but reproducible. The inclusion of ISTDs to correct for differences in protein binding, losses on protein precipitation, and adsorptive losses on glassware is likely to be unhelpful unless a SIL ISTD can be used.

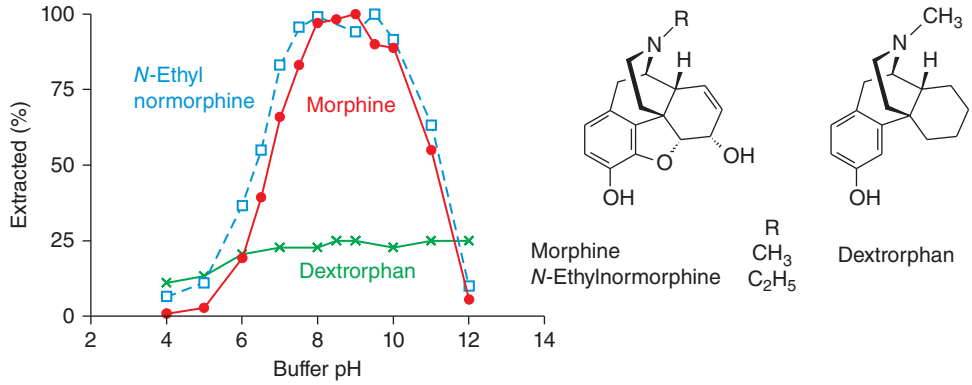


Figure 3.10 Extraction characteristics of morphine (●), *N*-ethylnormorphine (□), and dextrorphan (x) used as ISTD in a morphine assay. Aqueous phase: Britton–Robinson buffers (0.1 mol L^{-1}). Extraction solvent: butanol:toluene (1+9). Solvent:buffer ratio 10:1 (Whelpton, 2019–reproduced with permission of Elsevier)

3.3.1.5 Derivatization and non-stoichiometric reactions

Other factors being equal, only SIL ISTDs should be used in analyte derivatization as chemically distinct entities are less likely to react identically. The causes of any imprecision should be investigated and the reaction conditions modified to make them suitably robust rather than rely on the inclusion of a second compound. There may even be problems with SIL ISTDs, particularly if the bond(s) linking the isotope to the rest of the molecule are involved in the derivatization reaction. It may be necessary to use compounds with alternative sites of labelling.

3.3.2 Internal standard availability

Compounds suitable for use as ISTD may be available from chemical suppliers, pharmaceutical companies, or elsewhere. If the desired compound is not immediately available, it may be possible either to have it synthesized, or to prepare it in the analytical laboratory. An ISTD need not be 100 % chemically pure and a relatively small quantity will often suffice to assess the value of using a given compound in this way, prior to synthesizing a larger quantity. Often the reaction(s) required to synthesize a potential ISTD are not dissimilar to those used in common derivatization reactions. The ethyl homologue of dihydrocodeine can be prepared at room temperature by passing hydrogen through an aqueous solution of ethylmorphine (a marketed drug) containing a little platinum on barium sulfate as catalyst [Figure 3.11(a)]. The ethyl homologue of paracetamol is prepared easily from 4-aminophenol hydrochloride and propionic acid anhydride [Figure 3.11(b)].

3.3.3 External calibration

If a suitable ISTD cannot be found, it will be necessary to use external calibration. This does not mean that the method will inevitably have poor precision and accuracy. The increased use of reproducible injection systems, stable detectors, and extraction methods that gave good, reproducible recoveries can give acceptable results. The points highlighted in Box 3.4 are particularly relevant when working without an ISTD. Automated extraction systems are more amenable to external calibration provided that the extraction system has been properly validated.

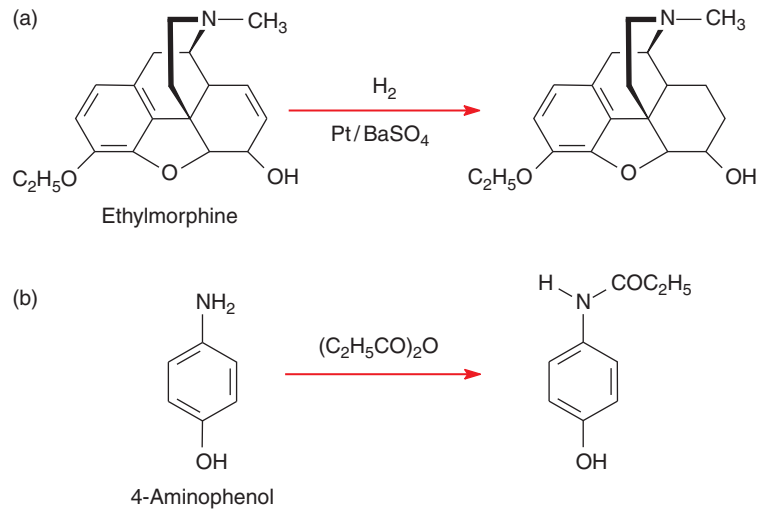


Figure 3.11 Synthesis of ISTDs from readily available starting materials to produce the ethyl homologues of (a) dihydrocodeine and (b) paracetamol

Box 3.4 Points to be considered when using external calibration

- Minimize the number of extract transfer and evaporation steps; consider injecting the sample or sample extract directly or after dilution, increasing the detector sensitivity/selectivity if necessary
- If analyte concentration steps are required, consider solid phase extraction (SPE) as this often gives high recovery with good reproducibility
- When LLE is used transfer volumes must be measured
- Avoid volatile extraction solvents as is difficult to assess the volume change during handling
- If feasible, concentration of the analyte in sample extracts by back-extraction into a small volume of liquid is preferable to solvent evaporation
- Completely (over) fill the sample loop when injecting in LC
- Inject as large a volume as possible in GC to reduce the errors associated with measuring small volumes

3.3.4 Potential disadvantages of internal standardization

It should not be assumed that the inclusion of an ISTD will inevitably improve the precision and accuracy of a GC or LC assay. An ISTD cannot reduce the imprecision caused by random errors. Indeed, the random errors associated with the quantification of the ISTD are likely to *increase* the overall imprecision rather than reduce it [Equation (3.28)]. From replicate assays of analyte and ISTD, mean responses (e.g. peak height or area) and their associated RSDs (RSD_a and RSD_{is}) can be obtained. The relationship between the deviations in the measurement of the responses of the individual compounds and the ratio as used in a typical assay is given by:

$$(RSD_r)^2 \approx (RSD_a)^2 + (RSD_{is})^2 - 2r(RSD_a.RSD_{is}) \quad (3.28)$$

where r is the correlation coefficient between the responses to the analyte and the ISTD. If the precision of the assay has been improved by the introduction of the ISTD then:

$$RSD_r < RSD_a$$

Substituting in Equation (3.28) and rearranging, gives:

$$RSD_{is} < 2r \,.\, RSD_a$$

Thus, depending on the correlation between the responses to analyte and ISTD, and the imprecision associated with those responses, inclusion of an ISTD may either reduce, or increase the overall imprecision. Even when $r = 1$, the RSD for the ISTD must be less than half that of the analyte to give an overall improvement in precision.

If the analyte can be measured with high precision in the absence of the ISTD, then the above equation confirms that addition of an ISTD will not improve the situation. A large RSD for the ISTD would indicate that the compound was a poor choice. The effect of an ISTD on assay accuracy and precision can be calculated using analyte response data.

Crucial general assumptions are that a plot of ISTD concentration versus detector response is linear and passes through a (0,0) origin. The plot of concentration versus response for the analyte need not be linear, but if the ISTD concentration–response plot is non-linear then the result of using a calibration graph of analyte response/ISTD response versus concentration will be bizarre.

3.3.5 Quantity of internal standard added

As a general guide, the amount of ISTD added to each sample for quantitative assays should be that which produces a signal, after sample preparation approximately equivalent that of the mid-point calibrator. Note that this is not concentration-based, but signal-based, and so accounts for any differences between analyte and ISTD, both in terms of (i) instrument response and (ii) extraction efficiency.

3.4 Method comparison

Methods may need to be compared for a number of reasons. It may be necessary to demonstrate that a new method is either equivalent, or superior to an existing method, either in the same laboratory, or in a different laboratory. Alternatively, the requirement may be to compare the same procedure carried out either by different analysts, or by different laboratories.

Linear (least squares) regression analysis assumes that all the errors are associated with the dependent variable, y, there being negligible error in the independent variable, x. However, it has been shown that the flaw inherent in this approach if used to compare methods is inconsequential if at least 10 data pairs, spread uniformly over the concentration range under study, are used. Results from the method with the greater precision should be plotted on the x-axis. If both methods, gave identical results then the regression analysis will give slope of 1 and $r = +1$. Such a line, referred to as the line of identity, may be drawn on the $x - y$ plot (Figure 3.12).

If there is a systematic error in the results from one laboratory, this will be clear from the x–y plot [Figure 3.12(a)], although there is no way of knowing which method is the more accurate. In the example there is a good correlation between the results from the two laboratories, but a clear relative bias. A possible explanation for this could include an error in the stock solution used to prepare the calibrators. For example, the stock solution used in one laboratory may

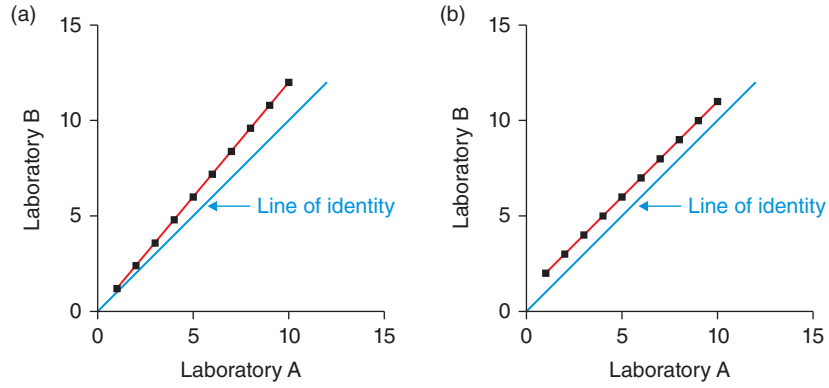


Figure 3.12 Linear regression to compare results from two laboratories showing systematic errors with proportionate bias (a) and constant bias (b). The line of identity is shown in blue. See text for the limitations of this approach

have partially decomposed, leading to overestimation of the test concentrations. Alternatively, one of the laboratories may have used a salt to prepare the standards and not taken this into consideration when reporting the data. In such situations, exchanging calibrators may shed light on the reasons for the discrepancy.

Linear regression should not be used without considering and stating the provisos above. For collaborative trials, i.e. when a method is tested in several laboratories, Youden plots (Youden & Steiner, 1975) can be used to detect bias. For a rigorous estimation of reproducibility, analysis of variance should be used.

3.4.1 Bland–Altman plots

The Bland–Altman plot is a method that is used to compare two measurement techniques. The *differences* between measurements performed using two methods are plotted against the averages of the methods. Alternatively, the differences can be plotted as the % of the average. This is useful when there is an increase in the variability of the differences as the magnitude of the signal increases. Finally, the ratios of the measurements may be plotted instead of the differences – this avoids the need for log transformation of the data, and is also useful when there is an increase in variability of the differences as the magnitude of the signal increases. The Bland–Altman plot is useful in revealing a relationship between the differences and the averages, to look for systematic bias, and to identify possible outliers. If there is a consistent bias, it can be adjusted for by subtracting the mean difference from the new method.

Comparisons of two methods of measuring plasma clozapine, LC-MS and LC-ED (Section 10.3.4), performed using linear regression analysis and a Bland–Altman plot are shown in Figure 3.13. Linear regression suggests that there may be both fixed and proportionate bias; the 95 % CI for the intercept (0.04–0.12 mg L^{-1}) and slope (0.79–0.94), do not encompass 0 and 1, respectively. Plotting the differences in concentrations measured by the two methods as a percentage or absolute value, a Bland–Altman plot [Figure 3.13(b)] confirms a bias of approximately –0.01 mg L^{-1} or –5 % for the LC-ED method.

With the Bland–Altman method, there are no formal statistical tests, so one has to decide whether the differences within the mean ±1.96 SD are sufficiently close (some 25 % in this example) for the two methods to be considered equivalent. The data show a tendency for

the differences to increase with increasing mean concentration that was confirmed by linear regression analysis (Giavarina, 2015).

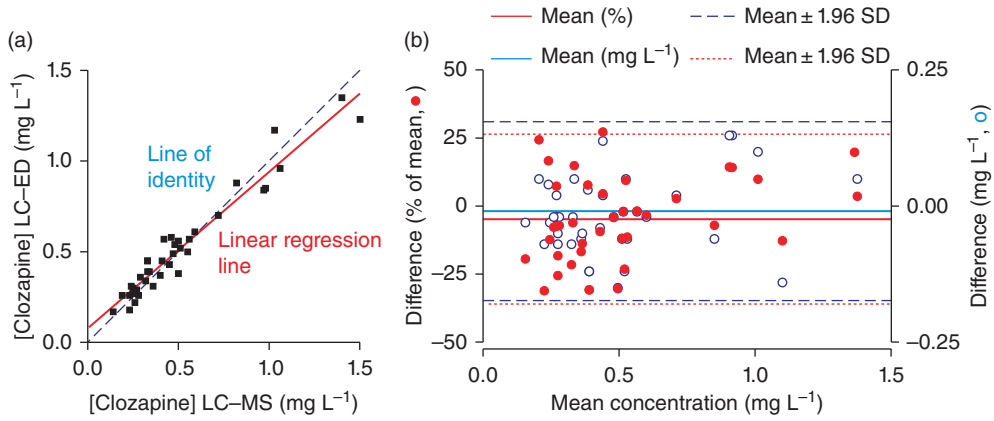


Figure 3.13 Comparison of linear regression (a) and Bland–Altman plot (b) using percentage difference (red) or absolute difference (blue) to compare two analytical methods (LC-ED versus LC-MS) for measurement of clozapine in plasma

Bland–Altman plots may also be used to assess the *repeatability* of a method by comparing a number of measurements using one single method on a series of subjects. The graph can also be used to check whether the variability or precision of a method is related to the size of the characteristic being measured. Because for the repeated measurements the same method is used, the mean difference should be zero. Therefore, the *Coefficient of Repeatability* (*CoR*) can be calculated as 1.96 times the SDs of the differences between the two measurements (d_2 and d_1):

$$CoR = 1.96 \times \sqrt{\frac{\sum (d_2 - d_1)^2}{n - 1}} \tag{3.29}$$

3.5 Non-parametric statistics

Parametric statistics require the data to be from a population that is normally distributed about the mean. In many instances it is assumed that the data are normally distributed, although it is possible to increase the sample size and to test for normality (Figure 3.1). If skewed data can be transformed to a normal distribution, for example by taking logarithms, then parametric statistics can be applied to the transformed data. If it is suspected that the data are normally distributed, but are skewed because of an outlier then Grubbs' test can be applied (Box 3.5) (free online calculation: https://www.graphpad.com/quickcalcs/grubbs2/). However, if it is not possible to prove normality then a range of non-parametric statistical tests are available that use ranked data and are thus less prone to the influence of outliers than parametric tests (Table 3.4).

For skewed data the *median* rather than the mean is a better indicator of 'central tendency'. The median is sometimes called the middle quartile, quartiles being the lines that divide a data set into four equal portions when the data are ranked in order. For an odd-numbered data set, the median is the $(n + 1)/2$ value, for an even-numbered data set it is the average of the 'middle two'. The dispersion of the data can be described by the range and/or the upper and lower quartiles, which can be presented visually using box-and-whisker plots.

Box 3.5 Grubbs' test for outliers

Compares the deviation of a suspect outlier from the sample mean with the standard deviation (s) that is calculated with the suspect value included:

$$G = \frac{|\text{suspected outlier} - \bar{x}|}{s}$$

If $G >$ critical value (from statistical table) then the suspect point may be omitted and the remaining data analyzed using parametric tests

Table 3.4 Comparison of parametric and non-parametric tests to compare means/medians

Reasons for test	Parametric	Non-parametric
Comparison of a mean/median with a standard value	t-test	Sign test Wilcoxon signed rank test
Comparison of means/medians of two sets of unpaired data	t-test	Mann–Whitney U-test
Comparison of two sets of paired data	Paired t-test	Wilcoxon signed rank test
Comparison of means/medians >2 sets of data	ANOVA	Kruskal–Wallis test
Outliers	Grubbs' test	

3.5.1 Sign tests

Although the sign test is the simplest and most insensitive non-parametric test, it is also the most convincing and easiest to apply. The null hypothesis is that in a distribution, values larger (+) and smaller (−) than the median are equally likely. When matched pairs are used, the probability of observing (A > B) is equal to that of observing (B > A), and the value of A – B has a median value of 0. If the number of positives is $n+$ and the number of negatives is $n–$, then these are binomially distributed, with $p = q = ½$, where p and q represent the probability of either a positive, or a negative, respectively. An alternative notation is to express the probability $P(r)$ of obtaining r observations (heads, for example, in coin tossing) from a total of n events:

$$P(r) = {}^{n}C_{r}p^{r}q^{(n-r)} \tag{3.30}$$

where:

$${}^{n}C_{r} = \frac{(n)!}{(r)!(n-r)!}$$

Alternatively, values of P can be obtained from statistical tables, derived using the binomial distribution with $p = q = ½$. With regular use it is possible to remember combinations that indicate significant differences – 6 out of 6, 8 out of 9, 10 out of 12, etc.

3.5.1.1 Wilcoxon signed rank test

The sign test takes no account of the size of the deviation – it is simply positive or negative. The Wilcoxon test assumes that the data are distributed symmetrically about the median. This is not to say that the data must be normally distributed, although of course for symmetrical distributions the mean and median will be the same. The test may be used to investigate whether a median/mean value has come from a given set of data (a t-test would be used with parametric

data), or paired data can be used (matched-pairs rank test, equivalent to a paired *t*-test for parametric data).

Differences either from the median or between data pairs are arranged in order without regard to the sign, ignoring any zero differences, and ranked. Any observations with the same value are given the same rank, i.e. the mean of the ranks that would have been assigned if they had been different. The original signs are given to the rank numbers, and positive and negative ranks summed, ($W+$) and ($W-$). The lower absolute value is used as the test statistic for comparison with critical values in the appropriate statistical table.

3.5.2 Runs test

The sign test examines the number of positive and negative results, but sometimes it is important to examine whether the *sequence* of results is random. In Figure 3.5(a), for example, the sequence is: – – + + + –. The data contain three negative and three positive values, and there are three sequences or 'runs'. Statistical tables provide values for the numbers of runs that would be considered to be significantly different from the number arising by chance. Unfortunately, the numbers of points required for data with three runs to be significant is >11. This is typical of non-parametric statistics, which generally require larger numbers of observations than parametric tests to demonstrate significant differences. Had more data points (≥12) been included in the calibration data of Figure 3.5, then the residuals could have been tested to detect non-linearity. Some linear regression programs include the option of performing a runs test, although of course the caveat about the number of data points still applies. The runs test can also be used to test for non-random fluctuations about the line, for example + – + – + – + – + – + –.

3.5.3 Mann–Whitney U-test

This test is used to compare two sets of data. To give an example, the data from Figure 3.14 were ranked according to the procedure outlined in Box 3.6 using Microsoft Excel and assigned a rank. This process is simplified if the data from the two treatments are colour coded and the Excel Sort function used. The ranks of the placebo ($n_1 = 7$) and the treated ($n_2 = 8$) groups were summed (Table 3.5) and U_1 and U_2 calculated, giving 50.5 and 5.5, respectively. Comparison of the lower value with critical values for U showed that the scores from the treated patients were significantly different from the scores from the group given the placebo.

Placebo	Treated
4.00	9.00
6.00	7.00
3.00	8.00
3.00	7.00
1.00	6.00
4.00	3.00
2.00	10.00
	5.00

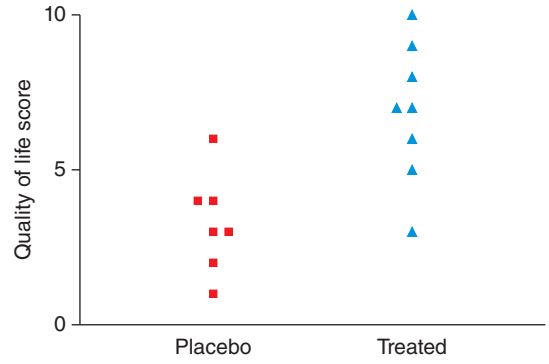


Figure 3.14 Quality of life scores from patients receiving placebo, or drug treatment

Box 3.6 Procedure for calculating U-values

- Define the smaller group as n_1 and the other as n_2
- Rank the combined scores of $n_1 + n_2$ from low to high, i.e. the lowest score gets a rank of 1. Where ranks are tied give each score the average rank of the tied scores
- Find the sum of the ranks of the smaller group (S_1)
- Find the sum of the ranks of the larger group (S_2).
- Calculate U_1 and U_2 from:

$$U_1 = n_1.n_2 + \frac{n_1(n_1 - 1)}{2} - S_1 \text{ and } U_2 = n_1 \,.\, n_2 + \frac{n_2(n_2 - 1)}{2} - S_2$$

Alternatively, U_2 may be calculated from:

$$\boldsymbol{U_2 = n_1 \,.\, n_2 - U_1}$$

- Use the lower of the U-values as the test statistic

Table 3.5 The data of Figure 3.14 ranked according to the procedure of Box 3.6

Treatment[a]	P	P	P	P	D	P	P	D	P	D	D	D	D	D	D	Sum
Quality score	1	2	3	3	3	4	4	5	6	6	7	7	8	9	10	
Rank (placebo)	1	2	4	4		6.5	6.5		9.5							33.5
Rank (treated)					4			8		9.5	11.5	11.5	13	14	15	86.5

[a]P = placebo, D = drug treated

3.5.4 Spearman rank correlation

The Spearman rank correlation coefficient is probably the best known non-parametric test for correlation between pairs of observations. It is used commonly for assessing clinical treatments and can be used when observations are expressed in rank order rather than quantitatively. Both sets of data are ranked and the difference in rank, d, obtained for each data pair. Average values should be used for tied ranks. The correlation coefficient is calculated from:

$$r_s = 1 - \frac{6\sum d^2}{n^3 - n} \tag{3.31}$$

where n is the number of data pairs. The significance of r_s is obtained from statistical tables.

3.5.5 Non-parametric regression

Although there are curve-fitting methods for non-parametric data, Theil's incomplete method is suitable for use with a spreadsheet. It is important to have an even number of calibration points (n) so that $n/2$ estimates of the slope can be calculated using pairs of points (Box 3.7, Figure 3.15). The median of the values for the slope is used to calculate n values of the intercept, using all the data points. The median values for the intercept and slope are used to calculate the unknown concentrations. Median values in a list can be located using the Median function in Microsoft Excel, so templates can be created to perform the necessary steps. Advantages of the

Box 3.7 Steps for Theil's incomplete method for non-parametric calibration curves

- Rank data in order
  - Must have even number of points [Figure 3.15(a)]
  - Discard median if odd number of points
  - Calculate new median value
- Obtain median slope, by calculating slopes:
  - 1st point (x_1, y_1) to first point beyond median
  - 2nd point (x_2, y_2) to second point beyond median
  - and so on ···
  - First point before median $(x_{(n/2)}, y_{(n/2)})$ to last data point [Figure 3.15(b)]
  - Rank the slopes in order
  - Calculate the median slope (b)
- Obtain median intercept
  - Calculate values (a_i) for every data point (x_i, y_i) using $a_i = y - (b.x_i)$
  - Rank a_i values in order and obtain the median value (a)
- Use median values of a and b to construct calibration line [Figure 3.15(c)]

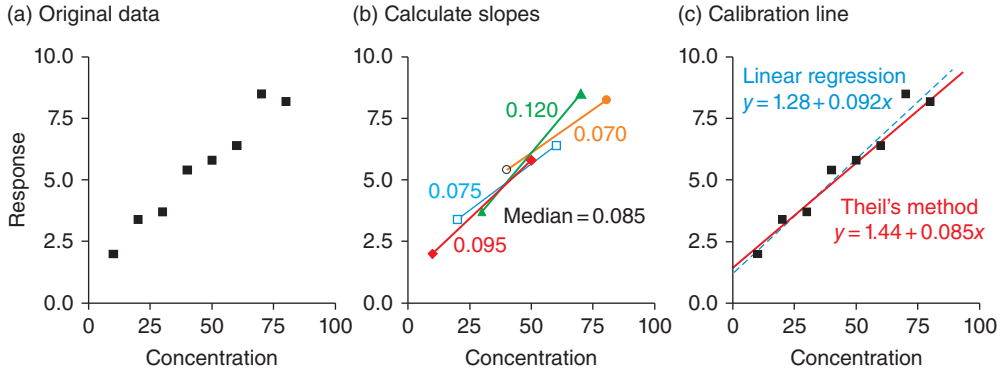


Figure 3.15 Example of Theil's incomplete method for non-parametric calibration

non-parametric approach to calibration include (i) it does not assume that the errors are normally distributed, (ii) it does not assume all the errors are in the y-direction with none in the x-direction, and (iii) it is less influenced by outliers when compared with least squares regression – the broken line in Figure 3.15(c).

3.6 Quality control and quality assessment

Having developed, or applied an analytical method and shown that it is 'fit for purpose' using appropriate validation procedures, it is important to demonstrate that the method continues to perform as required. QC guidelines may vary from discipline to discipline (e.g. clinical toxicology, forensic toxicology, doping control) and from country to country according to different accreditation guidelines and/or legislation.

IQC samples, ideally prepared independently by someone else using a different stock of reference material, different stock solutions, and different volumetric apparatus, should be

randomly distributed in a batch of samples to be analyzed. FDA guidelines require duplicate IQCs at three concentrations (high, medium, and low) to ensure that the assay is performing satisfactorily across the calibration range. The assay batch is deemed acceptable provided four of the six controls (with at least one at each concentration) are within specification. The FDA guidelines are applicable to batch processing – different assay acceptance criteria may apply for emergency work (Section 3.2.4.10).

3.6.1 Quality control charts

QC charts are valuable in that they (i) produce evidence of satisfactory assay performance, and (ii) give visual warning if assay performance begins to deteriorate. As with any QC method it is important that reliable estimates of the parameters defining the control material are established. The mean value, μ, should be obtained from a minimum of 10 observations and the SD, σ, should be the between-assay value (inter-assay value). Obviously, these parameters must be measured when the analysis is performing satisfactorily.

3.6.1.1 Shewhart charts

On the Shewhart chart lines are drawn representing the target value, and the 95 and 99.7 % confidence limits [Figure 3.16(a)]. Values between the lower and upper warning lines (99.7 % lines) are considered acceptable, but either if two consecutive values fall outside the same warning line, or a single IQC falls outside an action line then the assay batch should be rejected and the reasons for the assay failure investigated. Such charts can be used to detect trends, such as assays giving sequentially increasing (or decreasing) values. This can be built into the QC procedure, so that, for example, an assay is investigated if a defined number of IQC values in a row produce increasing results.

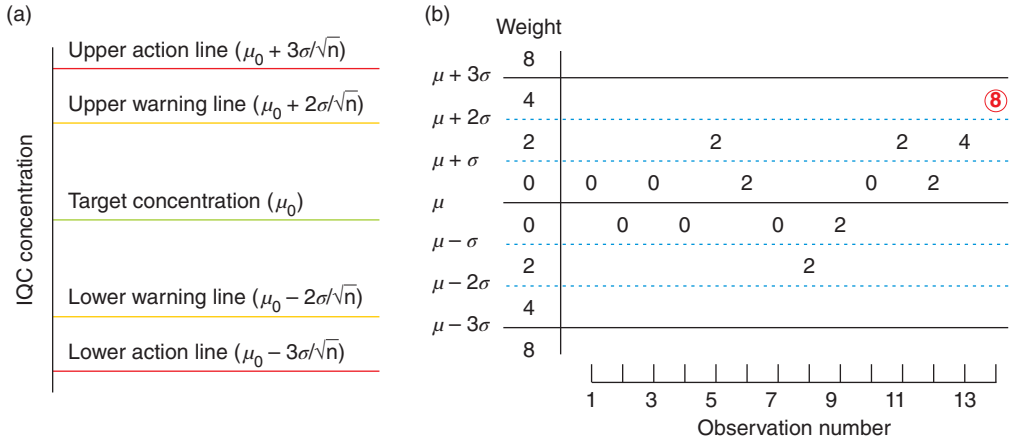


Figure 3.16 Examples of quality control charts: (a) Shewhart (b) J-Chart

3.6.1.2 Cusum charts

A limitation of Shewhart plots is they are relatively insensitive to a systematic drift of results. It may take as many as 50 analyses before an IQC value falls outside an action line. Cusum (cumulative sum) plots address this problem. Rather than plot the observed value, the difference

between the control and target value is calculated for each analytical sequence and the cumulative sum calculated. If the method is under control, the cumulative sum should fluctuate about zero, whereas a systematic trend would be evident as either a positive, or a negative divergence from zero. The ways of assessing when the process is out of control are either (i) the V-mask, or (ii) the tabular cusum, the latter being the preferred method (Montgomery, 2012).

3.6.1.3 J-chart

The J-chart, also known as a zone control chart, combines the properties of the Shewhart and cusum charts so that is it responsive to both abrupt changes and drift in results (AMC, 2003). The mean and three equal sized bands or zones (1 σ wide) are drawn on either side of the mean [Figure 3.16(b)]. Each observation is ascribed a weight, 0, 2, 4, or 8, depending on the band in which it falls. The weights of successive observations are cumulated and the value written on the chart at the appropriate point. When an observation falls on the opposite side of the centre line (mean) to the previous one the cumulative total is reset to zero and the process continues.

The process is considered out of control if the cumulative total is 8 or greater, so an isolated value greater than $\mu \pm 3\sigma$ would require the analysis be suspended and the reasons for the divergent observation investigated. The example of Figure 3.16(b) illustrates how a drift to high values triggers the alarm. Although observation number 12 was $<1\sigma$ from the mean, the previous observation was between 1 and 2 SDs away and on the same side of the centre line, giving a cumulative weight of 2. Observation 13, is also between $\mu + 1\sigma$ and $\mu + 2\sigma$, so the new total is 4. Observation 14, is between $\mu + 2\sigma$ and $\mu + 3\sigma$, which attracts a weight of 4, giving a cumulative value of 8, the critical number for the method to be deemed out of control. Had observation 14 remained between $\mu + 1\sigma$ and $\mu + 2\sigma$, the new cumulative total would have been 6, and a further value in this band would trigger the alarm, as the cumulative total for observation 15 would be 8.

3.6.2 External quality assessment

EQA schemes measure inter-laboratory performance and allow individual laboratories to detect and correct systematic errors. Portions of homogenous samples are distributed to participating laboratories. The samples should resemble clinical samples as closely as possible. The laboratories do not all have to use the same analytical method. The results of EQA schemes are usually reported as the z-score:

$$z = \frac{x - x_a}{\sigma_p} \tag{3.32}$$

where x is an individual result, x_a is the accepted, 'true' value, and σ_p is known as the 'target value of SD' which should be decided on the basis of what is required of the test and should be circulated in advance. If the result needs to be measured with high precision then a low value of σ_p would be chosen.

Coucke *et al*. (2015) have discussed the issue of choosing an appropriate value of target SD and how this is related to analyte concentration. Thus, z is a measure of a laboratory's accuracy and the organizer's judgement as to what is 'fit for purpose'. If the results of an EQA scheme are normally distributed with a mean of x_a and variance of 1, then z-scores <2 would be deemed acceptable whereas those >3 would not.

It is a condition of laboratory accreditation in many countries that laboratories offering services to patients participate in all relevant EQA schemes. It is important that the results from EQA analyses are recorded graphically, discussed regularly with laboratory staff, and

action taken in the event of poor performance. As with IQC, trends should be monitored. For instance, multiple consecutive results with negative z-scores might be suggestive of a negative bias. Consistently poor performance below an agreed performance threshold, or a poor EQA result return rate, is likely to result in the issue of a warning notice to the laboratory and if there are continued failings the laboratory may be subject to an intervention. In some countries such continued failure results in withdrawal of the right of the laboratory to be recognized to perform and be paid for analyses.

3.6.3 Toxicology external quality assessment schemes

Quantitative EQA schemes are available for a wide range of toxicology and TDM analytes. The European Proficiency Testing Information System (www.eptis.org) and European Network of Forensic Science Institutes (ENFSI) (//enfsi.eu/wp-content/uploads/2017/07/QCC-PT-001-_-Guidance-on-PT-CE.pdf) websites give guidance on EQA schemes for a range of analytes that are available in Europe, the Americas, and in Australia.

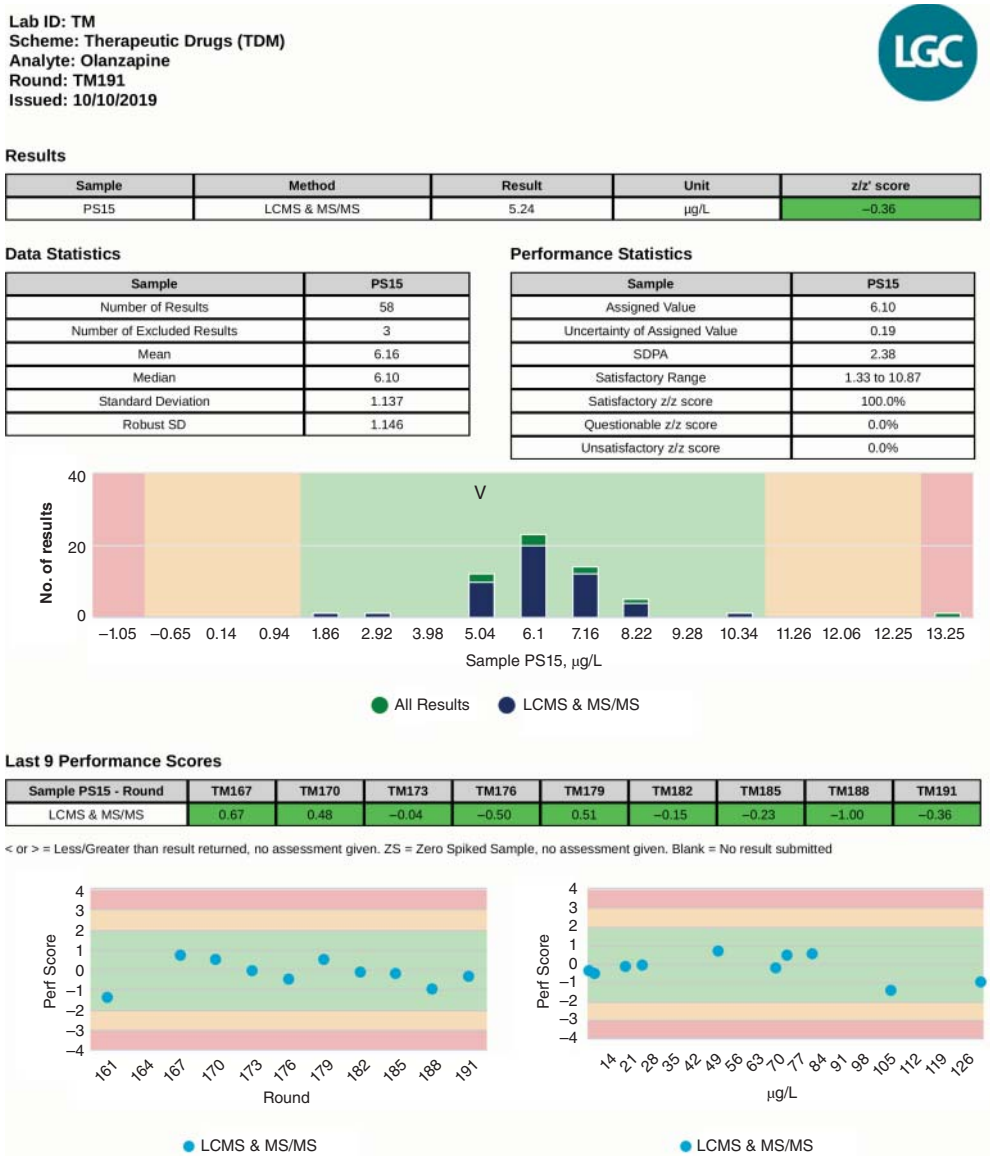
In the LGC quantitative EQA schemes, assessments are generally made using Robust statistics (Daszykowski *et al*., 2007). The assigned value (AV) is the 'consensus' of all results submitted including outliers. The intended ('spiked') values are also provided. For many analytes, the standard deviation of proficiency assessment (SDPA) varies with the analyte concentration. This value is derived by interpolation at the consensus mean concentration into a mathematical function fitted to historic data of variation in SD (y-variable) versus consensus mean (x-variable). Because the mathematical function is fixed, changes in laboratory performance with time can be identified. This is known as a concentration dependant model (CDM). When this model is not available, the SDPA is calculated using Robust statistics. The assessment is then provided in the form of a z-score [Equation (3.32)], which is colour coded for ease of understanding (Table 3.6).

Table 3.6 LGC EQA scheme: presentation of z-score results

z-score	Interpretation	Colour coding
$\|z\| \leq 2.00$	Satisfactory result	Green
$2.00 < \|z\|$ and < 3.00	Questionable result	Amber
$\|z\| \geq 3.00$	Unsatisfactory result	Red

Assessments are reported to participants in a number of reporting formats: (i) a main report detailing all results submitted, giving summary and method statistics and individual assessments; (ii) an individual report with a summary of the laboratory's results for the circulation; and (iii) an analyte report detailing all individual results from participating laboratories, statistics, and a method information histogram detailing all of the methods used, with the laboratory's method and result indicated. Trend information is also provided to identify potential bias over time and concentration (Figure 3.17).

There are of course sometimes concerns such as the possible effects of freeze-drying on analyte stability, and issues concerning the matrix used to prepare material for circulation. Due to the cost of analyte-free human plasma or serum, NBCS may be used for some analytes. Nevertheless, from the datasets generated, scheme organizers can ascertain the methods that give the best performance, investigate sources of interference or bias (Neef *et al*., 2006), and in



Lab ID: TM
Scheme: Therapeutic Drugs (TDM)
Analyte: Olanzapine
Round: TM191
Issued: 10/10/2019

Results

Sample	Method	Result	Unit	z/z' score
PS15	LCMS & MS/MS	5.24	µg/L	−0.36

Data Statistics

Sample	PS15
Number of Results	58
Number of Excluded Results	3
Mean	6.16
Median	6.10
Standard Deviation	1.137
Robust SD	1.146

Performance Statistics

Sample	PS15
Assigned Value	6.10
Uncertainty of Assigned Value	0.19
SDPA	2.38
Satisfactory Range	1.33 to 10.87
Satisfactory z/z score	100.0%
Questionable z/z score	0.0%
Unsatisfactory z/z score	0.0%

Last 9 Performance Scores

Sample PS15 - Round	TM167	TM170	TM173	TM176	TM179	TM182	TM185	TM188	TM191
LCMS & MS/MS	0.67	0.48	−0.04	−0.50	0.51	−0.15	−0.23	−1.00	−0.36

< or > = Less/Greater than result returned, no assessment given. ZS = Zero Spiked Sample, no assessment given. Blank = No result submitted

Figure 3.17 An example of an EQA scheme report (plasma olanzapine)

extreme cases report poorly performing methods to regulatory authorities. In the 30 years since the inception of these schemes, poorly performing assays have been identified and participants advised accordingly. The mean values reported have moved nearer to the intended value and the spread of results about the mean has been reduced.

EQA of qualitative work is also invaluable, particularly in substance misuse screening. Potential problems encountered include differing cross-reactivities of immunoassay methods to substances that may result in either false negative or false positive results, or elevated or decreased quantitative results. Other issues encountered may be sample manipulation prior to

analysis, thus affecting sample integrity. Therefore, well-run qualitative EQA schemes should not only assess routine performance, but include hard-to-detect compounds, samples with concentrations around nationally agreed 'cut-off' values, and substances that may cross-react with assays to produce a positive response. Sample integrity should also be assessed. Good screening and confirmatory analytical performance from laboratories are required to ensure that the correct result is reported. Studying trends for commonly encountered substances such as THC-COOH, morphine, 6-AM, and EDDP, for example, helps laboratories address any problems in their procedures.

These qualitative schemes are assessed by setting the AV as either positive or negative. Quantitative data are also collected and the statistical analysis is included in reports. These data can provide valuable information regarding the extent of any cross reactivity with specific methods and substances.

Urine is the matrix used most commonly, although many laboratories are now analysing oral fluid and/or hair (Chapter 18). Scheme 'cut-offs' are quite generous, but laboratories may also be scored against other agreed workplace cut-offs. A breakdown of the performance of each recognized method type is provided to help laboratories understand why false positive or false negative reports have occurred in a given distribution. Regular clinical/forensic toxicology case schemes have also been instituted. Provision of case specific samples and a brief history enable not only the assessment of analytical approaches, but also of the interpretation given with the results.

3.7 Operational considerations

3.7.1 Staff training

Clinicians, especially Accident & Emergency staff, need guidance not only as to what toxicological assays are clinically useful in a given set of circumstances, but also on sample collection, transport, and storage, local assay availability and turn-around time, and the interpretation of results.

Not only do laboratory staff in hospitals providing emergency analytical toxicology services need training in providing these services and in the interpretation of results, they will also need some training in dealing with requests for tests that will need to be referred to other analytical centres. Staff in regional analytical centres will require extensive training in the more complicated analytical methods they will be called upon to use. This training must encompass not only the techniques themselves (GC, LC, GC-MS, etc.), but also their application in analytical toxicology and related areas. Knowledge of the role of local hospital laboratories and the local poisons centre is also important.

There are no internationally recognized training programmes in analytical toxicology. However, the American Association Clinical Chemistry (www.aacc.org) and the GTFCh (www.gtfch.org/cms/index.php/en/fachtitelen), for example, offer postgraduate training programmes in various topics such as clinical and forensic toxicology, and LC-MS troubleshooting.

Participation in continuing education or continuing professional development (CPD) programmes is important when staff reach career grades (i.e. when initial and higher specialist training has been completed) and may be necessary for continued specialist registration in countries where such registration is mandatory. Compliance with CPD programmes necessitates maintenance and external audit of personal records listing educational activities such as attendance at scientific meetings, papers published, lectures given, etc.

Non-graduate scientific staff normally will be trained in-house in specific aspects of laboratory operation, although training in the operation of newer specialized instruments may sometimes be provided by manufacturers. Proper recording of training is important.

3.7.2 Recording and reporting results

All results should be recorded in laboratory notebooks or on worksheets together with information such as the date, the name of the analyst, the name of the patient, and other relevant information, the number and nature of the specimens received for analysis, and the tests performed. All specimens received in laboratories are normally allocated a unique identifying number. This number is used when referring to the tests performed on the specimen.

UV spectra, chromatograms, calibration graphs, and other documents generated during an analysis should always be kept for a period of time after the results have been reported. When reporting the results of tests in which no compounds were detected in plasma/serum or in urine, the LoD should always be known, at least to the laboratory, and the scope of generic tests (benzodiazepines, opioids) should be defined.

The results of urgent (emergency) analyses must be communicated directly to the clinician without delay, and should be followed by a written report as soon as possible. Ideally, confirmation from a second, independent method, or failing this, an independent duplicate, should be obtained before reporting positive findings. However, this may not always be practicable, especially if only simple methods such as colour tests are available. In such cases it is vital that the appropriate positive and negative controls are analyzed together with the specimen (Section 5.2).

Some information that may be important when reporting results is summarized in Table 3.7. When reporting quantitative results, it is important to state clearly the units of measurement used and the MU. The unit should be written out in full, e.g. milligrams per litre, if the report is for medico-legal purposes. In addition, any information necessary to ensure that the clinical implications of the result are fully understood must be available and should also be noted on the written report.

Although it is often easy for the analyst to interpret the results of analyses in which no compounds are detected, such results are sometimes difficult to convey to others, especially in writing. This is because it is important to give information as to the poisons EXCLUDED by the tests performed with all the attendant complications of the scope, sensitivity, and selectivity of the analyses and other factors such as sampling variations. Because of the potential medico-legal and other implications of any toxicological analysis, it is important not to use laboratory jargon such as ‘negative’ or sweeping statements such as ‘absent’ or ‘not present’. The phrase ‘NOT DETECTED’ should convey precisely the laboratory result, especially when accompanied by a statement of the specimen analyzed and the LoD.

However, it can still be difficult to convey the scope of analyses such as that of GC- and/or LC-MS for acidic, basic, and neutral drugs as discussed in Chapter 19. Even with relatively simple tests such as Trinder’s test normally used to detect aspirin ingestion, a number of other salicylic acid derivatives also react if the test is performed on the appropriate samples. One way of giving at least some of this information in a written report is to create a numbered list of the compounds or groups of compounds normally detected by commonly used procedures. If these groups are listed on the back of the report then it is relatively simple to refer to the qualitative tests performed by number and thus to convey at least some of the information required.

Table 3.7 Information important when reporting the results of toxicological analyses

For each specimen:	Name of subject or other identification
	Nature of specimen collected
	Time/date of specimen
	Sampling site (if appropriate)
	Specimen container used
	Specimen preservation/transport/storage
	Name of person collecting specimen
For each analytical report:	Time/date of analysis
	Nature of specimen (plasma, whole blood, etc.)
	Unambiguous nomenclature[a]
	Units used to report results
	Name and accreditation status of laboratory and analyst performing the analysis and reporting results
	Other poisons or groups of poisons looked for in the specimen
	Analytical method(s) and LoD or LLoQ
	Reference compound(s) used (source, purity, storage conditions, expiry date)
	Quantitative results accompanied by MU

[a]Some sources of potential confusion are 'free' (unconjugated) and 'total' morphine (includes conjugated metabolites) or 'free' (non-protein bound) and 'total' (includes protein-bound) phenytoin; 'arsenic' may represent both inorganic and organic arsenic species ('total arsenic')

3.8 Summary

The importance of a thorough knowledge of laboratory operations is crucial if reliable results are to be obtained and, if necessary, defended in court. Even in laboratories offering a relatively simple repertoire of tests, adoption of the principles of quality management as outlined above and expanded upon in the references will provide a framework to support the growth of the laboratory. With the increasing use of POCT devices, the principles of quality management also have to be applied to such procedures in order to ensure the reliability of results. Although quality management is more difficult to apply to the clinical interpretation of analytical results, it is vital that this aspect of the work of the laboratory is not neglected.

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4 Aspects of Sample Preparation

4.1 Introduction

Although in analytical toxicology simple colour tests (Section 5.2) and some immunoassays and enzyme-based assays, for example, may often be performed directly upon the specimen (homogenous assay, Section 6.4), some form of treatment of the sample is normally necessary prior to the analysis, even if this only consists of adding an ISTD. The complexity of the sample preparation procedure used will depend on the nature of the sample and the nature of the analyte (including whether it is unstable or extensively metabolized), whether analyte concentration or dilution is needed, and whether a chromatographic method is to be used and, if so, the method of detection. Clearly all these are factors are interdependent (Figure 4.1).

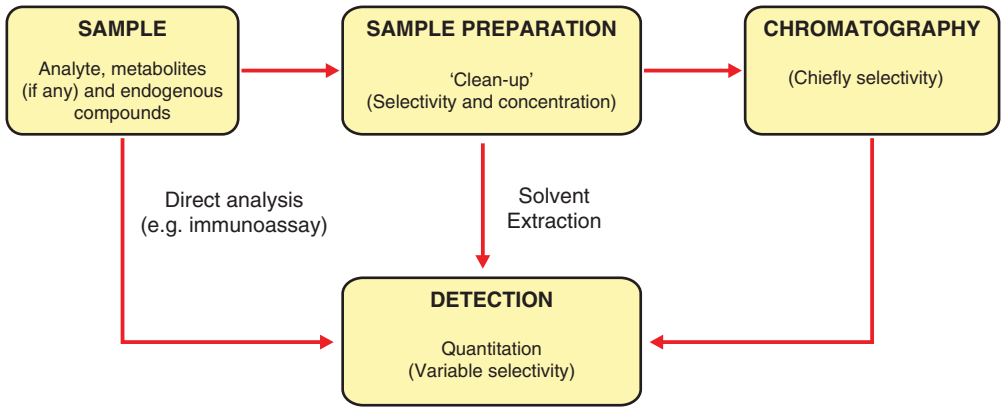


Figure 4.1 Summary of the steps that may be involved in an analysis

Additional aims of sample preparation may be removal of insoluble residues and interfering compounds, and sometimes either concentration, or even dilution of the analyte to adjust sensitivity (Box 4.1). Judicious choice of sample preparation can improve both selectivity and sensitivity. Traditionally sample preparation has been performed prior to a chromatographic or other step ('off-line'), but there is now interest in performing the sample preparation and analysis steps automatically ('on-line') to minimize errors and reduce (labour) costs.

The method chosen for sample preparation depends on the overall analytical strategy. If the analyte is thermally labile then GC is usually inappropriate, as is evaporation of an extraction solvent at an elevated temperature. If the analyte concentration is high, or a particular assay is very sensitive, then sample preparation may be minimal and may involve sample dilution.

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

Box 4.1 Aims of sample preparation

Achieve compatibility of analyte with the analytical system via:

- Solubilization/homogenization of solids/emulsions, etc.
- Disruption of protein binding
- Addition of ISTD(s)
- Removal of insoluble residues/interfering compounds
- Concentration/dilution of analyte
- Hydrolysis of conjugates
- Stabilization, decomposition, and/or derivatization of analyte to improve extraction, chromatographic, and/or detection properties

However, trace analysis may require a complex assay procedure with several concentration and clean-up steps. Urine and bile, on the other hand, may contain higher concentrations of compounds of interest and fewer insoluble residues than whole blood, plasma, or serum, resulting in simplified sample preparation, although hydrolysis of conjugated metabolites may be needed (Section 4.4). Provided that it is 'fit for purpose', the method should be as technically simple as possible to maximize reliability and reproducibility.

For large numbers of samples (e.g. routine assays or from multi-centre studies) throughput is important and time spent on method optimization is justified. If a drug is to be measured only occasionally, then consideration should be given to adapting a method used for a similar analyte even if the analysis time is extended. Liquid–liquid extraction (LLE) should be considered if TLC, GC, or derivatization is to be performed. Solid phase extraction (SPE) is often suitable for LC methods because the analyte may sometimes be eluted from the SPE cartridge with a hydrophilic solvent compatible with a reverse-phase eluent (Section 10.5.2). Only 'off-line' modes of sample preparation are discussed in this chapter (Box 4.2), direct analysis including headspace (HS) analysis being described as appropriate in subsequent chapters. Sample preparation for metals/trace elements analysis is detailed in Chapter 21. Derivatization to improve either chromatography, or analyte detection is also discussed in subsequent chapters.

Box 4.2 Modes of sample preparation

(a) Solids/tissues
- Physical disruption (homogenization, sonication, heat/microwave)
- Chemical disruption (enzymes, acid/base treatment)

(b) Liquids
- Direct analysis after off-line treatment (filtration, ISTD addition, protein precipitation)
- Direct 'on-line' analysis
- Headspace (HS) analysis (also purge/trap, microdiffusion)
- Liquid–liquid extraction (LLE)
- Supported liquid extraction (SLE)
- Solid phase extraction (SPE), also known as sorbent extraction (SE)
- Solid phase microextraction (SPME) of liquid or headspace
- Liquid phase microextraction (LPME)
- Supercritical fluid extraction (SFE) of solid matrix
- Accelerated solvent extraction (ASE)

The potential for loss of analyte during sample preparation such as adsorption on to the extraction vessel or loss during a solvent evaporation stage must be considered. Other factors being equal, miniaturization and the use of the minimum number of extract transfer steps will in general give the best recovery of analyte. Other losses may result from analyte instability – for example physostigmine is rapidly hydrolyzed if extracted at pH 9.5 or above, whilst norpropoxyphene, the major plasma metabolite of dextropropoxyphene, rearranges to an amide at pH 11 or above. Bupropion decomposes under strongly basic conditions. Many *N*-oxide metabolites are reduced to the parent compounds at pH 12 and above. LC has an advantage over GC in that the likelihood of thermal degradation during the analysis is minimized, although the possibility of analyte decomposition in a heated MS ion source remains (Section 13.4.1.2). Moreover, refrigerated autosampler trays and analytical columns may be used if decomposition at room temperature is a consideration.

Many drugs bind to plasma protein, chiefly albumin, α_1-acid glycoprotein (AAG), and lipoproteins. Protein-bound analytes must be released during the course of the analysis if 'total' (free + protein-bound) drug is to be measured. Analysis of calibration solutions of the analyte prepared in analyte-free plasma is a simple way of investigating the effect of protein binding on analyte recovery. When non-bound analyte concentrations are required, equilibrium dialysis or ultrafiltration may be used to separate the bound drug (Section 4.3).

Plasma, serum, and other fluids are normally sampled by pipetting. Whole blood or post-mortem blood is usually analyzed after a freeze/thaw cycle to disrupt erythrocyte membranes, followed by sedimentation of cell debris before sampling. As with other viscous fluids, use of a positive displacement pipette is recommended, or alternatively specimens can be apportioned by weight. In some jurisdictions blood ethanol in relation to driving a motor vehicle is defined by weight rather than by volume. If severe dehydration of a post-mortem specimen is suspected, haemoglobin can be measured and the analytical result corrected to a 'normal' haematocrit, although many forensic toxicology laboratories do not have access to pathology analytical facilities. Tissue specimens are normally analyzed wet after removing any surface blood by blotting gently with filter paper, for example.

Guidelines for sample preparation are summarized in Table 4.1. The myriad of different approaches to analytical toxicology problems might seem at first sight confusing, but with

Table 4.1 Guidelines for sample preparation

Aim to	Avoid
Keep the analysis as simple as possible	Unnecessary additives (e.g. preservatives)
Use high quality reagents, solvents, etc.	Use of ISTDs if these reduce precision
Understand the clinical pharmacology, metabolism, and toxicology of the analyte	Hazardous solvents and, if possible, toxic/hazardous reagents
Use the smallest sample possible compatible with the required sensitivity/selectivity	Using more sample than is needed
Increase sample size/injection volume to increase sensitivity, if needed	Using an extraction solvent that is too 'powerful' for the application
Use external standards in colour tests, UV/Visible spectrophotometry, and TLC	Using unnecessary extremes of heat or pH
	SPE if LLE is satisfactory
Use ISTDs in GC, LC, and MS	Developing over-sensitive methods

experience and in the knowledge of the instrumentation and expertise available in a particular centre, then standard approaches to similar problems will soon be developed. Those given here largely reflect personal experience. With the increasing requirement for high sensitivity assays, the use of high purity reagents, etc. has become even more important.

4.2 Modes of sample preparation

4.2.1 Protein precipitation

Plasma protein precipitation (generally off-line with analysis of the supernatant after centrifugation) is the simplest approach. The efficiencies of several precipitating agents have been compared (Table 4.2). Other mixtures used to precipitate plasma protein include aqueous zinc sulfate (5 % w/v):methanol (2+1), 5-sulfosalicylic acid (3.2 % w/v) in water:methanol (1+1), and methanol:acetonitrile (1+5). If strongly acidic reagents are used, the analyte and any ISTD

Table 4.2 The efficacy of some common protein removal procedures (Blanchard, 1981–reproduced with permission of Elsevier)

(i) Human plasma

Precipitant	Proportion of precipitant required to remove 99 % of protein
10 % w/v Aqueous trichloroacetic acid	0.2
6 % w/w Aqueous perchloric acid	0.7
Acetonitrile	1.3
Acetone	1.4
Ethanol	3.0
Methanol	4.0
Saturated aqueous ammonium sulfate	*ca*. 3.0
Ultrafiltration	not applicable[a]
Heat (80 °C)	not applicable[b]
Dialysis	not applicable[c]

(ii) Red cell extract[d]

Precipitant	Proportion of precipitant required to remove 97 % of protein
5 % w/v Aqueous trichloroacetic acid	4.5
10 % w/v Aqueous trichloroacetic acid	3.8
15 % w/v Aqueous trichloroacetic acid	3.3

[a] Provided there is no leakage ultrafiltration will remove 98 % of protein
[b] Only poor removal of protein (*ca*. 95 %, 10 min)
[c] Recipient stream is protein free, depends on membrane porosity
[d] Human red cells (initial protein content 311 g L^{-1})

must be stable at low pH values. Brief cooling to –20 °C before centrifugation may enhance protein precipitation.

When evaluating a protein precipitation procedure, it is always advisable to add a second portion of precipitation reagent to the supernatant obtained initially to ensure that no protein remains in solution. Supernatant filtration using a suitable system is a precaution sometimes adopted to minimize the risk of injecting particles, but the possibilities of loss of analyte on the filter and of introducing contaminants must be evaluated. In some cases, the supernatant may be analyzed directly by either LC or LC-MS, whilst in others further manipulation(s) such as LLE or SPE may be needed. The possibility of loss of analyte(s) with the precipitate must be considered.

Methanol containing 0.2 % v/v concentrated hydrochloric acid (2 volumes) when added to plasma or serum (1 volume) followed by vortex-mixing and high-speed centrifugation (10,000 *g*, 30 s) gives efficient protein precipitation. However, the sensitivity is less than if it had been possible to inject the sample directly because of the three-fold dilution, and selectivity is dependent on the chromatographic separation and the selectivity of detection because all solutes are injected. The high proportion of methanol in the supernatant also means that it is not normally possible to analyze more than 10–20 µL directly by LC unless an eluent with a similarly high organic solvent content is being used because unacceptable band spreading will result (Section 10.2.6).

If an organic solvent is used as the protein precipitant, an increase in the amount of the 'extract' that may be injected, with consequent enhancement of sensitivity, may be achieved by transferring the supernatant to a clean tube and evaporating to dryness under a stream of either compressed air, or nitrogen. This obviously takes time, but solvent:water mixtures are easier to evaporate than water alone because of the azeotropic effect. Compounds such as potassium carbonate may be added to plasma:organic solvent mixtures to 'force' an organic layer to form, thus simplifying extract concentration after removal. Evaporation to dryness is often necessary if analyte derivatization is to be performed (Section 9.6).

4.2.2 Liquid–liquid extraction

Traditionally, purification of lipophilic analytes has been performed by extracting the biological sample with an inert, water-immiscible organic solvent at an appropriate pH (Box 4.3). For targeted analytes, generally it is best to use the least polar solvent that will effectively extract the compound(s) of interest – selecting a solvent with too much 'extracting power' may reduce the selectivity of the assay by extracting interfering compounds. On the other hand, use of solvents such as butyl acetate or methyl *tert*-butyl ether (MTBE) in either poisons screening (Chapter 19), or generic methods has the advantage that analytes of differing polarity often can be analyzed using similar methodology.

Mechanical mixing of the aqueous and organic phases is normally necessary. Of the methods available, vortex-mixing is the quickest and the most efficient method for relatively small volumes, but is prone to forming emulsions. Strongly acidic or basic extraction conditions may also promote emulsion formation and so more moderate conditions should be used whenever possible. Rotary mixers capable of accepting tubes up to 30 mL volume are valuable for large volumes of plasma/serum, urine, or stomach contents, and are less prone to emulsion formation because vigorous shaking is unnecessary.

After mixing, the phases should be separated by centrifugation (e.g. using bench-top centrifuge that accepts test tubes of up to 30 mL volume and attaining speeds of 2000–3000 rpm).

Box 4.3 Properties of the 'ideal' extraction solvent

- Good 'extracting power'
- Low solubility in water
- Less dense than water
- Moderate volatility to facilitate removal by evaporation, but not so volatile as to evaporate during sample preparation
- Stable/inert (contains no added stabilizers)
- Low flammability
- Low toxicity (not only by inhalation, but also via dermal absorption)
- Inexpensive and readily available in required purity
- No UV absorption or electrochemical activity
- No response/deleterious effects on GC detectors

The centrifuge should have sealed buckets and be 'flash proof' to minimize the risk of explosion from ignition of solvent vapour. Use of sealed tubes and rotor units also minimizes the risks associated with centrifugation of potentially infective specimens. Regular centrifuge cleaning and maintenance programmes are important.

The RCF (Box 2.1) is not usually critical provided that adequate phase separation is achieved. The exact RCF and time of centrifugation will depend on local factors, such as the type of tube and the centrifuge being used. It is important that the tubes do not break or distort during centrifugation. Plastic tubes and organic solvents may introduce plasticizers and other contaminants hence glass tubes may be preferred. Use of high purity solvents is recommended. Swing-out rotors ensure that the interface between the organic and aqueous phases is at right angles to the tube, facilitating removal of the solvent.

After centrifugation, freezing the aqueous layer (–20 to –60 °C) in order to simplify phase separation by pouring is advocated by some authors. However, the extract is usually transferred to a clean vessel by pipetting. Once the extract has been transferred, the extraction solvent is often vaporized under a stream of either compressed air, or nitrogen prior to reconstitution of the dried extract in a suitable volume of an appropriate solvent. Back-extraction of acids or bases into an aqueous solution of appropriate pH can be used to remove neutral interferences. If derivatization is to be performed, then performing the extraction using a solvent that is compatible with the derivatization reagent may eliminate the need for a solvent evaporation step.

Evaporation may be performed either to concentrate the analyte prior to the analysis to improve sensitivity, or to remove solvents which would interfere in the analysis. Ethyl acetate, for example, has strong UV absorption at lower wavelengths. However, solvent evaporation is time-consuming, interferences may be concentrated, and relatively volatile analytes may be lost, unless precautions such as acidifying the extract to promote salt formation in the case of volatile bases such as amfetamine are taken. It may also be necessary to add a basic compound to the reconstituting solvent in order to remove adsorbed basic analytes from glass tubes.

Some commonly used extraction solvents are listed in Table 4.3. Mixtures of solvents may be used for specific purposes. Dichloromethane (DCM):2-propanol (9+1) (SG > 1), for example, has long been used to extract morphine and other opiates from urine, while mixtures such as DCM:heptane (1+1) (SG < 1) are useful if a chlorinated solvent is needed, but an upper layer is required to simplify extract removal. Alternatively, inorganic salts can be added in sufficient quantity to the aqueous phase to increase the density until the organic phase forms the upper layer.

Table 4.3 Some widely used extraction solvents

Solvent	SG	BPt (°C)	UV Cut-off (nm)	Dielectric constant	Polarity[a]	Solubility in water (g L^{-1})
Butyl acetate[b]	0.88	125	255	5.01	4.0	7.0
1-Chlorobutane	0.89	78	220	7.39	1.0	0.5
Cyclohexane	0.78	81	210	2.03	0	0.0
1,2-Dichloroethane	1.25	83	230	10.65	3.7	8.7
Dichloromethane	1.32	40	235	8.93	3.4	13
Ethyl acetate[b]	0.90	77	255	6.02	4.3	83
Heptane	0.68	98	210	1.92	0	0.5
Methyl *tert*-butyl ether[b]	0.74	55	220	4.5	1.3	48
Petroleum ether[c]	0.65	40–60	210	*ca.* 2	0	0.0
Toluene	0.87	111	285	2.38	2.3	0.53
2,2,4-Trimethylpentane	0.69	99	210	1.9	0.4	0.0

[a] According to Snyder (1974)
[b] Hydrogen acceptor
[c] Boiling range 40–60 °C (mixture of pentanes, hexanes, etc. – other boiling ranges available)

Care should be taken when adding salts, for example to either 'salt-out' analytes, or to adjust the pH, to ensure that the phases are not inverted accidentally. A further complication is that some hydrochloride salts of basic drugs are soluble in organic solvents, especially chlorinated solvents such as chloroform, DCM, and chlorobutane. Sulfates and phosphates are much less soluble in organic solvents, hence sulfuric acid is preferred if performing back-extraction into water as part of a sample preparation procedure.

The inhalational toxicity and other hazards associated with use of some solvents should not be ignored. Benzene, for example, is a proven human carcinogen, whilst occupational exposure to either hexane, or 2-hexanone (butyl methyl ketone) is associated with the development of peripheral neuropathy (Table 4.4). Isohexane (Fisher Scientific), containing less than 5 % v/v *n*-hexane, is a safer alternative to hexane itself. Ammonium hydroxide is unpleasant, if not overtly hazardous, and loss of ammonia from stored reagent may in time render the reagent unreliable. Buffers such as tris(hydroxymethyl)aminomethane [tris(hydroxymethyl)methylamine, 'Tris'] or sodium borate can give pH values in the 9–11 region and have the advantage over phosphate buffers in that bacterial growth is inhibited thus facilitating longer-term storage at room temperature.

In general, more 'polar' solvents will extract a larger number of compounds. Unfortunately, 'polarity' is not a simple property, but rather a composite of different physical characteristics, including the ability to form hydrogen bonds, dipole moment, and dielectric constant. Alkanes do not form hydrogen bonds, have little or no dipole moment and low dielectric constants, and are non-polar. Alcohols, which can form hydrogen bonds and have high dielectric constants, are polar (Table 4.3).

Solvents are sometimes ranked in order of their ability to elute analytes in adsorption chromatography – an elutropic series. In TDM and in PK studies the least polar solvent that will extract the compounds of interest should be used as this will generally give the cleanest extracts.

Table 4.4 Especial hazards associated with the use of some solvents

Solvent	Hazard
Benzene	Human carcinogen
Carbon disufide	Neurotoxin
Carbon tetrachloride, chloroform (also 1,2-dichloropropane, 1,1,2,2-tetrachloroethane)	Hepatorenal toxins; known carcinogens
DCM	Carboxyhaemoglobinaemia; possible carcinogen
Diethyl ether	Highly flammable; may form explosive peroxides
Di-isopropyl ether	May form explosive peroxides
Hexane, 2-hexanone	Peripheral neurotoxins
Trichloroethylene	Cardiotoxin

Analytes that can form hydrogen bonds, either as acceptor or donor, will be better extracted into solvents that support such bonding. Alcohols such as 2-propanol or 3-methylbutanol (isoamyl alcohol) may be added to aprotic solvents to encourage hydrogen bonding, and thus to enhance the extraction of relatively water-soluble analytes, and also to reduce adsorption of basic drugs onto glassware.

Simple LLE with direct analysis of the extract has been used for many years prior to TLC or GC [Figure 4.2(a)]. LLE has been replaced to a certain extent by SPE for LC sample preparation, but a well-defined LLE system is robust and cost effective. LC eluents that use a high proportion of an organic component also allow solvent extracts to be analyzed directly [Figure 4.2(b); Section 10.8.2]. Use of clear glass test-tubes (60 x 5 mm i.d., Dreyer tubes) as extraction vessels simplifies extract removal via a fine-tipped plastic Pasteur pipette and minimizes the risk of

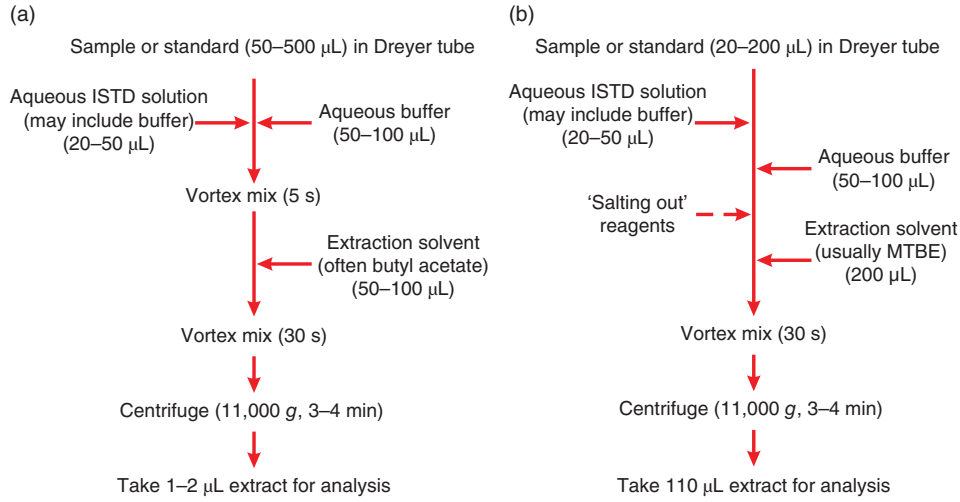


Figure 4.2 Microextraction procedure flow-diagrams: (a) prior to GC; (b) prior to LC

contamination with the aqueous phase. High-speed centrifugation gives rapid phase separation and minimizes the problems posed by emulsion formation.

This microextraction approach is simple, inexpensive, and suitable for emergency work and has been used for hundreds of thousands of analyses since it was introduced in the early 1970s. However, it is not amenable to automation and hence is labour intensive if large numbers of samples are to be assayed. Moreover, care must be taken to minimize the risk of glass tubes breaking in the centrifuge. The sample injection step, however, can be automated – extracts may be transferred to 0.5 mL (30 x 8 mm i.d.) capped disposable polypropylene tubes in an autosampler tray.

'Salting-out' relatively water-soluble analytes by increasing the ionic strength of the aqueous phase thereby encouraging partition of a relatively water-soluble analyte into an organic solvent is a further possibility. Substances used in this way include ammonium chloride, ammonium sulfate, sodium chloride, sodium citrate, sodium sulfate, and potassium carbonate. However, caution is needed because an emulsion may ensue if too much salt is added.

Care must be taken to ensure that the extraction solvent does not interfere in the analysis. It is difficult to remove all traces of solvent and even a small residual amount can affect the detection limit. Chlorinated solvents are best avoided if only on safety and environmental grounds. In addition, some solvents may react with the analyte. Ketones such as butanone will react with primary amines, for example. Some solvents may contain traces of decomposition products. For example, phosgene may be present in chlorinated hydrocarbons and is very reactive and highly toxic.

Antioxidants such as hydroquinone or pyrogallol are added to some ethers to limit the formation of explosive peroxides. Antioxidants are highly electroactive molecules that may react with the analyte and may interfere in the analysis, particularly if the antioxidant has been concentrated by evaporation of the extraction solvent. Diethyl ether is highly volatile and flammable, and is best avoided. Ethanol is frequently added to stabilize chlorinated solvents such as chloroform and DCM, and may react with derivatizing agents. Even ethanol stabilization may not prevent decomposition. LC grade DCM is available with pentene as a stabilizer as an alternative to ethanol.

Butyl acetate and MTBE give efficient extraction of many drugs and metabolites from plasma at an appropriate pH and form the upper layer thus simplifying extract removal for analysis. These solvents do not interfere in MS and the extracts are generally suitable for direct analysis on compatible systems except that butyl acetate cannot be used directly with LC-UV (MTBE has a relatively low UV cut-off, 215 nm). Unlike diethyl and di-isopropyl ethers, MTBE does not form peroxides at ambient temperature hence antioxidants are unnecessary.

4.2.2.1 pH-Controlled liquid–liquid extraction

The extraction of a weak acid or base into an organic solvent is a function of the pH of the aqueous solution, the pK_a of the analyte, and the partition coefficient describing the distribution of the analyte between the solvent and water. Thus, the fraction extracted can be calculated from a knowledge of these parameters (Whelpton, 2004). The pH–extraction curves are sigmoidal in shape with the extraction of basic analytes increasing as the pH increases with a resultant decrease in the degree of ionization (Figure 4.3). The converse is true for acids.

Lipophilic compounds are often extractable at pH values at which a large proportion of the analyte is ionized in the aqueous phase. Imipramine, for example, can be extracted from pH 7 buffer even though it is >99.6 % ionized at this pH, whist the more polar (less lipophilic) demethylated metabolites require higher pH values (Figure 4.3). 2-Hydroxyimipramine cannot

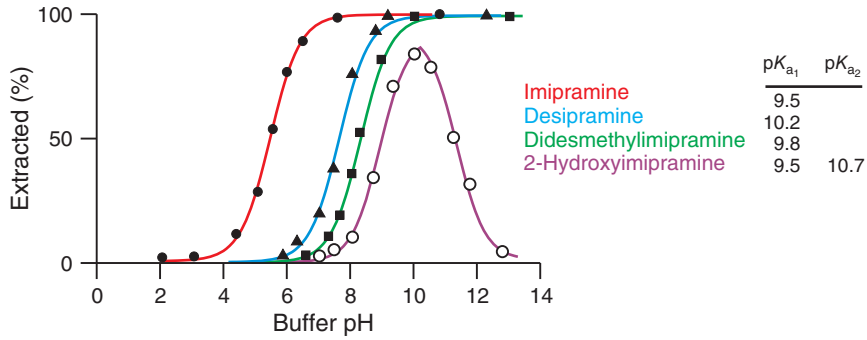


Figure 4.3 Extraction of imipramine and three metabolites from buffers of various pH and an equal volume of heptane. Solid lines represent data fitted as described previously (Whelpton, 2004–reproduced with permission of Springer Nature)

be extracted at high pH values because of ionization of the phenolic group. To extract the four compounds in a single step, the pH would have to be optimized at pH 10 and a larger volume of heptane, or a more polar extracting solvent, employed. Thus, by careful choice of extraction solvent, pH, and the relative volumes of the phases, targeted analytes may be separated.

Strong acids have low pK_a values, whilst the converse is true for strong bases. Typically, the extraction pH should be approximately 2 units less or greater than the pK_a of the acid or base, respectively. Under these conditions the analyte will be <1 % ionized, and there is little to be gained from using more extreme pH values as this increases the risk of decomposing the analyte.

4.2.2.2 Ion-pair extraction

Ionized lipophilic analytes can be extracted into organic solvents providing that an ion of the opposite charge (counter ion) is extracted to maintain electrostatic neutrality. The phase distribution is a function not only of the nature of the analyte and the extraction solvent, but also of the nature and concentration of the counter ion. Partitioning can be further affected by introducing a substance with which the analyte is able to complex. Ion-pair reagents include dodecyltrimethylammonium hydrogen sulfate for acidic compounds and sodium dodecyl sulfate for bases.

Aberrant partitioning in LLE can sometimes be a result of unintentional ion-pair extraction, the counter ions originating from either the buffer, or the biological matrix. Therefore, unless ion-pair extraction is required, solvents that promote ion-pair extraction are best avoided. DCM, ethyl acetate, and 4-methyl-2-pentanone have been used as solvents for ion-pair extraction. Hydrocarbons are less likely to extract ion-pairs than solvents that are hydrogen donors, such as alcohols. If ion-pairing is suspected, then addition of a different counter ion (e.g. bromide or iodide) may change the degree of extraction.

Ion-pair extraction may be used with SPE, employment of alkyl-modified or strong cation (SCX) or anion (SAX) exchange-modified silica stationary phases resulting in a simplified approach to that achieved with LLE.

4.2.2.3 Supported liquid extraction

LLE can be facilitated and/or automated by the use of polypropylene columns containing diatomaceous earth as a solid support for the aqueous phase (supported liquid extraction, SLE). These columns should not be confused with SPE columns (Section 4.2.3) because the aqueous

sample is retained in the column matrix and the extracting solvent (SG >1) allowed to run through the column.

Many different SLE columns are available, including 96-well plates (for example Agilent Combilut™ and Biotage Isolute™). Columns such as Tox Elut™ (Agilent) have been widely used in preparing urine extracts for drug screening. An advantage of this approach is that a wide range of basic and amphoteric compounds, including morphine, and also weak acids such as barbiturates, can be extracted in a single step. Disadvantages are the cost of extraction tubes and the loss of selectivity of separate acidic and basic extracts, but the time saved is considerable (Box 4.4).

Box 4.4 Use of Isolute columns to extract 12 drugs from whole blood prior to LC-MS/MS (Kristoffersen *et al*., 2018 – reproduced with permission of Elsevier)

- To 100 µL whole blood or calibrators/QCs in methanol:ethanol (2+1, v/v) in a sample tube, add 100 µL deionized water and 50 µL ISTD solution in deionized water:ethanol (2+1, v/v).
- Mix and place in a Tecan Freedom Evo 200
- Transfer to a 96-well plate and add 100 µL Trition® X-100/ammonium carbonate buffer (pH 9.3)[1]
- After mixing on a plate shaker, transfer to an Isolute 96-well SLE+ plate (400 µL bed volume)
- Apply vacuum (15 s) then add 700 µL ethyl acetate:heptane (80+20, v/v)
- Apply vacuum to elute to a 96-well collection plate.
- Repeat the elution step
- Remove the SLE plate, and add 30 µL 0.01 % v/v methanolic nitric acid to the extracts
- Evaporate to dryness under vacuum (45 °C, 22 min)
- Reconstitute in 100 µL methanol:deionized water (10+90, v/v)
- Seal with sealing tape (BioChromato) and place in the autosampler tray

[1]Triton®X-100 was used to increase the recovery of THC

4.2.2.4 Immobilized coating extraction

Immobilized coating extraction (ICE) aims to combine the benefits of LLE and fully automated 96-well plate technology (AC Extraction Plate™, Tecan). The inner surface of each well is coated with a layer of immobilized sorbent. This proprietary coating can extract low M_r analytes such as drugs, vitamin D, and steroid hormones efficiently from aqueous solution at an appropriate pH and as such eliminates the need for protein precipitation and phase separation steps (Baecher *et al*., 2014). After extraction the analytes are eluted from the sorbent and transferred to an autosampler plate for GC or LC assay (Box 4.5). A drawback of course is the cost of consumables (pipette tips, AC Extraction Plates, etc.), but the benefits in terms of operator time are clear.

4.2.3 Solid phase extraction

The use of siliceous or other materials with relatively close particle size distribution (15–100 µm) in disposable syringe barrels permits sequential extraction, clean-up, and finally reproducible

Box 4.5 LC-MS assay of plasma clozapine and norclozapine using ICE (Couchman *et al*., 2016 – reproduced with permission of Wolters Kluwer Health, Inc.)

- Position reservoirs containing (1) working ISTD solution (0.25 mg L^{-1} both $[^2H_8]$-clozapine and $[^2H_8]$-norclozapine), (2) wash solvent (10 % v/v methanol in deionized water), and (3) elution solvent (methanol) on the workdeck (Tecan Freedom Evo 100)
- Place a Tecan AC Extraction Plate™ on the orbital shaker, and position a clean microplate for extract collection
- Pipette samples, calibrators, IQCs/EQCs (100 µL), and working ISTD solution (100 µL) into individual wells of the AC Extraction Plate before mixing (1200 rpm, 10 min)
- Discard the contents of the plate manually by inversion and return the plate to the workdeck
- Pipette wash solvent (200 µL) into each well and shake the plate (1200 rpm, 2 min)
- Discard the contents of the plate manually by inversion and return the plate to the workdeck
- Add elution solvent (200 µL) to each well and shake the plate (1200 rpm, 10 min)
- Transfer the extracts (150 µL) robotically from the AC Extraction Plate to the microplate, seal manually with pre-cut, pierceable adhesive seals (Chromsystems), and transfer to the autosampler

elution of drugs and other analytes at relatively low pressures. A range of barrel geometries, reservoir volumes, bed sizes, and materials (glass columns, stainless steel frits) are available. Samples may be loaded using positive pressure (e.g. from a syringe), by drawing through with a vacuum, or by centrifugation using a swing-out rotor system. A variant is use of 250 µL syringes containing a SPE sorbent (microextraction in packed syringe, MEPS) to allow automated GC or LC operation (Altun *et al*., 2004). Disposable pipette extraction (DPX) and 'instrument top sample preparation' (ITSP) are further variants (Bordin *et al*., 2016; Lehmann *et al*., 2017).

Normally, SPE columns are washed and conditioned (e.g. with buffer of the appropriate pH) before the sample is applied. Once the analyte has been retained, a range of clean-up procedures may be used. For reverse-phase materials, water may remove residual hydrophilic materials, including proteins and buffer. Water–organic solvent mixtures may be used to remove interferences. Elution of the analyte in the minimum volume of solvent may negate the need for eluent concentration prior to the analysis (Figure 4.4).

Advantages of SPE are that batch processing can be simplified and that the extracts may contain fewer interfering compounds than with LLE. A further feature when screening for unknowns is that a range of analytes can be extracted either sequentially, or simultaneously. SPE is particularly useful for analytes that are difficult to extract by LLE (e.g. quaternary ammonium compounds – pyridostigmine, tubocurarine, paraquat), and other hydrophilic compounds such as glucuronides. Because ionized molecules can be isolated by SPE, it is possible to develop methods that do not require such extreme pH values as may be required by LLE, which makes SPE suitable for isolating drugs that are not stable under extremes of pH. Furthermore, it should be possible to perform SPE in an inert atmosphere by using syringes to load and elute samples, or nitrogen to provide positive pressure to SPE columns to facilitate elution. SPE may be combined with LLE, for example, when plasma protein binding reduces analyte retention

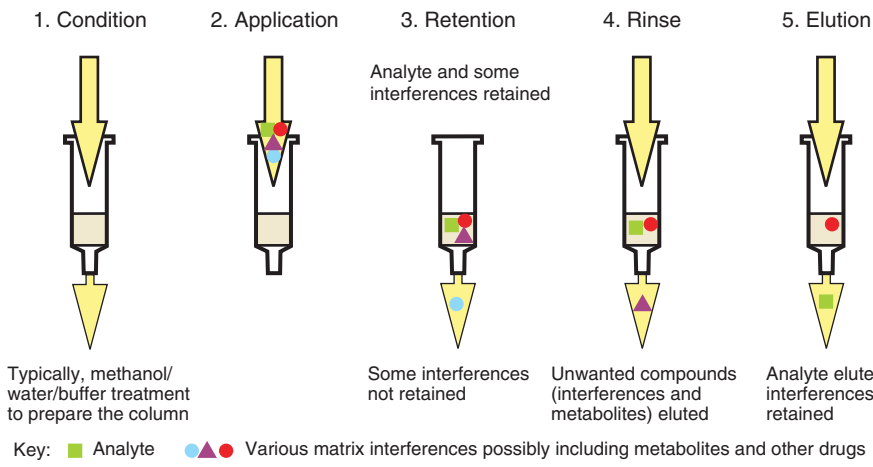


Figure 4.4 Schematic diagram of a solid phase extraction procedure

on SPE columns or to concentrate the extract thereby providing a quicker and possibly safer alternative to solvent evaporation.

SPE is not without its practical difficulties. Particles in the sample may cause blockage so it is advisable to filter or centrifuge samples before application to the SPE column. This should be *after* any pH adjustment because this may result in precipitation of material, particularly when urine is made alkaline. The trace enrichment capabilities of SPE mean that dilution of the sample before addition to the column is a practical option if this minimizes the risk of blockage. However, impurities in deionized water and in buffer solutions may be retained, so it is important to use high purity materials or to purify reagents and other elution solvents by filtration through suitable SPE columns before use. Haemolyzed whole blood can be assayed after centrifugation to remove cell debris, but post-mortem blood may require dilution in buffer or analyte-free plasma before application to the column to prevent column blockage. SPE is not amenable to extraction of analytes from tissues.

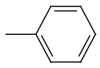
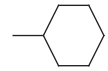
4.2.3.1 Types of stationary phase

In addition to unmodified silica, a range of bonded-phase materials analogous to those used as LC packings is available (Table 4.5). The stationary phase is attached by reaction of the appropriate chlorosilane with a surface silanol moiety on the base silica under anhydrous conditions in the same way as with LC packings and bonded capillary GC columns.

Different manufacturers use different base silicas, different bonding chemistries, and different phase loadings, and their products sometimes give very different results when used in a particular analysis, even though the bonded phase is ostensibly the same. The realization that interactions with residual silanol groups play an important, and sometimes subtle, role in many SPE methods, has resulted in manufacturers offering a greater choice of phases, with popular columns being available in ‘end capped’ (i.e. trimethylchlorosilane-treated to minimize residual silanols) and ‘non-end capped’ versions.

Phenylboronic acid (PBA) based SPE columns are unusual in that the analytes are retained by covalent bonding. The boronate moiety has a high specificity for 1,2- or 1,3-aliphatic and 1,2-aromatic or *cis*-1,2-alicyclic diols such as catechols, nucleic acids, some low M_r proteins,

Table 4.5 Some alkylsilyl-modified silica column packings for use in SPE and LC

Bonded Phase	Name	Bonded Phase	Name
$-(CH_2)_{17}CH_3$	Octadecyl (ODS, C_{18})	$-(CH_2)_3NH_2$	Aminopropyl
$-(CH_2)_7CH_3$	Octyl (C_8)	$-(CH_2)_3CN$	Cyanopropyl, nitrile (CN)
$-(CH_2)_5CH_3$	Hexyl (C_6)	$-CH_2COOH$	Carboxymethyl (WCX)
$-CH_2CH_3$	Ethyl (C_2)	$-(CH_2)_3\text{-}\overset{\oplus}{N}(CH_3)_3$	Trimethylaminopropyl (SAX)
$-CH_3$	Methyl (C_1)	$-(CH_2)_3\text{-}N(CH_2CH_3)_2$	Diethylaminopropyl (WAX)
	Phenyl		Cyclohexyl
$-(CH_2)_3OCH_2\underset{\displaystyle CH_2OH}{\underset{\vert}{C}}HOH$	Diol	$-(CH_2)_3-C_6H_4-SO_3^{\ominus}$	4-Sulfophenylpropyl (SCX)

and carbohydrates. Retention is by formation of 5- or 6-membered ring phenylboronates under neutral or basic conditions. PBA columns have proved to be effective in the isolation of catecholamines from biological fluids and for the separation of RNA from DNA. Elution is achieved under acidic conditions, for example using methanolic hydrochloric acid. The columns are expensive, but can be reused.

So-called 'mixed-mode' columns exploit two or more retention mechanisms to bring about the desired result. The use of non-polar and ion-exchange modifiers in one column, for example, allows efficient clean-up to remove non-polar impurities without the risk of prematurely eluting the compounds of interest. Using a strong cation exchange (SCX)-modified phase to retain a basic drug allows washing with methanol to remove interferences, prior to analyte elution with alkaline ethyl acetate. Adoption of 96-well plate technology and robotics increases throughput. The compatibility of SPE materials with LC systems means that 'on-line' extraction prior to liquid chromatography is an effective option for high-throughput analysis.

Molecularly imprinted polymers (MIPs) are prepared by forming a polymer in the presence of a template molecule, either the analyte or a suitable analogue. Once polymerized, the template molecule must be removed otherwise there is the danger of residual template molecule interfering in the assay. MIPs are frequently custom-synthesized, but some are available commercially. These include SPE columns for chloramphenicol, atrazine, β-agonists and β-blockers, and NSAIDs. Applications have been in food residues analysis and in the assay of environmental samples such as river water. MIPs for the analysis of 'amfetamines' (metamfetamine, MDA, MDMA, MDEA) in urine are available from several manufacturers. The use of molecularly imprinted polymer-sol-gel tablets that can be placed in urine with subsequent elution of the analyte of interest has also been described (El-Beqqali *et* al., 2017).

An important development has been the introduction of SPE columns designed to remove phospholipids from biological samples. Phospholipids are a major problem in LC-MS because not only may they be retained on the LC column thus altering analyte retention, but also may alter analyte ionization in the source if they co-elute with the analyte and/or the ISTD, thus compromising quantitative work (Section 13.10). Zirconium-modified silica SPE columns (HybridSPE-Phospholipid technology, Supelco) allow the simultaneous removal of proteins and

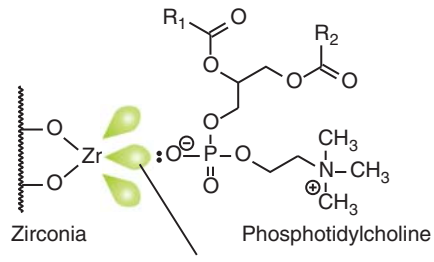


Figure 4.5 HybridSPE-Phospholipid technology

phospholipids (Figure 4.5). Other manufacturers also produce phospholipid removal columns. In one study use of the Oasis PRiME HLB (hydrophilic-lipophilic balance) in a 96-well μElution Plate (Waters) offered efficient, but not complete, phospholipid removal (Koller *et al*., 2019).

4.2.4 Solid phase microextraction

Solid phase microextraction (SPME), initially introduced for the analysis of environmental samples, has been used for the extraction of drugs and other lipophilic analytes from biological matrices either directly, or after derivatization (He & Concheiro-Guisan, 2019). In its simplest form, a spring-loaded solid probe (originally a fused silica fibre) coated with a polymer film is inserted through a septum into a sealed vial containing the sample (usually aqueous liquid or headspace above the liquid). After an appropriate period (normally 20–30 min) the fibre is retracted. Analyte or derivatized analyte partitions between the sample and the liquid phase supported on the fibre, usually polydimethylsiloxane (PDMS), PDMS-polydivinylbenzene (PDVB), polyacrylate, or other absorbent material [Figure 4.6(a)]. Some form of agitation is needed with liquid samples. Alternative approaches include a magnetic stir-bar sealed in glass and coated with PDMS [Figure 4.6(b)] and packed cartridges that can be used with a micro-syringe [Figure 4.6(c)].

Unlike LLE, the aim is not to extract all the analyte from the sample because SPME is an equilibrium extraction process. Indeed, the analyte concentration of the sample may be little changed if only a very small proportion of analyte is removed. Subsequently, extracted analytes are either thermally desorbed, in the case of GC/GC-MS, or injected via a modified sample loop, in the case of LC. Loaded probes may be sealed for transport to the laboratory for analysis.

Obvious attractions of SPME are that no solvents are required and all the material that is extracted by the probe may be analyzed directly. It is reported that probes may be reconditioned and reused 50–100 times. Metal SPME fibres are now available. The problem of variation in extraction conditions has been addressed by pre-loading of the extraction phase with an ISTD, and then measuring both analyte uptake and ISTD loss from the extraction phase.

When acids or bases are extracted it is normally necessary to buffer the pH to increase the proportion of non-ionized drug to optimize extraction efficiency. Salting out may be employed to increase the proportion of drug extracted. Differences in the ionic strength of urine samples may lead to variable recovery unless the samples are brought to similar ionic strength by adding salt. Increasing the temperature usually reduces the time to reach equilibrium, but may also reduce the amount extracted. Potential disadvantages include competition between drug and endogenous compounds for the fibre, particularly when the mechanism is adsorption rather than partitioning.

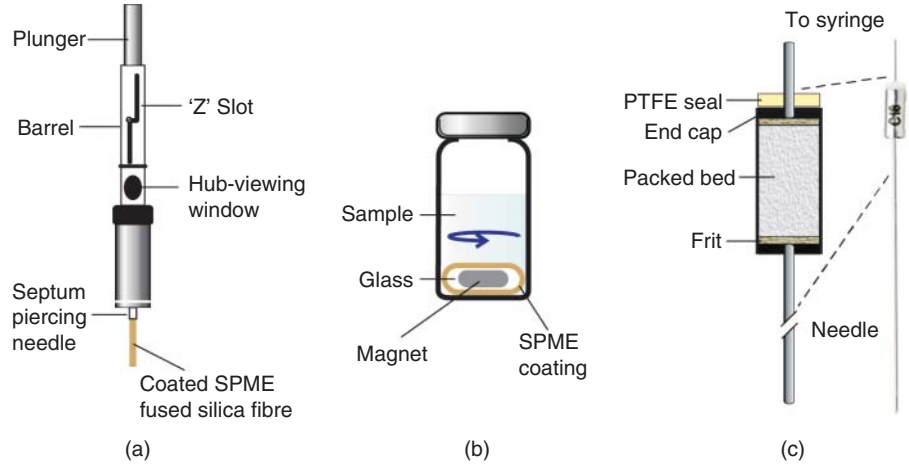


Figure 4.6 Solid phase microextraction: (a) spring-loaded solid probe coated with a polymer film, (b) polymer-coated magnetic stirrer bar, and (c) packed cartridge for use with a microsyringe

It is important to define the time required for equilibration. Protein binding is likely to have more of an effect on recovery than when practically all the analyte is extracted into an organic phase as in LLE. Plasma protein binding not only reduces the amount of analyte extracted, but also increases the equilibration time (Figure 4.7). By fitting the data of Figure 4.7 to exponential curves it can be calculated that >95 % of the equilibrium concentration in the non-deproteinated sample is reached in 70 minutes, but this is reduced to 30 minutes by deproteination.

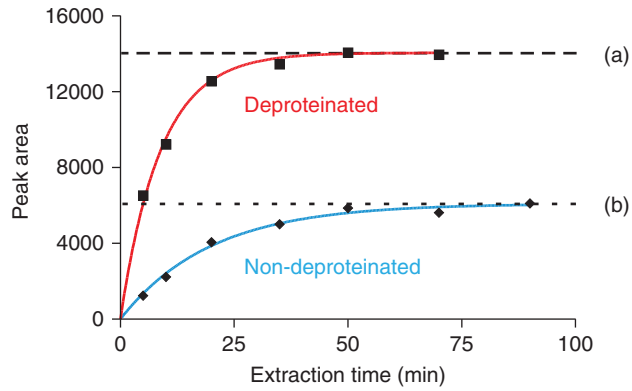


Figure 4.7 Equilibration time profile for SPME of lidocaine from plasma after buffering 1:1 to pH 9.5 (a) with and (b) without deproteinization. The solid lines represent the least-squares fit to: Peak area = Maximum peak area [1 – exp(-*kt*)] (redrawn with data from Koster *et al*., 2000)

Single-drop microextraction (SDME) using a non-volatile or immiscible solvent and thin-film microextraction using PDMS sheet are further variants on the microextraction theme. Each has unique features. Notably, the use of thin PDMS membranes has advantages of faster and more sensitive extraction when compared with conventional thick-film formats.

4.2.5 *Liquid phase microextraction*

Liquid phase microextraction (LPME) may allow high-throughput analysis of small sample volumes with automation and on-line coupling to analytical instruments. Because of (i) the high analyte capacity of the acceptor liquid and (ii) the substantial phase ratio difference that can be achieved, analyte enrichment of several hundred times is feasible. A further advantage is that only very small volumes of organic solvents are required. Several modes of LPME have been described including single drop/hollow fibre-based LPME and dispersive liquid–liquid microextraction (DLLME) (Barroso *et al*., 2011; Havlikova *et al*., 2019; Mansour & Khairy, 2017; He & Concheiro-Guisan, 2019).

LLE using single microdrops of solvent suspended from standard GC or LC microsyringe needles can be directly immersed either in the sample, or in the headspace above the sample. The drop is withdrawn into the needle and injected directly. Suitable solvents include octyl acetate, 2-methylbutanol, undecane, octane, nonane, and ethylene glycol. The use of a range of other liquids has been explored (An *et al*., 2017).

Difficulties include (i) the amount of analyte that can be extracted is small, (ii) the drop may be displaced, (iii) there may be dissolution of the drop in the sample, and (iv) the low surface area of the drop leads to long extraction times. Consequently, time has to be spent optimizing the extraction conditions. Three phase systems have been employed [Figure 4.8(a)], for example, Ebrahimzadeh *et al*. (2008) extracted fentanyl from urine into octane with back-extraction into 5 µL perchloric acid (1 mmol L^{-1}) giving an enrichment factor of 300.

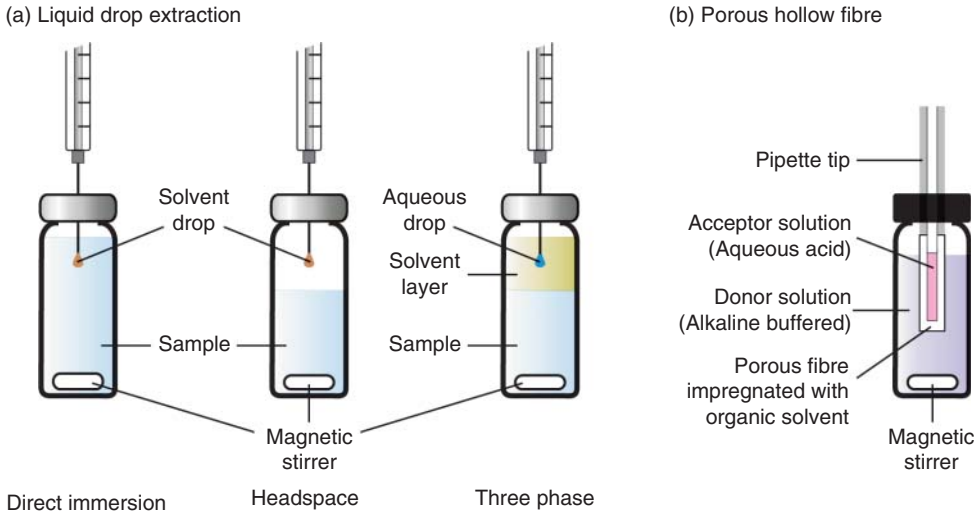


Figure 4.8 Schematic of modes of liquid phase microextraction: (a) single droplet extraction; (b) ion-trapping basic analytes using solvent impregnated hollow microfibre

Analytes have been extracted from biological samples (whole blood or plasma, 0.1–4 mL) into an acceptor solution contained in the lumen of a disposable porous hollow fibre supported in a sealed glass vial [Figure 4.8(b)]. Addition of ISTD to the acceptor solution eliminates the need for further extract handling. For GC, the tubing is pre-washed with acetone and extraction is into a water-immiscible solvent such as dodecyl acetate, dihexyl ether, or octanol (10–50 µL)

contained in the lumen of the tube. Conventional pH manipulation of the sample can be used if necessary. The extraction conditions can be further manipulated by impregnating the tubing with immiscible solvent and then placing an appropriate aqueous acceptor solution in the lumen. Analyte trapping can then be achieved [Figure 4.8(b)].

4.2.6 *Supercritical fluid extraction*

Above a certain critical temperature (T_c) and pressure (p_c), a vapour does not exist as a gas, a solid, or a liquid, but as a supercritical fluid [Figure 4.9(a)]. These fluids have densities, diffusivities, and solvent strengths similar to liquids, but viscosities comparable to gases, which means that mass transfer is faster and extraction times are reduced when compared with liquids. Use of supercritical fluids has advantages of mild conditions, no thermal degradation of analyte, and that the solvent readily evaporates after extraction. It is ideal for powdered samples such as soils, plant material, and hair, but is generally unsuitable for liquid or wet matrices unless the sample is adsorbed on, for example, silica gel prior to the extraction. Moreover, the cost of the equipment is high.

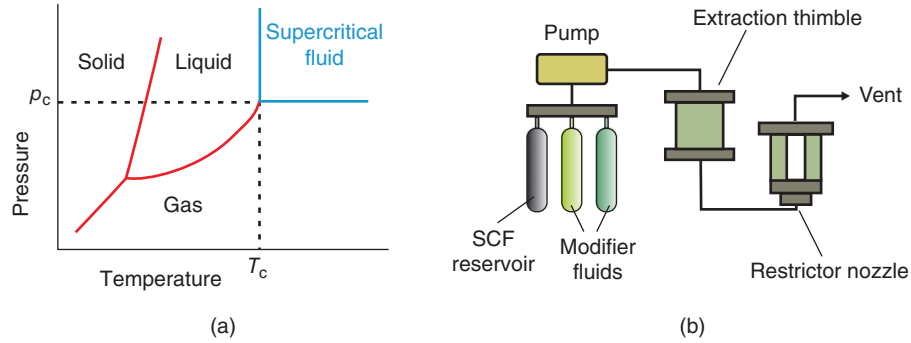


Figure 4.9 Supercritical fluid extraction. (a) Phase diagram for a fluid. (b) Schematic of typical apparatus

Most supercritical fluid extraction (SFE) processes are quite simple. A sample is placed in the extraction thimble, and supercritical fluid is pumped through the thimble [Figure 4.9(b)]. The extracted analytes are trapped as the fluid flows through a restricting nozzle. The fluid is vented from the collection trap, allowing the solvent to either escape or be recompressed for future use. The process is a leaching operation, but it is commonly referred to as extraction. Carbon dioxide is normally used because it is inexpensive, non-toxic (although an asphyxiant gas), available with high purity, and has a low T_c (31.3 °C). Typical operating conditions with supercritical carbon dioxide are 28,000 kPa (~4000 psi) at 50 °C.

The dominant factors that govern the SFE of an analyte are the solubility of the analyte in the fluid, the mass transfer kinetics of the analyte from the matrix to the fluid, and interactions between the fluid and the matrix. Altering the properties of the solvent system can change the extraction efficiency. Temperature and pressure are important influences. For example, raising the pressure increases the density of the fluid and often increases solubility. Obviously changing the solvent may provide different solvent properties. Supercritical water gives different extraction efficiency when compared to ethylene or carbon dioxide. Anhydrous ammonia is different again. Adding a small amount of a second material (co-solvent) such as a small amount

(a few %) of methanol to carbon dioxide enhances the solubility of hydrophilic compounds. Addition of hydrogen chloride or ammonia renders the fluid acidic or basic, respectively.

4.2.7 *Accelerated solvent extraction*

Accelerated solvent extraction (ASE), also known as pressurized liquid extraction (PLE), uses solvents under high pressures and at elevated temperatures (normally 100–140 °C) in an automated system similar to that of SFE. Microwave-assisted extraction is similar except that microwave heating replaces a conventional oven. Solid samples are placed in the extraction vessel and brought to operating pressure by pumping solvent or a mixture of solvents into the vessel that is heated to the desired temperature. When the pressure reaches a pre-set value, a pneumatic valve opens allowing the extract to be transferred to the liquid trap (Figure 4.10). Fresh solvent can be pumped into the extraction vessel and the process repeated if necessary.

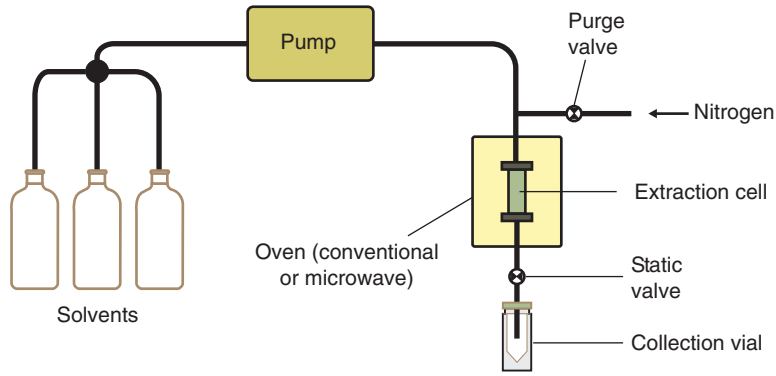


Figure 4.10 Schematic diagram of apparatus for accelerated solvent extraction

ASE can be useful because supercritical carbon dioxide does not possess the solvent strength needed to extract efficiently some polar analytes from complex matrices even after adding modifiers such as methanol. In addition, ASE uses less solvent than conventional Soxhlet extraction techniques and is faster. The higher temperatures used also make it easier for the solvent to overcome intermolecular interactions between the analyte and the matrix, but of course this could be a disadvantage with a labile analyte. ASE is not a selective extraction technique, however, and is essentially confined to solid matrices (Vincenti *et al*., 2019). There may also be a problem with loss of more volatile analytes when the extract is being transferred to the collection trap. A further disadvantage is there are no instruments currently available that can be interfaced directly to a chromatographic system.

4.3 Plasma protein binding

It is sometimes necessary to measure the concentration of non-bound ('free') drug in plasma or serum, usually as part of TDM of drugs such as phenytoin (Section 20.6.3). The plasma non-bound fraction may be in equilibrium with analyte concentration in oral fluid, but equilibrium with the CSF concentration requires that the drug is able to enter this fluid. For strongly (>90 %) plasma protein-bound analytes, non-bound plasma, oral fluid, and CSF concentrations are often very low and thus such measurements require sensitive analytical methods.

To measure plasma protein binding, non-bound drug is normally separated by either ultrafiltration, or equilibrium dialysis. There are a number of issues to be borne in mind when planning and conducting plasma protein binding studies (Box 4.6). Most methods perturb binding to some extent. The problem of protein leaking though the membrane used to separate the fractions applies to both ultrafiltration and equilibrium dialysis and should be monitored by protein assay. As an alternative, SPME of plasma or serum can be used to measure non-bound drug directly, providing the proportion extracted is small and does not perturb the binding (Peltenburg *et al*., 2015).

Box 4.6 Considerations in the measurement of plasma protein binding

(a) Ultrafiltration
- Relatively quick
- Change in protein concentration
- Adsorption of the analyte onto the apparatus
- Protein leakage

(b) Equilibrium dialysis
- Often slow to reach equilibrium
- Decomposition of the analyte and microbial growth
- Adsorption of the analyte onto the dialysis membrane
- Protein leakage
- Dilution of the sample

For highly protein bound drugs, measuring the non-bound drug concentration presents an analytical challenge. For some drugs, immunoassays may be sensitive enough for routine analysis of the non-bound fraction and kits are commercially available that contain suitable calibrators for quantifying the total and non-bound concentrations. Because phenytoin is approximately 90 % bound in plasma the concentration of the calibrators for the free drug need only be an order of magnitude less than the calibrators for measuring the total concentration. There is some doubt as to whether all the antibodies that are offered by some suppliers are specific enough for the purpose (Roberts *et al*., 2001).

4.3.1 Ultrafiltration

Several ultrafiltration devices are available commercially, one of which has been used in an automated system (Ye *et al*., 2017). The filtration membranes used are made from a variety of materials and have M_r cut-offs in the range 10,000–30,000. Most devices are centrifuged to provide the filtration pressure. During ultrafiltration the protein concentration in the retentate increases, potentially increasing the proportion of analyte bound. To minimize this problem as small a volume of ultrafiltrate should be collected as practicable, and the volume of retentate made up with an appropriate buffer solution periodically during centrifugation.

Binding of the analyte to the filtration membrane (a problem with many lipophilic drugs) will reduce the concentration in the filtrate. Control experiments to ascertain the magnitude of this problem should be conducted. It is good practice to collect serial samples of filtrate for analysis to ensure that the sample is representative of the non-bound concentration. Some filtration membranes need to be soaked in water or buffer before use and as a result the first ultrafiltrate collection(s) may be noticeably dilute.

4.3.2 Equilibrium dialysis

Equilibrium dialysis is the 'gold standard' for protein binding studies, but it is not without problems. The time for equilibration can be several hours, during which there may be microbial growth, particularly with plasma at 37 °C, possibly leading to changes in protein and analyte concentrations and analyte binding. If an antibiotic is added it cannot be assumed that it does not interfere with the binding.

An advantage of dialysis is that the problem of adsorption of analyte to the membrane and apparatus is largely overcome by measuring the concentrations on either side of the membrane. Adsorption will reduce the concentrations in the donor and recipient (dialysate) solutions, but at equilibrium the non-bound concentration in solution will be the same on either side of the membrane [Figure 4.11(a)]. Thus, it is possible to calculate the bound and free concentrations on the appropriate side of the membrane and to relate this to the initial plasma concentration of analyte.

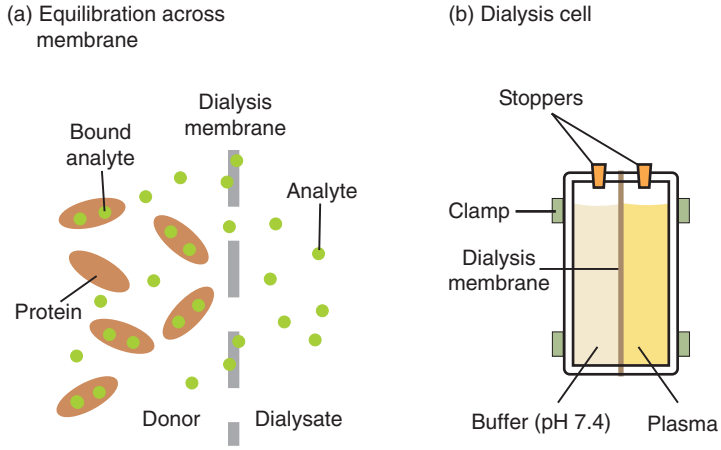


Figure 4.11 Diagrammatic representation of equilibrium dialysis. (a) Equilibration of a drug either side of a dialysis membrane. (b) Dialysis cell. Analyte, but not protein or protein-bound analyte, diffuses through the dialysis membrane until the non-bound concentrations on either side of the dialysis membrane are equal

Equilibrium dialysis cells [Figure 4.11(b)] may be made using Perspex or similar material, or commercially available cells may be used. On the protein side of the membrane there is bound and non-bound analyte so the concentration is the total concentration, C_t, and that on the buffer side is the non-bound (free) concentration, C_f, so the fraction bound, β, is:

$$\beta = \frac{C_t - C_f}{C_t} \tag{4.1}$$

Because analyte is transferred to the dialysate and possibly lost by adsorption to the apparatus, C_t is not the same as the initial plasma concentration. However, provided that the fraction bound does not markedly change with changes in total concentration, then the non-bound concentration in the original plasma sample can be derived from Equation (4.1).

4.4 Hydrolysis of conjugated metabolites

Cleavage of conjugates is an important consideration in toxicological analysis, especially of urine. Many drugs and metabolites (for example, benzodiazepines, laxatives, opiates, and steroids) are excreted in urine and in bile predominantly as conjugates with D-glucuronic acid and/or sulfate. In order to maximize sensitivity in drug/poison screening, and if appropriate in order to measure such conjugates indirectly, i.e. in conjunction with independent measurement of unconjugated analyte, either selective or non-selective hydrolysis of the sample can be undertaken. In quantitative work, calibration or QC samples containing the conjugate of interest should be carried through the chosen procedure in order to monitor the efficiency of hydrolysis.

Incubation with strong mineral acid such as an equal volume of 5 mol L^{-1} hydrochloric acid (15–30 min, 100 °C, or in a microwave or pressure cooker) is inexpensive and gives rapid, but non-selective hydrolysis of conjugates. Use of domestic microwaves for this purpose is potentially hazardous, but commercial instruments offering temperature control are available (Milestone Scientific, https://milestonesci.com/). Quartz hydrolysis vessels used as inserts abolish the risk of memory effects from adsorption of analytes into the PTFE vessels supplied with the instrument.

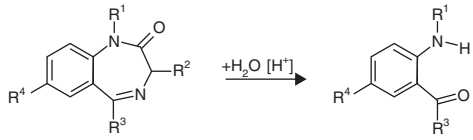
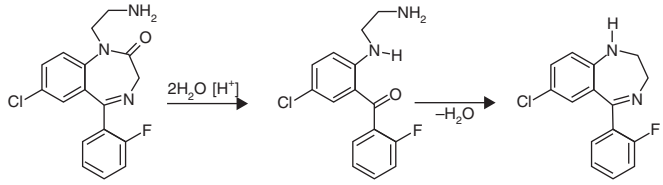
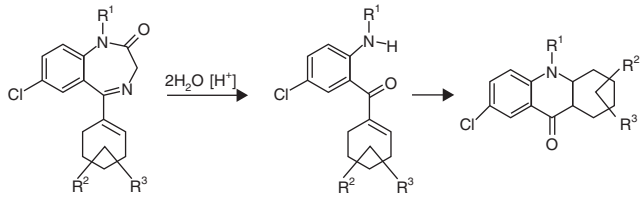
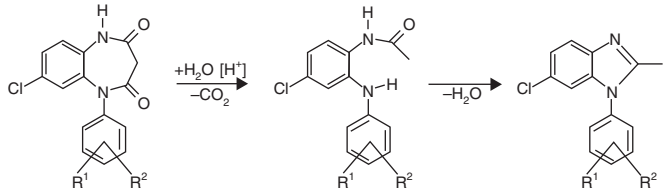
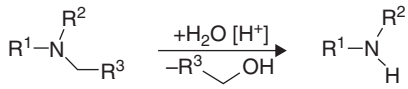
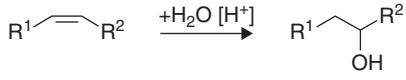
All strong acid hydrolysis procedures are likely to introduce additional, hitherto unseen compounds into sample extracts even if due care is taken to ensure effective neutralization/buffering during sample preparation. Destruction of labile analytes as well as conjugates may be advantageous as in the colour test for urine paracetamol (Section 5.2), but may lead to impaired selectivity as in the hydrolysis of certain benzodiazepines to aminobenzophenones (Section 8.2.4). Some typical artefacts formed during acid hydrolysis are listed in Table 4.6.

In contrast, incubation with β-glucuronidase (EC 3.2.1.31) and/or arylsulfatase (EC 3.1.6.1) (15 h, 35 °C) can give selective hydrolysis of conjugates under relatively mild conditions. Generally cleaner extracts result, but the incubation time is longer (usually overnight), there may be matrix effects from the enzyme solution, and the enzyme preparations are relatively expensive. A range of enzymes is available. Those from *Escherichia coli* or bovine liver lack arylsulfatase and therefore preparations of the digestive juice of *Helix pomatia*, which contain both enzymes, are widely used. β-Glucuronidase and arylsulfatase from *Patella vulgata* are also marketed. The use of β-glucuronidase from abalone has been described (Malik-Wolf *et al*., 2014).

The enzymes exhibit maximum activities at different pH values. The optimal pH for the *H. pomatia* enzyme is pH 4.5 whist that for *E. coli* is ~6.8. Furthermore, the *H. pomatia* enzyme can be incubated at temperatures up to 45 °C, which can reduce the incubation time markedly. Some temperature-tolerant preparations allow incubation up to 60 °C (2 h incubation).

Control experiments should be performed to ensure that the incubation conditions are optimum with regard to the degree of hydrolysis and stability of the analytes. It should also be borne in mind that prolonged incubation of potentially infective samples may increase the titre of the infective agent. Finally, note that β-glucuronidase is specific for β-D-glucuronides, and some glucuronides rearrange and therefore cannot be hydrolyzed enzymatically. There have been many attempts to prepare immobilized enzyme preparations, including preparations for on-line cleavage of conjugates in pre- or post-column reactors in LC, but none are in widespread use.

Table 4.6 Some examples of artefacts formed during acid hydrolysis (Maurer *et al*., 2016– reproduced with permission of John Wiley & Sons)

Summary	Chemical reaction	Example
Hydrolysis of 1,4-benzodiazepines to aminobenzoyl derivatives		Diazepam
Hydrolysis and rearrangement of didesethylflu-razepam		–
Hydrolysis and rearrangement of tetrazepam and its metabolites		–
Hydrolysis and rearrangement of *N*-desmethyl metabolites of clobazam to benzimidazole derivatives		–
Hydrolysis of ethers		Acebutolol, diphenhy-dramine
N-Dealkylation of ethylenediamine antihistamines		Antazoline
Hydration of an alkene		Alprenolol, pentazocine

4.5 Extraction of drugs from tissues

Conventionally, measurements in portions of organs such as liver and brain have been performed via mechanical homogenization using a PTFE-in-glass homogenizer, and/or acid digestion using, say, ~5 g wet weight of tissue followed by LLE at an appropriate pH. Other tissues such as lung or muscle generally require use of a cutting or mincing action.

Digestion (12–16 h, room temperature) of small amounts (approximately 100 mg) of tissue with collagenase, protease, or lipase often gives much improved recovery when compared with conventional procedures and has the advantage that, once the digest has been prepared, analogous methodology and calibration standards to those used with plasma can be employed. It is important to ensure that the enzymatic hydrolysis does not destroy the analyte(s), hydrolyze metabolites to re-form the analyte(s), or introduce other interferences. An ISTD can be added either to the homogenization buffer, or at a later stage.

Although papain has been reported to give the highest recoveries of added drug from liver homogenate, Subtilisin A gave similar recoveries for most compounds. Moreover, only a short incubation time (1 h) was required (Table 4.7). There has been tentative interest in using ASE (Section 4.2.7) in the analysis of drugs and other poisons in tissues and related samples (Abend *et al*., 2003).

Table 4.7 Recovery of added drug from liver homogenate by various digestion procedures (Shankar *et al*., 1987–reproduced with permission of Oxford University Press)

	Recovery (%)					
Drug	Papain	Subtilisin A	Neutrase	Trypsin	Acid hydrolysis	Stas-Otto
Chlordiazepoxide	96	60	58	40	56	47
Chlorpromazine	76	67	62	50	47	38
Diazepam	90	80	87	71	66	41
Diphenhydramine	67	57	61	54	39	–
Imipramine	78	82	81	55	54	34
Nitrazepam	80	74	38	57	52	49
Oxazepam	92	85	66	63	73	64
Promethazine	88	61	54	50	44	32

4.6 Summary

Sample preparation is an important part of many of the procedures discussed in subsequent pages. Even if confined to relatively simple tasks such as centrifugation of urine and addition of an ISTD, incorrect sample preparation, as with inappropriate sample collection, can negate any subsequent analytical work. Of all the procedures in common use, LLE has advantages of simplicity, efficacy, flexibility, reproducibility, and cost-effectiveness. Modern ‘off-line’ semi-automated approaches such as SLE and ICE have comparable throughput to SPE and as such as are applicable to sample preparation for both GC and LC. Moreover, such procedures have practical advantages over ‘on-line’ methods in LC.

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5 Colour Tests, and Spectrophotometric and Luminescence Techniques

5.1 Introduction

Some simple colour tests for poisons in biological specimens may find a place under certain circumstances, but chromogenic reactions are most valuable when used in the visualization of developed TLC plates (Chapter 8). Although not strictly toxicology, presumptive colour tests also find a role in testing suspected drug seizures, for example.

UV/visible absorption and spectrophotofluorimetry were amongst the pioneering techniques applied in qualitative and quantitative toxicological analyses, and whilst still used in their own right, they now find great application as detection systems in LC. UV spectrophotometry is also important for monitoring reaction rate in immunoassays and in monitoring NAD/NADH- or NADP/NADPH-linked enzyme reactions. Atomic absorption spectrophotometry and other spectroscopic techniques remain important in trace element and toxic metal analysis (Chapter 21).

The term luminescence is applied to the phenomenon whereby light is emitted when 'excited' molecules return to a lower energy, ground state. The three major subdivisions of luminescence are fluorescence, phosphorescence, and chemiluminescence. With fluorescence and phosphorescence, molecules are excited by absorption either of incident electromagnetic radiation (light), which is then re-emitted (usually as light of a longer wavelength), or by heat such as via a flame, as in flame emission photometry, respectively. Chemiluminescence, as its name implies, results when light is emitted from molecules excited as a result of a chemical reaction at ambient or near ambient temperatures without the introduction or generation of heat.

5.2 Colour tests in toxicology

Many drugs and other poisons give characteristic colours with appropriate reagents if present in sufficient amounts, and in the absence of interfering compounds. A few of these tests are for practical purposes specific, but usually compounds containing similar functional groups will also react, and thus interference from other poisons, metabolites, or contaminants is to be expected. Further complications are that colour description is very subjective, even in people with normal colour vision, while the colours produced usually vary in intensity or hue with concentration, and may be unstable. Recording the results of the tests is impossible without good colour photography (Box 5.1).

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

Box 5.1 Colour tests in toxicology

- Very quick/inexpensive: add reagent(s) and observe colour
- Usually homogenous assay – apart from some immunoassays, most other toxicology tests require some form of sample pre-treatment
- Mainly useful for urine/gastric contents/scene residues
- Usually carried out in clear glass test tubes, but a white, glazed tile is better (uniform background; uses less reagent)
- Must always analyze a reagent blank and positive control with samples
- Subjective – people vary in how they perceive/describe colours; also colour may vary in intensity
- Enormous range of tests available, but most have poor selectivity

The reagents for these homogenous assays typically contain strong acids or alkalis, or use other potentially hazardous chemicals. Appropriate safety precautions must be observed. Many tests can be performed satisfactorily in clear glass test tubes. However, the use of a 'spotting' tile (a white glazed porcelain tile with a number of shallow depressions or wells in its surface) gives a uniform background against which to assess any colours produced, and also minimizes the volumes of reagents and sample used.

Colour tests feature prominently in the WHO Basic Analytical Toxicology manual (Flanagan *et al.,* 1995) where common problems and sources of interference in particular tests are emphasized. The colours given by some commonly used tests are also illustrated. Badcock (2000) and Widdop (2011) have listed some further tests. When performing colour tests it is **always** important to analyze concurrently with the test sample:

1. A *'reagent blank'*, i.e. an appropriate sample that is known NOT to contain the compound(s) of interest. If the test is to be performed on urine then analyte-free ('blank') urine should be used, otherwise deionized water is generally adequate.
2. A *known positive sample* at an appropriate concentration, i.e. a concentration within the range likely to be encountered in patient samples. If the test is to be performed on urine, then ideally urine from a patient or volunteer known to have taken the compound in question should be used. However, this is not always practicable and then 'spiked' urine ('blank' urine to which has been added a known amount of the compound under analysis) should be used.

Colour tests are useful in that a minimum of equipment and expertise is required [Table 5.1; see Flanagan *et al.,* (1995) for full details]. However, the reagent has to be available and stable. The tests are usually applicable only to urine and/or other samples that may contain relatively large amounts of poison such as stomach contents. Sensitivity is limited especially if the urine itself, for example, is strongly coloured. It is possible to extract the poison and apply the colour test to the residue, though this is rarely done for biological fluids. False negatives are a risk even when a test is used for its intended purpose with appropriate samples.

Positive results with poisons such as trichloro-compounds or paraquat may indicate the need for a quantitative measurement in plasma, if available. With chloral hydrate, trichloroethylene, and trichlofos, 2,2,2-trichloroethanol is the principal plasma metabolite, a compound that may not be looked for if GC-MS alone is used in STA (Chapter 19). The test for paracetamol may detect recent paracetamol exposure when the parent drug has been cleared from plasma. Other tests such as the Forrest test for imipramine-type compounds and the FPN test for phenothiazines

Table 5.1 Some colour tests used in analytical toxicology

Test	Analyte(s)	Fluid	LoD (mg L^{-1}, deionized water)	[Additional] compounds detected
o-Cresol/ ammonia	Paracetamol	Urine	1[a]	Aniline,[b] benorylate,[b] ethylenediamine,[c] nitrobenzene,[b] phenacetin[b]
Diphenylamine	Oxidizing agents	Gastric contents[d]	10	Bromates, chlorates, iodates, nitrates, nitrites, peroxides
Dithionite	Paraquat (blue) Diquat (yellow-green)	Urine[e]	1 5	–
Diphenylamine	Ethchlorvynol	Urine	1	–
Forrest	Imipramine	Urine	25	Clomipramine, desipramine, trimipramine
FPN[f]	Phenothiazines	Urine	25[g]	–
Fujiwara	Trichloro-compounds	Urine	1[h]	Chloral hydrate, (alpha)chloralose, chloroform, dichloralphenazone, trichloroethylene, trichlofos
Trinder's[i]	Salicylates	Plasma Urine Gastric contents[d]	10	Aloxiprin,[b] aminosalicylate, aspirin,[b] benorylate,[b] methylsalicylate,[b] salicylamide[b]

[a] As *p*-aminophenol
[b] After metabolism or hydrolysis
[c] From aminophylline, for example
[d] Includes vomit, SWO, scene residues, and similar samples
[e] Can be used on plasma in severe cases
[f] Ferric chloride/Perchloric acid/Nitric acid
[g] As chlorpromazine
[h] As trichloroacetate
[i] Ketones interfere

are less useful and only indicate the need for confirmatory chromatographic analysis. These same reagents can be utilized as location agents following TLC and some have been developed for use in quantitative measurements.

5.3 Colour tests for pharmaceuticals and illicit drugs

Sometimes, the analytical toxicology laboratory will be asked to identify the drugs present in pharmaceutical dosage forms (tablets, etc.) or plant material. A series of manuals giving details of simple colour tests to confirm the identity of pharmaceutical preparations and more recently herbal medicines are available (WHO, 1986; 1991; 1998). However, these tests are aimed at confirming the identity of drug formulations, many of which have low intrinsic toxicity, prior to clinical use and a range of reagents is needed in order to perform the tests. Hence it is

usually easier to use a computer-based tablet identification system, for example, and/or dissolve a portion of material in an appropriate solvent prior to analysis using a chromatographic or other suitable method.

In contrast, presumptive tests for the presence of illicit drugs in seized materials, for example, continue to be used widely. Validation procedures have been described for 12 spot tests aimed at identifying traditionally abused substances (Table 5.2). The validation included selectivity and limit of detection. Up to 45 substances were analyzed in triplicate using each test. The colours resulting from reactions with known amounts of analytes were compared to two reference colour charts [Inter-Society Color Council and the National Bureau of Standards (ISCC-NBS) and Munsell charts].

The tests were very sensitive with LoDs of typically 1–50 µg depending on the test and the analyte. When used in combination selectivity was enhanced. Note, however, that drug mixtures inevitably complicate such tests. Levamisole, for example, a common constituent of illicit cocaine, gives a blue colour in the Scott test even in the absence of cocaine (de Jong *et al*., 2018). Other problems with this test have been documented (Tsumura *et al*., 2005).

Data on the colours produced on testing 40 NPS including synthetic cathinones, synthetic cannabinoids, piperazines, and phenethylamines as well as likely sources of interference using a commercially available colour test system (MMC International, Breda, the Netherlands) and with Mandelin, Marquis, Scott, Simon, and Mecke reagents (Table 5.2) are also available (Cuypers *et al*., 2016). The Duquenois–Levine colour test for the presumptive identification of THC gives a negative result with synthetic cannabinoids (Isaccs, 2014).

Other simple drug detection and identification systems have been described, for example DETECHIP (Burks *et al*., 2010).

5.4 UV/Visible spectrophotometry

When a compound is irradiated with electromagnetic radiation of an appropriate wavelength it will absorb energy. This absorbed energy may be emitted as radiation at a less energetic (longer) wavelength (fluorescence or phosphorescence), dissipated as heat, or give rise to a photochemical reaction. Gamma- and X-rays occupy the short wavelength (high energy) region of the spectrum. Ultraviolet (UV), visible, infrared, and microwave radiations come next and finally, radio waves.

The absorption of the different types of radiation produces different effects. UV (10–400 nm) and visible (approximately 380–740 nm) light excite electrons from their ground state to higher energy (excited) states. Wavelengths (λ) of maximum absorbance are denoted λ_{max}. Infrared radiation (IR, approximately 700 nm to 1 mm) induces molecular (bond) vibrations, whilst microwaves (1–10^3 mm) are used to induce electron spin transformations in electron spin resonance spectroscopy (ESR). Nuclear magnetic resonance (NMR) spectroscopy uses wavelengths between those of radio and television waves to detect nuclear electron spin flips.

5.4.1 The Beer–Lambert law

In UV/visible spectrometry, the analyte absorbs some of the incident light energy as a result of electrons in molecules being excited to higher energy levels.

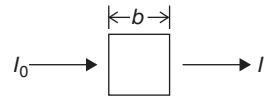

Table 5.2 Some colour tests used to identify drugs in suspect materials. See O'Neal *et al*. (2000) for full details. Reproduced with permission of Elsevier

Test	Reagent(s)	Procedure[a]	Target (colour)
1. Scott reagent	Dissolve 2.0 g cobalt(II) thiocyanate in 100 mL deionized water	–	Cocaine (strong greenish blue)
2. Dille–Koppanyi reagent, modified	A: Dissolve 0.1 g cobalt(II) acetate dihydrate in 100 mL methanol. Add 0.2 mL glacial acetic acid and mix B: Add 5 mL 2-propylamine to 95 mL methanol	Add two drops of A to the drug, followed by one drop of B	Barbiturates (light purple)
3. Duquenois–Levine reagent, modified	A: Add 2.5 mL acetaldehyde and 2.0 g vanillin to 100 mL 95 % v/v ethanol B: Concentrated hydrochloric acid C: Dichloromethane	Add three drops of A to the drug and shake (1 min). Then add three drops of B. Agitate gently, and note the colour produced. Add nine drops of C and note whether the colour is extracted from the mixture of A and B	Cannabis (THC) (purple/blue)
4. Mandelin reagent	Dissolve 1.0 g ammonium vanadate in 100 mL concentrated sulfuric acid	–	Opioids and other amines (yellow/orange/brown)
5. Marquis reagent	Carefully add 100 mL concentrated sulfuric acid to 5 mL 40 % v/v formaldehyde	–	Opioids and other amines (red/yellow/brown)
6. Nitric acid	Concentrated nitric acid	–	Opioids and other amines (green/orange/yellow)
7. *p*-Dimethylaminobenzaldehyde (*p*-DMAB)	Add 2.0 g *p*-DMAB to 50 mL 95 % v/v ethanol and 50 mL concentrated hydrochloric acid	–	LSD (deep purple)

(*continued overleaf*)

Table 5.2 (*Continued*)

Test	Reagent(s)	Procedure[a]	Target (colour)
8. Ferric chloride	Dissolve 2.0 g of anhydrous ferric chloride or 3.3 g of ferric chloride hexahydrate in 100 mL deionized water	–	Opioids and other amines (blue/green)
9. Froehde reagent	Dissolve 0.5 g molybdic acid or sodium molybdate in 100 mL hot concentrated sulfuric acid	–	Opioids and other amines (yellow/green/brown)
10. Mecke reagent	Dissolve 1.0 g selenious acid in 100 mL concentrated sulfuric acid	–	Opioids and other amines (Red/yellow/green)
11. Zwikker reagent	A: Dissolve 0.5 g copper(II) sulfate pentahydrate in 100 mL deionized water B: Add 5 mL pyridine to 95 mL chloroform	–	Barbiturates (light purple)
12. Simon's reagent	A: Dissolve 1 g sodium nitroprusside in 50 mL deionized water and add 2 mL acetaldehyde with thorough mixing B: 2 % aqueous w/v sodium carbonate	Add one drop of A to the drug, followed by two drops of B	Secondary amines such as metamfetamine and MDMA (deep blue)

[a]One or two drops of reagent(s) were added to the drug using a Pasteur pipette unless otherwise noted

If the incident light intensity is I_0 and the transmitted light energy is I, then transmission T is:

$$T = \frac{I}{I_0} \tag{5.1}$$

The law that governs the relationship between the intensity of light entering and leaving a cell is the Beer–Lambert Law. This states that, for a solution of an absorbing solute in a transparent solvent, the fraction of the incident light absorbed is proportional to the number of solute molecules in the light path (Beer's Law) and the path length (Lambert's Law), i.e.

$$\text{Absorbance} = \log\left(\frac{I_0}{I}\right) = \varepsilon cb \tag{5.2}$$

where I_0 is the incident light intensity, I is the transmitted light intensity, c the solute concentration (mol L^{-1}), ε is the molar absorptivity, previously known as the extinction coefficient (L cm^{-1} mol^{-1}), and b is the path length (cm).

The molar absorptivity is a fundamental property of the analyte, but it also depends on temperature, wavelength, and solvent. Absorbance (A) is linearly related to both solute concentration and path length for dilute solutions only. In older textbooks it was known as optical density (OD) or extinction (E), but these terms are now obsolete. The specific absorbance ($A_{1\%,\,1\,\text{cm}}$) is the absorbance of a 1 % (w/v) solution of the solute in a 1 cm path length cell, and is usually written in the shortened form A^1_1. There are a number of reasons why deviations from the Beer–Lambert Law may become apparent (Box 5.2).

Box 5.2 Beer–Lambert law: factors causing non-linearity

- Analyte concentration
  - At high concentrations (> 0.01 mol L^{-1}) electrostatic interactions between species reduces absorbance
  - Refractive index changes at high analyte concentration
- Interactions/decomposition
  - Analyte dissociates/associates or reacts with solvent
  - Particles in light path
  - Phosphorescence or fluorescence in the sample
  - Oxygen absorption becomes limiting at wavelengths below *ca*. 205 nm
- Instrumental
  - Polychromatic (not monochromatic) incident light. The incident slit is of finite width, and thus the incident light is of more than one wavelength. Because ε varies with wavelength, this leads to deviations in Beer's Law. For a given slit width the variation in ε will be greater on the slope of an absorption peak than at a maximum hence the effect is reduced by performing the measurement at λ_{max}
  - The effect of stray light from reflected radiation in the monochromator reaching the exit slit becomes more apparent at higher absorbance

5.4.2 *Instrumentation*

Spectrophotometers can be divided into two basic types: single beam or double beam. The simplest of the single-beam designs (Figure 5.1) are often referred to as colorimeters when their operation is limited to the visible region of the spectrum. The light source in such instruments is a tungsten filament lamp and the analytical wavelength is selected using a coloured filter.

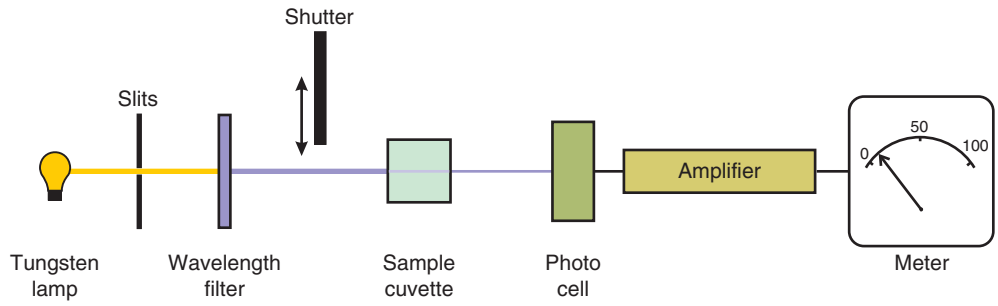

Figure 5.1 The basic components of a single-beam spectrophotometer

Box 5.3 Operation of a single-beam spectrophotometer

- Select the wavelength
- Close the shutter and adjust the meter to zero transmission
- Place the reference cuvette in the sample chamber and adjust the meter to 100 % transmission (zero absorbance)
- Replace the reference cuvette with the sample cuvette and record the absorbance

The light beam passes through the sample cuvette held in a light-tight sample chamber and the transmitted light intensity is measured with a photocell. After amplification the signal is monitored with a meter. Early colorimeters were calibrated in linear transmission, absorbance being superimposed as a logarithmic scale on the meter. Operation is simple (Box 5.3).

The introduction of hydrogen (later deuterium) lamps and monochromators (quartz prisms or diffraction gratings) extended the useable wavelength range into the UV region. Tungsten–halogen filament lamps, in which the presence of a small amount of iodine assists in redepositing vaporized tungsten onto the filament thus prolonging lamp life, are also useable into the far UV.

Single-beam spectrophotometers have the advantage of high energy and good signal-to-noise ratios, and are suitable for quantitative measurements at a single wavelength. Spectra can be obtained manually, but at each wavelength the zero and 100 % transmission must be set as detailed above, making the process tedious – the relatively complex absorption spectrum of benzene would take a skilled operator an entire day's concentrated effort to produce. Double-beam spectrometers overcome this problem by automatically compensating for differences in incident light intensities during the scan.

In optical-null instruments, the light beam is split into sample and reference beams by a rotating semicircular mirror (often referred to as the beam chopper or simply 'chopper'). The beams, which consist of pulses of light that are out of phase, are directed through either the sample or the reference compartments and then recombined before being directed to a single photomultiplier tube (Figure 5.2).

If the absorbance of the sample and reference cells are identical, the photomultiplier tube 'sees' a continuous beam of light. However, if the sample has a higher absorbance than the reference sample, the beam is no longer continuous, but stepped. The optical-null circuitry drives a servomotor that moves an attenuator (comb) into the reference beam until the signals are balanced and the movement of the attenuator is recorded as the spectrum. Wavelength accuracy is maintained by linking the recorder movement with the monochromator movement. At high

absorbance ($A = 2$), transmission is 1 % and little if any light reaches the detector, the recorder pen becomes 'dead', and no readings can be made.

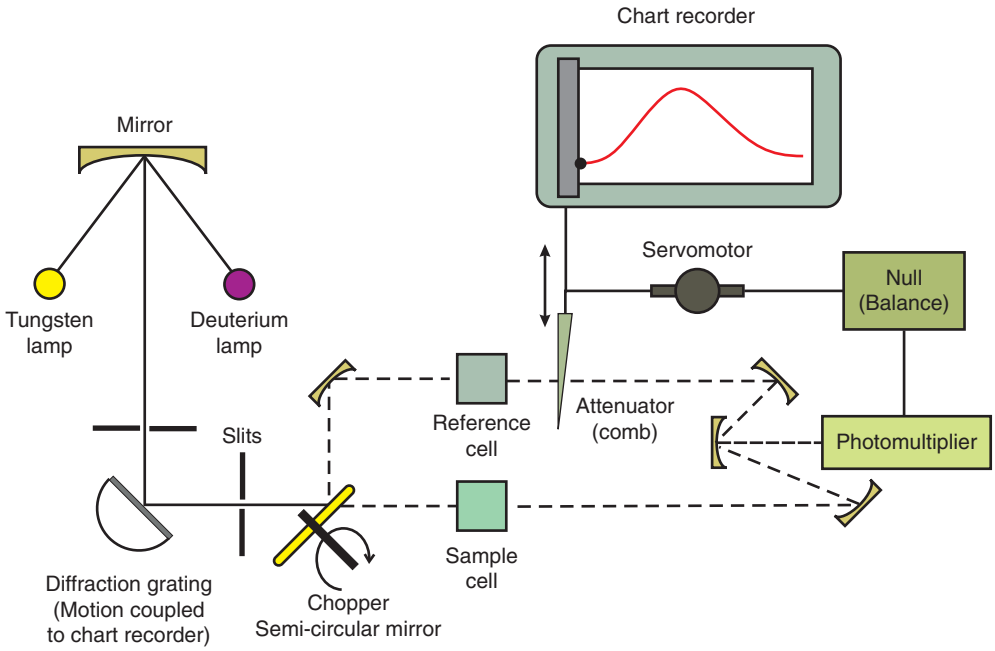

Figure 5.2 Diagram of a typical optical-null double-beam spectrophotometer

UV absorption by water limits the lowest wavelength that can be used with aqueous samples to *ca*. 195 nm. The presence of dissolved oxygen and solvents can raise the cut-off value even higher (Table 4.3). Lower wavelengths, down to 125 nm, a region of the electromagnetic spectrum giving excitation of all (including aliphatic) chemical bonds in an organic molecule, can be employed if analytes are measured in the gas phase (Zhang *et al*., 2019). This is the basis of vacuum UV (VUV) that finds use as a GC detector (Section 9.2.2.10).

Modern double-beam spectrophotometers use two photomultiplier tubes and ratio the signals. However, background electrical noise can be limiting at high absorbance values, so as with the optical-null instruments, no measurements can be made under these circumstances. Modern single-beam instruments use microprocessors so that background scans can be stored and subtracted from the sample data.

In a diode array detector (DAD, Figure 5.3), the detector is a linear array, typically 3–7 cm long, of over 1000 diodes (in modern instruments the wavelength resolution is of the order of 1 nm per diode, wavelength accuracy ±1 nm, and sensitivity better than 10^{-4} AU). Light from the source passes through the sample to a diffraction grating that disperses the beam onto the array and each diode records the absorbance at a defined wavelength. Thus, a spectrum is recorded almost instantaneously, which makes the diode array detector particularly useful for LC because several spectra can be scanned across an eluting peak.

With all types of spectrophotometer it is important to ensure that the monochromator is aligned correctly. This can be checked by observing the absorbance maxima (λ_{max}) of a known reference solution or material. A holmium oxide glass filter, for example, has major peaks at a number of important wavelengths (241.5, 279.4, 287.5, 287.5, 333.7, 360.9, 418.4, 453.2, 536.2,

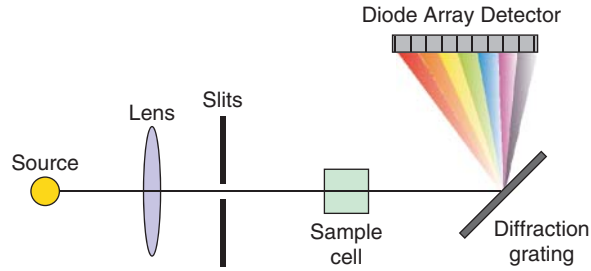

Figure 5.3 Diagram of a linear diode array detector

and 637.5 nm). A simple method of checking photometric accuracy is to measure the absorbance of an acidic potassium dichromate solution (Flanagan *et al.*, 1995).

The cuvettes used in the spectrophotometer must be of the correct specification and must be scrupulously clean. Traditionally, glass cells were used for measurements in the visible region (>400 nm), and fused silica or quartz cells were used for UV work (190–400 nm). However, disposable plastic cells are available for working from 220–900 nm. Normally, cells of 1 cm path length are employed, but 2 or 4 cm path length cells can sometimes be used to enhance sensitivity.

Cells for spectrophotometry usually have two optical faces and two ground or ribbed faces, and should be handled by the ground or ribbed faces only. The optical faces may be wiped with a soft cloth or photographic lens tissue if necessary. Samples should be introduced with the aid of a suitable pipette, taking care not to scratch the inside surface of the cell. Quartz cells should be marked, for example on a ground face, to ensure that they are always placed in the spectrophotometer in the same orientation. After use such cells should be cleaned carefully and allowed to dry.

Double-beam spectrophotometers have the advantage that 'background' absorbance from reagents, solvents, etc. can be allowed for by including a 'blank' extract in the reference position. Normally an extract of analyte-free plasma or serum will be used to provide the solution used in the reference cell, but purified water may be used in certain assays. A matched-pair of cells is necessary for use in double-beam instruments. When filled with identical solutions, the absorbance readings should be within ±1 %. Pairs of matched cells may be purchased and should be stored together in a dust-free environment.

5.4.2.1 Derivative spectrophotometry

Rather than plotting absorbance against wavelength, a plot of the first (or higher derivative) versus wavelength can reveal spectral detail that would otherwise be lost:

$$\frac{\mathrm{d}^n A}{\mathrm{d}\lambda^n} = \frac{\mathrm{d}^n \varepsilon}{\mathrm{d}\lambda^n}.c.b \tag{5.3}$$

where n is the order of the derivative [the other symbols are as defined for Equation (5.2)]. Because sensitivity is dependent on the rate of change of ε ($\mathrm{d}\varepsilon/\mathrm{d}\lambda$) rather than ε itself, the technique is suited to analytes with sharp absorbance peaks. Consequently, when the analyte signal is difficult to quantify because of interferences, as is often the case with extracts of biological samples, derivative spectrophotometry may be advantageous, particularly when the width of the overlapping peak is greater than twice the width of the analyte peak. Generally, the peak-to-peak heights are proportional to the concentration of the analyte. Note, however, that spectral information tends to degrade with increasing order of derivative, particularly when the

spectrum is poorly defined (noisy) to start with, and there is also an obvious increase in the cost of more complex instruments.

Derivative spectra can be obtained using electronic circuits to convert the analogue absorbance (zero-order) signal to the derivative of the appropriate order, or digital signals can be transformed mathematically. A third method for first-order spectra is to use wavelength modulation. Scanning the spectrum with alternate beams from two monochromators, with wavelengths set 2–3 nm apart, will produce something very close to the derivative spectrum (ΔA is approximately the slope at the particular wavelength). However, whilst derivative spectrophotometry may find the occasional niche application, it not been adopted widely in analytical toxicology largely because of the rapid growth of LC-MS.

5.4.3 Spectrophotometric assays

Spectrophotometry may be used in both qualitative and quantitative work (Box 5.4). However, one of the major problems in attempting to apply spectrophotometry *per se* to complex matrices is that it is not an inherently selective technique. Most endogenous compounds absorb in the UV region of the spectrum, if not in the visible part, and even those with low molar absorptivities may be present in such excess compared to the concentration of the analyte that selectivity and hence sensitivity is a problem.

Box 5.4 Visible and UV spectrophotometry

Visible spectrophotometry (colorimetry)

- Visible spectrometry was the first really reliable quantitative method used for drug/metabolite analysis in blood
- Aim is to perform chromogenic reaction directly in sample with minimum treatment
  - Can use protein precipitation or microdiffusion (Box 5.7)
- Problems:
  - Relatively few analytes undergo convenient chromogenic reactions
  - Relatively poor selectivity/sensitivity (interference from metabolites/other drugs)

UV spectrophotometry

- Double-beam spectrophotometers or linear diode array instruments give the most reliable results
- Can perform UV spectrometry directly on sample extracts
  - Care: use silica or appropriate plastic cuvettes
  - Generally poor selectivity
- Use of double-beam scanning instruments (210–400 nm) or linear diode arrays can aid in analyte identification
  - Many poisons have either a poorly defined UV absorption spectrum, or do not absorb in the UV, although compounds that have characteristic spectra can be readily identified
  - Always the likelihood of interference if more than one drug/metabolite present
  - Complex spectra may be resolved using derivative spectrophotometry
- UV spectrophotometry finds greatest application as a detector in LC, monitoring reaction rate in immunoassays, or in NAD/NADH- or NADP/NADPH-linked enzyme-based assays

Whilst spectrophotometric assays generally lack selectivity, some information as to the purity of a sample extract may often be obtained by examining the UV absorption spectrum using a scanning spectrophotometer; manual scanning can be performed on simpler instruments. UV spectra of extracts of urine, stomach contents, or scene residues can give useful qualitative information and can be used as an adjunct to more specific, and sensitive, drug screening procedures. UV absorption spectra of many compounds of interest are given in Moffat *et al*. (2011), but care is needed to ensure that the pH/solvent combination employed is the same as that used to produce the reference spectrum. Wavelength scanning at different pH values can be useful in identifying the constituents of tablets.

It may be possible with techniques such as protein precipitation, LLE, possibly with back-extraction at an appropriate pH, or microdiffusion to develop a spectrophotometric assay for a particular analyte in a particular biological fluid. Concentration of the analyte into a smaller volume during LLE or SPE, for example, may give adequate sensitivity provided there are no interferences. This being said, many spectrophotometric methods suffer from interference from metabolites to a greater or lesser extent. Other drugs are further potential sources of interference. These may have been either prescribed or obtained over-the-counter, and may be chemically unrelated to the analyte, although chemically related compounds and any metabolites are more likely to interfere. Compounds from the same pharmacological class as the analyte may be co-prescribed and may be chemically similar to the analyte hence metabolism, extraction, derivatization, and absorbance spectra are all likely to be similar.

Although spectrophotometric methods often suffer from a lack of selectivity when metabolites are present, this is not always the case. Of three spectrophotometric methods for paracetamol that were compared with LC, two methods that included a hydrolysis step (paracetamol is mainly metabolized by glucuronide and sulfate conjugation) overestimated the true concentration of parent drug by up to seven-fold. However, the third method, based on the relatively specific nitration of paracetamol, gave values that were in good agreement with LC. Thus, despite the cautions listed above, quantitative spectrophotometry can be used successfully when an analyte is present at relatively high concentration, when a characteristic colour reaction can be employed, or when the analyte can be isolated in relatively pure form prior to the analysis, as illustrated below.

5.4.3.1 Salicylates in plasma or urine

The principle of this test (Box 5.5) is the colour that often develops when phenols are treated with a ferric iron [iron(III)] salt. Different phenols give a range of colours, salicylate producing a purple-red colour. Plasma protein should be precipitated with mercuric chloride [mercury(II) chloride] so that the colour in the supernatant can be measured directly. The mercuric chloride may be omitted if only urine is to be assayed. Any interference from colour in the sample is reduced by the large dilution incorporated in the procedure.

5.4.3.2 Carboxyhaemoglobin in whole blood

The half-life of carboxyhaemoglobin (COHb) in blood is usually very short, especially if oxygen is given in treatment, so COHb assays are rarely indicated clinically. However, spectrophotometric COHb assay is simple if potentially interfering oxygenated haemoglobin is removed (Box 5.6). Haemoglobin is normally present in blood as deoxygenated (HHb) and oxygenated (O_2Hb) forms together with a small amount of methaemoglobin (MetHb) i.e. oxidized haemoglobin, in which the iron is present as iron(III) (Fe^{3+}). In the assay, oxyhaemoglobin, which has a spectrum similar to that of COHb, and MetHb are reduced to HHb with sodium

dithionite. COHb is unaffected and hence retains its characteristic spectrum that can be measured spectrophotometrically.

Box 5.5 Measurement of salicylates in plasma or urine

Reagents

- Trinder's Reagent [mercuric chloride (10 g) + 1 mol L^{-1} hydrochloric acid (12 mL) + ferric nitrate nonohydrate [$Fe(NO_3)_3.9H_2O$] (4 g) in 200 mL deionized water]
- Calibration solutions containing salicylic acid (0, 200, 400, and 800 mg L^{-1}) in deionized water. Store at 4 °C

Method

- Add Trinder's Reagent (5 mL) to plasma/serum or calibration solution (1 mL)
- Vortex-mix (30 s) and centrifuge (3000 rpm, 10 min)
- Measure the absorbance of the supernatant layer (double-beam spectrophotometer, 540 nm) against plasma 'blank'
- Construct graph of absorbance versus salicylate concentration in the calibrators, and calculate the plasma salicylate concentration
- Limit of quantification: 50 mg L^{-1} salicylate

Box 5.6 Measurement of carboxyhaemoglobin

Method

- Add 25 mL 0.1 % v/v ammonium hydroxide to 0.2 mL blood
  - Portion A – saturate with pure CO (needs a cylinder of CO)
  - Portion B – saturate with oxygen to dissociate COHb (needs a supply of compressed air or oxygen)
  - Portion C – test sample
- Add sodium dithionite, mix and read absorbance at 500–600 nm versus reagent blank
- Measure absorbance at 540 (COHb max) and 579 nm (isosbestic point) and calculate the ratio A_{540}/A_{579} for A, B, and C
- Calculate % COHb saturation

Absorbance readings are made at 497 nm (the isosbestic point, the wavelength at which both HHb and COHb have the same absorbance) and 540 nm, the wavelength where the difference in absorbance, $A_{COHb} - A_{HHb}$, is greatest (Figure 5.4). Note how the 100 and 0 % COHb values are obtained by treating portions of the sample with either carbon monoxide, or oxygen (compressed air), respectively. Commercial systems such as the IL 682 Co-Oximeter are available that measure total haemoglobin (tHb), metHb, and other haemoglobin species as well as COHb, and aim to compensate for the presence of sulfhaemoglobin in blood obtained post-mortem.

5.4.3.3 Cyanide in whole blood by microdiffusion

Microdiffusion (Box 5.7) is a form of sample purification that relies on the liberation of a volatile compound, e.g. hydrogen cyanide in the case of cyanide salts, from the test solution held in one

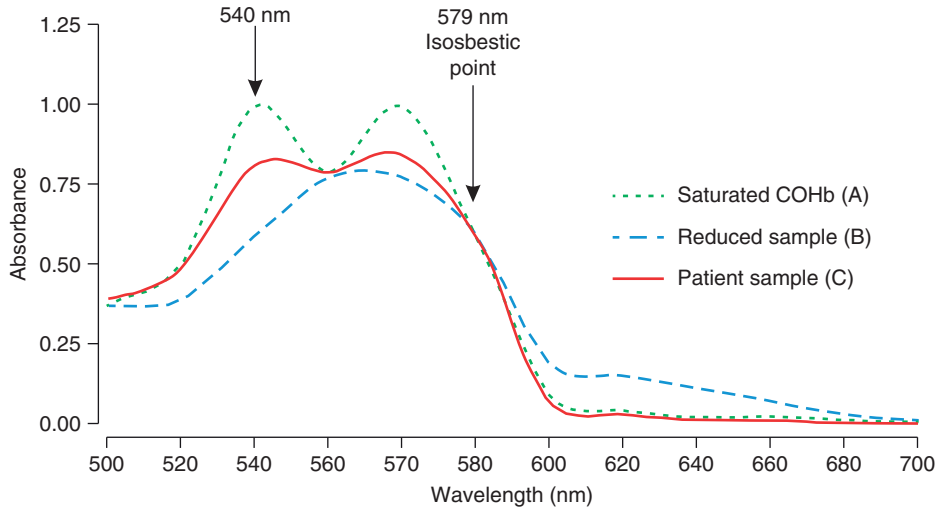

Figure 5.4 Measurement of carboxyhaemoglobin (note the wavelength expansion at 560–580 nm)

compartment of an enclosed system. The volatile compound is subsequently 'trapped' using an appropriate reagent (sodium hydroxide solution in the case of hydrogen cyanide) held in a separate compartment of the specially constructed (Conway) apparatus [Figure 5.5(a)].

The cells are normally allowed to stand for 2–5 h (room temperature) for the diffusion process to be completed, but sometimes shorter incubation times can be used. The analyte concentration is subsequently measured in a portion of the 'trapping' solution either spectrophotometrically or by visual comparison with standard solutions analyzed concurrently in separate cells. The Conway apparatus may be made from glass, but polycarbonate must be used with fluorides because hydrogen fluoride etches glass. Lightly smearing the cover with petroleum or silicone grease ensures an air-tight seal. In order to carry out a quantitative assay at least eight cells are needed: 'blank' sample, a minimum of three calibrators, test sample (in duplicate), and positive control sample (in duplicate). It is important to clean the diffusion apparatus carefully after use.

Cyanide ion is present in blood mainly in erythrocytes hence whole blood is used in cyanide assay, but care has to be taken to avoid interference from thiocyanate present largely in plasma.

Box 5.7 Microdiffusion

- Simple means of purifying volatile analytes prior to colorimetric analysis
- Enclosed system: sample/standard and 'releasing' reagent added to opposite sides of outer well; 'trapping' reagent added to inner well
- Contents of outer well mixed – released volatile product allowed to diffuse into 'trapping' solution
- Principal disadvantages:
  - Diffusion cells may be difficult to obtain
  - Time taken for diffusion to complete
  - Limited to volatile analytes (room temperature)

Cyanide ion is non-volatile at room temperature, but is converted into volatile hydrogen cyanide under acidic conditions; thiocyanate is unaffected.

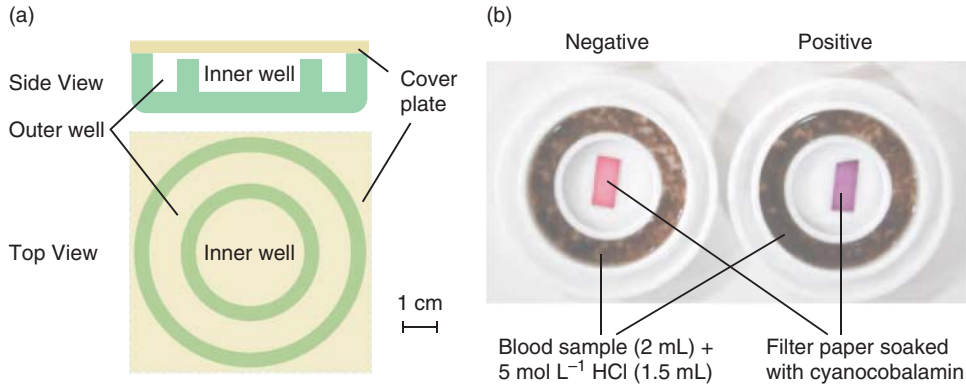

Figure 5.5 Conway microdiffusion. (a) Apparatus. (b) Qualitative detection of cyanide (Chaudhary *et al*., 2016–reproduced with permission of Taylor & Francis)

If acidification of a biological sample such as blood containing cyanide is performed in a Conway cell, the hydrogen cyanide that is released can be trapped in alkali as cyanide ion in the centre well. Reaction with 3-nitrobenzaldehyde/1,2-dinitrobenzene gives a coloured product that can be removed from the cell and the absorbance measured using the mixture from the 'blank' analysis in the reference position (Box 5.8). Assay calibration is by analysis of standard sodium or potassium cyanide solutions analyzed in separate cells. A simple modification of the method using filter paper impregnated with cyanocobalamin has been described [Chaudhary *et al*., 2016; Figure 5.5(b)].

Box 5.8 Cyanide measurement using microdiffusion

Method

- Place 3.6 mol L^{-1} sulfuric acid (1 mL) in Conway outer well
- Place sample, blank, or standard (0.5 mL) at opposite side of outer well
- Place 0.5 mL of 3-nitrobenzaldehyde solution + 0.5 mL of 1,2-dinitrobenzene solution (both 0.05 mol L^{-1} in 2-methoxyethanol) + 0.1 mL aqueous sodium hydroxide (0.5 mol L^{-1}) in the centre well
- Seal the cell, gently mix the contents of the outer well, and allow to stand (20 min, room temperature)
- Add 1 mL aqueous methanol (1+1) to the centre well, transfer contents to spectrophotometer cells, and measure absorbance (560 nm)

5.5 Fluorescence and phosphorescence

When molecules absorb light, the energy from the light is transferred to the electrons in the molecules so that the molecules are promoted from the lower energy, ground state, to a higher energy, excited state. The molecules usually remain in the excited state for a very short period of

time before they return to the ground state. When the molecules return to the lower energy state, they must lose energy. Most molecules lose this energy in the form of molecular vibration by dissipation as heat, but some molecules lose some of this energy via the emission of light. The emitted light is usually of a longer (less energetic) wavelength than that of the absorbed light. This is because excited molecules lose some of the absorbed energy by radiationless transitions, such as collisions with other molecules, or the electrons return to higher energy levels in the ground state, and so the energy of the emitted light is correspondingly less.

Fluorescence is the term used when light is emitted almost instantaneously, usually within 10^{-9} or 10^{-12} s of the energy being absorbed. *Resonance fluorescence* describes the situation when the frequency of the emitted light is the same as the absorbed light.

Phosphorescence occurs when an excited electron in the singlet state (paired electrons having opposite spin) crosses to a triplet state (paired electrons having the same spin). These transitions are known as 'forbidden transitions' because their probability is so low. The electron must return to the excited singlet state before it can return to the singlet ground state and emits light as it does so, and thus phosphorescence is much longer-lived than fluorescence, usually of the order of several seconds. Phosphorescence assays are not widely used in analytical toxicology, although a kinetic phosphorescence assay has been reported for uranium (Section 21.5).

5.5.1 Intensity of fluorescence and quantum yield

The fraction of excited molecules that emit light is the *quantum yield* or *quantum efficiency* (ϕ) of fluorescence. Highly fluorescent molecules will have quantum yields approaching 1. For dilute solutions, the intensity of the fluorescence, I_f, is a function of ϕ, the absorbance, and the intensity of the incident light, I_0. The absorbance is given by Beer's Law so:

$$I_f = 2.3 I_0(\varepsilon c b)\phi \tag{5.4}$$

From Equation (5.4), it is clear that the size of the signal is proportional to the intensity of the incident light as well as the concentration of the analyte, c, and as a consequence, the fluorescence, unlike absorbance, is 'relative' and not absolute. Thus, fluorescence is reported as 'relative intensity' or 'fluorescence (arbitrary units)'. This means that the concentration of an analyte cannot be calculated from the fluorescent intensity in the way that it can be calculated from absorbance and molar absorptivity in spectrophotometry. Analyte sample concentrations must always be obtained by external calibration (Section 3.3.3).

Equation (5.4) only holds true for dilute solutions. Quenching, caused by absorption of the incident or emitted light by other compounds, or by the analyte itself (internal absorption), reduces fluorescent efficiency, as does quenching caused by molecular interactions (collisions) leading to non-radiative relaxation of excited molecules to the ground state. A very important consequence of quenching is that as the concentration of analyte is increased the intensity of the fluorescence will reach a maximum and then decline. Thus, it is possible for two samples, one dilute and one concentrated, to show the same degree of fluorescence. If quenching is suspected, the sample should be diluted with analyte-free matrix and re-assayed.

Fluorescence is usually reduced at increased temperatures (increased number of collisions) and is often pH dependent. The ionized and non-ionized form of a weak electrolyte may both be fluorescent, but it is rare for both species to have the same quantum efficiency. Barbiturates, for example, fluoresce in 0.1 mol L^{-1} sodium hydroxide. At this pH these compounds are ionized and have strong absorption at approximately 255 nm. Phenols such as catechol, however, fluoresce when unionized, i.e. under acidic conditions.

5.5.2 *Instrumentation*

Although in fluorescence the emitted light is non-directional, it is measured at right angles to the excitation beam to minimize the amount of incident light reaching the detector (Figure 5.6). Some instruments have both excitation and emission filters, or the instrument may have a combination of a diffraction grating and a filter, or, as in the more expensive instruments, there may be two diffraction gratings.

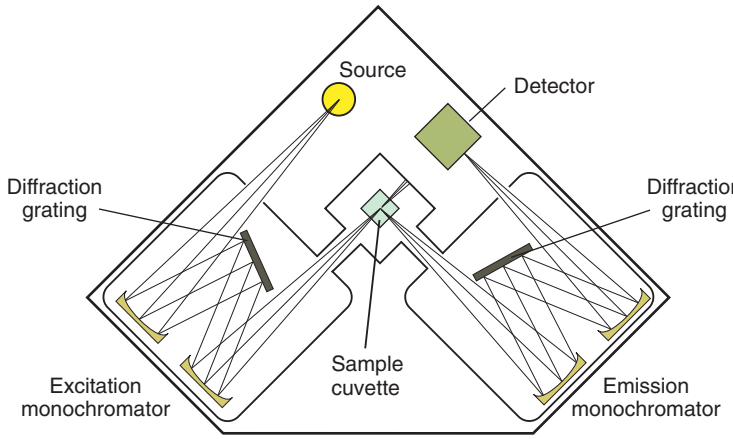

Figure 5.6 Diagram of a double-grating spectrophotofluorimeter (based on the AMINCO-Bowman design)

Xenon lamps are popular because they have high energy output between 350 and 1000 nm and so are useful general purpose light sources. Xenon lamps can be used down to 200 nm, but the light intensity is about an order of magnitude lower at this wavelength and for some applications, a deuterium lamp, or even a mercury lamp when the intense emission line at 254 nm can be used, may be better. Halogen lamps may be used for excitation in the visible region. Because the intensity of the fluorescence is dependent on the intensity of the incident light [Equation (5.4)] it is important in any report to state the type of lamp used. High intensity xenon lamps may be pulsed to reduce the risk of photodecomposition of an analyte.

Sample cuvettes may be square or circular in cross-section. The square ones have four optical faces and so these should be handled carefully by the top and not where the light beams pass through them. Although sample holders are often designed to take 1 cm square cuvettes, the area over which the measurements are made is defined by the width of the emission slits, and smaller cuvettes may be used without any loss of fluorescent signal; indeed smaller path length cells have the advantage that there will be less loss of light due to absorption.

Because the incident light intensity is generally much higher than the intensity of the emitted light, scattering of light from particles in the sample solution can affect assay sensitivity markedly. Thus, good analytical technique is important in high sensitivity fluorescence assays. Also, the more similar the excitation and emission wavelengths, the more likely it is that incident light will interfere with the emission signal. The use of dual diffraction gratings and narrow slit widths will reduce this problem.

An indication of the excitation wavelength for a particular analyte can be deduced from the UV spectrum. If the excitation wavelength is set to an absorption peak then the emission

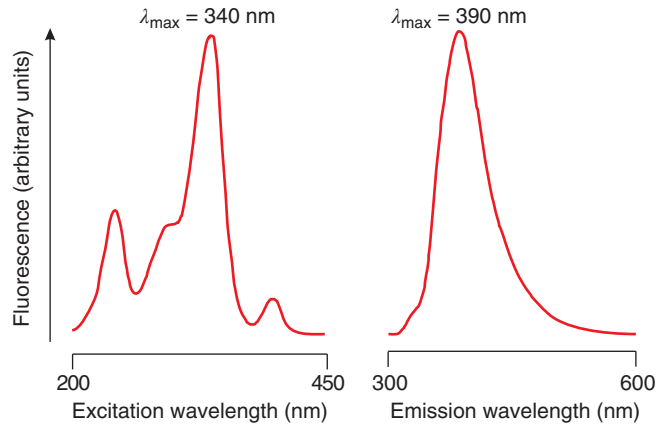

Figure 5.7 Excitation and emission spectra of quinine (1 mg L^{-1}) in deionized water

wavelength can be scanned until the emission maximum is found. With the emission monochromator set to its λ_{max}, the excitation monochromator can then be scanned to locate the exact excitation λ_{max}. The excitation and emission spectra will now be maximal and equal (Figure 5.7). The difference between the excitation and emission wavelengths is known as the Stokes shift.

5.5.3 Fluorescence assays

Fluorimetry gives enhanced sensitivity over spectrophotometric methods for those drugs that are naturally fluorescent. Generally, sensitivity is increased by 1 or 2 orders of magnitude because (i) only a limited number of drugs and other poisons possess natural fluorescence, and (ii) both excitation and emission wavelengths can be varied. High quantum efficiencies are associated with compounds that have low energy $\pi^* \rightarrow \pi$ transitions – these include conjugated aromatic compounds (generally the more rigid the structure, the greater the fluorescence). The substitution of an alkyloxy moiety on an aromatic ring usually gives rise to fluorescent properties. Substitution of electron-withdrawing groups (carbonyl, carboxylate, nitro), however, usually reduces the intensity of any fluorescence. Drugs with good fluorophores include several antimalarials and tetracycline antibiotics, propranolol, and alkaloids such as ergometrine, LSD, and physostigmine (Figure 5.8).

If an analyte does not possess native fluorescence, derivatization to a fluorescent product may be possible if the analyte possesses a reactive (functional) group amenable to chemical reaction. A number of fluorogenic reagents are available, including dansyl chloride and fluorescamine. 1,2-Phthalic dicarboxaldehyde (*o*-phthaldialdhyde, OPA) is particularly useful for primary amines and has been used to enhance detectability in LC (Section 10.7.1).

5.5.3.1 Fluorescence measurement of quinine

Quinine and its stereoisomer quinidine exhibit strong natural fluorescence under neutral or acidic conditions. Both drugs are relatively lipophilic and thus solvent extraction can be used to separate the parent compounds from fluorescent polar metabolites, notably the 3-hydroxy metabolite, and this forms the basis of sensitive fluorescence assays such as that for plasma quinine (Box 5.9). However, commercial quinine and quinidine can contain up to 10 % w/w of the respective dihydro derivatives, which have similar extraction and fluorescence properties to the

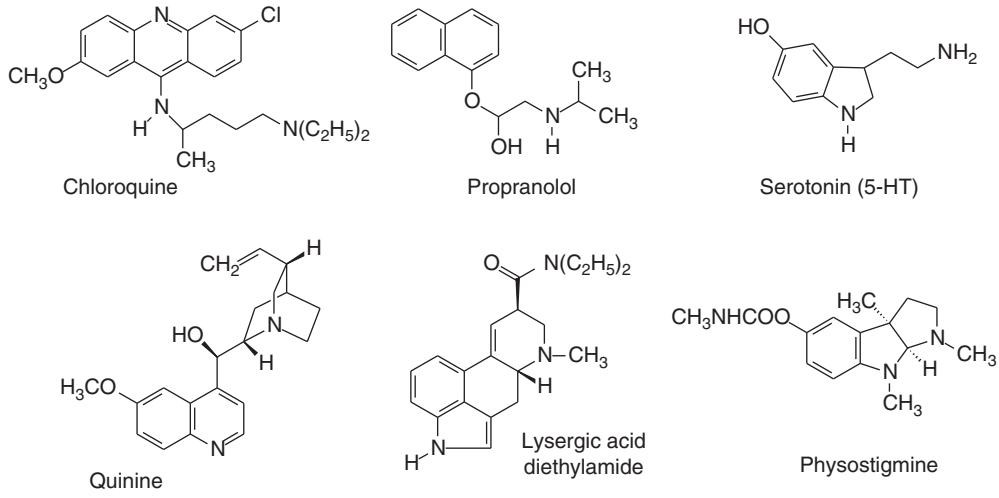

Figure 5.8 Some compounds that exhibit natural fluorescence

Box 5.9 Fluorescence assay for plasma quinine

Method

- Add analyte-free plasma, sample, or standard solution (0.5 mL) to a glass centrifuge tube, and add 0.1 mol L^{-1} sodium hydroxide solution (1 mL) and toluene (5 mL)
- Rotary mix (5 min) and centrifuge (3000 rpm, 10 min; bench centrifuge)
- Transfer 4 mL supernatant to a second glass tube and add 5 mL sulfuric acid (0.05 mol L^{-1})
- Rotary mix (5 min) and centrifuge (3000 rpm, 10 min; bench centrifuge)
- Discard the toluene layer (supernatant) and measure by spectrophotofluorimetry (excitation 350 nm, emission 450 nm)

parent compounds, and thus even the combination of solvent extraction and spectrophotofluorimetry is not totally selective in this case. A chromatographic method such as LC with fluorescence detection is thus needed to resolve the dihydro analogues of quinine and quinidine and permit selective measurement.

5.6 Chemiluminescence

Firefly luciferase, an enzyme from the North American firefly, *Photinus pyralis,* which is commonly used in laboratory work, catalyzes the oxidation of (*S*)-luciferin with hydrolysis of ATP and emission of light. When the quantity of ATP is limiting, the light emitted is proportional to the amount of ATP present and this forms the basis of chemiluminescent ATP assays. Commonly, it is oxidation of a suitable species that produces an excited state that decays to the ground state with emission of light.

Although chemiluminescence assays are not as widely applicable as spectrophotometric methods, the much-increased sensitivity (10–100 times) over other luminescence methods,

and the simple instrumentation required, make them attractive when high sensitivity is needed (Seidel & Niessner, 2014). Such assays are being used to increase the sensitivities of immunoassays (Chapter 6). Chemiluminescence LC detectors are also available (Section 10.3.3). In addition, chemiluminescence is important in specific detectors such as the flame photometric detector for GC (Section 9.2.2.6) for the detection of sulfur and phosphorus, and the chemiluminescent nitrogen detector for LC (Section 10.3.5).

Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) is often used in chemiluminescent assays as it can be oxidized by perborate, permanganate, hypochlorite, and iodine. However, the most useful reaction is that with hydrogen peroxide (Figure 5.9). When excited 3-aminophthalate decays it emits intense blue light (425 nm). Several enzyme-catalyzed reactions produce hydrogen peroxide and so luminol chemiluminescence can be used to monitor such reactions, including enzyme immunoassays. The reaction can be catalyzed by metals, including iron in haemoglobin, and this forms the basis of using luminol/hydrogen peroxide spray to detect blood stains.

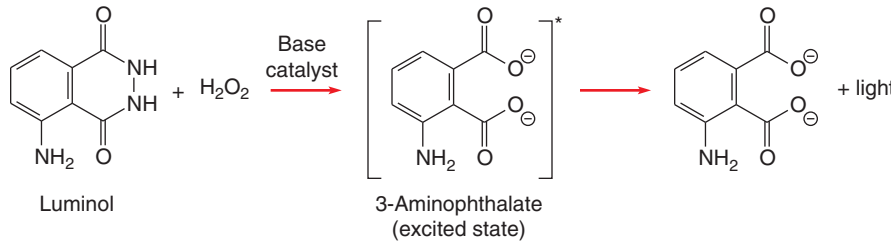

Figure 5.9 Chemiluminescent reaction of luminol with hydrogen peroxide

The luminol system can be used to quantify those ions that catalyze the reaction (for example Fe^{2+} and Cu^{2+}) as the luminescence is proportional to the concentration of the ions. Enhancers such as *p*-iodophenol can increase the light intensity by as much as 2500 times and the light is emitted as a prolonged glow, facilitating measurements, which may be made several minutes after the reaction has been initiated.

Chemiluminescent reactions can be direct as illustrated by luminol, or indirect via an energy transfer system as exemplified by peroxyoxalate derivatives. Oxidation of peroxyoxalate produces excited intermediates, such as 1,2-dioxetanedione (Figure 5.10), that react with a fluorophore that may be the analyte or a fluorescent derivative of the analyte.

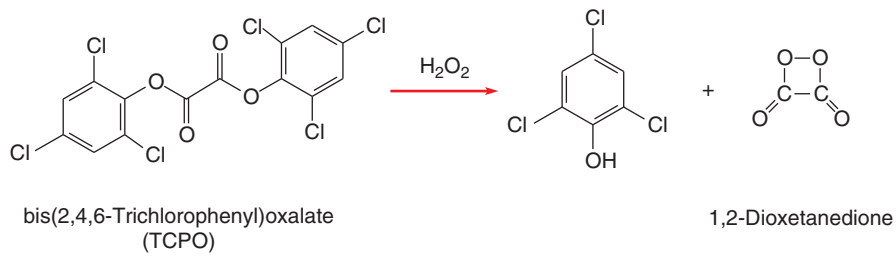

Figure 5.10 Hydrogen peroxide oxidation of TCPO

Electrochemiluminescence represents a further refinement that is being used increasingly, particularly with the tris(2,2′-bipyridyl)–ruthenium(III) system (Li *et al*., 2003). Reduction gives an excited complex that decays with the emission of light:

$$Ru(bpy)_3^{3+} + \text{reductant} \longrightarrow [Ru(bpy)_3^{2+}]^* \longrightarrow Ru(bpy)_3^{2+} + h\nu$$

where bpy = 2,2′-bipyridine. Lledo-Fernandez *et al*. (2014) claimed a sensitivity of 50 μg L^{-1} flunitrazepam in pH 4 buffer in such a system. A simple and selective on-chip chemiluminescence assay using Ce(IV) to oxidize $Ru(bpy)_3^{2+}$ in order to measure hydrochlorothiazide in plasma (limit of sensitivity 0.5 μg L^{-1}) has also been reported (Kadavilpparampu *et al*., 2017).

5.6.1 Instrumentation

A basic luminometer is very simple, consisting of a light-tight box in which to place the sample tube and a photomultiplier tube (Figure 5.11). There is no external light source and no need for filters or monochromators, although these may be included in more complex instruments. Refinements might include a mirror behind the sample tube holder, an injection port so that reagents to start the reaction may be added, and a magnetic stirrer. Replacing the sample tube with a coil of PTFE tubing in front of the photomultiplier tube converts the basic system into one that can be used either for FIA, or as an LC detector.

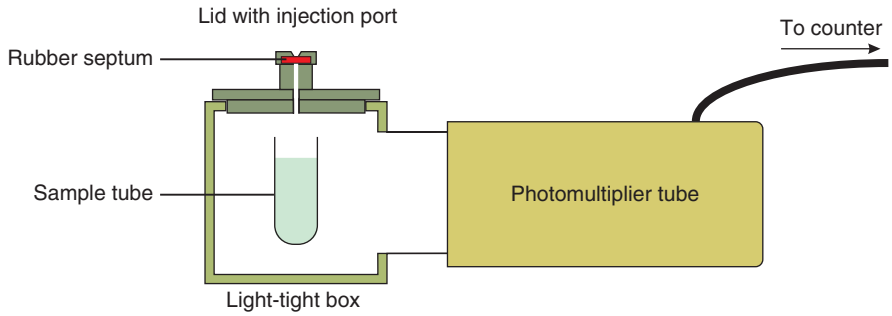

Figure 5.11 A simple luminometer

Each chemical event emits one photon of light. During the course of the reaction the photomultiplier tube and electronic circuitry ‘count’ the number of flashes and so the output is a numerical count, although with some instruments the signal can be integrated to give a curve in a manner analogous to LC radioactivity detectors. Luminometers that will read 96-well format microplates are available. Because the instrumental requirements are so simple, portable versions are also available for use in environmental applications, for example.

5.6.2 Chemiluminescence assays

To improve selectivity, chemiluminescent assays are usually combined with a separation technique such as chromatography or CE, or with an immunoassay. Enzyme reactions have also been used for this purpose. The acetylcholine assay in which acetylcholine is hydrolyzed to choline with acetylcholinesterase and the choline oxidized to betaine and hydrogen peroxide, with the

latter being measured via the luminol system described above, is an example. Several variations of this approach have appeared including construction of immobilized enzyme reactors for LC detection of acetylcholine. Although these can be used with luminol and a chemiluminescence detector, electrochemical detection of the hydrogen peroxide at platinum electrodes is a viable alternative.

As an example of the sensitivity that can be achieved, chemiluminescence detection has been used for the simultaneous measurement of promethazine, chlorpromazine, acepromazine, perphenazine, clonazepam, diazepam, nitrazepam, oxazepam, and estazolam added to pig urine. Two MIPs were coated in different wells of a conventional 96-well microtitre plate as the recognition reagents. After sample loading, the absorbed analytes were detected by using an imidazole enhanced bis(2,4,6-trichlorophenyl)oxalate–hydrogen peroxide system. The analysis time was 10 min. The limits of detection were in a range of 0.1 to 0.6 fg L^{-1}, and the recoveries from fortified 'blank' urine samples were in a range 80–95 %. Furthermore, the sensor could be reused six times (Xia *et al*., 2018). However, the practical use of the system is as yet untried as most of these drugs are excreted as metabolites in urine.

5.7 Infrared and Raman spectroscopy

Absorption of infrared (IR) radiation as a result of the bending or stretching of chemical bonds in a molecule allows identification of the types of bond present and hence provides information of the functional groups present. A spectrum may be plotted as absorbance versus wavelength. However, with IR, transmission [Equation (5.1)] may be plotted against either wavelength, or 'wavenumber', where wavenumber (ν) is the number of waves than can be accommodated in a length of 1 cm (there are other definitions). Therefore, if $\lambda = 1$ μm, $\nu = 10{,}000\,\text{cm}^{-1}$. For example, the major absorption in acetone as a result of stretching of the C=O double bond is at 5.83 μm which equates to 1715 cm^{-1}. For compound identification, IR peaks in what is known as the 'fingerprint' region of the spectrum (500–1450 cm^{-1}) are usually compared with those of known compounds via a suitable database such as that available from BioRad (www.bio-rad.com/en-uk/product/ir-spectral-databases?ID=N0ZXNZE8Z).

In Raman spectroscopy monochromatic radiation, usually from a laser source in the visible, near IR, or near UV part of the electromagnetic spectrum, excites bonds in sample molecules to higher vibrational energy states. The energy is lost as low intensity scattered light that can be measured, provided that the scattered incident laser light (Rayleigh scattering) is suitably filtered. When the wavelength of the Raman scattered light is longer than that of the incident light, this is referred to a positive Stokes shift. However, the wavelength of the emitted light may be shorter (more energetic) than the incident light and the difference is known as an anti-Stokes shift.

5.7.1 Instrumentation

The basic designs of IR spectrometers are similar to those of UV/Vis spectrophotometers (Figure 5.2), but with an IR source and a detector that is responsive to changes in temperature such as a thermocouple. Early monochromators used rock salt (NaCl) prisms, rather than the quartz ones used for UV/Vis, but more usually, diffraction gratings are used. The sample compartment does not have to be light-tight. Qualitative investigations of non-aqueous samples can be performed with a drop of the liquid sample between two rock salt plates, or liquid cells of known path length may be used for quantitative work. The spectra of the surfaces of solids can be

obtained using attenuated total reflection (ATR), for example human hair has been differentiated from that of other animals (Manheim *et al*., 2016).

Several lasers, including HeAg (224 nm), diode-pumped solid state (532 nm), HeNe (633 nm), and semiconductor (785 nm), are available for irradiation of samples in Raman spectroscopy. Raman radiation is usually measured at right angles to the incident beam and a narrow-band stop filter, known as a notch filter, is used to remove most of the scattered laser light but allow the weak Raman radiation to reach the spectrometer (Figure 5.12). A charge coupled device (CCD) that has light sensitive areas (pixels) is used as the detector. The spectrum may be plotted as the *shift* in wavelength or wavenumber relative to that of the laser light.

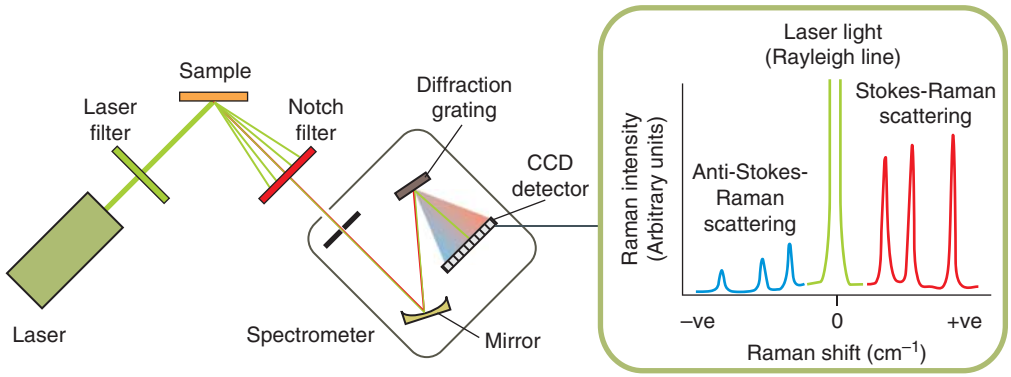

Figure 5.12 Schematic diagram of laser Raman spectroscopy

5.7.2 *Applications*

Laser Raman is particularly suitable for sampling solid materials and surfaces, and the availability of handheld devices allows point of contact testing (POCT, Chapter 17) of seized tablets and powders in the case of suspected illicit drugs or explosives (de Oliveria Pendio *et al*., 2016). The use of laser Raman spectroscopy for solutions is exemplified by its use to detect flunitrazepam in alcoholic beverages (Ali & Edwards, 2017).

In surface-enhanced Raman spectroscopy (SERS), the intensity of the Raman signal is enhanced (10^4–10^{10} times) upon intensification of an electric field at the surface of a nanometallic structure, typically gold or silver nanoparticles. Ben-Jaber *et al*. (2017) found silver nanoparticles to be superior to gold ones for detecting vapour phase concentrations of explosives down to femtomolar concentrations – 10^{-15} mol L^{-1} (182 fg L^{-1}) for 2,4-dinitrotoluene and 10^{-9} mol L^{-1} (222 ng L^{-1}) for RDX (1,3,5-trinitro-1,3,5,-triazine).

SERS has been used to measure low concentrations of fentanyl and carfentanil (Leonard *et al*., 2017). Cocaine in oral fluid has been quantified (LoD 25 µg L^{-1}) using the 1002 cm^{-1} Raman peak. After SPE, the reconstituted extracts were drawn into gold-sol capillary tubes for SERS (Dana *et al*., 2015). The same group (Farquharson *et al*., 2017) developed a method for the rapid identification of buprenorphine in oral fluid. Samples were extracted with dichloromethane, and the dried residues reconstituted with gold colloid and deposited onto a glass microfibre filter attached to a glass slide. The slides were scanned with laser light at 785 nm enabling buprenorphine to be identified from its Raman spectrum. To avoid the need for sample pre-treatment, lateral flow strips (Section 17.3.2) that incorporate a pad impregnated with gold nanoparticles

are being developed. The approach, which has been applied to whole blood, plasma, and oral fluid samples containing codeine and fentanyl (Shende *et al*., 2019), offers a potential advantage over immunoassay detection in that analyte identity can be confirmed from the spectral peaks, and possibly other drugs in the sample identified.

5.8 Summary

UV/Vis spectrophotometry, spectrophotofluorimetry, and chemiluminescence are valuable techniques in analytical toxicology, both in their own right and when used in conjunction with other techniques such as LC and immunoassay. Modern microprocessor-controlled spectrophotometers offer many advantages over older single- and double-beam instruments. These include not only increased stability and sensitivity, but also background correction to facilitate spectral scanning. Nevertheless, spectral scanning has been largely superseded in STA by GC-MS and LC-MS. With the exception of the urine tests for trichloro-compounds, paracetamol, and paraquat, colour tests too have been largely superseded, although such tests may still be valuable if used with due caution when other methods are not available.

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6 Immunoassays and Related Assays

6.1 Introduction

Immunoassays are competitive binding assays (ligand binding assays) that depend on the principle of competition between antigen and labelled antigen for binding sites on an antibody raised against the antigen. The proportionality of the resulting signal reflects the concentration of the antigen (analyte) in the sample. However, immunoassays are susceptible to a range of interferences that can include cross-reacting substances, heterophile antibodies (antibodies produced by external antigens), autoantibodies (antibodies produced against one or more of an individual's own proteins), and the high dose hook effect, i.e. when a high analyte concentration is associated with a anomalously low response (Honour, 2015; Ward *et al*., 2017).

Immunoassays may be classified on the basis of the need to separate the bound and free antigen prior to measuring the signal. Heterogeneous assays require separation of the antibody–antigen complex from the unbound antigen prior to signal measurement, whereas homogeneous assays do not. Homogenous assays, although technically simpler to perform, may suffer deleterious effects from the presence of other components of the sample (matrix effects). Nevertheless, many homogeneous immunoassays have been successfully automated and are in use in a number of different high and low throughput clinical chemistry systems.

Some practical aspects of the use of immunoassays in analytical toxicology are summarized in Box 6.1. The advent of therapeutic antibodies such as infliximab (M_r = 144,190) that pose difficulties in analysis by methods such as LC-MS (normally used for small molecules) on the one hand, and may generate anti-drug antibodies *in vivo* on the other, has given renewed importance to the development and understanding of immunoassays (Kim *et al*., 2015).

6.2 Basic principles of competitive binding assays

Immunoassays such as radioimmunoassay (RIA) are assays in which the analyte and a second molecule, the label, compete for binding sites on an antibody that has been raised against the analyte. The label is a molecule that can be quantified by counting radioactivity, by a change in spectral properties, or via an enzyme reaction in which the rate of the reaction is monitored. Because the number of binding sites is finite, competitive binding assays produce non-linear calibration curves and so have defined working ranges. The use of antibodies, rather than some other macromolecule, to which analyte and label bind, increases the selectivity of the test (Section 6.2.3).

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

Box 6.1 Immunoassays: practicalities

General

- Have to use confirmatory techniques to be certain of analyte identification (e.g. for medico-legal purposes)
- In practice need a commercial assay

Therapeutic drugs

- Many drugs (e.g. cardioactives, psychoactives) have active metabolites: need a separate antibody for separate assay or carefully crafted antibodies to measure both if appropriate
- Cannot raise antibodies to some drugs (lithium) or selective antibodies to others (e.g. amiodarone, cross-reacts with thyroxine)
- Insulin – variable cross-reactivity to insulin analogues used in therapy
- Some drugs are themselves antibodies that may provoke the formation of anti-drug antibodies *in vivo* that interfere in the analysis

Substance misuse

- Have to do separate test for each drug/drug group
- Need separate assays for metabolites (EDDP, norbuprenorphine)
- Amfetamine, benzodiazepine, and opiate assays poorly selective – need confirmatory techniques

Clinical toxicology

- Cannot raise antibodies to some inorganic or very small organic poisons (carbon monoxide, cyanide ion, ethanol, ethylene glycol)
- Condition of sample may affect result due to non-specific binding (matrix effects) or the presence of heterophilic antibodies, but may be able to minimize interferences by LLE, for example

RIAs are heterogeneous assays in which the analyte and label have to be separated. This step is avoided in homogenous assays such as enzyme-multiplied immunoassay technique (EMIT), although separation steps such as LLE may be used prior to the immunoassay to enhance either selectivity or reliability (Section 6.4.1).

6.2.1 Antibody formation

Many drugs are relatively small molecules and are therefore not inherently immunogenic. Hence immunogenicity has to be induced by conjugating the analyte, or a derivative of the analyte, the hapten, to a protein such as bovine serum albumin (BSA) (Figure 6.1). Anti-analyte antibodies can then usually be produced by injecting the modified protein into an animal. Usually rabbit, goat, or sheep, rather than rat or mouse, are used because relatively large amounts of blood need to be taken repeatedly to harvest antibodies. Antibodies raised against the analyte–protein complex are immunoglobulins (IgG). The heavy chain component, the F_{ab} portion, is the antigen-binding portion. The degree of antibody–antigen binding is known as the antibody titre.

The antibody recognizes the antigen by its three-dimensional structure and its peptide sequence. The degree of antigen binding, the affinity, reflects the 'goodness-of-fit' of the antigen and antibody. The selectivity of an immunoassay is dependent on the portion of the analyte

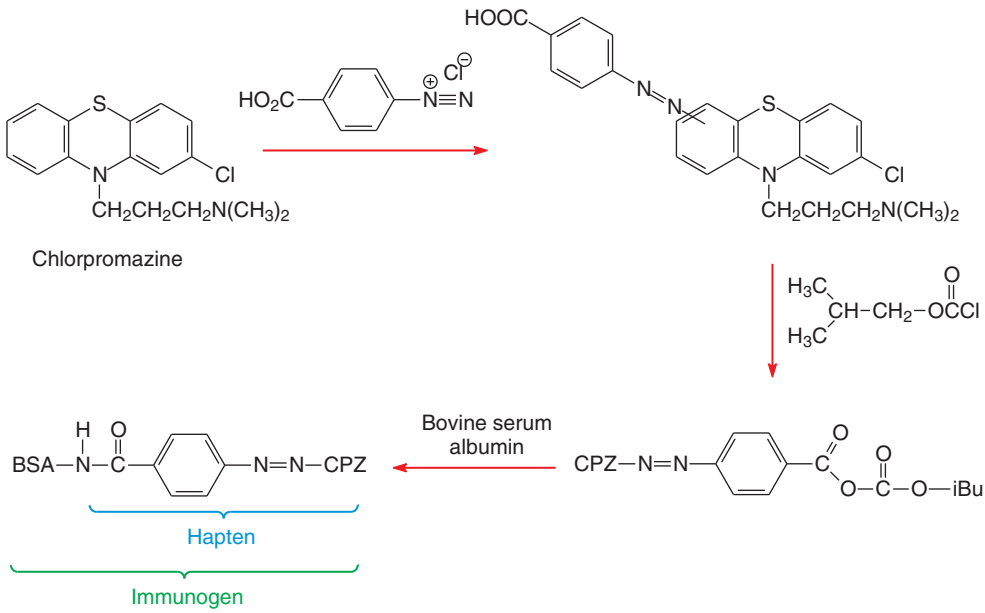


Figure 6.1 Preparation of immunogen for chlorpromazine radioimmunoassay

molecule exposed on the surface of the protein following hapten attachment and therefore available to promote antibody formation. Generally, this exposed portion of the analyte should be chosen to maximize selectivity, i.e. minimize cross-reactivity with structurally similar drugs and metabolites. Careful selection of the orientation of the analyte during immunogen formation to expose unique portions of the analyte molecule, if possible, improves selectivity. Selectivity may also be improved if the antibody is raised against an analyte that has several 'spacer bonds' added to its linkage with the protein.

Antibodies vary in their affinity for the antigen, and the mix of antibodies produced on inoculation varies between different animals and between species. This variability causes difficulties in maintaining a reproducible supply of such polyclonal antisera over time. The development of monoclonal antibodies proved an important advance. In the hybridoma technique, murine myeloma cells and lymphocytes from immunized mice are fused and propagated. Selection of a single cell for propagation provides reproducibility, continuity, and most importantly good selectivity, although sensitivity may be an issue. The affinity of the antibody for the analyte is greater for polyclonal antibodies than for monoclonal antibodies.

6.2.2 *Selectivity*

An important issue when evaluating an immunoassay is selectivity. For assays such as RIA, this is usually defined by cross-reactivity, i.e. the extent to which other molecules will displace the label and be quantified (in error) as the analyte. Cross-reactivity can be harnessed to detect a drug class, a feature that is particularly useful when screening for substance misuse. The percentage cross-reactivity (*CR*) is calculated as below:

$$CR\,(\%) = \frac{\text{Apparent concetration of analyte}}{\text{Concentration of displacing drug}} \times 100 \qquad (6.1)$$

Thus, if (–)-amfetamine (2 mg L^{-1}) cross-reacts with an antibody raised to dexamfetamine to give an apparent (+)-amfetamine concentration of 1 mg L^{-1}, then:

$$CR\,(\%) = \frac{\text{Apparent concentration of (+)-amfetamine (1 mg L}^{-1}\text{)}}{\text{Concentration of (-) – amfetamine (2 mg L}^{-1}\text{)}} \times 100 = 50 \qquad (6.2)$$

It is usual to test potentially interfering compounds at several concentrations, over several orders of magnitude, and to plot log (concentration)–displacement curves to assess the magnitude of any problem. It should be remembered that the potencies, and hence the concentrations of related drugs encountered in clinical samples, may vary widely (compare the potencies of fentanyl and codeine, Table 22.12) and even low cross-reactivity *in vitro* does not guarantee that interference from other drugs or metabolites, for example, will not occur with real samples.

The way in which the immunogen is produced can affect cross-reactivity. By linking a barbiturate via a 5-substituent, for example, antibodies can be raised that will recognize most barbiturates, whereas for a more specific assay the 5-substituent must be available to the antibody (Figure 6.2). However, in TDM interference by other drugs, drug metabolites or endogenous compounds can be problematic, as in the case of digoxin (Section 6.7.1). In addition, non-specific binding can occur in poorly designed systems limiting the sensitivity of the assay. This can often be counteracted to an extent by adding BSA or analyte-free NBCS to the incubation mixture to bind unwanted matrix components thus limiting interference in an assay. Serial dilution of the sample will usually give differing results if the binding is non-specific.

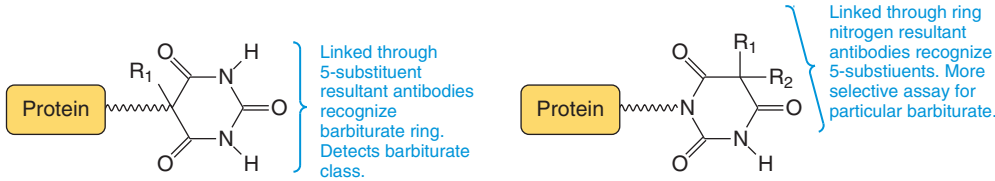


Figure 6.2 Directing antibody formation for a class-selective and an analyte-selective assay of a chosen barbiturate

6.2.3 *Performing the assay*

The principles of competitive binding assays can be understood most easily by considering RIA, which is a heterogeneous method, requiring separation of antibody-bound and non-bound label. Although homogeneous assays do not require a separation step, the principle of competitive binding is the same.

6.2.3.1 *Classical radioimmunoassay*

In classical RIA, a fixed amount of radiolabelled analyte is added to the samples (unknowns, calibrators, and blanks) which are pre-incubated to allow equilibration of analyte and label. Antiserum containing the antibody raised against the analyte is added. After further incubation, the bound label is separated, often by adding charcoal or other suitable material, to adsorb non-bound label. After centrifuging, the supernatant layer, containing the bound label, is taken for radioactive counting. Competition between label and analyte results in less label in the supernatant at higher analyte concentrations (Figure 6.3). The calibration graph is non-linear with a negative slope and a limited working range.

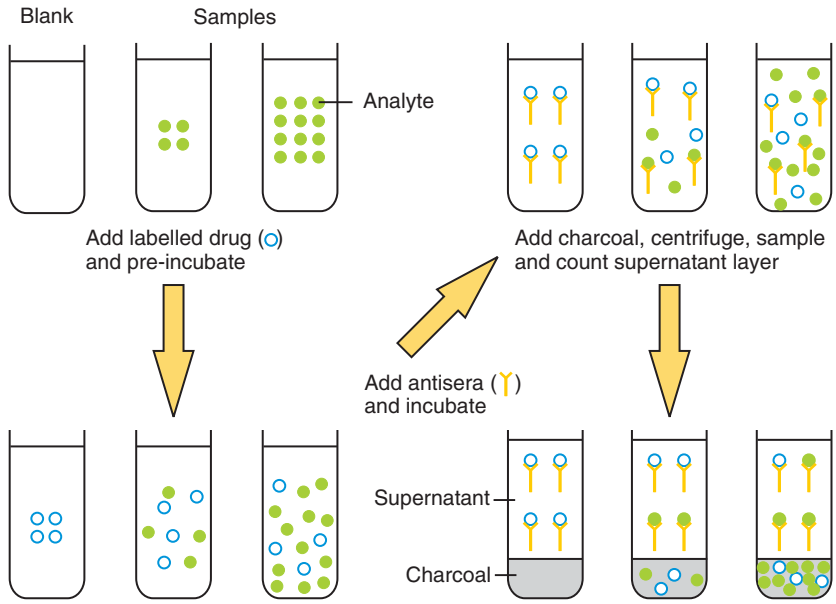


Figure 6.3 Principle of competitive binding

Initially, ^{3}H- or ^{14}C-labelled analytes were used. However, these are 'soft' β-emitters that have to be 'counted' (quantified) by liquid scintillation spectrometry, requiring large and relatively expensive apparatus. Moreover, the disposal of the scintillation cocktail can be a problem. Furthermore, for maximum sensitivity the amount of added radiolabel should be as low as possible requiring radioactive nuclides of high specific activity (number of disintegrations per minute per mole), a property that is inversely related to the half-life of the radioisotope. ^{3}H- or ^{14}C- ($t_{½}$ = 5568 yr) labels were largely replaced by ^{131}I ($t_{½}$ = 8.08 d) and ^{125}I ($t_{½}$ = 60.14 d). These γ- and X-ray emitting isotopes do not require the use of liquid scintillation counting, but disadvantages are that stock solutions require lead shielding and accidental ingestion is dangerous because radioactive iodine is sequestered in the thyroid gland thus giving a high dose of radiation to a small area. Shelf-life too may be a problem with rarely requested assays. Generally, ^{125}I is used because its longer half-life makes it more convenient and it gives faster and more efficient counting than ^{3}H.

6.2.3.2 Modern radioimmunoassay

Clearly, separating non-bound radioactive label by charcoal adsorption and centrifugation was not only inconvenient, but also the nature of the binding had to be controlled to prevent charcoal adsorption of the antibody-bound label. With the introduction of antibody-coated tubes, the analysis mixture could be decanted allowing easy separation of bound and free label. Using ^{125}I tracers, the tubes can be placed directly into a γ-counter (Box 6.2).

Use of a second antibody precipitation step can also accomplish separation of bound and free fractions. Centrifugation gives a pellet of material including bound analyte that can be counted after decanting or aspirating the free fraction. Magnetic beads and other particles coated with a second antibody have also been used to help separate bound and free fractions.

Box 6.2 Radioimmunoassay

- Tubes pre-coated with antibody to the analyte
- Standard, blank or sample, and radiolabelled (^{125}I) analyte added
- Analyte in the sample competes with the radiolabelled analyte for binding sites
- Fluid poured from tubes and residual radioactivity measured (γ-counter) – the more analyte added, the lower the residual radioactivity
- Problems: cost, short shelf-life, radioactive handling and disposal, selectivity of the antibody

6.2.4 Non-isotopic immunoassay

Immunoradiometric assays (IRMA) where the antibody was labelled resulted in significant improvements in sensitivity. However, difficulties associated with the safe handling and storage of radioactivity, the disposal of radioactive waste, the short shelf-life of some radioactive isotopes used as labels, lengthy assay times, and the difficulty of automating the assay have resulted in RIA being replaced largely by non-isotopic immunoassays for small molecules. Such systems offer reduced costs, speed, and simplicity of operation (Box 6.3).

Box 6.3 Non-isotopic immunoassays in analytical toxicology

- Fast, simple to use, automated equipment
- Various manufacturers of assay kits, but the kits can be expensive
- Ideal for preliminary screening ('presumptive screen')
- Important role especially in screening for substance misuse and in TDM of certain drugs
- Qualitative and quantitative
- Sensitivity adequate for purpose (reasonable 'cut-off' limits)

6.2.5 Assay sensitivity and selectivity

Immunoassays for small molecules such as most drugs are prone to cross-reaction with structurally related molecules. Assays designed to measure analytes in plasma are also vulnerable to interference from heterophilic antibodies (usually IgG or IgM), endogenous antibodies that bind assay antibodies, resulting in erroneous results (Bolstad *et al*., 2013).

All immunoassay procedures used for detecting misused drugs or other poisons require confirmation of positive results using a second method, ideally GC-MS or LC-MS. Poor selectivity can be turned to advantage when screening for a number of related compounds such as opiates/opiate analogues (Section 18.2.2.1), but can cause problems for the unwary. There can also be significant batch-to-batch variation in cross-reactivities for kits produced by the same manufacturer at different times.

A further factor is that not all compounds that cross-react do so to the same extent at the same concentration. For example, of three fentanyl immunoassays tested (the Thermo DRI® Fentanyl Enzyme Immunoassay, the ARK™ Fentanyl Assay homogeneous enzyme immunoassay, and the Immunalysis® Fentanyl Urine SEFRIA™ Drug Screening Kit), there was 33–95 % cross-reactivity for analyte-free urines spiked with acetylfentanyl,

acrylfentanyl, butyrfentanyl, 4-chloroisobutyrfentanyl, 4-fluorobutyrfentanyl, 4-fluorofentanyl, 4-fluoroisobutyrfentanyl, isobutyrfentanyl, methoxyacetylfentanyl, or tetrahydrofuranfentanyl, whereas 4-methoxybutyrfentanyl (all assays) and 2-fluorofentanyl (DRI assay) showed low cross-reactivity (Helander *et al*., 2018).

6.2.6 *Immunoassay development*

Immunoassay development requires a degree of expertise no longer readily found in routine analytical toxicology laboratories. Moreover, the rapid pace of change in the range of compounds available for misuse makes immunoassay development uneconomic. In consequence, commercial assays, either homogenous or immunochromatographic methods, abound despite sometimes having poor selectivity. Roche, Siemens, and Thermofisher (Microgenics), are amongst the principal manufacturers, although there are others, particularly of immunochromatographic devices utilized in POCT and related areas (Chapter 17).

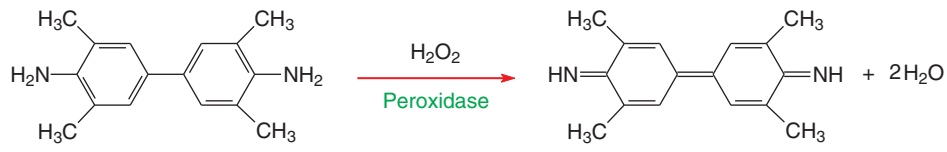
Immunoassay kits have advantages in that assay selectivity, sensitivity, and precision (reproducibility) will have been investigated beforehand to an extent, but may appear expensive and may not be readily applicable to specimens other than those for which they were developed, usually plasma or urine. Indeed, cross-reaction studies will often have been performed with pure compounds and may not have been performed at the concentrations encountered in practice (Birch *et al*., 2013).

6.3 Heterogeneous immunoassays

An enzyme immunoassay (EIA) is a non-isotopic assay that uses an enzyme and a suitable substrate as the end step to quantify the amount of analyte that was present in the original sample. EMIT is a homogeneous assay (Section 6.4.1) whereas an enzyme-linked immunosorbent assay (ELISA) is a heterogenous assay that requires a separation step that can be simplified by the use of 96-well plastic (polystyrene or PVC) microplates. Various formats of ELISA have been described including direct, indirect, sandwich, and competitive. Unfortunately, these terms may be misleading as the same method may be described by more than one term. An advantage of using heterogenous EIAs is that some interfering substances, including pigments from biological samples, are washed from the plate before colour development.

6.3.1 *Tetramethylbenzidine reporter system*

A frequently used system for quantifying enzyme activity in ELISA assays is 3,3′,5,5′-tetramethylbenzidine (TMB) with hydrogen peroxide (Figure 6.4). When reacted with horseradish peroxidase (EC 1.12.1.7) or phosphatase (EC 3.1.3.1), the rate of oxidation of



3,3',5,5'-Tetramethylbenzidine (TMB) Soluble blue dye

Figure 6.4 Reaction of tetramethylbenzidine with hydrogen peroxide in the presence of peroxidase

TMB can be monitored at 370 or 630–650 nm. Alternatively, the reaction can be stopped after 20–30 min by adding 1 mol L^{-1} sulfuric acid, which gives a shift in absorbance to 450 nm. The yellow colour is stable for at least an hour and can be measured using a standard microplate reader.

Horseradish peroxidase may be preferred to alkaline phosphatase because it has a lower M_r. However, it should not come into contact with solutions stabilized with sodium azide because it is very sensitive to the presence of this compound. Alkaline phosphatase, on the other hand, can be used with solutions containing sodium azide, but as zinc and magnesium ions are required as cofactors, concomitant use of solutions containing high concentrations of chelating agents such as EDTA must be avoided. TMB is also used with enzyme systems that produce hydrogen peroxide. It forms the basis of saliva alcohol test strips, when the intensity of the colour is used to quantify ethanol (Section 17.3.1.2).

6.3.2 Antigen-labelled competitive ELISA

This term is used to describe ELISA-type assays in which the antibody is bound to the microplate and the analyte (antigen) is labelled with enzyme. Preparation of the wells is relatively simple. A precise amount of antibody solution (in bicarbonate buffer, pH 9.0) is added to each well and incubated for an appropriate time, typically either 4 h at ambient temperature, or overnight at 4 °C. Once the antibodies are bound, the plate is washed and dried. In order to increase the stability of the bound antibody and to reduce non-specific binding of assay components, a second non-specific protein coating may be added. Commercial EIA kits are provided as dry microplates pre-coated with antibody.

Sample (10–50 μL) is added to an antibody-coated microplate well followed by a buffered solution (100 μL, for example) containing enzyme-labelled analyte. The plate is incubated at ambient temperature to allow the enzyme-labelled analyte, analyte, and antibody to equilibrate [Figure 6.5(a)]. After the incubation period, the plate is washed with buffer (the separation step) to remove unbound enzyme. Bound enzyme conjugate and bound drug are left on the microplate. TMB/hydrogen peroxide solution is added, and the plate incubated to develop the colour as described above. The higher the concentration of analyte in the sample, the less labelled enzyme will be bound on the plate and thus the calibration curve has a negative slope, there being no colour at very high analyte concentrations.

6.3.3 Antibody-labelled competitive ELISA

In this ELISA the antibody is directly coupled to the enzyme, the antigen being bound to the microplate. To do this the analyte is conjugated to a protein using a process similar to that used to prepare an immunogen, but the protein must be different from the one used to make the immunogen, otherwise the antibody may ‘recognize’ the protein and bind to it. The protein–analyte conjugate can be coated to the microplate well in the same way as antibodies are coated (Section 6.3.2).

During the assay, competition occurs between the analyte in the sample and the immobilized analyte for binding to the enzyme-labelled antibody [Figure 6.5(b)]. Following incubation, a washing step separates bound and free fractions, and leaves enzyme-labelled antibody bound to the analyte derivative in the well (Box 6.4). TMB/hydrogen peroxide can be used as the reporter system as described above, and because the amount of labelled antibody that is bound is inversely related to the analyte concentration in the sample, the calibration curve has a negative slope.

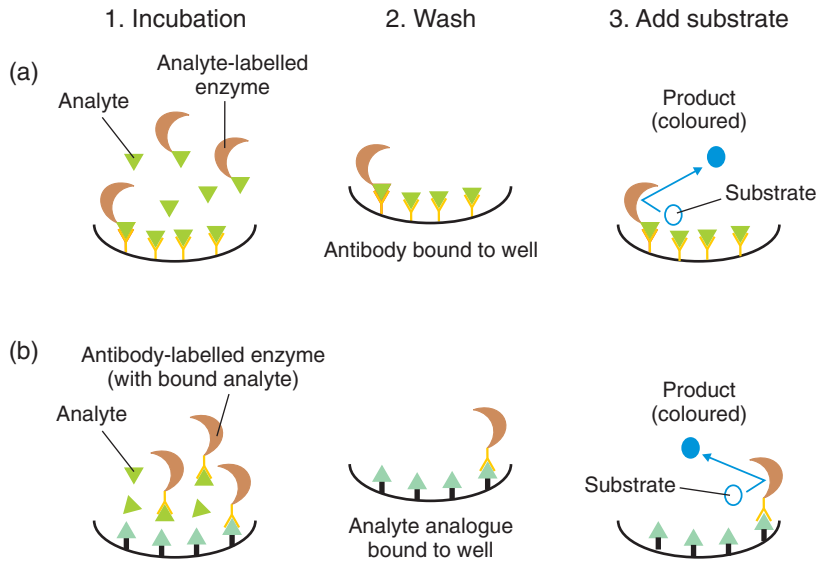


Figure 6.5 Comparison of (a) antigen-labelled and (b) antibody-labelled ELISA

Box 6.4 Enzyme linked immunosorbent assay

- Wells of ELISA plate pre-coated with immobilized analyte
- Sample and enzyme conjugate (analyte-labelled horseradish peroxidase) incubated in the well (30 min)
- Wells washed, then substrate (3,3′,5,5′-tetramethylbenzidine) added
- After further 30 min, reaction stopped by adding 1 mol L^{-1} sulfuric acid
- Absorbance of each well measured (460 nm)

ELISA kits are available for a range of drugs including: amfetamines, barbiturates, benzodiazepines, benzoylecgonine, cannabinoids, fentanyl, flunitrazepam, phencyclidine (PCP), opiates, methadone, and tricyclic antidepressants (TCAs). The Neogen SPICE ELISA kit that targets JWH-018 *N*-pentanoic acid has been evaluated (Spineili *et al.*, 2015). ELISA is sensitive and has a good dynamic range. As an ELISA is a heterogeneous assay there is less opportunity for interference than with many homogenous assays, and although easily automated, such assays are performed typically in 96-well plates and are not compatible with high-throughput clinical chemistry analyzers.

6.3.4 Sandwich ELISA

Sandwich (or indirect) ELISA uses two or more antibodies. This requires the analyte to have more than one binding site and so in general is not useful for small analytes such as most drugs. In the example (Figure 6.6), excess primary antibody has been bound to the well and incubated with the sample. After washing, a second enzyme-labelled antibody was added and the system was allowed to equilibrate. After a second wash to remove excess label, reporter solution was added as described above.

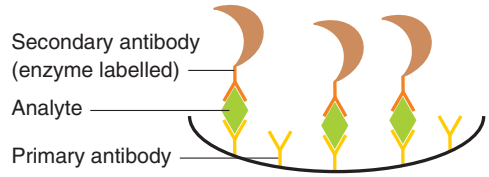


Figure 6.6 Representation of sandwich ELISA microplate well after its second washing. Note that the amount of enzyme on the plate is proportional to the amount of analyte present

Advantages of this approach are that the amount of enzyme bound to the plate is proportional to the amount of analyte in the sample hence (i) the calibration curve has a positive slope and (ii) the assays are potentially much more sensitive than competitive ELISAs. By using excess antibody, equilibration times are short, which leads to rapid assays. An obvious disadvantage is that generally more antibody is required. Sandwich ELISAs are frequently used to test for hormones, for example human chorionic gonadotrophin (HCG) in pregnancy testing kits.

6.3.5 Lateral flow competitive ELISA

Lateral flow systems are used in many POCT kits because the technique lends itself to miniaturization and are discussed more fully in Chapter 17. Briefly, drug is immobilized as a line across a test strip, which has a reservoir of labelled antibody towards one end. When sample (e.g. urine or oral fluid) is added, the antibody is carried along the strip by capillary action. In the absence of drug in the sample all the labelled antibody is free to react with the immobilized drug to produce a visible line. Any drug in the sample reduces the amount of free antibody and so reduces the intensity of the line. In such a system, a strong positive result would be manifest as the absence of a visible line.

6.3.6 Chemiluminescent immunoassay

Chemiluminescent immunoassays (CLIA) may be extensions of the ELISA assays described above, but use chemiluminescence as the endpoint, for example the luminol/hydrogen peroxide system described in Section 6.6. Advantages of chemiluminescence include greater sensitivity, i.e. less sample may be required or diluted samples may be used (which may have the benefit of diluting out potential interferences), lower background signals, and shorter analysis times. Furthermore, the number of chemiluminescent counts (relative chemiluminescent units, RLU) are usually greater than the counts obtained using radioactive labels and so competitive CLIAs have a larger working range, giving accurate concentration values up to 95 % analyte binding. A second mode of CLIA that uses superparamagnetic beads instead of an enzyme is described in Section 6.5.2.

Based on ELISA principles, solid-state devices (‘chips’) with discrete test sites onto which antibodies specific to different drugs/drug groups are immobilized and stabilized are available commercially (Randox). Competitive chemiluminescent immunoassays offer sensitive detection of some synthetic cathinones (Ellefsen *et al.,* 2014), some synthetic cannabinoids (Castaneto *et al*., 2014), and for a range of other drugs in a variety of samples including insect larvae and cardiac muscle (Basilicata *et al*., 2019).

A cell-based cannabis reporter bioassay for the activity-based detection of synthetic cannabinoids and their metabolites has been described (Cannaert *et al*., 2017; 2018). Activation of the

CB1 or CB2 receptor leads to β-arrestin 2 (βarr2) recruitment, which results in functional complementation of a split NanoLuc luciferase. This functional complementation restores NanoLuc luciferase activity, resulting in a bioluminescent signal in the presence of the substrate furimazine that can be measured with a standard luminometer. Activity-based opioid assays have also been described (Cannaert *et al*., 2019), although the presence of an opioid receptor antagonist such as naloxone can give rise to false-negative results unless this is tested for by adding an opioid agonist during the assay.

6.4 Homogenous immunoassays

6.4.1 Enzyme-multiplied immunoassay technique

EMIT assays were introduced by Syva, now part of Siemens. By attaching the analyte to an enzyme, it is possible to inhibit the activity of the enzyme by introducing an antibody raised to that analyte. Introducing analyte in a sample restores enzyme activity. Lysozyme was the enzyme used originally, but it has a low K_m, resulting in an insensitive turbidimetric assay that was subject to interference from urinary lysozyme. The commercial assay is now based on hydrolysis of glucose-6-phosphate (G-6-P) in the presence of bacterial G-6-P dehydrogenase (G-6-PDH) to which analyte has been conjugated. The coenzyme nicotine adenine dinucleotide (NAD) is reduced to NADH and the resulting increase in absorbance is monitored at 340 nm (Figure 6.7).

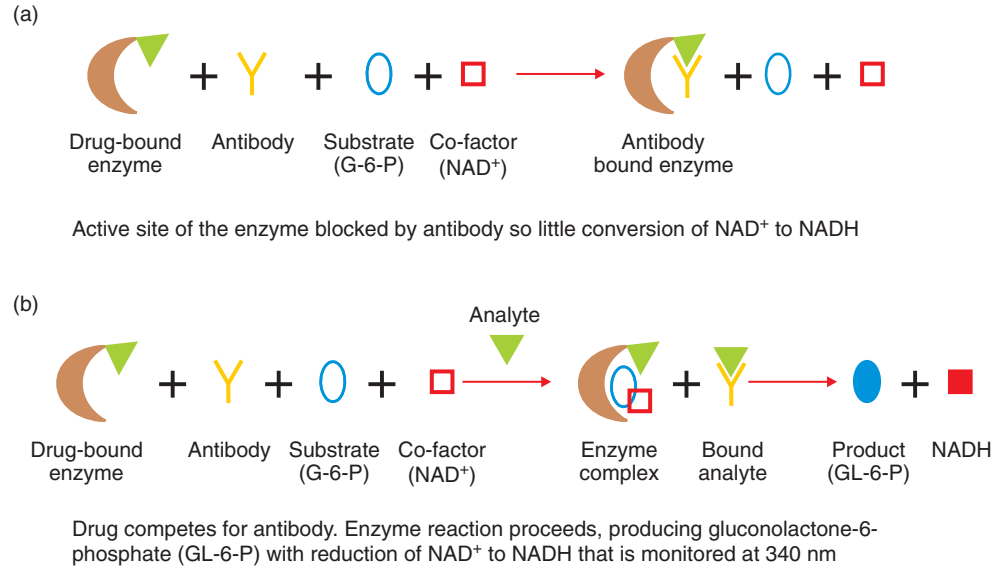


Figure 6.7 Principle of enzyme-multiplied immunoassay technique (a) without additional drug and (b) in the presence of drug

Antibody bound to the analyte–enzyme conjugate prevents substrate binding and reduces the rate of formation of NADH. Analyte in the sample competes with the analyte-labelled enzyme for binding to the antibody, which increases the fraction of unbound enzyme and thereby increases the rate of change of absorbance (Box 6.5). Use of bacterial G-6-PDH (NAD

Box 6.5 Enzyme-multiplied immunoassay technique

- Homogenous assay – little if any sample preparation
- Mainly used for urine or plasma (but can use for other samples after, for example, LLE)
- System: antibody to analyte, analyte bonded to glucose-6-phosphate dehydrogenase (G-6-PDH), glucose-6-phosphate, and NAD
- Antibody–enzyme complex inactive – added analyte in sample displaces antibody from enzyme giving enhanced enzyme activity
- Measure enzyme activity by monitoring NAD to NADH conversion spectrophotometrically (340 nm)

coenzyme), avoids interference from endogenous G-6-PDH, which requires nicotine adenine dinucleotide phosphate (NADP) as co-factor.

There is now extensive experience of EMIT, particularly as regards interference in substance misuse testing. The system is simple, has adequate sensitivity for compounds with M_r <200 present in biological fluids at moderate concentrations, and avoids the use of radioactive labels. Although the initial rate of the reaction is proportional to the concentration of enzyme, the amount of enzyme is not directly proportional to the analyte concentration. Rate monitoring can be performed using high-throughput clinical chemistry analyzers, which makes this technique and other homogenous assays attractive technically and commercially.

Plasma assays are available for a number of TDM analytes (Section 20.4). Whilst not particularly reliable for analytes in the μg L^{-1} range, there are also EMIT II Plus assays for a range of compounds in urine including: amfetamines, barbiturates, benzodiazepines, benzoylecgonine (BE), cannabinoids, opiates, methadone, (dextro)propoxyphene, and tricyclic antidepressants (TCAs). In general, these assays may be used with serum if assay calibrators of lower concentration are used, a stratagem made possible because of the lower background values obtained as compared with urine. Other modifications of the basic assay system have been suggested to facilitate use of EMIT assays with post-mortem whole blood, for example (Hino *et al*., 2003).

6.4.2 Fluorescence polarization immunoassay (FPIA)

When fluorescent molecules are irradiated with polarized light of the appropriate wavelength, freely rotating molecules emit light in different planes. However, slowly rotating antibody-bound fluorophores emit more light in a similar plane to the incident light and this can be measured via use of a polarizing filter. This is the basis of homogeneous competitive FPIA (Box 6.6; Figure 6.8). Fluorescein (excitation wavelength 485 nm, emission 525–550 nm) was chosen as the fluorophore. The background fluorescence present in biological samples means it is usual to take a reading of the sample and reagents before the addition of the fluorescent tracer.

Abbott introduced FPIA commercially as the basis of the AD_x analyzer for urine substance misuse testing and the TD_x analyzer for TDM. Urine assays included amfetamines, barbiturates, benzodiazepines, BE, cannabinoids, opiates, and (dextro)propoxyphene, and serum assays included carbamazepine, digoxin, paracetamol (for overdose work), phenobarbital, phenytoin, primidone, theophylline, and valproate.

Box 6.6 Fluorescence polarization immunoassay

- Use with plasma or urine – advantages/disadvantages similar to EMIT except that not compatible with clinical chemistry analyzers
- Fluorescein-labelled analyte rotates rapidly in solution – if irradiated with polarized light, emitted light not polarized
- When antibody to analyte added, bound analyte rotates more slowly – emitted light retains polarization
- Added analyte in sample competes with labelled drug for antibody sites, increasing depolarization of emitted light – measure on polarimeter
- Amount of depolarization related to the concentration of analyte in the sample

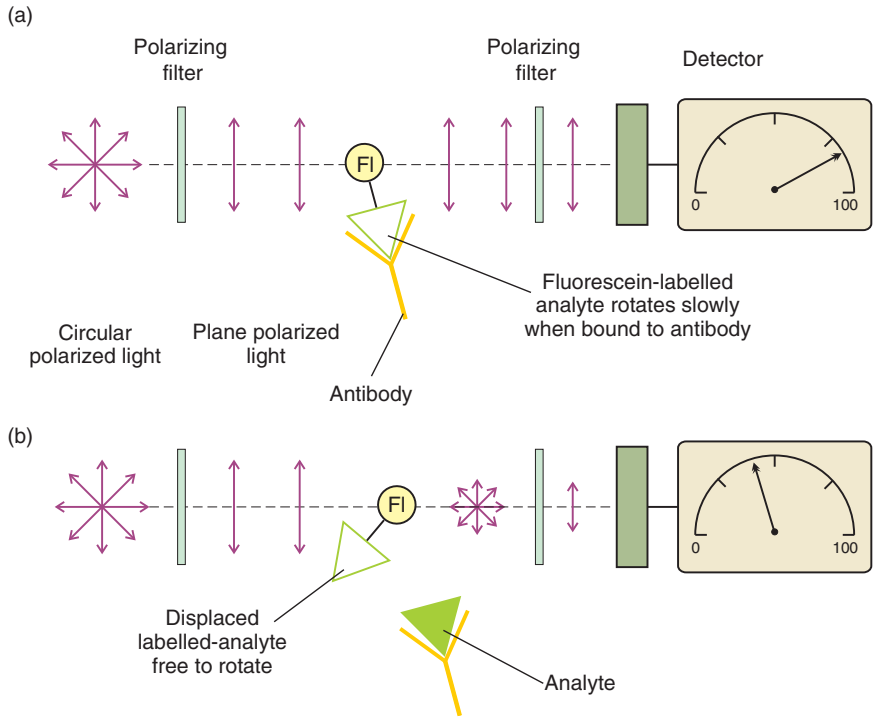


Figure 6.8 Principle of fluorescence polarization immunoassay: (a) without added analyte (b) with analyte

FPIA was used as the basis of assay systems in addition to those produced by Abbott, but such systems are no longer available commercially. The improved precision and reagent stability offered by FPIA are advantages over EMIT, but FPIA is not compatible with the clinical chemistry analyzers for which EMIT is readily suited. Moreover, although fluorescence methods are inherently sensitive, in FPIA sensitivity is limited by protein concentration and so the digoxin assay incorporated a protein precipitation step.

6.4.3 *Cloned enzyme donor immunoassay*

As with EMIT, CEDIA exploits antigen–antibody binding to influence spectrophotometrically measured enzyme activity. β-Galactosidase from *Escherichia coli* is supplied as inactive fragments. The large fragment (some 95 % of the enzyme) is termed the enzyme acceptor (EA), and the smaller fragment is termed the enzyme donor (ED). By conjugating hapten (analyte) to the ED fragment, antibodies to the hapten can prevent the formation of intact, active enzyme. Any analyte present in the sample competes for binding sites on the antibody. Thus, an increase in analyte concentration will decrease binding of antibody to the ED fragment and increase enzyme activity, which can be monitored by production of chlorophenol red (CPR) from CPR-β-galactoside (Box 6.7; Figure 6.9).

Box 6.7 Cloned enzyme donor immunoassay

- β-Galactosidase split into two inactive fragments: larger fragment enzyme acceptor (EA), smaller fragment enzyme donor (ED)
- When EA and ED mixed they combine to form the active enzyme
- Sample and Reagent 1 (EA/antibody) first placed in tube
- Reagent 2 (analyte labelled with ED and substrate) then added
- Analyte in sample binds to the antibody preventing ED/drug conjugate from binding
- The higher the analyte concentration in the sample, the lower the number of ED/drug conjugates bound to antibody
- More ED/drug conjugate available to combine with an EA fragment – this combination forms the active enzyme, which in turn hydrolyzes the substrate
- Hydrolyzed product (usually CPR) measured spectrophotometrically; absorbance proportional to analyte concentration

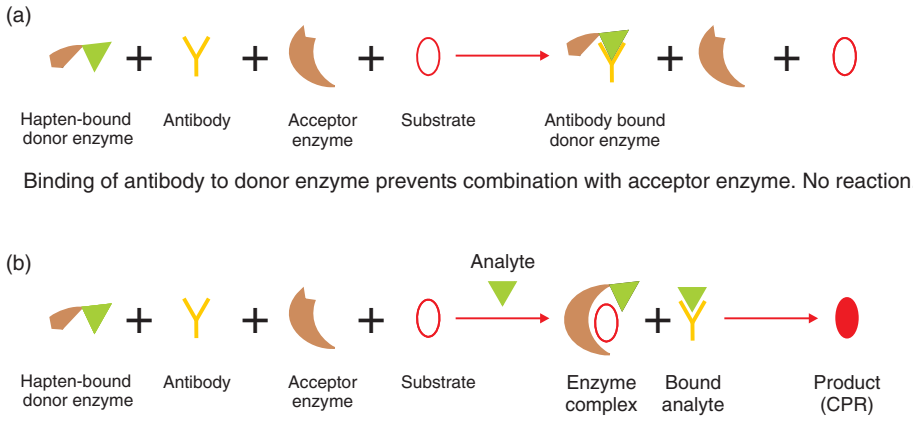


Figure 6.9 Principle of cloned enzyme donor immunoassay. (a) No added drug and (b) with added drug

As with EMIT, CEDIAs are reaction rate assays and may be performed on high-throughput clinical chemistry analyzers. The technique has a wide dynamic range. The repertoire includes amfetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, LSD, methadone, opiates, paracetamol, PCP, propoxyphene, salicylates, TCAs, and some therapeutic drugs. CEDIA assays for haloperidol and bromperidol have also been described. Weak points of CEDIA include the fact that the ED and EA fragments are not as stable as naturally occurring proteins, and the need to assemble the complex means that it is susceptible to physicochemical disruption.

6.5 Microparticulate and turbidimetric immunoassays

The aggregation of microparticles as a diagnostic test is by no means new. Latex agglutination tests (LAT) were described in the 1950s. LATs are portable, robust, efficient, and work under primitive conditions making them one of the first POCT techniques. Submicron sized polystyrene microspheres ('latex particles') are used to which antigen is bound. When antibody is introduced, several microspheres bind to each antibody producing aggregates that scatter incident light. Front- or back-scattered light can be measured either with a nephelometer, or by scanning laser microscopy, respectively, or the transmitted light can be measured with a spectrophotometer (Figure 6.10). The method can be used to measure small drug molecules if the antigen is a hapten of the drug in question, addition of unbound drug inhibiting the aggregation process (Latex Agglutination Inhibition Test, LAIT).

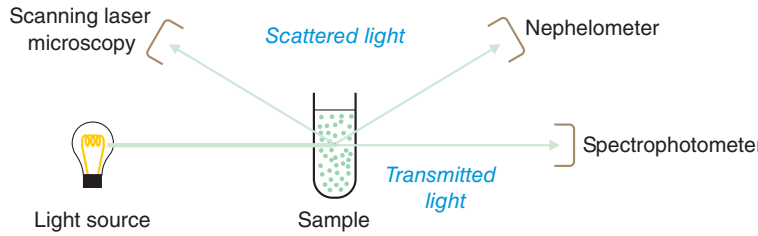


Figure 6.10 Different modes of measurement for turbidimetric assays

The greatest degree of scattering is obtained with particles with diameters approximately equal to the wavelength of the incident light. Thus, for visible light (λ = 390–760 nm) the optimal size of the aggregates should be in the range 0.4–0.8 μm. It would be possible to start with microspheres of this size and to observe the reduction in light scattering as they aggregate. However, it is more common to start with smaller (<0.1 μm) microspheres and to measure either the increase in light scattering, or an apparent increase in absorbance if using a spectrophotometer. Turbidimetric immunoassays (TIA) provide sensitive and rapid end points and have been adopted by a number of suppliers of commercial assay kits. The acronyms PETIA (particle enhanced turbidimetric immunoassay) and PETINIA (particle enhanced turbidimetric inhibition immunoassay) were introduced by Du Pont.

6.5.1 Microparticle enzyme immunoassay (MEIA)

MEIA is similar to a sandwich ELISA (Section 6.3.4). The beads, which are coated with antibodies against the analyte of interest, capture the analyte. The separation step is to add the

reaction mixture to a fibreglass mat. An anti-analyte antibody labelled with an enzyme such as alkaline phosphatase is incubated with the beads and a suitable substrate, 4-methylumbelliferone phosphate, for example, is added. The fluorescence of the product, 4-methylumbelliferone, is measured. Because it is not a competitive assay, the signal is proportional to analyte concentration in the sample.

6.5.2 Chemiluminescent magnetic immunoassay (CMIA)

CMIA avoids the use of an enzyme by labelling the antibody with a chemiluminescent precursor. The antigen is bound to superparamagnetic particles that are not magnets, but can be attracted to a magnet, making them very easy to separate and wash. The beads retain no residual magnetism when the magnet is removed. For example, in a competitive heterogeneous digoxin assay, a digoxin analogue was bound to superparamagnetic particles and a murine monoclonal antibody labelled with an acridinium ester added. After incubation with the sample, the beads were collected on a magnet, washed and a 'trigger solution' (hydrogen peroxide and a promoter) added to start the chemiluminescent reaction. Using acridinium labels, reaction times can be as short as 2 seconds, as opposed to the 30 minutes typical for colorimetric assays.

Alternative approaches include sandwich-type assays in which superparamagnetic beads with bound primary antibodies against the analyte are incubated with sample and, after washing, a second antibody labelled with a luminescent compound is added. Various acridinium derivates may be encountered as different manufacturers develop and patent their own labels. Superparamagnetic particles can be used for ELISAs rather than using microplates as described above.

6.6 Assay calibration, quality control, and quality assessment

Quantitative immunoassays require calibrators that are traceable to an identifiable primary standard. This is rarely the case with proprietary material. Assay calibrators need to be prepared in the same matrix as the sample to be assayed. Indeed, with proprietary assays this will be defined by the manufacturer and certified by CE marking (EU) and by the FDA (US).

6.6.1 Immunoassay calibration

Calibration graphs for immunoassays tend to be complex, particularly if the calibration range is large. For EIAs, the *rate* of the enzyme reaction should be directly proportional to the enzyme concentration. However, in competitive assays the *amount* of active enzyme present will not be directly proportional to the analyte concentration in the sample. Binding of analyte to antibody is analogous to drug–receptor binding and can be treated by the Law of Mass Action in the same way. The amount bound, B is:

$$B = \frac{B_{\max} C}{K + C} \tag{6.3}$$

where C is the concentration of analyte, K is the binding constant, and $B_{\max}$ the maximum amount that can be bound. This is the equation of a rectangular hyperbola [Figure 6.11(a)].

As can be seen from Figure 6.11(a), calibration plots of bound label versus analyte concentration are not easy to read and the data may be transformed, for example by plotting log C. This gives an approximately linear region between 20 and 80 % binding [Figure 6.11(b)]. A linear plot is obtained using the log–logit plot where:

$$\ln\left[\frac{B/B_0}{1 - B/B_0}\right]$$

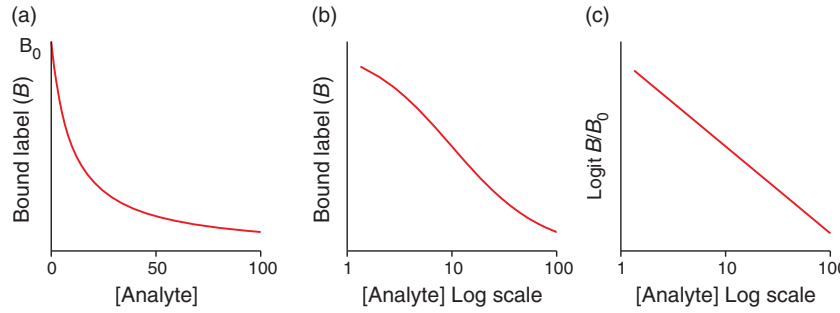


Figure 6.11 RIA calibration graphs showing original data (a) transformed to (b) log C versus B and (c) logit B/B_0 versus log C. B_0 = bound label in absence of added analyte

is plotted against log C [Figure 6.11(c)]. Such approaches were useful when calibration graphs were drawn manually and the B versus log C plot was helpful in defining the working range of the assay. However, the errors associated with the log–logit plot are complex and production of a linear calibration line does not mean that it should be extrapolated below or above the LLoQ and ULoQ, respectively. With the availability of curve-fitting programs there is no reason for data transformation.

A problem that can occur with calibration curves is the 'hook' effect. This may occur when samples having extraordinarily high concentrations of analyte, far exceeding the highest calibration standard, appear to have much lower concentrations, i.e. an extended calibration curve would be hook-shaped. This is similar to the situation with fluorescence assays when two concentrations can produce the same signal (Section 5.5.1). Several explanations have been suggested for the phenomenon, including, in sandwich-type assays, binding of analyte to the secondary antibody preventing binding to the analyte that is bound to the primary antibody.

6.6.2 Drug screening

Immunoassays are convenient and rapid if screening for the presence of drugs and other substances. The requirement for only small volumes of sample has made immunoassays popular for POCT and workplace testing (Chapters 17 and 18). The advent of NPS has heralded new approaches to developing immunoassays for these compounds (Section 6.3.6).

A full calibration line is normally unnecessary. The aim is to indicate if a substance is present, usually with the intention of confirming its identity with a more selective quantitative technique. 'Cut-off' limits have been defined, chiefly for misused substances, below which the drug is deemed not to be present. Thus, the complication of calibration curves is avoided as only a calibrator at the cut-off concentration need be used. However, assays using cut-offs have to have good reproducibility at the nominated limit and have good within-batch precision (of the order of 25 %).

The US Substance Abuse and Mental Health Services Administration (SAMHSA, 2017) have defined cut-off values for five drug groups (12 drugs) in urine (Table 6.1). These do not necessarily represent the LLoQ of an assay, but take account of other considerations. For example, the limit for codeine/morphine in urine was 300 µg L^{-1} for a number of years, but was changed to 2 mg L^{-1} to avoid confusing use of heroin with the ingestion of poppy seeds.

Table 6.1 SAMHSA 2018 'cut-off' values for drugs of abuse screening in urine (μg L^{-1})

Analyte		Screening[a]	Confirmation
Cannabis metabolite (THC-COOH)[b]		50[c]	15
Benzoylecgonine		150[c]	100
Phencyclidine (PCP)		25	25
Amfetamines	Amfetamine	500	250
	Metamfetamine	500	250
	MDMA/MDA	500	250
Opioids	Codeine/morphine	2000	2000
	6-Acetylmorphine	10	10
	Hydrocodone	300	100
	Hydromorphone	300	100
	Oxymorphone	100	100
	Oxycodone	100	100

[a]For grouped analytes (i.e. two or more analytes that are in the same drug class and have the same initial test cut-off):

Immunoassay: the test must be calibrated with one analyte from the group identified as the target analyte. The cross-reactivity of the immunoassay to the other analyte(s) within the group must be 80 % or greater; if not, separate immunoassays must be used for the analytes within the group.

Alternate technology: either one analyte or all analytes from the group must be used for calibration, depending on the technology. At least one analyte within the group must have a concentration equal to or greater than the initial test cut-off or, alternatively, the sum of the analytes present (i.e. equal to or greater than the laboratory's validated limit of quantification) must be equal to or greater than the initial test cut-off

[b]An immunoassay must be calibrated with the target analyte, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH)

[c]Alternate technology (THC-COOH and BE): the confirmatory test cut-off must be used for an alternate technology initial test that is specific for the target analyte (i.e. 15 μg L^{-1} for THC-COOH, 100 μg L^{-1} for BE)

6.7 Interferences and assay failures

Different systems are prone to different forms of interference. This is a particular issue in substance misuse work (Section 18.2.1) and in digoxin and insulin assay (Sections 20.6.7.1 and 22.4.4.1, respectively). Modern blood-collection tubes may contain a range of additives including surfactants that may interfere in immunoassays (Section 2.2.3.4).

Metabolites and other structurally related and indeed structurally unrelated compounds often cross-react in immunoassays to varying extents. This can be helpful in qualitative work such as substance misuse screening as noted above, but is obviously undesirable in quantitative work unless exploited as in cross-reaction of digoxin assays with other digitalis glycosides (Section 22.4.10). Many opiate immunoassays, for example, detect codeine as well as morphine and metabolites, necessitating further analysis ('confirmation') to prove the presence of morphine (Table 6.2). Some morphine immunoassays detect inactive metabolites such as morphine-3-glucuronide ('total morphine') whilst some just detect morphine ('free morphine').

Table 6.2 Urine 'opiates screen': some compounds detected

Codeine + metabolites	Hydromorphone	Pholcodine
Dextromethorphan	6-Acetylmorphine	'Poppy seed' metabolites (includes morphine)
Dihydrocodeine	Oxycodone/oxymorphone	
Hydrocodone	Morphine + metabolites	

6.7.1 Measurement of plasma digoxin after F_{ab} antibody fragment administration

Conventional serum immunoassays of glycoside concentration are no longer useful when the patient has been treated with anti-digoxin F_{ab} antibody fragments because the digoxin is already bound and not available for competition in an immunoassay system. Equilibrium dialysis or ultrafiltration (Section 4.3) is required to measure non-bound, pharmacologically active digoxin. The situation is analogous to that where autoantibodies to a drug are present in a sample.

Digestion of the F_{ab} antibody fragment–digoxin complex using a proteolytic enzyme is also required before measurement of 'total' digoxin because the affinity of the F_{ab} fragment for digoxin may well be similar to or greater than the affinity of the antibody used in the immunoassay. Plasma digoxin measurements using immunoassay may not be reliable for up to 2 weeks post-treatment especially in patients with impaired renal function.

6.8 Aptamer-based assays

Aptamers are synthetic oligonucleotides selected to bind a certain target. Aptamers, in contrast to antibodies, require the formation of a three-dimensional structure for target binding and thus are likely to have a much higher affinity for binding the target molecule rather than a modified form of the target such as an enzyme-labelled target. A number of enzyme-linked aptamer assays and aptasensors have been reported (Sharma *et al*., 2017; Wiedman *et al*., 2018).

6.9 Enzyme-based assays

Ease of automation and rapid throughput make homogeneous enzyme assays appealing, but specificity must be addressed. Two analytes for which enzyme-based assays are used quantitatively are paracetamol and ethanol; the procedures can also be adapted for qualitative use.

6.9.1 Paracetamol

Bacterial aryl acylamide amidohydrolase (EC 3.5.1.13) can be used to hydrolyze paracetamol to 4-aminophenol, which can be measured spectrophotometrically after reaction with *o*-cresol and ammoniacal copper sulfate. The method is easily automated. A modification uses 8-hydroxyquinoline in the presence of Mn^{2+} ions as the chromogenic reagent, forming a blue dye in the presence of 4-aminophenol. These assays have good specificity, but may suffer from interference (false lowering of plasma paracetamol concentration) from *N*-acetylcysteine (NAC) at the concentrations attained during the early stages of a NAC infusion in the treatment of paracetamol poisoning. Paracetamol immunoassays do not suffer from interference from NAC.

Precision, accuracy, linearity, and interferences of three enzymatic assays (Beckman Coulter AU Paracetamol, Abbott MULTIGENT Acetaminophen, and Sekisui Acetaminophen L3K) and two immunoassays [Beckman Coulter SYNCHRON ACTM (Acetaminophen) Reagent and Siemens SYVA Emit-tox Acetaminophen] have been evaluated on a Beckman Coulter AU680 chemistry analyzer (Chan *et al.,* 2018). Within-run and between-run precision of the enzymatic assays ranged from 0.3–0.8 and 0.5–2.9 %, respectively, while those for the immunoassay-based methods ranged from 1.0–6.3 and 1.5–11.3 %, respectively.

All assays except the SYNCHRON assay fell within performance specifications (±3 mg L^{-1} or 10 %) for EQA samples, with the highest positive bias (32 %) being observed in the SYNCHRON assay. Bilirubin interference was most significant in the Abbott assay (up to 13 mg L^{-1} positive bias in blank serum). Lipaemic interference on the SYNCHRON was significant (up to 110 % positive bias at 15 mg L^{-1}). The immunoassay-based methods were less susceptible to haemolytic interference, while the Abbott and Beckman Coulter assays were more susceptible to NAC interference. The immunoassays showed no hook effect up to 2.7 g L^{-1}.

6.9.2 *Ethanol*

A number of kits are available for enzymatic measurement of ethanol. Most are based on the oxidation of ethanol to acetaldehyde catalyzed by yeast ADH (EC 1.1.1.1) with subsequent reduction of the cofactor NAD to NADH and monitoring of the reaction at 340 nm (Figure 6.12). Generally, the reaction mixture is incubated (21–37 °C, 10–30 min) to allow the reaction to go to completion. A 'trapping agent', such as semicarbazide or hydrazine, which reacts with acetaldehyde, may be included.

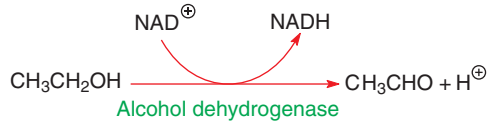


Figure 6.12 Alcohol dehydrogenase catalyzed oxidation of ethanol

Plasma or serum can be assayed directly, but whole blood has to be treated before analysis, with perchloric acid, for example. Calibration standards (0.5–4.0 g L^{-1}) should be prepared in heparinized whole blood containing sodium fluoride (10 g L^{-1}). Although ADH is insensitive to the presence of fluoride at this concentration, solutions of the enzyme should not be shaken, for example during reconstitution, to avoid foaming and denaturation. Methanol and acetone do not interfere, but 2-propanol, higher alcohols, and ethylene glycol may cross react to an extent. As these latter compounds react more slowly than ethanol, interference will be more apparent if the samples are incubated for longer than normal.

A colorimetric enzyme assay, which forms the basis of some POCT protocols for ethanol (Section 17.3.1.2), uses alcohol oxidase (EC 1.1.3.13) to produce hydrogen peroxide (Figure 6.13), which is detected by its reaction with horseradish peroxidase in the presence of a suitable chromogen such as TMB (Figure 6.4) or 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Roche). However, methanol shows cross reactivity in this type of assay.

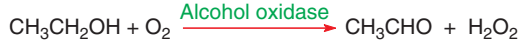


Figure 6.13 Oxidation of ethanol catalyzed by alcohol oxidase

6.9.3 *Anticholinesterases*

A qualitative test for exposure to organophosphorus (OP) and other anticholinesterases is based on the inhibition of plasma cholinesterase (pseudocholinsterase, EC 3.1.1.8). Plasma cholinesterase (ChE) activity can be assessed using acetyl- or butyryl-thiocholine as substrate and 5,5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent. This latter compound reacts with thiols to produce 5-thio-2-nitrobenzoic acid, which is yellow in alkaline solution (Figure 6.14). The presence of anticholinesterases inhibits the hydrolysis of the thiocholine esters and so reduces the intensity of the yellow colour formed on incubation. Oximes such as pralidoxime can be added to a further patient sample – such a sample should give the same reaction as samples from unexposed individuals provided that irreversible inactivation ('ageing') of the enzyme has not occurred.

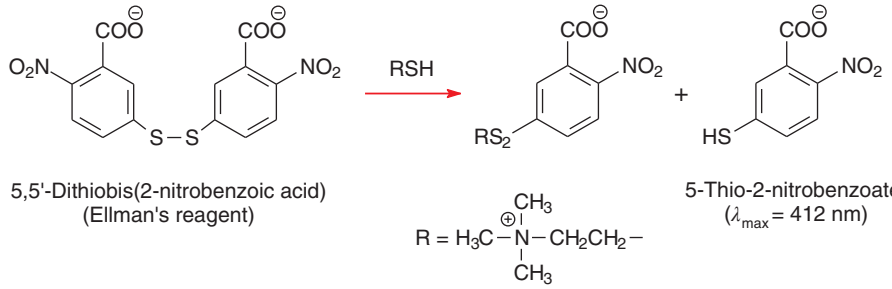


Figure 6.14 Reaction of Ellman's reagent with thiols

Measurement of the activity of membrane-bound erythrocyte acetylcholinesterase (AChE; EC 3.1.1.7) is used to assess the severity of OP exposure and to guide patient treatment. Monitoring at 436 nm minimizes haemoglobin interference (Worek *et al.*, 1999). The signal-to-noise ratio was further enhanced at lower pH and substrate concentrations. AChE activity was measured in whole blood in the presence of the selective butyrylcholinesterase inhibitor ethopropazine. Dilution of blood samples 1+99 prevents secondary reactions in the presence of an OP and of an OP reactivator (an oxime). Normalization of the AChE activity to the haemoglobin content measured as cyanmethaemoglobin minimizes dilution errors.

6.10 Summary

All immunoassays need to be validated for use. If an assay is to be used with patient samples, then EQA samples from patients should also be analyzed. The analyst must be aware of possible matrix effects, and of the likelihood of interference from metabolites, co-prescribed or illicit drugs, or dietary or other sources. Manufacturers sometimes change their product characteristics without warning, and this may alter the likelihood of interference in substance misuse assays. Finally, be aware of possible unexpected effects if in-house modifications are made to assays – dilution of reagents, for example, may affect selectivity and sensitivity. All immunoassay results need to be verified using a physical technique such as GC-MS or LC-MS if they are to stand scrutiny.

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Section B
Separation Science

7 Separation Science: Theoretical Aspects

7.1 General introduction

A detailed understanding of separation science is essential in order to make best use of the wide range of techniques used in analytical toxicology. As discussed in Chapter 4, in most cases the analyte(s) have to be separated from the matrix prior to further separation via chromatographic or other means. Even then detection may involve separation as in MS, where ions originating from the analyte are separated on the basis of their mass:charge (*m/z*) ratio.

In chromatography and electrophoresis, a mixture of components to be separated is applied as a narrow initial zone or bolus to an appropriate *stationary phase* or, in the case of electrophoresis, a column or gel containing an electrolyte. In chromatography, a suitable flowing *mobile phase* or *eluent* is introduced to the mixture or eluent flow is otherwise initiated and the components of the mixture are separated by differential physiochemical interaction with the stationary phase. The stationary phase may be a porous solid such as silica gel (adsorption chromatography), or an immiscible or non-volatile liquid held on a suitable solid support (partition chromatography). The technique has many similarities to SPE (Section 4.2.3), which in some ways can be viewed as preparative chromatography. In electrophoresis, an electrical potential is applied across the column or gel and separation is by movement of ionized species to the appropriate electrode. A compendium of terms related to chromatography and associated areas is available (IUPAC, 1997).

Chromatography and electrophoresis have developed into a range of modes including CE, GC, LC [including so-called ultra (high) performance liquid chromatography, U(H)PLC], and TLC (Table 1.1). In TLC, a technique that is sometimes referred to as *planar chromatography* or *development chromatography* (Chapter 8), the mixture to be separated is usually applied in solution as a small discrete spot or band near to the bottom of the thin-layer plate (the origin). The application solvent is evaporated before the bottom edge of the paper or plate is placed in the liquid mobile phase (the eluent), which is drawn up the plate (the stationary phase) by capillary action. Several analyses may be performed in parallel. All the analytes are detected (normally visualized) at the end of the development process.

In most other forms of chromatography, a sample (or sample extract) is either added to the eluent, which may be a gas, a liquid, or a supercritical fluid, or placed on a support material or otherwise concentrated before the eluent is introduced. The eluent containing the analyte and other components of the sample/sample extract is then allowed or made to flow through or past a stationary phase supported within a column. The mobile and stationary phases are chosen such that different components of the sample have different affinities for each phase.

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

A component that has poor affinity for the stationary phase will pass through the column quite quickly, and *vice versa*. As a result of these differences in affinity, sample components become separated as they travel through the column. This process is called *elution* chromatography and analytes are detected sequentially as they elute from the column. In both development and elution chromatography sample transport is by continuous addition of mobile phase. Various modifications of these techniques are possible, e.g. development of a TLC plate in a second dimension using a different mobile phase.

7.2 Theoretical aspects of chromatography

In the simplest forms of GC and of LC, the stationary phase is simply a rigid material packed within a column through which the eluent flows, but more usually the stationary phase is coated or bonded directly to a column, or to particles of a rigid support material packed within the column. In the mid-1960s, discussion of the parallels between LC and GC suggested that use of smaller particles in LC would lead to better efficiency hence greater speed of analysis and better sensitivity/selectivity.

7.2.1 Analyte phase distribution

The components of a mixture become distributed between the stationary and mobile phases as they are transported through the stationary phase in the mobile phase. Differences between their distribution coefficients (K_D) result in separation:

$$K_D = C_s/C_m \tag{7.1}$$

where C_s is the molar concentration of a component in the stationary phase, and C_m is the molar concentration of the same component in the mobile phase. In development chromatography, the distance moved by the analyte in relation to the distance moved by the eluent ('solvent front') is called the *retention fraction* (R_f). In elution chromatography, the time between sample injection and an analyte peak reaching a detector at the end of the column is termed the *retention time* (t_R). The time taken for the mobile phase itself to pass through the column is called t_M (Figure 7.1). In LC, multiplying the retention time by the eluent flow rate (which can usually be considered to be constant) gives the retention volumes, V_M and V_R. V_M is known as the void volume of the column and is the volume of fluid that surrounds the packing material. Because gases are much

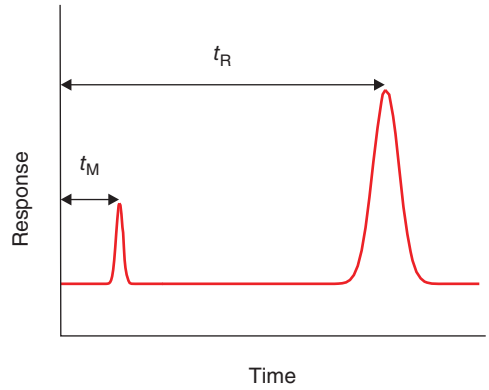

Figure 7.1 Elution chromatography: retention time

more compressible than liquids, the average flow rate in a GC column is less than the flow rate measured at the column outlet and the corrected retention volume is given by:

$$V_R^0 = \frac{3}{2}\left[\frac{(p_i/p_o)^2 - 1}{(p_i/p_o)^3 - 1}\right] V_R \tag{7.2}$$

where p_i and p_o are the inlet and outlet pressures, respectively. A different correction is required if the flow rate is measured at the column inlet rather than the outlet.

The term *retention factor (k,* sometimes called *capacity factor, k′)* is often used to describe the migration rate of an analyte on a column. The retention factor for analyte *A* is defined as:

$$k_A = \frac{t_R - t_M}{t_M} \tag{7.3}$$

Because analyte migration is defined in terms of the number of column volumes, this parameter is independent of column geometry and flow rate. One problem, though, is that it may be very difficult to measure t_M accurately – a small error here can give large errors in k especially with very short columns. In GC, t_M is normally taken as the first deviation of the baseline post-injection (it is assumed that some air gets into the system at the time of injection hence this is known as the 'air peak'), whilst in LC with UV detection, the first deviation of the baseline following the injection of a small quantity of non-retained solvent such as acetone is used as the t_M marker.

Accurate measurement of t_R, on the other hand, may be difficult with asymmetric peaks. If $k < 1$, accurate measurement of t_R may be difficult, and the analyte peak may be lost in the so-called 'solvent front' that is often seen with biological extracts. On the other hand, values of $k > 20$ are usually associated with long retention times and broad peaks. Temperature programming (raising the column oven temperature in a pre-planned way) in GC and gradient elution (altering the eluent composition systematically) in LC are ways of sharpening late-eluting peaks during the analysis and thereby extending the range of analytes that can be detected/measured. Ideally, in *isocratic* (constant eluent composition) LC and *isothermal* (constant column temperature) GC k should be in the range 2–5, or thereabouts.

7.2.2 Column efficiency

To obtain optimal sensitivity and selectivity, sharp, symmetrical peaks (so-called because of their appearance on a recording device) are the goal in elution chromatography, whilst small, discrete spots or bands are the aim in planar chromatography. Analyte peaks or bands that move most quickly in a chromatographic system tend to be sharper than those that elute later because there is less time for zone broadening (Section 7.2.3). The extent of zone broadening determines the *efficiency* of a column for a given analyte under the analytical conditions used.

For any meaningful comparison to be made between two columns, efficiency must be measured with the same analyte under the same conditions. Efficiency can be expressed either as the number of *theoretical plates* or *plate number* (N), or by the *height equivalent of a theoretical plate* (HETP, H). The theoretical plate model of chromatography postulates that the column contains a large number of separate layers or plates, a concept based on bubble-cap fractionation columns, in which the greater the number of plates in a given length, the greater the separation (Figure 7.2). Separate equilibration of the sample between the stationary and mobile phases is said to occur at each plate.

The width of a chromatographic peak is influenced by a number of random processes, each of which has its own variance, and so an ideal chromatographic peak will be of Gaussian shape

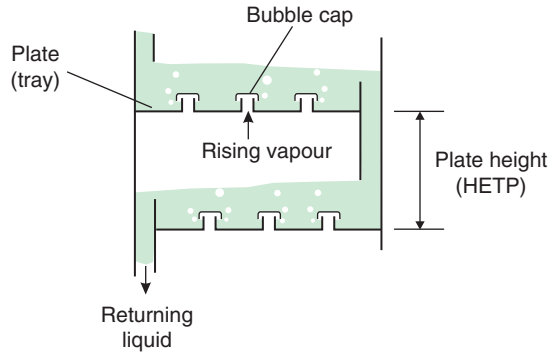

Figure 7.2 Schematic representation of a fractionating column showing the concept of plate height

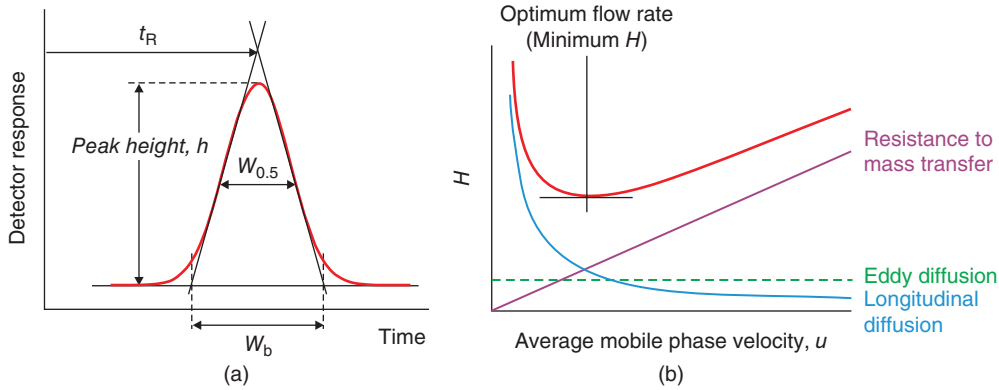

Figure 7.3 Chromatographic efficiency: (a) measurement of *H;* (b) effect of mobile phase velocity on *H*

with a variance, σ^2, equal to the sum of the total variances [Figure 7.3(a)]. There are two main ways to calculate *N*. The first is to estimate the 'peak width' ($W_b = 4\sigma$) by constructing tangents to the points of inflexion. The plate number is:

$$N = 16\left(\frac{t_R}{W_b}\right)^2 \tag{7.4}$$

A more practical method is to use the width of the peak at half the peak height ($W_{0.5} = 2.355\sigma$) so:

$$N = 5.54\left(\frac{t_R}{W_{0.5}}\right)^2 \tag{7.5}$$

The advantage of *H* is that it is independent of column length. If the length of the column is *L* cm, then:

$$H = L/N \tag{7.6}$$

An efficient column will have a small value of *H*. Efficiency can also be expressed as plates per metre:

$$\text{Plates m}^{-1} = \frac{100\,N}{L} \tag{7.7}$$

7.2.3 Zone broadening

The plate model assumes that analyte equilibration between the stationary phase and the mobile phase is virtually instantaneous. A more realistic description of the chromatographic process takes account of the time needed for analyte equilibration between the phases. The shape of a chromatographic peak is affected by the rate of elution, but it is also affected by the different paths available to solute molecules as they travel between particles of stationary phase. Considering the various mechanisms that contribute to band broadening gives the van Deemter equation:

$$H = A + B/u + C\,u \tag{7.8}$$

where u is the average linear velocity of the mobile phase and A, B, and C are factors ('terms') that contribute to band broadening.

7.2.3.1 Multiple path and eddy diffusion

Analyte molecules take different paths through the column at random. This will cause broadening of the solute band, because the different paths are of different lengths. The spreading is proportional to the particle size (diameter = d_p) and is independent of the mobile phase velocity. Van Deemter introduced a constant (2λ) to account for the inhomogeneity and quality of the packing so his expression for the multipath term contribution (the 'A term') was:

$$A = 2\lambda\, d_p \tag{7.9}$$

For an ideally packed column, $A = d_p$, i.e. $\lambda = 0.5$.

7.2.3.2 Longitudinal diffusion

Considering a band of analyte in a column, the concentration will be lower at the edges of the band than at the centre because the analyte diffuses out from the centre to the edges. The greater the amount of time that the band is in the column the greater will be the diffusion and hence the band broadening. High eluent velocities will decrease the effect of longitudinal diffusion by reducing the time the analyte is in the column, i.e. the contribution to H caused by longitudinal diffusion (the 'B term') is inversely proportional to u. Because the van Deemter equation was derived for packed column GC, the longitudinal diffusion in the stationary phase was negligible compared with that in the gas phase, so:

$$B = 2\gamma\, D_m \tag{7.10}$$

D_m is the diffusivity of the analyte in the mobile phase, and γ a correction factor for the geometry of the packing, which affects the diffusional path.

7.2.3.3 Resistance to mass transfer

The analyte takes time to equilibrate between the phases. If the eluent velocity is high and the analyte has a strong affinity for the stationary phase, then the analyte in the eluent will move ahead of the analyte in the stationary phase giving band broadening. The higher the eluent velocity, the larger will be the band broadening. The 'C term' used by van Deemter was:

$$C = \frac{8k}{\pi^2(1+k)^2}\,\frac{d_s^2}{D_s} \tag{7.11}$$

where D_s is the diffusivity of the analyte in the stationary phase and d_s is the thickness of the film of stationary phase. Again, because eddies in the eluent aided mixing, the resistance to mass transfer in the mobile phase was considered negligible and ignored.

Thus, the van Deemter equation can be written:

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{8k}{\pi^2(1+k)^2}\frac{d_s^2}{D_s}u \tag{7.12}$$

Despite the fact that the van Deemter equation was derived for packed column GC, the terms illustrate the importance of:

- particle size for fully porous particles (smaller particles give greater efficiency)
- the thickness of the porous layer for superficially porous particles (Section 10.4.1.5)
- the thickness of the stationary phase (thinner films give greater efficiency)
- eluent velocity.

If the van Deemter equation is to be used to explain chromatographic behaviour in LC, then a term for the resistance to mass transfer in the eluent must be included. Like the resistance to mass flow in the stationary phase, this is inversely proportional to u, and so the general form of the equation, although more complex, is the same as Equation (7.8). Van Deemter plots, in which H is plotted against eluent velocity, are very useful in ensuring that the mobile phase flow rate is optimal [Figure 7.3(b); Figure 7.4].

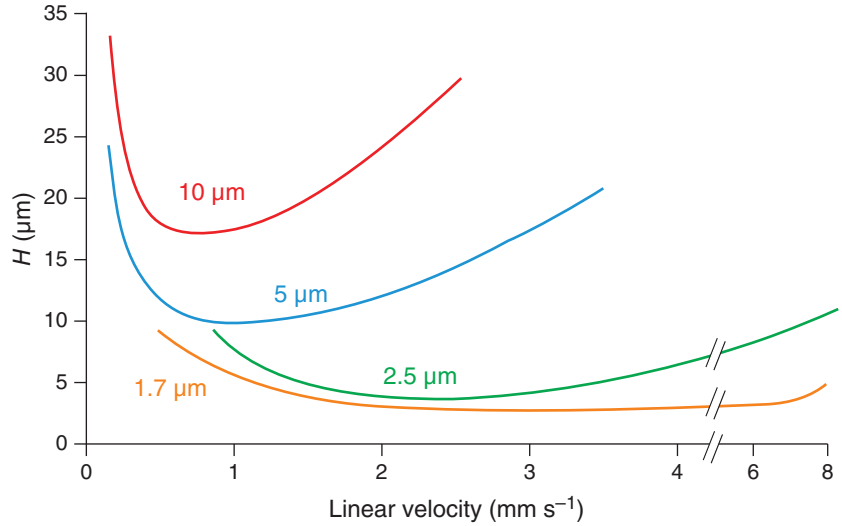

Figure 7.4 Effect of particle size and linear velocity on H using ~2 μm size packings

It is important to consider the effects of column diameter on the linear flow rate. If the column diameter is reduced by half, then the flow rate (mL min^{-1}) must be reduced to ¼ to maintain the original linear flow.

The Golay equation was formulated to describe dispersion in open tubular or capillary columns. Because there is no multipath term, it takes the form:

$$H = B/u + Cu \tag{7.13}$$

For a column of internal radius, r, it is:

$$H = \frac{2D_m}{u} + \frac{(1 + 6k + 11k^2)r^2}{24(1 + k)^2\, D_m}u + \frac{k^3 r^2}{6(1 + k)^2 K^2\, D_s}u \tag{7.14}$$

where K is the distribution coefficient of the analyte between the stationary and mobile phases. Whereas the size of the packing is a major contributor to band broadening in packed columns, it is clear that for capillary columns the resistance to mass transfer terms are proportional to the square of the radius.

The Giddings equation was derived using a random walk model and takes the general form:

$$H = \frac{A}{1 + (E/u)} + \frac{B}{u} + C \tag{7.15}$$

Where E = 'separation impedance'. Unlike the van Deemter equation, H becomes zero when there is no eluent flow, but as the flow increases the term E/u will tend to zero and the equation then takes the same form as the van Deemter equation.

The Knox equation is an empirically derived equation applicable to LC and is simpler than that of Giddings. H is replaced by the reduced plate height, h, which equals H/d_p (i.e. the plate height in terms of the particle size):

$$h = Av^{1/3} + \frac{B}{v} + Cv \tag{7.16}$$

and v is the reduced velocity and equals ud_p/D_m. The coefficient A normally ranges from 0.2 to 1.7 and decreases with increasing homogeneity of the packing. B ranges from 1.6 to 1.8, and C ranges from 0.05 to 0.03. Usually h has a minimum value of 2–3 at v = 2–3, so in LC, H = 2–3 d_p at the optimum. For example, if a 3 µm average particle size (aps) stationary phase is used, the optimum H is 6–9 µm, and in the case of a column 25 cm in length, the theoretical plate number that should be obtained is about 40,000.

7.2.4 Kinetic plots

If the plate number is plotted against the analysis time rather than against either linear velocity, or reduced velocity on the x-axis, the resulting curve describes the analysis time that generates the highest plate number for a particular column. Analysis time is a practical and useful tool when selecting a column, hence comparisons using kinetic plots can be used to ensure optimum performance. Kinetic plots can be used either to calculate the maximum number of plates that can be achieved in a given analysis time, or can be used to minimize the analysis time whilst generating a given number of plates.

For short analysis times, i.e. at high flow rates, the plate number will be low. As the analysis time increases, the plate number increases to the maximum for that column. Above this point, the plate number reduces because diffusion effects (the van Deemter B term) predominate. The minimum analysis time is determined by a predefined pressure limit – the maximum for either the column, or the LC pump – and forms the first point on a kinetic plot. If the experiment is repeated for columns of the same chemistry and particle size, but of increasing length, and a line fitted to these minimum analysis times, the resulting kinetic plot demonstrates the compromise between analysis time and plate number that can be achieved for a given column chemistry/particle size on a particular pump. These data can be used to compare columns, packings, etc.

Variants on kinetic plots (Desmet *et al.*, 2005) replace analysis time with 'plate time' (t_M/N), the amount of time taken to generate one theoretical plate, or t_M/N^2. A figure of merit used to

compare columns is the separation impedance, E,

$$E = \left(\frac{\Delta p_{\max}}{\eta}\right)\frac{t_M}{N^2} \tag{7.17}$$

and a plot of E as a function of N is minimal at the optimum plate number (Heaton & McCalley, 2014). Provided the pressure drop across the column (Δp) and eluent viscosity (η) remain constant, a plot of t_M / N^2 versus N will produce a minimum at the same value of N (Figure 7.5).

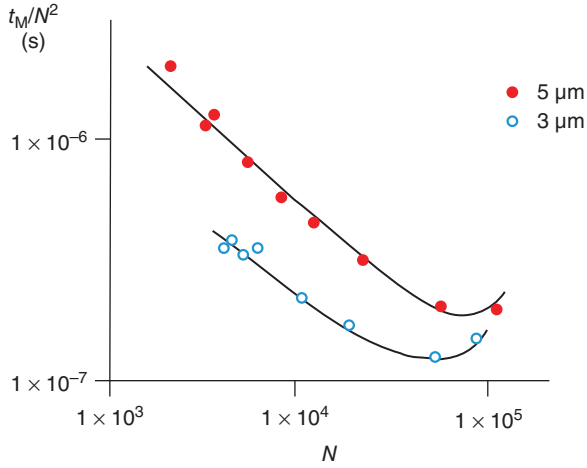

Figure 7.5 Kinetic plots (t_M/N^2 against plate number) for two packing materials. 5 µm and 3 µm Techsphere SCX using 35 mmol L^{-1} ammonium perchlorate in methanol, pH* 6.9, system pressure 200 bar (adapted from Morgan *et al*., 2010–with permission of John Wiley & Sons)

7.2.5 *Extra-column contributions to zone broadening*

Three factors should be taken into consideration: (i) sample volume, (ii) detector volume, and (iii) the connecting tubing between the injection valve and the column, and between the column and the detector. Band spreading due to the first two is proportional to their respective volumes. Generally, therefore, the volume injected should be kept as small as practicable. If detectors are connected in series, the detector with the largest internal cell (swept) volume should be placed last whenever possible. The internal diameter (d) of the connecting tubing can have a major effect, as spreading is proportional to d^4. Narrow-bore PTFE tubing is generally suitable for post-column connections in LC. Extra-column effects assume greater importance with small particle size columns and may negate the higher efficiencies attainable unless controlled.

7.2.6 *Temperature programming and gradient elution*

Another practically important variable in LC and, to an extent in GC, is 'peak capacity' i.e. the number of peaks that can be represented on a chromatogram. A typical packed LC column with $N = 5000$ plates yields a peak capacity between 17 for values of k from 0.2 to 2, and about 50 for k values from 0.5 to 20. However, when the sample contains a large number of analytes of differing volatilities or polarities, then temperature programming (GC) or gradient elution (LC) will be required, as discussed above (Section 7.2.1). Isothermal or isocratic analysis of such a

mixture would not only require very long analysis times, but also the late-eluting peaks would be so wide that they would be 'lost' in the baseline noise of the chromatogram.

With temperature programming, the analytes are condensed in a tight band at the top of the column and only begin to migrate when the temperature is high enough, i.e. some analytes are being separated whilst others are stationary. Thus, the time a particular analyte is actually moving in the column is similar to that of any other analyte and so the band spreading due to longitudinal diffusion (normally proportional to $t_R^{0.5}$) is very much reduced for later eluting peaks. The same principle applies to gradient elution LC, but in this case it is the eluent composition that is modified.

7.2.7 Selectivity

The column *selectivity factor*, α, describes the separation of two species (A and B) on the column. When calculating the selectivity factor, A always elutes before B, i.e. α is always >1.

$$\alpha = \frac{k_B}{k_A} \tag{7.18}$$

Although α describes the separation of band centres, it does not take into account peak width. Another measure of separation is provided by, *resolution (R):*

$$R = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B} \tag{7.19}$$

where W = baseline peak width. Baseline resolution is attained when $R = 1.5$ (Figure 7.6).

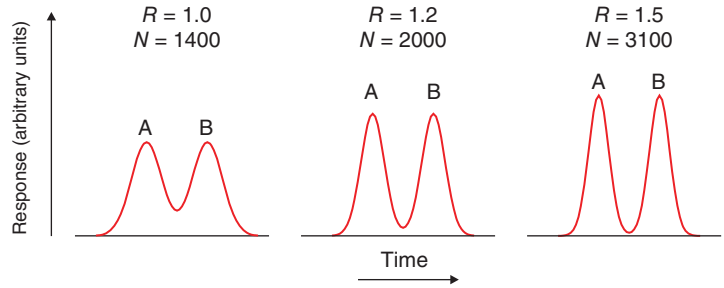

Figure 7.6 Effect of efficiency on resolution of A and B. Values calculated using $k_A = 10$, $\alpha = 1.2$; N calculated for peak B

It is also useful to relate resolution to the number of theoretical plates (N), the selectivity factor (α), and the retention factors (k) of the two solutes.

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_B}{k_B + 1}\right) \tag{7.20}$$

To obtain good resolution, all three terms of Equation (7.19) must be maximized. Increasing N by lengthening the column will cause some increase in band broadening. Alternatively, N can be increased by decreasing the size of the stationary phase particles, but there are practical limits to this because use of smaller particles increases the pressure needed to maintain eluent flow. In GC especially, this is self-limiting as increased gas pressure simply compresses the mobile phase. This is the great theoretical advantage of CE and CEC because eluent flow is not dependent on eluent inlet pressure.

An alternative measure of column efficiency is provided by the separation number (Trennzahl, *TZ*):

$$TZ = \frac{t_{R_B} - t_{R_A}}{w_{0.5_A} + w_{0.5_B}} - 1 \tag{7.21}$$

TZ represents the number of peaks that can be inserted between the peaks of two consecutive hydrocarbon homologues in a chromatogram and is related to resolution:

$$TZ = \frac{R}{1.18} - 1 \tag{7.22}$$

Separation number is very useful in temperature programming in GC and gradient programming in LC where conventional (isothermal or isocratic) measures of column efficiency cannot be used. If $TZ = 0$, an additional peak will not fit between the two peaks of interest, if $TZ = 1$ one peak will fit, and so on.

On a practical level, chromatographic separations can often be improved by changing the column temperature (mainly GC) or the eluent composition (LC). The selectivity factor, α, can also be manipulated to improve separations. Sometimes changing the composition of the stationary phase (mainly GC, but also LC) or using eluent additives such as a species that complexes with one of the analytes in the stationary phase are effective in improving resolution and/or peak shape.

It is important to understand that matrix components or other analytes may affect separations. In TLC co-extracted sample components, for example, may influence the analysis if present at much higher concentration than other analyte(s). In GC, LC, and CE the injection solvent, or the injection volume in the case of headspace GC, or indeed the presence of other analytes in high concentration, may alter the retention characteristics of other components of a mixture.

7.2.8 *Peak asymmetry*

The ideal chromatographic peak will be a perfect Gaussian shape, reflecting the additive combination of the random motions of the analyte in the system. In practice, symmetrical peaks are not obtained, and their deviation from that of a true Gaussian peak – the peak asymmetry – can be measured and compared. To do this, the peak is usually measured as shown in Figure 7.7.

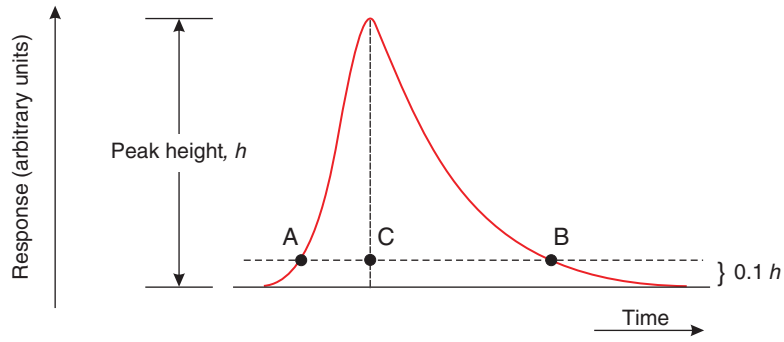

Figure 7.7 Measurement of peak asymmetry

Here, AC and CB are measured at 10 % of the total peak height (h) above the baseline, and the asymmetry factor, A_s, is calculated thus:

$$A_s = \frac{CB}{AC} \tag{7.23}$$

A Gaussian peak will have A_s equal to unity. Tailing peaks, usually caused by adsorption of analyte to 'reactive sites' on the column or column walls, will produce A_s values >1. 'Fronting' ('front-tailing', 'shark's fin peak') peaks, often a result of either overloading the column with analyte, or eluent flow through channels within the column, will have A_s <1. Peak shape can be considered poor if A_s exceeds 1.5–2, depending on the type of analysis and the conditions used, due to its impact on the resolution of components and the likely error in the measurement of retention times. Fronting peaks lead to an overestimate of the true retention time, whilst tailing gives an underestimate of the retention time compared with that of a Gaussian peak.

Column efficiencies can also be assessed in terms of the variance, σ^2 of a chromatographic peak, assuming that it is Gaussian, and equations have been proposed to include peak asymmetry, such as the Dorsey–Foley equation (Heinisch *et al.*, 2008):

$$\sigma^2 = w_{0.1}^2 \times \frac{A_{s_{0.1}} + 1.25}{41.7} \tag{7.24}$$

where $w_{0.1}$ and $A_{s_{0.1}}$ are the peak width and peak asymmetry at 10 % of the peak height, respectively.

7.3 Measurement of analyte retention

7.3.1 Planar chromatography

Retention factor (R_f, also known as retardation factor) is normally used to record TLC retention data. R_f is defined as:

$$R_f = \frac{\text{Distance the analyte has travelled from the origin}}{\text{Distance the solvent front has travelled from the origin}}$$

Thus, R_f is a fraction (0.30, 0.75, etc.) and is (reasonably) independent of the length of the chromatogram (distance the solvent front has travelled from the origin). hR_f values (R_f x 100) are even more convenient, especially if the length of the chromatogram is standardized at 10 cm (100 mm) as then hR_f is simply the distance (mm) the analyte has travelled from the origin.

There are many factors that influence the reproducibility of hR_f values including: (i) the TLC plate, (ii) the amount of analyte applied to the plate, (iii) the development distance, (iv) the degree of tank saturation with eluent vapour, (v) the ambient temperature, and (vi) the effect of co-extracted compounds (matrix effects). The influence of some of these factors can be minimized if reference compounds (standards) are analyzed with each sample.

When calculating hR_f values for unknown substances, it is usual to calculate a 'corrected hR_f value' from experimentally observed values of sample and reference compounds (Box 7.1). Alternatively, a graph of accepted literature values of hR_f and observed values for the reference standards (ideally four, equally spaced on the chromatogram) can be plotted against the observed values. The corrected value for the unknown can thus be obtained by interpolation.

Box 7.1 Calculation of corrected hR_f values

Calculation of corrected hR_f for an unknown analyte, U with an observed hR_f of U_{obs}. If U_{obs} is between two standards A and B, with uncorrected hR_f values of A_{obs} and B_{obs}, respectively then the corrected value for the unknown is:

$$\text{corrected } hR_f \text{ of unknown (U)} = A_{lit} + \left(\frac{B_{lit} - A_{lit}}{B_{obs} - A_{obs}} \right) (U_{obs} - A_{obs})$$

where A_{lit} and B_{lit} are the accepted literature (database) values for the hR_f of A and B

7.3.2 *Elution chromatography*

Retention factors (k), absolute retention times (volumes), and retention times relative to the retention of a given compound (usually an ISTD) can be useful ways of recording retention data in GC. However, the Kovats retention index provides a method of recording retention data that is independent of eluent flow rate, column length, phase loading, and operating temperature. Straight-chain (normal) hydrocarbons are assigned an index of 100 times the number of carbon atoms in the molecule (e.g. decane = 1000). The retention index, RI_x, of a given analyte, x, is calculated by difference from the retention indices of the normal alkanes eluting before and after the analyte. For isothermal elution:

$$RI_x = 100z + 100\frac{\log t'_{R_x} - \log t'_{R_z}}{\log t'_{R_{(z+1)}} - \log t'_{R_z}} \tag{7.25}$$

where $t'_R = t_R - t_M$. Retention indices can also be calculated from data generated on a temperature programme by applying the following formula during individual ramps of the programme:

$$RI_x = 100z + 100\frac{t_{Rx} - t_{R_z}}{t_{R_{(z+1)}} - t_{R_z}} \tag{7.26}$$

where z = number of carbon atoms in the alkane eluting before x, t_{Rx} = retention time of x, t_{Rz} = retention time of the alkane, and $t_{R(z+1)}$ = retention time of the alkane with $z + 1$ carbon atoms eluting after x. Note that accurate measurement of t_M is not required for Equation (7.26).

A practical problem in the use of retention index data in GC is that normal hydrocarbons do not give particularly good responses on MS and do not respond on NPD, hence attempts have been made to construct retention index schemes based on, for example, trialkylamines or nitroalkanes. However, in practice alkylamines are not pleasant compounds to work with and such schemes have found little favour. Thus, if GC retention index data are to be used an effluent splitter system to an FID will be needed and allowance must be made for the MS vacuum in altering retention times *en route* to the MS. This being said, published retention indices based on a solution containing typical drugs generated using a PDMS capillary column are more useful for compound identification in analytical toxicology, especially when EI spectra are non-specific, or when using selected ion monitoring (SIM) (de Zeeuw *et al*., 1992; Maurer *et al*., 2016).

The introduction of 'retention time locking' (RTL) for capillary GC (Agilent) has prompted a re-evaluation of the use of retention index data in STA. The long-term precision of three retention parameters: the absolute retention time, the retention time relative to dibenzepin, and a retention index based on the alkylfluoroaniline series, were studied with 14 basic drugs on HP-5 (Agilent; Table 9.2) and DB-17 (J&W, equivalent to OV-17, Table 9.2) columns with and without the use

of RTL. Using the constant flow mode, RTL gave better reproducibility with all three retention parameters when compared to the non-RTL method on both columns. RTL offered a significant advantage, not only within a single instrument, but also between methods, with RSDs of <0.1 % in relative retention time (Rasanen *et al*., 2003).

Many attempts have been made to develop a suitable retention index system for LC using, for example, homologous series of alcohols, ketones, or nitroalkanes, but in practice such methods offer no advantage over retention factors and other simpler ways of expressing retention in LC.

7.4 Summary

Chromatographic techniques, notably GC and LC, and to an extent SFC and TLC, are of unrivalled importance in analytical toxicology as discussed in the following chapters, but must be used with due care and attention to detail if reliable results are to be obtained. An appreciation of the theoretical aspects of chromatography as presented here is important in making best use of the resolving power of these systems. Capillary electrophoretic techniques, whilst of value in pharmaceutical QC and in separating enantiomers on an analytical scale, as discussed in Chapter 12, have neither the sensitivity nor the mechanical strength needed to provide robust systems for trace analysis.

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8 Planar Chromatography

8.1 Introduction

Planar chromatography includes paper chromatography, thin-layer chromatography (TLC), and high-performance thin-layer chromatography (HPTLC). The mobile phase, generally a mixture of organic solvents, moves through the stationary phase by capillary action, or in the case of forced-flow chromatography, the flow is maintained by either centrifugal force, or pumping (Section 8.3.1). Paper chromatography is now rarely encountered, but had the advantage that it could be performed either in an ascending mode, with the paper supported such that the mobile phase rises up the paper, or in the descending mode. In the latter mode, the paper could be continuously eluted to separate slowly migrating materials.

With TLC a uniform layer of stationary phase (usually hydrated silica gel, $SiO_2 \cdot nH_2O$) is held on a rigid or semi-rigid support, normally a glass, aluminium, or plastic sheet – the 'plate'. TLC is an inexpensive, robust, and portable technique that is relatively simple to perform, particularly if plates are dipped rather than sprayed for identification purposes (Box 8.1). Unless the analyte is volatile, everything that has been applied will be somewhere on the plate, even if it remains at the origin or elutes with the solvent front. Thus, TLC is useful for checking the purity of reference materials, particularly radioactively labelled material when the spots may be scraped off, or for plastic- or foil-backed plates, cut into segments for counting radioactivity.

Box 8.1 Application of TLC in analytical toxicology

- Robust qualitative technique
- Batch analysis – many extracts can be analyzed together
- Primarily used for urine, although extracts of other samples can be analyzed
- Non-destructive: can usually recover analyte if plate not treated with visualization reagent
- Does not require complex equipment (but may need fume cupboard or hood)
- Relatively low sample capacity – chromatographic system easily overloaded
- Quantitative measurements are possible using densitometric scanning

With advances in stationary phases for LC, HPTLC was a natural development, taking advantage of smaller particle sizes to obtain higher resolution with faster separation times on smaller plates. HPTLC can be performed in the same way as TLC, but using a smaller plate size has made instrumentation easier so that sample application, development, and visualization can be automated, leading to reliable quantification. Coupling HPTLC and MS, particularly the use of atmospheric ionization interfaces, allows high spatial resolution with identification as well as quantification (Section 13.7.2).

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

TLC and HPTLC remain widely used in pharmaceutical development (Shewiyo *et al*., 2012) and in the analysis of complex mixtures such as natural products, foods, and environmental samples. There are internationally accepted formal requirements/guidelines for validating TLC and HPTLC procedures. In addition, guidance on quality assurance procedures is available (Renger *et al*., 2011). This being said, TLC/HPTLC are labour-intensive techniques and are not readily automated. Although good results can be obtained and resource requirements are less than for GC and LC, these latter techniques are more robust in practice and have greater selectivity and sensitivity, and are thus preferred in STA.

8.2 Qualitative thin-layer chromatography

TLC can be a powerful qualitative technique when used together with some form of sample pre-treatment such as LLE (Section 4.2.2). However, some separations can be difficult to reproduce, even when they are performed in the same laboratory. In some respects, TLC is an extension of the colour tests discussed in Section 5.3 because the colours formed with various reagents form the basis of compound identification. However, incorporation of (i) a solvent extraction and concentration step, and (ii) a chromatographic step enhances both sensitivity and selectivity. If required, unreacted zones, i.e. zones not treated with a visualization reagent, can be removed and prepared for GC, LC, or MS analysis.

TLC of solvent extracts of urine, stomach contents, or scene residues has been used widely in STA and has also been used for the detection and identification of a number of specific compounds and groups of compounds. Generally, however, it is unwise to rely solely on TLC without corroboration of the results by GC-MS and/or LC-MS. This is because the resolving power of TLC is limited and the interpretation of the chromatograms obtained is, to an extent, subjective. HPTLC has superior resolving power when compared with conventional TLC (Section 8.3), and the combination of efficient chromatography with colour visualization can confer good selectivity.

It is important to note that many spray reagents used in analyte visualization are extremely toxic and spraying should ALWAYS be carried out in a suitable fume cupboard or hood. Disposal of sprayed TLC plates must also be undertaken with due care after recording the results. Some practical points are summarized in Box 8.2.

Box 8.2 Practical aspects of TLC in analytical toxicology

- Difficult to obtain reliable TLC plates – pretest a plate from every batch purchased
- Difficult to automate
- If developing solvent uses ammonium hydroxide then concentrated ammonia (SG 0.88, 28 % w/v) *MUST* be used; loss of ammonia from reused solvent is a frequent cause of inconsistent results
- Use ‘saturated’ TLC tanks to obtain reproducible results
- Must score columns on large plates for good results
- Many spray reagents are very toxic – TAKE CARE!!

8.2.1 Thin-layer plates

On a standard TLC plate the stationary phase is normally a uniform layer (0.25 mm thick) of silica gel (20 µm average particle size). The usual size of the plate is 20 × 20 cm, although smaller

sizes may be used. A large number of different plates are available commercially, varying in size, layer thickness, and composition. TLC plates can also be prepared in the laboratory from silica gel containing an appropriate binding agent (silica gel G contains gypsum – hydrated calcium sulfate – as binding agent) by using a simple slurry-spreading apparatus. Such plates generally give silica layers that are much more fragile than those of commercially available plates. In addition, chromatographic performance tends to be much less reproducible.

Stationary phases are typically silica gel, alumina, or cellulose. Plates incorporating a fluorescent indicator are available such that UV absorbing compounds can be located as dark spots on a fluorescent background before spraying with visualization reagents if required. Plates containing sodium hydroxide or potassium hydroxide are available (or plates can be soaked in methanolic potassium hydroxide and allowed to dry) to improve the chromatography of some basic compounds when using certain solvent systems. However, in general addition of concentrated ammonium hydroxide (SG 0.88) to the mobile phase has the same effect. When using developing solutions with a high water content, plates with organic binder may be used rather than gypsum ('wettable plates'). However, the binder will char if the spots are to be visualized by spraying with concentrated sulfuric acid.

Experience suggests that it is best to standardize on a particular brand of commercially available plates, such as Silica gel 60 F_{254} (E. Merck, Darmstadt, Germany). However, even with commercial plates batch-to-batch variations in retention, and also in sensitivity with certain analyte–spray reagent combinations, may be encountered. It is important to adhere carefully to the prescribed method with regard to, for example, activation (i.e. heating at 100 °C for 30 min before use), saturation of the developing chamber (or not) with eluent vapour, and development distance.

8.2.2 Sample application

Some commercially available plates are supplied with a special adsorbent layer to simplify application of the sample. Normally, however, the sample is loaded directly onto the silica gel layer. The plate should be prepared by marking the origin by drawing a light *pencil* line at least 2 cm from the bottom of the plate – care should be taken not to disturb the silica surface in any way. A line should then be drawn on the plate 10 cm above the origin to indicate the optimum position of the solvent front; other distances may be used if required. It is advisable when using 20 × 20 cm plates to score columns *ca.* 2 cm wide vertically up the plate leaving scored margins on either side of the plate to minimize 'edge effects' as discussed below.

The samples and any standards should be applied at the origin in the appropriate columns. Sample loading should be performed carefully using a micropipette or syringe so as to form 'spots' no more than 5 mm in diameter. If larger spots are produced, then resolution will be impaired when the chromatogram is developed. The volume of solvent applied should be kept to a minimum; typically 5–10 µL of solution containing *ca.* 10 µg of analyte is suitable. Sample extracts reconstituted as appropriate should be applied first followed by the required standard(s) or mixtures of standards; applying the sample extracts *before* the standards minimizes the risk of cross contamination. The use of a non-polar solvent to apply the spots will result in small concentrated spots as the analytes tend to be adsorbed onto the silica. However, this is not always practicable, and methanol will usually prove satisfactory. The spots should be dried with a stream of air or nitrogen prior to chromatography. Warm air blowers should be used with care, having regard for the stability and volatility of analytes such as amfetamines and the plate allowed to cool before development is commenced.

8.2.3 *Developing the chromatogram*

Glass TLC development tanks, in a range of sizes to suit different TLC plates, are available from many suppliers. Such tanks normally have a ground glass rim, which forms an airtight seal with a glass cover plate. A small amount of silicone lubricant jelly may be used to secure the seal. Some tanks have a well at the bottom to reduce the amount of solvent required. Tanks should be lined with filter paper or blotting paper on three sides and the solvent added at least 30 min before the chromatogram is to be developed to saturate the atmosphere with solvent vapour because this promotes reproducible chromatography. Faster separations are possible with saturated tanks, and the results are less dependent on the chromatographic conditions, such as the dimensions and shape of the tank and the ambient temperature.

Some TLC mobile phases consist of a single solvent, but most are mixtures. One of the most widely used mobile phases in analytical toxicology is ethyl acetate:methanol:concentrated ammonium hydroxide, SG 0.88 (85+10+5), 'EMA'. It is important to prepare mobile phases daily because the composition may change with time due to evaporation or chemical reaction. In particular, loss of ammonia not only from the mobile phase, but also from reagent bottles once opened has caused many problems.

The chromatogram is developed by placing the loaded plate in the equilibrated tank, ensuring that the level of the solvent is above the bottom edge of the silica layer on the plate, but below the level of the 'spots' applied to the plate, and quickly replacing the lid (Figure 8.1). The plate should be observed to ensure that the solvent front is being drawn up uniformly. The mobile phase movement is primarily due to capillary forces and, because the stationary phase is dry, the profile of the mobile phase is concave (the shape of an advancing meniscus). Consequently, the solvent front will show a degree of curvature, particularly at the edges of the plate; more serious curvature or bowing may be observed if the atmosphere in the tank is not sufficiently saturated with solvent vapour. This 'edge' effect can be minimized by dividing the plate into *ca.* 2 cm columns as discussed above.

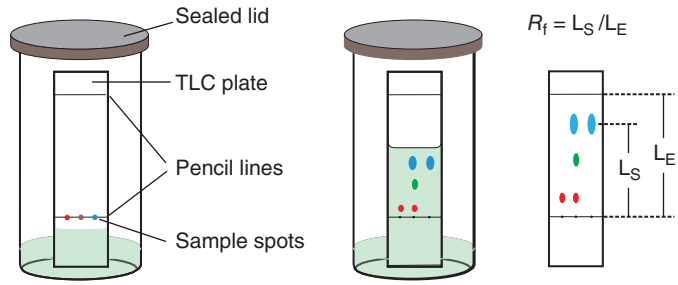

Figure 8.1 Thin-layer chromatography: schematic and calculation of R_f values

The velocity of the mobile phase decreases as it progresses up the plate because of its viscosity, loss of solvent through evaporation, and the increasing weight of the mobile phase held on the plate. Forced-flow systems (Section 8.3.1) overcome this by maintaining a constant flow rate (Figure 8.2).

The chromatogram should be allowed to develop for the intended distance, usually 10 cm from the origin, after which the plate should be taken from the tank, placed in a fume cupboard or under a fume hood, and allowed to dry. Drying may be hastened by blowing warm air (from an explosion-protected hair dryer) over the plate until all traces of solvent have been removed,

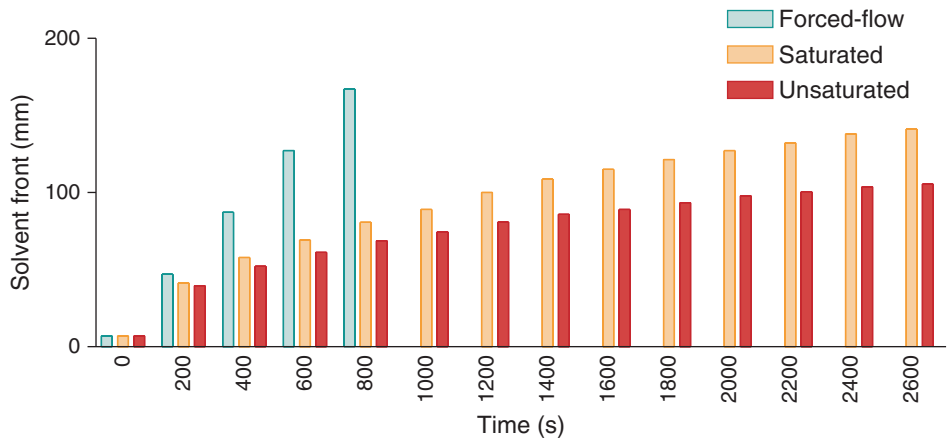

Figure 8.2 Comparison of the migration of the solvent front in saturated and unsaturated chambers. With forced-flow chromatography the solvent front moves at constant rate. Eluent: chloroform (from Kalász & Báthori, 2001–reproduced with permission of Elsevier)

although there is again the risk of losing volatile analytes such as amfetamine. Ensuring that the plate is dry before visualization can be especially important with ammoniacal mobile phases because the presence of residual ammonia affects the reaction with certain spray reagents.

8.2.4 Visualizing the chromatogram

When the chromatogram has been developed and the plate dried, the chromatogram should be examined under UV light (254 and 366 nm) in a suitable enclosed box and the positions of any fluorescent compounds noted. If a fluorescent marker has been added to the silica, many substances present appear as dark areas against a fluorescent background as noted above. Prior use of a visualization reagent normally quenches the fluorescence of the indicator.

In clinical and forensic toxicology, the use of chromogenic reagents (Table 8.1) generally gives more useful information than simply examining the plate under UV light. Plates can be 'dipped' in reagent, but unless special precautions are taken the structure of the silica tends to be lost and the chromatogram destroyed. Thus, the reagent is normally lightly applied as an aerosol using a commercially available spray bottle attached to a compressed air or nitrogen line. Varying the inlet pressure varies the density of the aerosol and thus the amount of reagent reaching the chromatogram in a given time.

When spraying a developed plate, it can be difficult to obtain even coverage, particularly where the plate is standing on a surface and excess spray reagent may be drawn up the plate by capillary action thereby destroying the lower part of the chromatogram. The simple expedient of inverting the plate before spraying overcomes this problem. Glass plates can be used to 'mask' portions of the plate if selected columns are to be sprayed with different reagents (Box 8.3, Figure 8.3). Alternatively, if plastic- or aluminium-backed plates are used then the appropriate regions can be cut-up and sprayed separately.

The appearance of certain compounds may change with time and so it is important to record results as quickly and carefully as possible, noting any time-dependent changes. A standardized data recording system is valuable for reference purposes – ideally a digital camera should be used and the files archived.

Table 8.1 Examples of chromogenic reagents

Reagent	Constituents	Target
Diazotized 4-nitroaniline	(i) 4-Nitroaniline (0.7 g) in hydrochloric acid (1 mol L^{-1}, 100 mL) (ii) Sodium nitrite (1 g) in deionized water (100 mL) Add 4 mL (i) dropwise to 5 mL ice-cold (ii) and make up to 100 mL with deionized water	Phenols
Dragendorff	(i) Bismuth subnitrate [basic bismuth nitrate, $4BiNO_3(OH)_2 \cdot BiO(OH)$] (0.85 g) dissolved in glacial acetic acid (10 mL) and deionized water (40 mL) (ii) Potassium iodide (8 g) in deionized water (20 mL) Mix 5 mL (i) and 5 mL (ii) and glacial acetic acid (20 mL). Make up to 100 mL with deionized water	Amines and quaternary ammonium compounds, particularly alkaloids
Folin–Ciocalteau	Available as a stock solution (2 mol L^{-1}). Dilute with deionized water or ethanol:deionized water (1+1) before use	Phenols
FPN (ferric perchloric nitric)	Iron(III) chloride (5 % w/v, 5 mL), perchloric acid (20 % w/w, 45 mL), nitric acid (50 % v/v, 50 mL)	Chiefly phenothiazines
Ninhydrin	Ninhydrin (0.3 g), pyridine (5 mL), methanol (95 mL)	Primary amines, amino acids
Nitroprusside–acetaldehyde	(i) Sodium nitroprusside (5 g) acetaldehyde (10 mL) + deionized water to 100 mL (ii) Sodium carbonate (5 g) in deionized water (100 mL) Mix equal volumes of (i) and (ii) before use	Secondary amines

Box 8.3 Visualizing reagents for acidic and basic extracts (see Figure 8.3)

- Mercury(I) nitrate reagent (A)
  - Mercury(I) nitrate (1g) + deionized water (100 mL)
  - Add concentrated nitric acid until the solution is clear
- Acidified iodoplatinate reagent (B)
  - Platinum(II) chloride (0.25g) + potassium iodide (5 g) + concentrated hydrochloric acid (5 mL)
  - Make up to 100 mL with deionized water
- Mandelin reagent (C)
  - Ammonium vanadate (finely powdered, 0.5 g) + concentrated sulfuric acid (100 mL)
  - Shake well before use
- Aqueous sulfuric acid (D)
  - Water (50 mL) + concentrated sulfuric acid (50 mL)
  - Add acid slowly with care and cooling

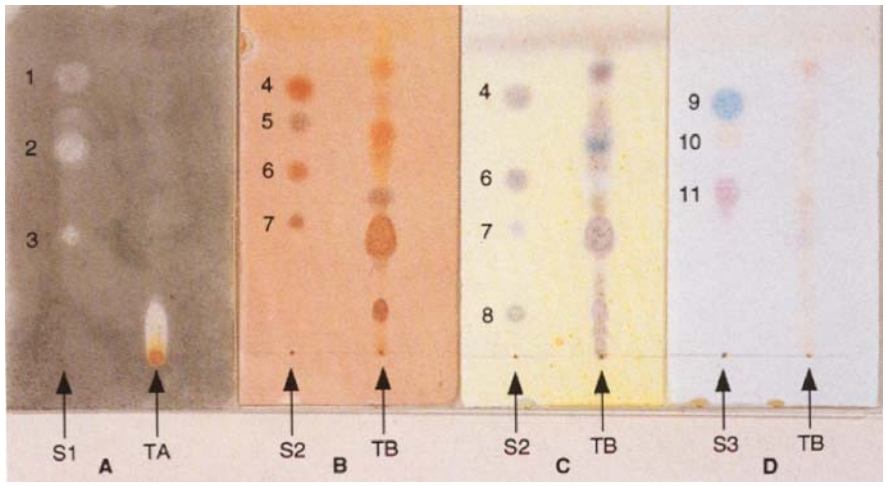

Figure 8.3 Example of differential visualization of a TLC plate (Flanagan *et al*., 1995). Key: TA = acidic extract of urine, TB = basic extract of urine. Spray reagents A–D as described in Box 8.3. Reference compounds: 1: amobarbital, 2: phenobarbital, 3: theophylline, 4: amitriptyline, 5: nicotine, 6: nortriptyline, 7: codeine, 8: mefenamic acid, 9: thioridazine, 10: trifluoperazine, 11: perphenazine. Stationary phase: silica gel, 20 μm aps. Mobile phase: ethyl acetate:methanol:concentrated ammonium hydroxide (85+10+5)

Various combinations of spray reagents may be used to aid identification of unknown substances and some agents may be useful for identifying different functional groups. For example, Dragendorff reagent gives orange to orange-red spots with amines, in particular tertiary amines and quaternary ammonium compounds, and so traditionally has been used to detect alkaloids. Ninhydrin reacts with primary amines, including amino acids. Secondary amines can be visualized using nitroprusside:acetaldehyde. Phenols usually react strongly with diazotized 4-nitroaniline forming azo dyes. FPN is usually used to identify phenothiazines and related compounds.

In the example shown in Figure 8.3, areas of the plate were masked with thin glass plates and each region sprayed separately. However sequential spraying with different reagents is a valuable identification technique. Quite complex reactions may be performed by spraying sequentially, for example Dragendorff reagent can be sprayed after the plate has been sprayed with ninhydrin and FPN reagent.

Benzodiazepines in urine have been identified after acid hydrolysis to aminobenzophenones. Those with a primary aromatic amine may be localized using the Bratton–Marshall reaction. Analytes are diazotized with nitrous acid and coupled with *N*-1-naphthylethylenediamine to give highly coloured azo dyes, the excess nitrous acid having been removed by reaction with ammonium sulfamate. Benzophenones from benzodiazepines with N_1-alkyl substituents can be dealkylated, for example by exposure to UV light (Schütz, 1989) before reaction with nitrous acid to ensure increased sensitivity (Figure 8.4).

Clearly, the method cannot distinguish between benzodiazepines that are hydrolyzed to a common benzophenone (diazepam, ketazolam, and temazepam, for example). However, the migration of 5-chloro-2-methylaminobenzophenone is different from that of the 2-amino-5-chlorobenzophenone formed from nordazepam and oxazepam. Generally, the spots

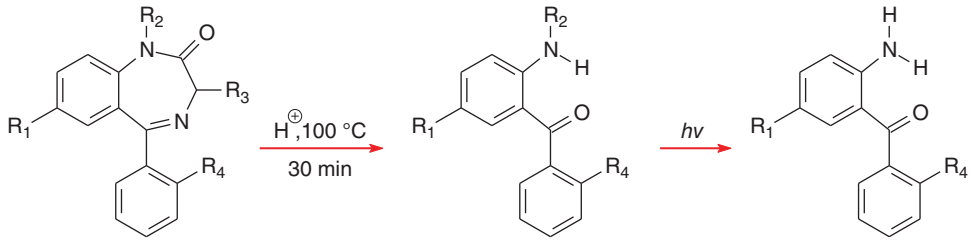

Figure 8.4 Hydrolysis of benzodiazepines and dealkylation of *N*-alkylbenzophenones

are purple in colour, but the 7-aminobenzodiazepine metabolites of nitrazepam and clonazepam, for instance, give diaminobenzophenones that appear blue with the Bratton–Marshall reagent. Interference from other primary amines such as sulfonamides can be minimized by using relatively non-polar eluents. The chromatography of benzophenones and related hydrolysis products of many benzodiazepines, including clonazepam, lorazepam, flunitrazepam, prazepam, and quazepam, has been described (Schütz, 1989).

8.2.5 *Interpretation of thin-layer chromatograms*

The correct interpretation of TLC data requires practice and experience, but there are some basic guidelines (Box 8.4). A major complication is that the chromatography of compounds present in extracts of biological samples is often different from that of pure substances (matrix effects). It is not practical to prepare standard solutions in every type of matrix that may be encountered, but assuming that there is enough sample, addition of a small quantity of a suspected component to an extract followed by reanalysis may help decide if that particular component is present in the extract. If the added material and the unknown do not co-chromatograph this is a powerful way of eliminating the proposed identification.

Box 8.4 Analytical toxicology: interpretation and storage of TLC data

- Analyze known reference compounds on the same plate as unknowns
  - Spot the extracts first!
- R_f data are not very reproducible
  - Compare results with authentic compound for uncommon drugs/poisons
  - Analyze on same plate as the unknown
- Interpretation depends on the complexity of the case and the experience of the operator
  - Many basic drugs give very similar colour reactions with certain sprays
  - Always correlate with GC, LC, and immunoassay data
- Difficult to store developed chromatograms – use digital photography

Identification of candidate molecules for further investigation can be made by the judicious use of a combination of development systems and visualization reagents. However, a problem with using colour as a means of analyte identification is describing a given colour unambiguously. The colour produced by fluphenazine with FPN reagent, for example, has been described as cameo, salmon, orange, amongst other colours. The perception of a given colour is also likely to change with the amount of analyte present. It is also noteworthy that metabolism, particularly

hydroxylation, may give rise to a different colour from that given by the parent compound when the plate is sprayed with a particular reagent.

In interpreting TLC results, as in any analytical toxicology results, it is important to understand the nature of the sample and chemistry of the analyte(s). Usually, only small quantities of very lipophilic compounds are excreted in urine, although their metabolites may be present at high concentrations. Thioridazine, for example, is prescribed as a racemate and because sulfoxidation and *N*-oxidation introduce additional chiral centres, this gives rise to diastereomeric metabolites that often chromatograph as characteristic pairs of spots (Flanagan *et al*., 1995; plate 11).

8.2.6 TIAFT-DFG R_f data compilation

The second edition of this publication (TIAFT-DFG, 2006) provides R_f values for some 1800 toxicologically relevant substances on 10 TLC systems that can be used for general screening purposes. There are also data on special systems for the analysis of pesticides and benzodiazepine hydrolysis products (benzophenones), in urine. Much of this information is available from other sources (Moffat *et al*., 2011).

8.2.7 Toxi-Lab

Toxi-Lab AB and Toxi-Lab AB-Plus (DRG Diagnostics, https://www.drg-diagnostics.de/42-1-TOXI-LAB+Drug+Identification+System.html) is a standardized TLC system that is available in kit form together with a compendium of colour plates showing R_f values, colour reactions, and additional information to facilitate interpretation (AB System). Cellulose impregnated with silica takes the place of the conventional TLC plate – a practical advantage is that dipping in visualization reagents can be used obviating the need for a fume hood.

Over 700 analytes and their metabolites have been documented for ease of identification. However, as with TLC in general, problems can arise when attempting to differentiate compounds with similar mobility and colour reactions, especially if more than one compound is present.

8.3 Quantitative thin-layer chromatography

There are two major approaches to quantification in TLC: either (i) continuous flow or (ii) quantification on the plate. If a densitometer is not available, then spots may be scraped from the plates and the analytes eluted for quantification. In metabolic studies, radiolabelled compounds are used frequently, and analytes can be located on TLC plates (for example by autoradiography) and removed for scintillation counting, or located and quantified by radioactive scanning. TLC is particularly useful for metabolic and toxicological studies during product development as all the metabolites in an extract will be somewhere on the plate, assuming that they are not volatile and hence may have been lost by evaporation.

8.3.1 Forced-flow planar chromatography

Rather than rely on the capillary action to draw eluent up the plate, several ways of ‘driving’ the flow have been described (Kalász, 2015), including rotational planar chromatography in which centrifugal force is used, electroplanar chromatography, which is the TLC equivalent of capillary electrochromatography (Section 12.5.2) and overpressured-layer chromatography (OPLC).

OPLC, introduced in the 1970s, has been given a new lease of life since 2000 with the introduction of improved apparatus and a change of name; the acronym now stands for Optimum Performance Laminar Chromatography™. With the flow being driven by high-pressure pumps and the possibly of on-line detection, OPLC allows continuous quantification with standard LC detectors (Mincsovics & Tyihák, 2011; Tyihák *et al.,* 2012). OPLC can be thought of as flat-bed LC (Figure 8.5). Indeed, the manufacturers refer to the sorbent layer as a column rather than a plate.

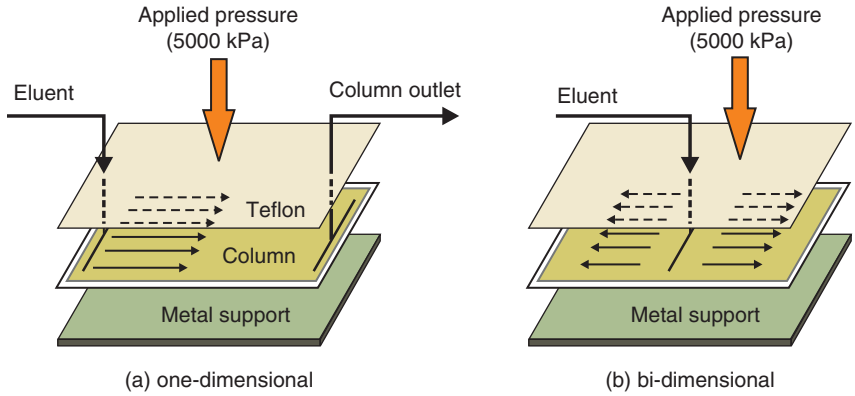

Figure 8.5 Two modes of forced-flow planar chromatography (Bryston & Papillard, 2004–reproduced with permission of John Wiley & Sons)

A basic OPLC™ unit can be connected in the same way as a cylindrical LC column and be used on-line with either isocratic or gradient elution. Or it can be used off-line, in much the same way as a conventional TLC plate. Thus, OPLC retains the advantages of TLC, including multiple channels and two-dimensional development. There are also many innovations, such as being able to perform two-dimensional chromatography on four samples on a single plate.

Methods for screening for basic drugs in 2 mL urine samples and in autopsy liver samples by dual-plate OPLC have been described (Pelander *et al*., 2003, 2007).

8.3.2 Quantitative high-performance thin-layer chromatography

HPTLC plates have a smaller average particle size (5–10 μm) and give greater efficiency than conventional plates. 'Reverse-phase' plates, where a hydrophobic moiety (usually C_2, C_8, or C_{18}) is bonded to the silica matrix, are also available. However, HPTLC and reverse-phase plates are more expensive and have a lower sample capacity than conventional plates, an important consideration in clinical toxicology when the amounts of analyte present can easily overwhelm even ordinary TLC plates.

HPTLC plates are typically 10 × 10 cm. HPTLC spots should be as discrete as possible (1–2 mm diameter). Automated spotting systems are available and should be used for quantitative work. As with TLC, the plates can be developed in two directions and modern systems allow automatic multiple development, where a number of eluents can be used sequentially (Pothier & Galand, 2005).

Provided care is taken, quantitative HPTLC can give highly reproducible results, with RSDs of 2–3 %. HPTLC with scanning densitometry, which may be used in several modes including transmittance, fluorescent quenching, and fluorescence, is probably the most reliable approach.

Automatic spotting or spraying ensures precise application of the sample. However, for quantitative work variability in application of the chromogenic reagent can be problematic. Dipping manually is extremely technique dependant and an automated dipper is the only acceptable approach.

8.4 Summary

TLC still can find a place in STA if resources are limited, and has the advantage that, provided analytes are not lost by either evaporation, or decomposition on the plate, all extracted substances are present on the chromatogram. Given adequate laboratory facilities such as a fume hood, TLC has the advantages over elution chromatography that (i) expensive analytical equipment is in general not needed and (ii) several sample extracts can be analyzed simultaneously. On the hand, GC and LC offer increased selectivity and sensitivity for most analytes, as discussed in subsequent chapters.

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9 Gas Chromatography

9.1 Introduction

In analytical toxicology, GC has a number of advantages over other widely used techniques such as immunoassay and LC. Firstly, GC has a sensitive, reliable 'universal' detector, the FID. Secondly, stable, high efficiency (capillary) GC columns are widely available. Thirdly, GC is easy to interface with techniques giving direct information about compound identity such as electron-ionization MS (EI-MS) and Fourier transform infra-red spectroscopy (FTIR) (Grob & Barry, 2004; Rood, 2007).

With GC, as with LC, qualitative and quantitative information can often be obtained in the same analysis provided that appropriate calibration and QC procedures are followed. Temperature programming in GC is analogous to gradient elution in LC, but is much simpler to perform and permits the analysis of compounds of different volatilities in one analysis. Moreover, the return to starting conditions is easy and the interdependence of M_r, retention time, and column temperature is valuable in aiding peak assignment in STA. In addition, normalized GC retention data are reproducible between different days, columns of the same type, instruments, operators, centres, etc. (Box 9.1).

Box 9.1 Advantages of GC in analytical toxicology

- Can inject aqueous mixtures in some applications such as ethanol analysis, although headspace almost as easy and applicable to wide range of volatiles
- Range of stable, efficient capillary columns
- Reliable, sensitive (almost) universal (FID) and selective (MS) detectors
- Qualitative and quantitative
  - Wide-bore capillary columns used with injection liners facilitate quantitative work
- Interdependence of elution time, column temperature, and M_r valuable in qualitative work
- Normalized retention data (Kovats indices) reproducible between days/columns of the same type/operators/laboratories/countries
- Temperature programming and interfacing to MS or FTIR easy
- Need few column types because of high resolving power of capillary columns
  - If necessary, can link columns to improve selectivity
- Can generate analyte 'fingerprint' using EI-MS
  - Principal peak index valuable in compound identification via databases
- Can use for a wide range of gases and solvents
- Chiral analysis using chiral stationary phase or chiral derivatization

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Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

Disadvantages of GC include the requirement that the analyte or a derivative should be volatile and stable at the temperature required for the analysis (in practice below approximately 400 °C). In addition, substances with highly polar or ionizable functional groups may give poor performance (broad, tailing peaks), and some form of sample preparation is normally needed to transfer the analyte from aqueous samples to organic solutions (Box 9.2). Moreover, some analytes may be thermally labile and the decomposition products may interfere in the analysis, although in certain instances the breakdown product(s) can be used to aid analyte identification. Examples include dehydration products from alcohols and decarboxylation of acids (Section 9.5).

Box 9.2 Limitations of GC in analytical toxicology

- Hardware/infrastructure relatively expensive – need experienced operators, pure gas supplies, consumables, support from instrument manufacturer
- Septum injection – need frequent maintenance
- Sequential analysis – need autoinjectors
- Usually need some form of sample pretreatment
- Cannot adjust selectivity by modifying eluent (cf. LC)
- Cannot analyze very polar/high M_r compounds (cf. LC), although can sometimes form volatile derivatives
- Cannot analyze carbon monoxide directly by FID
- Destructive detectors
- There are always the possibilities of analytes co-eluting and of thermal decomposition on-column

In addition to the choice of the sample preparation procedure, the column, and the chromatographic and detection conditions, due consideration must be given to other factors in the analysis including sample collection and storage, choice of ISTD, and quality assurance. This being said, GC remains the method of choice for gases and other volatiles such as ethanol, inhalational anaesthetics, and glycols after suitable derivatization. GC is also widely used in the analysis of other compounds, in STA, and as an interface to MS, an especial advantage being that EI spectra can be obtained (Section 13.3.1.1).

9.2 Instrumentation

Typically, a GC consists of a gas control unit, which governs the supply of carrier gas to the column as well as gases such as compressed air and hydrogen to the detector, a sample injection system, an analytical column, and a detector with associated data acquisition/processing (Figure 9.1). The injector, column, and detector are contained in ovens that are normally heated independently, the injector and detector generally being maintained at a slightly higher temperature than the maximum temperature attained by the column oven.

Gas chromatographs are usually purchased as a single unit, with gas supplies and data capture being separate. Today, instrument control is from a computer that also provides data capture and data analysis facilities. Although commercially supplied cylinders are a convenient source of the gases needed, alternatives are available for air (simple compressor), hydrogen (electrolytic hydrogen generator), and nitrogen (nitrogen generator based on a molecular sieve). However,

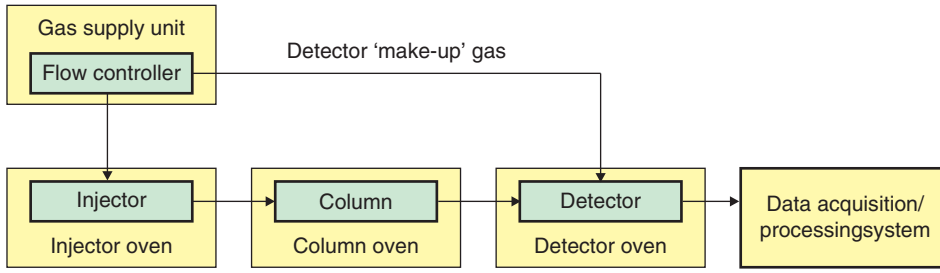


Figure 9.1 Block diagram of a gas chromatograph

regular monitoring and maintenance, and the use of appropriate filters to remove hydrocarbons, oxygen, and moisture are mandatory. Filters should always be used with gas from cylinders.

Nitrogen is normally used as the carrier gas with packed columns (flow rates 30–60 mL min^{-1}) and helium with capillary columns, especially if interfaced to MS (flow rates 1–10 mL min^{-1}). With capillary columns, for example, an additional gas supply ('make-up gas') is needed with some detectors. In all but the simplest systems, it is vital to use oxygen-free carrier gas as the presence of even very small amounts of oxygen can oxidize certain stationary phases. A constrictor fitted to the detector end of the column limits back diffusion of air from an FID, for example.

9.2.1 Injectors and injection technique

Because sensitivity is often limiting in analytical toxicology it is important that as much of a sample extract as possible is injected onto the column, other factors (the 'signal-to-noise ratio') being equal. In GC, sample capacity is limited, but this does not matter greatly because GC detectors are very sensitive. However, in most GC methods the difficulties inherent in reproducibly injecting relatively small volumes of an extract necessitate the use of an ISTD (Section 3.3). With packed or wide-bore capillary columns, sample injection is usually via a syringe through a silicone rubber septum in the injection port. It is important to use 'low-bleed' septa, especially with sensitive detectors such as MS(/MS). Modern GC(-MS) systems are coupled with an autosampler allowing better reproducibility and unattended operation.

Use of a glass injection port liner that can be removed and cleaned minimizes the accumulation of non-volatile residues on the column, but on-column injection may be preferred if labile substances are to be analyzed. In such cases contaminated packing at the top of a column or the first few centimetres of a capillary 'guard' column should either be replaced with fresh material, or removed if column performance is affected. Normally a minimal amount of solvent will be injected to reduce solvent effects at the detector. However, for gases and vapours much larger volumes can be injected via a gas-tight syringe or a gas-sampling valve.

A variety of injection devices can be used with capillary columns and the terminology employed can be very confusing. Nevertheless, the importance of using an appropriate injection technique when working with such columns cannot be overemphasized and the subject is worthy of specialized study if the full capabilities of modern columns are to be realized (Bailey, 2005). With relatively narrow-bore capillaries (<0.32 mm i.d.) some form of inlet splitter is normally needed to prevent overloading the column with injected material (Figure 9.2).

When using an inlet splitter, as with other GC injection devices, it is important that the portion of the injection solution passed to the column has the same composition as the rest

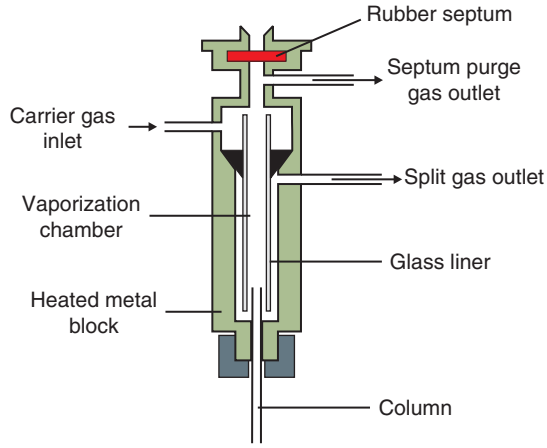


Figure 9.2 Split/splitless injector for gas chromatography

of the solution. With wider bore capillaries (0.32 mm i.d. or greater), then (splitless) syringe injection either through a septum into a glass liner, or directly onto the column via a fused silica capillary guard column or retention gap (a 1–10 m length of deactivated tubing without stationary phase) is relatively simple and ensures deposition of the analytes on the column. If efficiency deteriorates then removal of the first few cm or even an entire coil from the guard column often restores performance without altering analyte retention on the analytical column.

With on-column injection, it is usual to inject at a temperature below the boiling point of the injection solvent. As the solvent evaporates in the stream of carrier gas, the analytes are concentrated in a small band and are then volatilized by increasing the column oven temperature. A retention gap is sometimes needed to improve chromatographic performance when relatively large volumes (>2 μL) of solvent are injected, and of course can also serve as a guard column.

9.2.1.1 Cryofocusing/thermal desorption

The great flexibility offered by the ability to use temperature programming in GC can be exploited in the cryofocusing or 'purge-and-trap' of volatile analytes. Thermal desorption and refocusing techniques are simple to use, easily automated, and applicable to a wide range of samples. A further major benefit of thermal desorption directly coupled to GC is enhanced sensitivity because all of the desorbable components are trapped at low temperature, either in a short column held in the GC oven filled with adsorbent such as Tenax GC, or on the GC column itself, prior to flash vaporization by rapidly raising the oven temperature (Figure 9.3).

Using a trap may be necessary if the matrix contains water. In this case the analytes are trapped on the adsorbent, and then the water is removed from the system by purging with nitrogen prior to heating to allow rapid transfer of the analytes to the GC column. Alternatively, a Nafion (a sulfonated tetrafluoroethylene-based fluoropolymer-copolymer) trap can be used. A disadvantage is that thermal desorption techniques are not selective – all trapped volatiles are passed to the column unless removed by, for example, a nitrogen purge.

Cartridges filled with layers of different adsorbents can also be used. After an appropriate sampling time, trapped volatiles are flash vaporized into a stream of carrier gas and carried onto the GC column. Alternatively, cartridges filled with activated charcoal can be used to 'trap' the volatiles, which are then extracted into a small volume of carbon disulfide prior to the analysis.

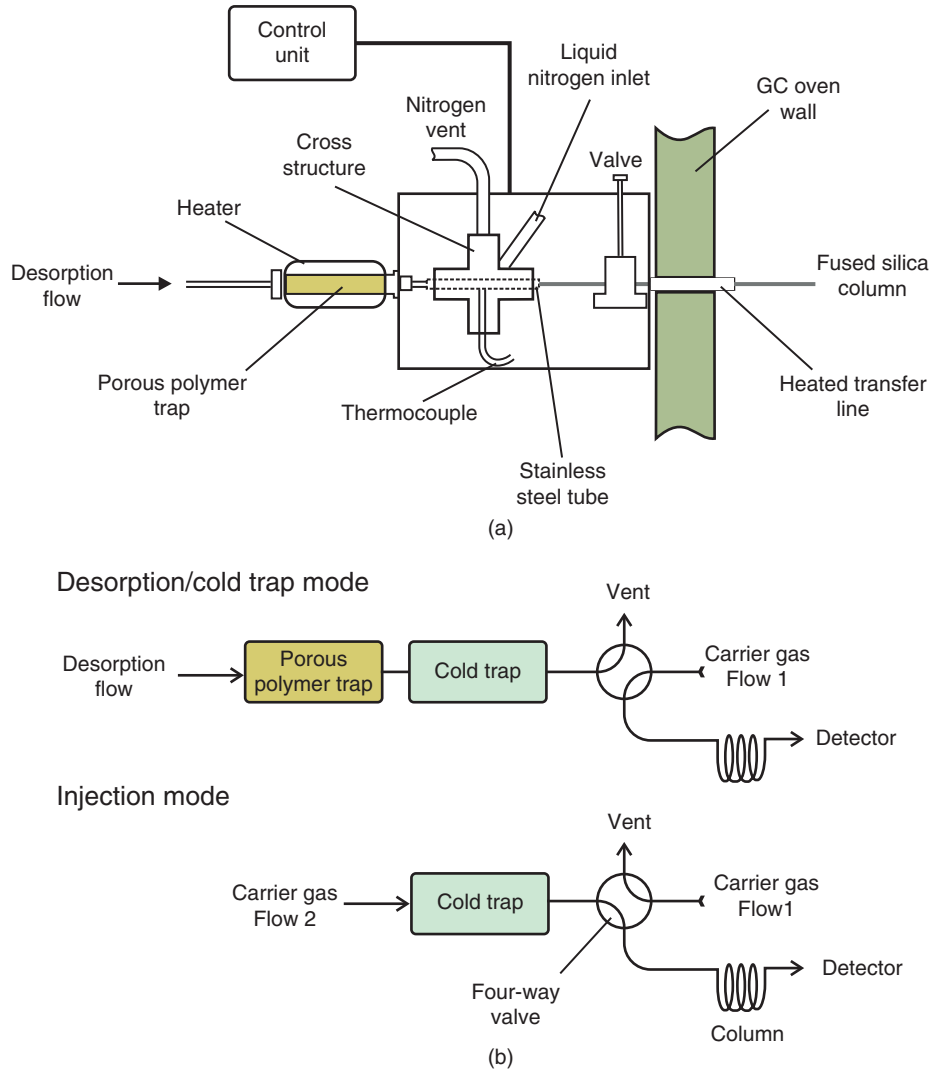


Figure 9.3 Schematic of (a) GC thermal desorption unit; (b) modes of use

This technique has been used widely in the analysis of volatile compounds in potable water samples, for example, but has not found wide application in the analysis of biological samples, mainly because of the difficulty in interpreting the results at concentrations below those which can be measured using ordinary headspace methods.

Sample delivery mechanisms may include headspace (HS) analysis (Section 9.4), purging liquids by bubbling with nitrogen, pulse heating extraction for liquids, thermal desorption for solid matrices, or simply pumping ambient air through the cryogenic trap. In the main, these cryofocusing and related techniques find application in occupational and environmental toxicology. However, fluorinated inhalation anaesthetics (halothane, enflurane, isoflurane, sevoflurane, and desflurane), for example, have been assayed in biological samples by GC with HS, purge and trap, or pulse heating extraction.

9.2.2 Detectors for gas chromatography

Of the two commonly used detectors, FID and MS, the latter has assumed such importance that it is discussed in detail in Chapter 13. With the exception of the FTIR detector, GC detectors are destructive. Thus, post-column splitter systems are usually employed if more than one detector is to be used simultaneously, the relative detector response offering an additional parameter to aid compound identification even though this is rarely used in practice. Note that use of MS may shorten retention times because of the effect of the vacuum necessary for its use.

The electron capture detector (ECD) gives a selective and thus enhanced response to compounds containing electronegative moieties such as halogen atoms (Poole, 2015). It is, however, little used today because traditionally it required a radioactive source, normally ^{63}Ni, and has largely been superseded by negative ion MS. The nitrogen–phosphorus detector (NPD) likewise gives a selective response to analytes containing either C—N bonds, or phosphorus, and although it can be difficult to use, still finds wide application.

Finally, the flame photometric detector (FPD) can give a selective response for phosphorus- or sulfur-containing compounds, whilst the photoionization detector (PID) gives a similar signal-to-noise ratio to the FID for most organic compounds and does not require gas supplies. As such it has found application in environmental analysis, for example (Zhou *et al*., 2018). However, nowadays the FPD and PID find little application in analytical toxicology.

9.2.2.1 Thermal conductivity detection

The first GC detector was the katharometer or hot-wire detector, also known as the thermal conductivity detector (TCD, Figure 9.4). It is relatively insensitive (typically not better than 1 ng on column) and has a poor linear range, but may be useful in analytical toxicology for permanent gases and for carbon monoxide, compounds that do not respond on FID and are difficult if not impossible to monitor on MS. It is based on the principle that the resistance of an electrically heated filament placed in the column effluent changes as the composition of the effluent changes.

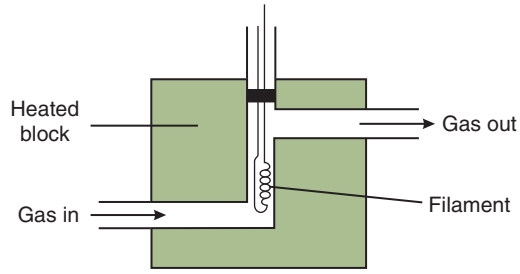


Figure 9.4 Schematic diagram of a GC thermal conductivity detector

9.2.2.2 Flame ionization detection

The FID is based on the principle that the ionization produced as the column effluent passes into a hydrogen/air flame changes as the effluent composition changes (Figure 9.5). The change in ionization is monitored as a change in the standing current maintained by applying a potential difference between the flame tip and a collector electrode. The high sensitivity (10^{-11}–10^{-12} g s^{-1} methane, typically 0.1–10 ng on column), good stability (low background current), and wide

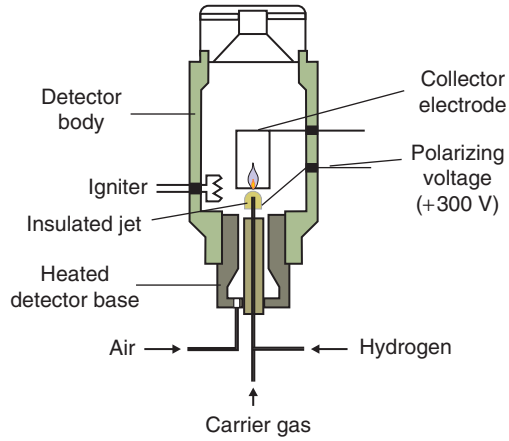


Figure 9.5 Schematic diagram of a GC flame ionization detector

linear range (up to 10^6) of this detector have ensured that it remains widely used. Moreover, all organic molecules containing C—H bonds except formate give a response. The magnitude of the response is roughly proportional to the number of carbon atoms present in an analyte, although this is reduced if oxygen or nitrogen are also present in the molecule.

The lack of response to water enables aqueous injections to be performed when measuring ethanol and similar compounds (Section 19.2.2.1). On the other hand, the risk of interference is high especially when analysing samples obtained post-mortem, and thus selective detectors, especially NPD and MS, are favoured. There may be some build-up of siliceous deposits if used with packed columns or silicon-containing derivatizing reagents, necessitating periodic cleaning. A minimum operating temperature of 125 °C is recommended to prevent the condensation of water vapour.

9.2.2.3 Nitrogen/phosphorus detection

The NPD is based on the FID, but contains either a rubidium, or caesium silicate (glass) bead situated in a heater coil a little distance from the flame. This introduces alkali metal vapour into the flame, which can facilitate a selective response to compounds containing phosphorus or C—N bonds via adjustment of the flame gases. Because many drugs and other poisons contain C—N bonds and many injection solvents and potential interferences do not, this detector, also known as the thermionic detector or the alkali flame ionization detector (AFID [Figure 9.6(a)], has been widely used in the nitrogen-selective mode (nitrogen:carbon response ratios approximately 5000:1).

The increased sensitivity to phosphorus is a potential disadvantage because phosphorus-containing plasticizers show a good response (phosphorus:nitrogen response ratios *ca*. 10:1 in N mode). In the phosphorus mode, grounding the polarizing potential increases the selectivity (phosphorus:carbon response ratios *ca*. 50,000:1), which can be exploited in a few instances, notably in the analysis of organophosphorus pesticides and their metabolites (Ko *et al*., 2014; Section 22.4.15), or by producing phosphorus-containing derivatives.

Heavily chlorinated solvents such as dichloromethane are not recommended for use with the NPD because they tend to volatilize the alkali metal from the source. Butyl acetate has proved a valuable extraction and injection solvent when used both with this detector and with the ECD.

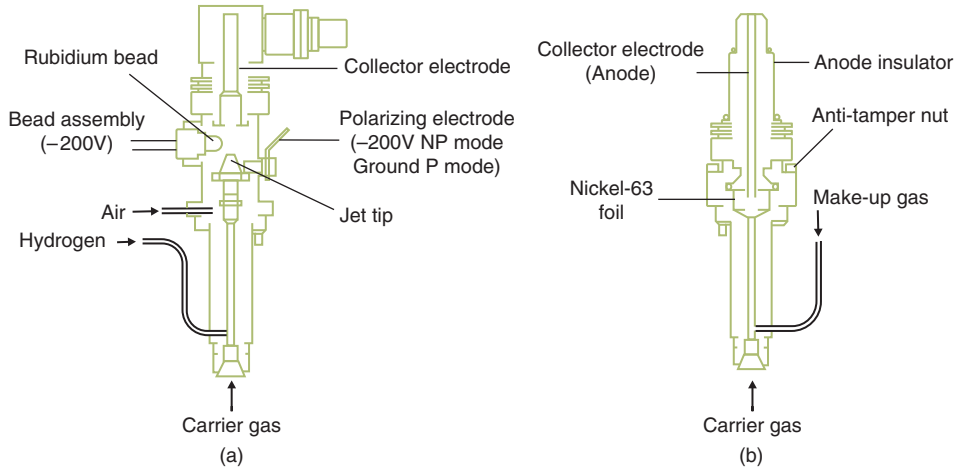


Figure 9.6 Schematic diagrams of (a) nitrogen–phosphorus detector and (b) electron capture detector

Modern NPDs use an electrically heated rubidium silicate source and are more stable than earlier versions, although operation of this detector is still technically demanding. Correct adjustment of the position of the bead and of the detector gas flow rates are critical. Bead life may vary depending on bead quality and usage. Nevertheless, for some analyses, notably in cyanide analysis (Section 19.2.2.2), and in STA (Rohrig *et al.*, 2018), the NPD is a useful complement to MS.

9.2.2.4 Electron capture detection

The ECD is based on the principle that electrons produced either from a ^{63}Ni source or by plasma discharge (Section 9.2.2.5) are selectively 'captured' by certain analytes in the column effluent, thus decreasing a standing current maintained between the source and a collector electrode. The ^{63}Ni version of the detector [Figure 9.6(b)] could be operated either with a constant DC potential (DC mode) or with a pulsed potential (pulsed mode) applied across the electrodes. In DC mode a constant potential (a few volts) was applied that was just sufficient to collect all electrons produced from the source and thus give a standing current.

The ECD is one of the most sensitive GC detectors available and shows an enhanced and selective response (*ca.* 10^3 times greater than the FID) to compounds containing a halogen, a nitro moiety and, to a lesser extent, to carbonyl-containing compounds. It was thus important for the analysis of halogenated solvents, pesticides, and some halogen- or nitro-containing drugs, notably benzodiazepines. For some compounds the sensitivity of this detector easily exceeds that of MS – limits of sensitivity of a few femtograms are possible for organochlorine pesticides, for example. All this being said, the ^{63}Ni ECD suffers from a number of problems in routine use (Box 9.3) and consequently this version of the ECD is rarely encountered nowadays.

9.2.2.5 Pulsed discharge detection

The pulsed discharge detector (PDD) can be operated in three modes: pulsed discharge helium photoionization (He-PDPID; Figure 9.7), pulsed discharge electron capture (PDECD), and

Box 9.3 Disadvantages of ^{63}Ni electron-capture detectors

- Limited number of analytes (must be electron deficient, for example halogen-containing compounds)
- Easily contaminated – with loss of standing current
- Large volume – requires make-up gas
- Limited linear range
- Safety issues
  - ^{63}Ni is a hard β-emitter
  - Maximum operating temperature generally 350 °C
- Legislative issues
  - Requires radioactivity 'wipe-test'
  - Difficulties in disposing of old instruments

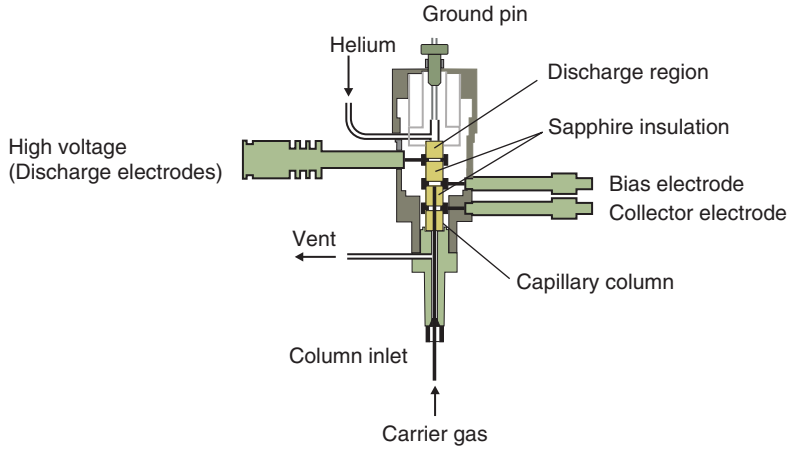


Figure 9.7 Schematic diagram of a pulsed discharge helium ionization detector. Reprinted from Poole, 2015–with permission from Elsevier

helium ionization emission (PDED) (Poole, 2015). The He-PDPID can detect permanent gases, volatile inorganic species, and other compounds that give little or no response with the FID and has lower limits of detection [with minimum detectable quantities (MDQs) in the low picogram range] than can be achieved with TCD and a wide linear range (Roberge *et al.*, 2004). In the He-PDPID, helium is passed through a chamber where a glow discharge is generated and high-energy photons are produced. These pass through an aperture to another chamber where they ionize the gas or vapour species in the sample stream. The resulting electrons are directed by the bias electrode(s) to the collector electrode and are measured using a standard electrometer.

When used as an ECD, a few percent of an additional gas ('dopant') is added, which is ionized and provides electrons to produce a standing current. Several dopants have been tested – the best appear to be methane and xenon. The PDECD has a sensitivity (minimum detectable quantities of 10^{-15} to 10^{-12} g) similar to, or better than, ^{63}Ni ECD, but does not require licensing, wipe tests, and other administrative or safety requirements.

9.2.2.6 *Flame photometric detection*

The FPD exploits the chemiluminescence of sulfur or phosphorus when heated in a flame to give relatively specific detection for substances containing either element. The λ_{max} for emission of excited S_2 (the emitting species for sulfur) is *ca*. 394 nm, whilst excited HPO shows a doublet at 510–526 nm. An interference filter placed between the flame and the photomultiplier tube determines selectivity. This detector does not find wide application in analytical toxicology, but is used in pesticide development and other relatively specialized areas.

9.2.2.7 *Atomic emission detection*

The microwave induced plasma emission detector or atomic emission detector (AED), once called a microwave plasma detector (MPD), can measure a number of elements, typically 15 or so, selectively and simultaneously in GC effluent. Analytes eluting from the GC column pass into a microwave plasma (or discharge) cavity where individual atoms are excited from ground state by the heat energy of the plasma. Each element emits light of a specific wavelength when decaying from the excited state and this can be measured via a photodiode array, usually in the range 170–800 nm. An associated computer can produce chromatograms made up of peaks from analytes that contain only a specified element. The AED has found application in conjunction with MS in pharmaceutical analysis if not in analytical toxicology *per se* (Laniewski *et al*., 2004).

9.2.2.8 *Chemiluminescent nitrogen detection*

The chemiluminescent nitrogen detector (CLND) produces a linear and equimolar response to nitrogen compounds. A stainless-steel burner is used to achieve high temperature combustion of nitrogen-containing compounds to form nitric oxide (NO). A photomultiplier tube detects the light produced by the subsequent chemiluminescent reaction of NO with ozone (Section 10.3.5). Because of the specificity of the reaction, complex sample matrices can be analyzed with little or no interference. GC-CLND can be used for quantification in the absence of reference materials if the number of nitrogen atoms in a molecule are known (Ojanperä *et al*., 2016), an important consideration given the continually changing NPS market.

9.2.2.9 *Fourier transform infra-red detection*

The idea of using IR spectroscopy as a GC detector is not new, but only became practicable with the development of Fourier transform (FT) instruments. FT converts data from an intensity versus time plot to an intensity (% transmission) versus frequency spectrum. GC-FTIR has not been widely applied in analytical toxicology largely because of cost and the fact that, with the exception of isomers and of simpler molecules such as volatile solvents, GC-MS often gives more information. However, FTIR detection is non-destructive and can be used in a quantitative as well as qualitative mode, although sensitivity is poor (at best 10 ng on-column).

GC with sequential FTIR/FID has been used to detect and measure volatiles in blood (Ojanperä *et al*., 1998). More recently, GC-FTIR has been used to help characterize analogues of the synthetic cannabinoids 1H-indol-3-yl(2,2,3,3-tetramethylcyclopropyl)methanone and 1H-indol-3-yl(adamantan-1-yl)methanone (Carlsson *et al*., 2016), and of synthetic cathinones (Carlsson *et al*., 2018). GC-FTIR has also proved helpful in the isolation and structural characterization of a tadalafil analogue (chloropropanoylpretadalafil) found in a dietary supplement (Kern *et al*., 2016) and in identifying the designer steroid androsta-3,5-diene-7,17-dione in a dietary supplement (Lorenz *et al.,* 2019).

9.2.2.10 Vacuum UV detection

VUV detection can provide spectral information that complements techniques such as MS (Section 5.4). In contrast to GC-FTIR, GC-VUV exhibits MS-like features with respect to sensitivity, linearity, and the ability to perform quantitative analyses. Although VUV spectra contain less structural information than IR spectra and do not allow structure elucidation, compounds can be identified by spectral library matching. A deuterium lamp allows spectra to be collected between 120–400 nm. The VUV-grade windows may be constructed of magnesium fluoride, calcium fluoride, or sapphire (Figure 9.8). Commonly used carrier- and make-up gases, helium, argon, and hydrogen, do not interfere. A commercially available instrument has a flow-cell with a pathlength of 100 mm and a volume of <80 μL and, because VUV is non-destructive, the detector can be connected in series with other detectors such as MS.

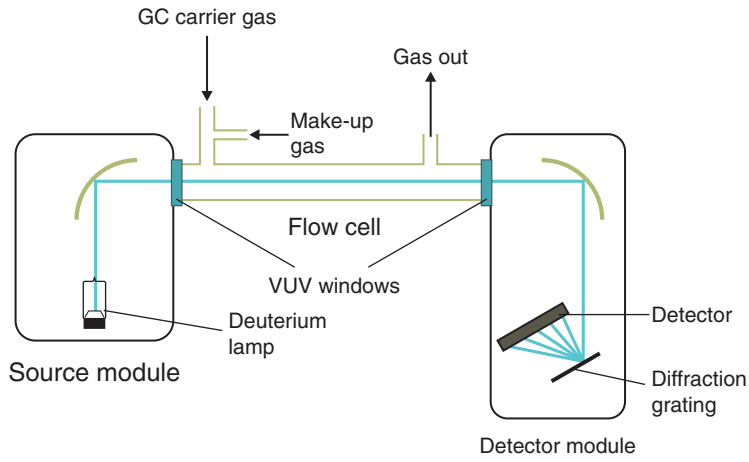


Figure 9.8 Schematic diagram of a GC vacuum UV detector

GC-VUV has proved useful in identifying isomers and other structurally similar compounds such as volatile organic compounds (VOCs). For example, GC-VUV has been used to identify nitrate ester explosives partly through thermally induced degradation in the VUV cell (Cruse & Goodpaster, 2019) and in the identification of substituted cathinones (Skultety *et al*., 2017). More recently, Buchalter *et al*. (2019) reported the coupling of GC-VUV and cold spray EI-MS (Section 13.3.1.1) in the analysis of fentanyl analogues. GC-VUV has also been used to help differentiate positional isomers of MDA, MDMA, and other compounds of interest (Kranenburg *et al*., 2019).

9.3 Columns and column packings

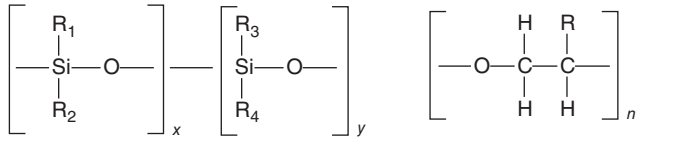
Originally, distinction was made between gas–solid chromatography (GSC) in which the stationary phase is an active solid and gas–liquid chromatography (GLC) in which the liquid stationary phase is coated onto an inert support. However, with the widespread use of capillary columns in which the stationary phase is chemically bonded onto the inner surface of the column, these distinctions are often less clear cut. Some advantages to the use of capillary columns are summarized in Table 9.1.

Table 9.1 Capillary versus packed columns in gas chromatography: practical considerations

	Packed	Capillary
Efficiency	Low (*ca.* 20,000 theoretical plates per column)	High (*ca.* 500,000 theoretical plates per column)
Peak symmetry	Poor for polar compounds	Good
Analysis time	Long	Short (for comparable resolution)
Temperature programming	Difficult (baseline drift)	Easy
Sample capacity	High	May be low
Sensitivity	Good (more sample injected)	Good (sharp peaks)
Quantitation	Yes	Direct injection
Interface to MS	Difficult (need splitter system)	Easy

9.3.1 Stationary phases

Several hundred GC stationary phases are available, but only a few, mainly those based on polysiloxane (often incorrectly called silicones) or polyethylene glycol, have found wide application in analytical toxicology. In the simplest of the polysiloxane phases, i.e. when $R_{1\text{-}4}$ = methyl (Figure 9.9), the polymer is known as PDMS or simply methylpolysiloxane. Substitution of methyl moieties by other groups of increasing polarity alters the selectivity of the material. In the stationary phase OV-17, for example, R_1 and R_2 = methyl, R_3 and R_4 = phenyl, and x and y both = 50 %. This phase can be referred to as 50 % dimethyl, 50 % diphenylpolysiloxane; 50 % methyl, 50 % phenylpolysiloxane; or even 50 % phenylpolysiloxane. In other phases, two different groups may be bonded to the same silicon atom. OV-225, for example, differs from OV-17 in that R_3 = cyanopropyl.



Polysiloxanes
R may be: CH_3, $CH_2CH_2CF_3$, $CH_2CH_2CH_2CN$, C_6H_5, C_8H_{17}

	R
Polyethylene glycol	H
Polyalkylene glycol	Alkyl

Figure 9.9 Structures of polysiloxane and polyethylene glycol stationary phases for GC (for explanation, see text)

Polyethylene glycol (PEG) phases are used either directly, or after substitution with alkyl moieties such as 2-nitroterephthalic acid in the phase FFAP (free fatty acid phase). Although PEG phases have unique selectivities, they are less robust than the polysiloxanes and have lower operating temperature limits and a shorter life expectancy. Carbowax 20M (polyethylene glycol of average M_r 20,000), for example, has a relatively low t_{max} (Table 9.2).

Table 9.2 Some GC stationary phases used in analytical toxicology

Phase[a]	Type	Suggested operating temperature range (°C)	USP[b]	Sum of McReynolds constants[c]
SPB-Octyl	50 % Dimethyl, 50 % octylpolysiloxane	−60/280	–	51
SE-30	100 % Methylpolysiloxane gum	−60/300	G2	217
OV-1	100 % Methylpolysiloxane gum	−60/320	G2	222
OV-101	100 % Methylpolysiloxane oil	−60/320	G1	229
DB-1	100 % Methylpolysiloxane gum	−60/320	G1	229
SP-2100	100 % Methylpolysiloxane oil	−60/320	G1	229
SE-52	95 % Methyl, 5 % phenylpolysiloxane	−60/320	G27	334
HP-5	95 % Methyl, 5 % phenylpolysiloxane	−60/320	G27	334
MS-5	95 % Methyl, 5 % phenylpolysiloxane	−60/340	G27	334
SE-54	94 % Methyl, 5 % phenyl, 1 % vinylpolysiloxane	−20/280	G36	337
OV-73	94.5 % Methyl, 5.5 % phenylpolysiloxane	−60/320	G27	401
RTX-1301	6 % Cyanopropylphenyl, 94 % methylpolysiloxane	−20/280	G43	592
OV-7	80 % Methyl, 20 % phenylpolysiloxane	−60/320	G32	592
OV-17	50 % Methyl, 50 % phenylpolysiloxane	30/310	G3	884
SP-2250	50 % Methyl, 50 % phenylpolysiloxane	30/310	G3	884
HP J&W 50+	50 % Methyl, 50 % phenylpolysiloxane	40/280	G3	884
RTX-50	50 % Methyl, 50 % phenylpolysiloxane	40/320	G3	884
OV-210	50 % Methyl, 50 % trifluoropropylpolysiloxane	40/230	G6	1520
OV-225	50 % Cyanopropylphenyl, 50 % methylpolysiloxane	40/230	G19	1813
Carbowax 20M	Polyethylene glycol (PEG) M_r 15,000	35/280	G16	2308
SP-1000	Terephthalic acid substituted PEG	60/200	G25	2409
FFAP	Polyethylene glycol-2-nitroterephthalic acid ester	50/250	G25	2546
DEGS (Hi-EFF-1BP)	Diethyleneglycol succinate polyester	20/200	G4	3504

[a]Key to suppliers: DB = J&W, OV = Ohio Valley, SP = Supleco, HP = Hewlett Packard (now Agilent), RTX = Restek (this list is not exhaustive)
[b]US Pharmacopoeia Convention liquid phase cross-reference
[c]Sum of individual constants

McReynolds, building on the work of Rohrschneider, used the retention of benzene, butanol, 2-pentanone, nitropropane, and pyridine to summarize the polarity of stationary phases. The sum of the difference of the retention indices (Section 7.3) of each of these compounds on the stationary phase under investigation as compared to the retention index of the compound on a standard non-polar phase (squalane) is used to derive the *McReynolds Constant* for the phase. This gives a measure of the polarity of the phase, which can be used for classification purposes. The system is explained fully elsewhere (Sigma-Aldrich, 1997).

Phases with McReynolds Constants <100 are known as non-polar, 100–400 intermediate polarity, and >400 polar. The McReynolds Constants for some stationary phases and the US Pharmacopoeia (USP) Convention liquid phase cross-reference are given in Table 9.2. Note, however, that the 'polarity' of stationary phases, as reflected in the retention of polar as compared to non-polar analytes, increases with temperature.

As a general rule, retention on relatively non-polar phases such as PDMS is influenced mainly by temperature and by analyte M_r, the dominant influence on volatility. However, hydrogen bonding and dipole interactions may also influence volatility and therefore GC elution time on a given system. As a guide, polar compounds, such as many drugs and pesticides, show increased separation and give better peak shapes and hence greater sensitivity on more polar phases.

SE-30, OV-1, and OV-101 (and also HP-1, DB-1, RTX-1, and SPB-1) are PDMS stationary phases that are broadly equivalent. Polar interactions are minimal with these phases and thus separations occur largely on the basis of M_r. SE-54, OV-7, OV-17 (and their broad equivalents HP-50+ and RTX-50), and OV-225 are amongst the more polar polysiloxane-based phases available. In general, the higher the polarity of polysiloxane phases, the lower the maximum operating temperature.

9.3.2 Packed columns

Conventional borosilicate glass, stainless steel, or glass-lined stainless steel packed columns (0.5–4 m × 2–4 mm i.d.) still find application in some areas. Although less robust than steel, glass is less likely to adsorb or react with polar analytes. In addition, any settling of the packing and column contamination can be checked visually.

GSC packings are mainly used in the analysis of gases and solvents. Molecular sieve (synthetic zeolite) or silica gel packings are useful for the analysis of permanent gases and carbon monoxide. The Chromosorb and Porapak series are cross linked divinylbenzene polystyrene copolymers with maximum operating temperatures of *ca*. 250 °C. Alcohols from methanol to pentanol can be separated on Porapak Q or Chromosorb 101.

Tenax-GC is a porous polymer of 2,6-diphenyl-*p*-phenylene oxide and is used both as a stationary phase material and as a trap for volatiles prior to GC. Carbopaks B and C are graphitized carbon blacks having surface areas of 12 and 100 $m^2\ g^{-1}$, respectively. The Carbopaks are usually used after modification with a light coating of a polar stationary phase such as Carbowax 20M (Table 9.3), and can give good peak shapes and separations for alcohols and other volatiles. However, these materials are very friable and batch-to-batch variations in the peak shapes attained may occur.

9.3.3 Capillary columns

Nowadays almost all applications in analytical toxicology use fused silica capillary columns. An outer polyimide coating protects the brittle silica giving mechanical strength and flexibility (Figure 9.10). The column may often be threaded through complex pipework to emerge, for

Table 9.3 Some stationary phases and stationary phase supports used in packed column GC (unless otherwise specified, intended mesh size is 80/100 or 100/120)

Material	Description	USP[a]
Gas Chrom 254, Chromosorb 101	Styrene-divinylbenzene copolymer (surface area <50 m^2 g^{-1}; average pore diameter 0.3–0.4 μm)	S2
Super Q, Porapak Q	Ethylvinlybenzene-divinylbenzene copolymer (surface area 500–600 m^2 g^{-1}; average pore diameter 7.5 nm)	S3
Porapak R	Styrene-divinylbenzene copolymer with aromatic -*O*- and -*N* groups (surface area 400–600 m^2 g^{-1}; average pore diameter 7.6 nm)	S4
Chromosorb T	High M_r tetrafluoroethylene polymer, 40/60 mesh	S5
Gas Chrom 220, Porapak P, Chromosorb 102	Styrene-divinylbenzene copolymers (surface area 250–350 m^2 g^{-1}; average pore diameter 9.1 nm)	S6
Graphpac-GC	Graphitized carbon (surface area *ca.* 12 m^2 g^{-1})	S7
Porapak S	Copolymer of 4-vinylpyridine and styrene-divinylbenzene	S8
Tenax TA	Porous polymer of (2,6-diphenyl-*p*-phenylene oxide)	S9
3 % w/w Carbowax 540 on Graphpac-GB, 80/120 or Carbograph 1, 80/120	Graphitized carbons (surface area of 100 m^2 g^{-1}), modified with polyethylene glycol	S11
Graphpac-GB, Carbograph 1	Graphitized carbons (surface area 100 m^2 g^{-1})	S12

[a]US Pharmacopoeia Convention GC packing cross-reference

example, at a detector inlet. Because fused silica columns are inherently straight they are usually coiled and held in a metal support (cage). They should be handled with care because when they break, they suddenly straighten and may cause injury. Typical dimensions are 0.05–0.53 mm i.d. and 10–50 m length. The polyimide coating imposes an upper operating temperature limit of 360 °C (400 °C for high-temperature polyimide coatings).

Column bleed and thus detector noise are reduced as compared to packed columns because (i) the amount of stationary phase present is low, and (ii) the phase is usually cross-linked and chemically bonded to the wall of the capillary via surface silanols. Cross-linking is brought about

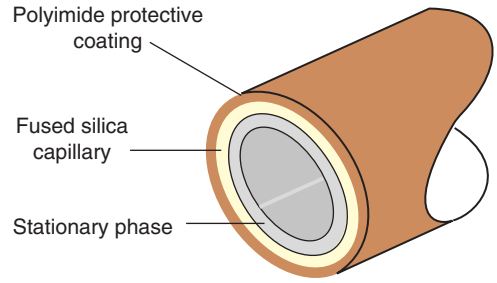


Figure 9.10 Schematic of a fused silica capillary GC column

via γ-irradiation, for example, which acts to initiate free radical reactions between adjacent stationary phase molecules. This has the effect of increasing the average M_r of the polymer, which in turn increases its three-dimensional stability, viscosity, and solvent resistance. Modified polysiloxanes in which a phenyl moiety, for example (arylene stabilized), is incorporated into the siloxane chain in place of some oxygen atoms [—O—Si(R_1R_2)—C_6H_5—Si(R_3R_4)—O—, cf. Figure 9.9] are also available. These phases give enhanced stability leading to a further decrease in column bleed, factors valuable in high sensitivity, high temperature analyses. The arylene substitution can be adjusted to maintain the same separation characteristics as the original, non-arylene stabilized phenylsiloxane.

An important advantage of capillary columns is that thermal mass is low and thus temperature programming is facilitated. It is usual to allow a short period ('solvent delay') at the start of the programme to allow an injection solvent to elute from the column before increasing the oven temperature. Interfacing to MS is simplified because carrier-gas flow rates (and column bleed) are low. A further advantage in comparison to packed columns is that when using polysiloxane stationary phases, accumulation of siliceous deposits on the detector is minimized. On the other hand, one complication is that GC detectors other than the MS require higher flow rates than normally used with capillary columns and thus a 'make-up' gas supply is needed (Section 9.2).

Capillary columns should be conditioned according to the manufacturer's instructions and tested by injecting an appropriate mixture before use for sample analyses. When conditioning columns it is obviously important to ensure that the GC oven temperature controller is working properly and that the carrier-gas flow is adequate because there is no detector output to help indicate if anything is wrong. If temperature programming is to be used, it is important that the column is not left at the maximum run temperature for a prolonged period.

PLOT (porous layer open tubular), SCOT (support coated open tubular), and WCOT (wall coated open tubular) are terms used to describe different types of capillary column. In PLOT columns a fine (10 μm or less) layer of stationary phase particles, for example activated alumina, is bonded to the internal surface of the capillary. Columns of this type, which are very retentive, are usually reserved for the analysis of very volatile substances such as permanent gases. They are not recommended for use with MS detection because of their tendency to shed particles of stationary phase.

In SCOT columns a liquid stationary phase is coated onto particles such as barium chloride microcrystals that are bonded onto the inner surface of the capillary, whilst in WCOT columns a liquid stationary phase is coated, or more usually, chemically bonded onto the capillary wall directly. Although glass capillary WCOT columns can be prepared in-house it is much more convenient to buy pretested columns. The stationary phases used are in general those developed for packed columns (Table 9.2), but better column deactivation and more uniform phase coating mean that good peak shapes are often obtained even when using low-polarity phases. Moreover, when used with temperature programming most phases are stable when used 20–50 °C above their packed-column temperature limit for short periods.

In general, efficiency is increased with narrower column diameters/thinner film thicknesses, but sample capacity is greater with wider bores/thicker films, and thus the risk of column overloading, usually manifest as 'fronting' (Section 7.2.8), is minimized. Columns of 0.32 mm i.d. and 30–50 m length with a moderate phase loading (0.2–0.3 μm film thickness) and used with an appropriate injection system are suitable for many purposes in analytical toxicology such as STA (Section 19.2).

Columns of 0.53 mm i.d. and 15–30 m length with relatively high phase loading (1–5 μm film thickness) have similar capacity and efficiency to packed columns at carrier-gas flow rates of 8–20 mL min^{-1}, but with the advantages of better peak shapes, reproducibility, and applicability

in temperature programming. When used at these flow rates, detector make-up gas may not be required. Alternatively, these columns can be used at lower flow rates (1–2 mL min^{-1}) that are more suitable for interfacing with MS (Box 9.4).

Box 9.4 GC capillary column selection

- Longer columns (25 or 50 m typical) give increased efficiency, but longer analysis times
- Narrower columns give more plates per metre, but other factors also important
  - 0.15 mm i.d. high efficiency with low carrier flow, but low capacity (split injection). Good for GC-MS
  - 0.32 mm i.d. reasonable efficiency and sample capacity (on-column injection). Good for STA and high sensitivity work
  - 0.53 mm i.d. gives efficiency and sample capacity at least equal to packed columns, but less tailing with polar compounds and temperature programming easier
- Film thickness (0.1–5 μm, often 0.25 μm) also important – influences both retention and sample capacity
- Cross-linked (immobilized) phases relatively stable. Large volume sample injections and column washing to remove non-volatile contaminants can be employed
- High efficiency means that fewer stationary phases needed. Commonly used polysiloxanes are:
  - OV-1/SE-30/OV-101, HP-5, OV-17, OV-225
- Other phases used include Carbowax 20 M, FFAP

Columns with a relatively high film thickness (5 μm film) can generally tolerate the injection of up to 1 μL of water and such columns may often be substituted directly for packed columns in a given method. Several manufacturers have supplied 'conversion kits' to enable these 'wide-bore' capillaries to be used in instruments configured for use with conventional packed columns. Some companies also produce capillary GC method optimization/simulation software for use with personal computers.

9.3.4 Multidimensional gas chromatography

In multidimensional GC (MD-GC) the sample is dispersed in different time dimensions. When a sample is separated using two dissimilar columns the total peak capacity will be the product of the individual column peak capacities. For maximum gain in resolution, separation in the individual time dimensions should be totally uncorrelated, i.e. orthogonal. Although this is rarely achieved in practice, significant gains in selectivity can be obtained without total orthogonality (Marriott *et al*., 2004).

In two-dimensional (2D) GC with heart-cutting, a *fraction* from the first retention axis is transferred for separation on the second retention axis. In contrast, in comprehensive 2D-GC ('GC×GC') fractions of the entire sample, after being separated on the first column, are passed for analysis on the second column. This is achieved with a longitudinally modulated cryogenic trap located between the columns. GCxGC is a continual sampling process that operates with a modulation period (P_M) faster than the peak elution width at the baseline on the first column, and normally uses a very short second column to ensure that the chromatography on column 2 is within the time of the P_M value. Thus, chromatography on the second column is typically

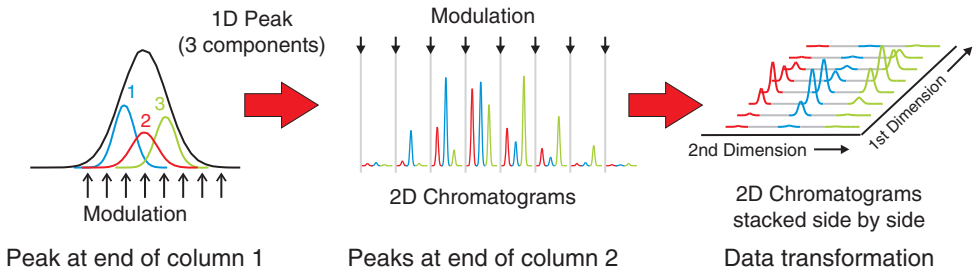


Figure 9.11 Schematic diagram of 2D-GC

between 2 and 10 seconds (Figure 9.11). A peak on the first column may consist of several compounds that are resolved on the second column, producing 'mini-chromatograms' for each modulation period (Adahchour *et al*., 2006). These can either be transformed into 2D chromatograms, or visualized as 2D, contour, or 3D plots.

The choice of columns is usually a non-polar phase with a polar phase, or *vice versa*, to achieve maximum orthogonality. A chiral phase may be used for the second column to resolve enantiomers that cannot be resolved on the first, achiral, column. The use of different phases makes the use of retention indices (Section 7.3) more complex. Alkanes may be suitable, particularly for the first column operated with temperature programming, when Equation (7.26) can be applied. However, the second column is usually either operated isothermally using a separate GC oven, or is considered to be isothermal (pseudoisothermal) because the change in oven temperature is so small during the few seconds the chromatography takes on column two. Consequently, Equation (7.25) is the more appropriate (von Mühlen & Marriott, 2011). Because alkanes may not chromatograph satisfactorily on the second column, retention indices based on other homologous series such as, alcohols, fatty acid methyl esters, and polyethylene glycols have been proposed (Veenaas & Haglund, 2018). The choice will be influenced by the nature of the analyte(s).

The potential for 2D-GC in ecstasy, heroin, and cocaine profiling (Mitrevski *et al*., 2011) and in doping control has been discussed (Kueh *et al*., 2003). However, these issues seem better tackled by conventional GC-MS or LC-MS procedures. On the other hand, MD-GC may have a role in chiral analysis (Elbashir & Aboul-Enein, 2018).

9.4 Headspace and 'purge and trap' analysis

Direct injection of blood after dilution with aqueous ISTD solution has been used for many years in the GC of ethanol and other low M_r volatile compounds using columns packed with a porous polymer such as Chromosorb 101 or Porapak Q, or a modified carbon black (Section 9.3.2). A large (5- or 10-fold) dilution with ISTD solution is needed to facilitate the use of aqueous calibration standards and QC solutions. Sensitivity is not an issue. However, static headspace (HS) analysis is generally preferred. The principle underlying HS-GC analysis is that in a sealed vial at constant temperature, equilibrium is established between volatile components of a liquid or solid sample in the vial and the gas phase above it – the 'headspace' (Figure 9.12). After allowing time for equilibration (normally 15 min or so) a portion of the headspace may be withdrawn via a rubber septum using a gas-tight syringe and injected onto the GC column.

Advantages of this method are that the risk of contamination of the column with non-volatile residues is virtually eliminated and that automation is relatively simple. An ISTD or other

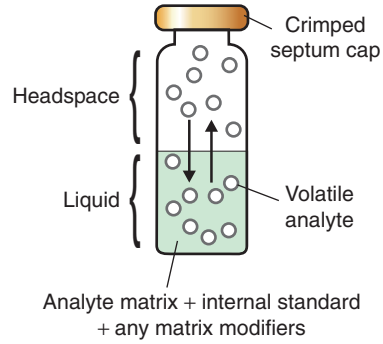


Figure 9.12 GC headspace vial

additive such as a matrix modifier, for example a concentrated salt solution to enhance partitioning of less volatile sample components into the gas phase, may be added prior to the incubation. Quantitative analyses may be performed after constructing a calibration graph. The technique is widely used in the analysis of ethanol and other volatile substances in biological samples and in the pharmaceutical industry for measuring solvent residues in tablets.

Generally, headspace vials are available in sizes of 6, 10, and 20 mL. A vial large enough to ensure an adequate phase ratio without excessive dilution of the components of interest in the headspace should be used. The phase ratio should normally be at least 50 % of the sample volume. Round-bottomed vials are stronger than flat-bottomed ones and tend to withstand higher pressures and thus are more suited to work at elevated temperatures, for example if derivatization is to be performed. Headspace vials must be correctly crimped (the cap should not turn after application) and septa suitable for the incubation temperature and the analyte(s) must be used. Poor quality septa may release volatile components into the headspace, and/or may adsorb analyte(s) from the headspace.

Sample preparation aims to maximize the concentration of volatile components in the headspace and minimize unwanted contamination from other compounds in the sample. The equilibrium distribution of the concentration of analyte in the sample phase (C_s) and that in the gas phase (C_g) is denoted by the partition coefficient (K):

$$K = \frac{C_s}{C_g} \tag{9.1}$$

Thus, sensitivity increases as K decreases, that is compounds with low K values tend to partition more readily into the gas phase giving relatively low LoDs. Conversely, compounds with high K values partition less readily into the gas phase and have relatively high LoDs. Ethanol has a relatively high K value of 1355 at 40 °C in an air–water system (Table 9.4).

K can be lowered by raising the incubation temperature. In the case of ethanol in water, K can be lowered from 1355 to 328 by increasing the incubation temperature from 40 to 80 °C, although 60–65 °C is used when measuring blood ethanol by headspace GC to minimize injection of water vapour.

K may also be lowered by adding salts to the analyte matrix. High salt concentrations in aqueous samples decrease the solubility of polar organic volatiles and promote transfer into the headspace. However, the magnitude of the effect is not the same for all compounds. Compounds with K values that are already relatively low, for example, show very little change in K after adding a salt. Generally, volatile polar compounds in polar matrices (aqueous samples)

Table 9.4 Partition coefficients (K) of some common solvents in an air–water system (40 °C)

Solvent	K	Solvent	K
Cyclohexane	0.077	Butyl acetate	31.4
Hexane	0.14	Ethyl acetate	62.4
Tetrachloroethylene	1.48	Butanone	139.5
1,1,1-Trichloroethane	1.65	Butanol	647
o-Xylene	2.44	2-Propanol	825
Toluene	2.82	Ethanol	1355
Benzene	2.90	Dioxane	1618
Dichloromethane	5.65		

will experience the largest shifts in K and have higher responses after the addition of a salt. Commonly used salts include ammonium chloride, ammonium sulfate, sodium chloride, sodium citrate, sodium sulfate, and potassium carbonate. A practical consideration is that it is not easy to add reproducible amounts of solid substances to the vials.

K is also dependent on the phase ratio, β, the relative volumes of the headspace (V_g) and the sample (V_s):

$$\beta = \frac{V_g}{V_s} \tag{9.2}$$

Sensitivity is increased as β is minimized, that is to say lower values of β (larger sample sizes) will yield higher responses for volatile compounds. However, reductions in β do not always yield the increase in concentration in the headspace (C_g) needed to improve sensitivity. When the sample size is increased, compounds with high K values partition less into the headspace compared to compounds with low K values and yield correspondingly smaller changes in C_g. Samples that contain compounds with high K values need to be optimized to provide the lowest K value before changes are made in β. Thus, partition coefficients and phase ratios together determine the final concentration of volatile compounds in the headspace.

The concentration of volatile compounds in the gas phase can be expressed as:

$$C_g = \frac{C_0}{K + \beta} \tag{9.3}$$

where C_0 is the original concentration of volatile analytes in the sample.

In addition to working with the partition coefficient (K), the phase ratio (β), and derivatization reactions, sensitivity in headspace GC can be improved by increasing the size of the sample that is withdrawn from the headspace for analysis. Increasing the sample size increases the time it takes to transfer sample to the column in proportion to the column volumetric flow rate. Sample size can be increased only to the point that increases in peak width, caused by longer sample transfer times, do not affect the chromatography.

Some ways of enhancing sensitivity in static HS-GC have been explored (Snow & Bullock, 2010). Larger sample sizes and longer transfer times can be offset to an extent by using cryogenic cooling and sample refocusing at the head of the GC column (‘dynamic sampling’). A range

of static and dynamic HS sampling techniques for GC have been compared (Kremser *et al*., 2016). In the 'purge and trap' method volatile compounds are liberated from liquid samples or suspensions by bubbling with an inert carrier gas such as nitrogen or helium and subsequently either condensed in a receiver cooled usually with solid carbon dioxide, or adsorbed on a cartridge filled with a material such as Tenax-GC (Section 9.3.2).

9.5 Formation of artefacts in gas chromatography

Some analytes may be thermally labile during GC and the decomposition products may interfere in the analysis. However, in some cases the decomposition product can be used to aid analyte identification if the mechanism of artefact formation is known (Table 9.5).

9.6 Derivatization for gas chromatography

Analytes with polar functional groups, particularly phenols, alcohols, and carboxylic acids, may give inefficient, tailing peaks on GC, leading to poor selectivity and sensitivity. Derivative formation is sometimes performed to achieve satisfactory chromatography, to improve the detection characteristics of an analyte, and on occasions to provide additional evidence of compound identity. Derivatization may be used to shorten or lengthen the retention time as required, and also to permit the separation of enantiomers (Box 9.5). Whilst derivative formation in GC still has a place, LC-MS has abolished the need to derivatize many analytes.

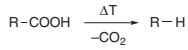
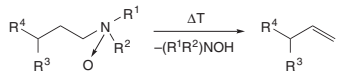
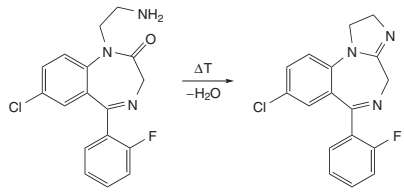
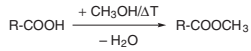
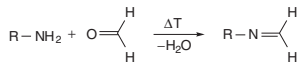
The choice of derivatization reagent and hence the reaction conditions will be based on the functional group to be derivatized, the presence of other functional or labile groups in the molecule, and the reason for performing the derivatization. Generally, three types of reactions are performed, i.e. silylation, acylation, and alkylation. In GC, silylation generally reduces retention, whereas acylation usually increases retention. The order of elution for derivatives of a homologous series will be the same as the underivatized parent compounds.

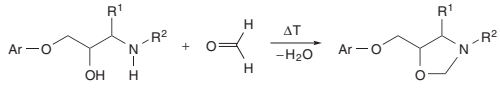
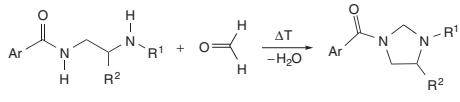
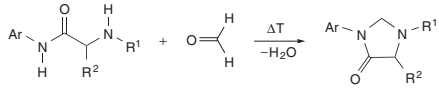
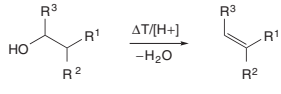
An obvious factor is that the analyte must be amenable to derivatization. It is also important that any ISTD undergoes the same derivatization reaction as the analyte. Use of a stable isotope-labelled ISTD in GC-MS is preferred (Section 13.11). The derivatization reaction may be carried out during the extraction or other sample preparation procedure, on a dried residue, in an autosampler vial, on a SPME fibre in a headspace vial, or after injection onto the GC column ('on-column'). If derivatization is to be performed prior to the injection, then the reaction needs to be rapid and quantitative if possible, as otherwise problems may arise in assessing the optimum reaction time.

There may be a number of confounding issues: (i) the need to remove excess derivatizing agent; (ii) the risk of decomposition of the analyte, the derivatizing agent, or other reagents, and of the derivative itself; and (iii) the risk of interference by contaminants, breakdown products, or reaction by-products. 'On-column' derivatization in GC may not suffer from these drawbacks and has the advantage that the analyte and derivatizing reagent can be injected directly. However, the analyte may not be amenable to this technique. Finally, care must be taken to ensure that artefacts such as acetylation of morphine to 6-AM or diamorphine, or decomposition of diamorphine or 6-AM to morphine, do not occur either during or as a result of the derivatization process used.

Most aromatic and aliphatic hydroxyls form silyl ethers when treated with trimethylsilyl (TMS) reagents at room temperature under anhydrous conditions or, in the case of sterically hindered compounds, on heating (Table 9.6).

Table 9.5 Examples of artefact formation during GC (Maurer *et al*., 2016–reproduced with permission of John Wiley & Sons)

Summary	Chemical reaction	Example
Decarboxylation of carboxylic acids		Carbendazim
Cope elimination of *N*-oxides		Amitriptyline *N*-oxide
Rearrangement of didesethylflurazepam		–
Thermal decomposition	Elimination of bromomethane, formaldehyde, hydrochloric acid, hydrocyanic acid, ammonia, sulfonamide moiety	–
Methylation of carboxylic acids in methanolic solution		–
Formation of formaldehyde adducts if methanol used as injection solvent		Amfetamine

		Acebutolol
		Flecainide
		Prilocaine
Dehydration of alcohols		Tramadol
Decarbamoylation of carbamates	-CHNO decarbamoylation -C_2H_3NO *N*-methyl decarbamoylation -C_3H_5NO *N,N*-dimethyl decarbamoylation -C_5H_9NO *N*-isobutyl decarbamoylation	Carbamate pesticides

Box 9.5 Summary of reasons for derivatization in gas chromatography

- To improve resolution and reduce tailing of polar compounds (–OH, –COOH, $–NH_2$, –SH, and other functional groups) and thereby increase sensitivity and selectivity
- To adjust retention and thereby improve resolution
- To facilitate enantiomer resolution
- To increase analyte volatility
- To increase detectability by introducing detector-specific moieties (e.g. $–COCF_3$)
- To improve analyte stability (for example in mass fragmentation)
- To confirm analyte identity

Table 9.6 Examples of TMS reagents used in gas chromatography

Agent	Name	Notes
BSA	*N,O*-bis(Trimethylsilyl)acetamide	Volatile by-products. Good solvent properties. Unhindered –OH. Alcohols, phenols, carboxylic acids, amides, amines, acid anhydrides
BSTFA	*N,O*-bis(Trimethylsilyltrifluoro)acetamide	More reactive than BSA. Good solvent properties. By-product HF reduces detector contamination (FID). Unhindered –OH. Alcohols, phenols, amides, amines, carboxylic acids, carbohydrates, acid anhydrides, sulfonamides
HMDS	Hexamethyldisilazane	Only by-product ammonia. Can be used with acid catalysts. Alcohols, phenols, carboxylic acids, and amines
TSIM	*N*-Trimethylsilylimidazole	More reactive than BSA/BSTFA. Alcohols, fatty/organic acids, carbohydrates, carboxylic acids, sulfonic acids, phenols, thiols
TBDMSIM	*tert*-Butyldimethylsilylimidazole	Unhindered alcohols/phenols. Produces more stable esters. Does not release HCl
BMDMCS	Bromomethyldimethyldichlorosilane	Used for electron-capturing derivatives
CMDMCS	Chloromethyldimethyldichlorosilane	Used for electron-capturing derivatives
TMCS	Trimethylchlorosilane	Added to other agents to increase reactivity

Carboxylic acids are usually analyzed as methyl or butyl esters. Methylation can be achieved by heating (60–100 °C, 30 min) with 14 % w/v boron trifluoride in methanol. After evaporating most of the solvent and adding water, the methylated derivative can be extracted into an organic solvent such as hexane. Diazomethane may also be used as a methylating reagent and reacts rapidly *in vitro* at room temperature; excess reagent may be removed by evaporation. However, care is needed because diazomethane is carcinogenic, highly toxic, and potentially explosive.

In addition, diazomethane will react slowly with phenols and this may be disadvantageous because mixtures of partially methylated products may result.

On-column methylation of barbiturates, hydantoins, and some carboxylic acids can be achieved by injecting the sample mixed with 0.2 mol L^{-1} trimethylanilinium hydroxide in methanol. However, 'ghost' peaks may arise on subsequent injection of derivatizing reagent if incompletely methylated material has accumulated on the column.

Commonly used reactions before GC analysis of primary and secondary amines, and of phenolic hydroxyls, include acylation using reagents such as acetic and propionic anhydrides, or their acid chlorides. Primary amines react with aldehydes or ketones, such as acetone, benzaldehyde, and cyclohexanone, to give imines, whilst secondary amines produce enamines. Hydrazines and hydroxylamines react with carbonyl compounds to give hydrazones and oximes, respectively. Imine formation is exemplified by the reaction between acetone and amfetamine (Figure 1.2).

Derivatization can also be used in headspace GC (Section 9.4) to increase sensitivity and improve chromatographic performance for specific compounds. Some acids, alcohols, and amines may be adsorbed not only on the analytical column, but also in the injection port giving rise to tailing peaks and a poor detector response. In addition, they may be very soluble in the matrix, causing poor partitioning into the headspace. Derivatization can improve volatility, as well as reducing the potential for adsorption onto the components of the GC system.

Common derivatization reactions that may be performed using the sample vial as the reaction vessel are esterification, acetylation, and alkylation. However, although derivatization may improve chromatographic performance and volatility, the derivatization reaction itself may introduce other problems. The derivatization reagent, as well as any by-products from the reaction(s), may be volatile and partition into the headspace along with the derivatized analyte(s), and may elute at similar retention times to the compound(s) of interest. The derivatization reaction may also need to be performed at an elevated temperature, and in such cases the pressure inside the sample vial may exceed the pressure handling capabilities of either the vial, or the septum. Specially designed caps are available that allow excess pressure to be vented during derivatization reactions.

There are a number of halogenated silylating reagents available that may be used to impart electron capturing properties to the derivative (Table 9.6). However, usually perfluorinated acylating and alkylating reagents are employed for this purpose. A drawback to using electron-capturing derivatives is that biological extracts often contain many compounds with reactive functional groups and so the resulting chromatograms may contain additional peaks, which may interfere.

9.7 Chiral separations

LC has been widely applied in this area, but GC has also been used (Gumustas *et al*., 2018). LC has the advantage that the resolved analytes are more easily recovered from the column eluate and preparative-scale systems are common, whilst GC detectors are in general more sensitive. Modified lipophilic cyclodextrin (CD) derivatives have proved superior to all previous chiral stationary phases (CSPs) for capillary GC and have an almost unlimited range of application (Menestrina *et al*., 2016).

CDs are cyclic, chiral, torus-shaped molecules consisting of six or more D-(+)-glucose moieties linked via α-(1–4) glycoside bonds. α-CDs contain six, β-CDs seven, and γ-CDs eight

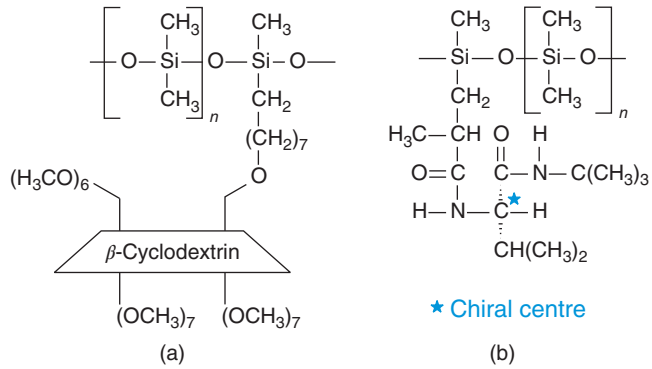


Figure 9.13 Examples of chemically bonded chiral phases. (a) β-CD bonded (Chirasil-β-Dex) (b) *N*-propionyl-L-valine-*tert*-butylamide (Chirasil Val)

amylose units [Xiao *et al.,* 2012; Figure 9.13(a)]. Peralkylated α-, β-, or γ-CDs can be used in fused silica capillaries dissolved in polysiloxanes to give chiral GC columns. Without the CD additive the columns are achiral. Enantiomers of polar compounds such as alcohols, diols, and carboxylic acids can be resolved underivatized. Moreover, racemic alkanes and cycloalkanes can be separated using CD-modified phases. Enantiomers of halogenated anaesthetics (enflurane, halothane, isoflurane), have been resolved using such systems (Schuring, 2013), as have enantiomers of ketamine and norketamine (Williams & Wainer, 2002). These columns will often separate positional isomers as well as enantiomers. The useable temperature range is *ca*. 30–240 °C.

As well as CD-modified phases that can be bonded to the column [Figure 8.13(a)], a number of chiral GC phases have been described including amides, diamides, dipeptides, and polysiloxanes with chiral substituents, but the low thermal stability of these phases precluded their widespread use. However, modification of phenylmethylpolysiloxanes by introducing L-valine-*tert*-butylamide [Figure 9.13(b)] or L-valine-*S*-α-phenylethylamide moieties can give chiral phases with temperature stability as high as 230 °C. PDMS phases modified with (*S*)-(–)-*tert*-leucine derivatives have also been used. In addition, a copolymeric stationary phase consisting of a chiral component, (1*R-trans*)-*N*,*N'*-1,2-cyclohexylenebisbenzamide and a PDMS phase with good temperature stability, has been developed (Juvanc *et al*., 2002).

If the analyte contains a reactive functional group, then derivatization using an optically pure reagent and analysis of the resulting diastereomers on an achiral GC column may prove adequate. Many derivatizing reagents have been used including, for amines, (*S*)-(–)-*N*-trifluoroacetylprolyl chloride, and *S*-heptafluorobutyrylprolyl chloride (Figure 9.14).

Further practical applications of the use of capillary GC in the analysis of enantiomers are (i) the measurement of amfetamine, metamfetamine, MDA, MDMA, and MDEA enantiomers from clinical toxicology and driving under the influence cases in plasma (Peters *et al*., 2003) or oral fluid (Peters *et al*., 2007), (ii) the separation of the enantiomers of eight novel amphetamine analogues after derivatization with either (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA), or (1*R*)-(–)-menthylchloroformate (Weiß *et al*., 2017), (iii) the identification of the (*S*)-enantiomer of pregabalin after methylation and derivatization with (*S*)-(–)-*N*-(trifluoroacetyl)pyrrolidine-2-carbonyl chloride (Hitchcock & Marginean, 2019),

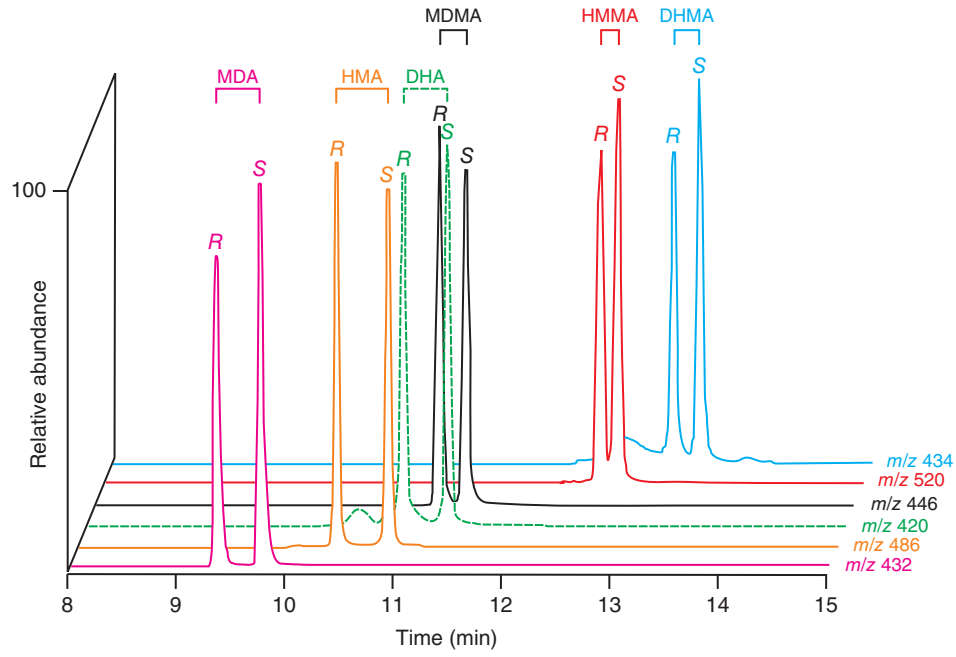


Figure 9.14 Extracted negative ion chromatograms (GC-MS) of *R*-/*S*-enantiomers of MDMA and its phase 1 and phase 2 metabolites in human urine after (*S*)-heptafluorobutyrylprolylation (Schwaninger *et al*., 2011–reproduced with permission of John Wiley & Sons)

and (iv) the measurement of synthetic cathinone enantiomers in urine and plasma, again after derivatization with (*S*)-(–)-*N*-(trifluoroacetyl)pyrrolidine-2-carbonyl chloride (Alremeithi *et al*., 2018).

9.8 Summary

There is no doubt that GC is the system of choice for the analysis of solvents and other volatiles, and that it is a reliable interface for MS. The high efficiency and stability of modern capillary columns together with other features of GC, such as the ease of temperature programming and the reproducibility and transferability of normalized retention data, are features that other chromatographic systems cannot match (Box 9.6). GC-MS thus remains widely used in testing for substance misuse and in STA (Chapters 18 and 19).

The drawbacks of GC are the need for dedicated instruments, experienced operators, and appropriate laboratory infrastructure. There is also the requirement to perform LLE or some similar method of sample preparation and the restriction on the M_r of analytes to those that are stable and volatile, either derivatized or underivatized, at the oven temperature required for the analysis (in practice up to M_r 750 or so). The great advantage of LC, especially when combined with MS, is that these latter restrictions are minimized. But all is not necessarily straightforward even with LC and LC-MS, as discussed in Chapters 10 and 13, respectively.

Box 9.6 Summary of the use of GC in analytical toxicology

- Modern fused silica capillaries offer unrivalled efficiency and stability, especially when used with temperature programming
  - MS interface easy (use in EI or PCI mode)
- In general, use HS-GC for volatiles with 60 m × 0.56 mm i.d., 5 µm film, OV-1 or equivalent capillary column with split FID and/or MS detection
  - For ethanol or methanol, direct injection often adequate
- For STA use 30 m × 0.35 mm i.d. chemically bonded fused silica capillaries (HP-5 or equivalent) with temperature programming and split FID/EI-MS detection
  - Take care to minimize losses of volatiles such as amfetamine during sample preparation
  - May need to derivatize polar analytes such as morphine
  - Split/splitless in splitless mode best for quantitative work

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10 Liquid Chromatography

10.1 Introduction

In the analysis of drugs and poisons, LC has practical advantages of flexibility, generally low running costs, a number of selective detectors that can sometimes be linked in series, and ease of automation. It is well suited to the analysis of hydrophilic, thermally labile, and/or high M_r compounds (Box 10.1). These properties can often be exploited to facilitate the analysis of several compounds (drug and metabolites, for example) at once. Major uses of LC include pharmacokinetic and metabolic studies, in TDM, in monitoring exposure to toxic chemicals, and increasingly, in STA.

Box 10.1 Advantages of liquid chromatography in analytical toxicology

- Rapid, efficient separations – can control selectivity by adjusting eluent composition
- Analyze high M_r and/or polar molecules that are not amenable to GC
- Sensitive, selective, non-destructive detectors (UV, fluorescence) that can be used in series
- Ability to perform post-column reactions
- Can interface to MS to give very selective detection for targeted analytes
- Can inject aqueous samples (but selectivity reduced compared with solvent extracts, for example)
- Running costs low
- Portable equipment – normally no gas supplies, etc.
- Relatively easy to automate
- Silica-based packings are robust – can be used with polar and non-polar eluents

Historically, LC of drugs and other poisons, and their metabolites, was performed isocratically (constant eluent composition). However, increasingly gradient elution is used if compounds with widely different polarities are to be measured in a single analytical sequence and/or a rapid analysis time is required. Operation at above ambient temperature using a column oven increases reproducibility of retention and improves efficiency by reducing eluent viscosity. However, caution is needed in order not to alter eluent composition or promote dissolution of the column packing by using too high a temperature.

LC is not without its difficulties (Box 10.2). Both sample volume and detector sensitivity may be limiting, and potential interferences are legion. Accumulation of sample components on the column and/or of particulate material on the top of columns may also contribute to

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

Box 10.2 Limitations of liquid chromatography in analytical toxicology

- Hardware/infrastructure expensive; need experienced operators, pure solvent supplies, consumables support
- LC resolves relatively few peaks in useful range ($k = 2$–10) without gradient elution
- Elution independent of M_r (cf. GC)
- Gradient elution comparable to temperature programming in GC, but more problems (baseline drift, re-equilibration especially with UV and ED detection)
- Only ED, fluorescence, and MS detectors give sensitivity comparable to GC detectors, but relatively selective
- No universal detector comparable to FID in sensitivity
- With LC-MS cannot use EI to generate principal peaks index
- Efficiency influenced by many extraneous factors (injection volume, injection solvent, 'dead' volumes in injectors/tubing/detector, detector sampling rate, void formation, blockage of frits, etc.)
- Always the possibility of co-eluting sample components

the loss of efficiency usually observed in routine use. With reaction detectors such as MS the presence of co-eluting compounds can alter the analyte signal dramatically. Ion suppression and/or enhancement are major considerations in LC-MS (Section 13.11).

Whilst analyte thermal stability is not as important as in GC, many compounds (especially metabolites) are unstable either in biological samples, or if subjected to extremes of pH, for example during sample preparation. Thus, as well as the choice of the column/eluent combination, due consideration must be given to the collection and storage of the sample (Chapter 2), the choice of sample preparation procedure (Chapter 4), and the detection conditions. In LC-MS, there may be analyte decomposition in the ion source, for example. Selection of a suitable ISTD is also an important step.

Operationally the big difference between GC and LC is the nature of the eluent. Liquids are not as compressible as gases. This means that in LC smaller particle-size packings can be used giving high efficiencies with relatively short columns at ambient or near-ambient temperature. In addition, the composition of the eluent can be altered to control retention and selectivity giving a very high degree of control over a given separation.

10.2 General considerations

A typical LC system consists of an eluent reservoir, a high-pressure pump (or pumps in the case of high-pressure mixing gradient systems) with flow controller, a sample injection system, stainless steel guard and analytical columns packed with stationary phase material, a detector usually with a low volume flow cell, and a data capture system (Figure 10.1). The most commonly used detectors in analytical toxicology are UV/Vis, including DAD, fluorescence, and MS.

Isocratic elution is the simplest mode of LC and is analogous to isothermal operation in GC. However, in gradient elution the eluent composition (organic solvent content, pH, or ionic strength) is made to change in a predetermined way by using a second solvent reservoir and pump, and a mixing chamber. Alternatively, solvents from up to four reservoirs can be combined using a proportionating valve and mixing chamber prior to a single pump (low pressure mixing).

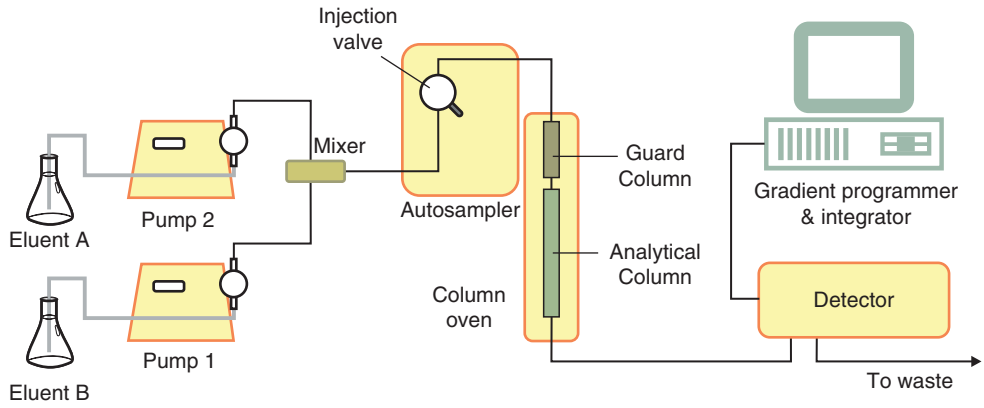


Figure 10.1 Schematic diagram of a dual pump gradient LC system with high-pressure mixing

The advantages of gradient elution are that later eluting peaks are sharpened and analysis times are shortened. Eluent delivery can be programmed to return to starting conditions, but this may take longer than cooling a GC oven. Baseline drift during gradient elution with UV detection can be minimized if the absorptivity of the components of the gradient at the detection wavelength is balanced beforehand. An alternative, use of an eluent splitter to divert a portion of the eluent to the reference cell in a double-beam detector, is rarely used. A major advantage of MS detection is that baseline drift during gradient elution is not normally a problem.

10.2.1 The column

As with other forms of elution chromatography, the LC column is the heart of the system. In order to maintain a column in good working order it must be used carefully, especially with regard to the integrity of the column bed. The higher efficiencies that are possible with LC as compared with traditional gravity-driven column chromatography result from use of column packing materials with particle sizes typically in the range 1.8–5 μm. The back-pressures that are generated require packings that can stand such forces, and because silica [silicic acid, hydrated silicon dioxide, $(SiO_2)_n.xH_2O$], can be used for long periods at pressures up to 28,000 kPa (280 bar) without physical damage, it is the most commonly used LC packing material. Furthermore, the surface silanol groups can be exploited to produce a large range of chemically modified phases (Section 4.2.3.1).

To withstand the operating pressures used, the column material is most commonly packed in stainless steel tubing with porous frits (typical pore size 0.5–2 μm, depending on the size of the packing material), at each end. Many manufacturers have also developed cartridge systems using stainless steel, glass, or polyetheretherketone (PEEK, Figure 10.2) columns in stainless steel or

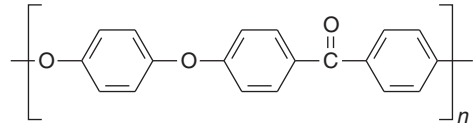


Figure 10.2 Structure of polyoxy-1,4-phenyleneoxy-1,4-phenylenecarbonyl-1,4-phenylene (polyetheretherketone, PEEK)

aluminium enclosures. If the inlet frit becomes partially blocked with particles not previously removed from the eluent and sample extracts, not only will the column back-pressure increase, but also column efficiency will likely be adversely affected because the sample will not be dispersed evenly onto the top of the column.

Columns should not be used with eluents that may, for example, dissolve the packing, or with samples or sample extracts that may introduce particles or cause precipitation of solid material in the system. It is important to ensure that organic solvents are only used with solvents with which they are miscible, and this should be borne in mind when changing eluents. Unmodified silica columns are normally supplied filled with hexane as they are usually used for normal-phase chromatography (Section 10.5.1). However, if used with methanol or methanol:aqueous buffer eluents, the hexane will have to be removed before use. Purging with MTBE or ethyl acetate, for example, which are miscible with hexane, and then with methanol, before using methanol:water ensures safe removal of the hexane. 2-Propanol can be used instead of ethyl acetate, thereby obviating the need for the methanol step, but is more viscous and tends to generate high back-pressures.

Use of too high an inlet pressure may crush silica packings, causing void formation and, in the case of chemically modified silica, revealing untreated surfaces with consequent impairment of selectivity and/or efficiency. Even at normal operating pressures there is a tendency for voids to form at the top of columns during use, although this seems to be not as common with spherical particles as it used to be with older, irregular shaped silica packings. The presence of a void will cause peak broadening, but the column may still give acceptable results. However, sudden changes in pressure or even dropping a column may cause channels to form in the packing with resultant loss of performance, which may be manifest as poor peak shapes or even double peaks.

Columns are supplied with an indication of the direction in which they were packed, and it is usual to maintain the flow direction in use. However, at least one manufacturer supplies columns that are designed to be used with the flow in either direction, the instruction being to reverse the flow regularly to reduce the risk of clogging of the frits. It is important not to connect to the detector until flow has been established for several column volumes to minimize the risk of particles being swept into the detector. It may be possible to reverse the direction of flow, particularly with columns packed with spherical particles, without appreciable deterioration in performance. However, it is better to stop the inlet frit becoming blocked in the first place. Monolithic silica columns (Section 10.4.1.4) should only be used in the indicated flow direction.

10.2.2 Column configuration

An in-line filter placed between the pump and the injector to trap particles from the piston seal can be beneficial, particularly if the outlet valve does not have its own filter. Filters have the advantage that they can be quickly and easily replaced. A guard column is a small column placed in front of the analytical or preparative column to protect it from impurities from the sample, wear particles from the injection valve, etc. It should be approximately 5 % of the size of the working column and should be changed frequently.

Use of a short column of narrower bore than the analytical column (say 2 mm i.d. if a 4.6 mm i.d. analytical column is being used) will help minimize peak broadening. The guard column should contain the same packing material as the analytical column. A sacrificial guard column packed with relatively large particle size unmodified silica, for example, may be inserted in-line between the pump and the injection valve to trap wear particles from the pump, to act as a pulse dampener, and to saturate the eluent with silica.

Low dead volume (LDV) tubing and fittings are used to connect the column to the injection device and to the detector. Smaller bore columns can give greater sensitivity and use less solvent, but if either the system dead volume, or the detector cell volume are too high, the extra-column band broadening will make the smaller bore column appear to perform poorly. Inert plastic (PEEK) finger tight male and stainless steel female fittings are sometimes employed. The plastic fittings do not lock onto the connecting tubing in the same way as stainless steel fittings and slide to fit any type of column fitting correctly.

If conventional male end-fittings are used then use of plastic (PTFE, Vespel, PEEK) ferrules may be possible even at the inlet end of the column as this minimizes the risk of physical damage to the stainless steel fittings and tubing from overtightening, and allows adjustment of the tubing as with finger tight fittings. Stainless steel or, increasingly, PEEK tubing may be used for pre-column connections. Use of narrow bore (0.15 mm i.d.) PTFE tubing for post-column connections is not only convenient, but also minimizes band broadening, particularly if several detectors are connected in series.

10.2.3 Column oven

For reverse-phase separations a stable column temperature of 35–40 °C improves efficiency and reproducibility. In general, a change in LC column temperature of 1 °C will change a retention time by 1–2 %. Thus, a column temperature change of 5–10 °C during a day is likely to be a source of significant variation in retention. Therefore, the use of column temperature control in LC not only increases column efficiency, but also improves retention time reproducibility and selectivity control.

Baseline drift, which may be observed with flow-sensitive detectors such as ED, can be eliminated by careful temperature control. Column ovens with high-velocity circulating air fans analogous to GC ovens can be used. Alternatively contact heating via aluminium blocks or by circulating fluid from a constant-temperature bath can be employed. Ideally, the column temperature should be controlled within ±0.2 °C to eliminate peak drift. If an oven is not available, the column should not be placed where it might be subject to excessive changes in temperature, e.g. near an open window or in direct sunlight for part of the day.

In the case of the immunosuppressant ciclosporin, LC column operation at 60 °C or so is necessary because at lower temperatures this drug exists as different conformers, each of which has a slightly different retention time on reverse-phase LC giving rise to a very broad peak (Section 20.6.8).

10.2.4 The eluent

Use of pure water is important particularly with gradient elution when impurities may be retained and concentrated on the column at the start of the analysis only to elute as peaks as the proportion of the organic component in the eluent is increased. Water purified by reverse osmosis may be suitable, particularly for isocratic separations. Deionized water is usually suitable for gradient systems, although contaminants may arise from impurities in the ion-exchange resins. Therefore, for high-sensitivity gradient work, use of certified LC-quality water is advisable.

Buffer salts and other reagents should be of the highest available purity. Generally, organic solvents should be LC grade, although this usually only indicates that the solvent has an appropriately low UV cut-off. Acetonitrile is available in several grades, a high purity grade being for use with UV detection down to approximately 205 nm.

It is important that LC eluent composition is reproducible if consistent separations and retention times are to be achieved. Solvent volumes should be measured in appropriate apparatus and liquids mixed carefully with cooling if necessary. Measuring cylinders may be suitable for large volumes, whereas for small volumes pipetting may be more appropriate.

Eluent pH may be controlled by: (i) using a buffer of known pH, (ii) adjusting the pH of the eluent just prior to making up to final volume, or (iii) adding a known volume of an acid or base such as trifluoroacetic acid or triethylamine to a set volume of eluent. Adding organic solvent to a buffer alters the hydrogen ion activity, such that the pH of the mixture differs from the buffer pH. The difference between the buffer pH and the 'apparent pH' (pH*) of the organic solvent–buffer mixture increases as more organic solvent is added. The terms pH and pH* are often used indiscriminately, and it not always possible from published reports to deduce how an eluent was prepared, which can result in failure to reproduce a given separation. Moreover, if the pH* is too high, the life of a silica-based column may be drastically shortened. Measuring accurate pH* in eluents with a high proportion of organic modifier requires a special pH electrode and thus the pH of the buffer is normally adjusted before mixing with organic solvent, which is acceptable provided the resulting eluent is suitable and reproducible.

Particles should be removed from the eluent before use by filtration through a 0.45 μm membrane. Some methods filter the buffer before it is mixed with organic solvent or even before the pH is adjusted. Because a precipitate may form on pH adjustment or addition of solvent, it may be better to filter the eluent just prior to use, providing this does not change the eluent composition significantly.

The final stage in eluent preparation is 'degassing'. Dissolved gas should be removed to prevent bubble formation in the pump head (cavitation), which affects flow control, and in detector flow cells, which affects baseline stability. Removal of oxygen from the eluent may also be important if low-wavelength UV is to be used. For some applications vacuum filtration may suffice. If additional degassing is desired after filtration, the vacuum pump can be left on and a stopper placed over the top of the flask containing the filtered eluent. Further eluent degassing will be indicated by the formation of small bubbles. Bubbling (sparging) with helium is the most effective way of degassing and a slow constant stream of helium should be maintained to prevent baseline drift due to oxygen redissolving in the eluent if working at 200–215 nm. However, both vacuum degassing and helium sparging may alter eluent composition by removing more volatile eluent components selectively. Placing the eluent reservoir in an ultrasonic bath for a few minutes is an alternative. In-line degassing units, which apply a vacuum to the eluent as it flows through semi-porous fluoropolymer tubing, have also been employed.

10.2.5 The pump

The pump has the role of delivering the eluent at a constant and reproducible flow rate independent of column back pressure. Although positive displacement (syringe) pumps were manufactured, nowadays usually eluent flow is induced by the reciprocating action of a sapphire piston and hence the pump is fitted with inlet and outlet check valves to maintain the flow in the required direction. During the filling stroke the inlet check valve opens as eluent is drawn into the pump head (Figure 10.3). On the displacement stroke the increased pressure closes the inlet check valve and opens the outlet valve so that high pressure eluent is pumped in the required direction. An outlet valve filter prevents pump seal debris from entering the valve where it might lodge in a ball valve, causing the valve to malfunction. A flexible, but pressure-resistant seal (often graphite filled PTFE) prevents leakage of eluent around the piston.

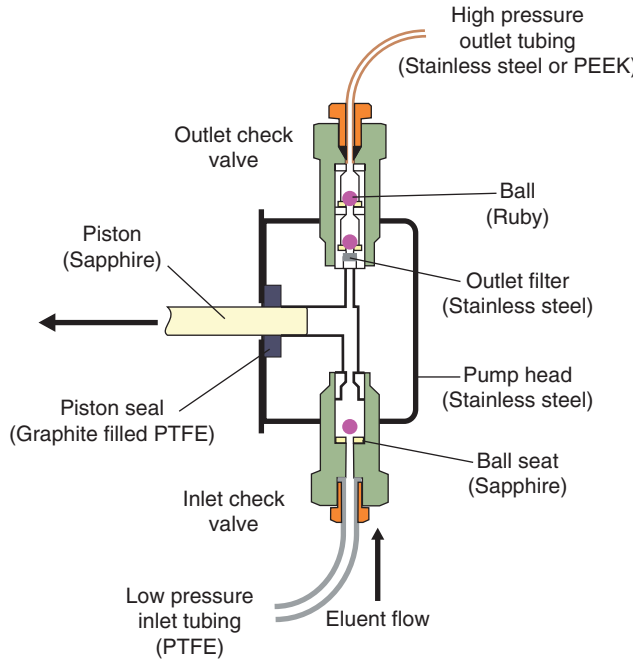


Figure 10.3 LC pump head during a filling stroke

The pump components that have contact with the eluent(s) have to be of inert materials, typically glass, ceramic, and/or stainless steel. Routine maintenance should include ensuring that the valves are functioning correctly and that the piston seal is not leaking. When working with aqueous buffer systems, the piston should be washed frequently with water to remove crystallized buffer salts thereby prolonging the life of the piston and the piston seal. The buffer should be flushed from the pump head using a compatible solvent before the pump is switched off. When changing a piston seal, any debris adhering to the piston may be removed by gentle polishing with toothpaste, for example. A scored (damaged) piston usually has to be replaced.

The filtered and degassed eluent should be placed into the eluent reservoir. The inlet tubing to the pump should be fitted with a filter to prevent particles that may have accidentally entered the reservoir passing through to the pump. Some pumps require the reservoir to be positioned higher than the pump to provide a static head pressure and this arrangement is useful when the pump is being primed. The reservoir may be pressurized, but this is not normally necessary. The eluent flow rates used are typically 1–2 mL min^{-1} with 2–5 mm i.d. columns containing 3–5 µm aps packings. Most pumps have an upper operational pressure limit of 34,500 kPa. Some pumps can be used with microbore or capillary LC columns (flow rates in the µL min^{-1} range).

Fluctuations in pressure (pulsations) when delivering the eluent are undesirable because of possible effects on baseline stability, hence LC pumps are designed to minimize such fluctuations (<1 % or better short-term variation). Use of a sacrificial guard column (Section 10.2.1) positioned between the pump and the injector can act as a pulse dampener if needed. Dual piston pumps, working either in parallel or in series, are a further way to minimize fluctuations in flow.

10.2.6 Sample introduction

Sample introduction is almost always via a sample injection loop operated electro-mechanically (as part of an autosampler). There are two types of LC sample valve: internal loop and external loop. Internal loops are designed to deliver sample volumes of less than 1 μL, and external loops can deliver sample volumes from a few μL to 20 mL or more. External loops use narrow bore tubing as the volume measure, and the internal type has a small hole or a narrow space in a disk which gives the fixed volume. Most valves work by having a stationary body, often made of stainless steel, known as the stator and a moving part, the rotor, which must be made of a softer material such as Vespel or PEEK to ensure a leak free seal between the two surfaces. If necessary, valves made of biocompatible materials such as PEEK or titanium rather than stainless steel may be used.

A diagram of a six-port LC sample injection valve is shown in Figure 10.4. In the load position the eluent flow passes via a slot on the rotor from the pump (port 2) to the column (port 3). The ends of the sample loop are connected to ports 1 and 4. The sample loop is charged by a syringe through the injection port which is aligned so the end of the needle abuts directly against one end of the sample loop (port 4). Thus, it is important to ensure the appropriate needle is used. Fluid in the loop is discharged via a slot in the rotor which, in this position, connects port 1 to waste (port 6). On rotating the valve to the inject position, the sample loop is placed between the column and the pump supplying eluent by connecting port (1) and (2) and ports (3) and (4). The eluent then sweeps the sample, in the opposite direction to the one in which it was loaded, onto the column. The injection port is now over the second waste port (5) allowing the injection port to be rinsed.

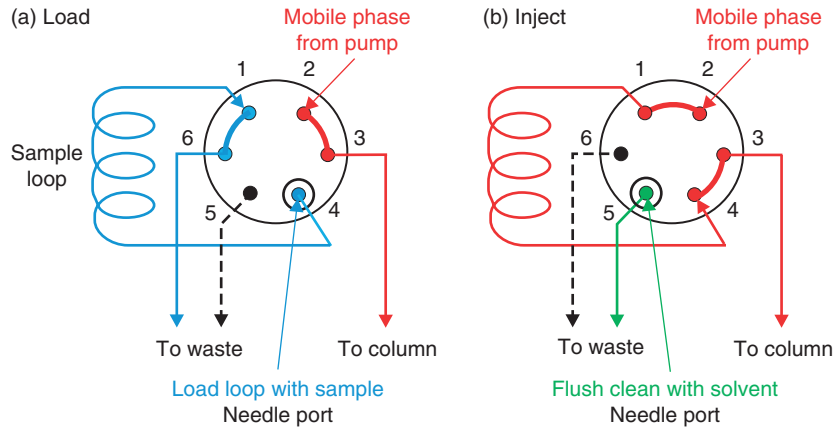


Figure 10.4 Six-port LC injection valve showing (a) load and (b) inject positions

After sampling, the rotor is returned to the load position, the external sample tube washed with solvent, and the sample loop filled with the next sample for analysis. Because the sample loop can be any length or diameter, and may even include a short column on which analyte can be concentrated, several configurations are possible. For maximum precision the sample loop should be partially filled, no more than 50 %, with a carefully controlled volume of sample or be overfilled (>2 loop volumes).

Automated sample injectors are widely used, typically with high precision motorized syringes for volume accuracy and precision. There is a risk of cross contamination (carry-over) in such

systems, particularly where there are wide differences in analyte concentration between one sample and the next, which must be guarded against, ideally by analysing 'blanks' between samples.

The possible effect of the injection solvent on chromatographic efficiency must be considered. In many published methods, the analyte is taken up for injection in LC eluent. However, if the injection volume is too large this will contribute to band broadening. Preferably, samples should be injected in a solvent of lower 'eluting power' than the eluent. This allows for concentration of the analyte(s) on the top of either the pre-column, or the column. For example, in reverse-phased systems, using an injection solvent containing less organic modifier than the eluent allows injection of larger volumes with no detrimental effect on efficiency and peak shape. Conversely, the use of an injection solvent with a significantly higher proportion of organic solvent than that of the eluent will lead to the analysis beginning well before the arrival of the eluent, unless a very small injection volume is used, which of course may limit sensitivity.

10.2.7 System operation

An accurate record of key column performance parameters such as system back-pressure, analyte retention time and LLoD for a test mixture, and baseline noise and drift, should be kept. Any deterioration in performance will then soon become apparent and preventative maintenance can be undertaken.

In reverse-phase LC and other situations where aqueous buffers are used, it is prudent to rinse a column periodically with deionized water to reduce the risk of precipitation of inorganic salts, and with organic solvent to remove retained lipophilic material. Organic solvents should not be used until inorganic salt residues have been removed. Typically, a column that is being used with an aqueous buffer as part of the eluent should be flushed sequentially with deionized water, methanol or acetonitrile, and deionized water before equilibration with fresh eluent. Similar flushing should be carried out before a column is stored, the final flush being with methanol or acetonitrile:water (1+1) to prevent bacterial growth.

If column performance is still poor after column reconditioning, then the fault may be due to dissolving or compression of the column inlet bed. To repair any column inlet, it is necessary to remove the fitting. Before proceeding with column repair on a reverse-phase column, it should be conditioned to methanol:water (1+1 v/v). This will solvate the C_8 or C_{18} phase with a moderately lipophilic mixture and so prevent the packing from expanding out of the column after the fitting has been removed. The fitting or frit closure should be removed from the inlet end of the column and the packing examined. It should be off-white.

If the packing is coloured, it may indicate that corrosion of steel components of the LC system has occurred, or that impurities present in the sample extracts have accumulated on the top of the column. If the inlet bed has some depression, then it has to be made level to prevent peak broadening or, possibly, peak splitting. The void should be filled with suitable packing material (5 or 10 μm column packing added as a dense slurry with a spatula and levelled off, or 30–40 μm glass beads). Attempting to use smaller sizes of packing material is rarely successful. A new frit should always be used in the fitting or closure when the column is reassembled.

Pump problems may be manifest as erratic retention times, noisy baselines, and/or spikes on the baseline due to pressure variations. Pump leakage usually occurs at inlet or outlet fittings, or at the piston seal. Salt crystals on a fitting usually indicate a leak. Problems attributed to solvents used as eluents include (i) fouling of the pump and/or clogging of the check valves from particles, (ii) low sensitivity due to contaminated solvent, and (iii) drifting baselines and/or retention times due to impurities, for example, in the eluent buffers.

Eluent filtration before use should be routine. If there is a danger of precipitation of buffer salts, the eluent can be filtered after the buffer and organic modifier have been mixed, although care must be taken to ensure that this does not change the composition of the eluent; a 1 % change in organic content could change retention by 5–10 % in a reverse-phase system. This will be more apparent when the eluent contains only a small proportion of a relatively volatile solvent, or if volatile buffer components such as ammonium hydroxide or trifluoroacetic acid are used.

10.3 Detection in liquid chromatography

The ideal LC detector would have (i) high sensitivity, (ii) either universal or specific response as required, (iii) wide linear dynamic range, (iv) minimal band broadening, and (v) a stable response with either temperature, or flow rate change. Currently, no detector fulfils all these requirements.

LC detectors are of two types: bulk property or selective. The former measure a change in a physical property of the eluent such as refractive index (RI), conductivity, or light scattering that is affected by the presence of an analyte. However, such detectors generally do not have sufficient sensitivity/selectivity for use in analytical toxicology. Thus, detectors that exploit chemical or physical properties of an analyte are used almost exclusively. UV/Vis and fluorescence detectors are non-destructive and are relatively insensitive to small fluctuations in flow rate.

Further options include post-column collection or modification of the analyte to improve selectivity and thus sensitivity. Whatever the detection system, it is important that an appropriate low dead volume detection cell and an adequate signal sampling rate or time constant (usually 0.2 s or less) are used to ensure that the high separating power of modern microparticulate packings at the commonly used flow rates of 1–2 mL min^{-1} is not lost.

LC does not have a simple, sensitive, universal detector analogous to the FID in GC. However, MS has become widely used. Although interfacing MS and GC is relatively simple, in LC interferences from the eluent must be eliminated. In order to overcome this problem, many interfaces have been developed for different purposes. The interfacing techniques available for LC-MS are discussed further in Section 13.4.

Combining LC with atomic spectroscopic methods can give information on the atomic composition of the analyte. An example is LC-ICP-MS (Section 21.9.1). This is a more powerful technique than LC-AAS because ICP has high sensitivity and can detect many elements simultaneously.

Detector problems can be electrical, optical, or mechanical. When a problem is encountered, turn the pump off – if there are still spikes on the baseline the problem is probably electrical. The presence of gas bubbles in the detector cell may also cause spiking on the baseline. Thorough eluent degassing should cure the problem by removing dissolved gas and thus prevent bubble formation. Detector cell bubble formation can also be reduced by using a flow restrictor to maintain a higher pressure post-column, given that the detector cell(s) can withstand that pressure.

10.3.1 UV/Visible absorption detection

Provided that the analyte has sufficient absorbance in the range 200–600 nm, the sensitivity of UV/Vis detectors can be less than a few ng injected if there is no contribution to baseline noise from small changes in eluent flow rate and temperature. UV/Vis detectors use either a

low-pressure mercury lamp as the light source, which is particularly useful for monitoring at 254 nm (an intense emission line), or more usually a deuterium discharge lamp (200–400 nm) and a tungsten lamp (400–700 nm). The most popular commercially available flow cells for UV/Vis detectors have cell volumes of 8 μL or less and a path length of 10 mm (Figure 10.5), but for microbore or capillary LC a lower cell volume (0.1–0.3 μL) must be used so as not to compromise the increased sensitivity generated by the low-volume column.

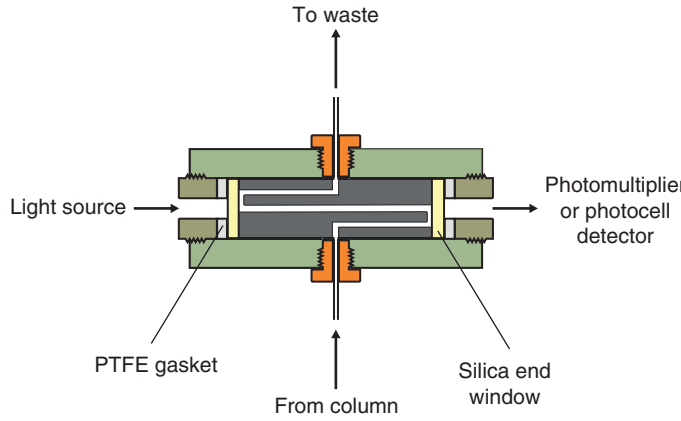


Figure 10.5 Schematic diagram of UV/visible detector flow cell for LC

Use of relatively non-selective detection wavelengths (200–220 nm) can pose problems if high-sensitivity work with biological extracts is contemplated; many reports advocating such wavelengths for particular analytes make no mention of potential interferences. Sensitivity may also be reduced when working near the UV cut-off of the eluent. The presence of oxygen dissolved in the eluent may also limit sensitivity at low wavelengths (200–210 nm).

Multi-wavelength detectors, i.e. detectors capable of generating absorbance data for more than one narrow band of wavelengths at once, have been evaluated for STA (Section 19.4.1). These instruments employ either a fixed grating/DAD combination (Figure 5.3), or a high-speed scanning monochromator in conjunction with an 8–13 μL flow cell. A data collection rate of one spectrum per second is normally optimal in conventional LC. A three-dimensional plot (absorbance against wavelength and time) is obtained, which can be used in both qualitative and quantitative work. Reference to a specific wavelength can be used to reduce baseline drift during gradient work.

Several protocols have been developed to help assess analyte identification and peak homogeneity using such instruments, including absorbance ratio data and peak purity parameters. If the ratio of two different wavelengths is not constant across a peak, then the peak is not homogeneous. In the main, however, DAD has not the identification power of MS and finds use nowadays in the analysis of seized drugs as well as biological specimens (Maurer, 2004; Strehmel *et al*., 2018). This being said, LC-DAD does not suffer from the ion suppression/enhancement problems that plague LC-MS and if used appropriately can give reliable results.

10.3.2 Fluorescence detection

Generally, mercury or xenon light sources are used in LC fluorescence detectors; such detectors fitted with a deuterium lamp are no longer available commercially. Fluorescence detection can

be very sensitive (typically a 10–100-fold increase as compared to typical UV/Vis detectors), but only a few analytes of toxicological interest fluorescence unless derivatized. The response obtained is dependent on both the energy of the incident light and the optical configuration of the detector. Some analytes, for example quinine, only fluoresce under acidic conditions. In such cases post-column adjustment of the eluent pH is easily accomplished and repeating the analysis without post-column flow gives an additional identification parameter.

Fluorescence detectors can be double monochromator, double filter, or a combination of monochromator (excitation) and filter (emission) instruments (Section 5.5.2). Some scanning instruments use a beam splitter to divert 50 % of the incident light to a reference photocell. The discrete excitation and emission wavelengths obtainable with double monochromator instruments provide maximum selectivity for the target analyte. However, because compounds with similar chemical structures, including metabolites, frequently have different fluorescence spectra, this may not always be an advantage. With some instruments, it is possible to programme excitation and emission wavelength changes during an analysis, but this presupposes that the analytes, their retention times, and their fluorescence spectra are known. The use of wide band-pass filters allows more compounds to be detected.

Detector cells vary in their design. Some are simply a narrow-bore quartz tube with inlet and outlet connections at the bottom and the top, the excitation and emission light paths being at right angles to the tube and each other. With others, the incident light is directed down the length of the tube and the emitted light detected at right angles. This arrangement has been used in combined UV/Vis-fluorescence detectors (Figure 10.6).

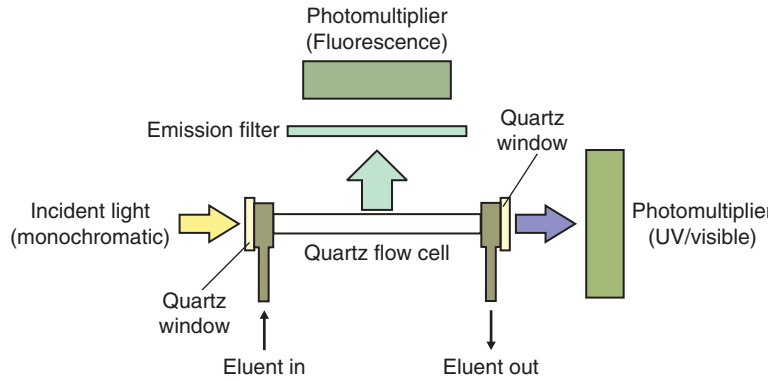


Figure 10.6 Schematic diagram of a combined fluorescence/UV detector cell for LC

10.3.3 Chemiluminescence detection

Chemiluminescence detection (CLD) is potentially more sensitive than fluorescence detection as it does not require irradiation of the flow cell, and so the problem of light scattering with consequent reduction in signal/noise ratio is avoided. The instrumentation is simpler and CLD has a wider linear dynamic range than conventional fluorescence detection. The reactants are usually introduced post-column (Section 10.3.9), or the chemiluminescence may be photo-induced. The use of CLD in LC and CE analysis has been reviewed (Li *et al.*, 2003). Chemiluminescence has found some use in environmental studies, for example measurement of nitrogen oxides, and as a signal in immunoassays (Section 6.3.6), but has not been widely adopted in analytical toxicology, in part because of the advances in LC-MS.

10.3.4 Electrochemical detection

ED offers high sensitivity for a limited number of compounds, but other factors being equal, UV/Vis, fluorescence, and more recently MS detectors are more robust and, therefore, preferred. There are two basic types of electrochemical detector for LC: (i) amperometric or coulometric, and (ii) electrical conductivity. The former are reaction detectors, that is the analytes are either oxidized, or reduced at the surface of the working electrode, and it is these that have found most application in analytical toxicology. To maintain the electric circuit a second (counter) electrode is required and this may be of the same material as the working electrode, or simply the stainless steel of the outlet tubing. A third electrode, frequently silver/silver chloride, having a known potential, is used as a reference by which the potential applied to the working electrode can be kept stable via feedback circuitry. Detection may be enhanced by derivatization of suitable analytes to produce electroactive species.

Amperometric detectors are used mainly with glassy carbon working electrodes in a wall jet or thin-layer arrangement (Figure 10.7). Porous graphite flow-through cells offer the possibility of coulometric detection, i.e. detection involving near quantitative conversion. Such cells were available in a dual-electrode configuration, but needed meticulous care to avoid blockage. However, the greatest drawback to the use of ED in LC is electrode deactivation. Loss of response is normally accompanied by a decreased standing current, while excessive noise due to, for example, a contaminated eluent is usually paralleled by an increase in the standing current. The ED is very sensitive to flow rate changes, so that pump pulsations are usually very apparent and even small changes in ambient temperature can lead to marked baseline drift.

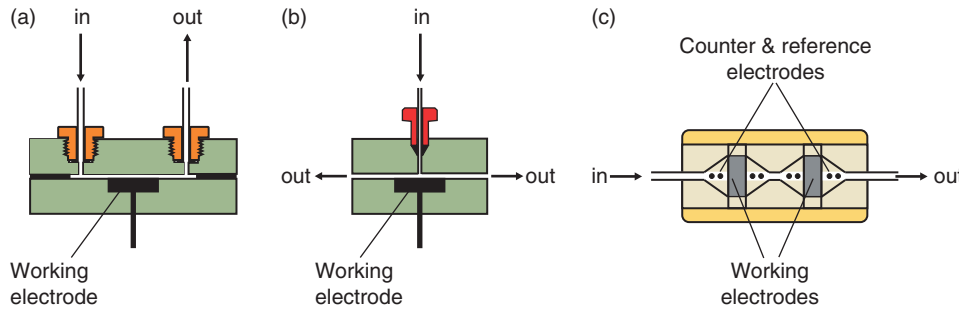


Figure 10.7 Simplified designs of LC-ED cells. (a) Thin-layer cell, (b) Wall-jet cell, (c) Porous flow-through cell

10.3.5 Chemiluminescent nitrogen detection

The CLND is a gas-phase detector that exploits the reaction of nitric oxide and ozone to produce excited nitrogen dioxide with subsequent emission of light:

$$NO + O_3 \rightarrow [NO_2]^* \rightarrow NO_2 + O_2 + h\nu$$

The nitric oxide is produced by heating nitrogen-containing compounds at 1050 °C in an oxygen atmosphere and, after separation of other pyrolysis products, it is mixed with ozone under reduced pressure (*ca.* 2.7 kPa) in a reaction chamber in front of a photomultiplier (Section 9.2.2.8). In LC, a PEEK needle valve is used to control the flow of gas to the reaction

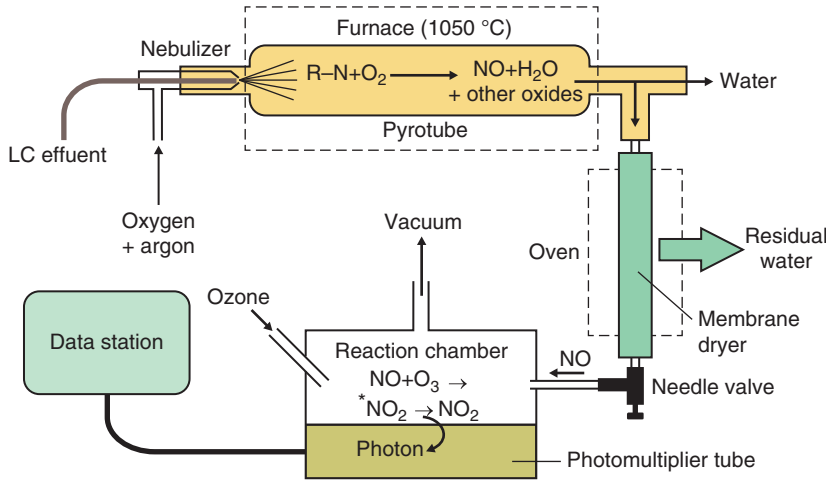


Figure 10.8 Schematic diagram of chemiluminescent nitrogen detector (Nussbaum *et al.*, 2002–reproduced with permission of Elsevier)

chamber (Figure 10.8). Obviously, CLND cannot be used with nitrogen-containing eluents (solvent or buffer).

The sensitivity of CLND is said to be 0.1 pg of nitrogen, with a linear range $>10^4$. For a compound such as imipramine (M_r = 280), which conveniently contains 10 % nitrogen by weight, this equates to 1 pg of drug. As this measurement was performed using flow injection analysis (FIA) giving very sharp peaks, chromatographic sensitivity is probably in the region 1–2 pg s^{-1} for imipramine. The reproducibly of response to equimolar amounts of nitrogen, irrespective of the analyte, means that this detector has been used to measure nitrogen-containing compounds without primary standards, such as screening combinatorial libraries (Taylor *et al.*, 1998), metabolic studies (Taylor *et al.*, 2002), and in the analysis of drug metabolites and of drugs in seizures without primary reference standards (Laks *et al.*, 2004).

10.3.6 Aerosol-based detectors

Aerosol-based detectors have increasingly addressed the need for sensitive, universal LC detection in a range of applications (Magnusson *et al.*, 2015).

10.3.6.1 Evaporative light scattering detection

In the evaporative light scattering detector (ELSD), effluent from the LC column is converted into a fine mist by a nebulizer and the solvent evaporated to leave a stream of solute particles onto which a beam of light is directed. An optical detector, set at an appropriate angle, measures the scattered light. Because detection is not dependent on any spectral properties of the analyte, the ELSD is often referred to as a universal detector, but clearly it cannot be used with volatile analytes.

The results from ELSD may be influenced by LC eluent composition, for example during gradient programming, but probably the biggest drawback is its lack of sensitivity. When evaluated for *in vitro* metabolism studies, the matrix was found to interfere, and even with less complex, 'contrived' samples, the LoD was 1–10 mg L^{-1}.

10.3.6.2 Condensation nucleation light scattering detection

As in ELSD, the condensation nucleation light scattering detector (CNLSD) uses nebulization of LC eluent followed by desolvation to produce a dry aerosol of analyte. Usually the aerosol then passes through a filter assembly, typically incorporating a number of diffusion screens that modify the aerosol particle size distribution and help extend the dynamic range of the detector. Supersaturated vapour, usually water, is condensed onto the dry particles to give droplets that are easily detectable by light scattering using a laser diode and a photodetector.

10.3.6.3 Charged aerosol detection

As with the ELSD, in the charged aerosol detector (CAD) the eluent is passed through a nebulizer and the solvent evaporated at ambient temperature. However, in the CAD the particles are charged by collision with a stream of positively charged nitrogen gas. High-mobility ions are removed by a negatively charged, low-voltage trap. The remaining analyte ions transfer their charge to a collector (filter-electrometer), the transferred charge being proportional to the analyte mass (Figure 10.9).

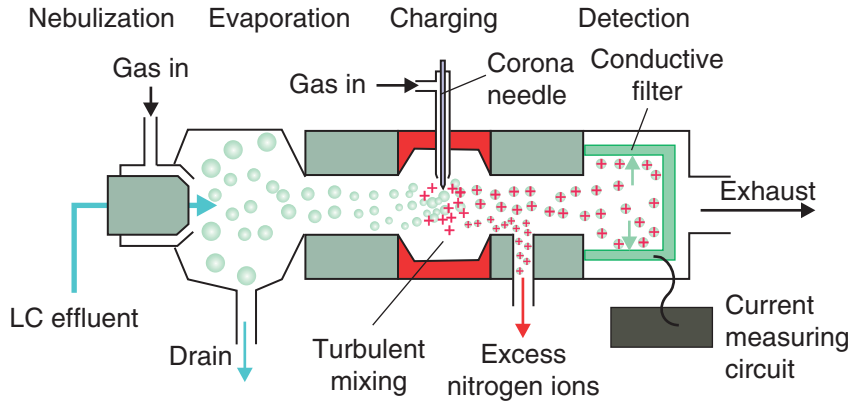


Figure 10.9 Schematic diagram of a charged aerosol detector (Magnusson *et al*., 2015–reproduced with permission of Elsevier)

The CAD has similar limitations to the ELSD, including those imposed by the volatility of the analyte and the LC eluent. However, the CAD is claimed to be more stable with regard to gradient elution, less influenced by differences in aerosol particle diameters, and at least 10 times more sensitive than ELSD. The CAD has found application in drug development work (Almeling *et al*., 2012; Soliven *et al*., 2017). A high throughput LC-MS/UV/CAD/CLND system for use in drug discovery has been described (Jiang *et al*., 2015).

10.3.7 Radioactivity detection

Radioactively labelled analytes can be monitored via a flow cell using various radioactivity (RA) detection systems such as a sodium iodide scintillation counter, a Geiger–Muller counter, or a liquid scintillation counter, depending on the radionuclide being used. Scintillation produces light via matrix ionization caused by the emitted radiation, and the light is amplified via

a photomultiplier tube. Usually, either ^{3}H- or ^{14}C-labelled substances will be used so that the labelled material is as similar as possible to the unlabelled compound. Other suitable radionuclides include ^{32}P and ^{35}S, if these elements are present in the compound being studied, but generally ^{14}C is preferred.

The basic design has features in common with CLDs. In order to enhance sensitivity, a large volume flow cell is needed, but too large a volume causes band broadening and thus loss of sensitivity. To some extent this can be minimized by constructing the cell as a coil of tubing placed in front of the photomultiplier. However, having to integrate the counts, i.e. counting at discrete time intervals, reduces resolution and clearly to achieve high sensitivity the counting interval will need to increased, resulting in further loss of resolution. Tritium, being a 'soft' β-emitter has to be counted in solution as the low energy electrons will not penetrate the detector cell walls. The added stream of liquid scintillator cocktail contributes to peak broadening and increases the volume of low-level radioactive waste for disposal.

Despite the limitations associated with RA detectors (Box 10.3), they are useful if large numbers of samples are to be assayed. However, for small scale studies, collecting fractions of eluent and counting off-line is a viable alternative. For targeted analytes, the non-labelled compounds can be added beforehand at a concentration that can be detected, say, by UV detection and individual peaks collected for RA counting.

Box 10.3 Practical considerations in the use of radioactivity detectors

- Not particularly sensitive
- Cannot recirculate the LC eluent
- ^{3}H-labelled analytes require addition of liquid scintillant
- Large volumes of low-level radioactive waste
- Band spreading leads to poor resolution and loss of sensitivity
- Integration of the signal produces a histogram
- Incompatible with high efficiency LC separations

10.3.8 Chiral detection

Chiral detectors are important in the analysis and purification of enantiomers. Several are available commercially and are based on either optical rotation of the plane of polarized light (OR detectors), or on measuring the difference between absorption of right and left-handed circularly polarized light [circular dichroism (CD) detectors]. CD detectors are generally 10–100 times more sensitive than OR detectors and are suitable for compounds that absorb at 220–420 nm. Laser CDs are said to be more sensitive than conventional instruments, but as yet do not seem to have the sensitivity required for monitoring chiral compounds in biological fluids (Kott *et al*., 2007).

Because there is an absorption signal as well as a CD signal, CD detectors can be used to measure an excess of one or other enantiomer without performing a chiral separation and, by measuring specific rotation of separated peaks, can give proper peak assignment (Lecoeur-Lorin *et al*., 2008). OR detectors, on the other hand, can be used to detect enantiomers that do not possess chromophores. The useful detection limit for typical pharmaceuticals ranges from 1–100 μg on column, depending primarily on the specific rotation exhibited by the analyte in the eluent, and secondarily on retention time and peak shape.

10.3.9 Post-column modification

By comparison with GC carrier gases, LC eluents are essentially non-compressible, and so LC also offers a range of options for post-column analyte modification, including post-column pH adjustment, hydrolysis, derivatization, and photolysis. Such modifications were normally employed to facilitate or enhance fluorescence because fluorescence detectors are relatively insensitive to flow rate changes. However, with the advent of LC-MS procedures, other post-column modification procedures are now little used in analytical toxicology.

To minimize band broadening during post-column reagent addition, a specially designed system is needed. The column effluent is mixed with reagent solution provided by an auxiliary pump using a T-piece or, preferably, a Y-connector, which gives better mixing (Figure 10.10). The reaction coil can be constructed of narrow bore stainless steel or PTFE tubing, or other suitable material, which can be placed in an oven or heating block if the reaction requires an elevated temperature. The reaction time is influenced by the geometry of the reaction coil and the flow rates of the eluent and reagent streams. For example, if the effluent and the reagent flow rate are both 1 mL min^{-1}, the length (L) of 0.2 mm i.d. tubing needed to give a residence time in the coil of 1 min is 63.7 m [volume of a cylinder $\pi r^2 L$, hence $L = 2 / (\pi r^2) = 63.7$ m].

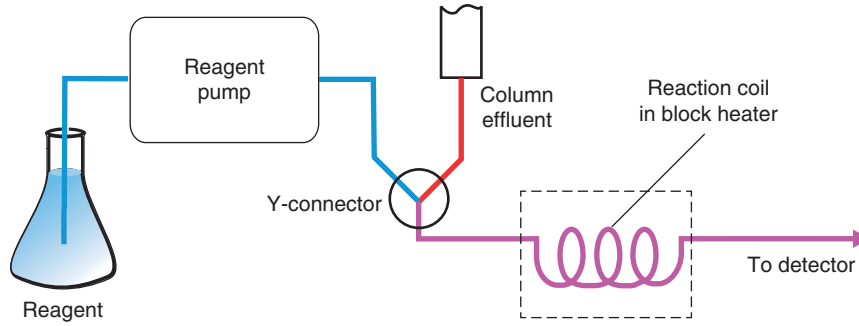


Figure 10.10 Arrangement for post-column reagent addition in liquid chromatography

Doubling the i.d. of the reaction coil will increase the residence time four-fold, but this is likely to reduce the system efficiency. Reducing the flow rate of either the eluent, or the reagent stream will increase the residence time and, provided sufficient reagent can be delivered, reducing the reagent flow rate to a minimum will maximize sensitivity.

Subjecting compounds to high energy light often yields highly fluorescent products. Advantages of photolysis over post-column derivatization are that a second pump is not required and the sample is not diluted. Thin-walled PTFE tubing, crocheted to form a flat mat, may be used for the reaction chamber, as this gives a large surface area for irradiation.

10.3.10 Immunoassay detection

Immunoassay techniques (Chapter 6) can sometimes be useful for off-line work. The column effluent is collected in small (approximately 1 mL) fractions, each fraction is assayed, and a chromatogram constructed by plotting assay result against fraction number. The procedure is lengthy, but very high sensitivity/selectivity can be achieved. Moreover, time may be saved as relatively simple sample preparation procedures may often be used as detection is so selective.

LC with immunoassay detection has been used for cannabinoids, opiates, LSD, and cardioactive glycosides in biological fluids, amongst other compounds.

10.4 Columns and column packings

In analytical toxicology conventional stainless steel columns (100 or 125 × 4.6 mm i.d.) packed with 5 µm materials have been preferred because at flow rates of 1.5–2.0 mL min^{-1} retention, analysis time, selectivity, and sensitivity can usually be adjusted within acceptable limits. Use of shorter columns packed with smaller (3 µm, or less) materials, or even microbore (1–2 mm i.d.) columns (Table 10.1), may give enhanced efficiency and hence sensitivity in specific applications, notably in LC-MS; 10 µm materials give lower back-pressures, but otherwise have few advantages and are rarely used.

Table 10.1 Broad classification of liquid chromatography column types

Type	Internal diameter (mm)	Length (mm)	Normal particle size (µm)	Eluent flow rate (mL min^{-1})
Preparative	100	600	10–50	>5
Semi-preparative	25	250	5–20	2–5
Normal	3.9–5	50–250	5–10	1–2
Minibore	2.1–3.9	50–200	3–5	0.5–1.5
Microbore	1.0–2.1	50–150	3–5	0.1–0.5
Capillary	0.05–1.0	50–250	2–5	0.0002–0.1

It should be remembered that the smaller the particle size of the packing, the greater the care needed in sample and eluent preparation to prolong column life because particles and other impurities can collect more rapidly on the smaller particle columns causing back pressure, selectivity, and resolution problems. Reduced analysis time is commonly achieved by using shorter columns and higher flow rates. Shorter columns require smaller particles in order to maintain efficiency. However, the increased column back-pressure when using smaller silica particles limits column length.

10.4.1 Column packings

Silica gel is the most widely used column matrix because it is cheap, relatively stable, inert, and has good mechanical strength. Moreover, it is available with various particle sizes (3-80 µm aps), pore sizes (4-33 nm), and surface areas (100-800 m^2 g^{-1}), and can be chemically modified via surface silanol moieties (Si-OH) to give a large range of stationary phases (Section 10.4.1.1). Other oxides (aluminium, magnesium, or zirconium oxides, for example) or cross-linked polymers (styrene/divinylbenzene, polyacrylamide, etc.) are used on occasion as they have good mechanical strength. Sometimes the matrix alone can be used as the stationary phase, e.g. silica is used in adsorption chromatography (Section 10.5.1) and styrene/divinylbenzene is used in reverse-phase LC (Section 10.5.3).

The use of good mechanical strength packings makes it possible to pack small particle size material into columns using high pressure to obtain high efficiency columns. Furthermore, the risk of the column packing collapsing in use, which would lead to high back pressures, void formation, and loss of efficiency, is minimized. Large pore polymer supports [pore size >50 nm (500 Å)], which are often used for size exclusion chromatography (SEC), do not have much cross-linking so they must be handled and stored carefully. Large pore silicas (>50 nm, used as the support in 'protein' columns) have thinner supporting walls and similarly need to be handled with care.

Silicas from different manufacturers differ in pore size, surface area, pore volume, and surface silanol pH. Silica gel is made by precipitation from silicate or silica sol solutions in the pH range 4 - 10, and the surface pH reflects the pH of the starting mixture. Most manufacturers try to make the surface pH as close to 7 as possible to minimize interactions with acidic or basic analytes.

The classical silica gel used in adsorption chromatography (Section 10.5.1) has a pore size of 6–8 nm and a surface area of 300–500 $m^2\ g^{-1}$. Because pore size may decrease after chemically bonding a stationary phase, some manufacturers use larger pore size material (10–12 nm) for their base silica to allow adequate access to the bonded phase. For separating analytes with M_r >2000 such as peptides and proteins, 30, 50, or 100 nm, or even larger pore diameter base silicas may be used. However, as pore diameter increases, surface area decreases, which can limit sample capacity.

Many silica gel packings will decompose if used outside of the pH range 2–8.5. In an attempt to solve this problem, packings have been prepared using highly purified silica and special bonding procedures with stable silanes. There has also been interest in macroporous cross-linked polystyrene-divinylbenzene (PSDVB) copolymer packings that are chemically inert and stable over a wide pH range. However, in general PSDVB columns are less efficient than those packed with siliceous materials; other problems are a tendency to instability at high pressures and an eluent-dependent tendency to swell or shrink.

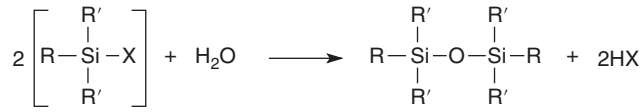
Porous graphitic carbon (PGC), commercially available as Hypercarb (Thermo Scientific), has also been suggested as an LC packing. Although chemically inert and with good mechanical strength, PGC columns are prone to void formation. In addition, peak shape is dependent to an extent on the nature of the eluent, and efficiency and peak shape both decrease very rapidly with increasing k. Trifluoroacetic acid is a useful modifier in the analysis of both acidic and basic compounds, but there is as yet no simple way of adjusting selectivity.

10.4.1.1 Chemical modification of silica

After washing (i) with acid to remove metal ions, and (ii) with deionized water to bring the silica to a neutral pH, the material is dried overnight at approximately 120 °C. For chemical modification, the silica is then placed in a flask with dried toluene or other suitable solvent and a mono-, di-, or tri-functional silane reagent added. The functional (leaving) group can be a halide, or a methoxy- or ethoxy- moiety. Generally, only one of the functional groups bonds to form a Si–O–Si bond. Less often two of the functional groups react to form adjacent Si–O–Si bonds.

The remaining functional groups on each reagent molecule hydrolyze to form silanols after the initial reaction has been completed. These additional silanols formed from hydrolysis of di- and tri-functional reagents can crosslink with one another near the surface of the silica support, and thus bonded phases made with any di- or tri-functional reagent are termed 'polymeric' phases. By analogy, any packing made with a monofunctional silane reagent is referred to as a 'monomeric' phase.

Monomeric and polymeric phases often have different selectivities. A monofunctional silane reagent can only bond to the surface silanols and any excess is removed by treatment with water:



where R = C_{18}, C_8, cyanopropyl, etc., and R′ = a shorter alkyl group (methyl-, ethyl-, or propyl-). This smaller alkyl group gives less steric bulk to the reagent, promoting more complete coverage of surface silanols.

Depending on the reaction conditions, which may include adding water to the reaction mixture, and post-reaction treatment, a range of stationary phase concentrations can be achieved at the silica surface. The surface carbon loading (%) can be ascertained by routine carbon and hydrogen analysis; nitrogen analysis may be helpful for amino- and nitrile-containing phases, as well as some nitrogen-containing chiral packings. However, surface coverage or bonding density will depend on the surface area of the silica as clearly columns of the same carbon loading, but different surface areas, will have different bonding densities. Furthermore, if the silica has a wide distribution of pore size, the surface coverage may be uneven, and calculation of bonding density based on carbon loading and surface area may be misleading.

Each manufacturer attempts to keep the carbon loading or bonded phase coverage numbers constant to give batch-to-batch reproducibility. The carbon loading varies with either the chain length, or the number of aromatic rings on the silane used; the higher the M_r the more weight added for each molecule bonded. The carbon loading is usually in the range 5–25 %, the surface coverage ranging from 1.19–3.8 μmol m^{-2}. It is not possible to react every surface silanol because of steric hindrance, which is more apparent when bonding higher M_r silanes.

If a packing is made from the same reagent and base silica, then generally the higher the carbon loading, the more retentive it will be for any given eluent/analyte combination. The column with the lower carbon loading will have a greater proportion of residual (unreacted) silanols. It is possible to take bonded material and derivatize residual silanols by using a reagent such as trimethylchlorosilane [$(CH_3)_3SiCl$, TMCS] or hexamethyldisilazane {$[(CH_3)_3Si]_2NH$, HMDS}. This process is called ‘endcapping’. Because the M_r of these reagents is small, they do not add much to the carbon loading (only 0.1–0.2 % by weight carbon) compared to the initial bonded phase. Of course, not all bonded phases can be endcapped. The above reagents would react with diol and amino moieties, for example.

Diol columns are prepared using a silane reagent with the structure Si–O–CHOH–CH_2OH. Nitrile and amino phases are most often made from dimethylcyanopropylsilane and dimethylaminopropylsilane, respectively. Many ion-exchange materials are manufactured via multistep reactions. Thus, after a phenylpropyl (Si–CH_2–CH_2–CH_2–C_6H_5) moiety has been attached to the silica surface, sulfonation of the ring gives a strong cation exchange (SCX) material. Similarly, a bonded secondary amine can be quaternized to form a strong anion exchange (SAX) phase.

10.4.1.2 Bonded-phase selection

Important parameters to be considered when deciding which stationary phases to evaluate for a new application include (i) the pore size and surface area, (ii) the type of bonding (monomeric or polymeric phase), (iii) the chain length bonded, (iv) the carbon loading (or, if available from the manufacturer, the μmol m^{-2} coverage of the bonded phase), and (v) whether the phase is

endcapped. Other parameters that might not be available except by direct measurement are the pH of the silica surface and the % coverage of the surface silanols.

The suitability of a column for a particular separation should be tested as part of method development. A different column of the same type from the same manufacturer should give the same results, but the same type of column from a different manufacturer may give different results. Such columns may need to be optimized as regards the eluent composition in order to bring the separation and analyte retention times to approximately the same range as the column used initially. The elution order on packings with residual silanols may be pH dependent. If the diameters of the columns tested are different, then the eluent flow rate will need to be adjusted to maintain the same linear velocity.

10.4.1.3 Stability of silica packings

The manufacturer's instructions regarding proper LC column storage and use should always be followed. A disadvantage of silica gel is that it is soluble in water and, to an extent, in methanol. This is true whether it is chemically modified or unmodified, although in theory bonded phase material should dissolve at a slower rate. The higher the eluent ionic strength and the higher the pH, the more rapid will be the dissolution of the packing. In general, silica-based packings should not be used above pH* 7.5–8.0. Mixing buffer solutions or ion-pairing reagents with organic solvents can alter pH*, possibly to values >8 (Section 10.2.4).

Stationary phases bonded via Si–O–Si bonds are generally stable, except under strongly acidic conditions (<pH 2) as noted above. However, amine-modified packings are particularly reactive (for example, with aldehydes and ketones) and should be used with care. Other silica packings produced by *in situ* bonding or coated polymer methods are more stable in the presence of water at extremes of pH, but may have other limitations as regards compatibility with organic solvents.

10.4.1.4 Monolithic columns

In GC the use of thin-film open tubular capillary columns with low thermal capacity and an inert carrier gas with low viscosity offers rapid mass transfer between phases and low back-pressures. This gives rise to very efficient separations and, especially when combined with temperature programming, is a powerful analytical technique. Whilst LC has many complementary features to GC, disadvantages of conventional LC are slow mass transfer between phases and limitations caused by column back-pressure. Moreover, slow mass transfer within and between phases limits the practicality of open-tubular columns.

In a conventional packed LC column, eluent must flow through pores in the packing material or through the voids between particles. In an attempt to avoid some of the problems associated with packed columns by providing an accessible flow path within the column, a variety of alternative matrices have been studied. Of the 'continuous media' developed, monolithic columns have been studied extensively and LC columns based on a porous silica 'monolith' have been marketed.

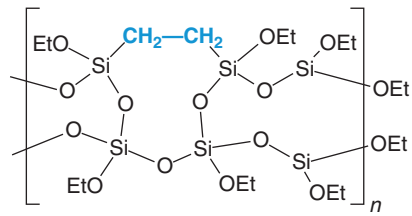
Monolithic columns consist of a single, rigid or semi-rigid, macroporous (2 μm or so average pore size) rod. Like other continuous media, monolithic columns can allow higher flow rates than conventional packed columns at reasonable column back-pressures. Analyte capacity is usually provided by smaller pores within the monolith structure. Two types of monolithic columns have been developed for chromatography: organic polymers based on polymethacrylates, polystyrenes, or polyacrylamides, and inorganic polymers based on silicates. Of these, the silicate monolith columns that are now available with a range of bonded phases offer enhanced possibilities for fast LC analysis.

10.4.1.5 Surface porous particles

Surface porous particles (SPP), silica particles with a solid core and a porous outer shell, have been investigated extensively with the aim of reducing LC back-pressure whilst maintaining high separation efficiency (Ahmed *et al*., 2018). Excellent performance has been recorded for a wide range of analytes including acidic and basic drugs, and peptides and proteins. These core–shell silica particles are also known as fused-core or solid-core particles. The main commercially available SPP columns include Poroshell (Agilent), Cortecs (Waters), Accucore (Thermo Fisher Scientific), Ascentis Express (Sigma–Aldrich), Halo (Advanced Materials Technologies), Kinetex (Phenomenex), and Ultracore (Advanced Chromatography Technologies). SPP columns with particles of size 2.6–2.7 µm have been found to show either similar, or improved efficiency when compared with a fully porous 1.7 µm column (BEH C_{18}), but with much lower back-pressure (Ludvigsson *et al*., 2016).

10.4.1.6 Hybrid particle columns

Hybrid particle technology (HPT, Waters) combines the properties of silica and organic polymers to produce columns with increased mechanical strength. UPLC™ (Waters) exploited second generation HPT packings based on bridged ethylsiloxane/silica hybrids (BEH) made by reacting tetraethoxysilane and bis(triethoxysilane)ethane (4+1) (Figure 10.11). As BEH packing can withstand packing pressures of up to 172,000 kPa (1700 bar) and operating pressures up to 103,500 kPa (1000 bar), UPLC utilizes 1.7 µm particles and narrow bore columns to give increased efficiency, resulting in increased sensitivity and reduced analysis times. These columns were developed for use with the Waters Acquity system, which was designed to cope with the higher pressures and increased efficiencies attainable.



Polyethoxysilane

Figure 10.11 Example of a bridged ethylsiloxane/silica hybrid LC packing

10.4.1.7 Restricted access media

It was envisaged that direct analysis of plasma or serum would prove possible with LC, but this hope has proved ill-founded. Of the approaches investigated, internal surface reverse-phase (ISRP) and shield hydrophobic phase (SHP) materials are types of restricted access media (RAM) that aim to permit plasma protein to elute from an LC column without precipitation, whilst retaining and separating hydrophobic compounds (Huang *et al*., 2018). However, the eluent pH has to be approximately 7 and the concentration of the organic component has to be low to minimize the risk of protein precipitation. Alternatively, one or more pre-columns, containing appropriate stationary phases, may be placed in-line with the analytical column. Computer controlled column switching of eluent flow between the columns is a method of on-line sample preparation; usually ISTD addition is performed off-line beforehand.

More recent ideas include the use of a RAM extraction column to remove protein and other unwanted high M_r compounds. An initial methanol concentration of 5 % v/v may be sufficient to release protein bound analytes without causing protein precipitation. In some cases, a second column packed with a mixed-mode stationary phase is used to further purify the analyte(s) and any ISTD prior to transfer onto the analytical LC column. Be all this as it may, these approaches have not found wide application.

10.5 Modes of liquid chromatography

Available modes of LC include adsorption, liquid–liquid partition, ion-exchange, ion-pair, size exclusion, and affinity chromatography. Each mode allows for different combinations of molecular interactions between the analyte and the stationary and mobile phases, and this gives the opportunity for almost infinite adjustment of selectivity. Partitioning or ion-exchange of an analyte between a stationary phase chemically bonded to an inert support material and the mobile phase is commonly used. In practice, several different mechanisms may operate simultaneously.

10.5.1 Normal-phase chromatography

Juxtaposition of a non-polar mobile phase and a polar stationary phase is called 'normal-phase' chromatography. This is exemplified by adsorption chromatography using silica gel as the stationary phase, analytes being retained by adsorption onto the silanols at the silica surface. Typical eluents in this mode are alkanes such as heptane, or alcohol-modified alkanes. Alcohols form a bilayer at the silica surface and such layers can be modified with miscible ions.

A systematic method for choosing a suitable LC solvent in such cases is (i) reject those with unsuitable physicochemical properties (boiling point, viscosity, UV absorption, etc.), (ii) from the remaining solvents select those that give appropriate retention (e.g. $2 \leq k \leq 10$), and (iii) perform the final choice on the basis of suitable separation (α) of the components of interest. Solvent polarity can be estimated by solvent strength. Solvent strength can be calculated by the additivity value of each solvent if a mixture is used. It is possible to recreate LC detector-compatible eluents from classical TLC systems. As the eluent does not contain water it is necessary to inject the analyte in a non-aqueous medium after, for example, LLE or SPE.

Other adsorbents used in normal-phase LC either directly, or after chemical modification include alumina (Al_2O_3) and zirconia (ZrO_2), but neither material is widely used in toxicological analyses. Polystyrene gel packings have also been studied. Here, retention is attributed to interaction between the analyte and π-electrons in the aromatic moiety, and although retention is said to be normal phase when heptane is used as the mobile phase, retention is said to be reverse-phase if methanol is used. This also applies if aminopropyl- or cyanopropyl-modified silica is used as the stationary phase.

10.5.2 Hydrophilic interaction liquid chromatography

Hydrophilic interaction liquid chromatography (HILIC) is a name that has been given to a variant of normal phase LC in which a polar stationary phase such as either unmodified silica gel, or silica modified by adding a polar bonded phase moiety such as diol or 4-sulfophenylpropyl (Table 4.5) is used together with a polar eluent, for example methanol or methanol:deionized water. Use of methanol containing an ionic modifier (non-aqueous ionic eluent), for example,

gives efficient phase transfer and thus good peak shapes for many basic drugs (Section 10.8.2.1), and is particularly useful when using ESI-MS (Section 13.4.1.1) because the analyte is ionized in solution, which gives good ESI efficiency. Moreover, the high organic content of the eluent ensures efficient droplet desolvation in APCI-MS (Section 13.4.1.2).

10.5.3 Reverse-phase chromatography

Reverse-phase LC uses a polar eluent and a non-polar stationary phase. Typically, the stationary phase is silica modified by the addition of octadecyl (ODS, C_{18}), octyl (C_8), ethyl (C_2), methyl (C_1), or phenylpropyl ('phenyl') silyl moieties. There are many different types of phases depending on the conditions of preparation and synthesis; even C_{18} phases differ when obtained from different manufacturers (Section 10.4.1.2).

Although reverse-phase is the most popular separation mode in LC, the retention mechanism is often unclear especially as far as polar analytes are concerned. Partition, adsorption, and ionic mechanisms may contribute to the retention of different analytes under the same eluent conditions, and *vice versa*. This is reflected in the fact that the effect on retention of altering the proportion of water in the eluent may not be predictable.

There are four major intermolecular interactions between solute and solvent molecules in liquid chromatography: dispersion, dipole, hydrogen bonding, and dielectric. Dispersion interactions are the attraction between each pair of adjacent molecules. Strong dipole interactions occur when both sample and solvent have permanent dipole moments that are aligned. Strong hydrogen bonding interactions occur between proton donors and proton acceptors. Dielectric interactions favour the dissolution of ionic molecules in polar solvents. The total interaction of the solvent and sample is the sum of the four interactions. The total interaction for a sample or solvent molecule in all four ways is known as the 'polarity' of the molecule. Polar solvents dissolve polar molecules, and, for normal phase partition chromatography, solvent strength increases with solvent polarity, whereas solvent strength decreases with increasing polarity in reverse-phase systems.

Typical eluent solvents in reverse-phase LC are methanol and/or acetonitrile modified by adding water to adjust retention and selectivity. Adding water increases the polarity of the eluent, which generally increases analyte retention. Addition of water to methanol also markedly increases viscosity and thus column back-pressure, other factors being equal. Water:acetonitrile mixtures tend to be less viscous than water:methanol mixtures and this, together with the lower UV cut-off of far UV-grade acetonitrile, are reasons why acetonitrile may be preferred. To separate compounds that are not soluble in methanol, acetonitrile, or tetrahydrofuran (THF) and water, dichloromethane:methanol, or THF:methanol mixtures can be used as eluent, but the use of such mixtures is uncommon.

10.5.4 Ion-exchange chromatography

Ion-exchange chromatography is a process whereby an ionized analyte is attracted by ionic (electrostatic) forces to species of opposite charge on the stationary phase. Ion-exchange chromatography has found wide application in the analysis of peptides and proteins including monoclonal antibodies (MAbs) (Cummins *et al*., 2017; Gentiluomo *et al*., 2019).

When the analyte has a positive charge and the stationary phase a negative charge the technique is called cation-exchange chromatography, and when the charges are reversed it is known as anion-exchange chromatography. Eluents used in conventional ion-exchange chromatography generally consist of aqueous solutions of a suitable salt or mixture of salts, with sometimes

a small amount of an organic solvent being added. The salt mixture may itself be a buffer, or a separate buffer can be used. The prime component of the eluent is the counter-ion, which has the role of eluting sample components from the column in a reasonable time. In cation-exchange chromatography, the presence of other positively charged ions in the eluent decreases analyte retention. Generally solute ions are retained in the following order:

$$Ba^{2+} < Ca^{2+} < Cu^{2+} < Zn^{2+} < Mg^{2+} < K^{+} < NH^{4+} < Na^{+} < H^{+} < Li^{+}$$

As a result, if K^+ is used as the counter-ion instead of Na^+, analyte elution is more rapid. In anion-exchange chromatography, the retention order is as follows:

$$\text{Citrate} < SO_4{}^{2-} < \text{oxalate} < NO_3{}^{-} < Br^{-} < SCN^{-} < Cl^{-} < CH_3COO^{-} < OH^{-} < F^{-}$$

Because the eluent is a solution containing various inorganic salts, retention can be changed by different kinds of salts, their concentration, and the eluent pH. Two main types of matrix are used, either silica gel or polymeric organic materials. Of these, silica gel is used in LC. Most silica-based ion-exchange materials are strong anion- or strong cation-exchange in nature (SAX- or SCX-, respectively).

LC of inorganic and organic anions such as chloride, nitrate, nitrite, oxalate, and sulfate, is often known as ion chromatography (IC). This is a specialized subset of ion-exchange chromatography in which a suppressor column is used to exchange the counter ion of the analyte ion after the separation column and, therefore, enhance the conductivity detection of the analyte ions. Anion measurements are sometimes necessary in analytical toxicology (Section 22.4.14).

10.5.5 Ion-pair chromatography

Although ionic compounds can be separated by ion-exchange chromatography, the ion-exchange mode may not give the desired selectivity. Instead, ion-pair chromatography can be used with, for example, a C_{18}-modified silica column. According to the simplest explanation, the analyte ion is paired with an ion of opposite charge added to the eluent to give a neutral species (ion-pair) that can be separated by reverse-phase partition. Typical ion-pairing agents include alkane sulfonates and alkane quaternary ammonium compounds. Because the analytes and counter-ions are ionic and thus hydrophilic, typical eluent solvents are methanol or acetonitrile–aqueous buffer mixtures. With the increased use of LC-MS, ion-pair chromatography is little used nowadays because of the need to minimize the use of eluent modifiers.

10.5.6 Affinity chromatography

The retention mechanisms described above are in the main based on small differences in the adsorptivity, hydrophobicity, or ionic interactions of analytes. The interactions are general and not specific for any given analyte, and therefore, various kinds of molecules can be separated by these separation systems. In contrast, affinity chromatography is based on a specific interaction such as an enzyme–substrate or antibody–antigen interaction. This kind of molecular recognition mechanism is an important interaction in LC separations, and the concept of affinity chromatography can extend to other separation problems such as isomeric and chiral separations.

To separate an antibody, for example, an immunoadsorbent has to be prepared (Arora *et al*., 2017). This consists of a solid matrix to which the antigen has been coupled (usually covalently). Agarose or Sephadex, i.e. modified cellulose, or other polymers can be used as the matrix.

When the sample is added, as long as the capacity of the column is not exceeded, those antibodies in the mixture that are specific for the antigen will bind (non-covalently) and be retained. Antibodies of other specificities and other serum proteins will pass through unimpeded (Figure 10.12). An elution reagent is passed through the column to release the antibodies from the immunoadsorbent. Buffers containing a high salt concentration and/or of low pH are often used to disrupt the antibody–antigen interaction. A denaturing agent, such as 8 mol L^{-1} urea, will also break the interaction by altering the configuration of the antigen-binding site of the antibody molecule. Another, gentler, approach is to elute with a soluble form of the antigen. These compete with the immunoadsorbent for the antigen-binding sites of the antibodies and release the antibodies from the matrix. The eluate is then dialyzed against, for example, buffered sodium chloride solution to remove the elution reagent.

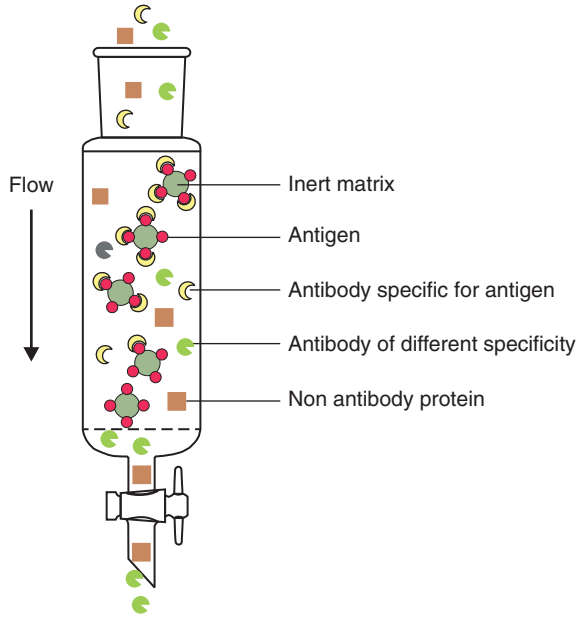


Figure 10.12 Schematic diagram of affinity chromatography

10.5.7 Size exclusion chromatography

Size exclusion chromatography (SEC) is based on the effective shape and size of molecules in solution. It is also called gel permeation chromatography (GPC) if used with organic solvents, or gel filtration chromatography (GFC) if used with aqueous solvents. The stationary phases are porous particles with closely controlled pore sizes. Unlike other modes of LC, in SEC there should be no interaction between the analyte and the surface of the stationary phase – if necessary, silica packings can be treated with TMCS to minimize adsorption effects.

When analytes are larger than the pore size they elute in the void volume of the column (V_0). Molecules that are much smaller than the pore size are able to freely diffuse in the internal volume (V_i) of the packing and elute in a volume equal to the total volume of the column (V_t).

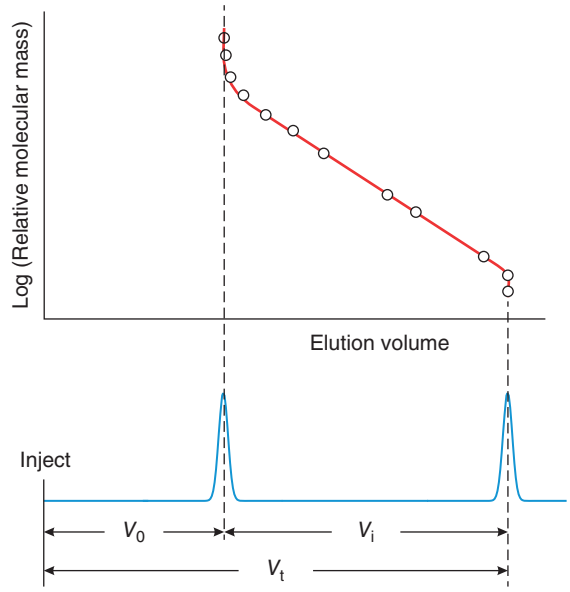


Figure 10.13 Typical calibration curve for size exclusion chromatography

Molecules of intermediate size will be able to penetrate some of the pores and will elute in a volume between V_0 and V_t, depending on their size (Figure 10.13). SEC columns have defined working ranges and should be selected on the basis of the size of analytes being investigated. The columns are calibrated using polystyrenes of known M_r (hydrodynamic volume is approximately proportional to M_r). The choice of eluent is simple because only one solvent is required. Although SEC was applied originally mainly to polymer characterization, it has now many uses in other fields including the analysis of proteins and peptides such as MAbs developed as drugs (Doss *et al*., 2019; Gentiluomo *et al*., 2019; Duivelshof *et al*., 2019).

10.5.8 Semi-preparative and preparative chromatography

Preparative chromatography is rarely used in analytical toxicology except in the form of solid phase extraction (SPE, Section 4.2.3), but could be used to obtain reference materials such as metabolites and ISTDs if these are not available from alternative sources. When the isolation of relatively large quantities of an analyte is required (more than 1 g), larger bore (10–150 mm i.d.), and longer length columns are needed. Above about 25 mm i.d. columns containing 3–5 µm packings not only become increasingly expensive because of the large amount of packing needed, but also difficult to pack because high flow rate pumps are needed to deliver large volumes of slurry.

In practice, preparative columns are always heavily overloaded to generate high product throughput, but this simultaneously decreases efficiency. Thus, these large columns (i.e. i.d. >25 mm) are often packed with larger particle size media (e.g. 25–40 or 40–63 µm) to minimize costs. Although efficiency is less, as long as the selectivity is the same as that used for the initial separation on an analytical column, the compound(s) of interest can usually be resolved although the analysis time may be longer.

10.6 Chiral separations

Chiral LC is more versatile than chiral GC because it can be used with non-volatile compounds and is generally the method of choice for enantiomer separations, and for semi-preparative and even preparative work. LC can provide fast and reproducible analyses, and allows on-line detection and quantitation of both mass and optical rotation of enantiomers if appropriate detectors are used. Problems in routine use are the very high cost of certain columns, and the fact that packings designed for enantiomer separations may not separate metabolites; a second, conventional LC column may thus be needed in series with the chiral stationary phase (CSP) column.

Chiral separation is a complex field (Patel *et al*., 2016; Sardella *et al*., 2018). The approaches adopted include the use of CSPs, the use of chiral additives in conventional reverse-phase systems (Section 10.6.2), and the formation of chiral derivatives (Section 10.7.2). Direct chiral separations using CSPs are more widely used and are often more predictable than those using chiral eluent additives. Some 100 LC CSPs have been marketed. However, no single CSP has the ability to separate all classes of racemic compounds.

Some CSPs are manufactured by immobilizing single enantiomers onto the column matrix. Enantiomer resolution relies on the formation of transient diastereomers on the surface of the column packing. The enantiomer that forms the most stable diastereomer will be more strongly retained, and *vice versa*. The forces involved are very weak (typically a free energy of interaction difference of only 0.03 kJ mol^{-1} between a pair of enantiomers and the stationary phase will lead to resolution) and require careful optimization by adjustment of the eluent and the column temperature to maximize selectivity.

To achieve discrimination between enantiomers there must be three points of interaction of one enantiomer with the CSP, at least one of which is stereochemically dependent (Figure 10.14). The intermolecular forces involved in chiral recognition are polar/ionic interactions, π–π interactions, hydrophobic effects, and hydrogen bonding. These can be augmented by the formation of inclusion complexes and binding to specific sites such as peptide or receptor sites in complex phases. These intermolecular forces may be manipulated by choosing suitable eluents – polar interactions, for example, may be controlled by adjusting eluent pH.

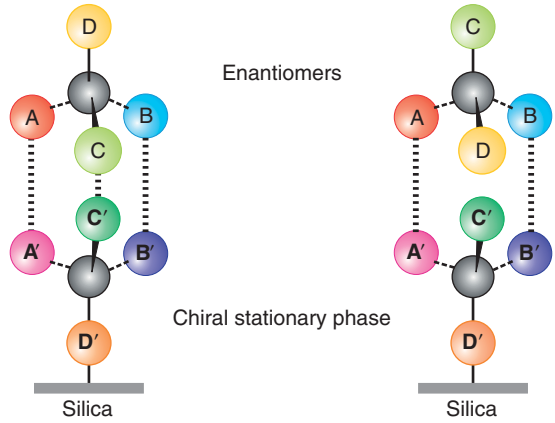


Figure 10.14 Representation of chiral recognition. The structure with three-point interaction (left) is retained more strongly than its enantiomer with only two (right)

The effect of temperature is important in chiral LC. Lower temperatures often increase chiral recognition, but reduce the speed of mass transfer between phases giving broader peaks. Thus, there is often an optimum temperature for a separation and this gives another factor to exploit in method development.

The type of column required to separate a particular class of enantiomers is often highly specific, making the choice of column for a given separation appear complex. Furthermore, the high cost of chiral columns may limit the number of CSPs available. Fortunately, study of the structure of chiral phases and visualizing the potential interactions with the analyte can narrow down the choice of column markedly. The key to a developing a successful enantioseparation of a particular class of racemates on a given system is the understanding of the possible chiral recognition mechanisms that may come into play. The nature of the analyte or derivatized analyte, the stationary phase, and the eluent must be taken into consideration. The most effective CSPs are those based on the macrocyclic glycopeptides and the cyclodextrins.

10.6.1 Chiral stationary phases

10.6.1.1 Amylose and cellulose polymers

These CSPs are derivatized amylose (starch) or cellulose polymers coated onto a silica support. The cellulose phases available include microcrystalline triacetate-, tribenzoate-, trisphenyl-, carbamate-, and tris(3,5-dimethylphenylcarbamate)-cellulose. These phases are used with hexane:ethanol, hexane:2-propanol, or even 100 % ethanol eluents. Chlorinated solvents are avoided because they could remove the cellulose from the silica support. Amylose CSPs show different selectivity to cellulose CSPs, but are less robust. Starch is water-soluble hence eluents must not contain water.

10.6.1.2 Crown ethers

Crown ether CSPs are ring structures based on repeating $–OCH_2CH_2–$ units. The name 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane; $M_r = 264$) indicates that there are 18 atoms in a ring, 6 of which are oxygen. The interior of the cavity is hydrophilic, whilst the exterior is hydrophobic. Varying the size of the crown ether varies the cavity size. Crown ethers are carcinogens, but have been immobilized to form LC stationary phases that complex with protonated primary amines, i.e. acidic eluents must be used. The most commonly used crown ether CSP, 18-crown-6, is available commercially (Crownpak, Daicel). Crownpak columns give very efficient separations and are available in both (+) and (–) forms, allowing reversal of elution order, which can be helpful, for example if trace analysis of one enantiomer is to be performed in the presence of an excess of the other enantiomer.

10.6.1.3 Cyclodextrins

Cyclodextrins bonded to 5 μm silica particles are popular CSPs. They can be used with either methanol:aqueous buffer, or non-aqueous eluents. Good sample capacity means they are useful in preparative work. Cyclodextrins are produced by partial degradation of starch followed by the enzymatic coupling of the D-(+)-glucose units via α-(1-4)glycoside linkages into crystalline, homogeneous, toroidal structures of different molecular size. α-Cyclodextrin (cyclohexamylose; M_r 973), β-cyclodextrin (cycloheptamylose; M_r 1135), and γ-cyclodextrin (cyclooctamylose; M_r 1297) contain six, seven, and eight glucose units, respectively. β-Cyclodextrin, for example, has 35 chiral centres.

The mouth of the torus-shaped cyclodextrin molecule has a larger circumference than the base and is linked to secondary hydroxyl groups of the C_2 and C_3 atoms of each amylose unit. The primary hydroxyl moieties are located at the base of the torus on the C_6 atoms and these groups are used to anchor the cyclodextrin to the silica matrix. The size of the cyclodextrin cavity increases with increasing number of amylose units in the molecule. When α-, β-, or γ-cyclodextrins are derivatized, the hydroxyl moieties on the 2-positions react first. However, the derivative remains size selective and interactions are determined by the size of the analytes as well as the functional groups present.

10.6.1.4 Ligand exchange chromatography

Chiral ligand exchange chromatography (CLEC) can resolve enantiomers via the formation of diastereomeric metal complexes. The method is used primarily in amino acid analysis. A chiral amino acid–copper complex is bound to silica or to a polymeric stationary phase, and copper ions are included in the eluent. Amino acids may be separated via formation of diastereomeric copper complexes. Water stabilizes the complexes, and steric factors then determine which of the two complexes is most stable; in one instance a water molecule is usually sterically hindered from coordinating to the copper. Other transition metals have been used in CLEC. The optimum temperature for such work is approximately 50 °C.

10.6.1.5 Macrocyclic glycopeptides

Immobilized macrocyclic antibiotics such as rifamycin, teicoplanin, or vancomycin chemically bonded to silica gel can be used as CSPs. Vancomycin (Chirobiotic V; $M_r = 1449$) contains 18 chiral centres surrounding three 'pockets' or 'cavities' (A–C) bridged by five aromatic rings. It has a basket-like structure with a single flexible carbohydrate 'flap'. Furthermore, it contains a range of functionalities useful for enantiomeric separations [hydrogen bonding, hydrophobic pockets (inclusion complexation), aromatic moieties (π–π interaction), and ionizable groups]. Vancomycin CSPs are stable, have a relatively high sample capacity, and can be used with either non-aqueous or aqueous eluents in the pH range 4.0–7.0. Ammonium nitrate, triethylammonium acetate, and sodium citrate buffers have all been used with these materials.

Tiecoplanin (Chirobiotic T; $M_r = 1885$) has 20 chiral centres, three carbohydrate moieties, and four fused rings surrounding four 'pockets'. Teicoplanin CSPs are said to complement vancomycin phases and can be used with similar eluents. Teicoplanin CSPs are stable over the pH range 3.8–6.5, although can be used outside these limits for short periods. Unusually, this CSP gives longer retention and increased resolution as the proportion of organic modifier in the eluent is increased. Avoparcin (Chirobiotic A), a 1:4 mixture of α- and β-avoparcin ($M_r = 1909$ and 1944, respectively), has also been used as a CSP. The avoparcin aglycone contains three connected semi-rigid macrocyclic rings (one 12-membered, two 16-membered) that form a possible inclusion pocket. This glycopeptide contains seven aromatic rings with four phenol moieties, four carbohydrate chains, 16 hydroxyls, one carboxylic acid, two primary amines, one secondary amine, six amide linkages, one or two chlorine atoms for α- and β-avoparcin, respectively, and 32 chiral centres.

10.6.1.6 Pirkle brush-type phases

These CSPs consists of low M_r chiral substances bonded to silica gel. Each bonded moiety has a limited number of chiral centres, but as the groups involved are small there can be a large number bonded to the silica surface. It follows that there is a relatively high probability of the

analytes interacting with a chiral centre. An advantage of brush-type CSPs is that, because the interacting stationary phase moiety is small and the analytes are not strongly retained, chiral selectivity is the dominant factor influencing retention.

There are two main types of Pirkle CSP, π-acceptors and π-donors. The most common π-acceptor phase is *N*-(3,5-dinitrobenzoyl)-phenylglycine bonded to propylamino-modified silica. These CSPs are capable of separating a large range of compounds that possess a π-donor aromatic group. This may be introduced by derivatization with naphthoyl chloride or other appropriate reagent (Section 10.7.2). A further advantage of brush-type CSPs is that they are easily synthesized. Urea-linked CSPs can be prepared by injecting the appropriate chiral isocyanate onto aminopropyl-modified silica columns. In the case of Chiral 1 and Chiral 2 (Figure 10.15) this can be achieved by injecting 1-phenylethyl isocyanate and 1-napthylethyl isocyanate, respectively (Whelpton & Buckley, 1992). It is not always easy to predict how even structurally related compounds will behave on these phases. Amfetamine (as the 3,5-dinitrobenzamide derivative), for example, was resolved on Chiral 2, but not Chiral 1 [Figure 10.15(a)]. In contrast, the norfenfluramine derivative was partially resolved on Chiral 1, but not at all on Chiral 2 [Figure 10.15(b)]. Tranylcypromine, again as the 3,5-dinitrobenzamide, was resolved on both stationary phases [Figure 10.15(c)].

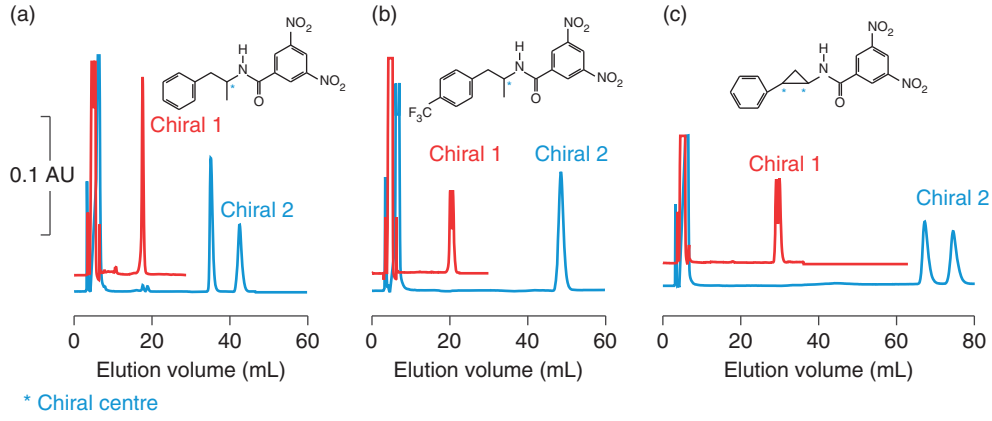


Figure 10.15 Analysis of 3,5-dinitrobenzamide derivatives of (a) amfetamine, (b) norfenfluramine and (c) tranylcypromine on Spherisorb Chiral 1 and Chiral 2 columns. Columns: 250 × 4.6 mm i.d. Spherisorb 5 Chiral 1 and Chiral 2. Eluent: hexane:dichloroethane:ethanol (50+10+1). Detection: UV, 254 nm. Adapted from Whelpton & Buckley (1992)

A CSP derived from dimethyl *N*-3,5-dinitrobenzoyl-*α*-amino-2,2-dimethyl-4-pentenyl phosphonate covalently bound to 5 μm mercaptopropyl-modified silica (Burke-II phase) has been used for the analysis of enantiomers of underivatized β-blockers. Lowering the temperature reduced the retention of the less retained enantiomer and increased the retention of the more retained enantiomer without appreciable band broadening. The separation between enantiomers (α) depends on the difference in the Gibbs free energy (ΔG) for the interactions with the CSP:

$$\ln(\alpha) = \frac{(\Delta G)_R - (\Delta G)_S}{RT} \tag{10.1}$$

where R is the gas constant and T the absolute temperature. If the interaction is enthalpy driven then lowering the temperature increases α, but predicting the effect of temperature change is not easy. Some separations have been shown to have an optimum temperature.

10.6.1.7 Protein-based phases

CSPs based on human serum albumin (HSA) or α_1-acid glycoprotein (AAG) have been described. Other protein CSPs include BSA and egg proteins (ovomucoid). For use as CSPs they are normally immobilized on spherical 5 μm silica. Eluent additives such as octylamine reduce overall retention and increase chiral selectivity. HSA-based columns are especially useful for the resolution of acidic, zwitterionic, and non-protolytic compounds. Enantiomers of acidic compounds can be resolved without derivatization. AAG may be more suitable for basic compounds. Racemates that do not bind to plasma protein will not be resolved with these columns.

10.6.2 Chiral eluent additives

Chiral eluent additives (CEAs) have advantages and disadvantages as compared with CSPs (Box 10.4). CEAs are of several types: ion pair, inclusion complex, ligand exchange, and protein interaction. Chiral ion pairing agents may be used with unmodified silica, although the loading capacity is very low. Enantiomers are resolved as diastereomeric ion pairs. Propranolol enantiomers, for example, have been resolved using tartaric acid as the counter ion. Other counter ions used in this way include quinine, *N*-benzoxycarbonylglycyl-L-proline, and (1*S*)-(+)-10-camphorsulfonic acid.

Box 10.4 Advantages and disadvantages of eluent additives in chiral LC

Advantages:
- Can use standard (less expensive) columns
- High loading capacities are possible
- Solute character may be modified (e.g. by ion pairing)
- Wide range of additives available
- Scaling up for semi-preparative or preparative work not limited by column availability

Disadvantages:
- May have to remove the chiral selector after the analysis
- Separations relatively difficult to develop
- Can be expensive on a large scale without recycling additive(s)
- Crown ethers too toxic for use as CEAs

Inclusion complexes may be formed by adding cyclodextrin to aqueous eluents. Resolution can sometimes be predicted from results on cyclodextrin CSPs, although the elution order is often reversed. Acidic or basic extraction can be used to remove cyclodextrins from the column effluent. The second approach to removing additives is to use SPE on a C_{18} column. After loading, the SPE column is washed with methanol:water (1+9 v/v) to remove the cyclodextrin and any buffer salts, and the analyte is then eluted with methanol.

Ligand chromatography can be carried out by using a chiral selector such as *N*-alkyl-L-hydroxyproline with copper(II) acetate in the eluent on a C_{18} column. The chiral selector is strongly adsorbed onto the stationary phase. If water-rich eluents are used there is very little column bleed and so the CEA may be omitted from the eluent for the actual analysis.

Both BSA and AAG have been used as CEAs. BSA may be added to an aqueous eluent at a concentration of around 3 g L^{-1} and used with C_{18}, CN, or diol columns. Although inexpensive, BSA has a UV cut-off at 340 nm that limits its use. AAG has the advantage of being UV transparent, but it is a human blood product and is expensive. As with protein stationary phases the preparative capacity is very low.

10.7 Derivatives for liquid chromatography

Except for facilitating chiral separations, derivatization in LC is usually performed to enhance sensitivity/selectivity for UV, fluorescence, or MS detection. The need for a derivatizable functional group on the analyte is self-evident. Many reagents can be used in either pre- or post-column mode. As in GC there is always the option of pre-column derivatization (Section 9.6), but this (i) alters the chromatographic characteristics of the analyte, (ii) may also derivatize unwanted sample components, and (iii) may make the choice of ISTD more difficult.

For post-column work, the ideal reaction would go to completion in a few seconds, but provided the reaction is first order with respect to analyte, and zero order with respect to reactants the signal will be directly proportional to the analyte concentration. However, it is crucial to ensure constant reaction times. Thus, reagent consumption is higher than with pre-column derivatization as a large excess must be maintained.

The use of derivatization to increase UV absorptivity is not widely used in analytical toxicology. Many of the available reagents were developed for use with early single-wavelength detectors. Consequently, several are based on *p*-nitrobenzoyl or *p*-nitrophenyl moieties that have a λ_{max} at approximately 254 nm. Carbonyl compounds treated with 2,4-dinitrophenylhydrazine, give hydrazones with λ_{max} 360 nm, $\varepsilon = \sim$20,000 L cm^{-1} mol^{-1}, and at 254 nm $\varepsilon = \sim$10,000 L cm^{-1} mol^{-1}.

10.7.1 Fluorescent derivatives

Primary amines may be derivatized using *o*-phthaldialdehyde (OPA) in the presence of either a thiol such as 2-mercaptoethanol, *N*-acetylcysteine, 3-mercaptopropionate, or *tert*-butylthiol, or sodium sulfite. 2-Mecaptoethanol has the disadvantage that the derivatives are unstable, but if the reaction is carried out in a suitable autosampler, the reaction time can be carefully controlled.

Fluorescence detection is relatively insensitive to changes in eluent flow rate hence an alternative approach is post-column derivatization (Section 10.3.9). The aim is to achieve rapid, selective derivative formation in the presence of the eluent with minimal reagent 'background'. OPA is a particularly good reagent for post-column procedures because it is 'silent', i.e. it has no native fluorescence.

10.7.2 Chiral derivatives

Chiral derivatization is always performed pre-column as the object is separation, not enhanced detection (Kraml *et al*., 2005). Requirements for an ideal chiral derivatization reagent are summarized in Box 10.5. Clearly chiral derivatization would be the last resort in semi-preparative or preparative work.

Box 10.5 Requirements for an ideal chiral derivatization reagent

- High chemical and optical purity
- Economic to purchase
- Stable (no racemization during storage)
- Rapid derivatization under mild reaction conditions to give a single reaction product
- Negligible racemization during derivatization
- Reaction rates and yields essentially the same for each enantiomer
- Resulting diastereomers have adequate detector response

Separations on π-donor brush-type (Pirkle) CSPs require the analyte to contain a π-acceptor such as an aromatic nitro moiety. Readily available reagents include 3,5-dinitrobenzoyl chloride, 4-nitrophenyl isocyanate, 2,4-dinitrophenylhydrazine, and 4-nitrophenacyl bromide, which may be chosen on the basis of the functional group (amine, alcohol, carboxylic acid, etc.) to be derivatized.

Carboxylic acids may be converted to acid chlorides and reacted with 2,4-dinitroaniline. Amide, urea, and carbamate derivatives are the most suitable as these provide sites for hydrogen bonding and dipole stacking, a second point of interaction. The third interaction is the spatial arrangement of groups around the asymmetric centre (Figure 10.16). Ester derivatives are not usually as successful as amides. Derivatization with a chiral reagent to form a diastereomer and subsequent analysis on a conventional, achiral stationary phase is a further option.

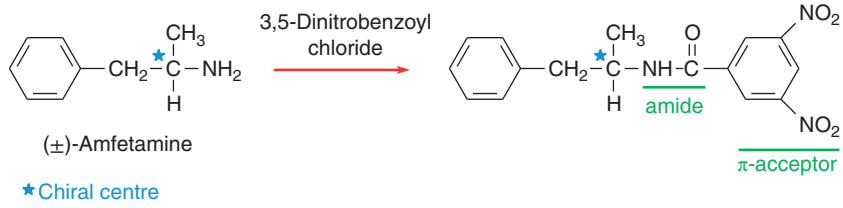


Figure 10.16 Derivatization of racemic amfetamine to provide points of interaction with a Pirkle-type π-donor column

The principal reactions used in chiral derivatization of amino acids and amino alcohols are based on the formation of amides, carbamates, urea, and isourea. Diastereomeric amide reactions are also widely used for the resolution of primary amines. For UV/Vis detection, derivatives that absorb in the visible region are of course preferable as this gives enhanced selectivity and hence sensitivity. Several chiral fluorigenic agents are also available.

10.8 Use of liquid chromatography in analytical toxicology

Many columns of various types are available, some of which are listed in Table 10.2. This list is by no means exhaustive. Consult manufacturers' catalogues for up-to-date information. Some general aspects of the use of LC in analytical toxicology are summarized in Box 10.6. Important considerations when selecting a column for a particular application are summarized in Section 10.4.1.2.

Table 10.2 Some commercially available liquid chromatography columns

Column name	Manufacturer	Particles[a]	Bonded ligand	Dimensions (mm)	Average particle size (μm)
ACQUITY UPC^2 HSS C18 SB	Waters	FPP	Octadecyl, non-endcapped	100 × 3.0	1.8
ACQUITY UPC^2 BEH	Waters	FPP	Unmodified hybrid silica	100 × 3.0	1.7
ACQUITY UPLC BEH Shield RP18	Waters	FPP	Alkyl with embedded carbamate group	100 × 3.0	1.7
ACQUITY UPC^2 BEH 2-EP	Waters	FPP	2-Ethylpyridine	100 × 3.0	1.7
ACQUITY UPC^2 CSH Fluorophenyl	Waters	FPP	Pentafluorophenyl	100 × 3.0	1.7
ACQUITY UPC^2 Torus 1-AA	Waters	FPP	1-Aminoanthracene	100 × 3.0	1.7
ACQUITY UPC^2 Torus 2-PIC	Waters	FPP	2-Picolylamine	100 × 3.0	1.7
ACQUITY UPC^2 Torus DEA	Waters	FPP	Diethylamine	100 × 3.0	1.7
ACQUITY UPC^2 Torus DIOL	Waters	FPP	Propanediol	100 × 3.0	1.7
Nucleodur C18 Gravity-SB	Macherey-Nagel	FPP	Octadecyl, endcapped	100 × 3.0	1.8
Hypersil Gold Silica	Thermo	FPP	Unmodified silica	100 × 3.0	1.9
Hypersil Gold CN	Thermo	FPP	Cyanopropyl-bonded silica	100 × 2.1	1.9
Synergi Polar RP	Phenomenex	FPP	Phenyloxypropyl	100 × 3.0	2.5
Accucore HILIC	Thermo	SPP	Unmodified silica	150 × 4.6	2.6
Accucore 150-Amide-HILIC	Thermo	SPP	Polyamide	150 × 4.6	2.6
Accucore Urea-HILIC	Thermo	SPP	Propylurea	150 × 4.6	2.6
Accucore Phenyl-X	Thermo	SPP	Phenylalkyl	150 × 4.6	2.6
Accucore Phenyl-hexyl	Thermo	SPP	Phenylhexyl	150 × 4.6	2.6
Accucore PFP	Thermo	SPP	Pentafluorophenyl	150 × 4.6	2.6
Ascentis Express OH5	Supelco	SPP	Pentahydroxyl	150 × 4.6	2.7
Ascentis Express F5	Supelco	SPP	Pentafluorophenyl	150 × 4.6	2.7
COSMOCORE Cholester	Nacalai Tesque	SPP	Cholesteryl	150 × 4.6	2.6
Cortecs HILIC	Waters	SPP	Unmodified silica	150 × 4.6	2.7
Kinetex HILIC	Phenomenex	SPP	Unmodified silica	150 × 4.6	2.6
Kinetex PFP	Phenomenex	SPP	Pentafluorophenyl	150 × 4.6	2.6
Kinetex F5 (2)	Phenomenex	SPP	Pentafluorophenyl	150 × 4.6	2.6
Kinetex Biphenyl	Phenomenex	SPP	Biphenyl	150 × 4.6	2.6
Kinetex XB C18	Phenomenex	SPP	Octadecyl	150 × 4.6	2.6
Nucleoshell HILIC	Macherey-Nagel	SPP	Sulfobetaine	150 × 3.0	2.7
Nucleoshell PFP	Macherey-Nagel	SPP	Pentafluorophenyl	150 × 3.0	2.7
Speedcore Diphenyl	Fortis	SPP	Diphenylalkyl	150 × 4.6	2.6

[a]FPP = fully porous particles; SPP = surface porous particles.

Box 10.6 Summary of the use of liquid chromatography in analytical toxicology

- Must consider selectivity from sample work-up (LLE often best) and detector as well as the column
- 5 µm packings often adequate if used with appropriate column/eluent combination
  - Routinely expect 3000–4000 theoretical plates per 125 mm column at 1–2 mL min^{-1} eluent flow with clinical sample extracts
- 3 µm packings or microbore/capillary systems can give higher efficiencies, but may also give higher back-pressures, longer analysis times, reduced solute capacity, and require modifications to injectors/detectors
- Use of methanolic or largely methanolic eluents in the LC of basic drugs (SCX-modified silica packings) gives good peak shapes and facilitates direct analysis of solvent extracts
- Use of LC-MS can be helpful in STA as an adjunct to temperature-programmed GC-MS

10.8.1 Acidic and neutral compounds

Generally acidic and neutral compounds are analyzed conveniently by reverse-phase chromatography using cyanopropyl-, phenylpropyl-, C_8-, C_{18}-, or similarly modified silica with aqueous methanol or acetonitrile eluents (Box 10.7). Ionic interactions with the silica matrix, itself weakly acidic, are relatively unimportant and the effect of altering the ratio of the organic component of the eluent to the aqueous component has by far the greatest impact and is generally predictable, more water giving increased retention and *vice versa*.

Similar separations can often be achieved using phenyl-modified packings as compared to ODS-modified materials, but retention of later eluting components may not increase as rapidly as with ODS. This can result in shorter isocratic analysis times and in greater sensitivity because peak broadening may be reduced. On the whole, therapeutic doses, and hence plasma concentrations, tend to be higher (w/w) with acidic and neutral drugs than with many basic drugs thus simplifying detection. However, this is not helpful with drugs such as barbiturates, which can only be detected by low-wavelength UV unless derivatized.

The analysis of some chlorophenoxy herbicides on modified and unmodified silica packings at constant eluent composition is illustrated in Figure 10.17. The elution sequence was the same in each case. S5 C_6, S5 C_8, and S5 ODS2 (C_{18}) columns gave essentially similar retention and peak shapes, whilst a 'mixed-mode' column (S5 OD/CN, 50 % C_{18}-, 50 % cyanopropyl-modified, a material designed for use with both acids and bases) gave more moderate retention and good

Box 10.7 Considerations in the liquid chromatography of acidic and neutral compounds

- Use on bonded-phase silica packing with aqueous methanol or acetonitrile eluent
- Need to buffer eluent in pH range 2–8 to give stable retention for ionizable compounds (higher pH values may encourage hydrolysis of packing)
- Can adjust retention/selectivity by:
  - Choice of bonded phase (and of manufacturer)
  - Eluent solvent composition (generally increasing water content = increased retention)
  - With ionic compounds can adjust pH, ionic strength, etc.

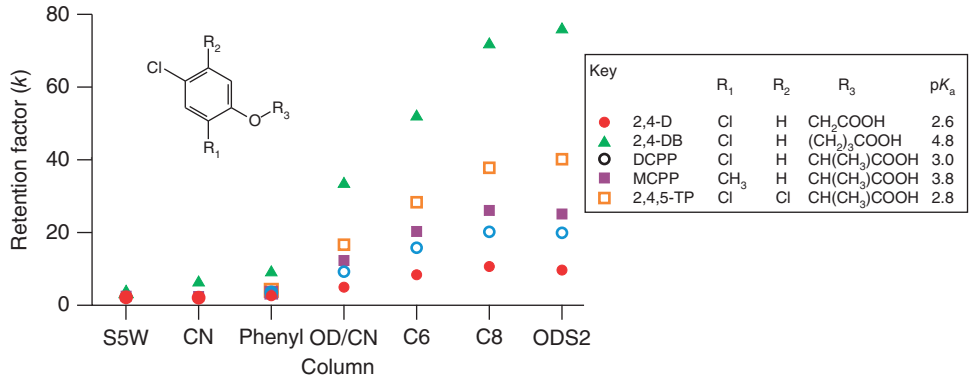


Figure 10.17 Effect of the nature of the column packing on the liquid chromatography of some chlorophenoxy herbicides. Column: 100 × 4.6 mm i.d. 5 mm Waters Spherisorb [S5 MM2 (50 % ODS, 50 % cyanopropyl)]. Flow rate: 1.8 mL min^{-1}. Detection: UV, 280 nm. Temperature: *ca.* 20 °C. Injection: 25 µL aqueous solution of 2,4-D, DCPP, MCPP, 2,3,5-TP, and 2,4-DB (50 mg L^{-1} each analyte). Eluent: aqueous potassium dihydrogen orthophosphate (50 mmol L^{-1}, pH 3.5):acetonitrile (75+25). [Key: 2,4-D = 2,4-dichlorophenoxyacetic acid, 2,4-DB = 4-(2,4-dichlorophenoxy)butyric acid, DCPP = 2-(2,4-dichlorophenoxy)propionic acid, MCPP = 2-(4-chloro-2-methylphenoxy)propionic acid, 2,4,5-TP = 2-(2,4,5-trichlorophenoxy)propionic acid)]

peak shapes. Retention was much reduced on the phenyl- and nitrile-modified materials, and was even less on unmodified silica (S5W). Nevertheless, the phenyl column gave the best compromise between peak shape, analysis time, and resolution.

In general, anticonvulsants, benzodiazepines, caffeine and other xanthines, and very polar amphoteric analytes such as catecholamines, being acidic, neutral, or weakly basic drugs, are analyzed on reverse-phase systems. In the case of the xanthines, addition of THF to the eluent has been advocated to ensure the separation of the caffeine metabolite paraxanthine and theophylline in some systems.

10.8.2 Basic drugs and quaternary ammonium compounds

Aspects of the LC of basic drugs are summarized in Box 10.8. Bonded-phase/aqueous methanol or acetonitrile eluent systems have been widely used. However, as well as buffer salts various

Box 10.8 Liquid chromatography of basic compounds

- Basic drugs (and quaternary ammonium compounds) may give poor peak shapes on bonded-phase silicas with aqueous methanol or acetonitrile eluents due to ionic interactions with surface silanols
- Can improve peak shapes by ‘end-capping’ or adding counter ions or other modifiers to the eluent
- Alternatively, can use unmodified silica in ion-exchange mode with methanol or aqueous methanol eluents of appropriate pH and ionic strength
- Microparticulate strong cation-exchangers (SCX) can be used in the same way as unmodified silica and give excellent results with many compounds

additives (pairing- or counter-ions such as alkylsulfonates, alkylamines, or quaternary ammonium compounds) have been advocated to give efficient performance. Moreover, in contrast to the behaviour of acidic/neutral compounds, the effect of altering the amount of water in the eluent on retention may not be predictable. This is probably because ionic interactions between the analyte and surface silanols are as strong, or stronger, than hydrophobic interactions with the bonded phase. Indeed, similar retention and elution sequences (and generally better peak shapes) to those obtained on a range of bonded phases can often be obtained on unmodified silica.

In practice, reverse-phase LC may permit the simultaneous analysis of basic drugs and neutral or even acidic drugs or metabolites (amides, glucuronides, or sulfates). Factors which may improve peak symmetry for bases are use of an eluent pH between 2.5 and 3.5, generally higher buffer concentrations, use of potassium instead of sodium salts, and addition of amine modifiers such as triethylamine (30 to 50 mmol L^{-1}) or dimethyloctylamine (5 to 10 mmol L^{-1}). Many manufacturers now produce special 'base-deactivated' bonded-phase silicas designed to minimize interactions with surface silanols, and thus permit more predictable reverse-phase behaviour and give better peak shapes.

10.8.2.1 Non-aqueous ionic eluent systems

As an alternative to the use of reverse-phase systems, efficient performance can be obtained for many basic drugs and quaternary ammonium compounds on unmodified silica using methanol or indeed aqueous methanol eluents (Section 10.5.2). Here retention is probably mediated largely by cation exchange with surface silanols. Eluent pH and ionic strength are thus major influences on retention (Flanagan & Jane, 1985; Jane *et al*., 1985). The peak shape of some analytes, notably alkaloids such as morphine, quinine, and strychnine, on non-aqueous ionic eluent systems is also influenced by eluent pH, higher pH values usually giving better peaks. Silica column/non-aqueous ionic eluent systems are useful for the analysis of basic drugs in biological extracts because only protonated bases and quaternary ammonium compounds are retained. In addition, *N*-dealkylated, phenolic, and other metabolites such as sulfoxides are usually resolved.

One problem with unmodified and indeed many modified silicas is that some compounds are poorly retained even if the eluent ionic strength is very low. Microparticulate strong cation-exchangers, however, give good retention and peak shape for many basic drugs (Croes *et al*., 1995; Figure 10.18). Practical advantages are that methanol is much less viscous than water/methanol mixtures and thus peak efficiency is high, and column back-pressures are relatively low. In addition, using methanol as the eluent solvent minimizes the risks of silica dissolution and of air-bubble formation; eluent degassing is not normally needed. A further advantage of non-aqueous ionic eluent systems is that relatively large volume solvent extracts can be injected directly in a 'non-eluting' solvent with no loss of efficiency.

10.8.3 Chiral analysis

The role of chiral analysis in forensic science has been reviewed (Ribeiro *et al*., 2018). Although chiral methodology is of primary interest in drug development, the possibility of inversion *in vivo* is real. Ibuprofen is normally marketed as a racemate, but a proportion of inactive *R*-(–)-ibuprofen undergoes inversion to the active enantiomer *in vivo* via the production of an unstable metabolite. Citalopram, a selective serotonin reuptake inhibitor (SSRI), is also prescribed as a racemate. Enantioselective LC of citalopram and *N*-desmethylcitalopram (norcitalopram) in femoral blood from 53 autopsy cases revealed an *S*:*R* ratio for citalopram of

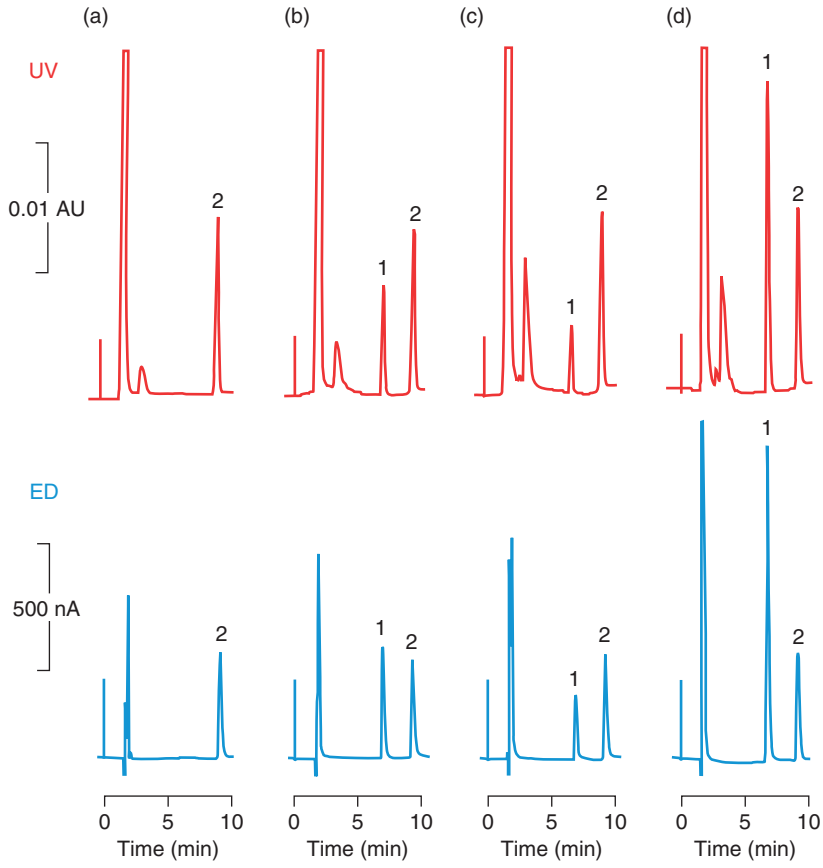


Figure 10.18 Liquid chromatography of lidocaine using Waters Spherisorb S5 SCX. Column: 250 × 5 mm i.d. Eluent: 20 mmol L^{-1} ammonium perchlorate in methanol (pH* 6.7). Flow rate: 2.0 mL min^{-1}. Detection: UV, 215 nm and ED, +1.1 V versus Ag/AgCl. Extraction: 200 µL sample + 50 µL aqueous Tris buffer (4 mol L^{-1}, pH 11) + 50 µL aqueous bupivacaine (internal standard, 10 mg L^{-1}) + 200 µL MTBE. Vortex-mix and centrifuge. Injection: 100 µL extract. Samples: (a) NBCS. (b) Standard in calf serum (1.0 mg L^{-1} lidocaine). (c) Equine serum (Gibco, Paisley, UK) found to contain 0.7 mg L^{-1} lidocaine. (d) Expired blood-bank human plasma found to contain 3.3 mg L^{-1} lidocaine. Peaks: 1 = lidocaine, 2 = bupivacaine

0.67 ± 0.25 and for norcitalopram of 0.68 ± 0.20 (Holmgren *et al*., 2004). The *S*:*R* ratios increased with increasing citalopram concentration and were also associated with high parent drug:metabolite ratios. In contrast, Rochat *et al*. (1995) found the ratios of *S*-(+)-citalopram and *S*-(+)-norcitalopram to their inactive enantiomers were only some 0.35 ± 0.05. A further factor is that escitalopram (*S*-citalopram) is available and is used at doses 50 % or so of those of (±)-citalopram.

10.9 Summary

LC has supplanted UV/Vis spectrophotometry and GC for measurement of many specific analytes and metabolites, especially when harnessed to the selectivity and sensitivity offered

by MS (Chapter 19). Basic limitations are that there is no sensitive 'universal' LC detector analogous to the FID in GC, and secondly that there is no parallel in LC-MS to EI in GC-MS. However, the great virtue of LC, the flexibility offered by the ability to modify the eluent, is also its greatest drawback in routine use. Not only may the column packing be dissolved, compressed, or otherwise physically altered, but also contaminants may accumulate at the top of (or in) columns leading to loss of performance. Use of eluent filtration and of pre- or guard-columns can help prolong column life.

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11 Supercritical Fluid Chromatography

11.1 Introduction

A supercritical fluid is neither a liquid nor a gas and only exists in conditions that exceed the critical temperature (T_c) and pressure (p_c), known as the critical point of a substance (Table 11.1). Increases in either temperature or pressure will not affect the state of the fluid whilst both are above the critical point.

Supercritical fluids have properties between those of gases and liquids (Table 11.2). Supercritical fluid chromatography (SFC), which has been known since the 1960s, utilizes the ability of a liquid to dissolve an analyte with the chromatographic interactions and kinetics of a gas. Like gases, supercritical fluids occupy the available space having higher diffusivity and lower viscosity than liquids – properties that give rise to high chromatographic efficiency. The increased diffusivity reduces the resistance to mass transfer, the 'C'-term in the van Deemter equation [Equation (7.11)], resulting in a shallower slope at higher linear flow rates [Figure 7.3(b)]. Hence, HETP (H) is reduced even at higher flow rates. Moreover, because supercritical fluids have densities similar to those of liquids, they have stronger solvating power.

In SFC, pressurized carbon dioxide is normally used as the major component of the eluent because the moderate critical point (T_c = 31.1 °C, p_c = 73.8 bar) makes instrumentation relatively straightforward. By comparison the critical point for water is much higher, both in terms of temperature and pressure (T_c = 374.2 °C and p_c = 221.2 bar). The triple point of carbon dioxide, the point at which solid, liquid, and vapour are in equilibrium, is –56.6 °C at 5.2 bar. Therefore, at atmospheric pressure solid carbon dioxide (dry ice) does not melt, but sublimes at temperatures just above –78.5 °C [Figure 11.1(a)]. The converse is true and at temperatures and pressures below the triple point, carbon dioxide vapour solidifies without the formation of any liquid.

As a solvent, supercritical carbon dioxide has properties similar to those of heptane but is easily removed at the end of the analysis. To promote the elution of polar analytes small amounts of methanol, ethanol, 2-propanol, water, or acetonitrile may be added. Adding 5.5 % v/v methanol can decrease diffusion coefficients by nearly 50 %. Acids, bases or ammonium acetate may be added, as required. Retention is adjusted by the choice of column, temperature, pressure, and choice and proportions of modifiers (Box 11.1). Increases in the pressure of a supercritical fluid increases the density, which in turn increases the solvation power and reduces retention. Thus, pressure is an additional variable affecting retention and hence analysis time.

Usually, changes in temperature have little effect on retention unless the eluent is no longer supercritical. Addition of modifiers will change the critical point of the eluent. For example,

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

Table 11.1 IUPAC definitions relevant to supercritical fluid chromatography

Term	Definition
Critical temperature (T_c)	The maximum temperature at which a gas can be converted into a liquid by an increase in pressure
Critical pressure (p_c)	The minimum pressure that would suffice to liquefy a substance at its critical temperature. Above the critical pressure, increasing the temperature will not cause a fluid to vaporize to give a two-phase system
Critical point	The characteristic temperature (T_c) and pressure (p_c) above which a gas cannot be liquefied
Supercritical fluid	The defined state of a compound, mixture, or element above its critical pressure (p_c) and critical temperature (T_c)
Reduced temperature (T_r)	The ratio of the temperature (T) in the system to the critical temperature (T_c) $T_r = T/T_c$
Reduced pressure (p_r)	The ratio of the pressure in the system (p) to the critical pressure (p_c) $p_r = p/p_c$

Table 11.2 Some physical properties of gases, supercritical fluids, and liquids

	Density (g cm^3)	Diffusion (cm^2 s^{-1})	Viscosity (g cm^{-1} s^{-1})
Gas	10^{-3}	10^{-1}	10^{-4}
Supercritical fluid	0.1–1	10^{-4}–10^{-3}	10^{-4}–10^{-3}
Liquid	1	$<10^{-5}$	10^{-2}

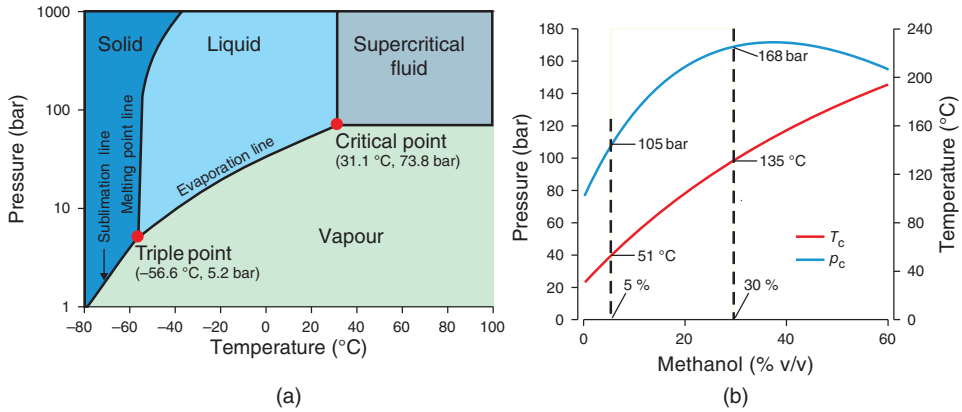

Figure 11.1 (a) Phase diagram for carbon dioxide. (b) Effect of addition of methanol to carbon dioxide on the critical point of the mixture (adapted from Saito, 2013–reproduced with permission of Elsevier)

Box 11.1 Supercritical fluid chromatography

- Use for high M_r, polar, and/or thermally labile analytes
- Use with capillary or packed columns
- Adjust retention by altering temperature/pressure/eluent composition/stationary phase
  - Column packings are those developed for LC
- Eluent supercritical carbon dioxide
  - Inexpensive, environmentally friendly – can be recovered and reused
  - Very low viscosity
  - Can modify with methanol, ethanol, 2-propanol, water, acetonitrile
  - Can also add acid, base, ammonium acetate
- Easy to interface to MS
  - Volatile eluent easy to remove

addition of 5 % v/v methanol to carbon dioxide increases the critical point to $T_c = 51$ °C, $p_c = 105$ bar, whilst at 30 % v/v methanol, the respective figures are 135 °C and 168 bar [Figure 11.1(b)]. The changes in the critical point of the mixture as a function of methanol content are not linear and will approach the critical point of methanol (240.3 °C, 82.2 bar) as the proportion approaches 100 % v/v. As a result, the final conditions chosen may result in subcritical operation but, provided the method is reproducible, this may be immaterial (Hofstetter *et al.*, 2019).

SFC has advantages over both GC and LC in some applications, and has acquired the name 'convergence chromatography', partly as an alternative to SFC when the eluent is not actually supercritical. SFC can be used either with open tubular capillary columns, very much like GC, or with packed columns analogous to LC. The use of sub-2 μm porous and sub-3 μm superficially porous particle size stationary phases can give improved efficiency and, hence, faster analyses, but without the problems caused by the use of viscous liquids in LC. Moreover, the distinction between SFC and LC is becoming less clear. Switching from one mode to the other, even within the course of a single analysis, is facilitated by modern instruments (West, 2018). In fact, the concept of 'enhanced fluidity LC' has been introduced where a significant amount (typically 20–95 %) of a viscosity lowering agent, such as carbon dioxide, is added to the mobile phase to provide higher eluent flow rates without a concomitant loss in chromatographic efficiency.

SFC has proved useful in the analysis and purification of low to moderate M_r, thermally labile compounds because operating temperatures for supercritical carbon dioxide are usually in the range 40–50 °C. This is a distinct advantage over GC (Box 11.2). Detectors normally

Box 11.2 Supercritical fluid chromatography versus gas chromatography

Advantages	Disadvantages
• Better solvation • Extended range of analytes including thermally labile analytes • Eluent strength can be controlled by a range of modifiers	• Unwanted reaction with the eluent • Hardware more complex • Use of modifiers may limit range of detectors that can be used (e.g. organic modifiers may preclude use of FID)

Box 11.3 Supercritical fluid chromatography versus liquid chromatography

Advantages	Disadvantages
• Lower eluent viscosity (higher efficiencies)	• Limited eluent choice
• Higher sample capacity	• Limited analyte solubility in the eluent
• High throughput	• Unwanted reaction with the eluent
• Can use chiral stationary phases	• Less suited to hydrophilic analytes
• Can use FID	• May require specialized detectors
• Can be interfaced to MS	• Gradient formation may be difficult

used for GC and LC can be used, after modification to withstand elevated pressures if necessary (Box 11.3). Interfacing with MS has become routine, but raises different issues from interfacing with LC (Section 13.5). Limitations such as poor UV sensitivity, limited reliability, and poor quantitative performance have been addressed by new instruments with low extra-column dead volumes (Nováková *et al*., 2014; Guillarme *et al*., 2018).

Disadvantages of SFC may include limited eluent choice, limited analyte solubility in the eluent, analyte reaction with the eluent (for example primary and secondary amines form carbamates with supercritical carbon dioxide), and difficulty in forming stable gradients with eluents containing a polar organic modifier (Boxes 11.2 and 11.3). A further drawback is that upon depressurization the carbon dioxide in the eluate boils rapidly and dissolved analyte may be lost as an aerosol. Cyclone separators may help capture aerosolized analyte.

11.2 General considerations

As indicated in Box 11.1, SFC can involve the use of either capillary, or packed columns, which have much in common with capillary GC and modern LC, respectively. Packed column SFC is the form used most widely. As with LC columns, most packed SFC columns are stainless steel filled with silica-based packing material. SFC fittings and accessories are those used in LC.

For packed column SFC, the instruments do not differ greatly from their LC counterparts (Figure 11.2), except for the obvious need to provide carbon dioxide and to maintain the pressure for supercritical conditions. This can be achieved by placing a back-pressure regulator between the column outlet and the detector when GC detectors such as a FID are used, or after the detector if LC detectors are used. Such detectors must be able to withstand the operating pressures. Automated back-pressure regulators (ABPR) have a valve controlled via a transducer to maintain the set pressure inside the system [Figure 11.3(b)]. Eluent flow rates are of the order of a few mL min^{-1}, and injection volumes can range up to hundreds of μL, thereby facilitating preparative SFC if desired. In view of this, modern instruments often feature a system to reuse carbon dioxide after allowing it to evaporate in order to remove modifiers and sample residues. Such systems can allow >90 % reuse of carbon dioxide. Adiabatic cooling as the carbon dioxide evaporates can reduce the temperature to the point where solid carbon dioxide forms [Figure 11.1(a)] resulting in blockages. Heaters are included to alleviate this problem (Figure 11.3).

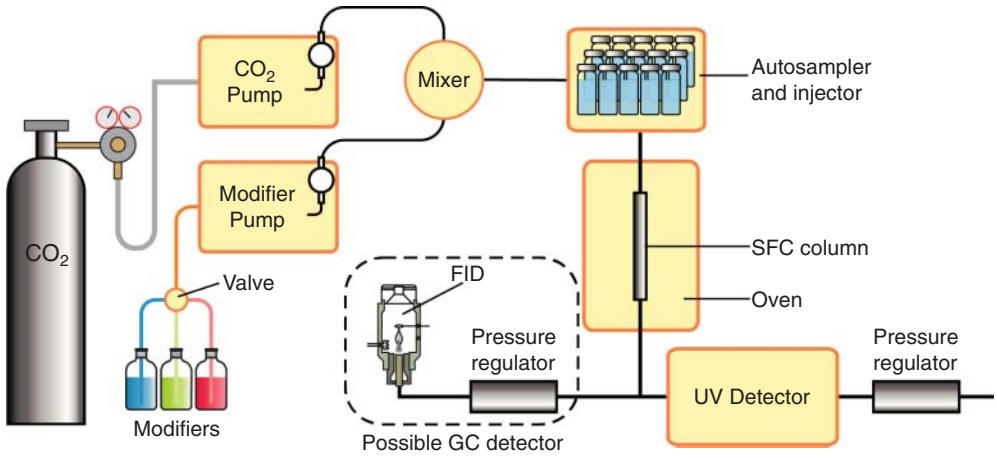

Figure 11.2 Schematic diagram of a packed column supercritical fluid chromatography system

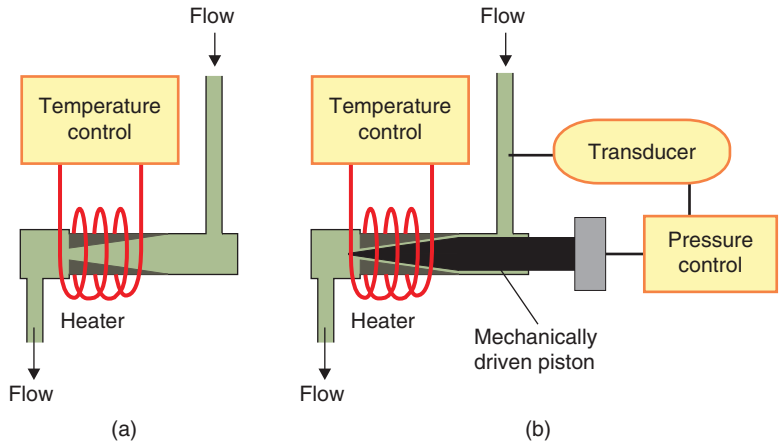

Figure 11.3 Schematic diagram of supercritical fluid chromatography pressure regulators (a) fixed and (b) variable

Capillary SFC uses modified GC equipment. Open tubular SFC capillary columns are similar to those used in GC, typically fused silica with an outer polyimide coating, and with the stationary phase bonded chemically onto the inner wall. In general, SFC capillary columns have smaller diameters than GC capillaries. Modified couplings are used to reduce dead volumes and to avoid leaks. Particular attention should be paid to the coupling between the column outlet and the ABPR. Eluent flow rates are of the order of a few μL min^{-1} and injection volumes are usually <1 nL.

11.2.1 The pump

Liquids are nearly incompressible, and their densities are constant despite changes in pressure. Supercritical fluids, on the other hand, are highly compressible and their physical properties

change with changes in pressure, such as the pressure drop across a packed column. Therefore, SFC pumps are designed to deliver eluent at constant pressure and use electronic speed control to alter the compressibility of the eluent. Pumps that lack this ability will deliver an accurate flow rate only at a pre-selected pressure and eluent composition so that gradient elution is not feasible unless constant pressure pumping systems are used.

SFC with carbon dioxide requires that the incoming carbon dioxide and pump heads be kept cold in order to maintain the carbon dioxide at a temperature and pressure that keeps it liquid so that the flow rate can be effectively metered. The carbon dioxide becomes supercritical in the column oven when the temperature and pressure are raised above the critical point.

11.2.2 The eluent

Carbon dioxide is the eluent fluid of choice in SFC because it is (i) inexpensive, (ii) available with high purity, (iii) safe and easy to use, and (iv) requires modest conditions to achieve supercriticality. Moreover, addition of polar modifiers to either increase analyte solubility in the eluent, or to facilitate elution is relatively straightforward. However, the addition of modifiers alters the critical point [Figure 11.1(b)]. Adding a short chain alcohol may raise the critical temperature to the point at which it may compromise the analysis of thermally labile compounds. Furthermore, addition of an organic modifier will limit use of FID, although the use of formic acid/formates may lessen the effect compared to use of acetic acid/acetates (Section 9.2.2.2).

Typically, gradient elution is employed in analytical packed column SFC using a polar co-solvent such as methanol, possibly containing a weak acid or base at low concentration (of the order of 1 % v/v). The effective plate count per analysis can exceed 500,000 plates per metre routinely with 5 μm packings. Eluent flow rate, co-solvent composition, system back pressure and column oven temperature, which must exceed 40 °C for supercritical conditions to be achieved with carbon dioxide, can be varied to adjust the separation.

11.3 Detection in supercritical fluid chromatography

SFC systems require pressure regulation (restrictors placed somewhere after the column) in order to maintain critical conditions. If the pressure regulator is placed after the detector then detection is as in LC, but the UV detector flow cell, for example, has to be designed to withstand the high pressures involved. Increased noise can arise because of variations in refractive index, which depends in part on the fluid density. Keeping the detector temperature below T_c and a modifier content above 5 % v/v can reduce the noise.

For ‘open cell’ detectors, such as FID, ELSD, CAD, and MS, the pressure restrictor is placed before the detector. ELSDs are usually used for non-volatile compounds having no chromophores and are often used for carbohydrates. The outlet from the pressure regulator is usually heated to prevent carbon dioxide solidifying and blocking the system. When using a CAD, the eluent is nebulized, and the analytes are ionized by the corona discharge source before being detected by an electrometer. When compared with interfacing MS with GC or LC, interfacing SFC with MS poses unique challenges because of the pressure within the system (Section 13.6).

11.4 Columns and column packings

As in GC, the polarity of the stationary phase is a guide to column selection in capillary SFC. Most of the column packings commonly used in SFC were developed for LC, although some

stationary phases have been designed specifically for SFC. Whilst the availability of different stationary phases may help to achieve successful SFC separations, selecting a column for method development has proved difficult (Galea *et al*., 2015). With this in mind, a classification of 31 stationary phases (Table 10.2) based on the analysis of 109 neutral and ionizable species using primarily a carbon dioxide:methanol (9+1, v/v) eluent at 25 °C has been proposed to facilitate method development in SFC (West *et al*., 2016). The packings studied include materials normally associated with normal phase, HILIC, and reverse phase chromatography, emphasizing the flexibility of SFC.

11.5 Chiral separations

It has been suggested that SFC is the method of choice when developing chiral separations (Kalíková *et al*., 2014). This is, in part, due to the belief that the separation factor (α) is greater at lower temperatures. However, the thermodynamic relationship for α is related to differences in standard enthalpy (ΔH°) and entropy (ΔS°) (Khater *et al*., 2018):

$$\ln(\alpha) = -\frac{\Delta\Delta H^\circ}{RT} + \frac{\Delta\Delta S^\circ}{R} \tag{11.1}$$

where R is the gas constant and T the temperature (K). $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ are the differences in standard enthalpy and standard entropy of the two enantiomers. At the temperature (T_{iso}) at which the isomers coelute ($\alpha = 1$) there exists a balance between enthalpy and entropy.

Below T_{iso}, the enthalpy term dominates and enantioselectivity increases as the temperature is reduced. However, above T_{iso}, entropy is the dominant factor and enantioselectivity *increases* with temperature. Thus, the important thing to note is that it is not always the case that reducing the temperature increases α and furthermore, a reversal of elution order may be observed when changing temperatures around T_{iso}. Having said this, T_{iso} may be outside of the working ranges of temperatures for a particular assay and analyte. The effect of temperature can be examined by plotting $\ln(\alpha)$ against $1/T$ (Van't Hoff plot).

The use of chiral SFC has been reviewed (Mangelings & Vander Heyden, 2008; De Klerck *et al*., 2012; Kalíková *et al*., 2014). The majority of the methods described use chiral stationary phases that are available for LC, although some use achiral columns with chiral mobile phase additives. For example, warfarin, lorazepam, and flurbiprofen enantiomers have been resolved using porous graphitic carbon columns and dimethyl β-cyclodextrin as chiral selector (Salvador *et al*., 2001). Although few of the methods have been applied to biological samples, Table 1 in Kalíková *et al*. (2014) and the tables in Lesellier & West (2015) may provide a useful starting point with regard to column and eluent selection for a large number of drugs.

Two dimensional approaches have been described. For example, Zeng *et al*. (2011) employed a pyridine column in the first dimension for achiral separation and either CHIRALPAK AD-H, or CHIRALCEL OD-H to resolve the enantiomers. A single quadruple MS was used to monitor the column effluent. This SFC/SFC-MS approach was applied in purifying pharmaceuticals.

Resolution and quantification of ketoprofen enantiomers in human plasma after either oral or topical application of the racemate have been reported (Hoke *et al*., 2000). (±)-[^{13}C,$^{2}H_3$]-Ketoprofen was used as ISTD and samples were prepared using automated SPE. The enantiomers were separated on a Chirex 3005 analytical column using 55 % v/v methanol in carbon dioxide at 5 mL min^{-1} for enhanced fluidity chromatography. The system was configured so that it could be converted from 'SFC' to LC mode in a few minutes. The LC eluent was 30 mmol L^{-1} ammonium acetate adjusted to pH 3.5 with formic acid in 95+5 v/v methanol:water delivered at 0.5 mL min^{-1}. Detection was by MS/MS (Section 13.2.4) using

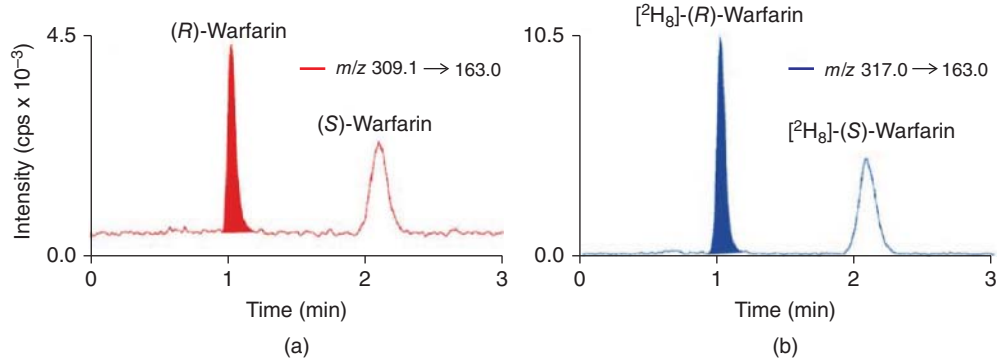

Figure 11.4 Resolution of warfarin enantiomers on a CHIRALPAK AD column in (Adapted from Coe *et al.*, 2006–reproduced with permission of Elsevier)

ESI (Section 13.4.1.1). The LLoQ was 50 ng L^{-1}. Analysis times were 2.3 and 6.5 min for SFC and LC, respectively.

SFC-MS/MS has been used to measure warfarin enantiomers in human plasma (Coe *et al.*, 2006). (*R/S*)-Warfarin was resolved on a CHIRALPAK AD column (250 × 4.6 mm i.d.) using 30 % v/v ethanol in carbon dioxide at a flow rate of 7 mL min^{-1}. The separation was carried out at room temperature and 240 bar. [$^2H_8$]-Warfarin was used as ISTD. Fragmentation of the pseudomolecular ions to a product ion *m/z* 163 was monitored. Plasma (0.2 mL) and ISTD solution (20 µL) were pipetted onto 96-well plates and extracted with diethyl ether after acidification with sulfuric acid using an automatic extraction system. After transfer and evaporation of the solvent, the residues were reconstituted in methanol (200 µL) and 5 µL taken for analysis. The enantiomers were well separated ($\alpha = 2.5$), with the (*R*)-isomer eluting first (Figure 11.4). At the LLoQ (13.6 µg L^{-1}) the inter-batch RSD was 7 %.

The SFC of the enantiomers of ketamine, and selected metabolites, norketamine, 5,6-dehydronorketamine, 6-hydroxynorketamine (Figure 11.5) on three different polysaccharide-based CSPs (Amylose-2, i-Amylose-3, and i-Cellulose-5) has been investigated (Hofstetter *et al.*, 2019). Methanol yielded lower analysis times, but superior chiral resolution compared with 2-propanol. Lower temperatures than those used conventionally did not impair phase homogeneity, but improved enantioresolution without affecting reproducibility. Thus, subambient temperature subcritical fluid chromatography – essentially low-temperature LC with subcritical carbon dioxide as eluent – was used. The same group used an Amylose-2 column to measure these compounds in fortified urine (Hofstetter *et al.*, 2018). Of particular

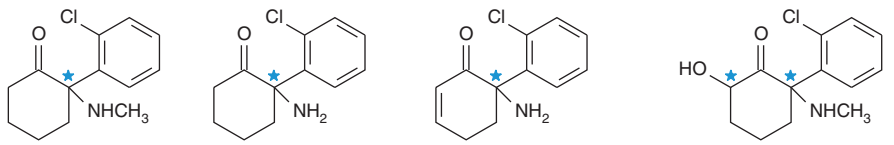

Figure 11.5 Chemical formulae of ketamine and selected metabolites

interest was quantification of (2*R*,6*R*)-hydroxynorketamine, a compound that has been shown to be of benefit in major depressive disorder.

11.6 Toxicological and forensic applications

The applicability of SFC in pharmaceutical analysis, including the measurement of drugs in formulations and biofluids, has been outlined (Desfontaine *et al*., 2015). Some of the considerations in SFC of drugs (caffeine, carvedilol, diclofenac, etodolac, haloperidol, hydrocortisone, ibuprofen, ipriflavone, nadolol, phenazone, terfenadine, theophylline, toremifene, and warfarin) have been reviewed (Al Bakain *et al*., 2017). Metabolite analysis using SFC-MS has also been discussed (Taguchi *et al*., 2014; Hofstetter *et al*., 2019; Section 13.5), an important feature being the ability to separate acidic and basic metabolites in the same analysis.

The application of SFC in the analysis of cannabinoids, amfetamines, and NPS, including synthetic cannabinoids, cathinones and phenylamines, and doping agents has been reviewed (Pauk & Lemr, 2018). As an example, Geryk *et al*. (2015) separated Δ^9-THC, cannabidiol (CBD), and 15 synthetic cannabinoids on a Zorbax Rx-SIL column (150 × 4.6 i.d. mm) in <14 minutes. The optimized conditions were a mobile phase consisting of 7 % v/v acetonitrile in carbon dioxide at 2.5 mL min^{-1}, with a temperature of 40 °C and a back pressure of 95 bar. A validated method was applied to the measurement of JWH-073 metabolites in the urine of someone who had smoked a herbal preparation containing JWH-073 (Figure 11.6). Conjugates were hydrolyzed with *β*-glucuronidase prior to LLE into acetonitrile after adding ammonium acetate to ensure phase separation.

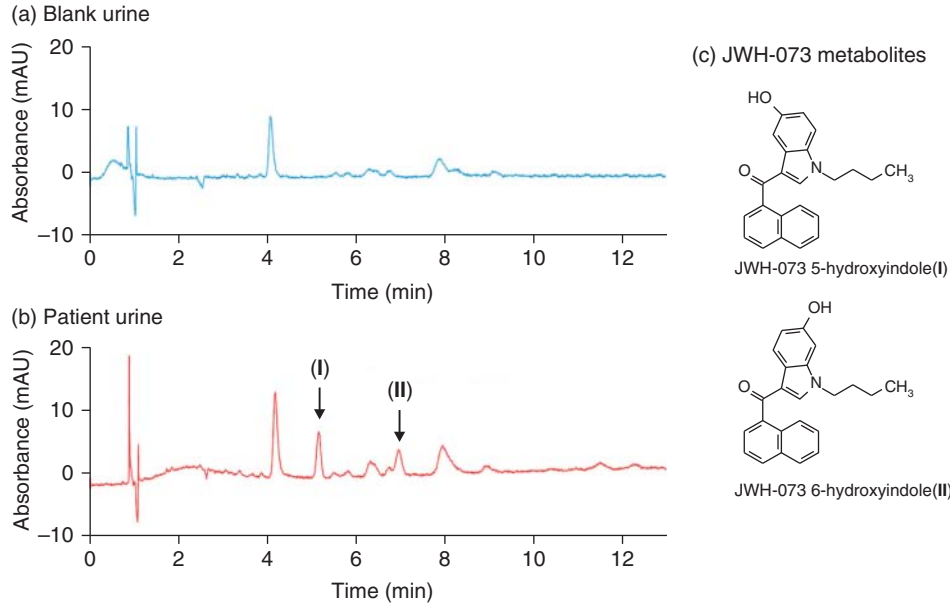

Figure 11.6 Supercritical fluid chromatography of JWH-073. (a) Blank urine; (b) metabolites extracted from urine; (c) structural formulae of hydroxylated JWH-073 metabolites. UV detection, 210 nm (redrawn from Geryk *et al*., 2015–reproduced with permission of the Royal Society of Chemistry)

Semi-preparative SFC has been applied to analysis of steroids in bovine urine (Doué *et al*., 2018). Initially, several columns were screened using carbon dioxide:methanol (95+5 v/v) at 25 °C, with 150 bar back-pressure, a flow rate of 3 mL min^{-1}, linear position and UV detection (210 nm) and ELSD. For untargeted compounds, a Cosmosil naphthyl-bonded column was used with carbon dioxide:methanol gradients to collect chiefly androgens (plus oestrogens) while use of a diol column allowed fractionation of (i) mono-hydroxylated androgens, (ii) di-hydroxylated androgens, and (iii) oestrogens. Use of carbon dioxide:methanol eluents gave a 14-fold saving on costs compared to a standard LC preparation method using hexane:2-propanol as well as environmental benefits.

11.7 Summary

SFC brings advantages over both GC and LC in certain areas. Modern instruments are robust, and SFC-MS has become as simple to use as LC-MS and complements both GC-MS and LC-MS in routine use. Interfacing to API is simple, and use of make-up flow solvent helps control ionization in ESI and APPI. Increasingly, methods are being developed that operate under subcritical conditions, the advantages of using liquid carbon dioxide as a component of the mobile phase being clear. Until a widely agreed name for such use is adopted it will continue to be known as SFC.

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12 Capillary Electrophoretic Techniques

12.1 Introduction

Of the many electrophoretic techniques available, the most widely investigated for use in analytical toxicology and related areas have been capillary electrophoresis (CE, also called capillary zone electrophoresis – CZE), micellar electrokinetic (capillary) chromatography (MEKC), and capillary electro(kinetic) chromatography (CEC). CE, sometimes referred to as free solution capillary electrophoresis (FSCE) to distinguish it from MEKC, refers to the separation of ions in free solution based on different velocities in an electric field. Selectivity is primarily due to differences in mass and charge – small charged molecules migrate more quickly than larger ones carrying the same charge.

In CE a fused-silica capillary, usually 25–75 μm i.d. (i.e. narrower than normally used in capillary GC (Section 9.3.3) coated with polyimide, is placed between two reservoirs of a buffer solution that is referred to as the background electrolyte (BGE). In non-aqueous CE, the aqueous buffer is replaced by a background electrolyte in (a mixture of) organic solvents. The capillary is filled with buffer and platinum electrodes are placed in both reservoirs so that a potential difference of around 5–30 kV can be applied across the capillary, in turn creating a current of 150–300 μA. With UV/fluorescence detection, the peaks are detected on-column, a small area of polyimide being removed to provide a window for the light path to the detector (Figure 12.1). The preparation and care of a capillary is summarized in Box 12.1.

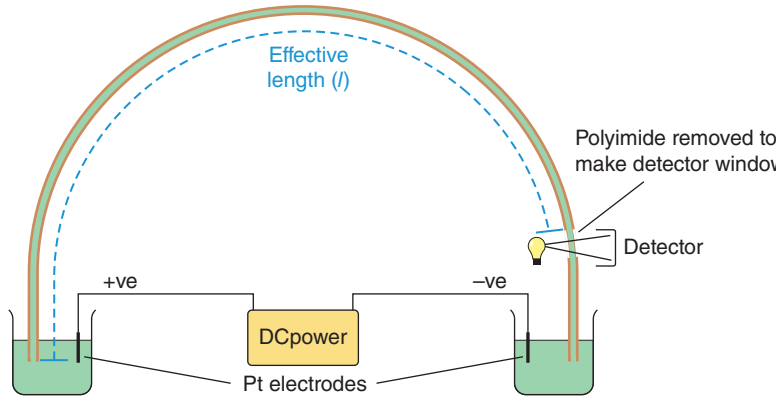

Figure 12.1 Schematic diagram of the arrangement for capillary electrophoresis

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

Box 12.1 Preparation, conditioning, and storage of capillaries for electrophoresis

Cutting to length
- Cut to length with ceramic cutter – pull lengthways to break capillary
- Burn off 1 mm of polyimide coating at each end[1]
- Check for clean cut with aid of a microscope

Detector window
- Measure appropriate distance to detector
- Burn off small area of coating[1]
- Carefully wipe off burnt coating with methanol-soaked tissue
- Inspect with microscope to ensure clear light path

Conditioning
- Flush with aqueous sodium hydroxide (1 mol L^{-1}, 30 min)
- Flush with deionized water (30 min)
- Equilibrate with BGE (20 min)

Storage
- Flush with aqueous sodium hydroxide and water as above
- Rinse with methanol
- Dry with a stream of nitrogen
- Store in a safe place – in a cartridge if possible

[1]Use a small flame such as a match held under a piece of aluminium sheet with a small hole drilled in it, or an electrically heated wire

The length of the capillary from the anode reservoir to the detector window is known as the effective length (l) and is usually a few centimetres shorter than the total length (L), which is typically 15–100 cm. The applied field, E (V cm^{-1}), is the applied potential difference (V) divided by L. The same basic apparatus and detectors are used for MEKC and CEC. The techniques depend heavily on the availability of high-quality fused silica capillary tubing and extremely sensitive detectors.

12.2 Theoretical aspects

12.2.1 Electrophoretic mobility

Ions are separated on the basis of size and charge. The electrophoretic mobility (μ_e) of an ion of radius r carrying a charge, q, is:

$$\mu_e = \frac{q}{6\pi\eta r} \tag{12.1}$$

where η is the viscosity of the buffer solution. Thus small, highly-charged ions migrate faster than larger ions carrying less charge.

Under alkaline conditions, the walls of a fused silica capillary are negatively charged because of ionization of the residual silanols and possibly because of the presence of adsorbed ions. Cations migrate to the wall, forming a layer of ions that is relatively immobile (Stern layer). There is a more diffuse layer of solvated ions that extends into the bulk liquid (Goüy layer). The potential between the Stern layer and the interface with the diffused double layer, is known as the

zeta potential, ζ. It is possible to calculate the thickness of the double layer, which is of the order of 1–10 nm depending on the conditions employed. When an electric field is applied, hydrated cations in the diffuse layer are attracted towards the cathode (negative potential) resulting in shearing in the region of the double layer. However, the bulk of the liquid in this sheath is carried towards the cathode. This is known as the electro-osmotic flow (EOF) and its velocity is unaffected by the diameter of the capillary, provided that it is *ca*. 20 times greater than the thickness of the double layer.

The EOF increases the rate of migration of cations and transports non-ionized molecules and even anions to the cathode provided that the EOF is strong enough to overcome electrophoretic migration of anions to the anode. Thus, cations and anions move in the same direction and can be measured as they flow past the detector (Figure 12.2). An important feature of the EOF is that, because the forces responsible for the flow are uniformly distributed across the capillary, the flow is uniform and the flow profile is perpendicular to the capillary wall (plug flow), unlike the parabolic flow profile in pressure driven systems such as GC and LC. This leads to less zone dispersion and in theory much greater efficiencies than those achievable by chromatographic techniques. The advantage of non-laminar flow is exploited in CEC (Section 12.5.2).

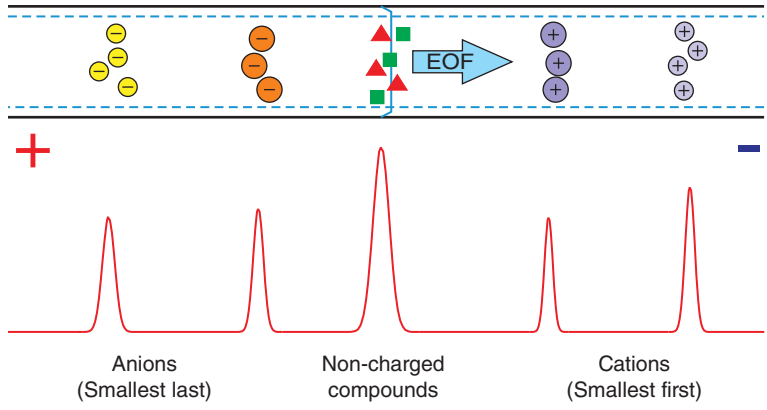

Figure 12.2 Diagram showing how electro-osmotic flow carries analytes to the cathode

The mobility of the EOF is determined to a large extent by the zeta potential:

$$\mu_{\text{EOF}} = \frac{\varepsilon\zeta}{\eta} \tag{12.2}$$

where ε is the dielectric constant of the BGE. The zeta potential, and hence the EOF, increases as the pH of the BGE is increased because this increases the number of ionized silanols on the capillary wall. The velocity of the EOF is proportional to the applied electric field, E. Other influences on EOF include temperature (via changed viscosity of the BGE), the proportion of any organic modifier, the concentration of any added surfactant, and changes to the nature of the capillary wall. It is important to be able to control the EOF: too high a flow rate and the analytes will not be resolved, too low a flow rate and cations may be adsorbed onto the negatively charged wall of the capillary.

Addition of cationic surfactants at concentrations below their critical micellar concentration (CMC) can reverse the direction of the EOF. A layer of surfactant is attracted to the negative charge on the capillary wall and a second layer forms (as a result of interactions of the

hydrophobic tails) with the positive charges facing the bulk fluid. Hence, the effective ionization is now positive rather than negative.

Increasing the ionic strength of the BGE decreases ζ and hence decreases EOF. This can improve resolution because migration times are prolonged. It also increases the pH stability of the BGE, which may be important as the pH is altered during electrophoresis. Consequently, the BGE should be renewed frequently, for example every 5 analyses, because pH is generally one of the major influences on migration time. Furthermore, this practice ensures that the liquid levels in the vials are equal because even a small degree of siphoning can have a marked effect on efficiency. The BGE should be filtered and degassed before use. Because of the small volumes involved, filtration can be performed using syringes fitted with membrane filters (0.2 μm) and degassing by sonication is usually adequate.

From the above discussion, it is clear that the migration time of an analyte will be a function of the electrophoretic mobility and the EOF. The apparent mobility, μ_a, is given by:

$$\mu_a = \mu_e + \mu_{EOF} \tag{12.3}$$

and is proportional to the effective length of the column (l) and indirectly proportional to the applied field:

$$\mu_a = \frac{l}{Et} = \frac{lL}{Vt} \tag{12.4}$$

where t is the migration time (s). Thus, the unit of mobility is $cm^2\ V^{-1}\ s^{-1}$. Non-ionized molecules will migrate with the EOF and so cannot be separated by CE. Anions have negative mobility.

12.2.2 *Efficiency and zone broadening*

The efficiency of a capillary electrophoretic separation can be expressed in terms of the number of theoretical plates, $N = 1/\sigma^2$ (Section 7.2.2). The main determinant of zone broadening is longitudinal diffusion because radial diffusion is negligible because of the plug-flow profile. The variance due to longitudinal diffusion is given by:

$$\sigma^2 = 2D_m t \tag{12.5}$$

where D_m is the diffusion coefficient of the analyte. Large molecules, such as peptides and proteins, have small values of D_m and usually produce sharp peaks on CE. Substituting Equation (12.4) for t gives:

$$N = \frac{\mu_e V l}{2D_m L} \tag{12.6}$$

Clearly, from Equation (12.6), high applied potentials result in high efficiency and therefore improved resolution. As with chromatography, the more quickly a compound migrates, the sharper the peak will be. Because N is directly proportional to V, applied potentials are not normally reduced as a means of reducing the EOF. There are a number of practical reasons why N is less than predicted by Equation (12.6), as discussed below.

12.2.3 *Joule heating*

It is important not only to understand the role of Joule heating, but also to minimize its effects. The heat generated by the electric current, which is proportional to the power (current × voltage), creates temperature gradients that cause local changes in viscosity and hence band broadening. Use of narrow-bore capillaries and temperature regulation (forced air or liquid cooling) can

reduce these effects, but very narrow capillaries limit sample size and the effective light path length when using spectrophotometric detection. Measuring current as a function of applied potential is used to check the working potential range of a method (Ohms Law plot). This will be linear whilst the contribution from Joule heating is acceptable.

12.2.4 Electrodispersion

A difference between the conductivity of the sample zone and the conductivity of the BGE causes peak distortion. For a cation, when the conductivity in the sample zone is high, the leading edge of the sample zone is diffuse, with a high concentration of the ions at the trailing interface. This leads to tailing peaks. Conversely, fronting occurs when the sample zone is of lower conductivity than the BGE. The reverse is true of species of the opposite charge. When the sample and BGE have similar conductivities then the peaks are more symmetrical. Neutral species are unaffected. These effects may be observed when a sample containing a range of species with different mobilities is injected. Although such distortions always occur with ions, the consequences may be small compared to other dispersive effects.

12.2.5 Adsorption of analyte onto the capillary wall

Interaction of the analyte with the capillary wall may lead to either peak tailing or complete loss of analyte. Proteins and peptides are frequently adsorbed by electrostatic attraction to the negatively charged capillary wall and by hydrophobic interactions. Adsorption of compounds will affect the zeta potential and hence the EOF, leading to changes in migration times. Thus, injection of biological samples containing protein may result in marked changes in analyte migration times, even if the effect on the analyte is negligible when aqueous standards are injected. It may be necessary to flush the capillary with aqueous sodium hydroxide (e.g. 0.1 mol L^{-1}, 10 min) and then to re-equilibrate with BGE (possibly 10 min) between injections. This can add considerably to the cycle time.

12.2.6 Reproducibility of migration time

A criticism of CE and related techniques is the problem of the reproducibility of migration time. Small changes in EOF have a considerably larger effect on the migration time of late migrating compounds than compounds migrating close to the EOF. For example, from basic electrophoretic equations it has been calculated that when the t_{EOF} in a CE or MEKC system changes from 3.0 to 3.1 min, the migration time of a solute first migrating at 4.0 min will increase to 4.2 min (5 % increase), whilst a compound migrating at 20.0 min will have a migration time of 25.5 min (27.5 % increase) (Hilhorst *et al*., 2001).

Thus, care must be taken to ensure minimal changes in EOF, for example by regular cleaning and conditioning of the capillary and frequently replenishing the BGE to maintain the correct operating pH. The use of dynamically coated capillaries may also be beneficial (Boone *et al*., 2001). However, these procedures alone may not be sufficient if migration times are be used to aid analyte identification in STA, and migration times may be reported relative to an ISTD used as a migration time marker. Better results may be obtained using more than one marker and some integration software packages allow electrophoretograms (and chromatograms) to be scaled to two retention time markers.

The use of mobility or migration time ratios has been proposed in order to obtain more consistent results. The use of markers to calculate migration indices has also been proposed, but this approach is likely to remain ‘in-house’ rather than be adopted universally as retention indexes

have been in GC. Use of corrected migration values, although improving between-laboratory migration time reproducibility in MEKC, had no effect on migration time reproducibility in CE (Boone *et al*., 2002).

Other ways of investigating peak identity include standard addition, i.e. adding an appropriate amount of a known compound and repeating the analysis to see whether the added compound co-migrates with the compound under investigation. Another way is to change the separation conditions. Changing the BGE is relatively simple in CE and this approach is particularly useful for amphoteric substances such as amino acids and peptides. By, for example, increasing the buffer pH, ionization of amines will be suppressed whilst the degree of ionization of acidic groups will be increased. Thus, such changes will result in altered migration times and migration order, which may give valuable information as to the nature of an unknown compound.

The use of detection systems such as DAD and MS, in particular, circumvents some of these problems as identification is not based solely on migration time. Sugimoto *et al*. (2010) used a model of migration time and CE-Time of Flight-(TOF)-MS to predict the migration times of metabolites.

12.3 Sample injection in capillary electrophoresis

In CE samples are introduced via either hydrodynamic, or electrokinetic injection. In either case, too large a volume of sample injected ('plug length') will have a deleterious effect on efficiency, just as in chromatography. The contribution of the plug length to the total variance is:

$$\sigma_{\text{inj}}^2 = \frac{w_{\text{inj}}^2}{12} \tag{12.7}$$

where w_{inj} is the injection plug length. Ideally the variance due to the injection should be less than that due to longitudinal diffusion [Equation (12.5)]. If possible, the injection plug length should not exceed 1–2 % of the length of the column. However, if sensitivity is a problem then it may be necessary to inject a larger volume of sample.

12.3.1 Hydrodynamic injection

Hydrodynamic injection is the most widely used method of sample introduction because it has the advantage that sample composition is unaltered by the injection. A small volume (a few nL) of sample can be introduced into the end of the capillary by hydrostatic pressure, which can be applied via (i) positive pressure at the injection end, (ii) vacuum at the exit end, or (iii) raising the sample vial above the height of the exit vial to cause siphoning. For the first two methods either the sample vial or exit vial needs to be sealed; this is usually achieved with septum caps through which the gas lines can be inserted. If siphoning is used, the difference in the heights of the vials should be 5–10 cm.

The quantity of sample injected (Q) will be influenced by the diameter of the capillary (d), the viscosity of the analysis buffer (η), the length of the capillary (L), and the pressure differential (Δp). For a sample of concentration, c, the quantity injected in t seconds is:

$$Q = \Delta p \frac{\pi d^4}{128 \eta L} ct \tag{12.8}$$

The pressure differential for siphoning is given by:

$$\Delta p = \Delta h g \rho \tag{12.9}$$

where Δh is the difference in height, ρ is the density of the buffer, and g is the gravitational constant. To obtain the highest reproducibility of injection, t should not be too short, yet compatible with not reducing efficiency by injection of too large a sample, and the viscosity of the BGE should be maintained by good temperature control. The viscosity of the sample solution has little effect because of the very small volumes injected.

12.3.2 Electrokinetic injection

By placing the injection-end of the capillary into the sample vial and applying a potential across the capillary (usually 3–5 times less than the running potential), a volume of sample will be drawn into the capillary by the EOF. However, ions will also be drawn in (or excluded) as a result of electrophoretic mobilities. The quantity of an analyte injected is given by:

$$Q = (\mu_{EOF} + \mu_e)\pi r^2 Ect \tag{12.10}$$

where the applied field, $E = V/L$. It is clear from Equation (12.10) that the composition of the fluid injected will differ from that of the original sample, there being an increase in the more mobile cations and a decrease in the concentration of anions, for which μ_e is negative. Furthermore, the composition of the residual sample will be changed – in some circumstances repeat injections from the same sample vial are clearly different, reflecting the changing sample composition with each injection.

12.3.3 Sample 'stacking'

When the conductivity of the sample buffer is less than that of the BGE, the ions migrate faster in the sample zone and become concentrated at the interface with the analysis buffer, a phenomenon commonly referred to as 'stacking'. Stacking is most easily achieved by injecting the sample (either hydrodynamically or electrokinetically) dissolved in water. If this is not feasible and the sample and BGE have similar conductivities, then a few nL of deionized water may be injected before the sample. Greater than 10-fold increases in the amounts injected can be achieved. A potential problem to be considered when using sample stacking is that most of the voltage drop occurs in the stacking zone, resulting in the production of a significant amount of heat with the attendant risk of destroying thermally labile analytes.

12.4 Detection in capillary electrophoresis

Detection systems for CE and related techniques are spectrophotometric (UV and fluorescence), ED, and MS. The low flow rates used in CE make MS detection an obvious choice and several commercial interfaces are available. There are, however, many problems (Section 13.7).

Optical detection systems normally detect analytes on-column. Using silica capillaries allows wavelengths as low as 190 nm to be used. An obvious problem is that the path length is limited to the internal diameter of the capillary unless bubble cell capillaries are used. In these, the capillary i.d. has been increased (approximately three-fold) in the detector region. UV detectors are usually fixed wavelength (a deuterium lamp with interference filters) or diode-array, which are particularly suited to CE because of their relatively small size and rapid response times (Section 5.4.2).

Of the fluorescence detectors, laser-inducted fluorescence (LIF) detectors offer greatly enhanced sensitivity, but the excitation wavelengths available depend on the types of laser

Table 12.1 Examples of gas lasers and appropriate derivatizing reagents

Laser	Excitation (nm)	Derivative
Helium Cadmium	325	Dansyl chloride *o*-Phthaldialdehyde (OPA)
Helium Cadmium	442	Naphthalene-2,3-dicarboxaldehyde (NDA) 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA)
Argon ion	488	Fluorescein isothiocyanate (FITC) 5-(4,6-Dichlorotriazinyl)aminofluorescein (DTAF) 3-(2-Furoyl)quinoline-2-carboxaldehyde (FQ) 7-Nitrobenz-2-oxa-1,3-diazole (NBD)
Argon ion	514	
Helium Neon	543 or 594	
Krypton	568	

employed. Although the wavelength range available is being extended by the introduction of diode-based lasers, it is still often necessary to derivatize the analyte to produce a suitable fluorophore (Table 12.1). In the case of argon-ion lasers, derivatives of fluorescein isothiocyanate (FITC) are suitable, but tertiary amines have to be *N*-dealkylated, for example with 1-chloroethyl chloroformate (CECF) before they can be derivatized (Alnajjar *et al*., 2004). Obviously, secondary and tertiary amines can be reacted with FITC without prior dealkylation.

Synthesis of FITC produces several isomers and the most abundant and the one that is generally used is known as FITC isomer I or 5-FTIC. With FITC derivatization, sensitivities of 50–100 ng L^{-1} for morphine, codeine, and 6-AM can be achieved using a LIF detector with excitation and emission at 488 and 520 nm, respectively (Alnajjar *et al.,* 2004), compared to sensitivities of 250–300 µg L^{-1} attainable using native fluorescence (excitation 245 nm, emission 320 nm). High sensitivity can be obtained using a xenon–mercury lamp in a specially designed fluorescence detector (Caslavska & Thormann, 2004). This approach has the advantage over LIF in that the excitation wavelength is adjustable and compounds can be detected using native fluorescence, thus obviating the need for derivatization.

The small cell volumes that can be achieved with ED makes the use of this detection mode with CE attractive. Rather than develop stand-alone detectors for CE, the trend has been towards separations 'on a chip' with combined electrophoretic separation and ED. The potential for miniaturization and rapid analysis times makes CE-ED on a chip of interest in clinical chemistry and possibly POCT for misused drugs, for example (Phillips, 2018 – see Chapter 17).

12.5 Other capillary electrokinetic modes

Other modes of separation related to CE include CEC, micellar electrokinetic capillary chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP). In CITP two buffer systems are used to create separated zones that move with the same velocity.

12.5.1 Micellar electrokinetic capillary chromatography

The inability of CE to separate neutral molecules has been addressed, in part, by the use of MEKC. Micelles are formed when surfactants (detergents) are added to solutions above their critical micellar concentration (CMC) and it is the interactions between the micelles and the neutral analytes that effects the separation. The surfactants are usually ionized so that the migration can be controlled by the electric field and/or the EOF.

Although negatively charged micelles migrate towards the anode, the EOF is usually strong enough to sweep them towards the detector. Neutral species partition in and out of the micelles and the greater the interaction of the analyte with the micelle, the longer the migration time. When the analyte is not in the micelle it is carried along by the EOF (Figure 12.3).

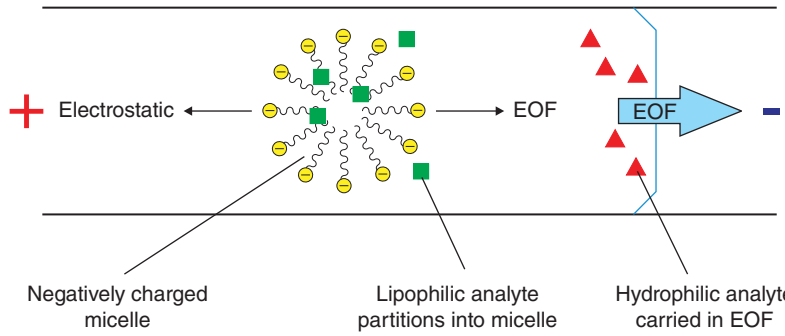

Figure 12.3 Principle of micellar electrokinetic capillary chromatography

If a neutral analyte does not partition into the micelles then it will not be resolved from the EOF, but on the other hand if it is highly bound to the micelles it will migrate with them. For a neutral analyte partitioning into the micelle, the retention factor, k, is:

$$k = \frac{\text{amount of analyte in micelle}}{\text{amount of analyte in buffer}} \tag{12.11}$$

and the migration time t_R is:

$$t_R = \frac{1 + k}{1 + (t_0/t_{mc})\, k} \tag{12.12}$$

where t_0 is the migration time of an analyte that does not interact with the micelle (i.e. the migration time of the EOF) and t_{mc} is the migration time of the micelle. Thus, there is a region (or time window) in the electropherogram, between the EOF and the micelles, in which the analytes must be separated (Figure 12.4).

The high efficiencies obtainable with MEKC result in high peak capacities (Section 7.2.6) hence multi-component mixtures can be analyzed (Figure 12.5). MEKC is also very versatile as cationic, anionic, or neutral surfactants may be used and partitioning can also be adjusted by the inclusion of organic modifiers. However, MEKC separations are, as the name implies, chromatographic and MEKC should not be considered to be orthogonal to chromatography.

12.5.2 Capillary electrochromatography

CEC is a hybrid separation method that uses an electric field rather than pressure to propel the eluent through a packed bed. Because there is little or no back-pressure it is possible to use

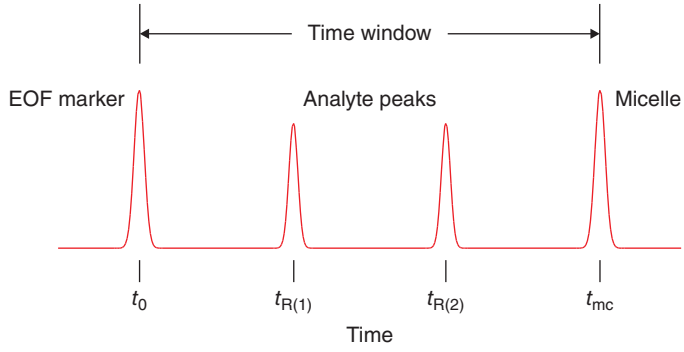

Figure 12.4 Elution time window for non-ionized species in MEKC

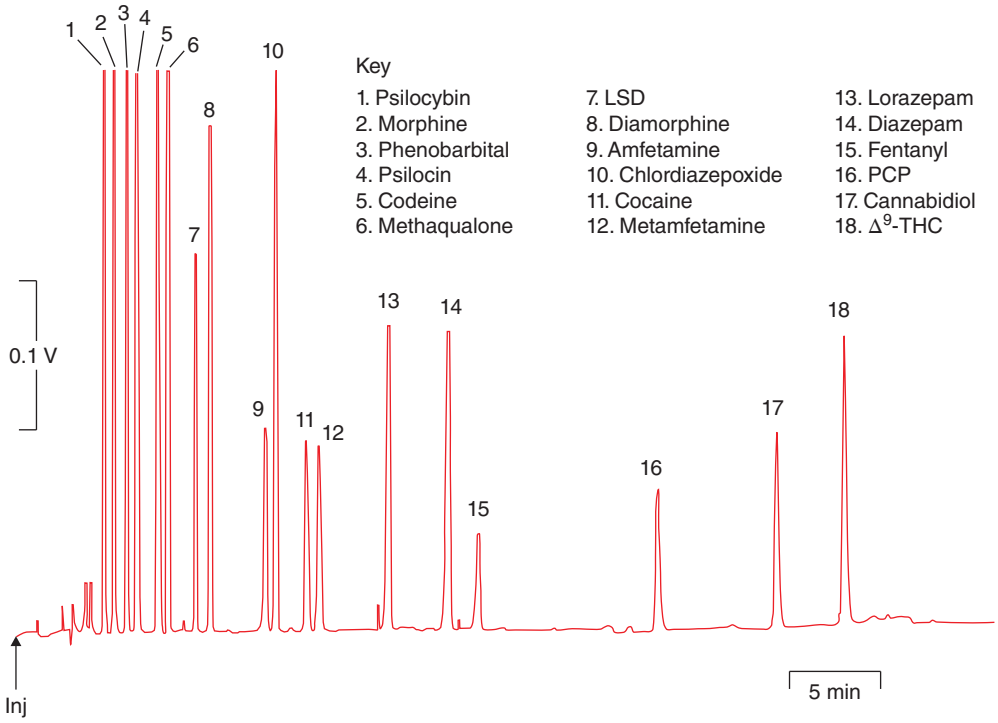

Figure 12.5 Micellar electrokinetic capillary chromatography of some misused drugs. Conditions: fused silica capillary (25 cm × 50 μm i.d.). BGE: 85 mmol L^{-1} SDS, 8.5 mmol L^{-1} phosphate, 8.5 mmol L^{-1} borate:acetonitrile (85+15), pH 8.5. Temperature: 40 °C. Potential 20 kV (redrawn from Weinberger & Lurie, 1991–reproduced with permission from the American Chemical Society)

small diameter particles, which when coupled with the plug-like flow profile, means that very high efficiencies can be attained. The A and C terms in the van Deemter equation (Section 7.2.3) are relatively small in CEC because the constant velocity of eluent between the particles means that the eddy diffusion (A) term is negligible and the ability to use small particles reduces the C term, so that efficiencies similar to those in CE are achievable. Typical particle sizes are 1–3 μm. Particle sizes >0.5 μm and very dilute aqueous buffers (<0.01 mol L^{-1}) should be used.

Capillaries are packed using a high-pressure pump (62 MPa). A retaining frit is made from silica and a slurry of the stationary phase pumped into the capillary. Once packed, a second retaining frit is burnt in place and excess packing removed by reversing the direction of flow. A detection window is formed just beyond the retaining frit. This technique is not easy to learn, but packed capillaries are now available from commercial manufacturers in a range of dimensions and with a range of packings. The problem of bubble formation in the region of the outlet frit, due to degassing of the BGE, has been reduced by pressurizing the column.

As with LC, C_{18}-modified silica has been widely used for CEC. However, in CEC an important contribution to the EOF arises from negatively charged residual silanols on the packing, a contribution that is reduced when using ODS-modified packings. Thus, the EOF is strongly dependent on pH, and this effectively makes rapid separations under acidic conditions difficult. Because of this drawback, there has been interest in many other types of bonded phase for use in CEC, including ion-exchange phases, particularly where a combination of ion-exchange and C_{18}-modified packing, so-called *mixed mode* phases, is used. SCX/alkyl chain mixed mode phases have been shown to produce an EOF that is stable over a wide pH range due to the SCX moieties retaining a negative charge even at low pH. Mixed mode phases have been used with chiral selectors to resolve enantiomers (Section 12.6.1). The EOF can be reversed using SAX-based CEC columns.

12.6 Capillary electrophoretic techniques in analytical toxicology

It was not until the 1990s that a number of techniques based on electrokinetic phenomena, including CE and MEKC, began to be applied to analytical toxicology (Hilhorst *et al*., 2001; Thormann, 2002). However, the application of the technique in this area remains limited despite further publications (Pascali *et al*., 2012).

The role of capillary electrophoretic methods in STA has been reviewed (Boone & Ensing, 2003). The poor reproducibility and low sample capacity, and hence poor sensitivity of current methods, remain major limitations. The high cost of CE instruments and the physical fragility of many systems are further disadvantages. However, orthogonality to chromatography and the requirement for very small sample size remain potential advantages. CE-MS may have a role in the analysis of monoclonal antibodies (mABs) and other antibody-based pharmaceuticals (Haselberg *et al.,* 2018).

12.6.1 Chiral separations

Chiral separations have been described for CE, MEKC, and CEC (Kapnissi-Christodoulou *et al*., 2016; Chankvetadze, 2018; Yu & Quirino, 2019). The approaches adopted parallel those described for LC (Section 10.6) and include the use of chiral additives, chiral surfactants such as bile acids (for MEKC) and, in the case of CEC, either by the use of additives or CSPs.

In CE, neutral chiral selectors have been used with the pH values of the BGE adjusted to ensure that the analyte was ionized, i.e. low pH for bases, high pH for acids. Modified cyclodextrins have been used widely (Saz & Marina, 2016), as have crown ethers, proteins, and other additives used in chiral LC. Baclofen enantiomers have been resolved after reaction with NDA using α-cyclodextrin as chiral selector (Chiang *et al.,* 2001). Naturally occurring and recombinant chicken AAGs (20 μmol L^{-1}) have been used to resolve racemic eperisone, pindolol, and tolperisone; the native protein was preferred, but even then, efficiency was poor with broad, tailing peaks (Matsunaga *et al*., 2003).

Vancomycin and teicoplanin have been assessed as chiral selectors in CE (Bednar *et al*., 2001). Pirkle-type compounds have been used as additives, for example (*R*)-DNBPG has been used to resolve the sulfoxide metabolites of albendazole. An advantage of CE is that, because only small volumes of BGE are required, the amounts of the relatively expensive chiral additives needed are also small.

Methadone enantiomers have been resolved using carboxylmethyl-β-cyclodextrin as chiral selector (Hamidi *et al*., 2017). The method, which was validated over a concentration range of 0.15–5 mg L^{-1}, was applied to exhaled breath condensate (EBC) from patients undergoing methadone maintenance therapy (Figure 12.6). Naghdi & Fakhari (2018) employed maltodextrin to resolve enantiomers of tramadol and methadone. The method was applied to analysis of tablets, and plasma and urine samples to which the drugs had been added.

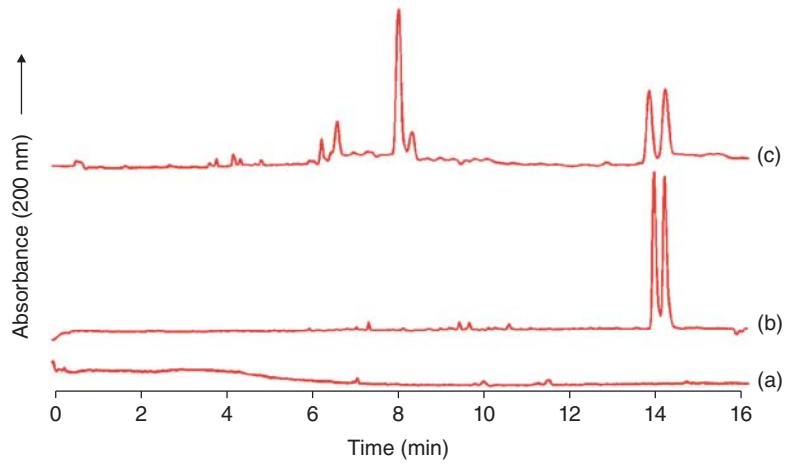

Figure 12.6 Sample electropherograms of (a) blank EBC, (b) an EBC sample spiked with 2.5 mg L^{-1} methadone, and (c) EBC sample of a patient undergoing methadone maintenance therapy (Hamidi *et al.,* 2017–reproduced with permission from the Royal Society of Chemistry)

Enantiomer resolution with CEC can be either by the use of chiral additives or CSPs. Silica-gel based 5 and 3 μm particles coated with cellulose tris(3,5-dimethylphenylcarbamate) have been used with a number of racemates including enilconazole, homatropine, ibuprofen, pindolol, propranolol, verapamil, and warfarin (Otsuka *et al*., 2000). Not all pairs of enantiomers, notably those of propranolol, could be resolved. Vancomycin, incorporated into diol-modified silica as a CSP for CEC columns, has been used to separate enantiomers of venlafaxine and *O*-desmethylvenlafaxine in plasma to which the analytes had been added (Fanali *et al*., 2001).

The synthesis of monolithic CEC columns incorporating chiral functional groups has been described, and more recently chiral monoliths with SCX groups have been developed (Lämmerhofer *et al*., 2000; Preinerstorfer *et al*., 2005). The role of CE, CEC, and MEKC in the resolution of enantiomers of selective serotonin reuptake inhibitors (fluoxetine, citalopram, paroxetine, sertraline); norepinephrine reuptake inhibitors (reboxetine); serotonin and norepinephrine reuptake inhibitors (venlafaxine, milnacipran, duloxetine); and noradrenergic and specific serotonergic antidepressants (mirtazapine) has been reviewed (Mandrioli & Raggi, 2006).

12.7 Summary

Despite the promise of unparalleled efficiency because flow is electrically rather than pressure driven, the practical problems caused by the need to limit and control heat production restrict column diameter, hence sample capacity and thus sensitivity. Moreover, the analytical system is not robust and CEC columns are very difficult to pack reproducibly. Therefore, whilst achieving prominence in specialized areas, the promise of CE remains largely unfulfilled as regards analytical toxicology. In contrast, recent developments in LC-MS especially have had a major impact.

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13 Mass Spectrometry

13.1 Introduction

Mass spectrometry (MS) is concerned with the vapour-phase separation of ionized atomic or molecular species according to their mass-to-charge ratio (*m/z*), where *z* is the charge number (1, 2, etc.). By convention, *m/z* is dimensionless. When an analyte is ionized, a characteristic ion, representing the intact atom or molecule, and/or a group of ions of different masses that represent fragments of the ionized species, are formed. These ions are separated, for example by manipulation of magnetic and/or electrostatic fields in a high vacuum (typically 10^{-5} Pa), and the plot of their relative abundance versus the *m/z* of each ion constitutes a mass spectrum. The mass resolution available is instrument dependent. Ideally, resolution should allow discrimination of molecules with less than 1 atomic mass unit (u) difference. Inductively coupled plasma MS (ICP-MS) is a specialized branch of MS used in the analysis of trace elements and toxic metals and is discussed further in Chapter 21.

MS can give more information about an analyte using less sample than other techniques. Identification of a molecule from its mass spectrum is much easier than with other types of spectral information, especially with the use of modern computerized databases. MS is also the most reliable technique for accurate mass measurement (high resolution MS, HRMS), i.e. mass measurement to four or more decimal places. Disadvantages of MS compared to some other techniques are that the sample taken for analysis is consumed and the investment in capital equipment and operator training are both relatively high, although with modern bench-top instruments and associated software GC-MS operation is now relatively simple and cost effective.

Accurate mass measurement allows the elemental composition of an analyte to be calculated. In addition, molecular fragmentation patterns ('fingerprints') can be used to identify analytes either empirically, or by comparison with published data. Even nominally isobaric compounds can be differentiated if they form different fragments. Alternatively, only ions with a particular *m/z* of interest can be monitored (selected ion monitoring, SIM). Controlled fragmentation, where ions issuing from one mass analyzer and subjected to further fragmentation/separation in subsequent analyzer(s) (MS/MS and MS^n, where n = the number of fragmentation steps), can be used in both qualitative and quantitative work (selected reaction monitoring, SRM).

Direct sample introduction can be performed with relatively simple matrices such as exhaled air (respiratory MS), but complex mixtures such as plasma and urine are best analyzed via 'hyphenated' techniques, notably GC-MS and LC-MS (Box 13.1). Some form of sample preparation is usually needed, although sample handling can sometimes be simplified when compared with that for conventional GC and LC procedures. A fundamental restriction with MS, however,

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

Box 13.1 Mass spectrometry in analytical toxicology

Qualitative work

- GC-MS and LC-MS valuable in STA (analyte identification/metabolite characterization)
  - Fragmentation patterns ('fingerprints')
  - Databases of GC or LC retention data and principal ion abundance or even full reference spectra
  - Accurate mass measurement (instrument dependent)
  - Daily system performance checks are essential

Quantitative work

- GC-MS and LC-MS can give sensitive/selective measurement especially if selected ion monitoring (SIM) and/or controlled fragmentation (MS/MS or MS^n) are used
  - Ideally need stable isotope ISTD (increased assay cost)
  - GC-MS restricted by volatility and thermal stability of analyte or derivative
  - Co-eluting components may interfere in LC-MS especially ('ion suppression', 'ion enhancement')

General disadvantages/restrictions

- May be high capital, running, and operator training costs (instrument dependent)
- Sample consumed
- Achiral

is that it cannot differentiate enantiomers, hence chiral separation by GC, LC, or CE has to be performed prior to MS in order to detect and measure such compounds (Speranza *et al*., 2005).

The first commercial MS (1948) used electron ionization (EI, often incorrectly called electron impact) to ionize volatilized analytes with a beam of electrons from a hot wire filament. A magnetic field (sector) with a mass range up to 300 u was used to separate the resulting ions. At this time, the measurement of ions by time-of-flight (TOF) as a mass analysis technique was also discovered. This is based on the principle that ions with different *m/z* values travel at different speeds in a vacuum when the same accelerating voltage is applied. TOF instruments have assumed new importance as robust instruments for HRMS, usually in association with LC.

In 1953 the quadrupole mass analyzer was patented, although it was work by Finnigan MAT in the 1980s that made the quadrupole ion trap (QIT) MS the versatile instrument it is today. Quadrupole instruments, together with advances in laboratory computers, have led to the relatively affordable 'benchtop' GC-MS systems that are in widespread use. The Orbitrap (OT) mass analyzer was developed for robust HRMS, initially for protein analysis, but is also used for small molecules such as many drugs.

Chromatographic and electrophoretic methods, particularly capillary GC, have proved ideal sample introduction methods for MS because (i) the analyte is presented in the vapour phase and (ii) low eluent flow rates are used. Ionization interfaces such as electrospray ionization (ESI) have been developed for LC that not only cope with the presence of relatively large amounts of eluent, but also permit the ionization of large molecules such as proteins. A major issue though is that the MS is a 'reaction detector', i.e. analyte ionization is dependent on chemical reaction(s) occurring in the ion source. The presence of other substances in the column effluent together with the analyte can either increase (ion enhancement) or decrease (ion suppression) analyte ionization and thus response. In LC-MS matrix effects are a major concern. Other techniques in which the analyte is introduced into the source directly may also suffer from matrix effects.

Matrix-assisted laser desorption ionization (MALDI) employs short pulses of laser light focused on the sample adsorbed on a matrix that absorbs UV light. Other ionization techniques such as surface enhanced laser desorption ionization (SELDI), desorption electrospray ionization (DESI) and direct ionization on silicon (DIOS), coupled with mass spectrometers that are not limited by a maximum *m/z* value (TOF instruments), have also extended the scope of MS. Paper spray ionization is another example of direct coupling to MS.

13.2 Instrumentation

The basic components of a mass spectrometer are an inlet device, an ion source, and an ion selection system (mass analyzer), both under vacuum, a detector (typically some kind of electron multiplier), and a data system. Analyte introduction to the source may be made with a probe inserted via a vacuum interlock, but in this case the only analyte separation that can be achieved is by heating the probe slowly to vaporize lower boiling point analytes first. Moreover, probe injection is not easily automated. Hence, more usually, analytes are introduced after a chromatographic or other separation step.

The ions from the inlet device enter the instrument via a focusing slit. The kinetic energy imparted by motion through an electric field gives the particles inertia dependent on their mass, and the analyzer either steers the particles to a detector on the basis of their *m/z* values by varying an electric and/or magnetic field, or measures the flight time. The smallest ions carrying the highest charge move most rapidly. The analyzer can be set to select a narrow range of *m/z* values, or to scan through a range of values to record the ions present.

There are several types of mass analyzer in addition to magnetic sector and TOF instruments (Box 13.2). Quadrupole mass analyzers and ion traps use electrical fields to selectively stabilize or destabilize ions falling within a narrow range of *m/z* values. Fourier transform ion cyclotron resonance MS (FTICR-MS) measures mass by detecting the image current produced by ions spinning in a magnetic field. The OT mass analyzer is a Fourier Transform mass analyzer analogue of FTICR technology, but it is smaller and easier to operate.

Box 13.2 Mass spectrometry: types of mass analyzers

- Sector or double focusing (magnetic/electrostatic)
  - Often higher resolution than quadrupole instruments
  - Upper *m/z* limit
- Quadrupole
  - Inherently higher sensitivity than sector instruments
  - Link in series to perform controlled fragmentation (MS/MS)
- Quadrupole ion trap (QIT)
  - Perform MS^n on single instrument
- Time-of-flight (TOF)
  - High *m/z* range
  - Suitable for high-resolution detection
- Fourier transform ion cyclotron resonance (FTICR)
- Orbitrap (OT)
  - High *m/z* range
  - Suitable for high-resolution detection

Box 13.3 Mass spectrometry: resolving power and mass error

- *Mass resolution:* there are two definitions in use (http://goldbook.iupac.org/html/R/R05318.html)
  - In the older '10 % valley definition', two peaks of equal height in a mass spectrum at masses m and $m - \Delta m$ can be separated by a valley, which at its lowest point is just 10 % of the height of either peak. For similar peaks at a mass exceeding m, let the height of the valley at its lowest point be more (by any amount) than 10 % of either peak height. Then the resolution (10 % valley definition) is $m/\Delta m$. It is usually a function of m. The ratio $m/\Delta m$ should be given for a number of values of m.
  - In the 'peak width definition' for a single peak made up of singly charged ions at mass m in a mass spectrum, the resolution may be expressed as $m/\Delta m$ where Δm is the width of the peak at a height that is a specified fraction of the maximum peak height. It is recommended that one of three values 50 %, 5 %, or 0.5 % should always be used. For an isolated symmetrical peak recorded with a system that is linear in the range between 5 % and 10 % of the peak, the 5 % peak width definition is technically equivalent to the 10 % valley definition. The common standard is the definition of resolution based upon Δm being 'full width of the peak at half its maximum height' (FWHM)
- *Mass error:* the difference of the exact mass (calculated based on the elemental composition) and the accurate mass (experimentally measured). It is typically given in parts per million (ppm) as the criterion for the accuracy of a measurement

Mass resolution is a measure of the ability to distinguish two peaks of slightly different *m/z* values in a mass spectrum (Box 13.3). Magnetic sector instruments can give resolution of up to 20,000. TOF instruments attain mass resolution of at least 30,000–40,000, whilst modern benchtop Orbitraps provide resolution of about 140,000 and FTICR-MS of about 1,000,000. HRMS should provide a mass resolution of at least 10,000.

13.2.1 Sector instruments

Sector 'double focusing' instruments combine an electrostatic focusing device ('E' sector) and a large electro-magnetic sector ('B' sector). Different manufacturers use different arrangements (Figure 13.1). TOF and OT instruments have largely replaced these systems.

Ions from the source are accelerated by applying an accelerating voltage and enter the electrostatic analyzer ('E sector'). Because of the physical nature of the ionization process, ions of the same *m/z* value are often produced with different energies. If uncorrected, this markedly reduces the resolution and mass accuracy attainable. The electrostatic analyzer focuses ions of the same *m/z* into a more coherent path through to the magnetic sector ('B sector'), which consists of a broad flight tube through a variable magnet that gives an angle of deflection of typically 60–120° to the ions according to Equation (13.1):

$$m/z = B^2 r^2 / 2V \tag{13.1}$$

where B is the magnetic field strength, r is the radius of path through the magnet, and V is the accelerating velocity. Changing the magnetic field strength focuses ions of differing *m/z* at the 'double focusing point'. A typical sweep time for the magnetic field across a mass range of 50–800 u would be 1 s, but faster scan speeds are needed if fast chromatography is used

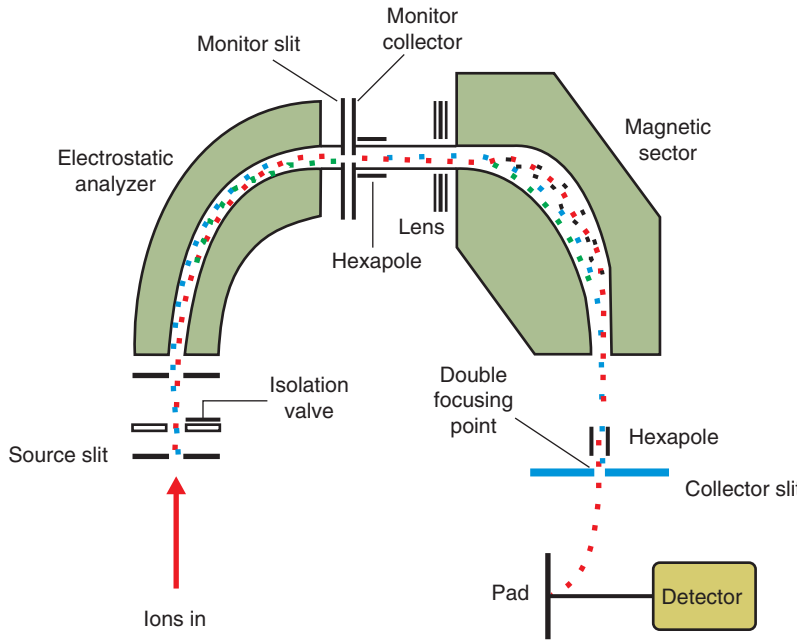


Figure 13.1 Schematic diagram of a double sector mass spectrometer

to introduce analytes to the source. After the ions have been separated, they enter a detector, the amplified output from which is sent to a computer that records the data, and converts the electrical impulses into formats suitable for display.

13.2.2 Quadrupole instruments

As its name implies, a quadrupole mass analyzer consists of four parallel rods (Figure 13.2) that have fixed direct current (DC) and alternating radio frequency (RF) potentials applied to them. Variants of the system use from one to eight devices or rods, but the principle of operation is the same. The mass range and resolution of the instrument is set by the length and diameter of the rods, larger diameter rods giving increased sensitivity, narrower or longer rods increasing the resolving power. In simple terms, ions are focused and passed along the middle of the space between the rods. Their motion depends on the electric fields so that only ions with a given

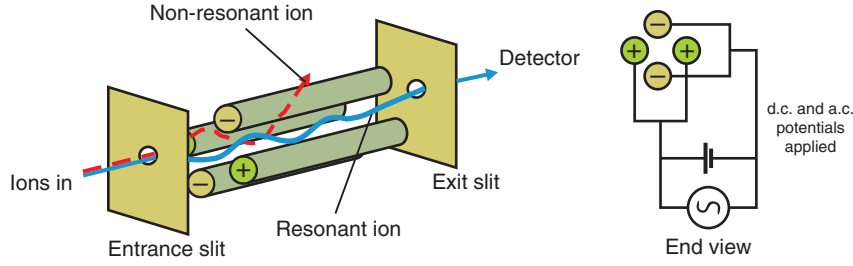


Figure 13.2 Schematic diagram of a quadrupole mass spectrometer

m/z will be in resonance and thus pass through to the detector. All other *m/z* values will be non-resonant and will hit the rods and thus not be detected.

Quadrupole analyzers do not require all the ions that enter the instrument to have the same kinetic energy and hence the sensitivity is inherently higher than in magnetic sector instruments. The RF can be varied to bring ions of different *m/z* into focus on the detector at different times, usually from lower to higher *m/z*, and thus build up a mass spectrum. Alternatively, the applied RF can be used to select only ions with a particular *m/z* value. SIM allows for a longer time to be spent monitoring a single ion, and also for rapid switching between other selected ions. The result is increased sensitivity towards the selected analyte(s) and reduced noise, but of course the overall amount of MS data collected is much reduced as other ions are not detected. Therefore, SIM is used primarily for high sensitivity quantitative analysis of targeted analytes, rather than for analyte identification.

13.2.3 Ion trap quadrupole instruments

The ion trap consists of three hyperbolic electrodes: a central ring electrode, and entrance and exit endcap electrodes (Figure 13.3). The ring electrode RF potential, a constant frequency/variable amplitude AC potential, produces a three-dimensional quadrupolar potential field in the trap. This captures incoming ions in a stable oscillating trajectory. The exact motion of the ions depends on the applied voltage and the individual *m/z* of the ions. The energy of the ions in the trap is quenched by helium at a pressure of 133 Pa so that they focus near the centre of the trap, i.e. their centrifugal energy is reduced. To detect the ions, the potentials are altered gradually to destabilize the ion motion resulting in ejection of the ions through the exit endcap, which also has a DC potential applied to it. The ions are usually ejected in order of increasing *m/z*. This stream of ions is focused onto the detector, an electron- or photo-multiplier tube to produce the mass spectrum.

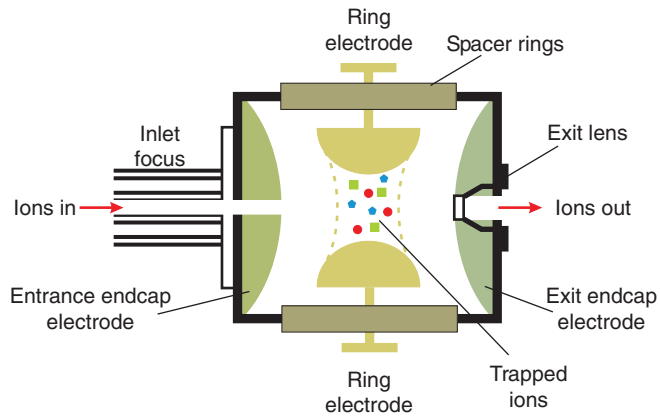


Figure 13.3 Schematic diagram of an ion trap quadrupole mass spectrometer

The distances ions travel in the ion trap are short and therefore the vacuum does not need to be as high to minimize the risk of collisions as in other types of mass spectrometer. However, because the ions are confined, interactions with other ions are more likely than in conventional

quadrupole instruments. Because such ion–ion reactions can generate atypical spectra, in modern ion traps not only is ionization performed outside the trap, but also the number of ions in the trap at any one time is controlled, thus reducing the incidence of ion–ion reactions. A big advantage of ion traps is the possibility of inducing further fragmentation of the fragments produced initially, which can be fragmented and, if necessary, selected ions can be fragmented further. This can be very useful in identifying metabolites, for example, particularly if used with HRMS (Welter *et al*., 2015).

13.2.4 Controlled fragmentation

The power of MS can be increased dramatically by linking mass analyzers in series [multistage MS, MS/MS, mass fragmentography (MF)]. In some instruments, quadrupole analyzers are linked in series. In 'triple quads', for example, three such analyzers are used (Figure 13.4). These instruments usually generate protonated (pseudo) molecular ions by chemical ionization (Section 13.3.1.2). The first quadrupole (Q1) is set to accelerate ions of a selected *m/z* into the second analyzer (Q2, also referred to as the collision cell) where they are fragmented by high energy collisions with helium or with other collision gases such as nitrogen or argon.

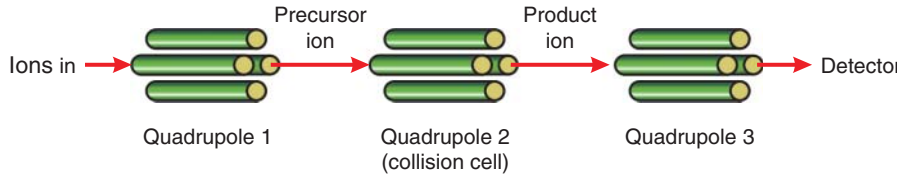


Figure 13.4 Schematic diagram of a triple-quadrupole mass spectrometer (Tandem MS or MS/MS)

The selected ion passed through from Q1 is referred to as the precursor ion, while those formed from the fragmentation of the precursor ion are product ions. In such cases the fragmentation produced is usually relatively reproducible. The third quadrupole (Q3) is used to scan the product ions, or selectively allow one or more such ions through to the detector. The fragmentation can be varied by changing the nature of the collision gas (argon, helium, nitrogen, or xenon), the energy of the ions emitted from Q1, and the temperature and pressure in the collision cell.

As explained above, MS/MS or MS^n may be performed in ion trap instruments. Firstly, all ions are ejected from the trap except for a chosen 'precursor' ion. A voltage is applied to the endcap electrode, 180 degrees from the field generated by the RF on the ring electrode. When the voltage applied to the endcap resonates with the energy of a particular *m/z* value, ions with that value are destabilized and fragment. The amount of energy used can be varied, thus varying the degree of fragmentation.

Once fragmented, the product ions are scanned out of the trap to the detector. The nature of ion trapping/ejection makes a QIT especially suited to MS^n experiments in structure elucidation studies. The isolation and fragmentation steps can be repeated several times, the precise number of repeats only being limited by the trapping efficiency of the instrument. MS^5 experiments are routine, as is the coupling of liquid chromatography to perform LC-MS^n studies.

MS/MS is a very powerful analytical tool (Table 13.1). If the first ion isolated is the molecular ion, the likelihood of interference from other compounds (i.e. from co-eluting peaks if a chromatographic method of sample introduction is used) is greatly reduced. This increases the certainty of analyte identification and reduces dependence on the pre-MS stages of the analysis

Table 13.1 Modes of triple-quadrupole MS (Pitt, 2009)

Mode	Q1	Q3	Applications
Product scan	Fixed	Scanned	Structural studies, identification of unknowns, confirmation by spectral matching with standard
Precursor scan	Scanned	Fixed	Detection of structurally related analytes that produce a common fragment ion
Neutral loss (or gain) scan	Scanned	Scanned with fixed offset relative to first quadrupole	Detection of structurally related analytes that eliminate (or gain) a common neutral molecule on collision
Multiple reaction monitoring (MRM)	Fixed	Fixed	High sensitivity detection of a panel of targeted analytes

to impart selectivity to the assay. MS^n methods also enhance the ability to elucidate molecular structures. Rather than seeing the total spectrum formed from the fragmentation of a molecule, individual ions can be isolated and their fragmentation evaluated. However, because the MS/MS spectra produced are almost always different from GC-EI spectra, established GC-MS spectral libraries are of little use and thus reference libraries are needed that have been produced under standardized ionization conditions.

13.2.5 Quadrupole ion trap

The SCIEX Triple Quadrupole Linear Ion Trap (the QTRAP™) MS systems are based on the capabilities of SCIEX Triple Quad™ systems, but with enhanced scan functions to enable simultaneous analyte identification and quantitation. QTraps are hybrid systems in which a linear ion trap is substituted for Q3 of a conventional triple quadrupole. Thus, ions emerging from Q2 are trapped in Q3 [Figure 13.5(a)] and can then be scanned on leaving Q3 [Figure 13.5(b)]. Alternatively, trapped ions can be fragmented and the fragments scanned.

13.2.6 Time-of-flight instruments

In TOF-MS, the analyzer is a vacuum chamber. Molecules are ionized in the source and accelerated by an electric field into the analyzer. The ions drift through the analyzer with the kinetic energy obtained from the potential energy of the electric field. Provided that all the ions attain the same kinetic energy, the ions of lower m/z will have greater velocity than ions of greater m/z. A microchannel plate detector is positioned at the end of the analyzer to measure the arrival time of the ions. Ions of lower m/z arrive first, followed by ions of greater m/z. Commercial instruments often incorporate an ion reflector to increase the effective length of the drift tube (Figure 13.6). The mass resolution of modern TOF instruments should be at least 30,000–40,000.

TOF analyzers are usually encountered in systems in which Q3 in a triple quadrupole is substituted by a TOF mass analyzer (QTOF, Figure 13.6). In some instruments the quadrupoles are replaced by hexapoles (Chernushevich *et al*., 2001). The instruments can be used in either single

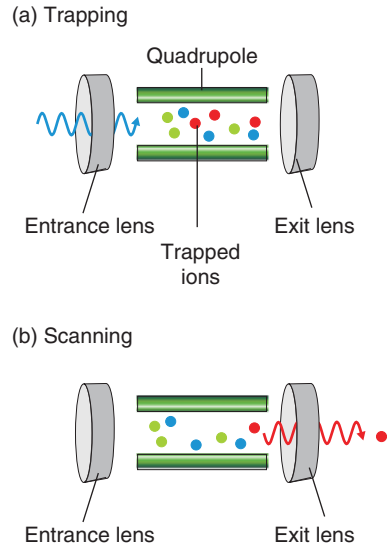


Figure 13.5 Schematic diagram of Q3 in a QTrap system. (a) Quadrupole and exit lens set to trap ions, (b) entrance lens set to prevent entry of further ions and exit lens adjusted to scan the trapped ions

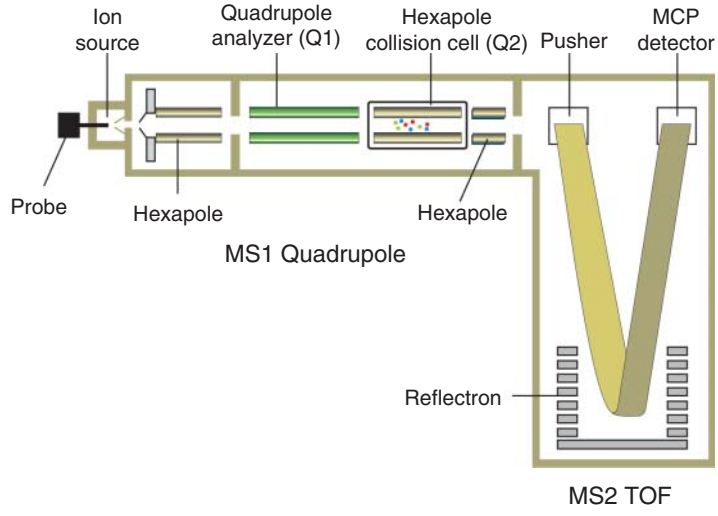


Figure 13.6 Schematic diagram of a common time-of-flight tandem MS (QTOF) (reprinted from van Bocxlaer *et al*., 2005–reproduced with permission from Elsevier)

MS, or tandem MS (MS/MS) modes. In single MS acquisition, Q1 is used in wide band pass mode to allow all the ions into the TOF, which acts as a mass analyzer. In MS/MS acquisitions, a precursor ion is selected in Q1, fragmented in Q2 by collision-induced dissociation (CID), and the fragment ions analyzed by the TOF producing full scan spectra. Any ion can be selected for generating ion chromatograms, or the full spectrum can be used for substance identification by searching suitable MS/MS libraries (van Bocxlaer *et al*., 2005).

13.2.7 Ion cyclotron resonance

FTICR-MS is analogous to Fourier transform nuclear magnetic resonance (FT-NMR) spectroscopy. As the ions pass close to two opposed electrodes they induce a small alternating current in the circuit encompassing the electrodes. The amplitude and frequency of this current is proportional to the number of circulating ions and the cyclotron frequency of the ions, respectively. When the circulating ions are excited by a RF pulse of radiation, the decay in the induced current is converted into a mass spectrum using Fourier transformation. This technique provides extremely high resolution and mass accuracy.

13.2.8 Orbitrap mass analyzer

The OT is an ion trap mass analyzer consisting of two outer electrodes and a central electrode, which enable it to act as both an analyzer and detector (Figure 13.7). Ions entering the OT are captured through 'electrodynamic squeezing', after which they oscillate around the central electrode and in between the two outer electrodes. Different ions oscillate at different frequencies, resulting in separation. By measuring the oscillation frequencies induced by ions on the outer electrodes, the mass spectra of the ions are acquired using image current detection. The mass resolution of OT analyzers is about 100,000–250,000. However, the resolving power decreases proportionately as mass increases and is inversely proportional to scan speed. Therefore, a compromise must be found between resolution and the number of scan points needed for better reproducibility, particularly with fast chromatography.

As with TOF analyzers, OTs are used mostly in tandem MS instruments (Thermo-Fisher Q-Exactive) coupled sometimes to GC, but mainly to LC. They are also hybrids in which Q3 is

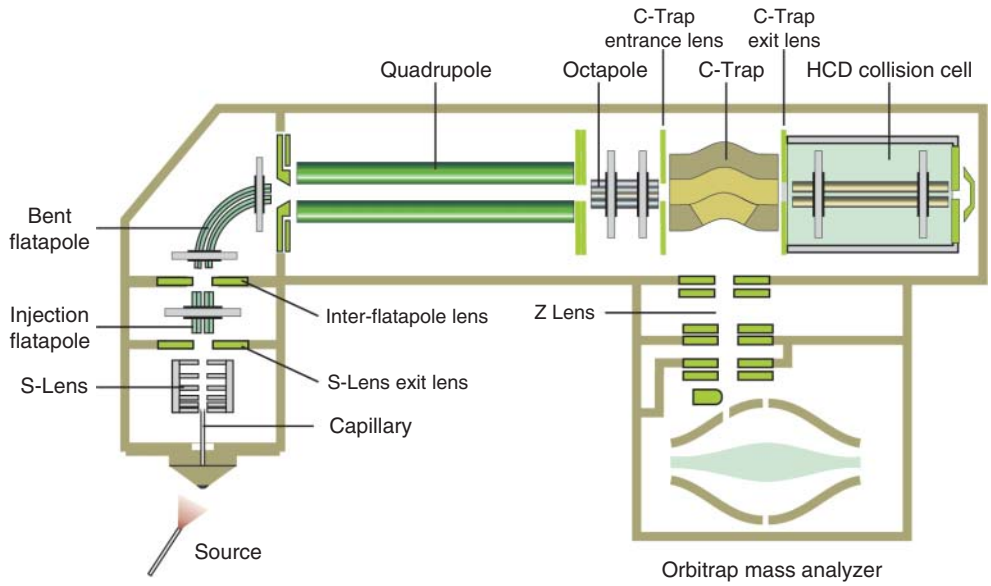


Figure 13.7 Schematic diagram of an orbitrap tandem mass spectrometer (Q-Exactive) (adapted from Michalski *et al*., 2011–http://www.asbmb.org/Page.aspx?id=47586. Licensed under CC-BY 4.0 https://creativecommons.org/licenses/by/4.0/)

substituted by an OT analyzer (Figure 13.7). It includes an atmospheric pressure ion source, a stacked-ring ion guide (S-lens) in the source region, a quadrupole mass filter, a C-trap, a high energy collision dissociation (HCD) cell, and an OT mass analyzer (Michalski *et al*., 2011). Ions formed by an ESI or nanoelectrospray ion source pass through a transfer tube to the S-lens followed by a multipole into a bent flatapole, which is open for clusters and droplets to fly unimpeded out of the flatapole. After collisional cooling, ions are transmitted via a lens into a hyperbolic quadrupole, capable of isolating ions down to an isolation width of 0.4 u at *m/z* 400.

Exit and spilt lenses gate the ion beam through a short octapole into the C-trap interfaced to a gas-filled HCD cell separated by a single diaphragm for HCD tuning. The HCD cell fragments the ions by adjusting the offset of the RF rods and the axial field to provide the required collision energy. All fragments remain trapped if this offset is maintained at a negative polarity relative to the C-trap and the HCD exit lenses. Thus, multiple precursor ions can be introduced and fragmented at optimum collision energy without compromising the storage of preceding injections. The whole ion population transferred back into the C-trap are ejected into the OT analyzer and analyzed in a single detection cycle. This arrangement gives the ability to acquire data from several precursor ions in the same scan without compromising the signal-to-noise ratio.

13.3 Gas chromatography-mass spectrometry

The results obtained in MS are fundamentally dependent on the way in which the analyte is introduced into the ionization source. Capillary GC, which provides the analyte in the vapour phase in relatively pure form in an inert gas is almost the ideal MS sample introduction because matrix effects, if any, are usually minimal. Modern vacuum systems can easily cope with the relatively low eluent flow rates used. The end of the column is simply passed through a heated transfer line to terminate at the entrance to the ion source.

13.3.1 Analyte ionization in gas chromatography-mass spectrometry

In GC-MS, both electron ionization (EI) and chemical ionization are used routinely in the analysis of relatively low M_r, volatile, thermally stable organic compounds. Both allow the same basic instrument and ionization source design. A range of other desorption ionization techniques (ESI, etc.) are available and are discussed in Section 13.5. EI produces positively charged ions via removal of an electron, whilst chemical ionization can lead to the production of either positively-, or negatively-charged species (Box 13.4). These techniques are in effect limited to the analysis of compounds up to M_r 1000 or so, although for GC, the practical mass limit is of the order of 750 u.

13.3.1.1 Electron ionization

Either the vaporized analyte, or its decomposition products if probe injection of a thermally labile analyte has been attempted, first pass into an EI chamber (Figure 13.8). Here interactions occur with a homogeneous beam of electrons produced from an electrically heated filament (a rhenium or tungsten wire). Typically, the electrons have an energy of 70 eV, much higher than the strength of the bonds within a typical analyte (*ca*. 10–20 eV). The use of 70 eV helps ensure that the resulting spectra are reproducible from instrument to instrument. The beam is directed across the source chamber to ground. A fixed magnet is placed, with opposite poles slightly off-axis, across the chamber to create a spiral beam to increase the chance of interactions between the

Box 13.4 Modes of ionization in gas chromatography-mass spectrometry

Electron ionization (EI)
- 'Hard ionization'
- Positively charged ions, may include molecular ion ($M^{+\cdot}$, a radical cation)
- Fragmentation pattern (fingerprint) often produced

Chemical ionization
- 'Soft ionization'
- Requires 'reagent gas' (usually ammonia, isobutane, or methane) in the ion chamber
- Protonated molecular ion (MH^+) usually produced in positive ion mode, with little fragmentation
- Negative ion mode may yield intact molecular anions with little fragmentation, but only applicable to electron-deficient analytes (e.g. halogen-containing analytes and/or derivatives)

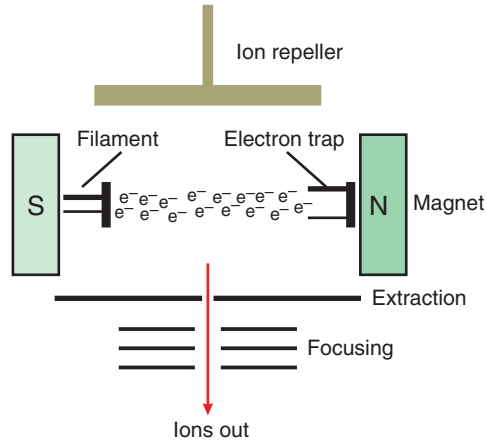


Figure 13.8 Schematic side-view of an electron ionization source

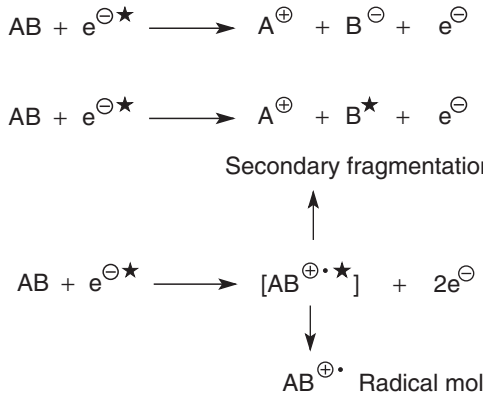
electrons and the vaporized analyte – even then typically only 0.1 % of the analyte is ionized. There are no actual collisions between either analyte molecules, or fragments and electrons.

The pathway leading to a charged molecule is initiated by either loss of one electron from the analyte, or by analyte decomposition. A molecule with one electron missing is called the *molecular ion* and is represented by $M^{+\cdot}$ (a radical cation). The peak resulting in the mass spectrum from this ion gives the molar mass of the analyte. The ions are expelled from the source by application of a potential to a repeller electrode, which is maintained at the same charge as the ions.

Because a large amount of energy is imparted to the molecular ion it often fragments producing smaller ions with characteristic *m/z* values and typical relative abundances, the plot of which provides the fingerprint for that molecular structure. This information may be used to identify compounds of interest even in the absence of the molecular ion, and to help elucidate the structure of unknown components of mixtures. However, in order to minimize fragmentation of labile analytes yet still allow use of EI spectral databases, reduction of the internal energy

of the analytes by vibrational cooling based on the implementation of a supersonic molecular beam prior to the ionization step ('cold spray EI') has been developed.

With an analyte AB, two processes that might occur are the direct result of energy transfer to the analyte, causing primary fragmentation [Reactions (13.1) and (13.2)]. This is the second cause (after thermal decomposition) of the presence of fragment ions in the spectrum. The third process is electron ejection from the analyte to create the energized radical ion. This can then lose energy either through 'ion cooling' and stabilize to give the radical molecular ion, or by further fragmentation [Reaction (13.3)]. Some instruments allow the ionization voltage to be adjusted, a lower voltage giving less ionization and less fragmentation, which can be useful in analyte identification. Be this as it may, the relatively high degree of analyte fragmentation in EI spectra often results in the technique being termed 'hard' ionization.



13.3.1.2 Chemical ionization

In positive ion chemical ionization (PCI), there is a reagent gas (usually ammonia, isobutane, or methane) in the ion chamber as well as the analyte, and analyte ionization is by reaction with ionized reagent gas rather than by direct interaction with the electron beam. Reagent gas is continually introduced at a concentration sufficient to allow the desired reactions to proceed, but the vacuum is not as high as that usually employed in EI, thereby increasing the likelihood that an analyte molecule will collide with ionized reagent gas. Analyte ionization is most commonly the result of transfer of a proton from the ionized reagent gas to the analyte. A small positive potential on the repeller electrode is used to repel the ions from the ionization chamber.

In PCI, although the initial reaction with the reagent gas is a high energy process, ionization is caused by proton transfer and it is therefore a less energetic process than EI. Because less residual energy is possessed by the protonated molecules, fragmentation is greatly reduced thus giving less information about the detailed structure of a molecule. However, it generally produces a protonated molecular ion (MH^+) so the M_r of the analyte can be obtained, which is not always the case when using EI. Furthermore, MH^+ is usually a suitable ion for SIM and hence quantitative analysis. However, PCI still requires the analyte to be present in the vapour phase, so thermal degradation can lead to varying degrees of analyte fragmentation. On the other hand, PCI is generally a much 'softer' ionization method than EI, and until the development of desorption ionization methods, was the preferred way to analyze small, polar analytes such as many drugs and drug metabolites, particular those with longer side chains (e.g. amfetamine derivatives), which typically do not form molecular ions in EI.

Ion formation in PCI with methane as the reagent gas is illustrated in Reactions (13.4)–(13.6). In Reaction (13.4), methane is ionized by an electron beam in the same way as with EI. There may be several further reactions [Reactions (13.5) and (13.6)]. Reaction (13.7) shows ionized reagent gas reacting with unionized gas to form the carbanion (protonated methane). This step requires the reagent gas to be at a critical pressure. If the pressure is too low, there will be no analyte ionization. Reaction (13.8) shows proton transfer from the carbanion to the analyte M to form protonated analyte (MH^+). If the pressure of the reagent gas is too high, then secondary reactions [Reactions (13.9) and (13.10)] may also occur, leading to formation of analyte adduct ions – these are seen as M+15 and M+29 *m/z* peaks in the spectrum (i.e. 14 and 28 *m/z* higher than the MH^+).

$$CH_4 + e^{\ominus} \longrightarrow CH_4^{\oplus\bullet} + 2e^{\ominus} \qquad \text{(Reaction 13.4)}$$

$$CH_4^{\ominus\bullet} \longrightarrow CH_3^{\oplus\bullet} + H^{\oplus} \qquad \text{(Reaction 13.5)}$$

$$CH_3^{\oplus} + CH_4 \longrightarrow C_2H_5^{\oplus} + H_2 \qquad \text{(Reaction 13.6)}$$

$$CH_4^{\oplus\bullet} + CH_4 \longrightarrow CH_5^{\oplus} + CH_3^{\bullet} \qquad \text{(Reaction 13.7)}$$

$$M + CH_5^{\oplus} \longrightarrow CH_4 + MH^{\oplus} \qquad \text{(Reaction 13.8)}$$

$$M + CH_3^{\oplus} \longrightarrow MCH_3^{\oplus} \qquad \text{(Reaction 13.9)}$$

$$M + C_2H_5^{\oplus} \longrightarrow MC_2H_5^{\oplus} \qquad \text{(Reaction 13.10)}$$

Ammonia is also a commonly used reagent gas and ionizes as shown in Reactions (13.11) and (13.12). However, subsequent reactions are generally dependent on the nature of the analyte. Amine-containing molecules often react as shown in Reaction (13.13), whilst non-basic polar molecules usually form adducts. Non-polar analytes do not usually ionize, and thus the use of ammonia as reagent gas confers a degree of selectivity on the ionization process. Reagent gases may also sometimes be combined to optimize ionization of a given analyte.

$$NH_3 + e^{\ominus} \longrightarrow NH_3^{\oplus\bullet} + 2e^{\ominus} \qquad \text{(Reaction 13.11)}$$

$$NH_3^{\oplus\bullet} + NH_3 \longrightarrow NH_4^{\oplus} + NH_2^{\bullet} \qquad \text{(Reaction 13.12)}$$

$$NH_4^{\oplus} + RNH_2 \longrightarrow RNH_3^{\oplus} + NH_3 \qquad \text{(Reaction 13.13)}$$

Negative ion chemical ionization (NCI) involves capture of a relatively low-energy electron such as those resulting from the ionization of methane [Reaction (13.4)] to generate a negatively charged ion. This process can yield intact molecular anions that are readily detected. Because the energy of the initiating electrons is low, high electron affinity analytes, i.e. molecules containing a halogen atom, or a nitro- or carboxyl-moiety, are the best candidates for this work. In essence, NCI-MS is a form of ECD and use of electron-capturing derivatives may be

advantageous (Section 9.6). Many biological molecules are not electron deficient and therefore are not ionized by this method. Hence the technique is often 100–1000 times more sensitive than PCI (Schwaninger *et al.,* 2011).

13.3.2 Gas chromatography-combustion-isotope ratio mass spectrometry

GC-combustion-isotope ratio MS (GC-C-IRMS) is a specialized technique that is used to measure the relative ratio of stable isotopes of carbon ($^{13}C/^{12}C$), hydrogen ($^{2}H/^{1}H$), nitrogen ($^{15}N/^{14}N$), or oxygen ($^{18}O/^{16}O$) in compounds separated from complex mixtures. The ratio of these isotopes may vary depending on the source of the compound, which may enable identification of, for example, use of exogenous testosterone in the presence of endogenous testosterone (Section 22.4.2). The primary prerequisite for GC-C-IRMS is that the compounds of interest are amenable to GC, i.e. that they are volatile and thermally stable. Polar compounds may require derivatization, in which case the relative stable isotope ratio of the derivatization agent must also be measured.

In GC-C-IRMS, carbon- and nitrogen-containing compounds eluting from the column pass through a combustion chamber (an alumina tube containing Cu, Ni, and Pt wires maintained at 940 °C). This is followed by a reduction reactor (an alumina tube containing three Cu wires maintained at 600 °C) to reduce any nitrogen oxides to nitrogen (Figure 13.9). For hydrogen and oxygen isotope ratio measurements a different thermal conversion reactor is required. Water, which would otherwise interfere, is removed in a water separator by passing the gas stream through a tube constructed from a water permeable Nafion membrane with a counter-current flow of helium. The sample is then introduced into the ion source of the MS by an open split interface.

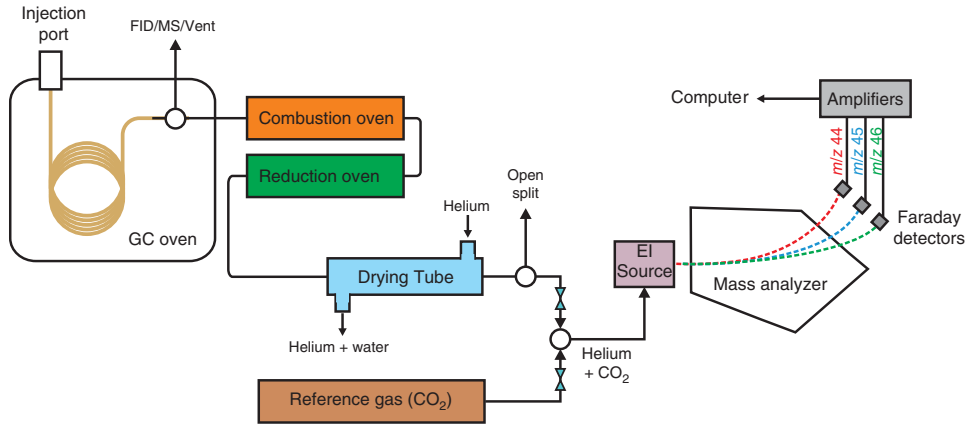


Figure 13.9 Schematic diagram of a typical GC-C-IRMS Instrument: configured to detect isotopes of carbon

Ionization of the analyte gases (CO_2, H_2, N_2, or CO) is achieved using EI. The ionized gases are separated in a single magnetic sector analyzer, the output from which is used to calculate the stable isotope ratio. Measuring the *absolute* abundance of a particular isotope is not a simple task and requires complex instrumentation, hence values are calculated relative to a standard of known isotopic composition (the reference gas of Figure 13.9). The result is usually expressed

as parts per thousand (‰) and is calculated from: [(Isotopic ratio in sample/Isotopic ratio in reference) – 1] × 1000 and given the symbol $\delta^{13}C$ in the case of ^{13}C, for example.

GC-C-IRMS is widely applied not only in doping control, but also in archaeology and biology research, and in foodstuffs analysis. For example, using the technique Janssens *et al*. (2016) have demonstrated progesterone administration to cattle, and van Leeuwen *et al*. (2018) could differentiate wood-derived vanillin from synthetic vanillin in distillates.

13.4 Liquid chromatography-mass spectrometry

The primary advantage of LC-MS over GC-MS is that it is capable of analysing a much wider range of compounds. Polar and thermally labile/high M_r analytes may all be chromatographed using LC-MS without derivatization. A simple syringe pump may be used to deliver a solution of the analyte if LC is thought unnecessary, although it is usual to use at least a short column. Selectivity, and hence sensitivity, is often increased over LC-UV, with the result that sample volumes may be reduced and sample preparation simplified. LC analysis times, particularly when using short columns with sub-2 μm packings, may also be shortened, giving reduced eluent consumption and increased sample throughput (Box 13.5).

Analytes eluting from the LC column have to be introduced to the MS via a specialized interface designed to cope with the presence of the eluent. However, the presence of co-eluting sample components may impair ionization of the analyte and/or any ISTD thus giving false results. To test for signal alterations, analyte can be infused continuously post-column, provided that there is an adequate stock, to provide a background signal against which the effects of various sample preparation methods can be assessed, ion suppression or ion enhancement showing as changes in the baseline (Figure 13.10).

Box 13.5 Some advantages and potential disadvantages of LC-MS

Advantages
- Wide range of analytes
  - Not limited by sample volatility or thermal stability
- High selectivity/sensitivity
  - May require less sample than with conventional LC
- Wide linear range
- Reduced sample preparation
- Use of short columns/sub-2 μm particles and gradient elution gives efficient separations, short analysis times, and reduced eluent consumption
- High flexibility

Potential disadvantages
- High capital cost
- Possibility of ion suppression or ion enhancement
- Possibility of interference from co-eluting compounds
- A unified ionization mode/conditions necessary to construct spectral libraries

13.4.1 Analyte ionization in liquid chromatography-mass spectrometry

The interfaces used most commonly for LC-MS are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) (Box 13.6). They are used with the same type of

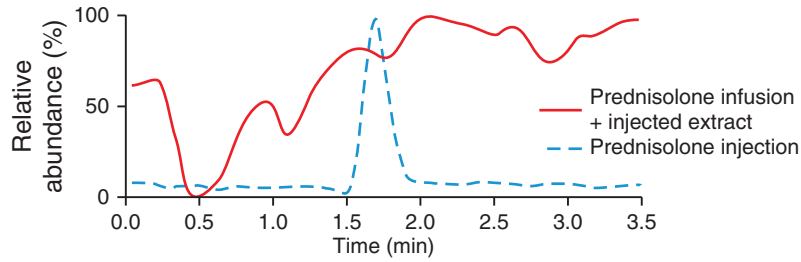


Figure 13.10 Ion suppression. Response after injection of (i) an extract of a prednisolone-free sample with a continuous prednisolone infusion post-column, and (ii) prednisolone injected in LC eluent (adapted from Adaway *et al*., 2015–reproduced with permission from Sage Publications)

Box 13.6 Some modes of ionization in liquid chromatography-mass spectrometry

Electrospray ionization (ESI)
- Employs the same interface as APCI except that the ionization source effluent spray needle is maintained at + or – 2.5–4 kV against a counter electrode
- Singly or multiply charged analyte molecules are repelled towards a sampling cone – solvent evaporation is assisted by nebulizer gas
- The extent of fragmentation depends on the collision energy
- The collision energy should be optimized for different applications and standardized for library construction

Ionspray ionization (ISI)
- As with ESI except that no external source of ionization is used – limited to flow rates of a few μL min^{-1}

Atmospheric pressure chemical ionization (APCI)
- Analogous to PCI in GC except that:
  - Corona discharge used as the ionization source
  - Nebulizer gas and vaporized solvent molecules are used instead of reagent gas
- Molecular species and adduct ions result, with minimal fragmentation

Atmospheric pressure photoionization (APPI)
- A gas discharge lamp generates vacuum-UV photons that ionize nebulized effluent molecules
- Dopant (e.g. acetone, anisole) added to the auxiliary or sheath gas may aid indirect ionization

source. In general, eluent flow rates of up to 1 mL min^{-1} can be used, although if an eluent with a relatively high water content is employed then a lower flow rate may be necessary. In addition, non-volatile salts and additives tend to deposit on the sampling cone resulting in a rapid decrease in instrument sensitivity. Thus, appropriate sample pretreatment procedures and volatile eluents are required. Acetic, formic, and trifluoroacetic acids may be used for acidic eluents and volatile (i.e. those that sublime) ammonium salts (ammonium acetate, carbonate, formate) for higher pH eluents. Additives, such as those used for ion-pairing in LC and surfactants in MEKC, can cause problems and are best avoided if possible.

All the modes of ionization used with LC are ‘soft’ methods, i.e. there is at present no true equivalent to EI in GC-MS. This is a disadvantage in analytical toxicology because spectral libraries are largely based on EI spectra, which benefit from the fragmentation that occurs

commonly with this ionization mode at 70 eV. However, the use of controlled fragmentation techniques compensates for this and allows the development of reproducible reference spectra (Maurer *et al*., 2018; Maurer *et al*., 2019).

13.4.1.1 Electrospray and ionspray ionization

In these methods, sprayed multicharged droplets are produced by high voltage charging of a nebulizer. When the droplets are vaporized at atmospheric pressure, analyte ions are ejected. Because little energy is required to produce analyte ions, hardly any fragment ions are produced. In ISI, gas pressure is normally used as the supplemental power for nebulization. The useful eluent flow rate is limited: electrospray can introduce up to 1 mL min^{-1}, whilst ionspray can only deal with a few µL min^{-1}. Therefore, an effluent splitter is usually required with ISI.

In ESI, the analyte solution flow passes through the electrospray needle maintained at a high potential with respect to a counter electrode (typically 2.5–4 kV). Charged droplets are sprayed from the needle with a surface charge of the same polarity as the charge on the needle. Droplet formation is assisted by a flow of nebulizer gas, usually nitrogen. The droplets are repelled from the needle towards the source sampling cone on the counter electrode. As the droplets traverse the space between the needle tip and the cone, solvent evaporation occurs assisted by the flow of nebulizer gas (Figures 13.11 and 13.12).

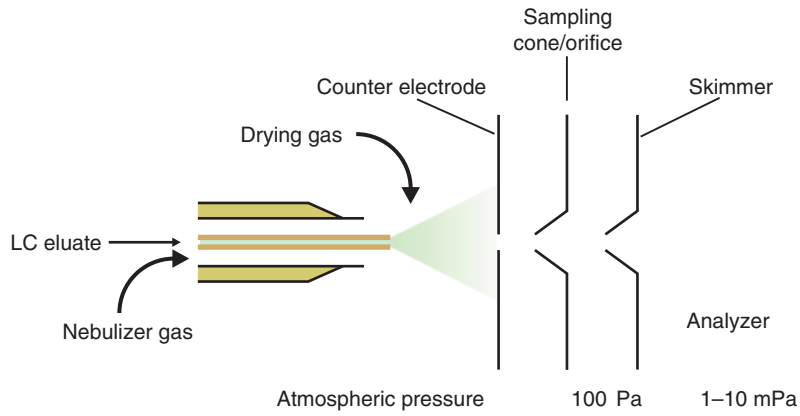


Figure 13.11 Schematic of an electrospray ionization interface

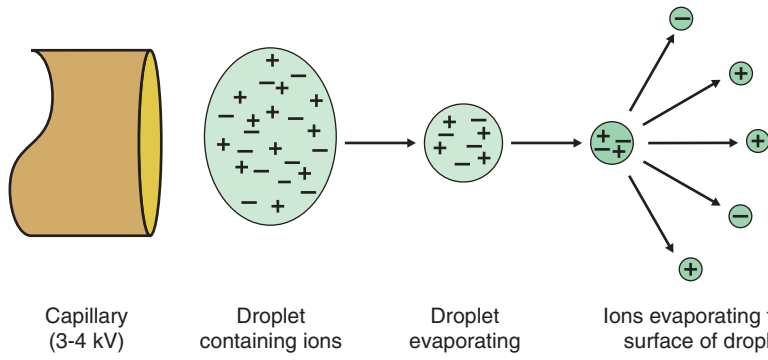


Figure 13.12 Electrospray ionization: schematic of the mechanism of ion formation

As the solvent evaporates, the droplets shrink until they reach the point when surface tension can no longer sustain the charge (Rayleigh limit) at which point the droplets disintegrate, producing smaller droplets that can disintegrate further as well as charged analyte molecules. This process is charge dependent and an electrolyte must be present in the eluent at a concentration of at least 50 μmol L^{-1} to promote ESI. Impurities found in lesser-grade reagents such as methanol can have significant effects on ionization in ESI (Annesley, 2007) and should be evaluated during method development and validation.

This is a very soft ionization method as very little residual energy is retained by the analytes. The charged analyte molecules (they are not strictly ions) can be singly or multiply charged. The generation of multiply charged molecules enables high M_r compounds such as proteins to be analyzed because *m/z* is measured rather than mass *per se*. The major disadvantage of the technique is that there is very little (usually no) analyte fragmentation, although this may be overcome by using either in-source fragmentation, or controlled fragmentation in a collision cell (Q2 in MS/MS) (Section 13.2.4).

13.4.1.2 Atmospheric pressure chemical ionization

APCI is analogous to PCI except that, as its name implies, the ionization occurs at atmospheric pressure. The primary applications of APCI are in the analysis of low mass, thermally stable compounds such as many xenobiotics. The interface consists of nebulizer, vaporizer, ionization source, exhaust, and electrical source control (Figure 13.13), and is the same as that used in ESI except for the introduction of a corona discharge needle.

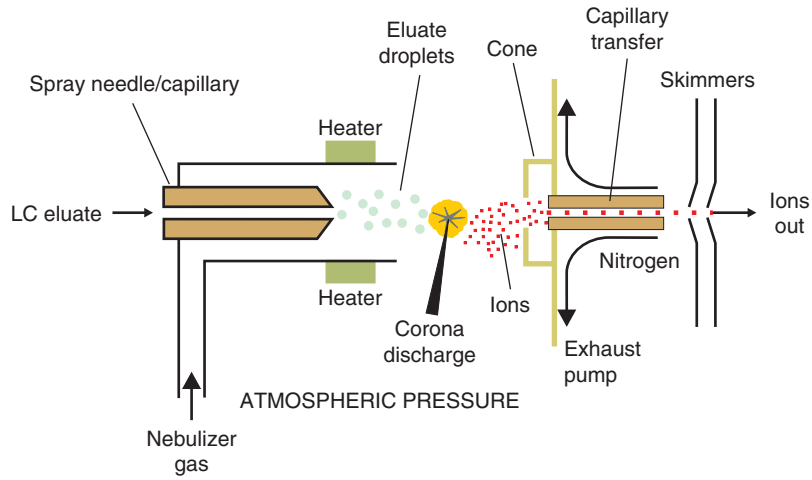


Figure 13.13 Schematic of the components of an atmospheric pressure chemical ionization source

The analyte solution (normally LC eluate) is introduced into a pneumatic nebulizer and desolvated in a heated quartz tube. The vaporized solutes reach a needle electrode and a high-voltage corona discharge (*ca.* 10 kV) ionizes eluent molecules. The corona discharge replaces the electron filament used in PCI and produces primary N_2^+ and N_4^+ ions by EI. These primary ions collide with the vaporized solvent molecules to form secondary reactant ions such as H_3O^+ and $(H_2O)_nH^+$ (Figure 13.14). These secondary reactant ions undergo repeated collisions with the analyte resulting in the formation of analyte ions. An advantage of APCI

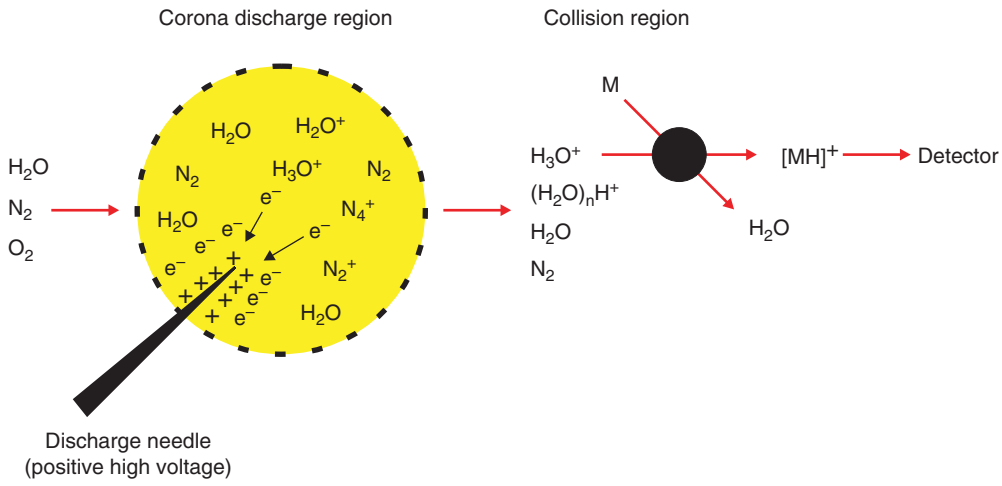


Figure 13.14 Atmospheric pressure chemical ionization: explanation of corona discharge (plasma) region and collision regions

over ESI is that the risk of either ion suppression, or ion enhancement is lower (Remane *et al.*, 2010a; 2010b).

The high frequency of collisions results in a high ionization efficiency and vaporization of the analyte ions. Once the ions are formed, they enter the pumping and focusing stage in much the same way as in ESI, although sensitivity tends to be less. Molecular species and adduct ions predominate in the spectra with little or no fragmentation. In order to collimate ions, send them effectively into the second small hole and then dissociate the cluster ions, the drift voltage ranging from 50 to 200 V is charged between the first and the second small holes. If this voltage is too high, the collision energy is increased and fragmentation is likely.

13.4.1.3 Atmospheric pressure photoionization

APPI is a relatively new ionization technique for LC-MS. The APPI source is based on a gas discharge lamp that generates vacuum-UV photons of 10–10.6 eV energy. The energy of the photons is normally greater than a first ionization potential (IP) of an analyte because many organic compounds have IPs in the range 7–10 eV. On the other hand, the IPs of the most common LC eluent solvents have higher values (water, IP = 12.6 eV; methanol, IP = 10.8 eV; acetonitrile, IP = 12.2 eV). This provides ionization of many analytes with lower IPs without interference from the eluent solvent. An example of an APPI interface is shown in Figure 13.15 (Robb *et al.*, 2000). Further enhancement of the ionization efficiency can sometimes be achieved by adding a dopant, such as acetone, anisole, benzene, or toluene, to the auxiliary or sheath gas to aid indirect ionization.

13.5 Supercritical fluid chromatography-mass spectrometry

SFC (Chapter 11) can also be coupled to MS with ESI, APCI, or APPI allowing direct infusion of the chromatographic effluents into a mass analyzer. A makeup flow of a suitable liquid is often required to ensure ionization with ESI, etc. APCI sources have the advantage that they

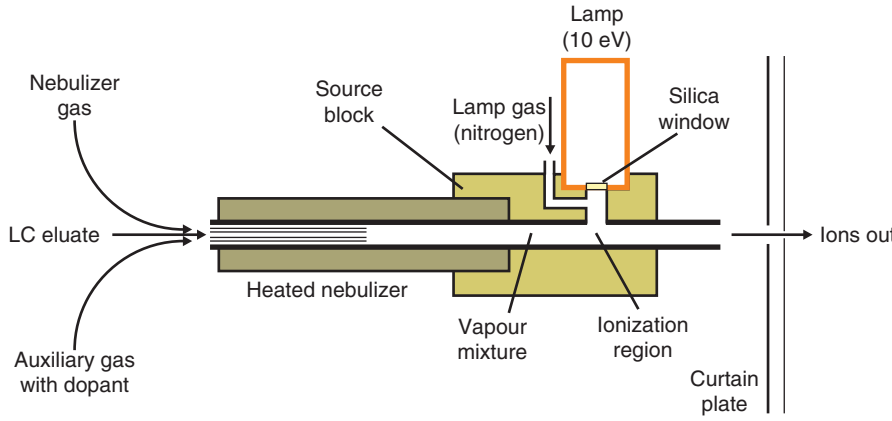


Figure 13.15 Schematic diagram of an APPI interface

are amenable to high flow rates, and the signal:noise ratio may be especially good with APPI because carbon dioxide and methanol do not photoionize (Tarafder, 2018).

A high throughput SFC-MS/MS screening method for 100 doping agents in urine has been described (Nováková *et al*., 2016). The classes of agents examined included anabolic agents, glucocorticoids, and synthetic cannabinoids. Urine samples were extracted with MTBE using Isolute SLE+ 48-well plates. SFC was performed using gradient elution on a diol column. The modifier was 10 mmol L^{-1} ammonium formate in methanol:water (98+2 v/v), with a gradient between 2–40 % with carbon dioxide. The column oven temperature was 40 °C and the BPR 150 bar. It was claimed that the method out-performed the Minimum Required Performance Levels for all the compounds apart from a metabolite of norethisterone, norbolethone, and turinabol, respectively.

On-line SFE-SFC-MS has been used to measure ketamine and its metabolites in dried human urine (Hofstetter *et al*., 2018). The method required a relatively small volume of urine (20 µL) and was validated for concentrations ranging from 25.0–1000 µg L^{-1} ($r^2 > 0.995$). The LLoQ for all compounds was found to be as low as 0.5 ng on column. In addition, the stability of analytes during the removal of water from the samples under different conditions (filter paper or ISOLUTE® HM-N) was studied.

13.6 Capillary electrophoresis-mass spectrometry

Most CE-MS detection systems are based on ESI, which is sensitive and can generate ions for a wide range of species. MS, particularly MS/MS, can provide information on the identity of peaks, and SIM can be used to resolve overlapping peaks if needs be. If volatile buffers are used, the coupling of CE with MS is relatively straightforward, but the presence of involatile surfactants makes coupling MEKC and MS more difficult, as surfactants may cause severe ion suppression.

Quadrupole and ion trap instruments have been successfully interfaced to CE. MDA isomers, for example, have been measured using ESI and MS/MS (Ramseier *et al*., 2000). In both cases a volatile BGE was used for CE-MS. Chip-based approaches have also been described (Deng *et al*., 2001).

13.7 Direct introduction mass spectrometry

MS coupled to chromatography is well established in clinical and forensic research and practice, whilst flow injection analysis (FIA-MS), and ambient MS (AMS) are rarely used routinely in these fields. Nevertheless, the latter technique offers higher throughput and minimal sample preparation (Figure 13.16). FIA-MS is recognized for rapid and reliable quantitation of, for example, pharmaceuticals, pesticides, and endogenous compounds in very complex matrices such as biological samples or foodstuffs in clinical diagnostics, metabolomics, and in nutritional and environmental sciences.

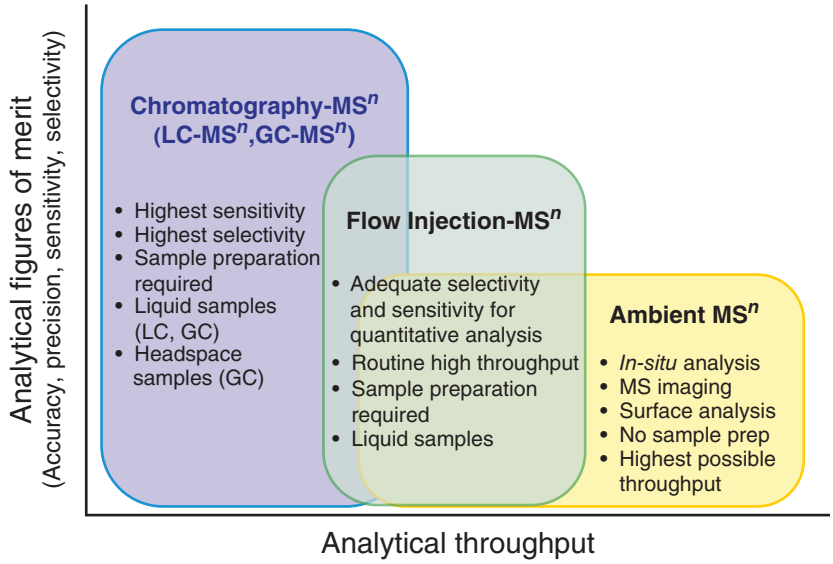


Figure 13.16 Analytical figures of merit versus analytical throughput of chromatography-coupled MS, flow injection MS, and ambient MS (reprinted from Nanita & Kaldon, 2016, with permission from Springer Nature)

AMS with different ambient ionization techniques is used increasingly in food analysis and clinical diagnostics. In the forensic sciences, AMS has been advocated for the direct analysis of seized drugs and for imaging hair and tissue samples. Besides established elution- or desorption-based HPTLC-MS, DESI, paperspray ionization (PSI), MALDI, and ICP-MS for metal analysis (Section 21.4.4) are commonly used techniques.

13.7.1 Flow injection analysis-mass spectrometry

FIA-MS offers high throughput with acceptable sensitivity, precision, and accuracy. Figure 13.17(a) shows a scheme with an LC autosampler connecting the sample injector directly to the MS ion source. The chronogram [Figure 13.17(b)] highlights the various steps of the very fast FIA process. Drugs, pesticides, environmental contaminants, and endogenous

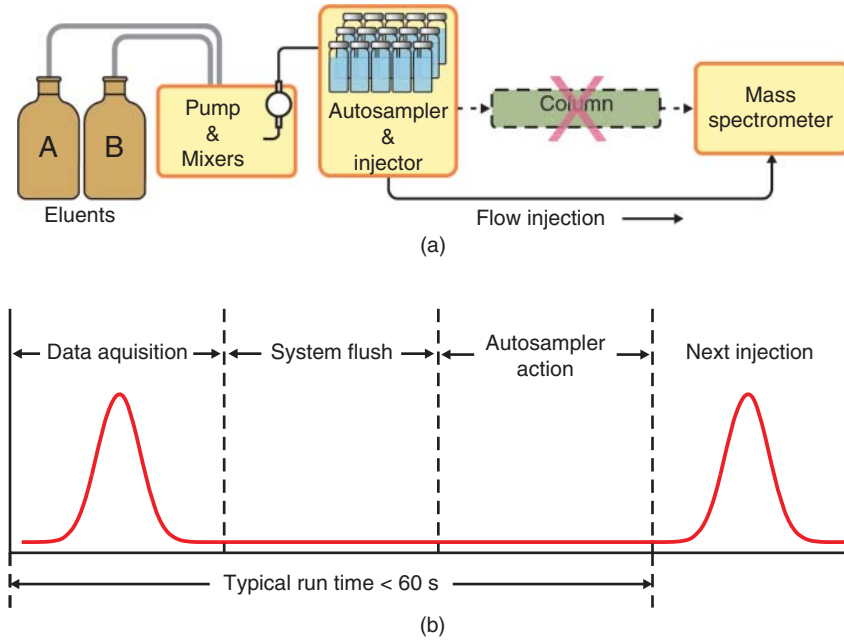


Figure 13.17 (a) Scheme of FIA-MS with LC autosamplers. (b). Diagram of a FIA-MS chronogram highlighting the various steps of the FIA process (reprinted from Nanita & Kaldon, 2016–with permission from Springer Nature)

compounds can be analyzed using FIA-MS (Nanita & Kaldon, 2016). The concentrations measured ranged from ppm to ppb even in complex matrices such as blood, urine, and foods. In summary, FIA-MS offers a balance between the advantages and disadvantages of chromatography-coupled MS and ambient MS.

13.7.2 High-performance thin-layer chromatography-mass spectrometry

Advantages of HPTLC-MS are that (i) plates can be assessed prior to MS allowing targeted recording of mass spectra of zones of interest, and (ii) the spectra are obtained at room temperature. A particular advantage is that a much wider range of eluents can be used compared with those suitable for LC-MS because the eluent is usually evaporated prior to MS. Several manufacturers offer 'MS-grade' plates that have been developed to produce minimal background signal. Coupling MS to planar chromatography has proved to be more difficult than coupling to capillary GC or LC, particularly when coupling to vacuum-based ionization techniques. The approaches can be thought of as being either 'off-line' and 'on-line', or 'elution' and 'desorption'.

Off-line techniques include scraping the spots from the plate, eluting the compounds of interest with suitable solvents, concentrating the eluate and inserting the residue into the MS. Clearly, this is very labour intensive and not amenable to high throughput screening. However, the choice of ionization methods includes EI, allowing structural identification. Of the scrape and elute approaches, spots on a developed plate may be located under UV light. The silica surrounding the spot corresponding to the analyte may be removed on three sides leaving a trapezoidal shaped zone. The analyte may then be concentrated along the narrow, closed edge of the trapezoid by applying methanol to the open end; an approach used by Tames *et al*. (1999)

to measure morphine. After treatment with glycerol, the silica band was removed to a fast atom bombardment (FAB) probe for MS analysis. Fully online approaches include forced-flow techniques such as OPLC and RPC (Section 8.3.1) and overrun chromatography.

13.7.2.1 Elution-based approaches

As well as the forced-flow methods mentioned above, several techniques for eluting analytes from TLC plates for coupling to MS have been proposed including the use of microcapillaries and surface sampling probes (Morlock & Schwack, 2010; Cheng *et al*., 2011). Elution head-based interfaces have been developed. The sampling head is placed over the zones to be eluted, where it forms a seal with the TLC plate and eluent is pumped through inlet and outlet tubes, there being a frit in the outlet tube to collect any particles that are dislodged from the plate. Three sampling heads are available, circular (4 mm diameter) and oval (2 × 4 mm), which are suitable for layers up to 300 µm thick, and circular (5 mm diameter), which is suitable for preparative layers up to 500 µm thick. The oval head gives better special resolution and is suited to samples applied as bands. Advantages of this interface are that it can accommodate 20 × 20 cm plates and can be used with any MS system that can be interfaced with LC.

13.7.2.2 Desorption-based approaches

Many of the early desorption techniques required the ionization to be performed under vacuum, and foil-backed TLC plates that could be cut into small regions for insertion into an evacuated interface were used. In the 1980s fast atom bombardment (FAB) and secondary ion MS (SIMS) were demonstrated and later the first report of MALDI combined with TLC appeared. A potential problem with these techniques is that the application of the matrix may lead to lateral diffusion along the plate resulting in some loss of resolution.

The development of atmospheric pressure (ambient) ionization techniques greatly simplified the interfacing of TLC with MS. Many of the approaches have employed laser desorption/ionization (LDI) or laser ablation (LA); these include ambient MALDI (Lafleur & Salin, 2008). Other ionization methods include direct analysis in real time (DART), which uses excited (metastable) helium atoms [Figure 13.18(a)], desorption electrospray ionization (DESI), and

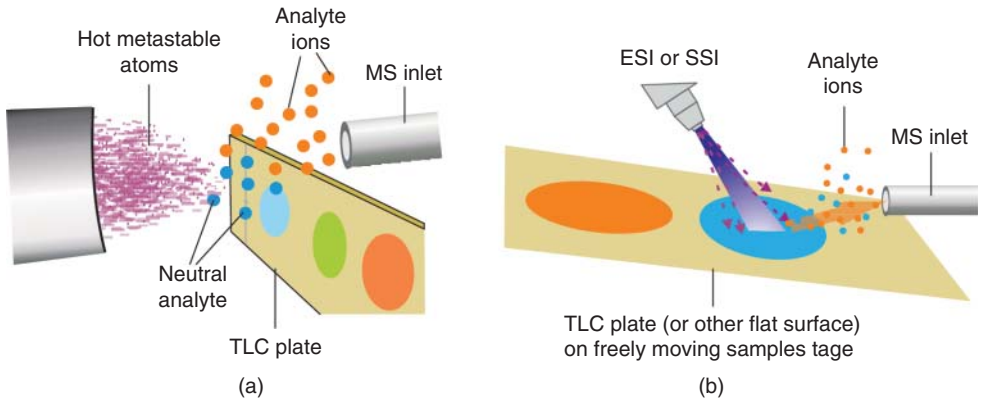


Figure 13.18 Schematic diagrams of (a) DART ionization and (b) ESI or EASI systems (reprinted from Cheng *et al*., 2011–with permission from Elsevier)

easy ambient sonic-spray ionization (EASI) [Figure 13.18(b)]. Some of these methods were developed for sampling solid surfaces and were later applied to TLC.

Many of the ambient desorption techniques allow high resolution continuous scanning of a plate. However, a disadvantage is the large amount of data that is collected and has to be processed. Zonal scanning (i.e. of identified spots) is less time consuming and so gives higher throughput. TLC-DART was applied in the forensic identification of the components of several pharmaceutical preparations (Howlett & Steiner, 2011). This combination of TLC with DART was reported to screen and subsequently identify analytes from the same TLC plate in less than 10 min. A method that utilizes an array of 12 SPME fibres for simultaneous isolation of drugs of abuse from urine followed by DART LC-MS facilitated rapid interrogation of each SPME fibre (Li & Musselman, 2018).

13.7.3 Desorption electrospray ionization mass spectrometry

DESI uses fast-moving solvent droplets to extract analytes from surfaces. The sample with the analytes is deposited from solution and dried onto a surface. Then, the solvent is sprayed onto the surface under the influence of high voltage, and desorbed ions are transferred to the MS [Figure 13.18(b)]. The spot can be scanned by moving the surface.

Misused drugs in urine have been detected by DESI after extraction and application of the concentrated extract to a PTFE surface (Kauppila *et al*., 2007). Morelato *et al*. (2013) reviewed applications of DESI in forensic sciences including drugs, explosives, chemical warfare agents, inks, and gunshot residues analyzed on various surface matrices such as tablets, filter paper, glass slides, and TLC plates.

13.7.4 Paperspray ionization-mass spectrometry

PSI-MS belongs to the family of ambient ionization or direct analysis methods providing sensitive analysis of complex samples without sample preparation. Extraction and ESI occur directly from the so-called paper substrate upon which a dried matrix spot is stored (Figure 13.19). The drop is applied directly to the triangular section of the paper substrate. The sample is wetted with a suitable solvent and a DC voltage is applied. The solvent extracts the analytes and forms a

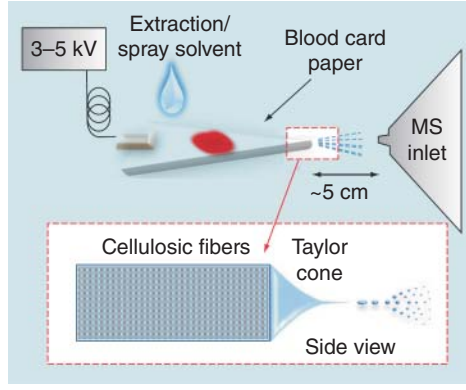


Figure 13.19 Analysis of a dried blood spot by paper spray-MS (Reprinted from Manicke *et al*., 2016–with permission from Newlands Press Ltd)

Taylor cone for ESI. Because analytes are not separated in this process, MS/MS or HRMS is required to perform the separation. Manicke *et al*. (2016) recently reviewed the use of PSI-MS for bioanalysis with critical discussion of its possibilities and limitations.

In clinical and forensic toxicology, PSI-MS has been used for drug measurements in whole blood, comprehensive urine drug screening, and for TDM (Espy *et al*., 2012; 2014; Michely *et al*., 2017).

13.7.5 Laser diode thermal desorption mass spectrometry

Laser diode thermal desorption (LDTD™, Phytronix Technologies) is a direct ionization method in which a small volume of sample is pipetted into a specially designed well plate with a stainless steel back (LazWell™, Phytronix Technologies) and allowed to dry. The 96-well plate will accommodate samples up to 10 µL volume. To vaporize the analyte(s) as gas-phase neutral molecules, heat is transferred instantly via an infrared laser diode aimed at the base of the well. The vaporized analyte(s) are carried through the transfer tube by a carrier gas together with water molecules and these flow into the corona discharge leading to an APCI source (Figure 13.20).

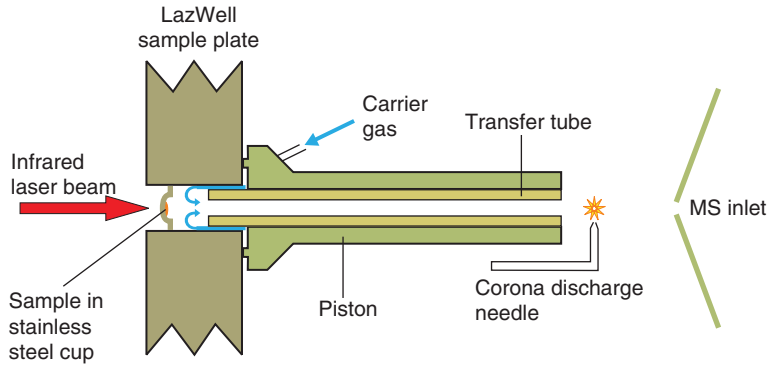


Figure 13.20 Schematic diagram of a laser diode thermal desorption source

This system gives very short analysis times hence high throughput and decreased costs. Some forensic applications have been described by Bynum *et al*. (2014). They recommended that the technique be considered as a supplement to conventional MS, but considered that for quantification, practicality and validity must be confirmed. Because LDTD provides no chromatographic separation, HRMS/MS should be used to differentiate isobaric compounds with the same precursor and product ions.

13.7.6 Matrix assisted laser desorption ionization mass spectrometry

In biomedical research, MALDI (Figure 13.21) coupled to either low, or high-resolution MS is widely used for either high-throughput quantification, or imaging, for example of tissues (Wu *et al*., 2013). With MALDI, the matrix absorbs laser light and converts it to heat energy. Rapidly heated matrix components vaporize together with the sample forming charged ions of various sizes directed to the MS.

MALDI-MS in drug analysis is of great interest for high-throughput quantification [Figure 13.22(a)] and particularly spatially resolved tissue and hair imaging [Figure 13.22(b)].

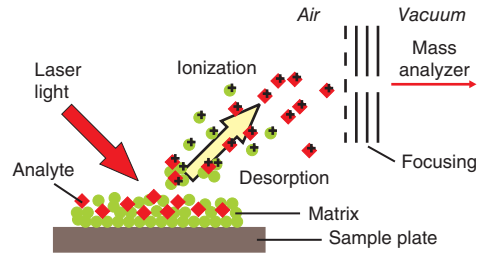


Figure 13.21 Principle of matrix assisted laser desorption/ionization mass spectrometry

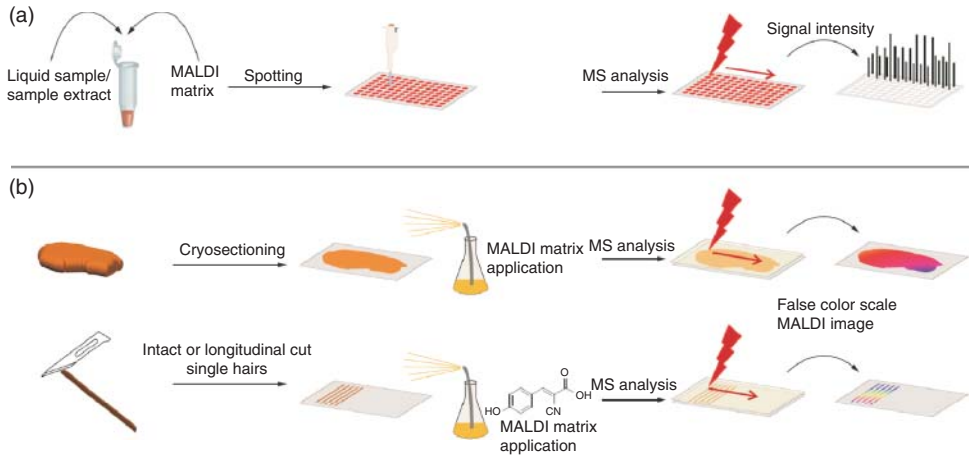


Figure 13.22 Typical MALDI applications in forensic toxicology: (a) high-throughput analysis and (b) imaging a tissue sample (reprinted from Steuer *et al.,* 2016–with permission from Newlands Press Ltd)

Liquid samples are mixed with a matrix solution and a small volume is applied to the surface of the MALDI target plate. Tissue or hair samples are fixed on the target plate and MALDI matrix is applied usually by either manual, or automatic spraying devices.

13.8 Presentation of mass spectral data

Mass spectra are constructed by plotting the intensity (equivalent to the number) of ions detected against their *m*/*z* value. It is usual for the ion intensities to be normalized as percentages of the intensity of the most abundant ion (base peak). With hyphenated techniques such as GC-MS, LC-MS, and CE-MS, mass spectral data are collected throughout the separation and can be displayed as mass chromatograms. These can be mass fragmentograms, i.e. plots of scan number (analogous to retention time) versus the intensity of an ion of chosen *m*/*z* ratio (SIM), or the sum of total ion intensity [reconstructed (total) ion chromatograms, R(T)IC] generated from the full-scan data. Either type of plot can be used for quantitative measurements. RTIC plots often look like GC-FID chromatograms (Figure 13.23).

When using SIM it is usual to monitor two or more ions, for example one characteristic ion derived from the analyte and, if possible two additional 'qualifier' ions, and another ion

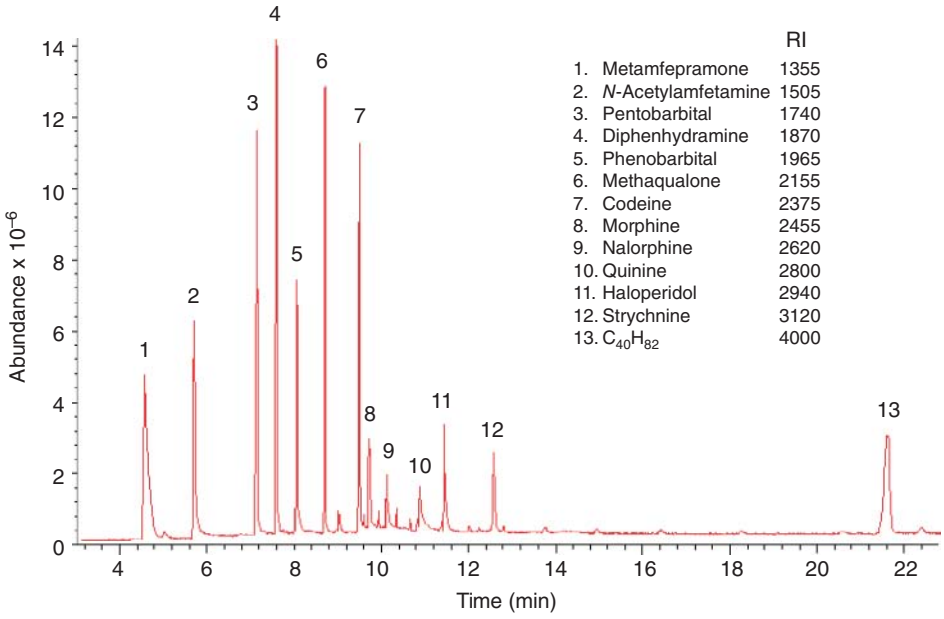


Figure 13.23 Typical GC-MS total ion chromatogram of a methanolic solution containing 50 mg L^{-1} of each analyte ($C_{40}H_{82}$ = tetracontane). Injection volume: 1 µL, column: cross-linked 5 MS, 12 m × 0.2 mm i.d., film thickness: 0.35 µm, column temperature: 3 min at 100 °C, then to 310 °C at 30 °C/min, and hold 5 min, ionization: EI, 70 eV (redrawn from Maurer *et al*., 2016–with permission of John Wiley & Sons)

characteristic of the ISTD, in separate channels to enhance the reliability of the analysis. The use of appropriate integration parameters such as threshold and peak width remains important, however.

GC-MS can be used in the EI mode to produce definitive information as to peak identity either by providing full spectra, or from the five or six principal peaks in the mass spectrum. Alternatively, PCI mode can be used to ascertain the M_r of the analyte, which is particularly useful in STA because many potential candidate structures can be eliminated. Quantitative information can also be obtained if the instrument is used in the SIM mode if appropriate calibration standards are available (Section 19.3.2).

An example of the use of GC-MS in compound identification is illustrated in Figure 13.24. The antidepressants citalopram and clomipramine have similar GC retention times (citalopram RI 2377, clomipramine RI 2388 on SE-30/OV-1) and NPD response if not derivatized. Although the presence of metabolites may help differentiate these compounds, the EI spectra are clearly different (citalopram principal peaks at *m/z* 58, 238, 208, 42, 324, and 190 u, clomipramine principal peaks at 58, 85, 269, 268, 270, 271, and 242 u). Note, however, that the principal peak on both spectra is at *m/z* 58, arising from fragmentation to $(CH_3)_2NCH_2^+$ or other ions with this *m/z*.

Not only citalopram and clomipramine, but also many other basic drugs and metabolites give rise to the $(CH_3)_2NCH_2^+$ fragment or $C_2H_4NHCH_3^+$ (also *m/z* 58) hence it is generally of little use in compound identification. In addition, in the case of citalopram this fragment carries almost all of the ion current, making it very difficult to see the other ions, including the molecular ion (324 u). Another common fragment is *m/z* 44 corresponding to $CH_3NHCH_2^+$ – this

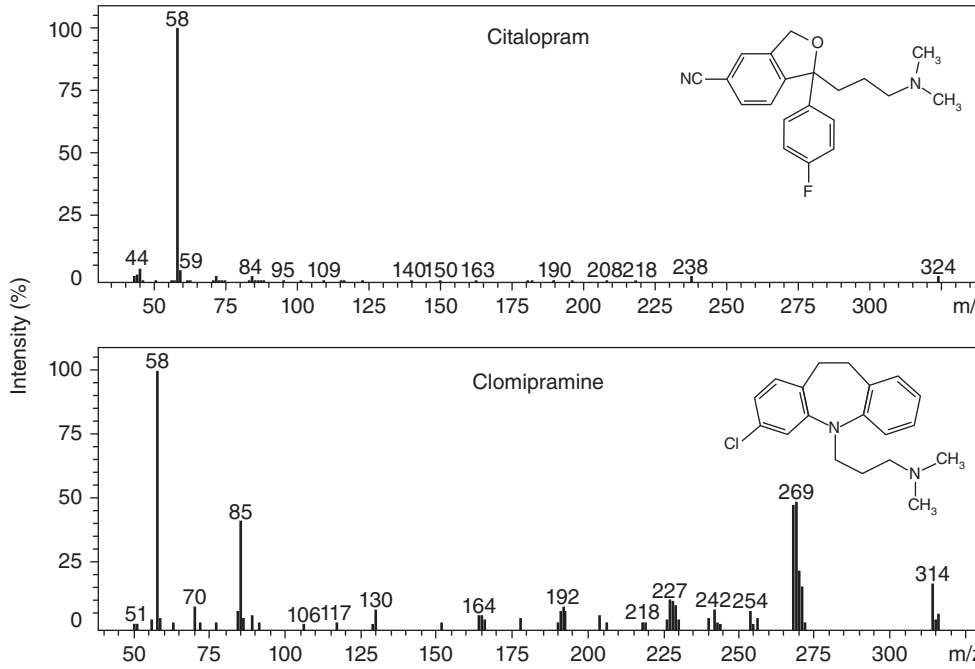


Figure 13.24 Mass spectra of citalopram (M_r 324.4) and clomipramine (M_r 314.9)

dominates the EI spectrum of another antidepressant, fluoxetine. These ions (*m/z* 44 and *m/z* 58) dominate the EI spectra of two important analytes, amfetamine and metamfetamine, respectively (Figure 13.25), and illustrate the need to derivatize these compounds to give reliable MS identification (Figure 13.26).

13.9 Interpretation of mass spectra

The availability of databases notwithstanding, interpretation of mass spectra has to be undertaken with knowledge of the type of ionization used and other factors such as sample preparation, derivatization, and LC eluent composition. The M_r of most organic analytes is an even number unless the molecule contains an odd number of (trivalent) nitrogen atoms. The presence of other elements, boron for example, can also give an odd number M_r, but in analytical toxicology such elements are encountered infrequently.

The presence of naturally occurring isotopes can aid identification (Table 13.2). With chlorine and bromine, the presence of ^{35}Cl and ^{37}Cl, and ^{79}Br and ^{81}Br, respectively, produces characteristic isotope patterns and fragments containing these elements can be identified easily. Frequently the average mass of a molecule in which isotopes contribute significantly is cited rather than an accurate mass. For example, hydrogen chloride has an average mass of 36.5 u as it contains 75 % ^{35}Cl and 25 % ^{37}Cl. The presence of these isotopes and others such as ^{13}C, ^{34}S, ^{29}Si, and ^{30}Si can lead to the occurrence of notable isotope peaks. A further source of information on the relative abundance of isotopes is the IUPAC Periodic Table of Elements and Isotopes (Holden *et al.*, 2018).

HR mass analyzers should have a mass accuracy of one part per million (ppm) or better, i.e. the mass of a fragment should be measurable to an accuracy of at least six significant figures. With

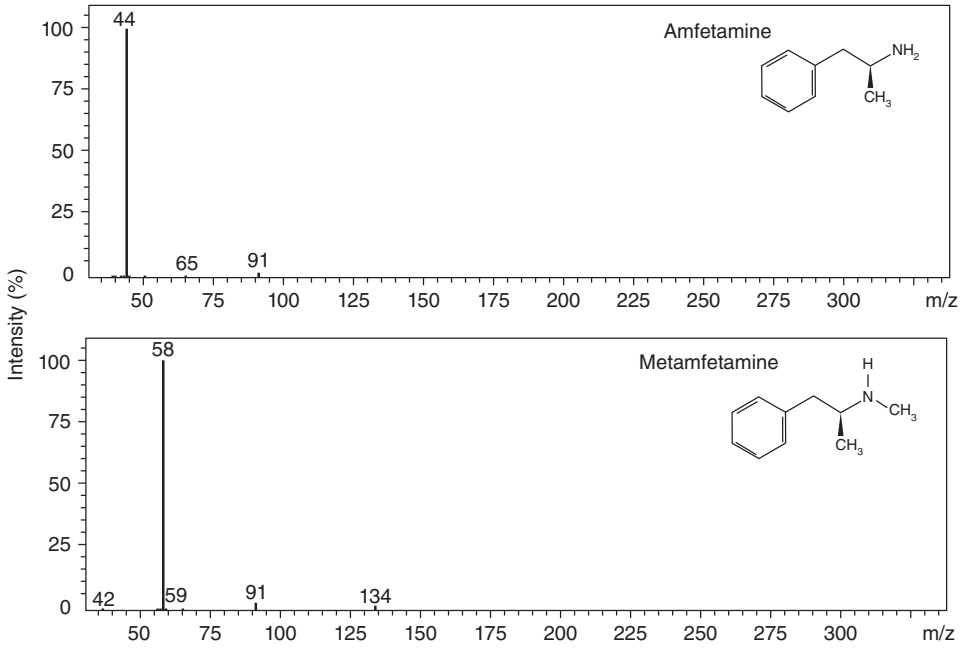


Figure 13.25 EI mass spectra of amfetamine (M_r 135.2) and metamfetamine (M_r 149.2)

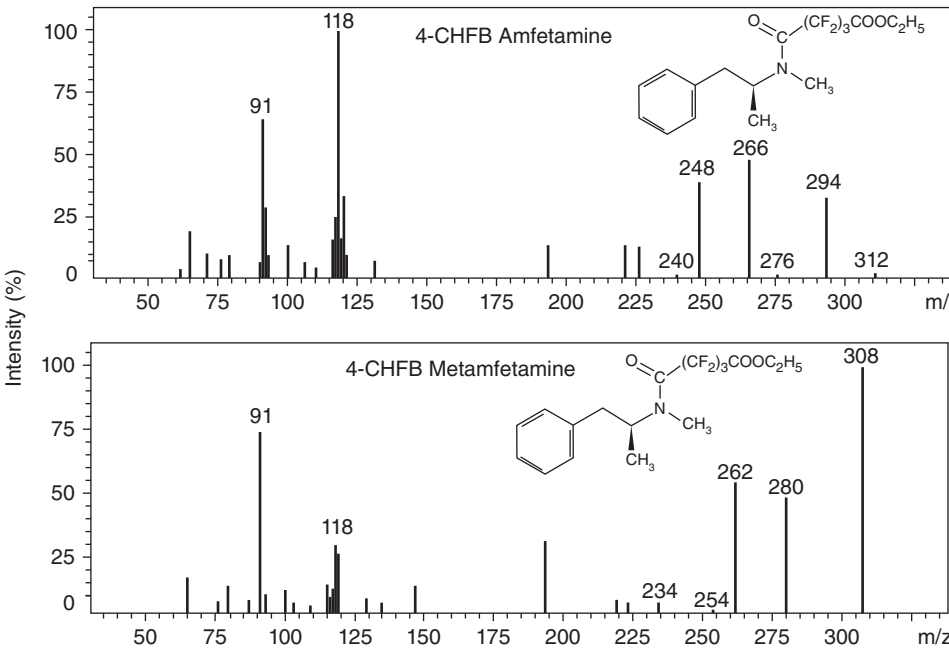


Figure 13.26 EI mass spectra of the 4-carboxyhexafluorobutyryl (4-CHFB) derivatives of amfetamine (M_r 385) and metamfetamine (M_r 399)

Table 13.2 Relative abundance of isotopes commonly encountered in analytical toxicology (Watson, 2011–reproduced with permission of the Pharmaceutical Press)

Isotope	M_r	Natural abundance (%)	Isotope	M_r	Natural abundance (%)
^{1}H	1.00782	99.985	^{29}Si	28.977	4.7
^{2}H	2.014	0.015	^{30}Si	29.974	3.1
^{12}C	12.0000000	98.9	^{31}P	30.974	100
^{13}C	13.003	1.1	^{32}S	31.972	95
^{14}N	14.0031	99.64	^{33}S	32.971	0.8
^{15}N	15.0001	0.36	^{34}S	33.968	4.2
^{16}O	15.9949	99.8	^{35}Cl	34.969	75.8
^{17}O	16.999	0.04	^{37}Cl	36.966	24.2
^{18}O	17.999	0.2	^{79}Br	78.918	50.5
^{19}F	18.998	100	^{81}Br	80.916	49.5
^{28}Si	27.977	92.2	^{127}I	126.904	100

this degree of accuracy, it is possible to identify the atoms present in a fragment unequivocally provided the accurate masses of the isotopes are known. Accurate M_r values are arbitrarily standardized to ^{12}C (12.000000). Accurate mass measurement can be useful, for example, in identifying either the source of illicit drugs, or the origin of compounds used in the synthesis of such compounds, based on their empirical formulae.

Mass calibration of the instrument needs to be performed at the time of the analysis. Calibrants such as glycerol, perfluorokerosene (PFK), perfluorotributylamine (PFTBA), polyethylene glycol (PEG), and Ultramark 1621 (perfluoroalkylphosphazine) are used depending on the requirement. PFTBA is commonly employed in GC-MS work because it can be used in positive chemical ionization (PCI) and in negative chemical ionization (NCI) mode and its fragmentation pattern covers a wide mass range. In addition, the M_r of fluorine is very close to its nominal mass of 19 (Table 13.2). Typically, the ions at *m/z* 69, 219, and 502 (EI), 219, 414, and 652 (PCI); and 302, 452, and 633 (NCI) are used as calibrant ions. With quadrupole, QIT, or other low resolution instruments (mass resolution $\pm$ 1 u), the nearest whole number is used, with exact masses to four decimal places being used in HRMS. In LC-MS instrument calibration is less well defined, and is usually developed for a particular instrument or application.

GC-MS fragmentation data are usually obtained under EI conditions at about 70 eV, although some such data may also be obtained by introduction of pure samples via a direct-insertion probe. A fundamental requirement for producing EI mass spectra is that it must be possible to introduce the analyte into the MS in the vapour phase under reduced pressure. The resulting ions and ionized fragments, and their relative proportions, should be characteristic of the analyte and independent of the instrument used and mode of sample introduction.

General GC-MS libraries, such as the Wiley Registry of Mass Spectral Data and that produced by the National Institute for Standards and Technology (NIST) in conjunction with the US Environmental Protection Agency (EPA) and National Institutes of Health (NIH) (McLafferty, 2017) are available complete with database programs that facilitate comparison of spectra from unknowns with those of known compounds. Chemspider (http://www.chemspider.com/)

and m/zcloud (https://www.mzcloud.org/) are further free resources that can aid in compound identification. There are also libraries specifically created for use in analytical toxicology (Maurer *et al*., 2016; Rösner, 2018; Maurer *et al*., 2018). If certain prerequisites are fulfilled, such libraries facilitate the identification of unknowns even in the absence of reference compounds (Maurer, 2006; Maurer & Peters, 2006).

When reference material is available, the ratios of the abundances of several (usually three to five) selected ions from the unknown are compared with the ratios of those ions obtained from the standard, typically analyzed in the same batch. The presence of these ions combined with good agreement in the ion ratios with the ratios given by the reference standard, are generally accepted as sufficient to identify a compound positively, especially when considered together with chromatographic retention data.

Use of selected ion ratios is less useful in identifying unknown compounds, and in such cases full-scan mass spectra are usually compared with library data, at least in the first instance. Several different search algorithms can be used to help identify compounds by comparison with the library spectra. If this approach fails, then study of the fragmentation pattern (Table 13.3) and consideration of other information such as any extraction and derivatization steps used can often help in compound identification. A knowledge of likely metabolic pathways for a given analyte (hydroxylation, *N*- or *O*-dealkylation, etc.), may also be useful. Even so, fragment losses that are readily explained usually occur only within about 100 u of the molecular ion.

Problems with MS also arise in the case of volatile materials such as gases and many solvents. Some instruments have a low mass cut-off of 50 u, whilst the compounds themselves have relatively simple structures and thus produce very few diagnostic ions. Aliphatic hydrocarbons give very similar EI spectra, for example.

Spectra obtained using soft ionization techniques such as PCI can result in protonated and other adduct molecules that have a charge. Fragmentation of these ions is interpreted using the same general principles used with EI spectra. Because these soft ionization techniques are much gentler than EI, a protonated molecular ion is much more likely to be present in the spectrum, but fragmentation is usually much reduced and hence published libraries of PCI spectra are generally less helpful than EI libraries in compound identification. However, this may help in identifying molecules (e.g. metabolites) with longer side chains, which typically do not form molecular ions in EI.

13.10 Quantitative mass spectrometry

Quantitative measurements in MS are not straightforward. Because of inevitable variations in the degree of ionization, the use of ISTDs is vital. SIL analogues are widely used for this purpose (isotope dilution MS). Appropriately labelled isotopic ISTDs have virtually identical chemical and physical properties to the analyte and thus extraction, derivatization if needed, chromatography, and fragmentation should be virtually identical. However, the site of isotopic labelling should be chosen such that the bonds linking the isotope are not broken during fragmentation. Bonds involving heavier isotopes are more stable than those of lighter isotopes (kinetic isotope effect) and the fragmentation pattern of the labelled compound could thus differ from that of the analyte.

There will often be a choice of SIL analogues available, either with different degrees of isotopic enrichment, or employing isotopes other than ^{2}H such as ^{13}C or ^{15}N. The purity of SIL analogues, i.e. the presence of unlabelled analyte and other impurities, should be assessed with each new batch of ISTD.

Table 13.3 Mass spectral interpretation: common fragment losses (Watson, 2011–reproduced with permission of the Pharmaceutical Press)

Mass loss (u)	Radical/neutral fragment	Likely origin
1	$H^{\bullet}$	Amine or aldehyde
15	$CH_3^{\bullet}$	Quaternary or TMS methyl
17	$OH^{\bullet}$	
17	NH_3	Primary amines
18	H_2O	Secondary or tertiary alcohol
19/20	$F^{\bullet}$/HF	Fluorine-containing molecules
28	CO	Ketone/carboxylic acid
29	$C_2H_5^{\bullet}$	Ethyl
30	CH_2O	Aromatic methyl ether
31	$CH_3O^{\bullet}$	Methyl ester/methoxime
31	CH_3NH_2	Secondary amine
32	CH_3OH	Methyl ester
35/36	$Cl^{\bullet}$/HCl	Chlorine-containing molecules
42	$CH_2{=}C{=}O$	Acetate
43	$C_3H_7^{\bullet}$	Propyl/2-methylethyl
43	$CH_3CO^{\bullet}$	Methyl ketone or acetate
44	CO_2	Carboxylic acid
45	$CO_2H^{\bullet}$	Carboxylic acid
57	$C_4H_9^{\bullet}$	Butyl
59/60	$CH_3COO^{\bullet}$/CH_3COOH	Acetate
73	$(CH_3)_3Si^{\bullet}$	Trimethylsilyl ether
90	$(CH_3)_3SiOH$	Trimethylsilyl ether

13.10.1 Stable isotope-labelled internal standards

Deuterium has been used widely as a stable isotope label partly because deuterium-labelled compounds are relatively inexpensive but, more importantly, because of the availability of high purity reagents with which to synthesize ^{2}H-ISTDs. The presence of each deuterium atom in the molecule increases the M_r of the molecule by 1 u. For high-sensitivity analyses it is recommended that the mass of the ISTD is at least 3 u greater than the analyte to reduce interference from naturally occurring isotopes ('cross-talk'). However, it is becoming increasingly clear that deuterated ISTDs are not as chemically similar to their unlabelled analogues as might be hoped.

^{2}H-Labelled analogues have been shown to separate from unlabelled material on GC and LC columns, and extract to different degrees with SPE. The extent of separation may be greater with increasing numbers of substituted atoms [Figure 13.27(a)]. Deuterium is also

prone to isotope exchange, particularly when substituted for acidic hydrogens. In the example of Figure 13.27(b), the electron-withdrawing groups of the sulfone in rofecoxib renders the hydrogen atoms of the adjacent methyl group acidic, and deuterium–hydrogen exchange was rapid when [$^2H_3$]-rofecoxib was dissolved in water (Chavez-Eng *et al*., 2002).

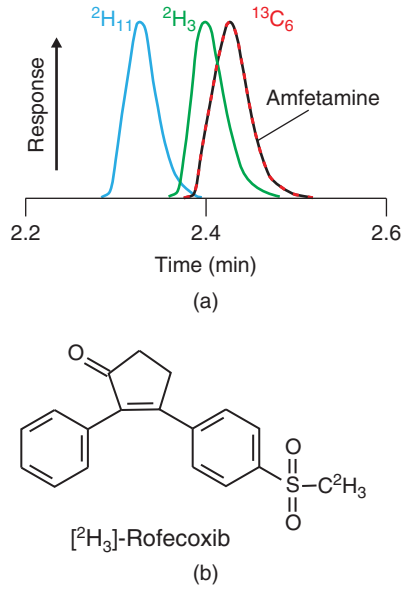


Figure 13.27 Problems associated with deuterated internal standards. (a) Separation of ^{2}H-labelled amfetamine (reprinted from Berg & Strand, 2011–with permission from Elsevier). (b) Inappropriate site of labelling: the proximity of electron-withdrawing groups results in the deuterium atoms being acidic and exchanging with hydrogen

To avoid the problems associated with deuterium, it has been suggested that whenever possible ^{13}C- and/or ^{15}N-labelled materials should be used. [$^{13}C_6$]-Amfetamine coeluted with unlabelled analyte on LC [Figure 13.27(a)]. However, synthesis is more difficult and expensive. With regard to ^{15}N-labelled materials it may be difficult to achieve the required mass difference between the analyte and ISTD, unless the analyte contains several nitrogen atoms. On the other hand, ^{18}O-labelled compounds are not widely used as ISTDs despite the mass difference of 2 u per atom, not only because of cost, but because oxygen in functional groups such as acids, esters, and ketones is readily exchangeable.

13.10.2 Assay calibration

The ISTD may add to the degree of ion suppression in LC-MS, but generally both ISTD and analyte are affected equally by such phenomena (Sojo *et al*., 2003). The fact that labelled and unlabelled compounds may be partially resolved during the chromatographic analysis must be borne in mind not only with regard to choosing the correct integration parameters, but also because any ion suppression may differ between the ISTD and the analyte(s). In addition, in multi-analyte LC-MS methods, an analyte in the calibrator may alter the signal of one of the

ISTDs if it co-elutes. Hence this must be tested for because this interaction will not occur in a real sample in which the interfering analyte is absent (Remane *et al*., 2010b).

For MS analysis, the ISTD may be added at a higher concentration than the analyte. This results in the assay being conducted over a narrower 'total' concentration range, thus minimizing the impact of possible concentration-related effects such as adsorption. Under these conditions the isotopic purity of the material will limit the amount that can be added for a particular LLoQ. Because the amount of non-labelled material in the ISTD is constant, a calibration graph with a significant positive intercept is permissible providing the blank and lowest calibration standard can be distinguished (Section 3.2.4.6).

Conversely, the presence of naturally occurring isotopes in the analyte can interfere with measurement of the ISTD ('cross-talk') by increasing the signal in the ISTD channel and thereby reducing the estimate of the concentration of analyte. This will be more prevalent at high analyte or standard concentrations, when the masses of analyte and ISTD are only different by 1 or 2 u, and when the analyte contains elements with a high abundance of heavier isotopes such as chlorine, bromine, or sulfur (Rule *et al*., 2013; Table 13.2). For example, if [$^{2}H_{2}$]-methadone is used as an ISTD, the signal given may increase at higher calibrator concentrations due to a greater contribution from the less abundant naturally occurring isotopes of carbon and nitrogen (^{13}C,^{15}N) in methadone. If this is the case, adjusting the amount of ISTD added can help, although a better solution is to source a more enriched standard, e.g. $^{2}H_{4}$ or $^{2}H_{5}$.

In the analysis of peptides and proteins, intact (i.e. full sequence) isotopically labelled proteins are commercially available for some analytes of toxicological relevance such as insulin. For others, isotopically labelled analogues can be synthesized, although this can be very costly and, for larger proteins, does not always ensure a product chemically and physically analogous to the analyte of interest. For instance, the isotopically labelled protein may neither have the same tertiary/quaternary structure as the analyte of interest, nor incorporate any post-translational modifications, which may influence its chemical and physical properties.

When SIL analogues are either not available, or the procedure is to be used to measure a large number of analytes simultaneously, ISTDs that have chemical properties close enough to the analyte(s) to yield reliable quantitative data are sometimes used.

There is no specific test of the sensitivity of a MS system other than those normally employed with a chromatographic system (LoD, LLoQ, etc.). Indeed, method validation and IQC/EQA procedures are the same as with other methods.

13.10.3 Isotopic internal calibration

Where analyte-free matrix is difficult to obtain and when charcoal-stripped matrices prove unsatisfactory, it may be possible to use a SIL analogue of the analyte added at different concentrations to a sample to produce a calibration graph. To measure testosterone, for example, calibrators containing [$^{2}H_{5}$]-testosterone could be prepared using pooled human serum. Whilst this will contain endogenous testosterone, it will effectively be 'blank' for [$^{2}H_{5}$]-testosterone. Provided that the instrument responses for the analyte and the isotopically labelled analogue are equivalent, then the concentration of endogenous testosterone can be calculated from the [$^{2}H_{5}$]-testosterone calibration curve (Li *et al*., 2013). Clearly use of ^{13}C- or ^{15}N-labelled analogues would be preferable given the problems with ^{2}H-labelled analogues discussed above.

A similar approach for plasma clozapine and norclozapine assay has been described (Couchman *et al*., 2013). Three different SIL analogues ([$^{2}H_{4}$]-clozapine, [$^{2}H_{8}$]-clozapine and [$^{2}H_{8}$]-norclozapine) at different concentrations were added to each specimen. The clozapine and norclozapine concentrations were calculated by interpolation from the internal calibration

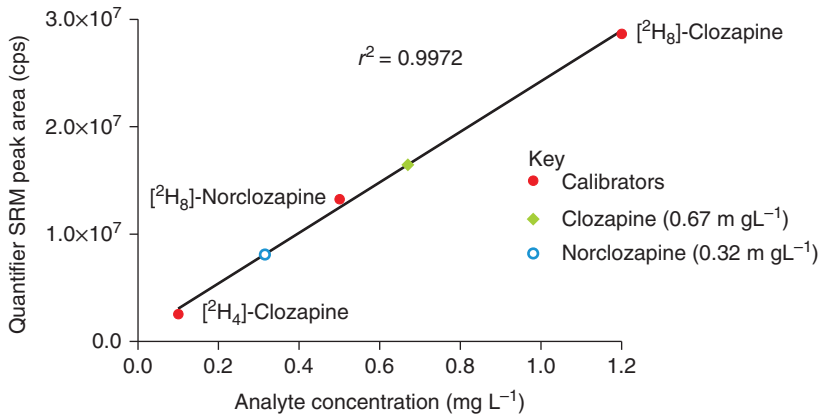


Figure 13.28 Isotopic internal calibration in LC-MS/MS: plasma clozapine and norclozapine assay

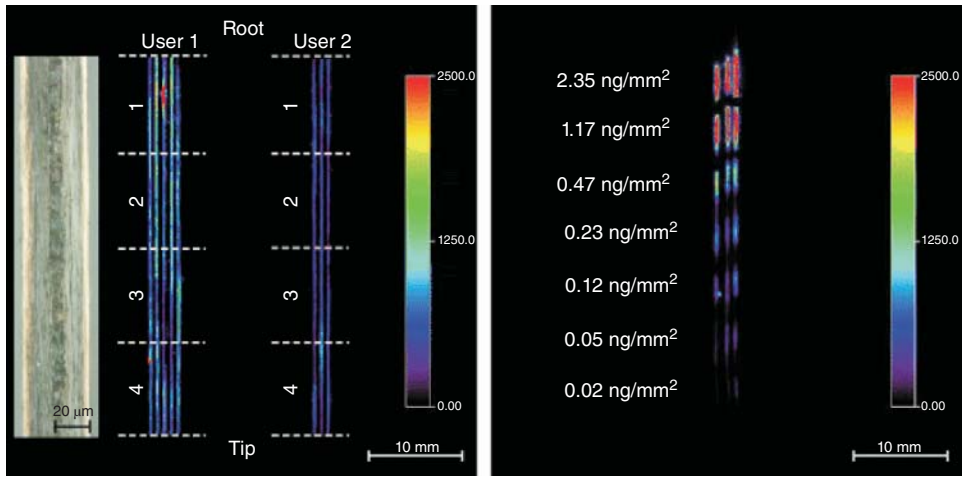
curve (Figure 13.28). This has been termed 'isotopic internal calibration' and is applicable to high-throughput assays where preparation and batch analysis of multiple-point calibration curves is time consuming. It was important to ensure that all aspects of the assays including not only instrument response, but also assay recoveries were comparable for both analytes.

An ICAL procedure has also been used in the analysis of cocaine in drug seizures. A solution containing [$^2H_3$]-, [$^{13}C_6$]-, and [$^2H_3{}^{13}C_6$]-cocaine at concentrations of 1.0, 0.4, and 0.2 g L^{-1}, respectively, was added to a diluted solution of the seized material prior to mixing and analysis by GC-EI-MS (Brockbals *et al.*, 2016). The method was fully validated, with recoveries compared to certified standards of 99–101 % and to an accredited method from an independent laboratory of 86–117 %.

13.11 Mass spectrometry imaging

Mass spectrometry imaging (MSI) is a label-free technique used to visualize the spatial distribution of the compound(s) of interest (drugs, endogenous compounds, drug delivery systems, etc.) in biological tissue [Fig 13.22(b)]. MSI provides the possibility to ascertain simultaneously the discrete distribution of the parent compound and any metabolites. One strength of MSI is the ability to directly overlay the molecular information from the MS analysis with the tissue section and allow correlation of molecular and histological information. MSI is a powerful tool for profiling the pharmacological and toxicological effects of drug candidates in tissues, for example. MSI encompasses a wide range of MS-based platforms all based on different surface sampling ionization techniques.

In forensic toxicology, MALDI-MSI has been applied successfully to assess the distribution and delocalization of misused substances in hair (Flinders *et al.*, 2017a). Tissue or hair samples are fixed on the target plate and MALDI matrix is applied by either manual, or automatic spraying devices [Figure 13.22(b)]. Quantification can be carried out in imaging mode by spraying standards on the same tissue. MS/MS analysis can confirm the identity of the imaged compound (Figure 13.29). DESI (Section 13.7.3) used in imaging mode can also target drugs and metabolites in tissue sections (Wiseman *et al.*, 2008; Lamont *et al.*, 2018).



(a) (b)

Figure 13.29 MALDI-MS/MS images of (a) longitudinal sectioned drug user hair and (b) longitudinal sectioned 'control' hair sprayed with dilutions of a cocaine standard. The MALDI-MS/MS image shows the distribution of the product ion at *m/z* 182, derived from the precursor ion of cocaine at *m/z* 304 (Flinders *et al*., 2017b–reproduced with permission of Springer)

13.12 Summary

A thorough knowledge of GC-MS and LC-MS is fundamental to modern analytical toxicology. Capillary GC-EI-MS used in conjunction with appropriate sample preparation and temperature programming provides sensitivity at best on a par with GC-FID, but this is combined with unparalleled selectivity. There are, however, problems for the unwary. Although valuable in analyte identification, some analytes give poor EI spectra unless derivatized. Moreover, many analytes are not amenable to derivatization. Daily mass calibration is essential. PCI with SIM gives better sensitivity with targeted analytes, but all the restrictions surrounding GC remain.

In LC-MS either ion suppression, or enhancement caused by co-eluting compounds, be they either matrix components, or other drugs, can alter the analyte signal. Nevertheless, LC-MS(/MS) with ESI and/or APCI is now used in many applications. LC-HRMS/MS is becoming more widely available in clinical and forensic toxicology laboratories and can provide very high sensitivity and selectivity. Accurate measurement of M_r can provide the elemental composition of analytes and with additional information such as fragmentation patterns may allow tentative identification of unknown compounds. Quantitative LC-MS is especially challenging. Use of stable isotope-labelled ISTDs, although expensive, is desirable if reliable results are to be obtained.

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14 Ion Mobility Spectrometry

14.1 Introduction

Ion mobility spectrometry (IMS; Box 14.1) is a technique that separates ionized molecules on the basis of their mobility in a carrier gas under the influence of an electric field. A drift gas flowing counter current to the flow of ions may be used to remove neutral molecules. IMS was originally known as plasma chromatography. Representation of IMS response–time data may be referred to as plasmagrams.

Box 14.1 Advantages of ion mobility spectrometry

- Portable, miniaturized devices
- Rapid analysis
- Separates on the basis of charge, mass, and shape
- Provides structural information
- Isomers and isobars can be identified
- Mobilities can be predicted via 3D molecular modelling
- Interfaced with GC and MS or coupled to give IMS-IMS or IMS^n
- Measure positive and negatively charged ions in the same analysis
- Selected ion monitoring
- Partially orthogonal to MS

Almost anything that can be ionized can be detected by IMS. Hence the technique encompasses a range of molecules with molar masses from that of helium to complex mixtures such as proteomes and even bacteria. It can be used in either stand-alone, or portable instruments and has been used to detect atmospheric compounds, chemical warfare agents, and petrochemicals. IMS technology has also been used to detect explosives and drugs using airport scanner devices. Such systems also find use in monitoring pharmaceutical preparations during manufacture. Coupling IMS with MS makes it possible to take advantage of IMS as a separation technique, allowing complex mixtures to be investigated and low-abundance species to be detected.

An ion mobility spectrometer has four regions: sampling, ionization, drift (separation), and detection (Figure 14.1). Ionization can be achieved with either radioactive sources, such as ^{63}Ni (c.f. ECD, Section 9.2.2.4) and ^{241}Am, or several of the ionization sources used in ambient pressure MS, including APPI, corona discharge, and laser desorption. The nature of the drift region varies depending on the type of IMS. Detection can be either a simple Faraday plate connected to an amplifier circuit, or ions may be passed to a second instrument. When coupled to MS, DART, DESI, paper spray, and laser ablation/desorption ESI (LADESI) have been used.

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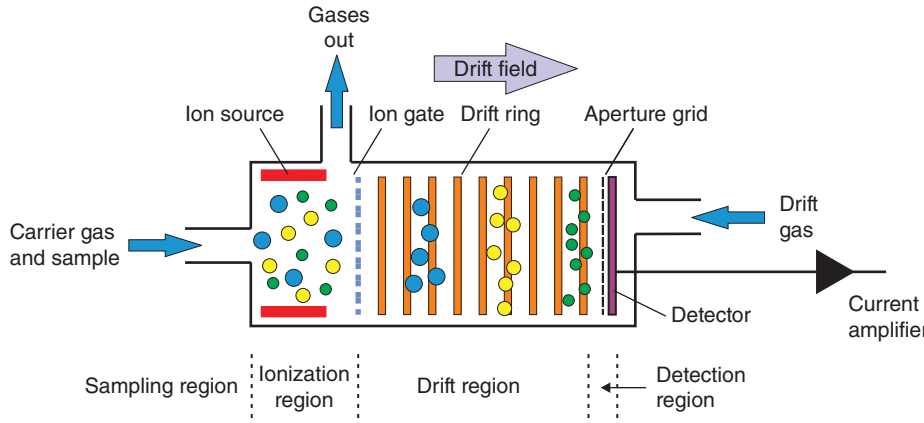

Figure 14.1 Schematic diagram of a basic ion mobility spectrometer

14.1.1 Interactions with buffer gas

IMS is conducted in the presence of a carrier gas and usually, but not always, a drift gas. The drift gas may be the same as the carrier gas. Other volatile materials ('dopants') such as acetone to react with primary amines or chiral selectors may be added. Dopants may either enhance, or supress the ionization. For convenience, the gas mixture may be referred to as the buffer gas. Ions are accelerated by the electric field, but collide with gas molecules, slowing them down or stopping them. Ions then accelerate again until they are slowed again, and so ions migrate with an average velocity. The mobility depends on the shape, mass, and charge on the ion.

Air and moisture may also be present. Depending on the ionization technique used, nitrogen and oxygen may be ionized, and reaction of these charged ions (N_2^+ and O_2^-) with water vapour produces a series of ions $(H_2O)_n(H_3O)^+$ and $O_2^-(H_2O)_n$, known as reactant ions. The peak(s) associated with these ions in the IMS spectrum is referred to as the reactant ion peak (RIP). Ammonium ions can also be formed, and the clusters with water, $(H_2O)_nNH_4^+$, may be seen as ammonia clusters before the RIP [Figure 14.2(a)].

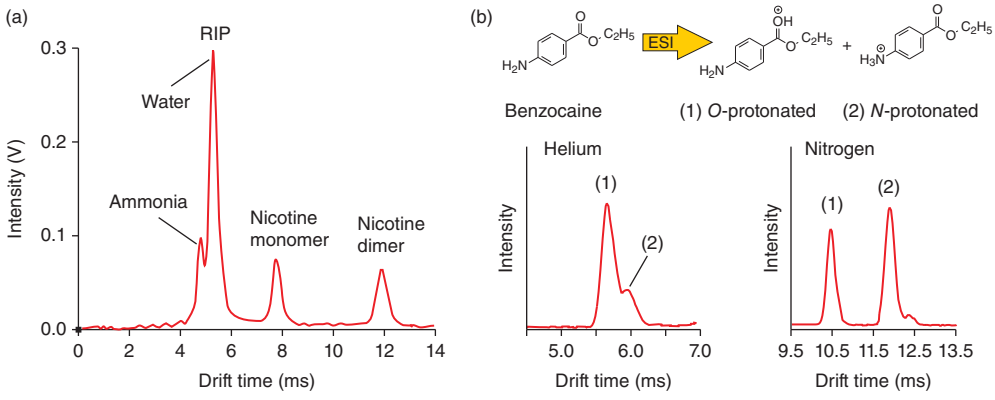

Figure 14.2 (a) Reactant ion peak and dimerization (O'Donnell *et al.*, 2008–reproduced with permission of Elsevier). (b) Effect of buffer gas on resolution of the tautomers of protonated benzocaine (Gabelica & Marklund, 2018–reproduced with permission of Elsevier)

Analyte molecules, M, may give positive and/or negative ions. They may also interact with reactant ions to form species such as MH^+, $(M–H)^-$, MO_2^-. At high analyte concentrations protonated dimers (M_2H^+) and trimers may be formed [Figure 14.2(a)]. The most commonly used dopants are acetone and ammonia for positive mode IMS, and chlorinated hydrocarbons to produce chloride ions for negative mode IMS. The use of dopants for IMS has been reviewed (Puton *et al*., 2008).

The selection of buffer gas can affect the separation markedly. For example, ionization of benzocaine in acetonitrile using ESI produces tautomeric *O*-protonated and *N*-protonated species that are not resolved if helium is used as the drift gas, but are resolved completely in nitrogen [Figure 14.2(b)]

14.2 Theoretical aspects

Ions moving in a gas are accelerated due to coulomb forces in the presence of the electric field and slowed down by collisions with gas molecules. At equilibrium, the ions have an average drift velocity, v_d, that is proportional to the electric field, E:

$$v_d = KE \tag{14.1}$$

where K is the ion mobility, usually expressed in $cm^2\ V^{-1}\ s^{-1}$. K can be calculated experimentally from the time, t_d, that it takes for an ion to transverse the length of the drift tube, L:

$$K = \frac{L}{t_d E} \tag{14.2}$$

Reduced ion mobilities, K_0, which are standardized for carrier/drift gas pressure, p and temperature, T (K) allow comparison of results across laboratories:

$$K_0 = K\frac{T_0}{T}\frac{p}{p_0} \tag{14.3}$$

where T_0 and p_0, are standard temperature (273.15 K) and pressure (1013 mbar), respectively. Ion mobility is also affected by the number density, N (the number of molecules per unit volume), and the collision cross-section (CCS, Ω) of the ion:

$$K = \frac{3}{16}\sqrt{\frac{2\pi}{\mu k_B T}}\frac{ze}{N\Omega} \tag{14.4}$$

where $\mu = mM/(m + M)$ is the reduced mass of the diffusing ions and carrier gas molecule (with respective masses of m and M), k_B is the Boltzmann constant, T is the gas temperature (K), z is the charge number and e is the elementary charge. Thus, at low field strengths, mobility is independent of E and the time that an ion needs to pass through the drift region is proportional to the inverse of the mobility. By rearranging Equation (14.4) a value for Ω can be calculated (Jiang & Robinson, 2013).

At higher field strengths, K becomes dependent on field strength and Equation (14.4) is no longer valid. Therefore, CCS can only be calculated in uniform low strength fields that are used in drift time ion mobility spectrometry (DTIMS). Field strength can be expressed in terms of N, the reduced electric field, E/N, usually expressed in Townsends ($Td = 10^{-21}\ V\ m^2$). The derived relationship is a Taylor series of the form:

$$K_0\left(\frac{E}{N}\right) = K_0(0)\left[1 + \alpha_2\left(\frac{E}{N}\right)^2 + \alpha_4\left(\frac{E}{N}\right)^4 \cdots\right] \tag{14.5}$$

Further terms in the series (not shown) make a negligible contribution to the value and are not usually included. Appreciating the complexity of this relationship is important for understanding the need for standardized conditions to generate databases to identify substances. The results from stand-alone IMS need to be referred to a suitable database of K_0 and Ω values (Gabelica & Marklund, 2018).

CCS can be derived from mobility experiments and values can also be calculated either from 3D-molecular modelling, or extrapolated from experiments using molecules of known CCS, to provide databases to aid compound identification. CCS has units of surface area and is usually reported as Å^2 (= 10^{-20} m^2 = 0.01 nm^2). Because structural isomers have different CCS values, IMS provides a way of identifying them that might not be possible by MS. Recommendations for standardizing the reporting K_0 and CCS values have been published (Gabelica *et al*., 2019).

14.3 Types of ion mobility spectrometry

Four modes of IMS are summarized in Table 14.1. With drift time- or temporally-dispersive IMS, the ions drift along a similar path and arrive at the detector at different times, thereby producing peaks as a function of time [Figure 14.3(a)], analogous to TOF-MS. Spatially-dispersive methods separate ions along different drift paths by scanning a voltage in a manner similar to scanning a quadrupole mass analyzer [Figure 14.3(b)]. Ion confinement and release methods are a recent addition to IMS because the ability to control the position of ions at elevated pressures with precisely tuned electrodynamic fields has become possible only recently. Ions are released individually as with ion-trap MS techniques [Figure 14.3(c)].

Table 14.1 Comparison of four modes of ion mobility spectrometry

	Drift time	High-field asymmetric waveform	Travelling wave	Trapped
Acronym	DTIMS	FAIMS	TWIMS	TIMS
Type	Temporal	Spatial	Temporal	Trapped
Electric field, *E*	Uniform Low *E*	Asymmetric High/Low *E*	Moving/ non-uniform Low *E*	Radio frequency Low *E*
Carrier gas	Yes	Yes	Yes	Yes
Drift gas	Yes	No	No	No
Dopant	Common	No	Uncommon	Common
Pressure	Ambient and above	Ambient and above	0.025–3 mbar	2.6–3.4 mbar
Analogous MS equivalent	Time of flight	Quadrupole	–	Ion trap
Hyphenated	Yes	Yes	Yes	Yes
Calculation of CCS	Yes	No	By calibration with species of known CCS	–
Orthogonal to MS	Partially	Yes	Partially	Partially

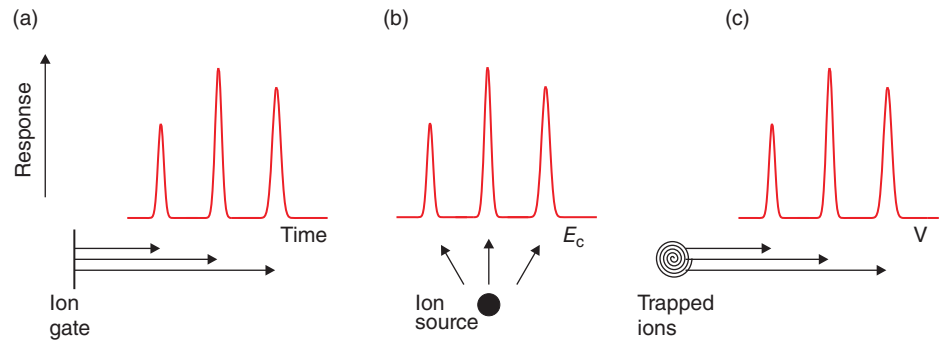

Figure 14.3 Basic ion mobility spectrometry operation. (a) Temporal. (b) Spatial. (c) Capture and release

14.3.1 Drift time ion mobility spectroscopy

In DTIMS the electric field is held more or less linear along the axis of the drift tube by a series of charged concentric rings (Figure 14.1). The sample is introduced into the ionization region in a carrier gas whilst a drift gas, often nitrogen, helium, or argon, is introduced at the detector end so as to flow in the opposite direction to the ion flow. The drift gas will remove neutral molecules from the drift tube and reaction chamber. Ions are held in the reaction region by an ion gate or shutter grid – parallel wires held at a potential to prevent passage of ions. When the potential is removed (typically for 10–100 μS) the ions are 'injected' into the drift tube. The duty cycle (i.e. the time before the next injection) is usually 20–100 mS.

The duration of the injection will affect resolution and the duty cycle may affect the number of scans that can be collected across a peak, for example. In the drift tube the field accelerates the ions, but they lose energy by colliding with neutral gas molecules. DTIMS is usually performed at low field strengths. The more mobile ions arrive at the detector first, this being an example of a temporal separation [Figure 14.3(a)].

Drift tubes are sold by several manufacturers as stand-alone devices for use as detectors in security applications. Modifications include miniaturization and resistive glass tubes rather than rings.

14.3.2 High field asymmetric waveform ion mobility spectroscopy

FAIMS utilizes the differences in the mobility of ions in high and low electric fields. The electrodes may be either cylindrical, or planar, when the technique may also be called differential mobility spectrometry (DMS). To avoid the use of excessively high potentials, FAIMS has been miniaturized so that the space between the electrodes in the drift tube is some 2–3 mm.

Two parallel electrodes form a channel through which ions can pass. One electrode is grounded and a high-frequency AC voltage is applied to the other electrode as a square waveform of a short-duration, high-amplitude positive potential followed by a long duration low-amplitude negative potential [Figure 14.4(a)]. The time period of each phase is such that the product of the electric field and the time period for which it is applied has a net value of zero over a single cycle.

As the ions are swept through the drift tube by the carrier gas, the field causes them to be displaced first towards one electrode, and then towards the other so that they travel in a saw-tooth

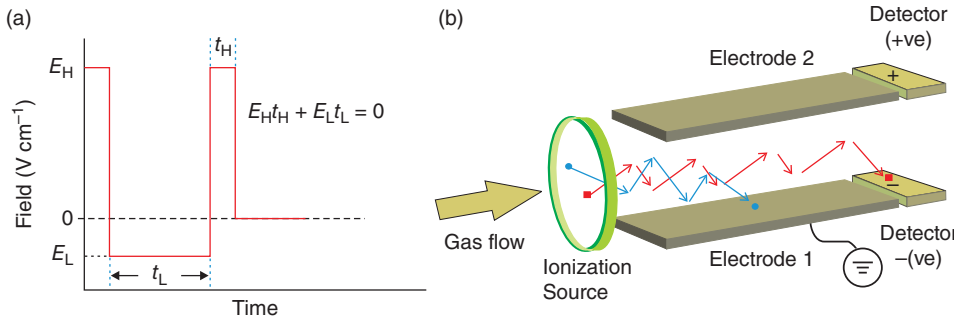

Figure 14.4 High field asymmetric waveform ion mobility spectrometry. (a) Waveform showing high (E_H) and low (E_L) field strengths. (b) Ions travelling in a saw-tooth path under the influence of the applied waveform to electrode 2 (data from Zrodnikov & Davis, 2012)

trajectory [Figure 14.4(b)]. Should an ion have equal mobilities in the high and low strength fields it will pass through the tube, otherwise it will have a net displacement toward one or other of the electrodes. DC compensation voltages (E_c) are applied to control the mobility of the ions. Each E_c value allows ion(s) with the corresponding specific differential mobility to pass through the drift tube. Ions are detected at a pair of electrodes that are either positively, or negatively biased, thereby allowing positive and negative ions to be measured in the same analysis. Alternatively, ions may be focused to another instrument ('ion filtering') such as a MS.

14.3.3 Travelling wave ion mobility spectrometry

TWIMS was developed for interfacing with MS. It is similar to DTIMS in that it has a series of concentric rings, but instead if a uniform field, RF frequency potentials are used to constrain the ions to the axis of the tube (Figure 14.5). Scanning is achieved by applying a pulsed DC voltage to each electrode in succession from one end of the drift tube to the other. After a fixed pulse dwell time, the voltage is stepped to the next electrode pair and so on through the device, creating a travelling wave.

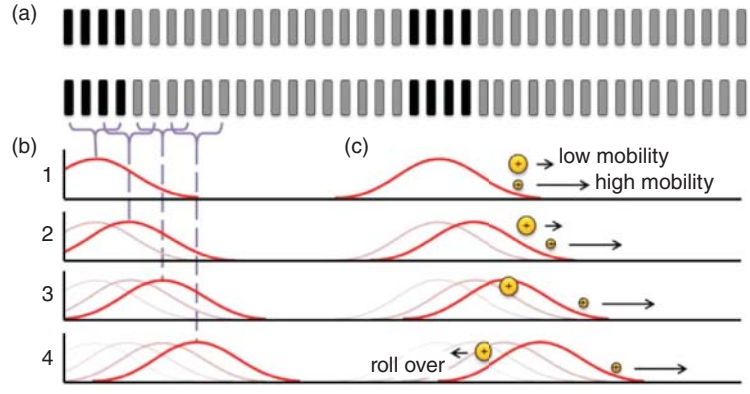

Figure 14.5 A stacked ring guide in travelling wave ion mobility spectrometry: generation of the wave (May & McLean, 2013–reproduced with permission of Springer Nature)

Ions in the drift tube experience the field of an approaching wave and start to drift through the gas in the corresponding direction. By altering the speed and magnitude of the travelling voltage wave, ions can be separated. The velocity of an ion is the product of its mobility and the field strength [Equation (14.1)], and when this equals the velocity of the wave, the ions travel with the wave. Less mobile ions are left behind or 'roll over' to the area of the tube at low potential. This delay results in the separation of different species. In a commercial instrument the DC potential is applied to rings 1–4 and then to rings 2–5, and so on, providing overlap and a smoother wave. Complex mixtures can be separated by sending several travelling waves through the device in quick succession.

14.3.4 Trapped ion mobility spectrometry

TIMS (Box 14.2) uses an ion funnel (Figure 14.6) to concentrate ions into the analyzer section where they are held against a flow of drift gas (Hernandez *et al*., 2014). Ions from an ESI source are deflected at right angles into the entrance funnel and from there into the separation region, where the weak electric field increases axially along the 'tube'. Ions are trapped in regions where their mobility is balanced by the force of the drift gas. Consequently, larger ions move further into the analyzer. When the field strength is decreased, ions emerge in order of largest to smallest size to charge ratios.

Box 14.2 Advantages of trapped ion mobility spectrometry

- High transmission efficiencies in MS and IMS-MS modes
- Compact design – integration into many MS instruments
- Calculation of K_0 or CCS from a simple calibration
- Low operating potentials
- Ability to alter ion mobility resolving power and duty cycle
- Superior mobility resolving power >250

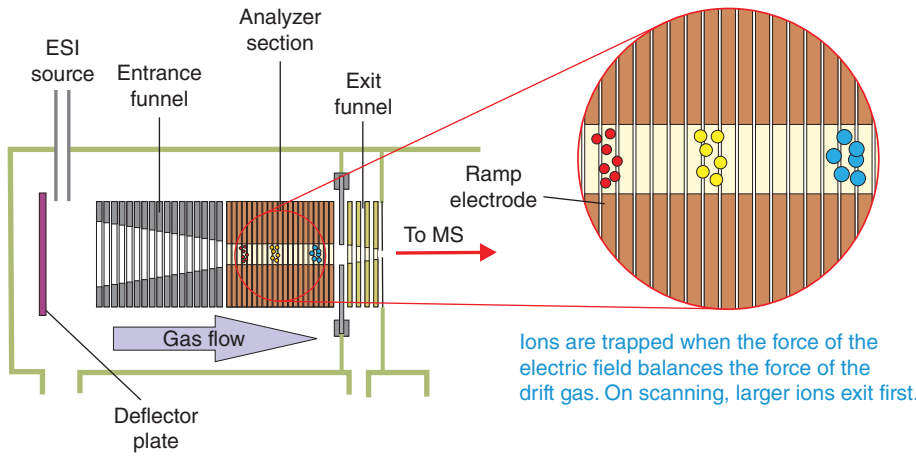

Figure 14.6 Trapped ion mobility spectrometry

14.4 Resolving power

In MS, the resolving power can be defined as the peak centroid divided by the width of the peak at half height and represents the upper mass value for which two ions differing by 1 u can be resolved at half their peak height (Section 13.2). Thus, a MS with a resolving power of 800 can separate (at half height) two ions with masses of 800 and 801 u. However, for IMS different separation techniques require different definitions of resolving power.

For DTIMS, temporal terms ($t/\Delta t$) may be used and for TWIMS, resolving power may be reported in terms of the ion collision cross-section ($CCS/\Delta CCS$), whilst in FAIMS and DMS, the compensation potential ($E_c/\Delta E_c$) is commonly used. Although a universal definition of ion mobility resolving power based on either the collision cross-section, or reduced mobility would be desirable, deriving such parameters from mobility measurements requires a correction or calibration procedure that introduces additional errors and complexity. Because direct comparisons of resolving power values across different ion mobility techniques is difficult, it has been suggested that such comparisons should be avoided unless a common frame of comparison is used (May & McLean, 2015).

14.5 Interfacing ion mobility spectrometry

Coupling IMS to a gas phase separation technique such as GC would seem a logical development. However, there were problems initially including column bleed, carrier gas impurities, and large void volumes (Kanu & Hill, 2008). Early studies showed that IMS operated in the positive mode generally had greater sensitivities than FID, while the results from negative IMS were comparable to ECD.

As well as conventional packed and capillary columns, multi-capillary columns (MCC), which consist of 1000 or more short capillaries in parallel, have been used, particularly for VOCs. MCCs allow rapid separations at high flow rates (>150 mL min^{-1}) that are compatible with IMS. The columns have been used with FAIMS for the detection of explosives (Buryakov, 2004) and with DTIMS for breath analysis to measure VOCs as an aid to disease diagnosis (Junger *et al*., 2010). A tutorial on GC-IMS has been published (Vautz *et al*., 2018).

Headspace sampling with SPME has been employed, sometimes in conjunction with GC (Alizadeh 2008). Thermal desorption interfaces have been developed especially for coupling to IMS (Perr *et al*., 2005; Rainsberg & Harrington, 2005).

Laser desorption has been used to sample analyte on TLC plates (Ilbeigi *et al*., 2016). The materials examined included explosives, nicotine, diphenylamine, alanine, and isoleucine.

There are several reasons for coupling IMS and MS. One is during IMS method development to identify the ions produced by IMS. Others include reduced noise, increased peak capacity, structural identification without reference compounds, and to distinguish isomers and isobars. Use of IMS prior to MS has proved to be a means of reducing noise by filtering ions that are not of interest. This is usually applied when there is no chromatographic separation. Increased peak capacity is achieved when the IMS and MS separations are orthogonal so that a large number of compounds can be represented on a 2D-plot of mobility versus m/z.

Kaszycki *et al*. (2019) have described coupling of DTIMS to an OT-MS. Development of techniques to produce electrodynamic fields to confine, transfer, and focus ions across different pressure regions into high vacuum regions has facilitated the coupling of IMS and MS. Interfacing IMS and MS has been reviewed (Cumeras *et al*., 2015a,b), as has interfacing MS with temporal IMS (May & McLean, 2015). The IMS may precede the MS (IMS-MS)

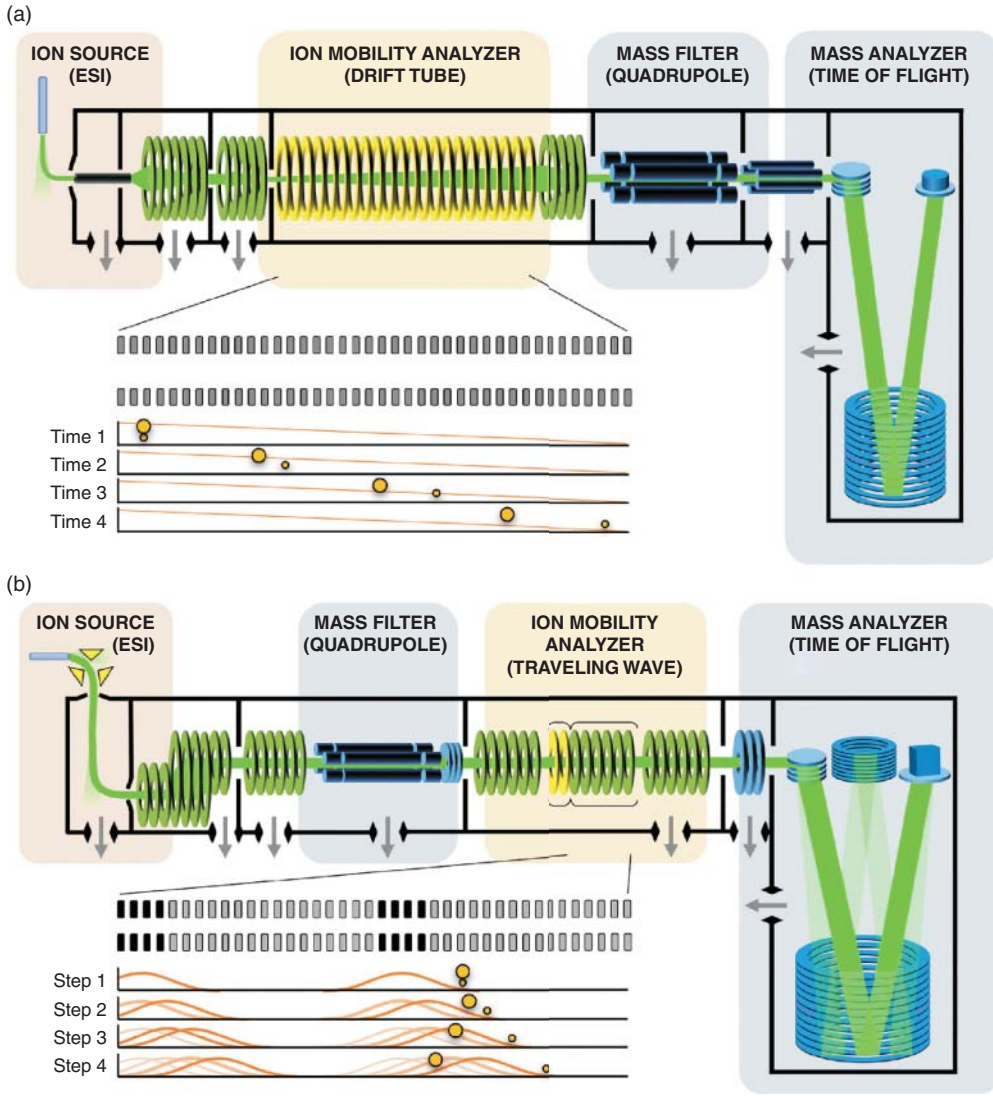

Figure 14.7 Ion mobility spectrometry-mass spectrometry. (a) Drift time ion mobility spectrometry. (b) Travelling wave ion mobility spectrometry (May & McLean, 2015–reproduced with permission of the American Chemical Society)

[Figure 14.7(a)] or may be positioned to examine the mobilities of MS fragment ions (MS-IMS) [Figure 14.7(b)].

Calculation of CCS values allows identification of unknown compounds when reference material is not available. In a study of ondansetron metabolism, Dear *et al.* (2010) used LC-TWIMS-TOF-MS to assign the position of the hydroxyl group in three isomeric metabolites. CCS values were calculated for 6-, 7-, and 8-hydroxyondansetron, from which IMS mobilities were calculated and compared with the experimental values. Cuyckens *et al.* (2011) used LC-MS-FAIMS to differentiate 2- and 3-hydroxycarbamazepine, and carbamazepine-10,11-epoxide via investigation of the mobilities of MS product ions.

14.5.1 Selected ion flow tube mass spectrometry

SIFT-MS is a gas phase analytical method that can be applied to moist ambient air and exhaled breath samples and has found use in environmental monitoring and as a non-invasive tool in clinical diagnosis and therapeutic monitoring. Unlike IMS, reactive ions, typically H_3O^+, NO^+, or $O_2{}^+$ that do not react with the major components of air or breath, are produced in a microwave plasma ion source and selected using a quadruple mass filter before being directed into the flow tube via a helium carrier at ~100 Pa. A selected reactant ion reacts with analyte molecules and the product ion(s) are diverted to a quadrupole mass analyzer (Figure 14.8).

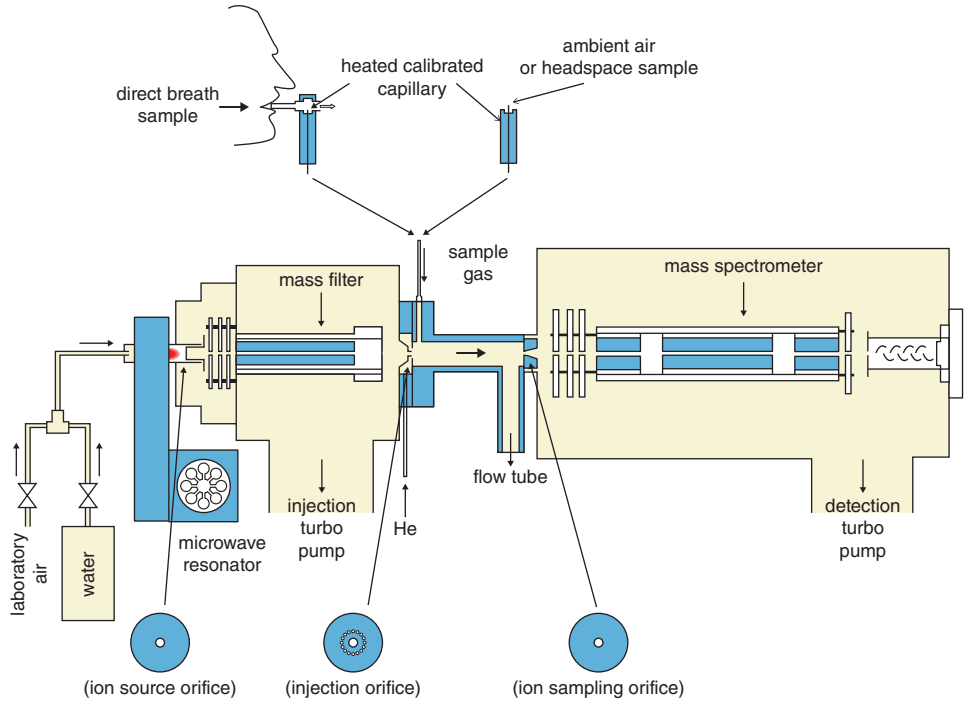

Figure 14.8 Schematic diagram of SIFT-MS (Smith & Španěl, 2011–reproduced with permission of the Royal Society of Chemistry)

Because ionization is very soft there is little fragmentation. Selectivity can be imparted by use of different reactant ions, for example methane ionizes with $O_2{}^+$, but not with H_3O^+, whilst methanol, hydrogen sulfide, hydrogen cyanide, and ammonia do not react with NO^+. Furthermore, different product ions may be formed, for example, dimethyl sulfide forms a pseudomolecular ion, MH^+, with H_3O^+, but introduction of NO^+ results in charge transfer giving M^+. Thus, by judicious selection of reactant ions different analytes may be distinguished.

SIFT finds applications in experimental physiology and clinical diagnosis for which volatile biomarkers for cancer, inflammatory bowel disease, renal function, and bacterial infection can be monitored. Environmental, health and safety, and security applications include monitoring of exhaust gases, atmospheric pollutants, inside cargo containers, and explosives residues. Volatile

drugs and their metabolites (ethanol, the components of cannabis and tobacco smoke, etc.) may be monitored during substance misuse treatment programmes (Smith & Španěl, 2015). Breath may be sampled directly (real-time analysis), via a Tedlar collection bag, or samples may be introduced by headspace analysis.

14.6 Applications of ion mobility spectrometry in analytical toxicology

14.6.1 Direct analysis

Eiceman *et al*. (1995) monitored exposure to nicotine during the manufacture of transdermal patches. Personnel wore individual air sampling devices and sampling devices were stationed in regions of interest within the facility. IMS was conducted using a ^{63}Ni ionization source and water vapour as a reactant gas, which produced protonated nicotine monomers and dimers similar to those shown in Figure 14.2(a). The use of IMS allowed continuous monitoring (every 60 s) of air quality with LoDs of 0.17 and 0.02 μg m^{-3} of nicotine in 1 h and 8 h samples, respectively. The previous method required analyte to be collected on XAD-2 resin, which was transferred to autosampler tubes and eluted with ethyl acetate, prior to analysis by GC.

Air sampling has been used to detect drug residues on the skin of patients suspected of drug overdose (Nanji *et al*., 1987). The residues were collected from the palms, fingers, injection site, and nostrils if insufflation was suspected by suction into a nickel tube with a platinum mesh. The samples were vaporized by thermal desorption and analyzed directly by IMS, allowing drug identification within 20 s. The number of drugs taken by 74 patients was 101. Of these, 50 involved ingestion of tablets, 47 were ingestion of film, sugar-coated, or varnished tablets and capsules. Four patients had sniffed cocaine, all instances of which were detected by IMS. The success rate with tablets was 42 % while that for the coated tablets and capsules was 29 %. The success rate was higher when sampling was carried out within 30 minutes of arrival at the emergency room.

Thermal desorption is not suitable for all analytes, but ESI provides a way of desolvating and ionizing samples prior to IMS. Midey *et al*. (2013) used a commercially available bench-top ESI-IMS system to develop a method for codeine and morphine, which was validated using samples in synthetic urine. ESI solution was prepared by diluting urine 1+9 with methanol:water (9+1, v/v) (Figure 14.9). However, the authors did point out that to avoid charge suppression further sample clean-up such as MEPS (Section 4.2.3) might produce better results. Literature values of reduced mobilities for codeine, morphine, metamfetamine, MDMA, MDEA, PCP, and Δ^9-THC were given. Positive mode K_0 values were calibrated against nicotinamide, whilst negative mode IMS (which was used for phenobarbital) reduced mobilities were calibrated with 1,2,4,5-benzenetetracarboxylic acid (pyromellitic acid, 1.32 cm^2 V^{-1} s^{-1}).

Sheibani & Haghpazir (2014) extracted added tramadol from plasma, serum, and urine samples. Tramadol in plasma was extracted with ethyl acetate, but C_{18} SPE columns were used for serum and for urine. Extracts dissolved in methanol were injected (1 μL) into the ionization region (corona discharge) and the ions separated by DTIMS. LLoQs were 0.4, 1.0, and 0.3 μg L^{-1}, for plasma, serum, and urine, respectively. Patient samples were not tested, and the authors admitted that further sample preparation might be needed for authentic samples.

The K_0 values of NPS from several classes including phenethylamines, cathinones, cannabinoids, and tryptamines have been measured using IMS with thermal desorption and ^{63}Ni ionization using dry air as drift gas (Armenta *et al*., 2015). The substances were also characterized by

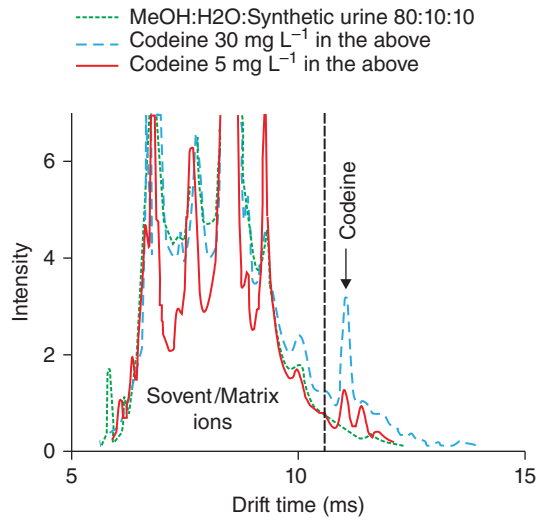

Figure 14.9 Ion mobility spectrometry of codeine in synthetic urine (Midey *et al.,* 2013–reproduced with permission of Elsevier)

GC-MS, LC-DAD, and IR spectroscopy. The mobilities of some primary amines were measured before and after derivatization with several ketones, chiefly acetone.

Metternich *et al*. (2019) used a commercially available DTIMS instrument with corona discharge ionization to measure the mobilities of 25 synthetic cannabinoids with a view to detecting these when they had been trafficked into prisons in herbal mixtures, food, cosmetics, or on paper. K_0 values were calibrated against nicotinamide. When tested in the field, synthetic cannabinoids were detected in 12 out of 36 samples. The major limitation of the method was the inability to detect more than one analyte in a sample, although a positive result was enough to alert the authorities to trafficking.

IMS has found use in POCT. One such application is monitoring propofol during surgery. Plasma was applied to glass fibre filter papers from which the drug was thermally desorbed into the IMS. Total analysis time was 1 min. The LoD was 0.1 mg L^{-1}, and comparison with conventional LC-UV showed a fixed bias of 0.15 mg L^{-1} (Wang *et al*., 2016).

14.6.2 Interfaced techniques

HS-SPME-IMS has been used to detect volatile substances (‘signature odours’) associated with some drugs. The presence of cocaine, marijuana, and MDMA was demonstrated by identifying the volatile markers methyl benzoate, piperonal, and terpenes (limonene, and α- and β-pinene), respectively (Lai *et al*., 2008).

MDMA and metamfetamine have been assayed in synthetic serum samples after HS-SPME and thermal desorption. Ions were produced by corona discharge and separated by DTIMS. LoDs of 8 and 5 μg L^{-1} were reported for MDMA and metamfetamine, respectively (Alizadeh, 2008).

Codeine and morphine have been measured in urine with LoDs of 20 and 60 μg L^{-1}, respectively, using ESI-FAIMS-MS. Urine samples were diluted 1+99 with methanol prior to introduction into the ESI. The compounds produced a peak in the IMS spectrum at the

same compensation voltage and, therefore, were identified from their pseudomolecular ions. However, the use of FAIMS markedly reduced the signal to noise ratio when compared with direct ESI-MS (McCooeye *et al*., 2001).

The structural isomers hydromorphone, morphine, and norcodeine have been differentiated using ESI-FAIMS-MS (Manicke & Bedford, 2015), something that is difficult when using MS/MS alone. Compound identities were confirmed using a triple quadrupole and the appropriate ion transitions (*m*/*z* 286 → 165 and 286 → 185). The isomers can be separated by LC, but this work illustrates the separations and rapid analysis times that are achievable with FAIMS (Figure 14.10).

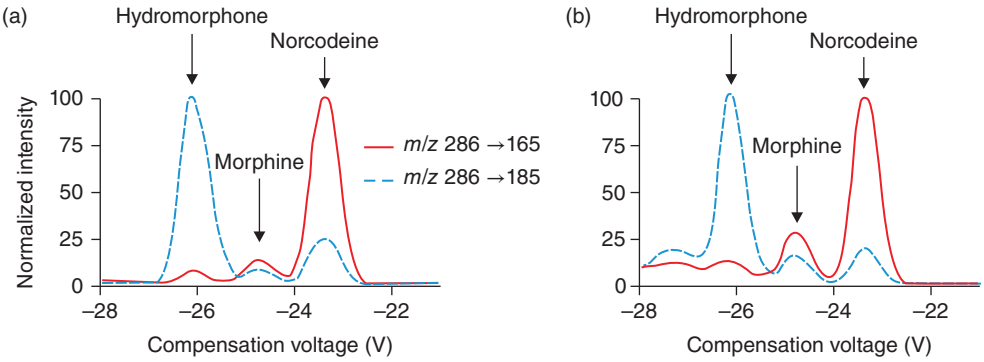

Figure 14.10 FAIMS compensation voltage scans for a mixture of hydromorphone, morphine, and norcodeine using (a) H-ESI and (b) paper spray (redrawn from Manicke & Bedford, 2015–reproduced with permission of American Society for Mass Spectrometry)

Dempsey *et al*. (2018) have described a FAIMS-MS/MS method for BE, cocaine, ecgonine methyl ester (EME), and cocaethylene in human plasma. Samples were prepared by adding acetonitrile (200 µL) to serum (100 µL) and centrifuging. The supernatants were transferred to autosampler vials. ^{2}H-Labelled analogues were used as ISTDs. Samples were introduced into the IMS via an autosampler (compare FIA-MS, Section 13.7.1).

The method was fully validated and applied to serum samples that had been shown to be positive for cocaine and/or BE. The results were compared with those from an LC-MS/MS study (Bland–Altman plots). Variability between the two methods was ≤30 % for cocaine and ≤25 % for BE. The mean bias between FAIMS-MS-MS and LC-MS-MS was 15.3 µg L^{-1} for cocaine and −1.0 µg L^{-1} for BE. EME was detected, but at concentrations (≤10 µg L^{-1}) below the LLoQ. Cocaethylene was not detected in any sample.

14.6.3 Chiral separations

Gas phase chiral separations have been achieved by adding a chiral gas to the drift gas. Dwivedi *et al*. (2006) used *S*-(+)-2-butanol as a chiral modifier to investigate enantiomeric separations of a range of compounds including atenolol, serine, methionine, threonine, methyl-α-glucopyranoside, glucose, and penicillamine. The separation of methionine enantiomers increased with increasing flow rates of modifier up to a maximum (~ 10 ppm in nitrogen drift gas) and was slightly greater with *S*-(+)-2-butanol than *R*-(–)-2-butanol. The order of mobilities of the methionine isomers was reversed when *R*-(–)-2-butanol was substituted for

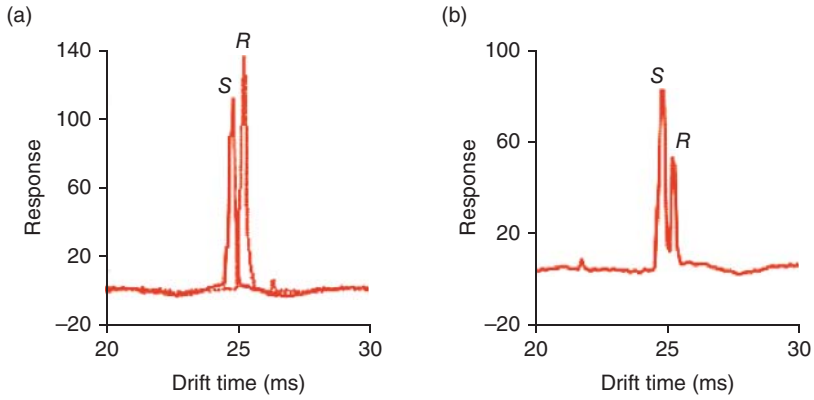

Figure 14.11 Ion mobility spectrometry of atenolol. (a) Analysis of individual enantiomers. (b) Analysis of a mixture of (*R*)-atenolol and (*S*)-atenolol (adapted from Dwivedi *et al.,* 2006–reproduced with permission of the American Chemical Society)

its antipode, as would be expected for a chiral interaction. Despite the low separation ratio, near-baseline separation of atenolol enantiomers was achieved (Figure 14.11).

Other gas-phase chiral separations have involved adding chiral selectors to the sample before ionization. Amino acid enantiomers have been separated as copper complexes (Yu & Yao, 2017). Zhang *et al*. (2018) used *N-tert*-butoxycarbonyl-*O*-benzyl-L-serine as a chiral selector to separate amino acids without complexation with metal ions.

14.7 Summary

IMS is a powerful analytical technique that has several capabilities as a stand-alone method to detect atmospheric compounds, explosives, chemical warfare agents, and drugs. Breath sampling allows detection of VOCs. Interfacing with MS allows analysis of complex mixtures, providing structural information and increased peak capacity.

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Section C
Essential Pharmacokinetics

15 Absorption, Distribution, Metabolism, and Excretion of Xenobiotics

15.1 Introduction

The absorption, distribution, metabolism, and excretion of substances in the body is often given the acronym ADME, and may be referred to collectively as xenobiotic or drug disposition. Almost all substances can be poisons depending not only on the dose, but also on other factors such as the way in which exposure occurred, whether the subject has been exposed to the compound previously, or whether other substances have been taken at the same time. Efficacy, in the case of drugs, and toxicity, in the case of poisons, can be affected markedly by ADME. There may be different effects when a substance is inhaled rather than swallowed, for example. Metabolism may 'detoxify' some xenobiotics, but activate others. Organ specific distribution, may in part, explain the localized toxicity of some xenobiotics. Metabolism and excretion (elimination) will influence the duration of any effects. The study of the rates at which these various processes occur is pharmacokinetics (Chapter 16).

In order to help to interpret the results of analytical toxicological measurements in body fluids, it is important to understand the ADME of xenobiotics, and the factors that affect these processes, such as enzyme induction and inhibition, drug–drug interactions and, intra- and inter-subject variations. Some of these variations may be a result of genetic differences. Pharmacogenetics is the study of how polymorphisms in gene expression (the phenotype) affect responses to xenobiotics whereas pharmacogenomics is the study of which genes are present – an individual's genome.

15.2 Movement of drugs and other xenobiotics around the body

To be effective, drugs have to travel from the site of administration to their site(s) of action. They are also distributed to other sites, which can influence the fluids and tissues that are taken for analysis (Table 2.2). Drugs are carried in the bloodstream and other flowing fluids, such as lymph, entering some tissues, but being excluded from others. This process is influenced not only by the physicochemical nature of the drug, but also by the nature of the membrane. There are three basic types of blood capillary: continuous, fenestrated, and sinusoidal. Small M_r drugs readily diffuse through peripheral capillary walls that have gaps or fenestrations. HSA

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Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

(M_r approximately 66,500) is too large to diffuse from plasma and is excluded from interstitial fluid. At the glomerulus substances up to about the size of albumin pass freely into renal tubular fluid, while the gaps in liver sinusoids are large enough for macromolecules such as lipoproteins to enter hepatocytes.

Some membranes, such as the GI epithelium, the blood–brain barrier (BBB), the blood–CSF barrier (BCSFB), blood–retinal barrier (BRB), and placenta, prevent or limit the entry of substances, Consequently, the concentrations of drugs and drug metabolites on either side of such barriers may be markedly different. Furthermore, the composition of the fluids on either side of a barrier is often different, one side perhaps lacking high concentrations of proteins, for example. Thus, some xenobiotics may be found in high concentrations in some tissues and not in others. Generally, drugs and other xenobiotics cross these membranes by either passive diffusion, or carrier-mediated transport.

15.2.1 Passive diffusion

Passive diffusion refers to the situation when a substance dissolves in the lipid membrane to enter cells. The direction of transfer is controlled by the concentration gradient on either side of the membrane, net movement being from high to low concentration. The rate of transfer is proportional to the concentration gradient and the surface area over which transfer is occurring, but inversely proportional to the thickness of the membrane. The specialized epithelial cells of the small intestine have rows of microvilli that vastly increase the effective surface area for absorption.

15.2.1.1 pH-Partition relationship

Generally, lipophilic (lipid-loving) substances readily dissolve in the lipid bilayers of cells and are rapidly absorbed as well as also being widely distributed in tissues, and extensively metabolized prior to excretion. Partition coefficients, usually measured using octanol/water, are used to access lipophilicity. Generally, partition coefficients (and lipophilicity) increase with increasing hydrocarbon chain length, numbers of aromatic moieties, and so on, and decrease with increasing numbers of polar groups, such as hydroxyl and carbonyl.

Ionization markedly reduces lipophilicity and can have major effects on passive diffusion. Acids lose protons (deprotonate) to ionize, and bases become ionized when they are protonated. The degree of ionization of a weak acid or base (weak electrolyte) is a function of the pH of the environment in which it is dissolved and the pK_a of the ionizable moiety, and can be calculated from the Henderson–Hasselbalch equation. For an acid, AH:

$$\mathrm{p}K_a = \mathrm{pH} + \log \frac{[\mathrm{AH}]}{[\mathrm{A}^-]} = \mathrm{pH} + \log \frac{(1-\alpha)}{\alpha} \tag{15.1}$$

where α is the degree of ionization.

The distribution of aspirin between gastric contents and plasma water illustrates the effect that differences in pH can have on the tissue distribution of a drug. Aspirin (pK_a = 3.4) is largely unionized (99.01 %) at gastric pH, but highly ionized (99.99 %) at the pH of plasma (Figure 15.1). At equilibrium, for every ionized aspirin molecule in gastric acid there will be 100 unionized molecules. Because it is the unionized aspirin that equilibrates across the membrane, there will be the same number (100) of unionized molecules in plasma. However, in plasma there will be 1,000,000 ionized molecules for every 100 non-ionized aspirin molecules. Thus, aspirin is absorbed from the stomach. Despite the favourable pH of the stomach and the less favourable pH of the small intestine, approximately 75 % of an oral dose of aspirin is absorbed from the small intestine, illustrating the importance of the large surface area of this part of the GI tract.

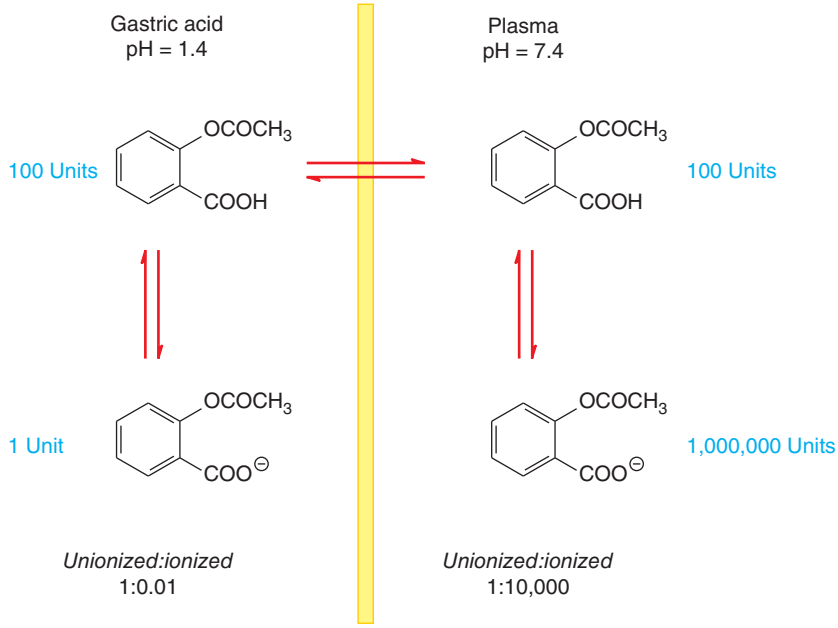

Figure 15.1 Distribution of aspirin ($pK_a = 3.4$) between gastric fluid and plasma water

The converse is true for bases. Amfetamine (pK_a 9.8) cannot be absorbed from the stomach because it is almost totally ionized at pH 1.4 ($\alpha = 4 \times 10^{-9}$). On the other hand, diffusion of basic drugs into the stomach can result in higher gastric concentrations than in plasma, a phenomenon referred to as 'ion-trapping'. Morphine, $pK_a = 8.0$, plasma protein binding <50 %, would be expected to attain higher gastric acid concentrations than those in plasma after i.v injection. Consequently, the presence of a basic drug in stomach contents does not necessarily indicate that the drug has been taken orally. Ion-trapping may be important with regard to distribution (Section 15.4.1), renal clearance (Section 15.6.1), and treatment of overdosage (Section 16.6.1).

15.2.1.2 Other physiochemical properties

Properties that determine the ability of a molecule to penetrate cells include the number of groups able to accept or donate hydrogen bonds (Lipinski *et al.*, 2001), the polar surface area (PSA), and the molar refractivity (i.e. the ability to form instantaneous dipoles). PSA is the total surface area over all polar atoms in a molecule, primarily oxygen and nitrogen, including any attached hydrogen atoms. Generally, molecules with PSA >140 $Å^2$ are not well absorbed after oral administration and to cross the BBB, PSA need to be <90 $Å^2$.

15.2.2 Carrier-mediated transport

There are two important superfamilies of transporters. The solute carrier (SLC) superfamily includes organic cation transporters (OCTs) that transport small cations such as choline, histamine, and norepinephrine, and the organic anion-transporting polypeptides (OATPs) of which there are several human isoforms found in brain, liver, kidney, small intestine, and many other

tissues (Table 15.1) (Roth *et al*., 2012, Zamek-Gliszczynski *et al*., 2018). OATPs in the small intestine are important for the absorption of some xenobiotics, for example, statins from the intestinal lumen. Levodopa, gabapentin, and methyl mercury (as an L-cysteine complex) are substrates for the large amino acid transporters, LAT1 and LAT2. Saturation of these transporters with increasing oral doses of such drugs can lead to non-linear kinetics (Section 16.6) as exemplified by gabapentin (Gidal *et al*., 1998).

Table 15.1 Selected examples of solute carrier (SLC) and ATP-binding cassette (ABC) transporters and their locations

Transporter	Gene	Selected localizations	Selected substrates	Selected inhibitors
OATP1A2	*SLCO1A2*	Brain Enterocytes (luminal) Kidney PCT (luminal)	Atenolol Ciprofloxacin Fexofenadine	Grapefruit juice Orange juice Verapamil
OATP2B1	*SLCO2B1*	Enterocytes (luminal) Hepatocytes (sinusoidal)	Aliskiren Atorvastatin Pravastatin	Ciclosporin Grapefruit juice Orange juice
OATP1B1	*SLCO1B1*	Hepatocytes (sinusoidal)	Pravastatin Rifampicin Simvastatin	Ciclosporin Lopinavir Rifampicin
OATP1B3	*SLCO1B3*	Hepatocytes (sinusoidal) Kidney PCT (basolateral)	Paclitaxel Pravastatin Rifampicin	Clarithromycin Ciclosporin Rifampicin
OAT1	*SLC22A6*	Kidney PCT (basolateral)	Captopril Cidofovir Furosemide	Indometacin Probenecid
OAT3	*SLC22A8*	Kidney PCT (basolateral)	Benzylpenicillin Furosemide Methotrexate	Indometacin Probenecid Salicylate
OCT2	*SLC22A2*	Brain Kidney PCT (basolateral) Lung	Cimetidine Cisplatin Metformin Ranitidine	Cimetidine Procainamide Ranitidine Trimethoprim
MATE1	*SLC47A1*	Hepatocytes (canalicular) Kidney PCT (basolateral) Skeletal muscle	Cefalexin Metformin Procainamide	Cimetidine Pyrimethamine Quinidine
PepT1	*SLC15A1*	Enterocytes (luminal) Kidney PCT (luminal)	Penicillins Cephalosporins Enalapril Valaciclovir	Glibenclamide Tolbutamide

Table 15.1 (*Continued*)

Transporter	Gene	Selected localizations	Selected substrates	Selected inhibitors
P-gp	*ABCB1*	Adrenal gland Blood–brain barrier Blood–retinal barrier Enterocytes (luminal) Hepatocytes (canalicular) Kidney PCT (luminal) Placenta	Digoxin Docetaxel Fexofenadine Ritonavir Vinblastine	Clarithromycin Ciclosporin Itraconazole Quinidine Ritonavir Verapamil
MRP2	*ABCC2*	Enterocytes (luminal) Hepatocytes (canalicular) Kidney PCT (luminal)	Glucuronide and GSH conjugates Bromosulfophthalein	Ciclosporin Probenecid
BCRP	*ABCG2*	Blood–retinal barrier Enterocytes (luminal) Hepatocytes (canalicular) Mammary glands	Doxorubicin Methotrexate 5-Fluorouracil	Ritonavir Omeprazole

BCRP: breast cancer resistance protein; MATE: multidrug and toxin extrusion protein; MRP: multidrug resistance-associated protein; OAT: organic anion transporter; OATP: organic anion transporting polypeptide; OCT: organic cation transporter; PepT: peptide transporter; P-gp: P-glycoprotein; PCT: proximal convoluted tubule

The adenosine triphosphate (ATP)-binding cassette family (ABC transporters) are efflux pumps that carry substrates from the intracellular to the extracellular side of cell membranes (Yang, 2013). The energy is derived from the binding of ATP, allowing transport of substrates against their concentration gradients. The most studied member of the family is P-gp, which was first recognized in tumour cells that were resistant to a number of cytotoxic agents as a result of over-expression of what was referred to as the multi-drug resistance (*MDR1*) gene, now known as the *ABCB1* gene.

P-gp is highly expressed in the apical membrane of many cells. Other efflux pumps include multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP) (Mao & Unadkat, 2015). Because some transporters have been renamed, reference to the appropriate gene may be helpful (Table 15.1). Many of the observations that were once ascribed to enzyme induction or inhibition (i.e. to changes in drug metabolizing enzyme activity, Section 15.5.5) may in fact be due to changes in transporter activity.

15.3 Routes of administration

15.3.1 Oral dosage

Once swallowed a drug enters the normally acidic environment of the stomach where acid labile drugs may be destroyed, and proteins and peptides digested by proteolytic enzymes (Figure 15.2). Most absorption occurs in the small intestine. Highly ionized and polar molecules may not be absorbed unless they utilize specific transport mechanisms, as described above.

Important points about carrier-mediated absorption are (i) it can become saturated at high substrate concentrations, and (ii) it can be inhibited by other molecules. In some situations, after drug overdose for example, this can result in non-linear kinetics (Section 16.6).

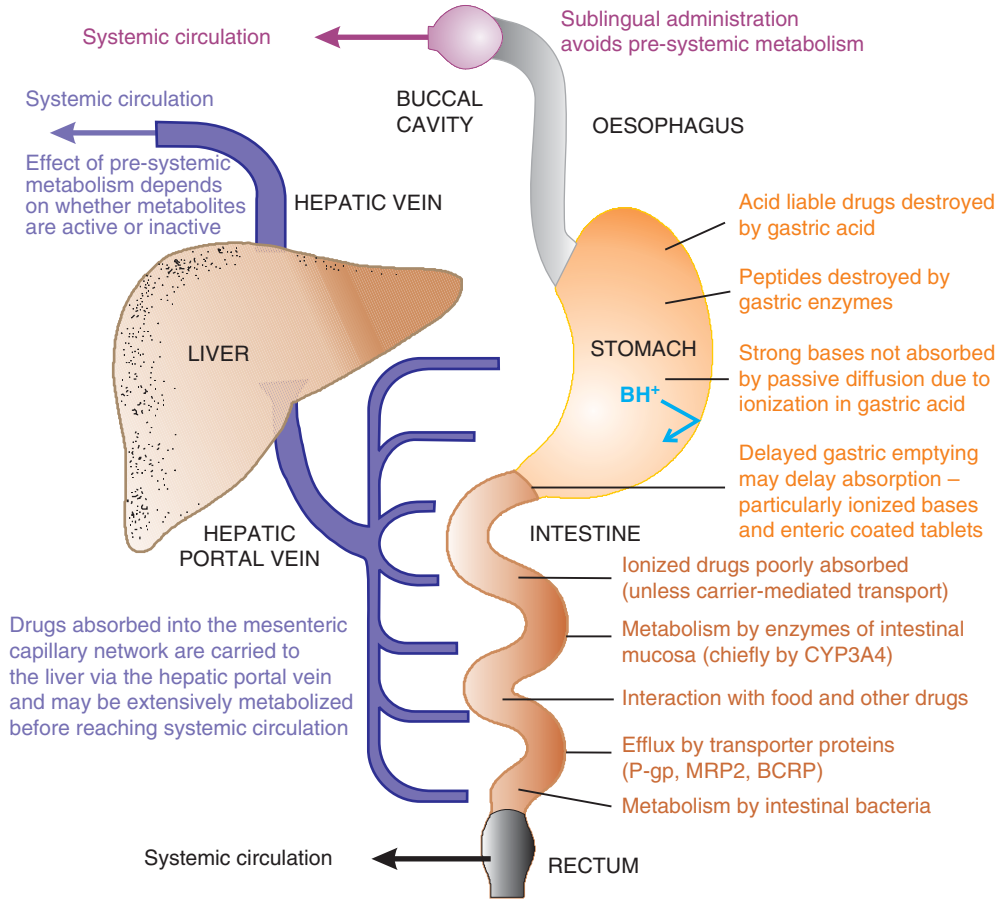

Figure 15.2 Some of the factors that may reduce oral availability. Other factors include GI motility, absorptive surface area, and splanchnic blood flow. MRP2, multidrug resistance-associated protein 2; BCRP, breast cancer resistance protein (Curry & Whelpton, 2017–reproduced with permission of John Wiley & Sons)

Alterations in pharmacokinetics after overdosage can have major implications for the interpretation of analytical results, and importantly, treatment. The duration of effect may be considerably longer when compared with what might be predicted from kinetic parameters derived from studies employing the doses used in therapy, therefore requiring prolonged treatment (Section 16.6.1). Moreover, substances absorbed across the mucosal membrane may not reach the systemic circulation. Either efflux pumps (Table 15.1) may return them to the gut lumen,

or those that reach the hepatic portal vein may be subject to extensive pre-systemic metabolism (Section 15.5.1.1).

Drug–drug interactions are another source of delayed or erratic absorption. Opioids and anti-muscarinic drugs may delay gastric emptying and reduce gut motility, and this may reduce the bioavailability of co-administered compounds. Drugs may interact in other ways (Table 15.2).

Table 15.2 Examples of reduced oral bioavailability or delayed absorption

Mechanism	Example	Notes
Destroyed by gastric acid	Benzyl penicillin (penicillin G) Methicillin	Penicillins with electron-withdrawing groups (e.g. penicillin V) less affected
Digested by proteolytic enzymes	Insulin Oxytocin	
Poorly/erratically absorbed	Tubocurarine Aminoglycoside antibiotics Pyridostigmine	Give i.v. or via large oral dosage (e.g. pyridostigmine)
First-pass metabolism	Glyceryl trinitrate	Section 15.3.1.1
Interaction with other drugs/food	Tetracycline antibiotics/divalent metal ions (Fe^{2+}, Ca^{2+}, Mg^{2+})	Absorption of both reduced
Delayed gastric emptying and reduced gut motility	Opiates, anticholinergic drugs	May reduce amount absorbed
P-glycoprotein efflux	Fexofenadine	

15.3.1.1 Pre-systemic metabolism

A substance absorbed into the mesenteric capillaries is carried via the hepatic portal vein to the liver where it may be so extensively metabolized that little of it reaches the systemic circulation (Figure 15.2). The phenomenon is frequently referred to as ‘first-pass’ metabolism reflecting that this is the first time the drug has entered the liver. Some drugs and poisons may be inactivated by first-pass metabolism if the metabolites are inactive. This is the case with glyceryl trinitrate (GTN) so it is usually administered via alternative routes. Large doses of some drugs, for example propranolol, saturate first-pass metabolism and the oral bioavailability increases with increasing doses. Clearly, this may be important after overdosage.

Pre-systemic metabolism may occur at sites other than the liver such as the gastric mucosa and the lungs, the metabolism of β-adrenoreceptor agonists being an example. Some drugs (pro-drugs) are not active until they have been metabolized and pre-systemic metabolism may play an important role in this activation. Inhibition of first-pass metabolism may produce unwanted effects. The non-sedating antihistamine terfenadine normally underwent extensive pre-systemic metabolism to an active metabolite, fexofenadine. However, when the enzyme responsible (CYP3A4, Section 15.5.1.1) was inhibited by drugs such as erythromycin or ketoconazole, sufficient terfenadine was absorbed to cause fatal ventricular arrhythmias in susceptible patients.

15.3.2 Intravenous injection

Drugs may either be injected as a single (bolus) dose or infused over extended periods. Drugs given i.v. include *N*-acetylcysteine (NAC), an antidote to paracetamol poisoning, which has low oral bioavailability, general anaesthetics (e.g. propofol), other drugs used in surgery such as skeletal muscle relaxants (e.g. atracurium), and neostigmine (used to reverse the effect of atracurium). The latter drugs are quaternary ammonium compounds and are extremely poorly absorbed from the GI tract if given by mouth.

15.3.3 Intramuscular and subcutaneous injection

Intramuscular (i.m.) and subcutaneous (s.c.) injections avoid the problems of oral administration but, unlike i.v. injection, the drug must be absorbed from the injection site. There is no immediate peak plasma concentration, and the onset of action may be either quicker, or slower than after oral dosage. Slow release from muscle is exploited in the case of depot injections of drugs such as procaine penicillin G and fluphenazine decanoate. Low (<5000) M_r drugs, including quaternary ammonium compounds, are readily absorbed after i.m. injection.

15.3.4 Sublingual and rectal administration

Drugs absorbed from the buccal cavity and lower rectum do not enter the hepatic portal circulation and so avoid first-pass metabolism (Figure 15.2). Sublingual (under the tongue) administration is particularly useful for GTN, which is rapidly absorbed from the buccal cavity to give relief from the pain of angina. Buprenorphine and fentanyl are available as sustained-release (SR) lozenges for sublingual administration.

Rectal administration may be used to give antiemetics such as prochlorperazine when the oral route is unlikely to be suitable. Diazepam suppositories are available for use in epileptic infants when attempts to establish i.v. access in a convulsing child may be dangerous. The rectal route is also used for compounds that cause gastric irritation and to give a prolonged duration of action, as is the case with aminophylline, which can be given rectally to asthmatic children.

15.3.5 Intranasal administration

Although drugs may be applied to the nasal passages for local effect, lipophilic drugs are rapidly absorbed into the systemic circulation. The structure of the nose, with its rich blood supply, highly fenestrated capillaries, and an epithelium layer with gaps around the goblet cells (Bourganis *et al*., 2018), allows absorption of molecules that cannot be given orally, such as peptide hormones including insulin, calcitonin, and desmopressin (Türker *et al*., 2004). Intranasal neostigmine has been shown to protect against Indian cobra envenomation (Lewin *et al*., 2014).

With regard to misused drugs, cocaine is rapidly absorbed from the nasal cavities after insufflation of crystals. Some of the dose bypasses the systemic circulation, reaching the brain via the olfactory bulb and CSF.

15.3.6 Transdermal administration

Transdermal drug administration rather than topical administration for local effect is primarily limited to potent, lipophilic drugs. Skin is a major barrier for many drugs and even very lipophilic

drugs may be slowly absorbed. Consequently, transdermal administration, either as ointments or sticking plasters, is a useful way of increasing the duration of action of these drugs. Available drugs include hyoscine (motion sickness), oestrogens (hormone replacement therapy, HRT), nicotine (tobacco withdrawal), and buprenorphine and fentanyl (analgesia).

15.3.7 Inhalation

The large surface area of the lungs leads to rapid absorption of inhaled substances and hence a rapid onset of effect. Inhalational general anaesthetics are given in this way. The lungs are also the major route by which these drugs are eliminated. Therefore, the correct level of anaesthesia can be maintained by adjusting the concentration of the anaesthetic in the apparatus used to administer the drug.

Bronchodilators, such the β_2-adrenoceptor agonists (salbutamol, terbutaline), muscarinic antagonists (ipratropium), and steroids (beclomethasone), are also given by inhalation. The advantage is that because the drug is being administered to the site of action the dose is less than required if it were given orally, reducing unwanted systemic effects.

15.3.8 Other routes of administration

Intraperitoneal (i.p.) injection provides a convenient way of dosing laboratory animals. Many drugs are rapidly absorbed if given by this route, and injecting into the peritoneal cavity reduces the risk of damaging a major organ. Intravaginal applications of fungicidal creams are usually for a local effect on C*andida* infections (thrush), whereas prostaglandin pessaries (preparations designed to release drug after insertion into the vagina) are used to induce labour. Drugs applied to the cornea are usually for effects on the eye, but timolol eye-drops, applied for the treatment of glaucoma, have been known to cause bradycardia.

15.4 Distribution

Small molecules such as amoxicillin and tubocurarine that do not bind to plasma protein and do not penetrate cells are distributed in extracellular fluid. Molecules that enter cells, but do not bind to plasma protein or cell constituents such as ethanol are uniformly distributed in body water. Ethanol crosses the BRB and vitreous humour can be a useful post-mortem sample. After equilibration, ethanol tissue concentrations will be in proportion to the proportion of water in the tissue. Serum and plasma concentrations are some 1.16 times higher than those in blood, whilst a ratio of 1.18 has been reported for vitreous humour:femoral blood concentrations, although use of this latter ratio in post-mortem work is not advisable (Section 22.4.1.1).

The greater the proportion of drug in tissues the smaller will be the proportion, and hence for a given amount in the body, the concentration in plasma. The mechanisms by which substances are sequestered in tissues include dissolution in lipids, ion-trapping, binding to macromolecules, and the effect of transporter systems.

Lipophilic substances concentrate in lipid membranes and fat cells. For some drugs, such as thiopental and propofol, distribution into lipid is a major determinant of their duration of action. Generally, lipophilic drugs are highly distributed, but not necessarily into the CNS; amiodarone, for example, is mainly found in fat, lung, and liver, with little being present in brain.

15.4.1 Ion-trapping

Differences in pH may result in intracellular (pH 6.8) concentrations of basic drugs being higher than the concentrations in plasma water (pH 7.4) in a manner analogous to the sequestration of bases in gastric acid discussed above. Under normal physiological conditions one would expect the concentrations of acidic substances such as salicylate to be lower in intracellular fluid than in plasma water (Figure 15.3). However, in salicylate overdose a resulting acidosis can shift the equilibrium from plasma to tissue.

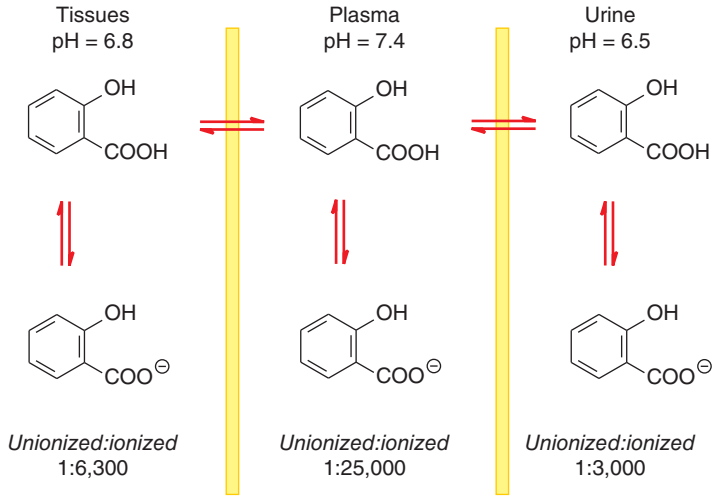

Figure 15.3 The effect of pH on the distribution of salicylate (pK_a = 3.0) between plasma water, extracellular fluid (tissues), and urine. The pH values represent normal physiological conditions

15.4.2 Binding to macromolecules

Mepacrine (quinacrine) and chloroquine are very highly localized in cells as a result of interaction with DNA. Basic drugs accumulate in melanin-containing tissues including hair, which in turn can be used to attempt to assess chronic exposure to such compounds (Section 18.5). High erythrocyte concentrations of chlortalidone are a consequence of binding to carbonic anhydrase. Digoxin binds to sodium/potassium-ATPase and thus tends to concentrate not only in cardiac tissue, but also in skeletal muscle. Neostigmine and pyridostigmine bind to acetylcholinesterase on the surface of erythrocytes. Similarly, lead and other toxic metals such as mercury bind to thiols on erythrocytes, and thus they are measured in whole blood rather than plasma.

Carbon monoxide binds to haemoglobin and cytochromes, whilst cyanide ion binds to cytochromes containing iron(III) such as cytochrome c, thereby inhibiting cellular respiration. Cyanide also binds to methaemoglobin, i.e. oxidized haemoglobin, which contains iron(III), and this is the rationale for administering nitrites to increase blood methaemoglobin concentrations in the treatment of cyanide poisoning.

15.4.2.1 Plasma protein binding

In blood, acidic and neutral drugs are often bound to plasma albumin, and bases to albumin and also to AAG (Huang & Ung, 2013). Protein binding is normally reversible, and bound

and non-bound drug exist in equilibrium. Plasma protein binding is an important mechanism by which drugs and other xenobiotics are transported in blood. Lipophilic molecules tend be bound extensively and the concentrations in plasma (i.e. bound + non-bound) can exceed the aqueous solubility of the drug. Because there is very little protein in CSF, vitreous humour, and saliva, drug concentrations in these fluids are often very close to the non-bound plasma concentration. For highly bound substances, measuring low non-bound concentrations in these fluids can be challenging analytically. However, oral fluid is convenient for non-invasive sampling, and vitreous humour is an accessible sample for post-mortem analysis of xenobiotics (Bévalot *et al*., 2016).

Binding to plasma protein reduces the distribution of a drug by 'holding' the drug in plasma, thereby reducing the concentration that is able to diffuse into tissues. More importantly, drug activity and, possibly toxicity, may increase if plasma protein binding is decreased (Dasgupta, 2007). This may occur in some disease states that result in reduced plasma protein concentrations. Displacement of one drug by another from plasma protein binding sites is a potential mechanism of drug–drug interactions, but rarely is the interaction clinically important (Rolan, 1994).

Many *in vitro* studies have demonstrated displacement of one drug by another, but *in vivo* the situation is more complex. The 'total' concentration of a displaced drug in plasma will be reduced as some of the liberated drug diffuses into tissues as new equilibria are established. The increased concentration of non-bound drug may lead to greater, possibly toxic, effects. Consequently, measurement of the 'total' (bound + non-bound) concentration of a drug in plasma may be misleading in certain circumstances and TDM of some drugs, phenytoin for example, is better performed using non-bound concentrations.

15.4.3 Carrier-mediated transport

Active transport systems can maintain higher concentrations of a drug inside a cell against a prevailing concentration gradient. Carrier-mediated transport of paraquat into and out of the lungs is an important factor in its pulmonary toxicity. It is concentrated in lung by a spermidine/putrescine transporter system, but its efflux is dependent on P-gp. Entry of paraquat into the brain is mediated by a system that can be blocked by L-valine, a high affinity substrate for the neutral amino acid transporter, although this cannot be exploited in the treatment of paraquat poisoning.

15.5 Metabolism

The liver is the most important site of drug metabolism, but most tissues are capable of metabolizing drugs, and hydrolysis by plasma esterases may also occur. Metabolism by intestinal flora and the intestinal mucosa is important in influencing the oral bioavailability of many compounds, as discussed above. Drug metabolism often reduces the biological activity of the drug. However, some drugs (i) have active metabolites that contribute to the overall pharmacological profile, (ii) are prodrugs that are activated by metabolism, and (iii) have highly toxic metabolites, as in the case of the postulated intermediary paracetamol metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI).

Drug metabolism is considered under two headings. Phase 1 reactions involve chemical modification of the molecule by oxidation, reduction, or hydrolysis, whilst phase 2 reactions are conjugations in which, a second hydrophilic molecule such as D-glucuronic acid is added

to the molecule. Phase 2 reactions cannot occur unless a suitable reactive group (e.g. hydroxyl or primary amine), is present. For example, diamorphine cannot be conjugated with glucuronic acid, whereas its phase 1 metabolite, morphine, can be conjugated without prior metabolism (Figure 15.4).

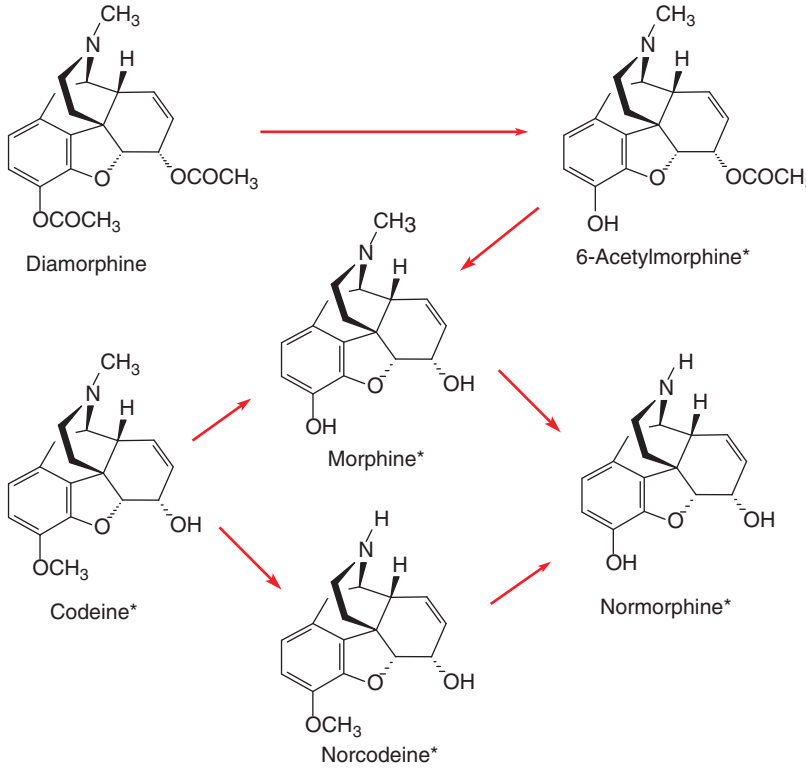

Figure 15.4 Some metabolic pathways of diamorphine, morphine, and codeine

15.5.1 Phase 1 metabolism

Phase 1 reactions can be subdivided on the basis of the types of products that are formed: aromatic hydroxylation, *N*-oxidation, *N*-demethylation, and so on. Mixed function oxidases (MFOs) are membrane bound enzymes found in the smooth endoplasmic reticulum (SER) of cells. When liver cells are homogenized the SER forms small vesicles known as microsomes. Consequently, the enzymes, and the reactions they catalyze, are referred to as being microsomal. Enzymes that are not bound to membranes (for example ADH) are referred to as soluble or cytosolic enzymes.

15.5.1.1 The cytochrome P450 family

Cytochromes are a superfamily of related enzymes, which are classified on the basis of cDNA cloning according to similarities in amino acid sequence. A family contains genes that have at least a 40 % sequence homology. The three major drug metabolizing families are CYP1, CYP2,

and CYP3. Members of a subfamily (denoted by a letter) must have at least 55 % identity. The final number denotes the individual gene products (Table 15.3). A comprehensive listing can be found at: https://drug-interactions.medicine.iu.edu/Main-Table.aspx with links to the original literature.

Table 15.3 Some drug metabolizing isoforms of cytochrome P-450

Isoform	Substrates	Inhibitors	Inducers
CYP1A2	Caffeine, clozapine, theophylline	Ciprofloxacin, cimetidine	Polycyclic aromatic hydrocarbons, rifampicin, phenobarbital, phenytoin
CYP2A6	Nicotine		Ritonavir
CYP2B6	Cyclophosphamide[a]	Orphenadrine	Cyclophosphamide, phenobarbital
CYP2C9	(*S*)-Warfarin, phenytoin, losartan[a]	Ketoconazole, sulfaphenazole, amiodarone	Rifampicin, phenobarbital
CYP2C19	Diazepam, omeprazole	Omeprazole, sulfaphenazole	Rifampicin, phenobarbital
CYP2D6	Haloperidol, codeine[a] phenothiazines, SSRIs[b], tramadol[a]	Quinidine, cimetidine, phenothiazines, SSRIs	Glutethimide
CYP2E1	DCM, enflurane, ethanol, halothane, paracetamol	Ethanol (acute), disulfiram, miconazole	Ethanol (chronic), isoniazid, rifampicin
CYP3A4	Clozapine, nifedipine	Ketoconazole, erythromycin, SSRIs, cimetidine	Rifampicin, phenobarbital carbamazepine

[a] prodrug
[b] selective serotonin reuptake inhibitors

CYP3A4 is the most prevalent drug metabolizing CYP in the body and has many substrates. It constitutes approximately 30 % of the total CYP content of the liver and 70 % of the total CYP in the GI mucosa. It is found in tissues that express large amounts of P-gp, with which it appears to work in concert; xenobiotics that are not removed by P-gp, being metabolized by CYP3A4.

Although CYP2D6 represents only 2 % of hepatic CYP, it is responsible for the metabolism of a large number of drugs, including many antipsychotics, antidepressants, and β-blockers. It is also responsible for activating tramadol and for the *O*-demethylation of codeine to morphine (Figure 15.4).

15.5.1.2 Other phase 1 oxidases

Flavin-containing monooxygenases (FMOs) are microsomal enzymes that catalyze the NADPH-dependent oxidation of a large number of sulfur-, selenium-, and nitrogen-containing compounds, such as the *N*-oxidation of tertiary amines and stereospecific oxidation of sulfides.

Monoamine oxidase (MAO), which is bound to the surface of mitochondria, is located in aminergic nerve terminals, liver, and intestinal mucosa. As well as deaminating endogenous neurotransmitters (norepinephrine, dopamine, and serotonin), chemically similar molecules such as tyramine are also substrates. Amfetamine, having an α-methyl substituent, is a poor substrate for this enzyme, however.

Other important phase 1 enzymes include xanthine oxidase, which metabolizes 6-mercaptopurine (Figure 15.5) and ADH, the mammalian form of which oxidizes several alcohols as well as ethanol, including methanol, ethylene glycol, and 2,2,2-trichloroethanol, the active metabolite of chloral hydrate.

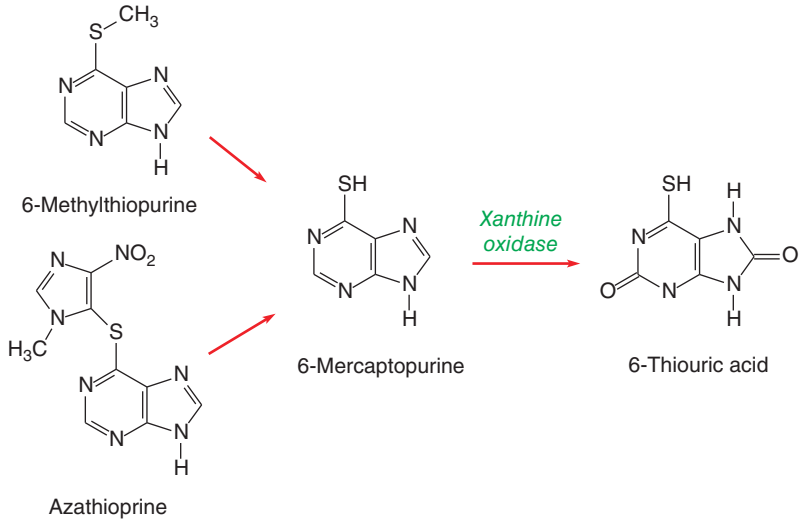

Figure 15.5 Role of xanthine oxidase in the metabolism of purines

15.5.1.3 Enzymatic reductions

Aromatic nitro- and azo-compounds are reduced by systems that require NADPH and are stimulated by flavins. The nitro moieties in chloramphenicol and nitrazepam are reduced to primary aromatic amines, which may then be acetylated (Section 15.5.2.2). Azo-reductase reduces and cleaves the double bond in azo compounds with the formation of two aromatic primary amines. The azo dye prontosil is converted to triaminobenzene and sulfanilamide, the active antimicrobial metabolite.

Under anaerobic conditions, enzymes that require NADPH and oxygen reductively dehalogenate halothane and methoxyflurane. These compounds also undergo oxidative dehalogenation (Section 15.5.4.4).

Nitrazepam is reduced by human cytosolic aldehyde oxidase (AOX1). The reaction proceeds via the potentially hepatotoxic hydroxylamine intermediate to the primary amine (Figure 15.6), which is acetylated by *N*-acetyltransferase type 2 (NAT2) (Section 15.5.2.2). 7-Acetamidonitrazepam may be a teratogen in rodents (Konishi *et al.*, 2017). Clonazepam and flunitrazepam undergo similar biotransformations.

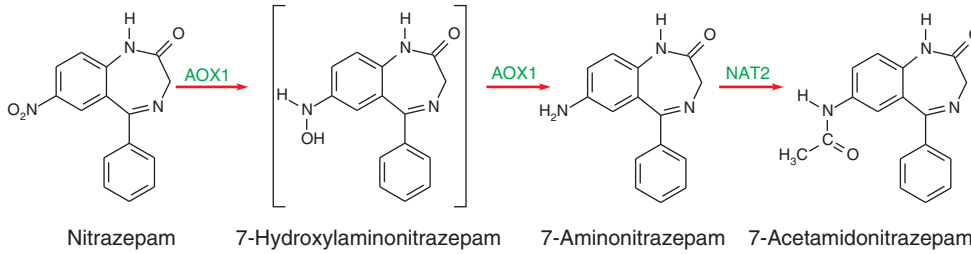

Figure 15.6 Reduction of nitrazepam by human aldehyde oxidase (AOX1)

15.5.1.4 Hydrolysis

Esterases and amidases hydrolyze substrates to reveal reactive groups that may then undergo phase 2 conjugations. Plasma contains a number of esterases, including butyrylcholinesterase (EC 3.1.1.8, pseudocholinesterase or plasma cholinesterase), which rapidly hydrolyzes cocaine, procaine, suxamethonium, and physostigmine. Acetylcholinesterase (EC 3.1.1.7) is a membrane-bound enzyme located primarily at cholinergic synapses and on erythrocytes.

Two important intracellular human carboxylesterases are CE1 and CE2. The former is found chiefly in the liver whilst the latter is primarily distributed along the length of the small intestine, with only a relatively small proportion in the liver. Thus, these enzymes are involved in the first-pass metabolism of many endogenous and exogenous substrates including esters, thioesters, carbamates, and amides. Cocaine and methylphenidate are inactivated by hydrolysis, but ester prodrugs including several angiotensinogen-converting enzyme inhibitors (ACEI), statins, and clopidogrel are activated. As well as distributional differences, CE2 shows substrate specificity for esters derived from large alcohols, for example valaciclovir, prasugrel, irinotecan, and flutamide. Polymorphisms (Section 15.7) in the genes (*CES1* and *CES2*) encoding CE1 and CE2 and subsequent changes in pharmacokinetic and pharmacodynamic responses have been reported (Wang *et al*., 2018).

15.5.2 Phase 2 reactions

These reactions include glucuronidation, sulfation, *N*-acetylation, and *N*- and *O*-methylation, as well as conjugation with reduced glutathione (GSH) and with amino acids such as glycine.

15.5.2.1 D-Glucuronidation

D-Glucuronides are usually very polar, water soluble, inactive metabolites that are rapidly cleared by the kidney or via the bile. The increase in size (glucuronidation increases M_r by 207) and their acidic nature make glucuronides good substrates for active efflux into bile. Morphine is unusual in having a glucuronide metabolite (morphine-6-glucuronide) that is at least as active as an analgesic as the parent molecule.

Glucuronidations require a donor molecule, uridine diphosphate glucuronic acid (UDPGA). UDP-glucuronosyltransferases (UGT) are microsomal enzymes and the reaction always produces β-D-glucuronides (Figure 15.7).

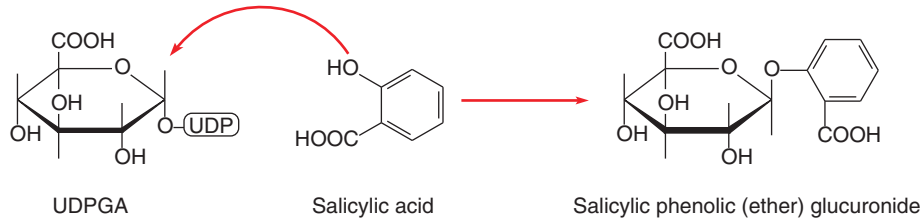

Figure 15.7 Conjugation of salicylic acid to produce the β-D-glucuronide

Ether *O*-glucuronides can be formed with phenols and alcohols, whereas ester glucuronides can be formed with carboxylic acids. Acyl glucuronides may undergo intramolecular rearrangement to give 2-, 3- or 4-*O*-β-glucuronides, which are reactive and have been implicated in idiosyncratic toxicity (Van Vleet *et al*., 2017). *N*- and *S*-Glucuronides are less stable than *O*-glucuronides. Conjugation with tertiary amines produces quaternary ammonium *N*-glucuronides, for example olanzapine 4′-*N*-glucuronide (Figure 15.12).

15.5.2.2 O-Sulfation and N-acetylation

Ethereal sulfate conjugation may be catalyzed by a number of cytoplasmic sulfotransferases depending on the substrate. The sulfate donor is 3′-phosphoadenosine-5′-phosphosulfate (PAPS). Sulfates are normally very water soluble and readily excreted. Sulfation is important in the excretion of several drugs, including paracetamol.

Aromatic primary amines and hydrazines are *N*-acetylated by a soluble enzyme, *N*-acetyltransferase 2 (NAT2), acetyl-CoA being the donor molecule. Genetic polymorphism in the expression of NAT2 can be a major determinant of the plasma concentrations of drugs acetylated by this enzyme (Section 15.7.5). Drugs that are acetylated include isoniazid, procainamide, and several sulfonamide antimicrobials, including sulfadimidine, which can used to test acetylator status (Figure 15.8).

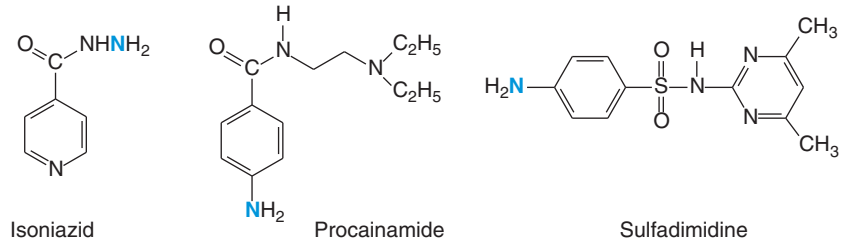

Figure 15.8 Examples of drugs metabolized by *N*-acetylation. The acetylated nitrogen atoms are shown in blue

Generally, the metabolites are less pharmacologically active than the parent drug, although *N*-acetylprocainamide (acecainide, NAPA) has similar activity to procainamide. Unlike the products of glucuronidation and sulfation, acetyl metabolites may not show increased water solubility and the acetyl metabolites of older sulfonamide antimicrobials were implicated in causing crystalluria.

15.5.2.3 O-, N- and S-Methylation

The methyl donor for these reactions is *S*-adenosylmethionine (SAM). Several methyl transferases catalyze the methylation of endogenous substances. Catechol *O*-methyl transferase (COMT) methylates endogenous catechols (epinephrine, norepinephrine, dopamine) and some drugs, for example isoprenaline (isoproterenol) to give 3-methoxy metabolites.

The biosynthesis of epinephrine from norepinephrine is catalyzed by phenylethanolamine *N*-methyl transferase. Other *N*-methylations include that of histamine to 1-methylhistamine and the methylation of acetylserotonin to give melatonin. Examples of xenobiotics that undergo *S*-methylation include 2-mercaptoethanol and thiouracil.

Microbiological methylations of toxic metals such as mercury are of toxicological significance; mercury salts are toxic to the kidney, whereas methylmercury is neurotoxic and more difficult to remove from the body by the use of chelating agents. Non-volatile inorganic compounds of sulfur, selenium, and tellurium are metabolized to volatile dimethyl metabolites that are excreted via the lungs.

15.5.2.4 Conjugation with glutathione

The thiol group in reduced glutathione (GSH) usually acts as a nucleophile and may react chemically or enzymatically via glutathione-*S*-transferases (GST), a family of cytosolic enzymes. Substrates include aromatic halogen and nitro compounds, and reactive phase 1 metabolites such as epoxides. Thus, GSH conjugation normally has a protective role by removing potentially toxic metabolites, as with paracetamol. At adult therapeutic doses (500–1000 mg) most of a dose of paracetamol is conjugated with glucuronic acid or sulfate (Figure 15.9). However, a small proportion of the dose is thought to be oxidized by CYP2E1 to a reactive intermediate, NAPQI, which reacts with GSH.

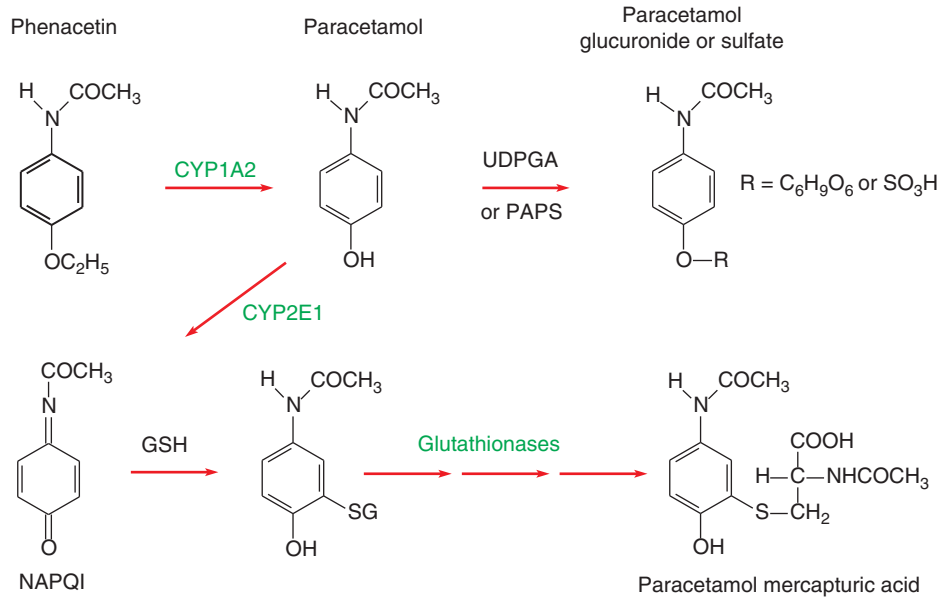

Figure 15.9 Metabolic pathways of phenacetin and paracetamol

This GSH-NAPQI conjugate undergoes further metabolism via glutathionases (sequential metabolism by γ-glutamyltransferase, cysteinylglycinase, and *N*-acetyltransferase) to give the mercapturate, the *N*-acetylcysteine conjugate of paracetamol, which is excreted in urine. However, in adults larger doses (>10 g = 20 × 500 mg tablets, perhaps less in susceptible subjects) may saturate the sulfate and glucuronide pathways and the increased amount of NAPQI produced exceeds the capacity for GSH detoxification. In the absence of antidotal therapy, the intermediate reacts with macromolecules in the hepatocytes and in other ways leading in some cases to liver failure, which may prove fatal.

15.5.2.5 Amino acid conjugation

Conjugation with amino acids is typified by the formation of salicyluric acid from salicylic acid and glycine (Figure 15.10). Reaction with coenzyme A produces salicyloyl-CoA, the acyl donor for glycine. In man, over 80 % of a 250 mg dose of aspirin can be eliminated as salicyluric acid in the urine. However, because this pathway is saturable, the proportion falls with increasing dose (70 % for a 1000 mg dose, 60 % for a 2000 mg dose).

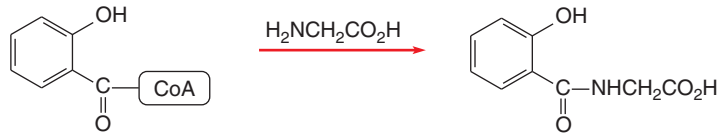

Figure 15.10 Conjugation of salicylic acid with glycine

15.5.3 Stereoselective metabolism

Many stereoisomers, particularly enantiomers, may be metabolized at different rates and by different enzymes. For example, the biotransformation of the enantiomers of warfarin is complex. The pharmacologically active *S*-isomer is rapidly hydroxylated at position 7 by CYP2C9, whereas the major sites of hydroxylation of (*R*)-warfarin, catalyzed by different CYP isoforms, are 6, 8, and 10 [Figure 15.11(a)]. Both enantiomers are hydroxylated at other positions, including 4′, but these are minor pathways. Dehydrogenation at position 10, with the formation of a double bond, gives *cis*- and *trans*-isomers, whilst the reduction of the carbonyl group to 'warfarin alcohol' complicates matters further by introducing a second chiral centre, resulting in four stereoisomers.

Attempts to overcome problems associated with the use of racemic drugs by administering only the active enantiomer are often thwarted by racemization and inversion. Thalidomide was responsible for birth defects in the 1950s and 60s after pregnant mothers took the drug to relieve morning sickness. The *S*-isomer is teratogenic, whilst (*R*)-thalidomide is sedative. However, use of the single *R*-isomer was unsuccessful as it rapidly racemized *in vivo*. The drug is used now to treat certain cancers (multiple myeloma) and for complications of leprosy, after careful assessment of, and instructions to, the prospective patient.

In humans, (*R*)-ibuprofen is converted to the active *S*-isomer. This inversion proceeds via the CoA metabolite (Section 15.5.2.5), which is hydrolyzed, probably by acyl-CoA-thioesterase (ACOT), to (*S*)-ibuprofen [Figure 15.11(b)]. The reaction is believed to be unidirectional in humans because they cannot form (*S*)-ibuprofenoyl-CoA. Other 2-acylpropionic acid analgesics undergo similar inversions and so there is little point in going to the expense of developing single enantiomers.

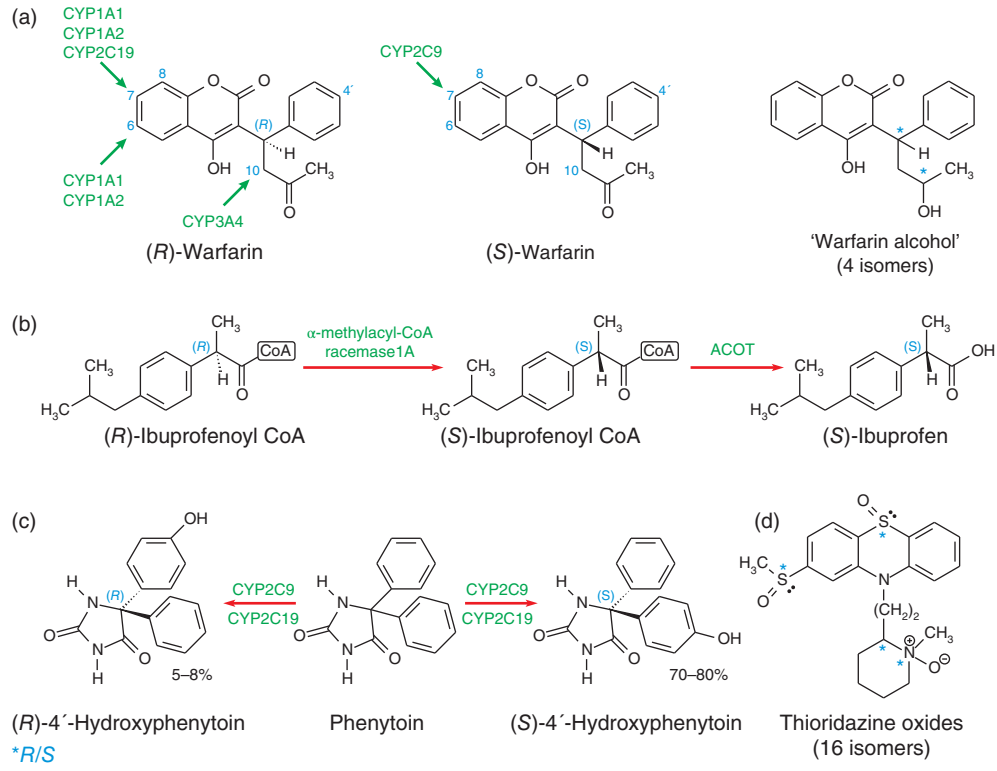

Figure 15.11 Stereochemical biotransformations. (a) Hydroxylation and reduction of warfarin. (b) Inversion of (*R*)-ibuprofen. (c) Hydroxylation of phenytoin. (d) *N*- and *S*-oxide metabolites of thioridazine

Achiral molecules can be metabolized to enantiomers. In the case of phenytoin, considerably more (*S*)-4′-hydroxyphenytoin than (*R*)-4′-hydroxyphenytoin is produced [Figure 15.11(c)]. Introduction of new chiral centres in enantiomers results in diastereomers that may be separable by achiral chromatography, which can lead to complications of metabolite identification. This is often the case with β-D-glucuronidation (e.g. oxazepam glucuronides). Oxidation of (*R/S*)-thioridazine can lead to 16 isomers [Figure 15.11(d)] because the 2-sulfoxide, the 5-sulfoxide, and the *N*-oxide are chiral. Asymmetric tertiary amines are chiral, but because of rapid molecular vibrations (inversion) they are not separable. However, *N*-oxidation slows the rate of inversion so that it may be possible to separate enantiomers of *N*-oxides.

15.5.4 Metabolic reactions of toxicological importance

15.5.4.1 Oxidative dealkylation

N-Dealkylation frequently leads to the formation of pharmacologically active metabolites, which may have different pharmacodynamic and/or pharmacokinetic properties to the parent compound. Tertiary amines may be didealkylated; amitriptyline is demethylated to nortriptyline and further demethylated to desmethylnortriptyline. Morphine and codeine are *N*-demethylated to normorphine and norcodeine, respectively (Figure 15.4). *N*-Demethylation of olanzapine results in *N*-desmethylolanzapine (Figure 15.12).

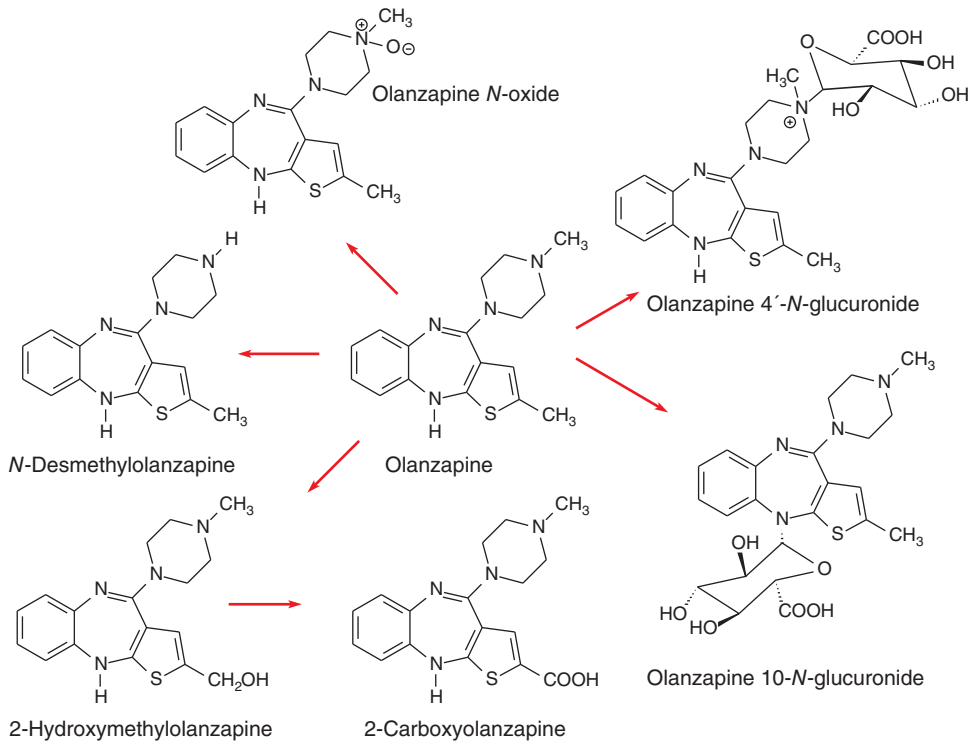

Figure 15.12 Some metabolic pathways of olanzapine

O-Dealkylation is exemplified by the demethylation of codeine to morphine (Figure 15.4) and of phenacetin to paracetamol (Figure 15.9). Methylmercaptopurine is *S*-demethylated to mercaptopurine (Figure 15.5).

15.5.4.2 Hydroxylation

TCAs such as imipramine are hydroxylated in the 2-position. Imipramine also undergoes aliphatic hydroxylation to give 10-hydroxyimipramine. The side chain methyl in olanzapine is metabolized to the alcohol, further oxidization giving 2-carboxyolanzapine (Figure 15.12).

Aliphatic hydroxylation and *N*-dealkylation are important pathways in the metabolism of benzodiazepine anxiolytics in humans, although aromatic hydroxylation is more prevalent in rodents, for example. Although the clinical use of 1,4-benzodiazepines has declined, the misuse of so called 'designer benzos', substances that can be synthesized so as not to contravene current controlled drug legislation, has led to increased toxicological interest in such compounds. Many of these compounds were synthesized originally as drug candidates, but are either not approved for medicinal use, or are in use in some countries only (Figure 22.4). Some examples are prodrugs. Because the same metabolites may be produced by several commercially available drugs and by 'designer' drugs, interpretation of results can be difficult. The 3-hydroxylated compounds are chiral and form diastereomeric glucuronides, a potential complication during an analysis (Figure 15.13).

Imidazobenzodiazepines (midazolam) and triazolobenzodiazepines (alprazolam, triazolam, etc.) form 4-hydroxy metabolites – this is equivalent to the 3-hydroxy metabolites of

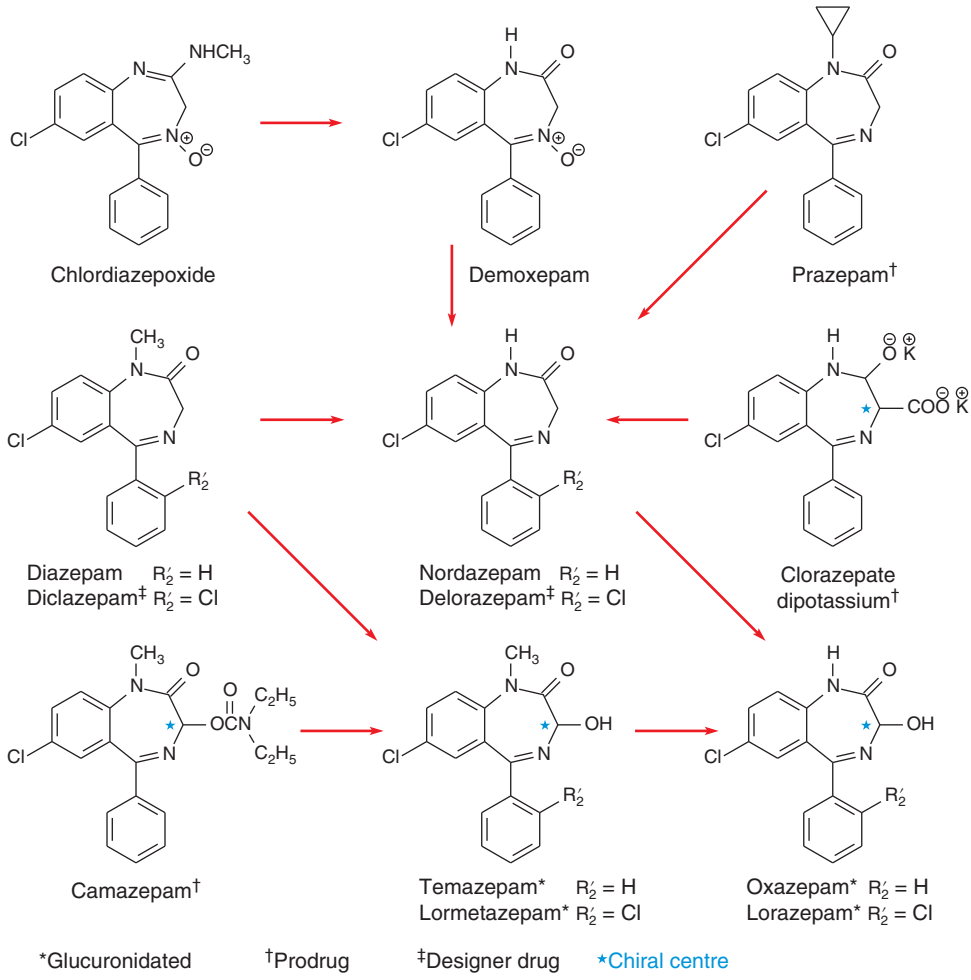

Figure 15.13 Metabolism of selected 7-chlorobenzodiazepines and their prodrugs

Figure 15.13, but reflects the different numbering in these compounds. Side chain oxidation (e.g. 1′-hydroxymidazolam) also occurs. The hydroxylated metabolites are excreted in urine as glucuronides. *N*-Glucuronidation is also possible (Figure 15.14)

15.5.4.3 S- and N-oxidation

Sulfides may be oxidized to sulfoxides and, in some cases, further oxidized to sulfones. Asymmetrically substituted sulfides will give chiral sulfoxides. *S*-Oxidation of methionine, for example, gives diastereomeric methionine sulfoxides. Reduction of sulfoxides, either *in vivo*, or *in vitro* (i.e. after the sample has been taken) can cause problems if the parent drug is to be measured.

Some drugs are sulfoxides and may be further oxidized or reduced. Sulindac (a prodrug) is reduced to the active sulfide metabolite, which is rapidly oxidized, resulting in the sulfoxide and sulfide being in equilibrium (Figure 15.15). Oxidation of sulindac results in an inactive sulfone.

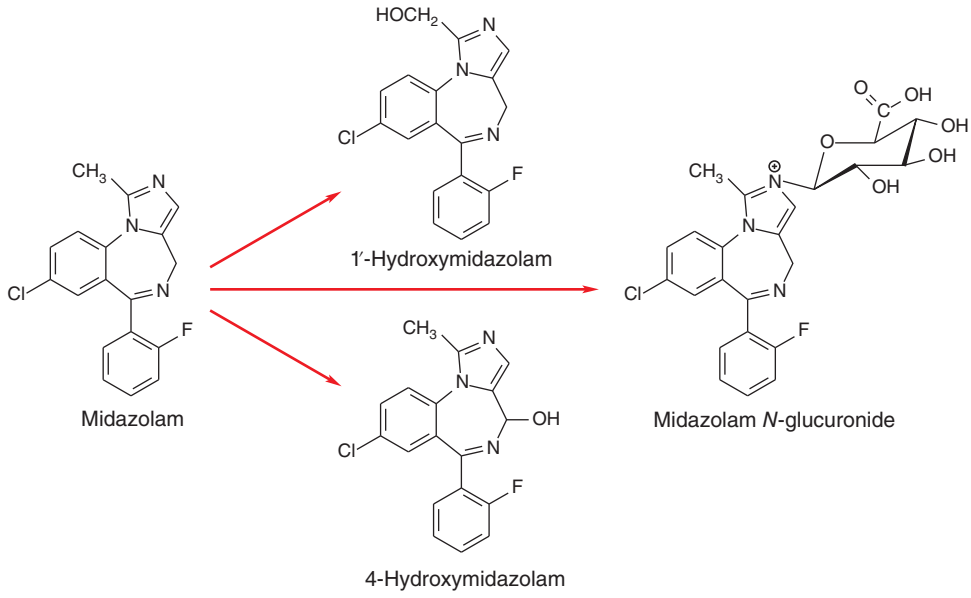

Figure 15.14 Selected metabolic pathways of midazolam in humans

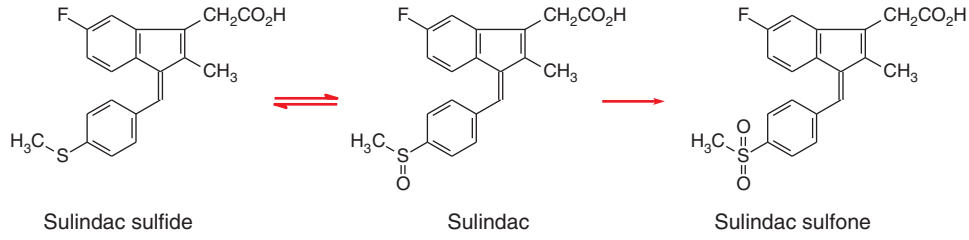

Figure 15.15 Reduced and oxidized metabolites of sulindac

Most tertiary amines, whether aliphatic or alicyclic, form *N*-oxides. The reaction may be catalyzed by FMO (Section 15.5.1.2) or by a CYP (Table 15.3). *N*-Oxidation of olanzapine, for example, is catalyzed by CYP3A4 (Figure 15.12).

N-Oxides are labile and relatively easily reduced to the parent amine by antioxidants (for example, if ascorbic acid or metabisulfite is added to a sample), exposure to alkaline conditions, or simply on storage (Section 2.4). Reduction may be accompanied by dealkylation, which should be born in mind when samples that might contain *N*-oxides are to be analyzed. On heating, *N*-oxides undergo Cope elimination (Table 9.5).

15.5.4.4 Oxidative dehalogenation

Halothane undergoes oxidative dehalogenation to trifluoroacetyl chloride and bromide ion. The acid chloride may be hydrolyzed to trichloroacetate, or may acylate neighbouring proteins to produce antigens (Figure 15.16). The trifluoroacetylated proteins are found chiefly in the endoplasmic reticulum, but some are found in the cell membrane, which is why repeat exposure

to halothane (or to a structurally related compound) can produce a potentially fatal anaphylactic reaction.

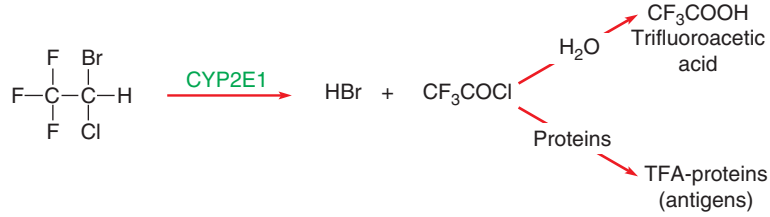

Figure 15.16 Oxidative debromination of halothane

The metabolism of DCM in mammals is dose dependent and proceeds via two major pathways. The predominant human pathway at low doses involves mainly CYP2E1-dependent oxidation to carbon monoxide and carbon dioxide, probably via an unstable intermediate, formyl chloride. Alternatively, cytosolic GST-dependent metabolism leads to the formation of formic acid presumably following the formation of a GSH conjugate (Figure 15.17). COHb derived from DCM has a longer half-life in blood than if the carbon monoxide were derived from an exogenous source, presumably reflecting continuing carbon monoxide production *in vivo*. Other dihalomethanes (dibromomethane, bromochloromethane) also give rise to carbon monoxide *in vivo*.

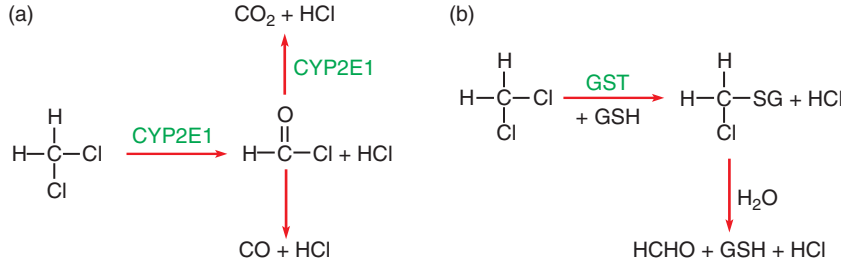

Figure 15.17 Metabolism of dichloromethane: (a) microsomal and (b) cytosolic

Aromatic oxidative dechlorination of clozapine gives 8-hydroxydechloroclozapine (Dain *et al*., 1997), in other words the chlorine atom is replaced by a hydroxyl moiety, but dechlorination of chlorpromazine gives promazine. Therefore, it is important not to conclude that promazine has been administered to patients taking chlorpromazine simply because some promazine has been detected in a biological sample.

15.5.4.5 Desulfuration

The thiobarbiturate thiopental is converted to pentobarbital. Desulfuration of malathion and parathion to organophosphates increases the toxicity of these insecticides. In mammals, malathion is chiefly hydrolyzed to the mono- and di-acid metabolites, whereas in insects it is converted to the considerably more toxic malaoxon (Figure 15.18).

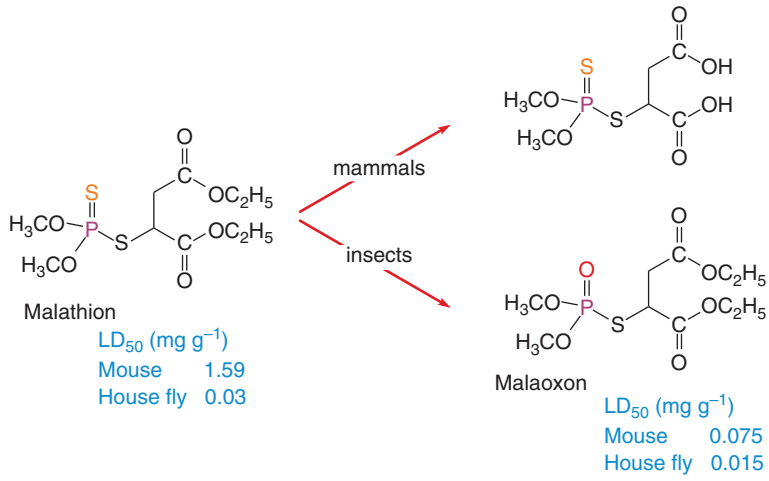

Figure 15.18 Comparative metabolic pathways of malathion in mammals and in insects

15.5.4.6 Trans-sulfuration and trans-esterification

Oxygen–sulfur exchange is a mechanism by which small quantities of cyanide may be detoxified. The sulfur donor is thiosulfate, and the reaction is catalyzed by the widely distributed enzyme rhodanese:

$$CN^- + S_2O_3^{2-} \rightarrow CNS^- + SO_4^{2-}$$

This is the rationale for giving thiosulfate to treat cyanide poisoning. The reverse reaction is catalyzed by thiocyanate oxidase, an enzyme found in erythrocytes.

When ethanol is co-ingested with methylphenidate, a small quality of ethylphenidate may be detected (Figure 15.19). This interaction also occurs with cocaine to give the ethyl homologue, cocaethylene, and detection of this metabolite may be used to confirm co-ingestion of ethanol (Section 22.4.21.2).

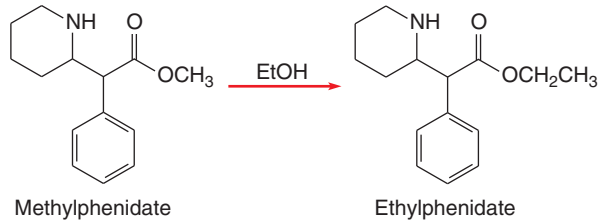

Figure 15.19 Trans-esterification of methylphenidate in the presence of ethanol

15.5.5 Enzyme induction and inhibition

15.5.5.1 Enzyme induction

Exposure of xenobiotic metabolizing enzymes to certain substances can lead to induction of synthesis of these same enzymes. The increased enzyme activity may lead not only to enhanced metabolism of the inducing agent, but also of those of other substrates of the enzyme(s). This

is an important mechanism underlying some drug–drug interactions. Phenobarbital induces a number of drug metabolizing cytochromes (Table 15.3) and also increases UGT activity, as evidenced by increased bilirubin conjugation. Rifampicin (rifampin) is a potent enzyme inducer and several drug–drug interactions have been described including pregnancies following failure of contraceptive pills.

Induction of CYP2E1 by ethanol may explain why chronic alcohol consumption is associated with an increased risk of paracetamol toxicity after overdosage because the production of NAPQI is catalyzed by this enzyme (Figure 15.9). However, reduced hepatic GSH concentrations in chronic alcoholics may be a further factor here. Acute alcohol ingestion, on the other hand, may be protective, presumably because of competition for the enzyme.

Drug metabolizing enzyme activity returns to pre-treatment levels, usually over a few days, when the inducing agent is withdrawn. Consequently, dosage of other drugs metabolized by the affected enzyme(s) may need to be reduced. Clozapine is metabolized by several enzymes including CYP1A2, which is induced by cigarette smoke. On cessation of smoking, clozapine plasma concentrations may rise leading to serious adverse effects if the dosage is not adjusted.

15.5.5.2 Enzyme inhibition

Some drugs act by inhibiting enzymes and thus drug–drug interactions with drugs that are metabolized by the same enzyme should be predictable. Allopurinol is a xanthine oxidase inhibitor that potentiates the action of mercaptopurine and azathioprine (Figure 15.5). Similarly, suxamethonium is hydrolyzed by plasma cholinesterase and interaction with an anticholinesterase is predictable. However, some inhibitors may not be specific for the target enzyme. For example, disulfiram, used to inhibit aldehyde dehydrogenase (ALDH) in aversion therapy for alcoholism, also inhibits CYP2C9 leading to interactions with other drugs, including warfarin.

Monoamine oxidase inhibitors (MAOIs) such as tranylcypromine and phenelzine potentiate the effects of indirectly acting sympathomimetic drugs such as ephedrine and phenylpropanolamine. The interaction of MAOIs with tyramine, found in cheese and other fermented foods, is potentially life threatening. Tyramine normally undergoes first-pass metabolism by intestinal and hepatic MAO. However, when these enzymes are inhibited, the tyramine is absorbed and displaces vesicular norepinephrine, the concentrations of which have been elevated by the effect of MAOI. This can lead to a ‘hypertensive crisis’ with the subsequent risk of stroke.

Other interactions have been observed. Several drugs, including erythromycin, ketoconazole, and also some components of grapefruit juice, inhibit CYP3A4. This enzyme acts in partnership with P-gp to reduce the bioavailability of many xenobiotics and the reduced activity may result in potentially dangerous interactions with a large number of drugs. The H_2-receptor blocker cimetidine inhibits several CYPs (Table 15.3).

15.6 Excretion

The major excretory organs are the liver, lungs, and kidneys. The liver not only eliminates drugs by metabolism, but also by biliary excretion of drug and metabolites, which if not reabsorbed, are excreted in faeces. The lungs are important for the excretion of gaseous anaesthetics and some other volatile compounds and metabolites such as dimethyl selenide (Section 15.5.2.3). The proportion of a dose of ethanol excreted by the lungs is small, but this route is exploited when performing breath alcohol measurements (Section 17.3.1.1).

Excretion via fluids other than urine and bile may be insignificant in terms of the amounts excreted, but could have important consequences – excretion of drugs via breast milk, for

example, may transfer significant quantities of a drug such as lithium to a breast-fed infant (Moretti *et al*., 2003). Similarly, sweat, oral fluid, hair, and nail, although usually of minor importance in terms of amount of drug excreted, may be important samples for detecting exposure to xenobiotics such as misused substances and toxic metals as discussed in Chapters 18 and 21, respectively.

15.6.1 The kidney

When plasma is filtered at the glomerulus, the ultrafiltrate is essentially plasma water, plasma proteins being too large to pass through the basement membrane. Drugs enter tubular fluid either in the ultrafiltrate, or by carrier-mediated transport from the cells of the renal PCT (Table 15.1). As the filtrate passes down the nephron up to 99 % of the sodium, water, and accompanying anions may be reabsorbed, resulting in a marked increase in the concentration of the remaining solutes in the lumen of the nephron so that those drugs that are able to diffuse across the tubular membrane are passively reabsorbed. However, the greater the ionization of a molecule, the less it will be reabsorbed and so the renal clearances of weak electrolytes are affected by changes in urine pH. This is the basis for treating salicylate overdose with sodium bicarbonate; the increased urine pH (as high as pH 8) increases the ionization of the acid and reduces tubular reabsorption, thereby increasing salicylate clearance. Alkalinization is important because it corrects the metabolic acidosis and reduces the amount of drug in tissues. Sodium bicarbonate can be used to increase the renal clearance of other weak acids such as chlorophenoxy herbicides (Section 16.6.1).

Although the renal clearance of drugs may be enhanced by increasing the flow of urine, as was once done for phenobarbital overdosage, the approach is not generally applicable. Increasing urine flow by administering large volumes of fluid and possibly a diuretic may cause electrolyte imbalance and pulmonary oedema due to fluid overload. In the case of salicylate simple adjustment of urinary pH is sufficient to increase renal excretion without the need for diuresis.

15.6.1.1 Tubular secretion

The transporters of the PCT are in the basolateral and apical membranes. OATs, OATPs, and OCTs are in the basolateral membrane. Efflux pumps, including P-gp and multidrug resistance-associated proteins MRP2 and MRP4, have been identified in the apical (luminal) membrane. Drugs and metabolites including penicillins, most diuretics, and glucuronide and glycine metabolites, are substrates for these transporters. Probenecid is a competitive inhibitor of OATs 1, 3, and 4, thereby blocking the secretion of penicillins, for which it was once used. Probenecid protects against cidofovir nephrotoxicity by blocking its uptake into PCTs.

Organic bases such as metformin and cimetidine are transported into PCT cells by OCT2, efflux being via MATE1. Digoxin is carried into the cells by OAPT1B3 and secreted into the tubular fluid by P-gp in the luminal membrane. Several drug–drug interactions involving these transport proteins have been reported (König *et al*., 2013).

15.6.1.2 Renal excretion of metabolites

Lipophilic drugs tend to be plasma protein bound, which reduces glomerular filtration. Any drug that is filtered is likely to be reabsorbed, so that, in general such compounds must be metabolized to polar metabolites to be excreted. Therefore metabolites, rather than parent compound, are

found in the urine. The urine of a subject taking diazepam may contain only small amounts of the drug, but oxazepam is found in large amounts and oxazepam glucuronide in even larger amounts. Hence detection of metabolites in urine, BE from cocaine for example, is often a useful indicator of exposure to the parent compound.

15.6.2 Biliary excretion

Small quantities of drug may diffuse passively into bile, but transporters are responsible for much higher rates of excretion. Cationic and anionic transporters (OCT, OAT, OATP) carry molecules into hepatocytes, whilst efflux proteins (P-gp, BCRP, MRP2, MATE1) secrete them into bile. This can result in some drugs (e.g. buprenorphine) having bile concentrations that are one or two orders of magnitude greater than their concentrations in blood (Ferner & Aronson, 2018). Consequently, it may be possible to detect these drugs in bile when they are not detectable in plasma.

Any drugs and metabolites that are not reabsorbed will remain in the GI tract and be removed from the body in faeces. For some drugs this may represent a large proportion of the dose. Temoporfin, which is given by i.v. infusion, is rapidly secreted into the GI tract via the bile and over 99 % of a dose can be recovered from faeces. Thus, finding large quantities of an orally administered substance in faeces cannot be taken as irrefutable evidence that the substance was not absorbed – it could have been absorbed and subsequently eliminated via faeces.

15.6.2.1 Recycling of xenobiotics

Xenobiotics may be detected in GI contents, even when they have not been administered orally. Firstly, xenobiotics may enter the stomach as predicted by the pH-partition hypothesis (Section 15.2.1.1). Secondly, they may diffuse into saliva, which when swallowed will introduce the compound to the GI tract. Thirdly, xenobiotics, including drugs and metabolites, may enter the GI tract via the bile. In each of these cases, a proportion of the xenobiotic may be (re)absorbed, thereby creating a cycle.

Enterohepatic cycling (EHC) usually refers to the situation where drugs such as morphine, digoxin, and carbamazepine form glucuronides that are transported into bile, which is secreted spasmodically into the GI tract via the bile duct. The salient features of EHC are (i) the glucuronides may be present in large quantities and (ii) there are microorganisms in the intestine containing β-glucuronidases that release the parent drug, which is then reabsorbed. Because bile is released in response to food, increases in plasma drug concentrations may be observed after meals, making pharmacokinetic modelling difficult (Malik *et al*., 2016). Genetic abnormalities, disease, and certain co-administered drugs may affect EHC. After overdosage, adsorbents such as activated charcoal may be administered to reduce reabsorption of the drug. On the other hand, EHC may be reduced inadvertently by giving antibiotics that destroy GI bacteria.

15.7 Pharmacogenetics and pharmacogenomics

Most of the recorded polymorphisms in drug response arise because of genetic differences in the expression of drug metabolizing enzymes and transporters. Phenotyping is carried out using probes, that is, test substances – debrisoquine, midazolam, sulfadimidine, and the like. A ‘cocktail’ containing up to six probes may be administered as a single oral dose to determine a subject’s metabolizer status. Genotyping uses standard molecular biology techniques such as

polymerase chain reactions (PCR) to sequence genes and identify variant alleles. An issue with determining the genome is demonstrating (i) that the gene is active and that the gene product (the enzyme or transmitter) is expressed, and (ii) that the gene can be related to the phenotype. Several databases offer guidance on which genetic tests should be conducted for particular drugs (Bousman *et al*., 2019).

If a compound is pharmacologically active, subjects who lack 'normal' drug metabolizing enzyme activity may be more likely to suffer dose-related adverse effects, whereas those with multiple copies of the intact gene may show no response unless the dose is increased. The converse may be true for prodrugs.

Knowledge of which enzymes and transporters exhibit polymorphism is important when predicting or explaining adverse effects of xenobiotics and when analyzing samples (Musshoff *et al*., 2010; Ahmed *et al*., 2016). Furthermore, differences in the distribution of 'mutant' genes is often related to ethnicity, so knowledge of an individual's racial grouping may also be informative.

15.7.1 Cytochrome P450

Several important polymorphisms in drug metabolizing enzymes, chiefly CYP2D6, CYP2C9, and CYP2C19, have been identified. Poor metabolizers (PMs) are homozygous with two copies of the defective gene and lack a functional enzyme, whereas extensive metabolizers (EMs) are homozygous for the 'normal' enzymes. Intermediary metabolizers (IMs) are heterozygous with one defective and one normal allele, and ultra-rapid metabolizers (UMs) have several copies of the 'normal' gene. Such differences can have a major influence on plasma concentrations at a given dose, and hence effect (Figure 15.20). Other examples of polymorphic CYPs are given in Table 15.4.

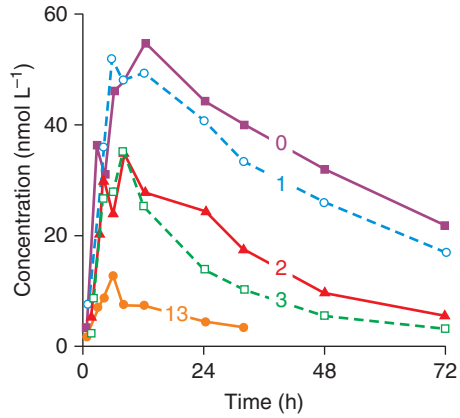

Figure 15.20 Mean plasma concentrations of nortriptyline after a single oral dose in subjects with varying numbers of *CYP2D6* genes (0–13) as indicated. Five subjects in each group apart from the individual with 13 genes (redrawn from Dalén *et al*., 1998–reproduced with permission of John Wiley & Sons)

Table 15.4 Selected examples of polymorphic CYPs

Enzyme	Substrates	Notes
CYP2A6	Nicotine	Reduced metabolism, particularly in some Asians
CYP2B6	Cyclophosphamide[a]	Rapidly metabolized to active 4-hydroxy in EMs
CYP2C9	Losartan[a]	
	Phenytoin	Increased adverse effects in PMs particularly those with two defective alleles, approximately 0.4 % in Caucasians
	(*S*)-Warfarin	Mutant allele reduces metabolism by approximately 40 %
CYP2C19	Omeprazole	Increased efficacy in PMs. High incidence in East Asians (13–23 %)
CYP2D6	Debrisoquine	Hypotension in PMs. Used for phenotyping
	Codeine,[a] tramadol[a]	Reduced analgesia in PMs
	Tamoxifen[a]	PMs do not respond as well as EMs

[a] prodrug

15.7.2 Atypical pseudocholinesterase

There are several genotypes of plasma cholinesterase. Approximately 94 % of the population are homozygous for the 'normal' allele and are designated EuEu. Of the atypical alleles, the one coding for a dibucaine-resistant form of the enzyme (Ea) is probably the most important. EaEa homozygotes (frequency 1:3000) show prolonged paralysis (*ca*. 2 h rather than approximately 6 min) when given the muscle relaxant suxamethonium (succinylcholine) and will require respiratory support until the drug is eliminated via other routes. Such individuals may be sensitive to other drugs including procaine, cocaine, pilocarpine, and physostigmine.

A more serious situation arises in homozygotes carrying the 'silent' Es gene who have no pseudocholinesterase activity and the duration of apnoea following suxamethonium may be over 8 hours. The frequency of this polymorphism is 1:100,000 of the population.

15.7.3 Alcohol dehydrogenase and aldehyde dehydrogenase

ADH is a dimeric enzyme made up of six separate sub-units, encoded by three genes, ADH_1, ADH_2, and ADH_3. Many combinations of isoenzymes exist, leading to different rates of metabolism amongst Caucasian, black African, and Asian populations.

ALDH is a mitochondrial enzyme. Some Asians have ALDH that differs from that of Caucasians and about 50 % of Asians (principally Chinese) have inactive ALDH, leading to flushing and other unpleasant effects when these individuals consume ethanol. These effects are similar to those seen with disulfiram.

15.7.4 Thiopurine methyltransferase

Phenotyping or genotyping of thiopurine methyltransferase (TPMT) is used to guide treatment with azathioprine to avoid life-threatening acute toxicity. The incidence of very low TPMT activities is relatively high (1:300), whilst 11 % of subjects have intermediate activity. 6-Mercaptopurine, derived from azathioprine is normally metabolized via one of three pathways: (i) methylation by TPMT, (ii) oxidation by xanthine oxidase, or (iii) by hypoxanthine phosphoribosyltransferase to active thiopurine metabolites including 6-thioguanine nucleotides. Patients with low TPMT activity have unusually high levels of 6-thioguanidine incorporated into DNA, which is, in part, responsible for the observed toxicity.

15.7.5 N-Acetyltransferase

Plasma isoniazid concentrations were shown to be bimodally distributed in the 1950s. Subjects were given identical doses and familial studies confirmed that this polymorphism was due to a genetic trait. Subjects were either 'rapid', or 'slow' inactivators. The rapid gene (R) is autosomal dominant, and thus only homozygotes (rr) are slow acetylators. The distribution of fast:slow acetylators is approximately 50:50 in white and black Americans, whereas Inuits and Japanese are fast acetylators (95 %), but some Mediterranean Jews are mainly (80 %) slow acetylators.

Fast acetylators may require higher doses of isoniazid for therapeutic efficacy. Slow acetylators may develop peripheral neuropathy due to depletion of pyridoxal by imine formation with isoniazid. Vitamin B_6 supplements may be given because this has no effect on antibiotic activity. Isoniazid-related hepatotoxicity was first described in Japan and is thought to be due to *N*-acetylhydrazine released from *N*-acetylisoniazid, which of course is present in much higher concentration in fast acetylators. The antidepressant iproniazid (*N*-isopropylisoniazid) shows similar hepatotoxicity.

Phenelzine, hydralazine, procainamide, and sulfadimidine (Figure 15.8) are amongst the drugs that are *N*-acetylated by NAT2. Sulfadimidine can be used to assess acetylator status using the Bratton–Marshall colorimetric method or by LC-UV (Whelpton *et al*., 1981).

15.7.6 UDP-Glucuronosyltransferases

UGT1A1 catalyzes the glucuronidation of bilirubin. The wild-type allele is *UGT1A1*1*, but a common mutant, *UGT1A1*28* leads to a mild form of hyperbilirubinaemia, known as Gilbert's syndrome that occurs in 5–10 % of the population. People with Gilbert's syndrome may be prone to the adverse effects of drugs or metabolites metabolized by UGT1A1, as in the case of the anti-neoplastic drug irinotecan.

3-Glucuronidation of morphine is catalyzed by several UGTs, but only UGT2B7 has been shown to catalyze both 3- and 6-glucuronidation. The rate of glucuronidation is higher in individuals with an allelic variant, *UGT2B7*2*.

15.8 Summary

An understanding of the factors affecting the disposition and fate of xenobiotics after different routes of administration is important to understanding clinical effect. Metabolic and excretory processes play a vital role in limiting the duration of drug action and in removing drugs and other potentially harmful substances from the body. However, biotransformation in some cases

produces metabolites that are considerably more toxic and/or have a longer duration of action than the parent substance. Knowledge of xenobiotic disposition and metabolism informs not only the choice of body fluid or other sample for analysis, but also the target analyte and the interpretation of the results obtained.

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16 Pharmacokinetics

16.1 Introduction

Pharmacokinetics (PK) is the study of the rates of the processes involved in the absorption, distribution, metabolism, and elimination of xenobiotics. By subjecting the change in plasma concentration of a drug as a function of time to mathematical analysis (mathematical modelling), parameters such as plasma half-life ($t_{½}$) and apparent volume of distribution (V) can be calculated. In forensic toxicology, knowledge of these parameters may allow either the timing, or the size of a dose to be estimated. However, there are usually caveats that must be applied in such circumstances. This chapter provides most of what is likely to be required by way of pharmacokinetic calculations in clinical and forensic toxicology. Additional material can be found in standard texts (Curry & Whelpton, 2017).

16.2 Fundamental concepts

The equations used in PK modelling are mainly those used in chemical kinetics. At the plasma concentrations encountered in therapy, most compounds exhibit first-order elimination, although the elimination of some, notably higher concentrations of ethanol, can be described using zero-order equations (Section 16.2.1.2). The kinetics of others, phenytoin for example, can only be described adequately using the Michaelis–Menten (M–M) equation (Section 16.6). Many drugs that exhibit first-order elimination kinetics at the doses used in therapy require the use of M–M kinetics to describe their time course following overdosage.

The plasma $t_{½}$ is a convenient way of summarizing the kinetics of a substance, but it is important to realise that plasma $t_{½}$ is controlled by clearance (CL) and V (Section 16.2.2).

16.2.1 Rates, rate constants, and orders of reaction

A general equation relating rate of decline in concentration ($-\mathrm{d}C/\mathrm{d}t$), rate constant (λ), and concentration (C) is:

$$-\frac{\mathrm{d}C}{\mathrm{d}t} = \lambda C^n \tag{16.1}$$

where n is known as the order of the reaction. In chemical kinetics n would be measured experimentally and is often close to an integer, frequently 0 or 1, and so such reactions are referred to as zero or first order, respectively.

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

16.2.1.1 First-order elimination

For a first-order reaction, substituting $n = 1$ in Equation (16.1) gives:

$$-\frac{dC}{dt} = \lambda C \tag{16.2}$$

indicating that the rate of the reaction is directly proportional to the concentration (or amount) of substance present. As the concentration of substance falls the rate of the reaction decreases. This is exponential decay [Figure 16.1(a)], analogous to radioactive decay where the probability of disintegration is proportional to the number of unstable nuclei present. The first-order rate constant has units of reciprocal time (e.g. h^{-1}). Integrating Equation (16.2) gives:

$$C = C_0 \exp(-\lambda t) \tag{16.3}$$

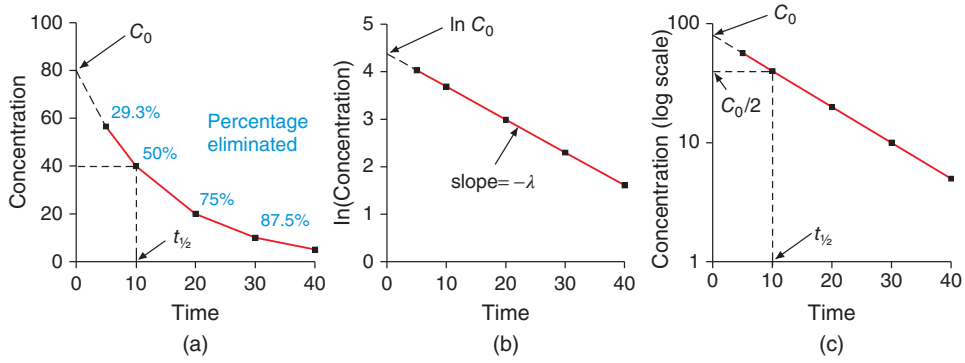

Figure 16.1 First-order elimination curves: (a) C *vs* t, (b) ln C *vs* t, and (c) C *vs* t using a semi-logarithmic scale

Taking natural logarithms of Equation (16.3):

$$\ln C = \ln C_0 - \lambda t \tag{16.4}$$

gives the equation of a straight line of slope $-\lambda$ [Figure 16.1(b)]. Often the data are presented by plotting C on a logarithmic scale when $t_{½}$ can be read from the semi-logarithmic plot [Figure 16.1(c)]. Because $t_{½}$ is the time for the initial concentration (C_0) to fall to $C_0/2$, then substitution in Equation (16.4) gives:

$$t_{½} = \frac{\ln 2}{\lambda} = \frac{0.693}{\lambda} \tag{16.5}$$

Note that ln 2 = 0.693. This important relationship, where $t_{½}$ is constant (i.e. independent of C_0), is *unique* to first-order reactions. Because $t_{½}$ is constant, 50 % is eliminated in $1 \times t_{½}$, 75% in $2 \times t_{½}$, and so on. Therefore when 5 $t_{½}$ have elapsed <4 % of the substance remains, and after $7 \times t_{½}$ <1 % remains.

16.2.1.2 Zero-order elimination

For a zero-order reaction $n = 0$, and substitution into Equation (16.1) shows that a zero-order reaction proceeds at a *constant rate*, so that a plot of C versus t is a straight line of slope $-\lambda$. Thus, zero-order rate constants have units of rate (e.g. g L^{-1} h^{-1}). The half-life is given by:

$$t_{½} = \frac{C_0}{2\lambda} \tag{16.6}$$

Table 16.1 Comparison of zero-order and first-order elimination

Reaction order	Concentration versus time plot	Rate of reaction	Half-life	Dimensions of rate constant[a]
Zero	Linear	Constant	Proportional to concentration	$M\ T^{-1}$
First	Exponential	Proportional to concentration	Constant	T^{-1}

[a] M = mass; T = time

The zero-order $t_{½}$ is inversely proportional to λ, but $t_{½}$ is also directly proportional to the initial concentration (Table 16.1). Therefore, the greater the amount of drug present initially, the longer the time taken for the amount to be reduced by 50 %.

16.2.2 Dependence of plasma half-life on volume of distribution and clearance

The plasma half-life is dependent on two fundament parameters: V and CL:

$$t_{½} = \frac{0.693V}{CL} \tag{16.7}$$

Therefore, changes in $t_{½}$ may be a result of changes in one or both of these parameters; increased V results in increased $t_{½}$, while increased CL results in decreased $t_{½}$.

16.2.2.1 Apparent volume of distribution

The simplest approach is to consider the drug or other xenobiotic in body as being in a single homogenous solution (single-compartment model). V is the volume of fluid that the amount (X) of such a substance in the body would have to be dissolved in to give the same concentration as the plasma concentration (C) of the substance at the time in question:

$$V = X/C \tag{16.8}$$

Therefore, V is a *constant of proportionality* that allows calculation of the amount of drug in the body from knowledge of the plasma concentration. It is normally measured in an experiment in which a dose (X_0) of drug is injected i.v. and timed blood samples are taken so that C_0 can be obtained by back extrapolation to $t = 0$ [Figure 16.1(c)]:

$$V = X_0/C_0 \tag{16.9}$$

Suitable markers, such as the dye Evans Blue, inulin, and deuterium oxide, can be used to measure anatomical volumes (Table 16.2). Some substances are confined to these volumes, but many have values of V much larger than total body water (TBW) because they are extensively distributed in tissues. V is often normalized to body weight (e.g. $L\ kg^{-1}$).

16.2.2.2 Clearance

The term clearance can be applied to individual organs, when it is known as organ clearance (CL_{org}) and is the volume of plasma from which a substance is completely removed per unit time as it flows through that organ. Hence, CL_{org} has units of flow (usually $mL\ min^{-1}$ or $L\ h^{-1}$).

Table 16.2 Examples of apparent volumes of distribution

Substance	V (L kg^{-1})	V (70 kg subject) (L)	*Notes*
Heparin	0.06	4.2	Too large to enter interstitial fluid
Evans Blue[a]	0.05	3.5	Dye used to measure plasma volume
Amoxicillin	0.2	14	Does not penetrate cells/CNS
Tubocurarine	0.2	14	Quaternary ammonium salt – does not penetrate cells/CNS
Inulin[a]	0.21	14.5	Used to measure extracellular fluid
Ethanol	0.55–0.7[b]	38–50	Distributes in total body water
Deuterium oxide (^2H_2O)[a]	0.55–0.7[b]	38–50	Used to measure total body water
Digoxin	5	350	Binds to Na^+/K^+ ATPase in muscle
Chlorpromazine	20	1400	Lipophilic – sequestered in tissues
Mepacrine (quinacrine)	500	35000	Intercalates in DNA

[a]Compounds used to measure anatomical volume
[b]Higher in men

Whole body clearance, also known as systemic clearance, CL, is the sum of the all the CL_{org} values. CL can be derived from:

$$CL = V\lambda \tag{16.10}$$

CL can be measured experimentally because λ can be obtained from the slope of a plot of ln C versus t (Figure 16.1) and V from Equation (16.9). The rate of elimination of a substance from the body is its plasma concentration, C, multiplied by CL.

CL is measure of how well the eliminating organs can metabolize or excrete a substance. Enzyme induction (Section 15.5.5) may increase CL, as may manipulation of urine pH to increase the excretion of weak electrolytes, such as salicylic acid and amfetamine. Conversely, enzyme inhibition may reduce CL. Liver or kidney disease may also reduce CL, but there may be accompanying changes in V, so predicting the effect on $t_{½}$ is not straightforward.

16.3 Absorption and elimination

16.3.1 First-order absorption

Other than following i.v. or intra-arterial (i.a.) injection, administered drug has to be absorbed, and so the plasma concentration–time curve must have a rising phase. The kinetics of absorption may be complex, but often first-order absorption is a reasonable approximation. The equation for the plasma concentration as function of time in single-compartment model with simultaneous first-order input and output is:

$$C = F\frac{Dose}{V}\frac{k_a}{k_a - \lambda}[\exp(-\lambda t) - \exp(-k_a t)] \tag{16.11}$$

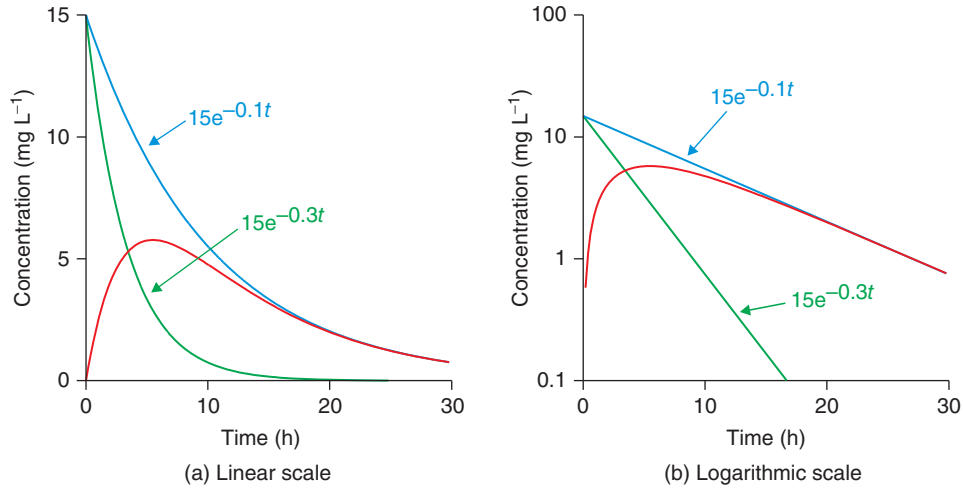

Figure 16.2 Concentration–time curves showing first-order input into a single-compartment model with (a) linear *y*-axis and (b) logarithmic *y*-axis. Model based on Equation (16.11) with *y*-intercept = 15 mg L^{-1}, $k_a = 0.3\ h^{-1}$, and $\lambda = 0.1\ h^{-1}$

where k_a is the first-order rate constant of absorption and F is the fraction of the dose that reaches the systemic circulation. The concentration is maximal (C_{max}) when the rate of absorption equals the rate of elimination, after which elimination dominates and the plasma concentration declines (Figure 16.2).

16.3.2 Quantification of F

In Equation (16.11) the term F is included because in many cases not all of the dose administered reaches the systemic circulation because of limited absorption or pre-systemic metabolism (Section 15.3.1.1). When input and output are first order, F can be calculated by dividing the area under the plasma concentration–time curve (AUC) after a test dose given, for example, by mouth, by the AUC obtained after giving the same size dose i.v. on a second occasion:

$$F = \frac{AUC_{po}}{AUC_{iv}} \tag{16.12}$$

It is very important to consider the effect of F when estimating expected plasma concentrations. Even if a literature value of F is known, the extent of absorption may be altered either after overdose, or by the presence of other substances. Measurement of AUC using the trapezoidal rule is explained below (Section 16.8).

16.3.3 Maximum concentration (C_{max})

C_{max} is often taken as the maximum concentration in the plasma concentration data. However, it can be calculated. For a single-compartment model, the time the maximum concentration occurs, t_{max}:

$$t_{max} = \frac{1}{k_a - \lambda} \ln \frac{k_a}{\lambda} \tag{16.13}$$

and

$$C_{max} = F\frac{Dose}{V}\exp(-\lambda t_{max}) \tag{16.14}$$

Note that t_{max} is dose independent, but C_{max} is directly proportional to the dose – an important feature of first-order pharmacokinetics.

16.4 Drug accumulation

It is usual for drugs to be given in therapy as several doses over a period of time. Because subsequent doses are given before all of a previous dose has been eliminated, the amount of drug in the body will accumulate. However, provided the drug is not eliminated according to zero-order kinetics, the plasma concentration will not increase indefinitely. This is most easily understood by considering a constant rate i.v. infusion.

16.4.1 Intravenous infusion

When a drug is infused at a constant rate, R_0, the plasma concentration will increase as the infusion progresses, but as the plasma concentration increases, the rate of elimination also increases until the elimination rate equals the infusion rate. When this steady state is reached the plasma concentration will be constant, C^{ss}, and providing that elimination is first order:

$$R_0 = VC^{ss}\lambda \tag{16.15}$$

The concentration during the rising phase is given by:

$$C = C^{ss}[1 - \exp(-\lambda t)] \tag{16.16}$$

Equation (16.16) represents an exponential curve that starts at 0 and asymptotes to C^{ss}. In essence, it is a decay curve that has been 'flipped over'. Therefore, because the decay curve goes from C_0 to $C_0/2$ in $1 \times t_{½}$, the infusion curve goes from 0 to $C^{ss}/2$ in $1 \times t_{½}$. In other words, the concentration increases to 50 % of C^{ss} in $1 \times t_{½}$, to 75 % in $2 \times t_{½}$, and to 87.5 % of the steady-state value in $3 \times t_{½}$. Because the rate of attainment of steady-state conditions is a function of $t_{½}$, a drug with a short half-life reaches steady-state before a drug with a longer half-life. The plasma concentration will be >99 % of C^{ss} within seven half-lives [Figure 16.3(a)].

16.4.1.1 Loading doses

To reduce the time required to reach steady-state conditions, one or more loading dose(s) (*LD*) may be given [Figure 16.3(b)]. An i.v. bolus dose, chosen to give C^{ss} instantaneously, will result in the rates of elimination and infusion being equal at $t = 0$, therefore *LD* should be:

$$LD = VC^{ss} \tag{16.17}$$

However, by rearranging Equation (16.15), *LD* can be calculated from:

$$VC^{ss} = R_0/\lambda \tag{16.18}$$

The steady-state concentration can be calculated from:

$$C^{ss} = R_0/CL \tag{16.19}$$

Note how *CL* is used to choose the infusion rate for a required C^{ss} and *V* is used to calculate *LD*.

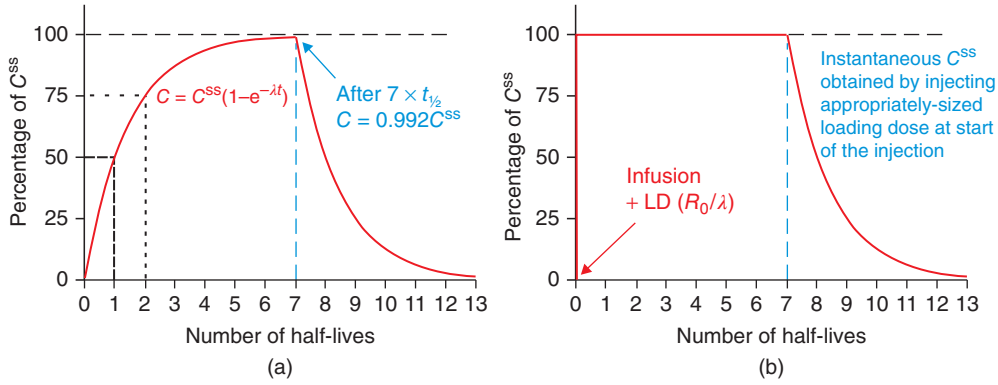

Figure 16.3 (a) Constant rate infusion into a single-compartment model showing the percentage C^{ss} as a function of number of half-lives elapsed (b) with an additional loading dose. The infusion was stopped after seven half-lives

16.4.2 Multiple dosage

A drug given as equal sized doses at equal intervals will produce a plasma concentration–time plot similar to one of those illustrated in Figure 16.4, depending on the $t_{½}$ of the drug. The average plasma concentrations will asymptote to a steady-state value, C^{ss}_{av}, in the same way as during a constant rate infusion, but now the concentration will fluctuate between doses. The fluctuations will be greater when the drug has a short $t_{½}$ because a greater proportion of the dose will be eliminated before the next dose. It is important, if possible, to use dosage regimens that maintain the steady-state concentrations within the 'therapeutic window', that is, above the threshold concentration for the required effect, but below concentrations at which adverse effects may become apparent. Some drugs have a narrow therapeutic window and it may prove difficult to maintain the concentrations in the required range, particularly when they also a have short $t_{½}$. Morphine, for example, may cause respiratory depression at the peak concentration, but patients may experience pain before the next dose.

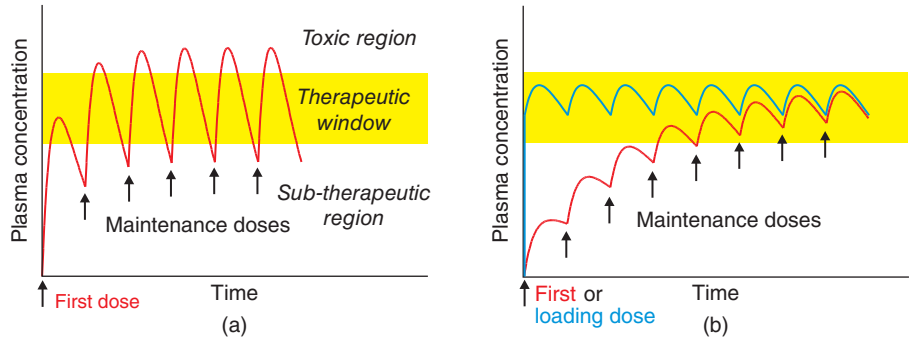

Figure 16.4 Plasma concentration–time plots following repeated doses at equal intervals for a drug with (a) a relatively short half-life, and (b) a relatively long half-life

The fluctuations in concentration are less with a drug with a long $t_{½}$, but it takes longer to reach steady-state conditions than with a drug with a shorter $t_{½}$. One or more loading doses may be given to ensure a more rapid onset of effect [Figure 16.4(b)] as described for i.v. infusions. Because (i) the maximum (peak) and minimum (trough) concentrations can vary so much, (ii) the peak value may be influenced by external factors (e.g. food in the stomach), and (iii) there is uncertainty about when the peak occurs, the timing of taking samples for TDM is usually standardized. Thus, samples may be taken just before the next dose (i.e. the 'trough' concentration) or some defined time (e.g. 12 h after the last dose) (Section 20.2).

16.5 Sustained-release preparations

SR preparations are designed to deliver drug at a constant rate over a prolonged time period thereby simplifying life for the patient and hopefully improving the efficacy of therapy. By making the absorption rate constant (k_a) smaller than the elimination rate constant (λ) it is possible to prolong the duration of action. As with any sequential reaction, the rate constant of the slowest step determines the overall rate, and under these conditions, k_a becomes rate determining (Figure 16.5) and the terminal $t_{½}$ of a plasma concentration–time plot is the $t_{½}$ of absorption. This apparent switching of k_a and λ can occur after acute overdosage if there is prolonged absorption due to either a drug delaying its own absorption, or when the presence of other drug(s) reduces k_a to the point it becomes rate determining. Thus, the time for the drug to be eliminated from the body can be greatly enhanced.

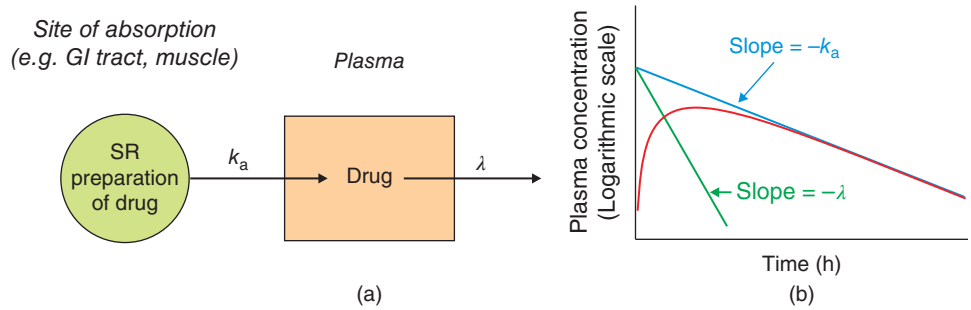

Figure 16.5 Principle of sustained-release preparations. $k_a < \lambda$ so it is rate determining, and the plasma concentration declines with a half-life determined by k_a

SR formulations are available for most routes of drug administration, including oral, subcutaneous (s.c.), i.m., and transdermal. Oral SR preparations make use of different particle sizes, wax matrixes, or tablets made of layers of material, so that different rates of dissolution give prolonged drug release. Several formulations of insulin are available, including soluble insulin and several crystalline forms that release insulin at different rates after s.c. injection. Long-acting i.m. depot injections are exemplified by fluphenazine decanoate, a very lipophilic ester of fluphenazine. It is slowly hydrolyzed to fluphenazine *in vivo*, which is released into plasma from the injection site. Doses may be given 2–4 weekly.

Transdermal delivery of drugs for systemic effect is a relatively new phenomenon, although absorption of toxic solvents though the skin has been recognized for many years. Glyceryl trinitrate (GTN) is readily absorbed through the skin and may be applied either as an ointment

rubbed on to an area of skin, or as 'sticking plaster' patch. In some preparations the plaster incorporates a membrane to control the rate of GTN release. Hyoscine (scopolamine), nicotine, buprenorphine, and some steroid hormones may be given in this way.

Transdermal patches of fentanyl are designed to be applied over three days, and with such a large dose of drug are prone to misuse. Patches have either been chewed, or attempts made to extract the opioid by boiling, or extracting with organic solvents. Alternatively, the patches have been heated to increase the rate of absorption to induce a 'high', or smoked (heating patch on a foil and inhaling the smoke through a tube) (Kuczyńska *et al*., 2018). There have also been serious accidents when the patches have been heated inadvertently whilst applied to the skin (Voigt, 2013; Hessulf, 2019).

16.6 Non-linear pharmacokinetics

First-order elimination results in simple relationships, which makes dosing and interpretation of analytical results relatively simple. CL, $t_{½}$, t_{max}, and time to reach C^{ss} are constant, and AUC, C_{max}, and C^{ss} are directly proportional to dose. Consequently, drugs that exhibit first-order elimination, when used at normal doses are more likely to be marketed than those with more complex pharmacokinetics. However, there are situations when first-order models are inappropriate. The kinetics of drugs such as phenytoin are best described using the Michaelis–Menten (M-M) equation:

$$\text{rate} = \frac{V_{max}C}{K_m + C} \tag{16.20}$$

If the concentration of drug is low, then $K_m \gg C$ and denominator $(K_m + C)$ in Equation (16.20) approximates to K_m (C is making a negligible contribution to the sum) so:

$$\text{rate} \approx \frac{V_{max}}{K_m C} \tag{16.21}$$

which is a first-order equation where $V_{max}/K_m = \lambda$. Therefore, even for drugs that are extensively metabolized, the elimination kinetics will be first order provided that drug metabolizing enzyme activity is greatly in excess of the amount of drug present.

However, if the drug concentration is very high compared with drug metabolizing enzyme capacity, $C \gg K_m$, and $(K_m + C) \rightarrow C$, so that:

$$\text{rate} \approx V_{max} \tag{16.22}$$

This is a zero-order equation because the reaction rate is constant. The enzyme is saturated with substrate and the rate of reaction is maximal, V_{max}. For first-order reactions, steady-state concentrations are proportional to dose, but as one moves from first to zero order the concentration rises disproportionately with increasing dose [Figure 16.6(a)]. Unlike the first-order case, CL is not constant, but declines with increasing plasma concentration. AUC is *not* proportional to dose and Equation (16.12) *should not* be used to calculate F, because the value will be less than the true value (Section 16.11.1.3).

Drugs whose pharmacokinetics can only be adequately described by M–M kinetics include phenytoin, voriconazole, ethanol (Section 16.6.1), and (at higher doses) salicylate. Adjusting the dose of a drug such as phenytoin to ensure that plasma concentrations remain in the therapeutic window is complicated by the fact that there are large individual variations in K_m and V_{max} [Figure 16.6(a)]. Although 'population' values of K_m and V_{max} could be used to calculate doses to obtain a required steady-state concentration, it is clearly better to use individual values.

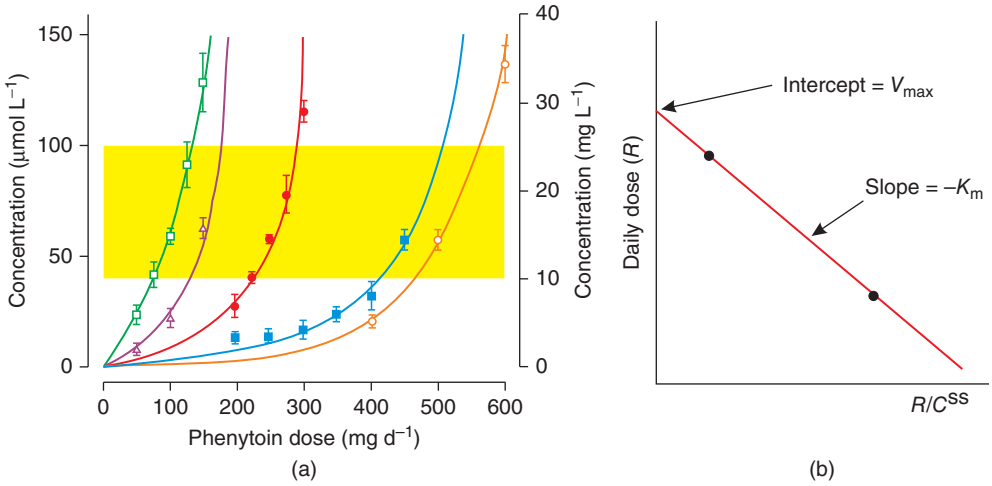

Figure 16.6 (a) Steady-state serum concentrations of phenytoin in five subjects showing the interpatient variability in K_m and V_{max} (Richens & Dunlop, 1975–reproduced with permission of Elsevier). (b) Graphical solution for V_{max} and K_m

Because there is a need to solve equations for two unknown values, steady-state concentration data for at least two doses are required. If the daily dosing rate is R, then at steady state the M–M equation can be written thus:

$$R = \frac{V_{max} C^{ss}}{K_m + C^{ss}} \tag{16.23}$$

Rearrangement gives:

$$R = V_{max} - \frac{R}{C^{ss}} K_m \tag{16.24}$$

which is the equation of a straight line, of slope $-K_m$, and y-intercept, V_{max}. Therefore, values for V_{max} and K_m can be obtained graphically [Figure 16.6(b)]. Once V_{max} and K_m have been estimated then the required dosing rate for a chosen value of C^{ss} can be calculated from Equation (16.23), or C^{ss} can be calculated by rearranging Equation (16.24):

$$C^{ss} = \frac{K_m R}{V_{max} - R} \tag{16.25}$$

Disproportionate increases in plasma concentration with increasing dose of drugs such as phenytoin require the dose to be carefully adjusted, and although a daily dose might be typically 300–500 mg as the sodium salt, small (25 and 50 mg) tablet sizes are available to facilitate this. Similarly, anything that changes the 'effective' dose, for example, changes in bioavailability, or enzyme induction or inhibition (Section 15.5.5), is likely to have a large effect on the plasma concentration and hence pharmacological action.

Because C^{ss} is proportional to K_m [Equation (16.25)], changes in this constant, as a result of competitive enzyme inhibition by a second xenobiotic, for example, will lead to proportionate changes in C^{ss}. The example of phenytoin illustrates not only the complexity of prescribing a drug that exhibits M–M kinetics, but also the difficulty in interpreting plasma concentration data if an overdose of drug or other xenobiotic has saturated either metabolism, or carrier-mediated transport.

Despite the fact that zero-order equations are used to predict blood alcohol concentrations (BAC) (Section 16.11.1.3), the kinetics of ethanol exemplify how, over a large range of concentrations as might be encountered in drug overdose, the kinetics of a drug can apparently change from zero to first order. The slope of the ln(concentration)–time plot is shallow initially while the kinetics are approximately zero order – a high rate of change (approximately V_{max}), but a small proportionate change in concentrations. The slope becomes progressively steeper as the kinetics approach first order, eventually approximating to $-\lambda$, as exemplified in Figure 16.7.

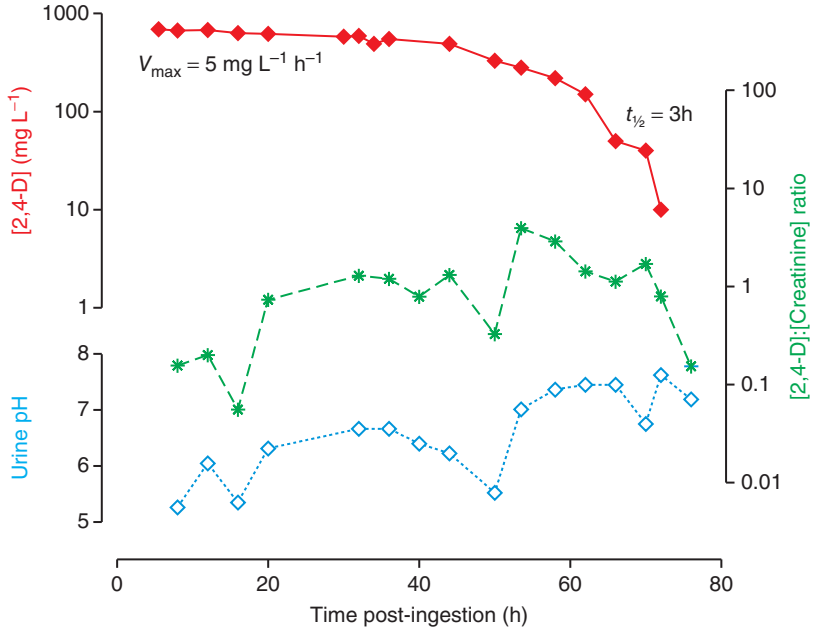

Figure 16.7 Plasma 2,4-D concentrations (♦), urinary excretion (*), and urine pH (◊) in a patient poisoned with 2,4-D and treated with i.v. sodium bicarbonate

16.6.1 Example of non-linear kinetics in overdose

Chlorophenoxy herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) are largely eliminated unchanged by the kidney. Saturable transporters, probably OAT1/3 in the basolateral membrane and OAT4 in the luminal membrane, are involved. Unless ionization of the acid is promoted to increase renal clearance by giving sodium bicarbonate, prolonged coma with the attendant risks of pulmonary complications, rhabdomyolysis, and renal failure may result.

Plasma 2,4-D, urinary 2,4-D excretion, and urine pH during treatment of a patient who had ingested 2,4-D are shown in Figure 16.7. The initial decline in plasma 2,4-D concentration was zero order, $V_{max} = 5$ mg L^{-1} h^{-1}. The apparent $t_{½}$, calculated using the first eight plasma points was approximately 70 h. When the plasma concentration had fallen by 2 orders of magnitude, the data showed first-order kinetics with $t_{½}$ approximately 3 h. Without further data it is impossible to be sure that the first-order plasma $t_{½}$ of 2,4-D was not even shorter (2,4-D was not detected in a 76 h sample).

However, in this case the situation has been complicated by the effect of therapy. 2,4-D has a low pK_a (2.6), hence 99.8 % will be ionized at pH 5.3 and 99.9996 % at pH 8.0. Cautious treatment with sodium bicarbonate markedly reduced the hydrogen ion concentration in the urine, and this was reflected in an increased urinary 2,4-D excretion rate, as indicated by the 2,4-D:creatinine ratio (Figure 16.7). The correlation between pH and urinary excretion rate can be seen clearly. At later time points the excretion rate is reduced somewhat because it is also a function of the plasma concentration. Sodium bicarbonate is important in treating any acidosis, and in reducing the amount of herbicide in tissues, because the increased degree of ionization shifts equilibrium towards plasma, as with salicylate (Figure 15.3). In effect, this *reduces* the apparent volume of distribution, thereby introducing a second mechanism by which $t_{½}$ is reduced.

16.7 Multi-compartment models

Thus far it has been assumed that a drug in the body can be treated as if it were in a single homogenous solution. Many basic PK concepts can be understood using such a model. However, it is difficult to find 'real' examples of drugs that conform to a single-compartment model, and more complex models may be required to explain some observations.

If after an i.v. bolus injection, the plasma concentration decay curve can be best described by the sum of *two* exponential terms [Figure 16.8(a)]:

$$C = C_1 \exp(-\lambda_1 t) + C_2 \exp(-\lambda_2 t) \tag{16.26}$$

then a two-compartment model is required. The number of exponential terms describing the *decay* defines the number of compartments for the model. The last (lowest) rate constant may be referred to as the terminal rate constant, λ_z, and for a two-compartment model, $\lambda_z = \lambda_2$.

The two-compartment model requires the concept of a second compartment with an apparent volume of distribution, V_2, into which the drug moves more slowly (determined by k_{12}) than the rapid ('instantaneous') distribution of drug to the tissues of the central compartment (V_1)

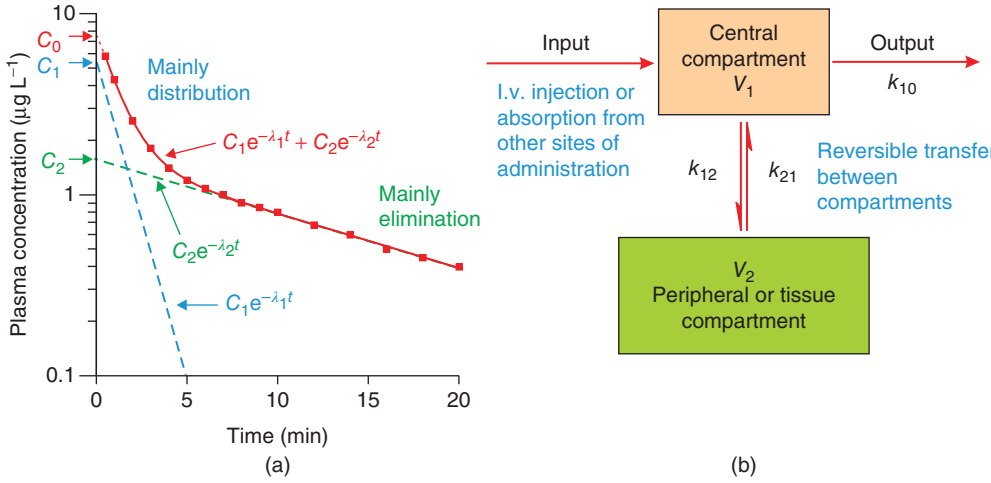

Figure 16.8 Decay from a two-compartment kinetic model following a i.v. bolus injection, (a) decay in plasma concentrations (b) schematic representation of the model

[Figure 16.8(b)]. Distribution to the second, peripheral compartment, explains the initial steep slope of the plasma concentration–time curve. Later, after equilibration between compartments, the slope of the decay curve is shallower, and the main mechanism of decay is elimination of drug from the body [Figure 16.8(a)].

The central compartment always includes plasma and often includes well-perfused tissues, such as liver, heart, and lung. Tissues of the peripheral compartment(s) may be skeletal muscle and adipose tissue. The brain may be in either compartment depending on the drug. For small, non-protein bound molecules, it is to be expected that ECF will be part of the central compartment because the fenestrations in peripheral capillary walls provide little in the way of a barrier to the movement of such molecules. After i.v. infusion, morphine plasma concentrations were fitted to a three-compartment model, the volume of the central compartment being 12.7 L, in keeping with the idea that non-bound drug in plasma rapidly enters ECF via fenestrations in the capillary walls (Meineke *et al*., 2002).

Lipophilic molecules rapidly cross cell walls and the rate of equilibration is largely controlled by the blood flow to the tissues. Distribution of a lipophilic substance might be predicted as: central compartment comprising plasma and well-perfused tissues and peripheral compartment comprising tissues, which, for their size, receive less of the cardiac output (e.g. muscle, fat, bone, etc.). I.v. injections of thiopental and fentanyl produce rapid effects because they are rapidly transferred to the brain, but may also have short durations of action because they are distributed from brain, via plasma, to peripheral tissues, with subsequent falls in brain concentration and cessation of effect.

16.7.1 Calculation of rate constants and volumes of distribution

The rate constants λ_1 and λ_2 [Figure 16.8(b)] can be solved either graphically, or by iterative curve fitting of the data. The values of the other variables, k_{10}, k_{12}, and k_{21} (known as micro-constants), and V_1 and V_2 can be calculated (Curry & Whelpton, 2017). The sum of V_1 and V_2 is referred to as the volume of distribution at steady-state, V_{ss}:

$$V_{ss} = V_1 + V_2 \tag{16.27}$$

V_{area} takes into account the fact that the peripheral-compartment concentrations are higher than those in plasma. It is calculated from:

$$V_{area} = \frac{Dose}{AUC\ \lambda_2} \tag{16.28}$$

V_{area} is a constant of proportionality that should be used to derive the amount of drug in the body from the plasma concentration post-equilibrium following a single dose of drug.

16.7.2 Multiple-compartment models in analytical toxicology

After rapid i.v. dosing it is usual to observe a distributional phase, provided samples are collected soon enough after dosing. However, finding literature values for V_1, V_2, and the microconstants, etc., may prove difficult. Databases are more likely to have values of CL and V and/or $t_{½}$. Therefore, any modelling and predictions will have to utilize a limited data set and will have to use equations largely derived for a single-compartment model. However, it must be remembered that blood samples taken before equilibration of drug with tissues may have much higher concentrations than those predicted for a single-compartment model. This could lead to a gross overestimate of the amount of drug in the body and hence the dose that has been taken.

A useful equation for calculating average steady-state concentrations following multiple dosing is:

$$C_{av}^{ss} = F\frac{Dose}{V_{area}\lambda_2\tau} \tag{16.29}$$

where τ is the dose interval. Of course, peak and trough concentrations will fluctuate about the average concentration, the fluctuations being greater for drugs with short $t_{½}$ values (Figure 16.4).

16.8 Non-compartmental methods

If the compartmental model approach is inappropriate, some pharmacokinetic parameters can usually be derived from *AUC* and λ_z, without establishing the number of compartments. *AUC* is usually obtained using the trapezoidal method (Figure 16.9).

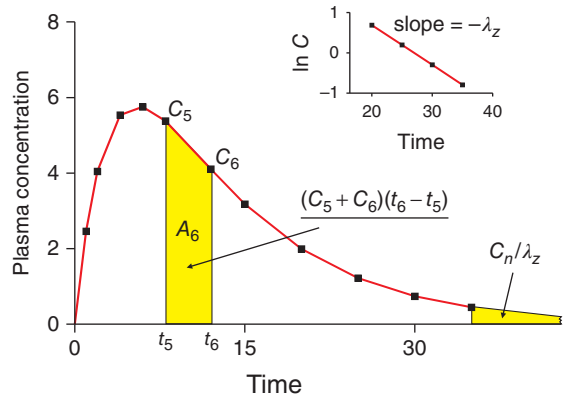

Figure 16.9 The trapezoidal method of measuring *AUC* (Inset: measurement of terminal elimination rate constant, λ_z)

The plasma concentration–time data are treated as a series of trapeziums and the areas of each calculated and summed:

$$AUC_{(0-t_n)} = \frac{(C_1 + C_2)(t_2 - t_1)}{2} + \cdots + \frac{(C_{(n-1)} + C_n)(t_n - t_{(n-1)})}{2} \tag{16.30}$$

where n is number of samples. The area from the last time point, t_n, to infinity is calculated from C_n/λ_z and added to $AUC_{(0-t_n)}$ to give $AUC_{(0-\infty)}$. λ_z is calculated from the slope of ln C versus t plot of the terminal points (Figure 16.9, inset).

After an i.v. bolus dose V_{area} can be calculated using Equation (16.28): For an oral dose the equation is:

$$V_{area} = F\frac{Dose}{AUC\lambda_z} \tag{16.31}$$

An intravenous dose will be required to estimate a value for F.

CL can also be obtained from knowledge of *AUC* using Equation (16.32). For a parenteral dose:

$$CL = \frac{F\ Dose}{AUC} \tag{16.32}$$

Note that F will have to be obtained from a separate i.v. investigation.

16.9 Factors affecting pharmacokinetic parameters

Increases in *CL*, as a result of enzyme induction for example, will result in decreases in $t_{½}$ and *AUC* for a given dose. The converse is also true. The effect of pharmacogenetics on drug disposition was considered in Section 15.7. Other factors that need to be considered are the gastrointestinal contents (i.e. food, liquids, and other substances that affect absorption of orally administered xenobiotics), age, sex, and disease.

16.9.1 Gastrointestinal contents and gastrointestinal motility

Substances, including food, liquids, drugs and other xenobiotics, in the GI tract can influence the rate and extent of absorption of not only the substances themselves, but also of other substances that have been taken orally. Xenobiotics must dissolve in the luminal fluids before they can be absorbed, and the volumes of such fluids can influence the rate of absorption. A high volume may facilitate dissolution of poorly soluble compounds, but lower volumes will produce greater concentration gradients leading to increased rates of passive absorption. After overnight fasting (8–10 h) the volume of stomach contents is approximately 10–50 mL, however in the presence of food the volume may be more than 10-fold greater (Koziolek *et al*., 2019).

Generally, food delays gastric emptying and so may delay the absorption of xenobiotics that are normally absorbed from the intestines, but not necessarily the extent of absorption [Figure 16.10(a)]. Furthermore, fibre in food may adsorb some drugs (digoxin, for example) thereby reducing their bioavailability. The increase in gastric acid and the delay in gastric emptying that normally accompanies the ingestion of food will increase the decomposition of acid labile drugs such as ampicillin, isoniazid, and azithromycin. On the other hand, poorly soluble weak bases, such as itraconazole and ketoconazole, are better absorbed when gastric pH is low [Figure 16.10(b)]. It is assumed that when such drugs are released into the intestine the dissolved proportion is absorbed rather than being precipitated at the higher pH values of the small intestine.

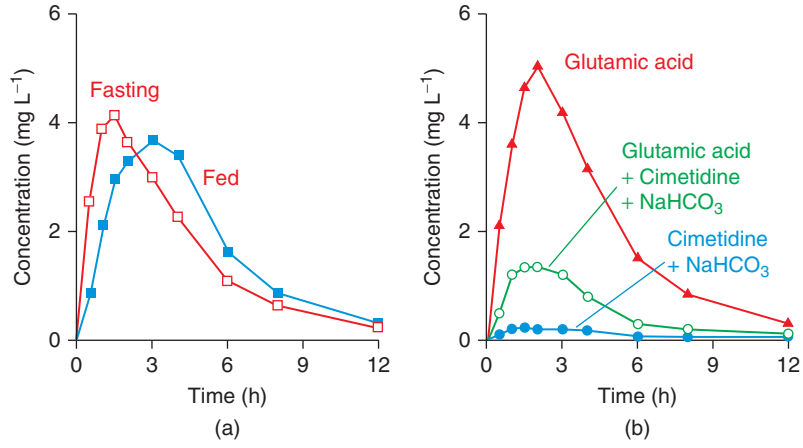

Figure 16.10 Effect of (a) food and (b) pH on the oral absorption of ketoconazole. Gastric pH was lowered by administration of glutamic acid and achlorhydria was simulated by a combination of cimetidine and sodium bicarbonate (Charman *et al*., 1997–reproduced with permission of John Wiley & Sons)

Specific components of food may affect bioavailability. The oral absorption of bisphosphonates, quinolones, and penicillamine, as with tetracyclines (Table 15.2), is reduced if they chelate calcium, for example from dairy products. The absorption of some drugs (carbamazepine, griseofulvin, posaconazole, saquinavir, tacrolimus) is increased when they are taken with fatty meals. Lipids in the upper small intestine increase secretion of biliary and pancreatic fluids and this is believed to either solubilize drugs, or facilitate absorption via micelles formed from bile acids. Dietary components of several fruit juices, orange and apple as well as the well documented grapefruit juice, herbal extracts, and green teas have been implicated in the inhibition of OATP1A2, with subsequent reduction in the absorption of substrates that are taken up by this transporter (Table 15.1). Thyme inhibits acetylcholinesterase.

Increasingly, it is becoming clear that the trillions of microbes residing in the GI tract (the biota), of which there are some 700–1000 different bacterial species, make an important contribution to the overall PK parameters of many xenobiotics (Walsh *et al*., 2018). These bacteria, which are concentrated in the colon, secrete a diverse range of enzymes capable of metabolizing various drugs, including by reduction, hydrolysis, dehydroxylation, denitration, dehalogenation, acetylation, and deacetylation. The reactions are often different from those encountered with human drug metabolizing enzymes (Wilson & Nicholson, 2017). The microbiome, i.e. all of the genetic material of the biota, reacts rapidly to changes in diet, for example a diet high in animal protein favours bile-tolerant bacteria. Antibiotics and laxatives will disturb the microbiome and may alter the metabolites that can be detected in plasma and in urine.

GI tract motility may be affected by food and by drug treatment. When food is ingested, the proximal stomach relaxes to accommodate the incoming food and then contracts to push it to the distal stomach, which mixes and grinds the food by powerful and regular contractions. Solids are normally not released into the duodenum until they are *ca*. 2–4 mm in size. Metoclopramide, which decreases gastric emptying time by an action on the pyloric sphincter and increases peristalsis in the duodenum, increases the oral absorption of paracetamol, pivampicillin, and ethanol, but decreases the absorption of digoxin.

Opioid and anticholinergic drugs, including antipsychotics and TCAs that have anticholinergic activity, reduce GI tract motility, and increase transit times, resulting in decreased absorption of lidocaine, paracetamol, and pivampicillin, but increased absorption of digoxin. Clozapine has an especially marked effect on GI motility. In one study, patients treated with clozapine had a median colonic transit time of 104.5 h, over four times longer than those on other antipsychotics (Every-Palmer *et al*., 2016).

Changes in GI motility may be mediated by drugs acting centrally. Reduced CNS activity, for example in brain trauma, markedly reduces GI tract motility (Bor *et al*., 2016), as does drug-induced coma. An overdose of a modified-release preparation or a drug that slows GI tract motility may result in a substantial amount of drug remaining in the GI tract should the patient die (Livshits *et al*., 2015).

16.9.2 Age

In neonates and in a relatively large proportion of the elderly, gastric pH values are high (achlorhydria) and the bioavailability of acid labile drugs such as penicillin G may be much higher than expected. However, some drugs, for example ketoconazole, are more rapidly absorbed when gastric pH is low.

In neonates the proportion of body water, particularly extracellular water, is high and the proportion of fat low [Figure 16.11(a)]. These factors will influence the distribution of both lipophilic and hydrophilic drugs. Neonates have lower plasma pH values than adults and this will affect the distribution of weak electrolytes. The proportion of body fat is higher in the elderly,

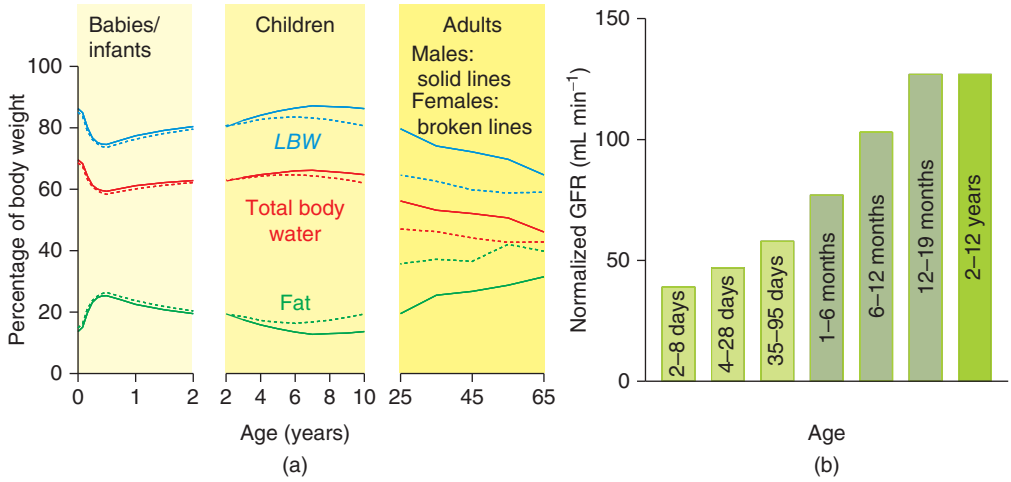

Figure 16.11 Effects of age on (a) relative distribution of lean body weight (LBW), total body mass (TBM) and fat (b) glomerular filtration rate (GFR) normalized to 1.73 m^2 of body surface area (Curry & Whelpton, 2017–reproduced with permission of John Wiley & Sons)

and changes in tissue perfusion, possibly caused by arteriosclerosis and a reduction in cardiac output, may be responsible for changes in tissue distribution. Plasma protein binding may be less in both neonates and the elderly, and this may lead to a fall in *total* blood concentrations, but to an increase in the non-bound fraction in plasma, and hence an increased activity.

In neonates, several drug metabolizing enzyme systems are not fully developed, including glucuronidation, acetylation, and plasma esterase activity. Morphine has a long $t_{½}$ in neonates and this drug is not used normally in obstetrics. Chloramphenicol (metabolized by acetylation) is safe provided the dose is appropriate, otherwise cardiovascular collapse ('grey-baby' syndrome) may ensue. The $t_{½}$ of diazepam is longer in neonates, particularly premature neonates, than in children because of reduced drug metabolic capacity. In adults the terminal half-life (20–90 h) increases linearly with age (20–80 yr) being due primarily to V increasing with age.

16.9.2.1 Effect of age on renal function

The renal clearance of a substance (e.g. inulin) that is totally filtered at the glomerulus and not reabsorbed can be used to measure glomerular filtration rate (GFR). Alternatively, creatinine clearance, or the serum creatinine concentration may be used to estimate a value (eGFR). Normal creatinine clearance as a function of age (yr) and weight (kg) can be calculated from Equation (16.33):

$$CL_{\text{Creatinine}} = (140 - Age)\frac{Weight}{Factor} \tag{16.33}$$

The *Factor* is to account for differences between men (70) and women (85). GFR increases after birth, reaching adult values by about 12 yr [Figure 16.11(b)]. In healthy young men, GFR is 125 mL min^{-1}, but is 50 % of this at age 75 yr.

16.9.3 Sex

Although a genetic phenomenon, the effects of sex on drug disposition are normally considered separately from pharmacogenetics *per se*. Men are generally heavier and have larger muscle

mass and organ blood flow than women, factors that can influence drug disposition. Women have a higher percentage of body fat than men [Figure 16.10(a)] and for lipophilic drugs such as diazepam and trazodone, V may be higher in women. For drugs that are distributed in TBW (e.g. fluconazole, ethanol) V is lower in women. Women also have reduced gastric ADH activity, resulting in higher plasma concentrations of ethanol for a given dose. GFR is directly proportional to body weight, and so differences in renal clearance may be attributable to differences in average weight. Men have higher serum creatinine concentrations (larger muscle mass) and so if serum creatinine is used to estimate GFR, a correction must be applied [as with Equation (16.33)].

Although women have not been studied as frequently as men, there is evidence to suggest that women have higher CYP3A4 activity than men, but that UGT activity is greater in men than women (Soldin *et al*., 2011). These findings may explain the sex-related differences that have been reported in the clearance of some drugs (Table 16.3). Erythromycin is one of the few compounds for which studies have shown consistently higher clearance in women when compared with men.

Table 16.3 Sex-related differences in drug clearance reported in the literature

Greater in women	Greater in men	No difference
Diazepam[a]	Clozapine, olanzapine	Amatadine
Erythromycin[a]	Digoxin[c]	Lidocaine
Midazolam[a]	Fluorouracil	Nitrazepam
Prednisolone[a]	Oxazepam, temazepam	
Theophylline[b]	Paracetamol	
Verapamil[a]	Salicylic acid	

[a]CYP3A4 substrate
[b]During menses
[c]Renal

There is some evidence that, apart from CYP3A4, men have higher activities of other drug-metabolizing cytochromes than women. Women attain higher plasma clozapine concentrations than men on a given clozapine dose (Couchman *et al*., 2010).

The menstrual cycle, pregnancy, menopause, hormone replacement therapy, and the use of oral contraceptives may all influence xenobiotic disposition. Drug concentrations may fluctuate with the menstrual cycle. For example, the metabolic clearance of methaqualone is approximately two-fold higher at mid-cycle. Similarly, the plasma half-life of paracetamol is shorter mid-cycle. GI transit time varies during the cycle, elevated plasma progesterone concentrations being associated with relaxation of smooth muscle and increased transit times. Absorption of alcohol and salicylates may be slowed mid-cycle.

16.10 Disease

Disease can cause both pharmacokinetic and pharmacodynamic changes in the response to drugs and poisons. The major systems where disease might be expected to affect PK parameters are the

GI tract, the liver, and the kidney. However, the cardiovascular system should not be overlooked. Reduced blood flow to the eliminating organs, including shock in acute poisoning, may affect rates of elimination. Oedema in congestive heart failure can lead to distributional changes and reduce drug absorption if it affects the GI tract.

Achlorhydria, arising from pernicious anaemia or gastric carcinoma, reduces the absorption of some acidic drugs such as aspirin, salicylamide, and cephalexin. Gastric stasis and pyloric stenosis delay the absorption of paracetamol. Coeliac disease delays the absorption of some drugs, rifampicin, for example, but increases the absorption of others such as clindamycin, erythromycin, and sulfamethoxazole.

Although it may seem reasonable to assume that diseases of the liver would reduce the metabolism of drugs, increase $t_{½}$, and lead to elevated plasma concentrations, the situation is much more complex. Even for drugs that are extensively metabolized, it should be remembered (i) that there may be extrahepatic metabolism and (ii) that for many drugs there appears to be excess metabolic capacity under normal conditions, such that liver failure may have to be extreme before an effect is apparent. Furthermore, $t_{½}$ is a function of the volume of distribution and clearance, therefore changes in plasma protein concentration in liver disease may change drug distribution and hence elimination.

Care should be taken clinically and when interpreting the results of toxicological analyses if renal function is impaired. The elimination of drugs that are normally cleared unchanged by the kidney will be most affected if the urine flow is markedly reduced. If the drug is normally metabolized prior to urinary excretion, metabolites may accumulate as with morphine-6-glucuronide.

Changes in the concentration of binding proteins can markedly influence the pharmacokinetics and pharmacodynamics of highly bound drugs. In otherwise healthy subjects, plasma total (free and protein bound) quinine concentrations of 8–16 mg L^{-1} may be associated with serious toxicity, but in acute malaria, plasma AAG is increased and total quinine concentrations in this same range are attained during effective treatment without manifestation of toxicity (Section 20.6.4.3). Protease inhibitors used in treating acquired immunodeficiency syndrome (AIDS) patients are also strongly bound to AAG hence the renewed interest in this area (Barrail-Tran *et al*., 2010).

16.11 Pharmacokinetics and the interpretation of results

16.11.1 Back-calculation of dose or time of dose

Assuming that a drug or other xenobiotic, or a metabolite, has been detected in a biological sample, questions that may be posed are:

(i) How much substance was administered?
(ii) When was the substance administered?
(iii) Did the substance cause or contribute to clinically apparent toxicity (including behavioural disturbance) or cause death?

Always given that many other factors have to be taken into account in attempting to interpret analytical results in blood samples taken post-mortem (Section 22.3.2), providing estimates in an attempt to answer the first two questions relies on being able to apply PK equations with appropriate caveats as to the inherent uncertainties in such matters. Answering the third, although requiring knowledge of the pharmacodynamics and toxicology of the substance, will also be influenced in part by knowledge of the drug's PK.

To help decide whether a given plasma concentration indicates that an overdose has been administered, the result can be compared with published plasma concentration data (Baselt, 2020). In attempting to calculate the time of dosing, the two most useful parameters are V and CL. If these are known then λ and $t_{½}$ can be calculated from Equations (16.10) and (16.7). Some indication of F is vital for the interpretation of results after ingestion. If a typical range of plasma concentrations is not known, a mean value for C^{ss} can be calculated from the dose rate [Equation (16.32)].

If whole blood has been analyzed, then for a drug that does not partition into erythrocytes, the plasma concentration may be calculated by assuming a haematocrit of 0.45–0.55. However, there will be a large discrepancy between blood and plasma concentrations for substances that are sequestered in erythrocytes. If the sample is taken some time (more than 2–4 h for most drugs) after the substance was administered then it is probably safe to assume that drug concentrations in body compartments have equilibrated and the simpler equations used for single-compartment models may be used. If blood or plasma samples were collected pre-equilibrium (i.e. during the distributional phase), the analyte concentration may be considerably higher than it would have been had equilibrium been attained, and the calculated dose may be a gross overestimate, particularly if $V_1 \ll V_2$.

Probably the most important caveat when there has been an overdose is that most pharmacokinetic parameters are derived from healthy, usually male, volunteers or patients given therapeutic or sub-therapeutic doses. Continued absorption and non-linear kinetics following overdose may make the use of published pharmacokinetic parameters unreliable. For drugs that normally have a high oral bioavailability ($F > 0.9$) then this is unlikely to change after overdosage, but for drugs with low oral bioavailability, saturation of first-pass metabolism and efflux proteins could markedly increase the proportion of the dose entering the systemic circulation. There may also be saturation of excretory mechanisms (hepatic metabolism, renal excretion) or inhibition by co-ingested compounds.

16.11.1.1 How much substance was administered?

If a blood sample was collected *during life* and the time of sampling and of dosage are known, then it may be possible to calculate the dose. For an i.v. bolus the concentration at $t = 0$ can be obtained from rearranging Equation (16.3):

$$C_0 = C \exp(\lambda t) \tag{16.34}$$

where t is the time since dose was taken. An alternative approach is to calculate the number of half-lives that have elapsed, N, because:

$$C_0 = C \times 2^N \tag{16.35}$$

N need not be an integer. A published value of V is used to convert C_0 to dose. The calculation for an oral dose is more complex. However, provided that absorption is essentially complete and $k_a \gg \lambda$, then:

$$C_0 = \frac{C \exp(\lambda t)}{F} \tag{16.36}$$

It may be possible to take serial blood samples to obtain a value of $t_{½}$ and hence λ for the patient, rather than using a population value.

16.11.1.2 When was the substance administered?

Provided that $t_{½}$ is known and there is a reasonable estimate of the amount of substance taken, then Equation (16.4) can be rearranged to calculate t:

$$t = \frac{(\ln C_0 - \ln C)}{\lambda} \tag{16.37}$$

Because $C_0 = Dose/V$ and $\lambda = 0.693/t_{½}$, for an i.v. bolus injection, this gives:

$$t = \frac{1}{\lambda} \ln\left(\frac{Dose}{VC}\right) = \frac{t_{½}}{0.693} \ln\left(\frac{Dose}{VC}\right) \tag{16.38}$$

For an oral dose the equation is:

$$t = \frac{1}{k} \ln\left(\frac{Fk_aDose}{VC(k_a - \lambda)}\right) \tag{16.39}$$

However, if $k_a \gg \lambda$, Equation (16.39) can be simplified as before:

$$t = \frac{1}{\lambda} \ln\left(\frac{FDose}{VC}\right) \tag{16.40}$$

As explained above these equations will only give approximate answers. Although more rigorous calculations would use equations applicable to multi-compartment models, (i) large individual variation in the parameters, (ii) uncertainties about the size or time of dose, and (iii) difficulty in finding published values for λ_1, k_a, V_1 , etc., mean that the conclusions are unlikely to be any more reliable than those obtained using the simpler approach.

16.11.1.3 Prediction of ethanol concentrations

In England and Wales, Canada, and in most US states for drivers aged 21 years and over the legal BAC limit for driving a motor vehicle is 0.80 g L^{-1} (80 mg per 100 mL, 0.080 % w/v). The limit in Sweden and Norway is 0.20 g L^{-1}. In Scotland, most other European countries, and Australia it is 0.50 g L^{-1}.

Ethanol is also metabolized by microsomal CYP2E1, which has a higher K_m than ADH, and which is important at higher BAC values (>0.6 g L^{-1}). Consequently, the rate of ethanol elimination increases with increasing doses, something not to be expected if the elimination kinetics were solely zero order. Induction of CYP2E1 as well as ADH, by chronic exposure to ethanol is thought to be the reason why heavy drinkers often have higher ethanol elimination rates than moderate drinkers.

Widmark (1932) showed that after moderate drinking (0.5–1.0 g kg^{-1}) the decline in the BAC could be described as zero order. The experiments were carefully controlled. The subjects (20 male and 10 female) were fasted overnight and alcohol consumed as spirits within 5–15 minutes. BAC values were measured in capillary (finger prick) samples. Extrapolation of the linear portion of the time–concentration curve to $t = 0$, gave the y-intercept, which Widmark designated C_0. We have chosen BAC_0, (Figure 16.12) to avoid confusion with C_0 (Figure 16.1). Widmark's rho factor, ρ, was a dimensionless ratio:

$$\rho = \frac{\text{ethanol concentration in body}}{\text{ethanol concentration in blood}} \tag{16.41}$$

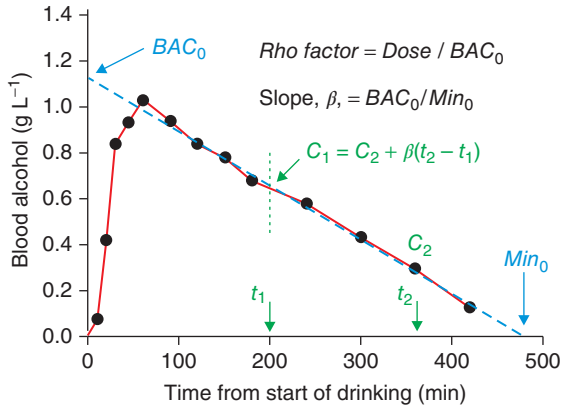

Figure 16.12 Example of a typical BAC versus time curve (Jones, 2010–reproduced with permission of Elsevier)

Because ethanol is evenly distributed in total body water:

$$\rho = \frac{\text{percentage of water in body}}{\text{percentage of water in blood}} \tag{16.42}$$

Men have a higher proportion of body water than women and the values of ρ (mean ± SD) were 0.68 ± 0.085 in men and 0.55 ± 0.055 in women. Using mass/volume units for ethanol concentrations the units of ρ are L kg^{-1} and it is often referred to as the volume of distribution of ethanol, even though V is usually assessed using plasma samples following i.v. injection. The values can be converted using the density of blood, 1.055 kg L^{-1}.

The amount of alcohol in the body, A_t, when the blood sample (BAC_t) was measured was calculated from:

$$A_t = BAC_t \times \rho \times W \tag{16.43}$$

where W is body weight. The amount of alcohol consumed, A_0, was obtained by substituting BAC_0 in Equation (16.43), from which Widmark was able to calculate the number of drinks consumed. It is, of course, necessary to know the amount of alcohol in each drink (Maskell *et al.*, 2017). The process can be reversed to predict a *BAC* value at a given time from the amount of alcohol consumed:

$$BAC_t = \frac{A_0}{\rho W} - \beta t \tag{16.44}$$

where β is the slope of the BAC versus time curve (Figure 16.12). Subsequent research has been to refine population estimates of ρ. Watson *et al.* (1981) computed ρ from estimates of TBW and the water content of blood, whereas Forrest (1986) produced a computer program to derive values of ρ based on body mass index (BMI). The values were tabulated by Barbour (2001). Maudens *et al.* (2014) confirmed that V (and hence ρ) declines with increasing BMI, and that the often-used values of 0.7 L kg^{-1} for men and 0.6 L kg^{-1} for women are too high for obese individuals.

Food delays ethanol absorption with a consequent increase in t_{max} and decrease in C_{max}. Usually *AUC* is reduced and although this has been ascribed to increased pre-systemic metabolism by gastric and hepatic ADH, it should be noted that for drugs displaying non-linear elimination kinetics the *AUC* increases disproportionately with increasing doses. Furthermore, the *AUC* is

greater when a given dose is absorbed more rapidly. After rapid drinking on an empty stomach, F is usually assumed to be close to unity. The effect of absorption rate can be seen by adding a first-order absorption rate term to Equation (16.44):

$$BAC_t = \frac{A_0[1 - \exp(-k_a t)]}{\rho W} - \beta t \tag{16.45}$$

Not only is the time to the peak increased, and the maximum concentration decreased, as would be expected for delayed absorption, but also *AUC* is decreased (Figure 16.13) even though there is no term for F in Equation (16.45). Using the values of Figure 16.12, the ratio of $AUC_{(0-\infty)}$ values was 0.78 even though the bioavailability was unchanged. Thus, food may reduce the *AUC* by delaying absorption, but not necessarily by reducing bioavailability. This is a feature of non-linear PK that is not always appreciated.

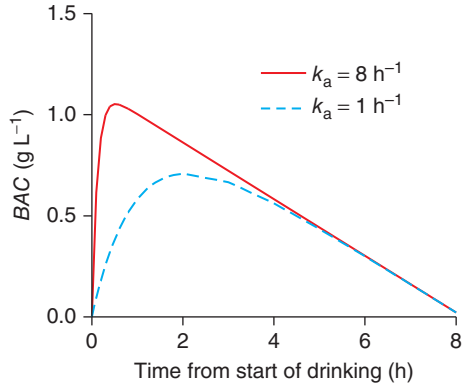

Figure 16.13 Simulated curves showing the effect of the rate of absorption of ethanol on the *AUC*. Curves were constructed using Equation (16.45), with $A_0 = 0.8$ g L^{-1} in 75 kg male, $\rho = 0.7$ L kg^{-1} and $\beta = 0.14$ g L^{-1} h^{-1}. It was assumed that the ethanol was consumed immediately and there was no lag until absorption started

The *BAC* at an earlier time, t_1, can be estimated from the concentration, C_2, measured at t_2 assuming that C_1 is on the linear portion of the decay curve (Figure 16.11):

$$C_1 = C_2 + \beta(t_2 - t_1) \tag{16.46}$$

Implementation of Equation (16.46) requires appropriate estimates of β. The rates of elimination (g L^{-1} h^{-1}) of ethanol are 0.12–0.15 in moderate drinkers, 0.15–0.25 in regular drinkers and can be up to 0.35 in alcoholics. In malnutrition, persons either on a low protein diet, or with liver cirrhosis, the range may be 0.08–0.12 g L^{-1} h^{-1}. Furthermore, not only are there inter-individual differences in rates of elimination, but also studies have shown intra-individual variances that can be higher than inter-individual variances. Some of these uncertainties may be reduced by taking two or preferably three samples an hour apart in an attempt to determine an individual value for β when the samples were taken. In one study the rates in drunken drivers ranged from 0.2–0.62 g L^{-1} h^{-1} (Jones, 2008).

Thus, 'backtracking' or back-calculation of BAC to some prior time point is fraught with difficulties, including the assumption that zero-order kinetics apply, the likelihood of individual variation in the rate of ethanol metabolism, including whether the subject is either a naïve, or

a heavy drinker (Paton, 2005), the effect of continued ethanol absorption from the GI tract (Jackson *et al*., 1991), whether the subject vomited, and whether consumption was over a prolonged period (Cowan *et al*., 2016). When reporting calculations of BAC from the volume of alcohol consumed, or conversely the number of drinks consumed, it is important to provide an estimate of the uncertainty of the result. However, how this is done is open to some debate (Gullberg, 2007; 2015; Searle, 2015).

16.12 Summary

For some drugs, notably anticonvulsants, cardiac glycosides, and lithium, TDM may be used to adjust the dose to individual need. In the absence of other information, the apparent volume of distribution and dose may be used to predict plasma concentrations, so that, for example, assay calibrators may be prepared over an appropriate concentration range. The predicative value of PK (estimating the size or time of dose) is more limited in analytical toxicology because of the caveats discussed in Chapter 22.

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Section D
Analytical Toxicology

17 Toxicology Testing at the Point of Contact

17.1 Introduction

Point of contact testing (point of care testing, POCT), as its name implies, refers to testing carried out in close proximity to the subject, usually with the aim of providing an almost immediate result. It can be considered as any testing performed outside of a central laboratory and may also be referred to as point of care laboratory testing (POCLT), rapid oral fluid testing (ROFT), bedside testing, near patient testing (NPT), home testing, and patient self-management (Table 17.1).

Table 17.1 Examples of point of contact testing

Type of Monitoring	Analyte	Test sample
Emergency department, trauma clinic	Blood gases, carbon monoxide, ethanol, paracetamol, salicylates, substance misuse, snake envenomation	Blood, urine, oral fluid, breath
Therapeutic monitoring	Lithium, theophylline	Blood, plasma
Roadside testing	Cannabis, cocaine, ethanol	Breath, oral fluid
Workplace testing		
Pre-employment	Substance misuse	Urine, oral fluid
On-site	Ethanol, substance misuse	Breath, oral fluid, sweat
Detention centres	Substance misuse	Urine, oral fluid, sweat
Drug treatment clinics	Buprenorphine, ethanol, methadone, substance misuse	Urine, oral fluid, sweat
Self-management	Warfarin	Blood

With the use of POCT systems for testing for driving under the influence (DUI) of drugs (including ethanol) or screening for possible impairment in the workplace, roadside, and employment and pre-employment testing can be added to the list. Consideration of issues such as staff training, QA, and the interpretation of results are inherent in any decision to implement testing (Shephard, 2016; Nichols & Petersmann, 2019).

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

17.2 Use of point of contact testing

The use of a POCT system should be given careful consideration before such a programme is initiated (Box 17.1). POCT may be performed to aid diagnosis of disease, guide therapy, or detect poisons. However, in the context of analytical toxicology the term is more generally applied to screening for, or assessing the concentration of, illicit or otherwise proscribed substances.

Box 17.1 Questions when deciding to implement point of contact testing

- **What are the reasons for testing?**
  - Emergency department testing/drug treatment clinic
  - Law enforcement
  - Post-incident
  - Random or scheduled workplace testing/pre-employment screening
- **Why are immediate results required?**
  - Initiate early treatment/alter treatment
  - Effect an arrest
  - Suspend from work
- **What is the window of detection to be?**
  - May influence choice of fluid to be sampled
  - Currently under the influence
  - Detect a regular substance misuser
- **Which drugs should be detected?**
  - Ethanol
  - Misused drugs
- **Where are the tests to be done?**
  - Clinic
  - Roadside
  - Workplace
- **What tests are available?**
  - Are the kits suitable for the task/venue?
  - Will the operators be able to use the system correctly?
  - Is storage/shelf-life a consideration?
- **Will it be cost effective?**
  - Avoid need to transport samples to the laboratory for analysis
  - Avoid need for employee to visit the test laboratory
  - Reduce the number of samples to be assayed by the laboratory – only test putative positives
  - Cost of kits/numbers of samples

The nature of the tests performed will depend on where they are being carried out. An emergency department will have access to blood gas analyzers, and these may be able to perform COHb assay. On the other hand, roadside or point-of-entry testing kits for use by trained police or customs officials need to be portable, robust, and reliable, whilst POCT kits for workplace testing need to be simple to use and give results promptly within the limitations of the device.

The immediacy of the result is the major difference between POCT and laboratory-based testing and this in turn can affect the doctor/patient or tester/subject relationship, particularly if the results may lead to punitive action (George, 2004). Under these circumstances, it is important that samples testing positive for substance misuse are taken for confirmatory analysis by GC-MS or LC-(HR)MS.

17.2.1 Samples and sample collection

POCT uses samples that require minimal manipulation prior to the analysis, common matrices being urine, blood, oral fluid, or sweat. The issues surrounding the collection of such fluids are the same as, or similar to, those when laboratory-based analyses are to be conducted. Additionally, breath is used to detect and quantify ethanol and carbon monoxide.

Blood is used for home glucose measurements, but it is not generally employed for drug POCT, although assays for lithium, and for theophylline have been described. The effects of the non-vitamin K antagonist oral anticoagulants (NOACs) dabigatran and rivaroxaban have been assessed using low-range activated coagulation time (ACT) and INR, respectively (Table 17.2). Although there is interest in POCT for clozapine using electrochemical sensing (Kang *et al*., 2017; Shukla & Ben-Yoav, 2019), these devices have not found clinical application as yet. Other matrices, such as hair and nail, are not amenable to current POCT technology.

Table 17.2 Examples of quantitative point of contact testing for drugs

Analyte	Assay	Test sample	Reference
Lithium	Microchip CE	Whole blood	Floris *et al*. (2010); Staal *et al*. (2015)
NOACs	Low range ACT (dabigatran), INR (rivaroxaban)	Whole blood	Padayattil Jose *et al*. (2018)
Theophylline	Microchip CEDIA	Whole blood	Nishiyama *et al*. (2019)

Identification of the snake responsible for an envenomation can guide the use of antiserum and help assess prognosis. Several approaches to this problem have been reported for use in blood, plasma, urine, or wound exudate. As an example, a sandwich ELISA (Section 6.3.4) and lateral flow assays to discriminate between neurotoxic and haemorrhagic snake venoms have been described (Liu *et al*., 2018). The LLoQs of the ELISA for neurotoxic and haemorrhagic venoms were 0.39 and 0.78 μg L^{-1}, respectively, whilst the lateral flow strips were capable of detecting neurotoxic and haemorrhagic venoms at concentrations lower than 5 and 50 μg L^{-1}, respectively, in 10–15 min. Tests of lateral flow strips in 21 snakebite cases showed 100% specificity and sensitivity for neurotoxic envenomation, whereas the sensitivity for detecting haemorrhagic envenomation samples was only 36 %.

Laboratory-based substance misuse testing is usually performed with urine, using systems based on immunoassays with confirmation of positives using GC- or LC-MS (Section 18.2). Oral fluid is preferred for POCT testing because supervised sample collection is more acceptable to the donor, there is less possibility of tampering and adulteration, and the result is obtained quickly (Box 17.2). However, oral fluid samples are more likely to be infectious than urine and the volume of sample may be limited. Note that a distinction needs to be made between ROFT devices designed to give an immediate answer, and oral fluid collection devices that collect samples for subsequent laboratory analysis.

Box 17.2 Advantages and disadvantages of using oral fluid to detect substance misuse

Advantages

- Less intrusive than urine collection
- Less opportunity for adulteration
- Relatively easy to collect
- Detects more recent drug use
- Prompt result

Disadvantages

- Flow- and pH-dependent effects on drug partitioning
- Narrower window of detection than urine
- Greater potential for passive exposure to smoke, e.g. cocaine/cannabis
- May detect residues of insufflated drugs
- Some drugs and diseases (and anxiety) reduce saliva flow
- May be more difficult to assay (viscous sample)
- Potentially infective
- No effective correlation between drug concentration and impairment

POCT oral fluid kits usually target different analytes to those used when testing for substance misuse in urine and certainly the analyte concentrations encountered will be different. Active transport of ions results in saliva (S) being hypotonic, having less sodium, but higher concentrations of potassium, than plasma (P). Oral fluid:plasma ratios for lithium are 2–3 and vary with plasma lithium concentration, indicating that there is carrier-mediated transport, probably via the potassium pump.

Excretion of drugs in saliva appears to occur largely by passive diffusion. The pH of saliva can vary from 5.5, the lower limit for parotid saliva, to 8.4 in certain ruminants. The average pH of 'mixed' saliva is approximately 6.5, about 1 unit lower than plasma pH. Thus, the unbound concentrations of basic drugs are usually higher in oral fluid than in plasma water, whilst the converse is true of weak acids. The saliva:plasma equilibrium ratio for an acid will be:

$$\frac{S}{P} = \frac{(1 + 10^{[pH(s) - pK_a]}) f_{u(p)}}{(1 + 10^{[pH(p) - pK_a]}) f_{u(s)}} \tag{17.1}$$

where pH(s) is the saliva pH, pH(p) is the plasma pH and $f_{u(p)}$ and $f_{u(s)}$ are the unbound fractions in plasma and saliva, respectively. There is less protein in saliva than in plasma, saliva protein being chiefly antimicrobial and digestive enzymes. Thus, the amount of drug-binding protein in oral fluid is thought to be insignificant, and hence $f_{u(s)}$ is taken to be zero. For salicylic acid (pK_a 3.0), which is 50–90 % protein bound in plasma, depending on the dose, the S:P ratio would be expected to be 0.06–0.01 for a salivary pH of 6.5. The equation for bases is:

$$\frac{S}{P} = \frac{(1 + 10^{[pK_a - pH(s)]}) f_{u(p)}}{(1 + 10^{[pK_a - pH(p)]}) f_{u(s)}} \tag{17.2}$$

Pethidine, a weak base (pK_a 8.7), is 40–50 % bound to plasma protein hence, at a salivary pH of 6.5, the S:P ratio is calculated to be in the range 4.6–3.8. Other factors being equal, this

means that overall there is longer detection window for basic drugs in oral fluid than in blood (Di Fazio *et al*., 2018).

Three pairs of glands are responsible for most of the saliva that comprises oral fluid. Stimulation, either mechanical, or with citric acid, changes the percentage produced by the different glands and so changes the composition of the fluid. Salivary flow is under autonomic control. Stimulation of β-receptors increases the amount of protein and mucin, and results in a more viscous fluid whereas α-adrenoceptor excitation increases protein. Muscarinic stimulation increases saliva flow, which is accompanied by increases in sodium and bicarbonate concentrations leading to increased pH values. Clearly drugs acting at these receptors will also influence the composition of the oral fluid. Given these variables it is not surprising that oral fluid:whole blood ratios show much variation (Table 17.3).

Table 17.3 Oral fluid:whole blood and theoretical saliva:plasma ratios for selected analytes (Langel *et al*., 2014–reproduced with permission of John Wiley & Sons)

Analyte	Oral fluid:whole blood ratio			Theoretical saliva: plasma ratio
	Mean	Median	Range	
Amfetamine	23	19	3.3–78	7.0
Amitriptyline	1.5	1.3	0.75–2.7	0.59
BE	3.2	1.7	0.18–31	–
Codeine	8.8	4.8	0.17–47	7.0
Cocaine	20	17	1.2–63	–
Diazepam	0.06	0.04	0.02–0.34	0.02
Metamfetamine	29	22	5.6–86	8.5
Methadone	2.9	1.8	0.6–71	0.71
Morphine	9.8	6.4	0.58–37	5.7
Nordazepam	0.05	0.05	0.02–0.12	0.03
THC	31	14	1.0–190	0.03
Tramadol	13	11	1.4–34	7.2
Zolpidem	0.43	0.28	0.05–18	0.12
Zopiclone	2.5	2.4	1.3–4.7	1.3

As noted above, saliva has an average pH of 6.5, but stimulation of salivary flow can increase this to pH 8. This will clearly affect the plasma:oral fluid ratios of ionizable compounds and in addition parent drug:metabolite ratios may be different in oral fluid when compared with those not only in plasma, but also in urine. Moreover, absorption of drug into oral mucosa provides a reservoir for drugs such as cannabis, cocaine, and metamfetamine that may be either insufflated, or smoked – clearly this will alter drug:metabolite ratios in oral fluid. Cocaine:BE ratios, for example, tend to be higher in oral fluid than in plasma and urine, especially if cocaine has been insufflated.

The European Workplace Drug Testing Society (EWDTS) has proposed cut-off limits for workplace testing using urine and oral fluid (Tables 18.7 and 18.9, respectively). For roadside testing, an advantage of oral fluid is that the result may relate more directly to concentrations of drug in blood than the amount of drug in urine, especially if a drug is metabolized extensively prior to excretion. On the other hand, although ethanol readily diffuses into saliva and blood:oral fluid concentrations equilibrate within about 30 min of stopping drinking, use of oral fluid to assess ethanol exposure is used rarely, principally because breath ethanol can be measured so easily (Kummer *et al*., 2016).

17.3 Toxicology testing at the point of contact

17.3.1 Ethanol

In order to establish impairment whilst driving or operating machinery it is necessary to measure or derive the concentration of ethanol in blood. HS-GC-FID is the most reliable method for measuring blood ethanol (Section 19.2.2.1), but this is clearly unsuitable for either roadside, or workplace testing. However, at equilibrium, breath, urine, and oral fluid alcohol concentrations are correlated with blood concentrations, and these matrices may be used as alternatives to blood.

17.3.1.1 Breath ethanol

The rationale for breath ethanol analysis is Henry's Law, which states that in a closed container at a given temperature and pressure, a solute in solution will be in equilibrium with air in the space above. The distribution of ethanol between blood and alveolar air at 34 °C is 2100:1 and this is the factor that is used to convert the breath alcohol concentration (BrAC) to the blood alcohol concentration (BAC). Thus, on average 2100 litres of alveolar air will contain the same amount of ethanol as 1 litre of blood, but this varies substantially and also varies with BAC

Modern POCT instruments use fuel cells in which oxidation of ethanol produces a current (e.g. the Intoxilyzer) or semiconductor oxide devices in which an ethanol-specific sensor is used. Semiconductor oxide sensors are claimed to offer many benefits, including low cost, low power consumption, and small size, although they need calibrating more frequently than the fuel cell devices. Methanol may interfere in POCT ethanol devices (Caravati & Anderson, 2010). Police station-based or other evidential breath ethanol instruments use infrared absorption at two wavelengths (3.37 and 3.44 μm) to identify and measure ethanol. Using the ratio of results obtained at two wavelengths reduces the risk of interferences.

17.3.1.2 Oral fluid ethanol

The ethanol oral fluid:blood ratio equilibrates about 30 minutes after cessation of drinking and remains at 1.077 for at least 6 hours. Thus, oral fluid provides an alternative to blood and breath ethanol measurement and reagent sticks for semi-quantitative measurement of ethanol have been produced, although such procedures are not suitable for evidential purposes. With the Alco-Screen, ethanol is oxidized by alcohol oxidase and the resulting hydrogen peroxide reacted with TMB in the presence of peroxidase (Section 6.3.1). Methanol, ethanol, and allyl alcohol give positive results; strong oxidizing agents will give false positives and reducing agents, including ascorbic acid and levodopa, will reduce the signal.

The OraSure QED (quantitative ethanol detector) is based on ADH oxidation and production of a coloured end point. Basically, it consists of a capillary tube, with an oral fluid reservoir at one end and a 'control spot' at the other. The spot turns purple when wetted with oral fluid, thereby

ensuring that sufficient oral fluid has been applied. As the sample is drawn up the capillary the ethanol is oxidized turning a marker dye purple until all the ethanol has been consumed. The length of colour in the capillary is proportional to the original concentration of ethanol and the concentration in the oral fluid can be read off a scale. Acetone, methanol, butanone, and ethylene glycol in aqueous solution (1 g L^{-1}) do not interfere, but propanol and 2-propanol (both 1 g L^{-1}) gave apparent ethanol results of 0.6 and 0.2 g L^{-1}, respectively, when tested.

The QED is available in two sizes, 0–1.45 g L^{-1} (A150) and 0–3.45 g L^{-1} (A350) for use in roadside testing and the emergency room, respectively. Several studies have been conducted comparing the QED with alternative ethanol assays, and good agreement between HS-GC and the QED for aqueous standards and for venous blood and end-expired breath samples has been reported. However, in a clinical setting there may be difficulty in obtaining sufficient oral fluid.

17.3.2 Substance misuse

More recent devices for substance misuse screening are based on lateral flow immunoassays (Wiencek *et al.,* 2017; Plouffe & Murthy, 2017; Figure 17.1). The strips usually have a wick for dipping into the sample or a reservoir (well) into which sample is pipetted. As the sample migrates along the strip, antibodies labelled with colloidal gold, latex beads, or some other suitable visualizing label, are carried in the stream of liquid. Immobilized antigen, usually bound as a line on the strip at a suitable distance from the origin, will capture any non-drug bound antibody to produce a visible line of labelled antibody. The intensity of the line will be maximal when there is no drug in the sample. When there is enough analyte in the sample to bind all the antibody, no line will be visible. Thus, by selecting the appropriate amount of antibody, devices can be manufactured to provide the required cut-off values or other end point such as a line.

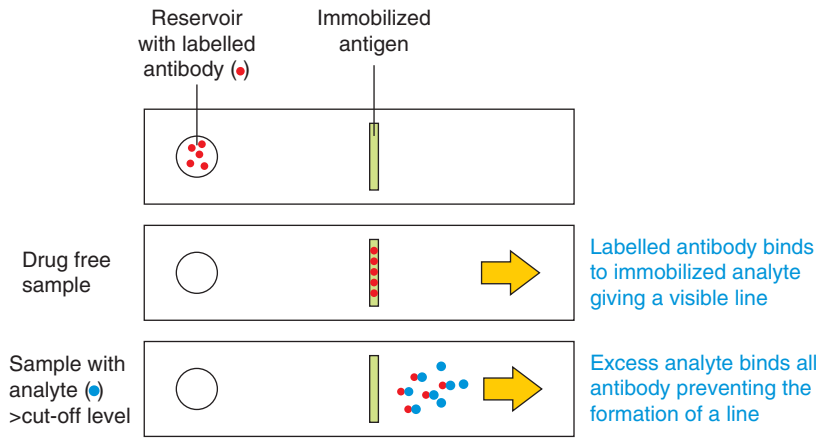

Figure 17.1 Principle of lateral flow immunoassay

A control strip or spot is frequently included beyond the bound antigen(s). A ‘positive’ reaction indicates that the sample has reached the zone (sufficient sample) and the reactants are functioning properly. Several antigens can be placed on one strip, giving an array of tests. One system uses labelled drug derivatives, the immobilized antibodies being bound onto the membrane, so that a positive result is indicated by the appearance of a line. With either type of device faint lines should be read as negative. As with other immunoassay methods

(Section 6.6.2) the specificities of the antibodies vary and many of the devices react to classes of drug, points that need to be remembered when interpreting and reporting results. However, given the continuing emergence of NPS it is never going to be possible to use POCT to test for all substances that may be misused (Øiestad *et al*., 2016; Graziano *et al*., 2019).

17.3.2.1 Oral fluid testing

Oral fluid testing devices are changing rapidly not only as regards performance, but also with respect to the availability of the devices themselves, hence manufacturer's literature and current reports from regulatory bodies should be accessed for up-to-date information. In assessing the performance of different devices, it must be remembered that analyte detectability is dependent on analyte concentration. Oral fluid concentrations for basic drugs are often much higher than in blood, whereas the concentrations of acidic or neutral drugs such as benzodiazepines are much lower hence use of amfetamines, cocaine, opioids, etc. is much easier to detect than might be expected.

The performance of the Rapid STAT (Mavand), DrugWipe 5/5 (Securetec Detektions-Systeme), and Dräger DrugTest 5000 (Draeger Safety) on-site oral fluid devices was evaluated using random oral fluid specimens from car drivers in Nord Rhine-Westphalia, Germany. Some drivers were also checked using an on-site urine device (DrugScreen). Over 11 months, 1212 drivers were tested. Both oral fluid and urine on-site tests were compared with serum results. The sensitivities for methadone 33–63 %, and benzodiazepines (BDZ) 0–33 % (both with a low number of positives) were low, and THC specificity was especially low (Table 17.4). The urine screening device showed a good % sensitivity [THC 93, opiate 94, amfetamine 94, metamfetamine 75 (low number of positives), cocaine 100] and acceptable specificities (39, 86, 63, 77, and 47 %, respectively).

Differences in sensitivity and selectivity between nine POCT devices have been assessed in a meta-analysis (Table 17.5). The overall sensitivity for amfetamines was found to be 0.808 (2266/2567). Selectivity was 0.945 (4240/4420). However, overall there was much variability depending on the device used. For example, amfetamine sensitivity was lowest for the Cozart DDS 801 device (0.336) and highest for OralLab (0.966). Amfetamine selectivity was the lowest in DrugWipe 5 (0.747) and highest in RapidStat and DDS (0.994). Comparable differences were found for other substances or classes of substances.

The performance of the Alere DDS®2 oral fluid roadside testing device for amfetamine, benzodiazepines, BE, metamfetamine, opiates, and THC, has been compared with (i) drug recognition expert (DRE) opinion, and (ii) the results of blood and oral fluid laboratory analyses in 88 detainees assessed as being under the influence of drugs other than alcohol. The results showed that (i) there was a good correlation with DRE observations and (ii) the device performance was >80 % in all drug categories compared to laboratory-based analysis, both in oral fluid and blood (Rohrig *et al*., 2018). The converse is, of course, that in some 1 in 5 instances there were significant discrepancies. Specific needs identified included the need to test for oxycodone and tramadol.

The performance of the Alere DDS®2 system has also been compared against the results of an oral fluid sample collected using the Quantisal™ device and analyzed in the laboratory. Of 124 paired samples, DDS®2 field screening yielded positive results for THC ($n = 27$), cocaine ($n = 12$), amfetamine ($n = 3$), metamfetamine ($n = 3$), and benzodiazepines ($n = 1$). For cocaine, amfetamine, metamfetamine, and benzodiazepines, the DDS®2 gave sensitivity, specificity, and accuracy of 100 %. For THC, the DDS®2 gave sensitivity, specificity, and accuracy of 90, 100, and 97.5 %, respectively (Krotulski *et al*., 2018).

Table 17.4 Sensitivities and specificities for selected analytes using three POCT devices (Musshoff *et al*., 2014–reproduced with permission of Elsevier)

Device	THC		Opiates		Cocaine		Amfetamine		BDZ	
	Sensitivity	*Specificity*	Sensitivity	*Specificity*	Sensitivity	*Specificity*	Sensitivity	*Specificity*	Sensitivity	*Specificity*
Dräger DrugTest	87	*47*	95	*91*	76	*74*	84	*90*	33	*100*
Rapid STAT	91	*9*	100	*100*	100	*100*	90	*43*	0	*100*
DrugWipe 5/5	71	*29*	100	*50*	100	*40*	100	*33*	NA[a]	*100*

[a]Not applicable

Table 17.5 Mean sensitivity and specificity (range) of nine POCT devices using oral fluid (Scherer *et al.*, 2017–reproduced with permission of Elsevier)

	Sensitivity			Selectivity		
	Overall	Low	High	Overall	Low	High
Amfetamines	0.808 (0.739, 0.862)	0.336 (0.010, 0.962)	0.966 (0.796, 0.995)	0.945 (0.909, 0.968)	0.747 (0.685, 0.800)	0.964 (0.715, 0.997)
Benzodiazepines	0.772 (0.536, 0.909)	0.696 (0.311, 0.921)	0.902 (0.455, 0.990)	0.884 (0.823, 0.926)	0.854 (0.646, 0.949)	0.951 (0.481, 0.998)
Cannabis	0.817 (0.723, 0.885)	0.615 (0.309, 0.851)	0.946 (0.933, 0.957)	0.814 (0.747, 0.866)	0.478 (0.066, 0.923)	0.893 (0.249, 0.995)
Cocaine	0.849 (0.759, 0.909)	0.325 (0.99, 0.484)	0.95 (0.676, 0.995)	0.953 (0.902, 0.979)	0.632 (0.119, 0.956)	0.999 (0.971, 1.000)
Opioids	0.844 (0.735, 0.914)	0.301 (0.151, 0.512)	0.967 (0.909, 0.988)	0.960 (0.932, 0.977)	0.779 (0.712, 0.834)	0.993 (0.982, 0.997)

Results from the Dräger DrugTest 5000 have been compared with the results from blood and oral fluid from drivers (Gjerde *et al*., 2018). In blood at the Norwegian legal limits the proportions of false positives ranged from 15 % for THC to 87 % for cocaine. On the other hand, amongst drivers who had drug concentrations above the legal limits in blood, the proportion who tested positive using DDT5000 was 37 % (benzodiazepines), 76 % (metamfetamine), 83 % (THC), 92 % (amfetamine), and 100 % (cocaine and opiates). Clearly there were large proportions of false positive and false negative results compared to results in blood.

A comparison of three different devices (DrugWipe 6, Ora-Check, and SalivaScreen) showed differences in device cut-offs for cocaine, ketamine, and THC. Depending on the cut-offs, the number of false negatives showed significant differences. Overall, the specificity and accuracy of the devices were satisfactory (>80 %,) but sensitivity varied (Tang *et al*., 2018). All the devices showed poor performance for THC (Figure 17.2), an important issue because cannabis is the drug misused most frequently in many countries.

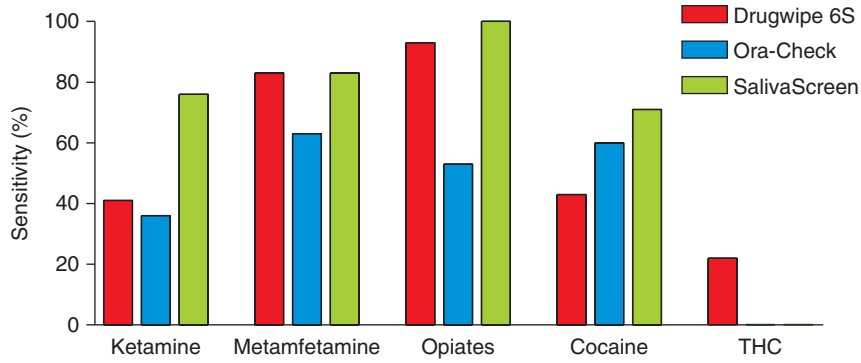

Figure 17.2 Comparison of the sensitivities of three oral fluid testing devices (data from Tang *et al*., 2018)

Drugwipe (Figure 17.3) is a pen-size detector that can be used to detect drugs on surfaces, in sweat, or in saliva. It was developed for customs use in identifying illicit drugs. Second generation Drugwipes, with increased sensitivities, were developed specifically for roadside testing. Although forehead sweat may be sampled, the device is used principally to collect a small sample of oral fluid through a wipe either of the tongue, or of mucosal surfaces within the mouth. Several devices are available: Drugwipe II detects single analytes: opiates, cocaine, amfetamines (metamfetamine/MDMA), cannabis, or benzodiazepines. Drugwipe II Twin detects pairs of drug types: opiates/cocaine or cannabis/amfetamines. Drugwipe 5 simultaneously detects five analytes. Quoted sensitivities are 20–300 μg L^{-1} for sweat or oral fluid, and 2–50 ng cm^{-2} for surfaces.

17.3.2.2 Autopsy specimens

The use of four Randox arrays (Section 6.3.6) designed for use with whole blood in a range of post-mortem body specimens has been described (McLaughlin *et al*., 2019). Liver, psoas muscle, femoral blood, vitreous humor, and urine from 261 post-mortem cases were analyzed and the results were obtained within the time taken to complete the post-mortem. Specimens were

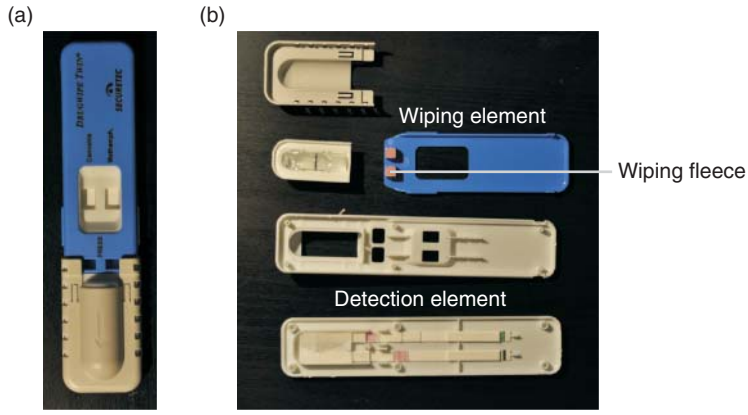

Figure 17.3 (a) Example of a Drugwipe Twin II. (b). Exploded view showing wiping fleeces for sampling surfaces, sweat, or saliva (adapted from Zapacit – Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=42609560)

screened for amfetamine, barbiturates, benzodiazepines, BE, buprenorphine, cannabinoids, dextropropoxyphene, fentanyl, ketamine, LSD, methadone, metamfetamine, methaqualone, MDMA, opiates, paracetamol, phencyclidine, salicylate, zaleplon, zopiclone, and zolpidem. The equivalent of 12,978 individual drug-specific, or drug-group, immunoassays were undertaken. Overall >98% of the 12,978 screening tests undertaken agreed with confirmatory tests performed on femoral blood.

17.4 Interferences and adulterants

The problems of interferences and adulterants are not of course confined to POCT, but the use of POCT in pre-employment and employment testing in particular makes consideration of these issues important. Immunoassay-based kits would be expected to suffer from the same, or similar, interferences to those seen when immunoassays are used in the laboratory. Some sources of interference are not obvious. For example, ecgonine methyl ester interacts with some benzodiazepine assays, and diphenhydramine with methadone screens. Known sources of interference should be quoted in the manufacturer's literature.

The three means used by substance misusers in attempting to avoid giving a positive urine test are dilution, adulteration, and substitution (Section 18.2.1). It is good practice to check routinely for evasive measures, and test strips are available that will measure creatinine, nitrite, glutaraldehyde, pH, SG, and two oxidants, including bleach and pyridinium chlorochromate, simultaneously. Three of these test kits have been evaluated (Peace & Tarnai, 2002). Some urine POCT kits for detecting substance misuse include tests for adulterants.

As a consequence of testing for adulterants, either urine substitution, or drinking large quantities of water in order to produce more dilute urine ('water loading') have become more prevalent. Urine substitutes are also commercially available – such as a product described as 'ultra pure pre-mixed synthetic unisex (*sic*) laboratory urine' that can be supplied with a heating pad to avoid detection by collection cups with built in temperature strips. Greater supervision (i.e. invasion of privacy) during sample collection may overcome some of these measures.

17.5 Quality assessment

The fact that POCT devices are designed to be simple to use by non-laboratory staff does not mean that that such devices are foolproof and therefore that rigorous QA measures are not needed. Some of the considerations surrounding POCT are addressed in the ISO standard (ISO, 2016). A major QA issue is staff training. Unlike automated immunoassay-based laboratory methods, most of the steps in POCT require operator intervention, including sample application, timing of the reaction, reading/interpreting a visual end point, and recording and documenting the result. Usually, trained laboratory staff will have evaluated POCT kits, and no study to date has shown 100 % concordance with validated laboratory tests.

Suppliers of POCT kits generally provide staff training, but in general staff with no laboratory experience have more difficulty in performing the tests and reporting results than laboratory staff. Manufacturers have tried to address the issue of incubation time by providing devices that include indicators to show when the end point of the test has been reached. POCT devices should be designed to facilitate the required regulatory agency documentation and retrieval of data, including QA data. It is important that users understand the limitations of POCT kits and the interferences that may occur. For example, because a test kit is labelled 'morphine' it does not necessarily mean that a positive result unequivocally indicates the presence of morphine.

IQC samples should be analyzed when a new batch of test kits arrives and at specified intervals thereafter, for example every 30 days or as otherwise directed by the manufacturer. The kits should be stored under appropriate conditions. All sites where POCT is used should be included in EQA schemes. The most effective approach is through circulation of samples of known composition with known amounts of substance or interfering compounds, and assessment of the results against (i) those of all participants and (ii) the known sample composition.

17.6 Summary

POCT applied to drugs and other poisons has made important contributions to road safety, detection of drug trafficking, and emergency clinical care. However, it finds its principal application in testing for substance misuse, and is widely used in employment and pre-employment screening, in prisons, in the enforcement of Drug Treatment and Testing Orders (DTTOs), and in drug treatment and rehabilitation centres.

The management of the use of POCT devices is of vital importance in order to ensure that reliable results are obtained within the limits of the devices employed. Training in the use of the devices, QA, and both confirmation and interpretation of results may fall within the responsibility of the analytical toxicology laboratory. Hence it is important that laboratory staff are up-to-date with developments in both the technology used in POCT devices, and their use in the area the laboratory serves.

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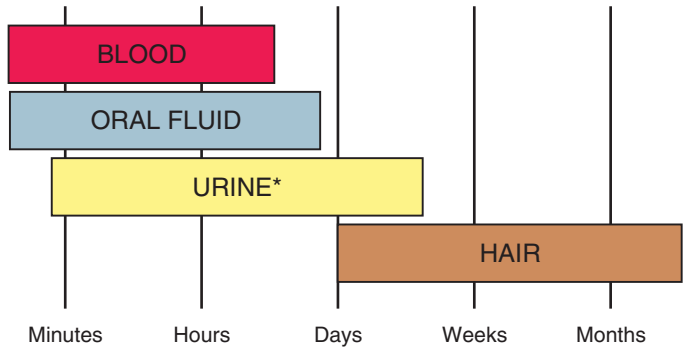
18 Laboratory Testing for Substance Misuse

18.1 Introduction

This chapter provides information on aspects of laboratory testing for substance misuse. Such testing may be performed for either clinical, or forensic purposes and may be undertaken either as an adjunct to POCT (Chapter 17), or as the sole form of testing. Clinical testing for ethanol and illicit/controlled drugs has much in common with the investigation of acute poisoning (Chapter 19). However, there are a number of important differences not only in the range of substances that may be of interest, but also in chain of custody and other requirements that may be more usually associated with forensic toxicology.

Testing for substance misuse has assumed much greater complexity in recent years, not only because of the vast range of NPS that have been marketed, but also because many countries have adopted limits for some illicit and prescription drugs analogous to those enacted for blood ethanol for driving a vehicle on the public roads. Added to this have been moves to use oral fluid as it is easier to collect in public areas and more difficult to adulterate than urine, and hair analysis because segmental analysis has been claimed to give a historical record of drug use (Figure 18.1).



*Except cannabis which may be detected up to a month after cessation in chronic users

Figure 18.1 Typical detection window for drugs of misuse in various common biological matrices

It is important to keep in mind the reasons for substance misuse testing. For example, if in roadside testing the aim is to detect impairment as a result of drug use, the matrix of choice

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

is exhaled air in the case of ethanol, and oral fluid or in some cases blood for other misused substances (Marillier & Verstraete, 2019). However, when either a company wants to check whether an employee is using drugs, or a clinic wishes to monitor a drug treatment programme, the matrix of choice is either urine, or oral fluid (Miller *et al*., 2017). Hair analysis is used under certain circumstances such as when checking for abstinence, the cost usually being borne by the individual supplying the sample. Hair testing has also been used in an attempt to detect illicit drug administration to children. However, the interpretation of results remains difficult because of the lack of comparative data (Pragst *et al*., 2019).

Sometimes the question of possible covert misuse of drugs such as diuretics and laxatives may be raised, thereby extending the scope of the analysis requested (Section 22.4.12). Testing for the misuse of volatiles such as butane and nitrous oxide is discussed in Section 19.2.2.1. Testing for the use of prohibited substances in sport and in food production are specialized areas (see Sections 22.4.2 and 22.4.4.3).

Finally, there is the question of monitoring alcohol use and misuse. Whilst acute ethanol intoxication in living subjects can be monitored easily by breath, blood, or urine ethanol assay, ethanol has a relatively short plasma half-life. Ethanol is metabolized principally by hepatic cytosolic ADH, a polymorphic enzyme (Section 15.7.3). Two further oxidative routes, the highly inducible CYP2E1 system and peroxisomal catalase, may play a part under specific circumstances. In addition, ethanol gives rise to several minor metabolites such as ethyl glucuronide (EtG), ethyl sulfate (EtS), ethyl phosphate, ethyl nitrite, phosphatidylethanol species (PEths), and fatty acid ethyl esters (FAEEs). These metabolites represent alternative biomarkers of ethanol exposure because they can be detected several hours or days after drinking (Dinis-Oliveira, 2016; Maenhout *et al*., 2017).

Monitoring of alcohol consumption over time is important in alcohol withdrawal and driving licence regranting programs, monitoring foetal alcohol exposure, child custody disputes, fitness to practise/employment tribunals, and organ transplant programs. Plasma carbohydrate deficient transferrin (% CDT) has been used as an indirect measure of ethanol exposure for many years (Schellenberg *et al*., 2017). More recent interest has focused on the measurement of ethanol and minor ethanol metabolites in a variety of matrices in order to assess ethanol ingestion. These include DBS (% CDT, EtG/EtS, PEths), dried urine spots (EtG/EtS), sweat and skin surface lipids (ethanol, EtG, FAEEs), oral fluid (ethanol, EtG), exhaled air (PEths), hair (EtG, FAEEs; Section 18.5.5), nail (EtG), meconium (EtG/EtS, FAEEs), and umbilical cord and placenta (EtG/EtS and PEth 16:0/18:1) (Kummer *et al*., 2016a; 2016b).

18.1.1 Matrix and sampling

18.1.1.1 Urine

Urine is the sample used most commonly for substance misuse testing. Urine collection is non-invasive and urine may contain higher concentrations of some drugs and, in certain cases metabolites, than blood, thus extending the time after which substance misuse may be detected. In addition, the sample volume is usually adequate and special collection devices are not needed. However, because collection has to be performed in private albeit under supervision, appropriate facilities are required and procedures to prevent tampering/substitution/adulteration of the sample are needed. Moreover, it is not possible to relate urinary drug or metabolite concentrations to biological effect except under certain circumstances, such as is sometimes the case with ethanol.

18.1.1.2 Oral fluid

Oral fluid is also a non-invasive sample. Moreover, oral fluid collection for forensic purposes, drug-impaired driving for example, has the advantage that sampling can be carried out by suitably trained staff while the donor is under observation, making it more difficult to adulterate or substitute the specimen (Gjerde *et al*., 2015; Veitenheimer & Wagner, 2017). However, analytically oral fluid is a more complex matrix than urine and concentrations of acidic and neutral drugs may be very low. Moreover, oral fluid concentrations of substances such as cocaine and BE will usually be higher than plasma concentrations after ingestion or insufflation of the drug, so that direct comparison of oral fluid and plasma concentrations is not viable (Section 17.2.1).

18.1.1.3 Hair

Hair has been studied extensively as a matrix for detecting drug use. Because head hair grows on average about 1 (range 0.5–2) cm per month, hair may contain a 'timeline' of drug use. This matrix is thus potentially very attractive in substance misuse testing. Hair is easily sampled (Figure 2.2). However, there are many problems, such as the possibility of incidental contamination or deliberate manipulation prior to collection, the effect of cosmetic treatment, and inter-individual rates of drug incorporation into hair (Kintz *et al*., 2006; Cuypers & Flanagan, 2018).

18.1.1.4 Blood

Blood is normally only used for substance misuse testing if a quantitative result is required in connection with road traffic or other legislation. Whole blood collected by venepuncture is used most commonly, with 'action limits' being set by legislation in some countries. The main practical issue is the inevitable delay caused by transfer of the suspect to a suitable location and organizing blood sampling, although as regards blood ethanol this problem has been minimized by evidential breath testing (Section 17.3.1.1). For evidential testing for drugs other than ethanol, whole blood is also used because of the practical difficulty of organizing on-site centrifugation.

18.1.1.5 Exhaled air

Exhaled air sampling devices have been developed for use in substance misuse testing, but have not found wide application except of course in the case of ethanol (Section 18.6). Sampling for illicit drug use requires a special sample collection device, and is said to be easy to perform on-site, and not susceptible to adulteration. However, transfer to a laboratory is required for the analysis and it is not known if exhaled air analyte concentrations relate quantitatively to blood concentrations.

18.1.1.6 Sweat

Sweat is a matrix that was originally used in a clinical context as in the chloride sweat test used to diagnose cystic fibrosis. Because sweat is a route for drug excretion, the use of sweat in drug testing has been studied within the last two decades. Sampling is easy with a compliant subject (Section 2.3.6) and can be done over a period of several days by use of sweat-collection patches. However, sweat testing has found little use in monitoring substance misuse (Section 18.7).

18.1.2 'Cut-off' concentrations

'Cut-offs' have their origins in immunoassays for drugs such as opiates and were set either by manufacturers, or by assay users at concentrations that aimed to minimize the likelihood of false positives and to minimize the risk of false negatives. Originally developed with workplace testing in mind, positive immunoassay results required 'confirmation' by a second technique, usually GC- or LC-MS. If the presence of a drug or metabolite was confirmed, the immunoassay 'cut-off' was still sometimes employed in reporting the result of the second analysis, although nowadays lower 'cut-off' values tend to be used for MS-based confirmatory methods. Whatever 'cut-off' is used it is important to remember that this is an arbitrary value, and not necessarily the analytical limit of detection (LoD). There are also of course uncertainties associated with any cut-off value.

Clinical substance misuse testing in either urine, or oral fluid tends to adopt 'cut-offs' based on those adopted for workplace testing for illicit/controlled drugs, for example, rather than analytical LoDs. There are thus questions of assay calibration and whether a positive finding of a drug or metabolite with a method such as LC-MS/MS or LC-HRMS should be reported if the concentration is below the workplace 'cut-off'. In clinical substance misuse testing, analysis by a chromatographic technique is almost always needed to differentiate use of over-the-counter medications and of foodstuffs incorporating poppy seeds, which contain similar alkaloids to those found in opium, from use of illicit/controlled substances such as amfetamine and diamorphine.

A further complication is that some immunoassays measure drug and cross-reacting metabolites, whilst chromatographic methods may measure either parent compound alone, or total drug after hydrolysis of glucuronides, for example. 'Free' and 'total' morphine is a case in point. GC methods invariably can only measure 'total' morphine after hydrolysis and solvent extraction. LC methods may be able to measure drug and glucuronide metabolites separately and thus report a 'total' concentration by adding the results in molar terms and converting to a 'cut-off' in mass units if sample preparation is minimal. However, if solvent extraction or other sample preparation procedure is performed to minimize the risk of ion suppression, hydrolysis of glucuronides will be required to give a 'total' figure.

With the increasing emphasis on direct GC- or LC-(HR)MS analysis, cut-offs are still used (i) to reduce the chance of reporting drug use when the drugs were taken involuntarily, for example either via cannabis smoke in coffee shops, or via ingestion of poppy seeds, and (ii) to minimize the detection of drugs that were used some time ago. On the other hand, detection of drugs below a 'cut-off' that were taken days or even weeks before can be valuable if drug-facilitated assault is suspected, for example (Section 22.3.1). It cannot be emphasized too strongly that reporting of results must take into account the reason for performing the test. Testing for the presence of a drug in emergency medicine is different to monitoring adherence to a drug-treatment regime, and is again different from testing for abstinence after withdrawal of a driving licence, for example.

18.2 Urine testing

As discussed above, urine is the sample that is used most frequently for screening for substance misuse, whether it be by POCT (Section 17.2.1), laboratory-based methods, or both if confirmation of a POCT result is required. In a clinical setting, urine samples can be directly

analyzed using colour or immunoassay tests without pre-treatment other than dilution of the sample (Box 18.1), often leading to a short turn-around time.

Box 18.1 Urine testing for substance misuse: summary

Validity testing
- Creatinine
- pH
- Specific gravity (SG)
- Temperature at the time of collection
- Nitrite

Sample preparation
- For immunoassay: centrifugation and 5- or 10-fold dilution with blank matrix
- For GC-MS: LLE or SPE with or without prior hydrolysis of conjugates
- For LC-MS: centrifugation and dilution with ISTD solution, or LLE or SPE with or without prior hydrolysis of conjugates

Screening (to quantitative 'cut-off')
- Immunoassay (with GC- or LC-MS confirmation of positive finding)
- GC- or LC-MS

Calibration and quality assurance
- Calibrators and IQCs prepared using certified reference materials or certified standard solutions appropriate for the matrix

Reporting results
- 'Cut-off' concentrations (reporting of positive results) may vary depending on the reason for the test (roadside testing, workplace testing, clinical testing, etc.)
- If only a metabolite/breakdown product has been detected, the parent drug(s) should be indicated
- The uncertainty of the measured value should be taken into account when reporting positive findings

18.2.1 Sample adulteration

Adulteration is described by the European Workplace Drug Testing Society (EWDTS) as any process by which an individual knowingly interferes with, or attempts to interfere with, specimen collection, transport, or analysis with the intention of avoiding a legitimate test result. The actions undertaken can include, but are not limited to, the addition of water or foreign substances to the specimen, specimen substitution, damaging bottle seals or packaging, and the deliberate consumption of either interfering substances, or copious volumes of water prior to specimen collection (www.ewdts.org).

Because urine screens are based on *concentration* cut-offs rather than the *amount* of drug present in the sample, the simple expedient of drinking large amounts of water can dilute the urine so that the cut-off is not exceeded. Counter measures are to measure the temperature, specific gravity (SG), and pH of the sample, and the creatinine concentration. Urine samples with creatinine concentration <1.8 mmol L^{-1} (200 mg L^{-1}) should be reported as 'too dilute'

and the test repeated using a more concentrated specimen (Table 18.1). Dyes to colour the water in toilet bowls are available, and access to running water during a test can be limited to remove obvious sources of sample diluent.

Table 18.1 EWDTS: guidelines for testing urine to detect sample manipulation

Test	Result	Action	Report
Creatinine	>2.0 mmol L^{-1} (>226 mg L^{-1})	–	As normal
	0.5–2.0 mmol L^{-1} (56–226 mg L^{-1})	SG 1.001–1.020	Negative drug test: report dilution
			Positive drug test: report as normal
	≤0.5 mmol L^{-1} (56 mg L^{-1})	SG out of range	'Specimen validity failed'
pH	4–9	–	As normal
	≥9	–	'Specimen validity failed'
	<4	–	'Specimen validity failed'
Nitrite	≥500 mg L^{-1}	–	'Specimen validity failed'
			Negative drug test: should not be reported
			Positive drug test: report as normal

Although low creatinine and SG values indicate dilute urine, a dilute sample does not necessarily indicate that the urine was intentionally diluted (Chaturvedi *et al*., 2013; Holden & Guice, 2014). However, the complete absence of creatinine does indicate that the specimen is, for example, tea, orange squash, or simply water. Creatinine measurement may also be useful for comparing drug use in one individual over a period of time because analyte concentrations can be normalized to creatinine to account for differences in hydration at the time of sampling. This may be useful for assessing whether the re-use of drugs, most notably cannabis due to the persistence of THC-COOH in urine after high-dose cannabis use, is a possibility (Smith *et al*., 2009).

Chemicals may be added to urine either in an attempt to mask a test result, or to destroy the analyte. The chief objective of adulteration is to produce a negative result, but even an invalid test may lead to a second collection, thereby 'buying time' for drug and/or metabolites to be eliminated before the second test. There is a large 'underground' sale of adulterants. Commonly used compounds include household chemicals, such as bleach, table salt, laundry detergent, toilet bowl cleaner, vinegar, lemon juice, eyedrops, and products that are commercially available via the Internet (Dasgupta, 2007; Fu, 2016). Soap and bleach disrupt the screening process and are easily detectable. Glutaraldehyde is also detectable by the effects observed on the screening process.

EMIT is particularly prone to disruption by the presence of non-physiological concentrations of salts, acid, and bleach and other oxidizing agents (Jaffee *et al*., 2007). Adding zinc to urine can reduce the detection of metamfetamine, cocaine, THC, and opiates (Lin & Strathmann, 2013). Other immunoassay systems are also susceptible to sample adulteration to either a greater or a lesser extent. Conversely, drugs such as buprenorphine, hydrocodone, or methadone may be

added to make it appear as if individuals are taking their prescribed medication (Feldhammer *et al*., 2019).

Nitrites are used to conceal THC metabolites, primarily in GC-MS confirmatory tests, although at low pH values they may also reduce the concentration to below cut-off values in screening tests. Another oxidizing agent, pyridinium chlorochromate, which can be detected by the presence of chromium or pyridine, has been shown to interfere with EMIT assays and in some cases Roche Abuscreen assays. 'Stealth' (Table 18.2), which has been described as a mixture of peroxidase and peroxide, has been shown to reduce the concentrations of morphine and codeine in some samples. The presence of these oxidizing agents was detected when the amounts of analyte and ^{2}H-labelled ISTDs present in GC-MS confirmatory tests were reduced markedly (Cody *et al*., 2001).

Table 18.2 Examples of adulterants used to mask urine drug screens

Adulterant	Brand
Potassium nitrite	Klear, Whizzes
Glutaraldehyde	UrinAid, Instant Clean ADD-IT-ive
Pyridinium chlorochromate	Urine Luck
Peroxidase/peroxide	Stealth

Analyte concentrations may indicate sample adulteration, and parent drug to metabolite ratios may also help in this context (Belsey *et al*., 2014). Immunoassays used in substance misuse testing are not designed to give quantitative results, and may be unable to differentiate between parent drug and metabolites in some cases because of structural similarities. Some immunoassay manufacturers have tried to overcome this by targeting metabolites, e.g. 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP) as opposed to methadone, in separate assays, but this increases costs.

An attempt to identify i.v. use of methadone or buprenorphine via disaccharide detection in urine has been described (Jungen *et al*., 2013; 2017). The rationale for this is that sucrose is used to increase viscosity in liquids, whilst lactose is needed for pressing tablets. After ingestion, disaccharides are broken down into monosaccharides by disaccharidases in the small intestine. Disaccharidases do not exist in blood, thus sucrose and lactose are not hydrolyzed if these compounds are injected i.v. and can be measured in urine.

18.2.2 Analytical methods

18.2.2.1 Immunoassay

Immunoassays offer the benefit of speed, high throughput, and ease of automation. Some immunoassays target metabolites such as BE, whilst others target the parent compound. The picture may also be confused by false positive immunoassay results. Manufacturers' assay cross-reactivity data are not always easy to interpret (Reschly-Krasowski & Krasowski, 2018), and may not include information on the cross-reactivity of metabolites.

Some urinary amfetamine immunoassays give positive results with tranylcypromine, proguanil, isoxsuprine, labetalol, and phenethylamine (2-phenylethylamine), amongst other compounds (Tables 18.3 and 18.4). This poor selectivity, which varies between assays (Saitman *et al*., 2014; Liu *et al*., 2015), is because (*S*)-amfetamine [(*S*)-1-methylphenylethylamine] is a relatively simple molecule. Small changes to the structure, while markedly affecting pharmacological activity, do not facilitate the production of antibodies of greater selectivity.

Table 18.3 Urinary amfetamine immunoassays: potential cross-reactants

(*R*)-Amfetamine	(*R,S*)-Methylenedioxyethamfetamine (MDEA)
Atomoxetine	(*R,S*)-Methylenedioxymetamfetamine (MDMA)
Benzathine phenoxymethylpenicillin	Metformin
Brompheniramine	Mexiletine
Bupropion	Nefazodone (metabolite *m*-CPP)
Chlorpromazine metabolites	Ofloxacin
Dimethylamylamine (DMAA)	Phenmetrazine
Diethylpropion	Phentermine
Ephedrine	Phenylethylamine
N-Ethylaniline	Phenylpropanolamine
Fluoxetine	Proguanil
Isometheptene	Promethazine
Isoxsuprine	Pseudoephedrine
Labetalol (dextrorotatory metabolites)	Ranitidine
(*R,S*)-Metamfetamine	Selegiline
(*R,S*)-Methylenedioxyamfetamine (MDA)	Tranylcypromine
	Trazodone (metabolite *m*-CPP)

With regard to benzodiazepines, efavirenz and sertraline are reported to give false positives in some assays; efavirenz is also noted as an interference in some cannabinoid assays. Oxaprozin unexpectedly interfered in most urine benzodiazepine immunoassays. On the other hand, assays for BE are relatively selective.

The ability of immunoassays to detect NPS is unclear. Stimulant NPS are often structurally related to either amfetamine, or MDMA. However, amfetamine-group immunoassays often may not have sufficient cross-reactivity to these novel compounds and thus drug misuse may go undetected (Beck *et al*., 2014). Similarly, because of the structural differences in synthetic cannabinoids from THC, these compounds are unlikely to cross-react in cannabis immunoassays, where antibodies have been raised against THC derivatives.

Immunoassays for detecting synthetic cannabinoids are being marketed, with some having targeted JWH-018 and structurally related cannabinoids that are not usually seen nowadays (Barnes *et al*., 2014; 2015). Thus, because of the time taken to develop an immunoassay it is likely that by the time such assays are available they will not detect the NPS in current use.

Table 18.4 Some recent reports of interferences in urine immunoassays for misused substances

Immunoassay	Interference	Reference
Amfetamine	Dimethylamylamine (DMAA)	Vorce *et al*. (2011)
	Labetalol (via 3-amino-1-phenylbutane)	Yee & Wu (2011)
	Mebeverine	Kraemer *et al*. (2001)
	Bupropion	Casey *et al*. (2011); Vidal & Skripuletz (2007)
	Chlorpromazine/promethazine	Melanson *et al*. (2006)
	Fenofibrate	Kaplan *et al*. (2012)
	Phentermine	Marin *et al*. (2009)
	Trazodone (via metabolite *m*-CPP)	Logan *et al*. (2010)
Buprenorphine	Amisulpride	Birch *et al*. (2013)
	Opioids (high concentration)	Pavlic *et al*. (2005)
	Sulpiride	Birch *et al*. (2013)
	Tramadol	Shaikh *et al*. (2008)
Cannabis	Efavirenz	Oosthuizen & Laurens (2012)
Opioid	Fluoroquinolone antibiotics	Saitman *et al*. (2014)
	Gatifloxacin	Straley *et al*. (2006)
	Pholcodine	Svenneby *et al*. (1983)
	Rifampicin	de Paula *et al*. (1998)
Phencyclidine	Ketamine analogues (e.g. 2-ketoeticyclidine)	Skaugen *et al*. (2019)
	Metronidazole	Fernández *et al*. (2018)
	Tramadol	Ly *et al*. (2012); King *et al*. (2013); Hull *et al*. (2006)

18.2.2.2 Chromatographic methods

The need to confirm immunoassay-positive results through use of more specific techniques increases turn-around time and laboratory expenditure. In addition, not all misused substances, for example GHB, can be detected by currently available immunoassays. In drug rehabilitation either buprenorphine, or methadone may have been prescribed leading to positive immunoassay results. However, it is important to detect covert use of non-prescribed drugs, some of which may not be detectable by immunoassay methods. For these reasons, laboratories with GC-MS and/or LC-(HR)MS facilities will often omit preliminary immunoassay screening.

18.2.2.3 Assay calibration and acceptance criteria

In LC-MS/MS urine substance misuse screening, calibration solutions ($n = 3$, Table 18.5) and a matrix blank (analyte-free human urine) should be included at the beginning and end of each batch analysis, with 'low' and 'high' IQCs included after the first set of calibrators

Table 18.5 Suggested nominal concentrations of analytes in calibrator and IQC solutions in LC-MS/MS urine substance misuse screening

	Nominal concentration (μg L^{-1})				
Analyte	Low calibrator	'Cut-off' calibrator	High calibrator	Low IQC	High IQC
Amfetamine	100	200	1000	150	250
Metamfetamine	100	200	1000	150	250
MDMA	100	200	1000	150	250
Mephedrone	100	200	1000	150	250
BE	75	150	1000	112.5	187.5
Morphine	150	300	1000	225	375
Morphine-3-glucuronide	150	300	1000	225	375
Codeine	150	300	1000	225	375
Codeine glucuronide	150	300	1000	225	375
Dihydrocodeine	150	300	1000	225	375
Pholcodine	150	300	1000	225	375
6-AM	5	10	50	7.5	12.5
Street heroin markers[a]	5	10	50	7.5	12.5
Methadone and EDDP	125	250	1000	187.5	312.5
Buprenorphine and metabolites[b]	2.5	5	50	3.75	6.25
Ketamine and norketamine	25	50	100	37.5	62.5
Tramadol and metabolites[c]	100	200	1000	150	250

[a] Street heroin markers: papaverine, noscapine, and acetylcodeine
[b] Norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide
[c] *N*-Desmethyltramadol and *O*-desmethyltramadol

and immediately before the last set of calibrators. Alternate IQC solutions should be analyzed once after every 10 patient samples throughout a batch. EQC samples should be analyzed with each batch.

The assays should include a SIL ISTD (Table 18.6). The intercept of the calibration graph must not be forced through zero. All calibration and IQC solutions should be stored at −20 °C until required. Suggested assay acceptance criteria are: (i) linear (r^2 >0.98) calibration curves for each analyte, and (ii) at least 67 % (4 out of 6) IQC samples within 20 % of their respective nominal value.

18.2.3 'Cut-off' concentrations

'Cut-off' concentrations vary between countries, especially as regards drugs and driving legislation. For workplace drug testing, the EWDTS has published recommended maximum 'cut-off' concentrations for screening and for confirmatory tests in urine (Table 18.7).

Table 18.6 Internal standards and associated analytes for LC-MS/MS substance misuse screening

Internal standard	Analyte(s)
[$^{13}C_6$]-Amfetamine	Amfetamine
[$^{2}H_{14}$]-Metamfetamine	Metamfetamine
[$^{2}H_5$]-MDMA	MDMA
[$^{2}H_3$]-Mephedrone	Mephedrone
[$^{2}H_3$]-Morphine	Morphine, morphine-3-glucuronide, pholcodine
[$^{2}H_6$]-Codeine	Codeine, codeine glucuronide
[$^{2}H_6$]-Dihydrocodeine	Dihydrocodeine
[$^{2}H_3$]-6-AM	6-AM, acetylcodeine, noscapine, papaverine
[$^{2}H_3$]-Methadone	Methadone, EDDP
[$^{2}H_3$]-BE	BE
[$^{2}H_4$]-Buprenorphine	Buprenorphine, buprenorphine glucuronide
[$^{2}H_3$]-Norbuprenorphine	Norbuprenorphine, norbuprenorphine glucuronide
[$^{2}H_4$]-Ketamine	Ketamine
[$^{13}C_6$]-Norketamine	Norketamine
[$^{2}H_3$,$^{13}C_6$]-Tramadol	Tramadol, *O*-desmethyltramadol, *N*-desmethyltramadol

Table 18.7 EWDTS recommended 'cut-offs' for screening and for confirmation in urine

Screening	'Cut-off' (μg L^{-1})	Confirmation	'Cut-off' (μg L^{-1})
Amfetamine group	500	Amfetamines	200
Barbiturates	200	Barbiturates	150
Benzodiazepine group	200	Benzodiazepines	100
Buprenorphine	5	Buprenorphine or metabolite	2
Cannabis metabolites	50	THC-COOH	15
Cocaine metabolites	150	BE	100
EDDP (methadone metabolite)	100 (300)	EDDP	75
		Methadone	250
LSD or metabolites	1	LSD or metabolites	1
Opiates (total)	300	Morphine	300
		Codeine	300
		6-AM	10
		Dihydrocodeine	300
Phencyclidine	25	Phencyclidine	25
Propoxyphene or metabolites	300	Propoxyphene or metabolites	300

18.3 Oral fluid testing

Guidelines for workplace drug testing in oral fluid that discuss collection devices, collection procedures, chain of custody, and validity testing have been prepared by the EWDTS (Brcak *et al.,* 2018). Reliable oral fluid collection requires a co-operative individual and even then is not without problems (Crouch, 2005; Cohier *et al*., 2017). Use of devices that stimulate saliva flow will alter the concentrations of ionizable compounds in the oral fluid collected, thus casting doubt on the validity of 'cut-off' concentrations.

Unstimulated normal human salivary glands do not secrete saliva. However, many stimuli will cause salivation and even during sleep there is usually sufficient stimulation to elicit a very small flow of saliva (typically 0.05 mL min^{-1}). Spitting is usually sufficient to elicit a flow of saliva of about 0.5 mL min^{-1}. Although saliva may be collected from, for example, the parotid gland by cannulation of the glandular duct, the collection of 'oral fluid' (mixed whole saliva) is normally the only practical alternative. In healthy subjects, gingival crevicular fluid may constitute up to 0.5 % of the volume of oral fluid; this proportion may be markedly increased in patients with gingivitis. Plasma exudate from minor mouth abrasions may also contribute. Therefore, subjects should not brush their teeth or practice other methods of oral hygiene for several hours before oral fluid is collected.

Because oral fluid is a viscous mixture it is less easily poured or pipetted than plasma or urine – routine dilution with aqueous collection buffer is advisable to minimize this problem. If dilution with buffer is performed, then the dilution must be factored into any quantitative report. Some drugs and medical conditions, anxiety for example, can inhibit saliva secretion and so the specimen may not be available from all individuals at all times. Moreover, problems may also be encountered in regard to adsorption on collection devices, particularly for lipophilic drugs, giving low analyte recovery (Bosker & Huestis, 2009).

18.3.1 Sample collection and storage

There are differences between POCT devices that use oral fluid and oral fluid collection devices. POCT devices provide a qualitative assessment as to presence (above cut-off) or absence (below cut-off) of drugs in a sample and are used to eliminate 'negative' samples from further investigation (Chapter 17). If the screening tests indicate the possible presence of a compound of interest at a concentration above the 'cut-off' a confirmation analysis using a portion of the same sample is indicated (Brcak *et al*., 2018). Oral fluid collection devices, on the other hand, are simply used to collect a sample that is to be tested in the laboratory.

18.3.1.1 Oral fluid collection devices

Chewing paraffin wax, *Parafilm*, rubber bands, pieces of PTFE, or gum will usually elicit a salivary flow of 1–3 mL min^{-1}. Use of either lemon drops, or a few drops of 0.5 mol L^{-1} citric acid were amongst other stratagems adopted to stimulate salivary flow. The oral fluid should be allowed to accumulate in the mouth until the desire to swallow occurs before being expelled into the collection vessel.

Obviously stimulating salivary flow can facilitate relatively large volume collection in a short time. Moreover, the pH of physically stimulated saliva is approximately 7.4, whereas the pH of unstimulated saliva shows a larger variability that may affect the secretion of weak acids and bases. However, any physical or chemical stimulus used during the collection should neither modify the concentrations of the compounds to be measured, nor introduce interferences into

the assay procedure. For example, paraffin wax and Parafilm may absorb highly lipophilic molecules, and the use of citric acid, will cause changes in salivary pH that will alter the secretion of ionizable compounds.

With the Salivette™ (Sarstedt) a dental-cotton (polyester) roll is chewed for 30–45 s. The device is available either with, or without citrate. The fluid-soaked roll is placed in a container and closed with a plastic stopper. The container is centrifuged (3 min, 1000 *g*) inside a polystyrene tube. During centrifugation the fluid passes from the dental-cotton roll into the lower part of the tube from whence it is collected. Cellular and other debris are retained at the bottom of the tube in a small sink (Figure 18.2). The advantage of the Salivette over many other devices is that it reliably absorbs a relatively large volume of oral fluid (1.5 mL), although a disadvantage is that the dental cotton may interfere with some assays. Another collection device (OraSure) absorbs only 1.0 mL and, moreover, may adsorb analyte unless special precautions are taken (Kauert *et al*., 2006).

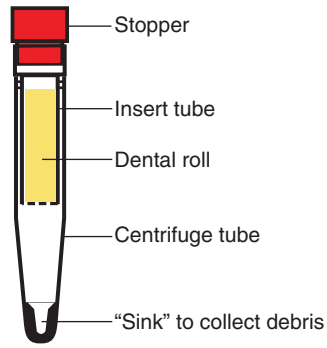


Figure 18.2 An example of an oral fluid collection device (Salivette™, Sarstedt)

Intercept™ and StatSure Saliva Sampler™ stability tests showed that 6-AM, cocaine, and zopiclone were the least stable compounds. In the short-term stability test, StatSure Saliva Sampler showed better results. This indicated that oral fluid samples should be analyzed as soon as possible after collection (Lund et al., 2011). Testing after one year of storage at –20 °C showed that most other compounds were stable in samples collected with both sampling devices.

18.3.2 Road-side testing procedures and 'cut-off' concentrations

The use of oral fluid in roadside testing is now commonplace. After a brief checklist indicating whether a driver might be under influence of drugs, oral fluid road-side tests (e.g. DrugWipe®) are used. If a test is positive, this needs to be confirmed by laboratory analysis of either blood, or oral fluid. 'Cut-off' concentrations may be defined in legislation and may differ between countries. Blood:oral fluid partitioning data are given in Table 17.3.

The sampling method used to collect oral fluid may influence analyte concentrations in the collected sample markedly. Cohier *et al*. (2017) evaluated two collection devices, Quantisal® and Certus®. Four parameters were studied including (i) the collected fluid volume; (ii) the recovery efficiency using oral fluid fortified with opiates, cannabinoids, amfetamines, cocaine

and its metabolites; (iii) analyte stability after storage for 1, 7, and 14 days at –20 °C, +4 °C and room temperature; and (iv) the impact of mouth cells present in the collected fluid on analyte stability. The Certus collector allowed the collection of significantly larger (0.94 ± 0.18 mL versus 0.84 ± 0.06 mL), but less reproducible fluid volumes (19 versus 6.7 %) compared with the Quantisal collector. Drug recovery was significantly better with Quantisal than with Certus, especially when used to detect cannabinoids (0.94 versus 0.54 %, for THC). For both devices, storage at 4 °C was preferable except for methadone, the stability of which was altered by adsorption on the device. If mouth cells were present in the oral fluid sample, THC concentrations were significantly decreased at Day 7 in comparison with Day 1 with both devices.

18.3.3 Analytical methods

The steps in the analysis of oral fluid in substance misuse testing are outlined in Box 18.2. In general, many drugs are detectable in oral fluid soon after ingestion. Moreover, oral fluid concentrations may be much higher than corresponding plasma concentrations if the drug has been either insufflated, or smoked because of the persistence of drug residues in the oral cavity.

Box 18.2 Steps in the analysis of drugs and metabolites in oral fluid

Validity testing
- Sample volume
- Endogenous biomarker results (amylase, cortisol) in accordance with reference values
- Checking for adulterants (unusual colour or texture, unusual odour, missing ‘device pads’)

Sample preparation
- For immunoassay: direct analysis of the sample
- For GC-MS, LC-MS, LC-MS/MS: solid-phase extraction (SPE) or preparation based on microextraction by packed sorbent (MEPS) can be used

Screening
- Immunoassay
- GC-MS(/MS) or LC-(HR)MS
- CE-MS

Confirmation
- GC-MS or LC-(HR)MS. If the first analysis is performed by MS, the positive findings must be confirmed and quantified by reanalysis of another portion of the sample
  - All confirmations must be quantitative

Validation and QA
- Calibrators and IQCs should be prepared using certified reference materials or certified standard solutions appropriate to the matrix

Reporting results
- Results should be considered taking into account current ‘cut-off’ concentrations.
  - ‘Cut-offs’ may vary depending on the jurisdiction and the purpose of testing

Although drug screening devices are widely used, there may be sensitivity issues (Table 18.8). Amfetamines generally occur at much higher (some 20-fold higher) concentrations in oral fluid when compared with blood, and there is a similar detection window when compared with blood (blood: 46 hours; oral fluid: 20–50 hours; urine 4 days). Several cross-reactions have been described leading to a high number of false positives and thus a low specificity. A study comparing amfetamine blood and oral fluid concentrations showed no correlation (Vindenes *et al*., 2012).

Table 18.8 Performance evaluation of DrugWipe 5/5+ (Pehrsson *et al*., 2011–reproduced with permission of Springer)

	Amfetamines	Cannabis	Cocaine	Opiates
True positive	1460	84	12	4
False positive	149	209	26	18
True negative	149	1402	1762	1749
False negative	49	112	7	36
Prevalence (%)	84	11	1.1	2.2
Sensitivity (%)	97	43	63	10
Specificity (%)	50	87	99	99
Accuracy (%)	89	82	98	97
Positive predictive value (%)	71	46	22	8.3
Negative predictive value (%)	92	86	100	99

Cannabis tests in oral fluid show low sensitivity. Moreover, oral fluid concentrations are very high after cannabis has been smoked recently, leading to a high variability in detection times. In one study the concentrations of low-prevalence drugs in oral fluid could not be used to accurately identify drivers with drug concentrations above the selected 'cut-offs' in blood in a cohort of drivers (Gjerde *et al*., 2015). Moreover, a very poor positive predictive value was found for THC (36 %).

Detection of 6-AM in oral fluid may be better than in urine. However, low sensitivity for opiate detection was observed in one study (Table 18.8). Morphine was not detected in oral fluid after codeine intake. Compared to urine, oral fluid was more sensitive for recent cocaine use. However, cocaine insufflation may lead to persistence in oral fluid after the drug has been largely cleared from blood.

18.3.4 'Cut-off' concentrations

The EWDTS recommended 'cut-offs' for screening and for confirmation in unstimulated oral fluid are given in Table 18.9.

Table 18.9 EWDTS recommended 'cut-offs' for screening and for confirmation in unstimulated oral fluid

Screening	'Cut-off' (μg L^{-1})	Confirmation	'Cut-off' (μg L^{-1})
Amfetamines	40	Amfetamines	15
Benzodiazepines	10	Benzodiazepines	3 (temazepam: 10)
Buprenorphine	5	Buprenorphine or metabolite	1
Cannabis (THC)	10	THC	2
Cocaine and metabolites	30	Cocaine	8 (BE 8)
Methadone	50	Methadone	20
LSD or metabolites	1		
Opiates (morphine)	40	Morphine	15
6-AM	4	6-AM	2
		Codeine	15
		Norcodeine	2
		Dihydrocodeine	15
Propoxyphene or metabolites	40	Propoxyphene or metabolites	5

18.4 Blood testing

18.4.1 Legislative limits drugs and driving

The UK government has taken a 'zero tolerance' approach to 'illegal drugs' and a 'risk-based approach' to 'medicinal' drugs, including amfetamine (Table 18.10). In the case of medicinal

Table 18.10 UK blood limits for drugs/driving (2015)

'Illegal' drugs	Threshold limit (μg L^{-1})	Prescribed drugs	Threshold limit (μg L^{-1})
Amfetamine	250	Clonazepam	50
6-AM	5	Diazepam	550
Cocaine	10 (BE 50)	Flunitrazepam	300
Ketamine	20	Lorazepam	100
LSD	1	Methadone	500
MDMA	10	Morphine	80
Metamfetamine	10	Oxazepam	300
THC	2	Temazepam	1000

drugs, the limits do not apply if the drug was being taken as prescribed and the driver was not impaired as a result. In the case of the 'illegal' drugs simply driving above the threshold limit is an offence.

18.4.2 Analytical methods

Steps in the analysis of drugs in whole blood are given in Box 18.3. Pelletti *et al*. (2019) have evaluated the use of CEDIA of whole blood as a screening test to triage samples for confirmatory analysis. General issues include the selectivity and reliability of the methodology employed, the instability of certain analytes, and the need for sample storage and the associated stability studies in the event that re-analysis is required. Clonazepam, cocaine, flunitrazepam, and LSD, for example, are known to be unstable in stored blood (Table 2.9). Moreover, with all quantitative methods there is the uncertainty associated with the result. A pragmatic approach, assuming the analyte is stable in the sample collected, is to allow +3 SDs depending on the validation data for the particular method used.

Box 18.3 Steps in the analysis of drugs in whole blood

Pre-analytical
- Analyze as soon as possible or store at –20 °C

Sample preparation
- GC-MS, LC-MS
  - Protein precipitation
  - LLE, SPE, or SPME, with derivatization if necessary

Assay calibration
- Commercially available or in-house calibrators and IQCs

Reporting
- Report quantitative result if above the legislative limit after allowing for analytical error

18.5 Hair testing

Segmental analysis can sometimes help distinguish between either episodic, or continuous drug use. Surface contamination is possible, although deliberate adulteration of the specimen is unlikely. Acidic and neutral drugs and metabolites, such as THC and THC-COOH, are in general more difficult to detect than basic drugs. Sample preparation is currently labour-intensive and obtaining appropriate QA material is difficult. There have been many publications on the detection of commonly misused substances in hair in recent years (Table 18.11). However, neither assessment of dose, nor delineation of an exact time of exposure are possible from hair analysis alone.

18.5.1 Surface contamination

Human hair consists of protein (65–95 %, mainly keratin), water (15–35 %), and lipid (1–9 %) together with some other components including melanin and trace elements. Drugs and

Table 18.11 Analysis of commonly misused substances in hair: recent reports

Compound class	Compound examples	Application	Reference
Alcohol biomarkers	Ethyl glucuronide (EtG), fatty acid ethyl esters (FAEEs)	Workplace testing, abstinence testing (driving licence suspension, liver and kidney transplantation)	Pragst *et al*. (2010); Andresen-Streichert *et al*. (2015); Cappelle *et al*. (2015); Oppolzer *et al*. (2017)
Illicit drugs	Cannabinoids, amfetamines, cocaine, GHB, ketamine, opioids	Workplace testing, driving impairment, chronic intoxication, substance misuse history and drug withdrawal control, criminal liability, drug facilitated crime, post-mortem toxicology	Dufaux *et al*. (2012); Concheiro *et al*. (2013); Imbert *et al*. (2014); Joya *et al*. (2015); Busardo (2016); Cappelle *et al*. (2017); Taylor *et al*. (2017); Wang *et al*. (2018); Matey *et al*. (2019)
NPS	Cathinones, phenethylamines, synthetic cannabinoids	Workplace testing, driving impairment	Strano-Rossi *et al*. (2014); Salomone *et al*. (2016); Alvarez *et al*. (2017); Kyriakou *et al*. (2017); Salomone *et al*. (2017)
Prescribed drugs	Antidepressants, antipsychotics, benzodiazepines, methadone, zolpidem	Workplace testing, monitoring adherence to therapy, drug-facilitated crime, post-mortem toxicology	Dufaux *et al*. (2012); Concheiro *et al*. (2013); Binz *et al*. (2014); Ramirez Fernandez Mdel *et al*. (2015); Ramirez Fernandez Mdel *et al*. (2016); Sim *et a*l. (2017); Hoiseth *et al*. (2018); Wang *et al*. (2018)
Doping agents	Androgenic steroids	Doping control	Strano-Rossi *et al*. (2013)

metabolites may be incorporated in the structure of the hair as it is synthesized in the hair follicle, but may also arise in hair due to diffusion from skin and sebum, an oily secretion of the sebaceous glands that helps to preserve the flexibility of hair. Note that drugs may arise in the systemic circulation from the subject simply being present in a contaminated environment via inhalation of drug in aerosol form, by skin absorption from surfaces, or in food, for example.

It is important to be able to differentiate between compounds incorporated into hair from within the body from substances that may contaminate the hair once it has grown away from the skin. Thus, after measurement of the length of the hair sample and segmentation if required (normally segments no less than 10 mm in length are used), hair analysis protocols should incorporate a ‘decontamination’ step prior to the analysis (Box 18.4).

Box 18.4 Steps in the analysis of drugs and metabolites in hair

Pre-analytical
- Decontamination [for example with detergents such as shampoos, surgical scrubbing solutions, surfactants such as 0.1 % w/v SDS, phosphate buffer, organic solvents such as acetone, diethyl ether, methanol, ethanol, DCM hexane, or pentane (various volumes, various contact times)]
- Analysis of washings to monitor removal of any surface contamination
- Measurement of length from scalp/body end and segmentation if required
- Accurate weighing of dried hair segments and fragmentation (fine cutting, ball mill)

Sample preparation
- For immunoassay
  - Incubation in aqueous buffer
- For GC-MS, LC-MS, LC-MS/MS, LC-HRMS
  - Incubation with ISTD solution
    - Incubation in acidic or basic aqueous solution, and LLE, SPE, or SPME, with derivatization if necessary
    - Incubation in organic solvent (often DCM, methanol, or acidified methanol) and LLE, SPE, or SPME, with derivatization if necessary
    - Enzyme digestion and LLE, SPE, or SPME, with derivatization if necessary

Assay calibration and reporting results
- Incubate 'blank' hair with known amounts of added analyte across the anticipated concentration range
- Calculation of results (e.g. mg kg^{-1} hair)

Usually, the efficacy of decontamination is assessed by measuring analyte concentrations in the wash fractions, but it remains difficult to know if what is found in the wash fractions comes from the medulla, from the surface of the hair, or from both areas. Generally, a single washing step is used, although a second identical wash is sometimes performed. If external contamination is suggested by analyzing the initial washings, repeated washing/analysis cycles may be taken to demonstrate that any surface contamination has been removed prior to analysis.

Using secondary ion MS (SIMS), a distinction can be made between contaminated or drug user hair by imaging single hairs. This can help differentiate between substance misuse and external contamination without the use of sample preparation procedures that might affect the drug distribution in the hair (Figure 18.3).

It has been suggested that the analyte concentration in the hair after washing should show a 10-fold increase over the concentration in the last wash for a positive result to be accepted, but this is simply a pragmatic attempt to justify the procedure. Decontamination solvents, including procedures adhering to Society of Hair Testing (SoHT) guidelines, cannot completely remove external cocaine and metamfetamine contamination from hair (Mantinieks *et al*., 2019). Indeed, commonly used decontamination protocols have been shown to move cocaine from the surface into the medulla of individual hairs (Cuypers *et al*., 2016). The implications are that using hair testing results alone in court proceedings, for example, is fraught with danger without other supporting information.

18.5.2 Analytical methods

After the washing stages, the dried hair or hair segments may be chopped finely with a razor blade or pulverized in a ball mill prior to solubilization of drug and any metabolite(s). Use

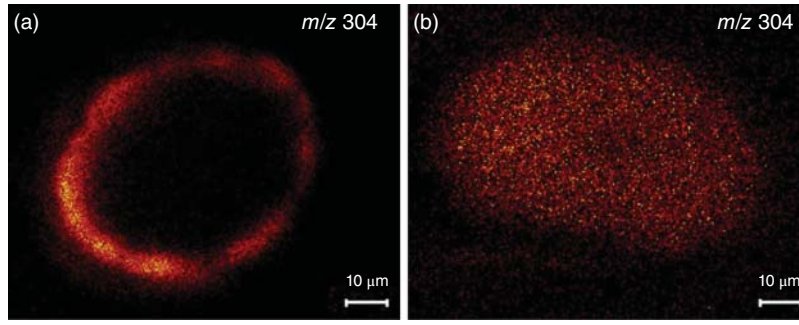


Figure 18.3 Differentiation between (a) contaminated hair and (b) cocaine user hair using SIMS. Cocaine concentration 0.5 µg mg^{-1} (adapted from the data of Cuypers *et al.*, 2016)

of ceramic balls in disposable plastic tubes is likely the best method. The choice of sample preparation procedure and the precise conditions used (such as the pH and the concentration of aqueous incubation solutions, and the duration and temperature of incubation) depends on (i) the analyte(s) and (ii) the analysis system to be used (GC-MS, LC-MS, etc.). If the sample is incubated in aqueous sodium hydroxide solution, solubilization of the hair is complete, but compounds such as cocaine and 6-AM will be hydrolyzed.

Use of enzymatic digestion with, for example, proteinase K, Biopurase, or β-glucuronidase/arylsulfatase, is a further option. LLE into an organic solvent has the advantage that GC-MS, with or without analyte derivatization, could be carried out directly following solvent evaporation (Breidi *et al.*, 2012). Hydrolysis of labile analytes was minimal. Use of LC-MS can further simplify sample preparation because conjugates can be analyzed directly.

The use of SFE in hair analysis was discussed in Section 4.2.6. Even if SFE permits rapid and efficient extraction of drugs and metabolites, all the considerations as to decontamination, segmentation, ISTD addition, derivatization, and assay calibration (use of hair to which analyte has been added), remain (Box 18.4).

18.5.3 Assay calibration and quality assessment

Participation in EQA programmes is mandatory for all laboratories offering hair testing services and is particularly important as a means of assessing the performance of in-house methods (Box 18.5). There are currently three European proficiency testing schemes for hair analysis, SoHT, HAIRVEQ (an external proficiency testing programme set up in Italy and Spain), and the German Society of Toxicological and Forensic Chemistry (GTFCh). The benefit of these schemes is that the samples are prepared using hair from known substance users.

Box 18.5 SoHT assay validation, IQC and EQA recommendations

- Use authentic hair samples to validate the extraction efficiency of methods
- Participate in EQA schemes that use authentic hair samples
- Always analyze both negative and positive hair QCs with each batch of samples
- Provide data to support technical competence in generating valid test results

Assessing analyte recovery from solid samples is simply not possible. If analyte is added to analyte-free hair by soaking in solvent, for example, it cannot be assumed that recovery would be the same as if the analyte had been deposited in the hair as the hair was growing. Therefore,

all quantitative work lacks certainty. IQCs should be prepared from hair from known drug users. If IQC samples are prepared in house it is difficult to ensure homogeneity and it may be difficult to obtain old case samples. A list of current suppliers of QC material, using both authentic and hair to which analytes have been added, and drug-free hair, is maintained on the SoHT website (www.soht.org).

18.5.4 'Cut-off' concentrations

The EWDTS recommended cut-off concentrations for substance misuse screening in hair are given in Table 18.12. Note that these are simply pragmatic recommendations and bear no relation to either dose, or source of drug/metabolite, but aim to minimize the risk of reporting concentrations that are likely incidental findings rather than indicating personal use.

The interpretation of results is based on knowledge (i) of the anatomy, physiology, and biochemistry of growing hair, (ii) of the PK and metabolism of the compound in question, and (iii) of the factors influencing the incorporation, storage, and removal of drugs and other poisons from hair. There are differences in hair growth rate depending on anatomical region, age, sex, ethnicity, and inter-individual variability and thus interpretation of parent drug and/or metabolite concentration data in hair is not straightforward.

Table 18.12 EWDTS recommended cut-off concentrations for screening in hair

Screening	'Cut-off' (μg kg^{-1})	'Confirmation'	'Cut-off' (μg kg^{-1})
Amfetamines	0.2	Amfetamines	0.2
Benzodiazepines	0.05	Benzodiazepines	0.05
Buprenorphine	0.01	Buprenorphine	0.01
		Norbuprenorphine	0.01
Cannabis (THC)	0.1	THC	0.05
		THC-COOH	0.0002
Cocaine	0.5	Cocaine	0.5
		BE	0.05
		Cocaethylene	0.05
		Norcocaine	0.05
Ethanol	N/A[a]	EtG	0.03
		FAEE	0.5
Opiates	0.2	Morphine	0.2
		Codeine	0.2
		6-AM	0.2
Methadone	0.2	Methadone	0.2
		EDDP	0.05
Ketamine	0.5	Ketamine	0.5
		Norketamine	0.1

[a]N/A – not applicable, cannot screen directly

Cosmetic treatment, natural and artificial hair colour, differences in hair structure, and the selectivity of the analytical methods used may all influence the results (Table 18.13). For example, MS imaging (Section 13.7.5) has shown the effect of hydrogen peroxide treatment on cocaine in hair (Cuypers *et al*., 2014). The images revealed decreased detectability of cocaine in hair, most likely through disruption of melanin–cocaine bonds, and degradation of cocaine into reaction products. Unbound cocaine as well as the more hydrophilic reaction products, including BE, were easily washed out, thus removing evidence of cocaine use.

Table 18.13 Some factors that might influence the interpretation of hair analysis results

Factor	Recommendation/effect on interpretation
Specimen collection (head hair)	Pluck (include root) or tie and cut; label cut end
Hair colour, location on the scalp, diameter, rate of growth	Collect a pencil thickness sample from posterior vertex of scalp, cut as close as possible to skin, and tie together
Storage	Room temperature wrapped in aluminium foil
Surface contamination (smoke, collection procedure, laboratory atmosphere)	Use validated sample pre-treatment (washing) method
Cosmetic treatment (shampoos, bleach, dyes, permanent waving, hair relaxers, hair styling products/sprays, hot dryers/curlers)	Many drugs degraded by these or related procedures, but useful qualitative results may still be obtained
Assay reliability	Use GC-MS or LC-(HR)MS with validated sample preparation procedure. Participate in pre-analytical and analytical quality assurance/assessment
Nature of analyte	Basic drugs better sensitivity than acidic/neutral drugs
Information on ‘normal’ and ‘abnormal’ ranges	Base interpretation on comparable methodology
Sex, ethnicity/race, diet, age, geographical location, season	May influence LoD

Detection of drug metabolite(s) such as cocaethylene and norcocaine in hair that cannot be explained by either surface contamination, or decomposition of surface contaminants, provides evidence that positive findings have arisen as a result of systemic exposure. Systemic exposure may of course include inadvertent exposure from being present in a contaminated atmosphere. Similarly, seepage from a cadaver may contaminate hair and of course may contain metabolites. There is still debate as to the problems posed by external contamination, particularly when ‘crack’ (cocaine free base), cannabis, and heroin have been smoked. It is known that it is not possible to remove contamination from such sources from hair by conventional washing procedures.

18.5.5 Ethanol markers

The direct measurement of ethanol is not possible in hair. Instead, ethanol metabolites (EtG and FAEEs, and also EtS) have been measured as indirect markers of alcohol consumption. In assessing chronic excessive alcohol consumption, EtG and FAEEs can be used either alone, or in combination.

The SoHT 2016 consensus for the use of alcohol markers in hair for assessment of abstinence and of chronic excessive alcohol consumption is as below. However, it should be emphasized that these are simply guidelines and need to be used with caution in individual cases. Indeed, this is the fourth revision of the guidelines, suggesting that interpretation provided in some earlier cases where evidence was presented to the Courts may have been misleading (Pragst *et al*., 2017).

18.5.5.1 Ethyl glucuronide

An EtG concentration <7 μg kg^{-1} does not contradict self-reported abstinence during the specified time period before sampling. On the other hand, a concentration ≥7 μg kg^{-1} EtG in the proximal scalp hair up to 6 cm in length strongly suggests repeated ethanol consumption in the last 6 months. Segmentation may provide additional information. If samples <3 cm or >6 cm in length are used, the results should be interpreted with caution.

A concentration of >30 μg kg^{-1} EtG in proximal scalp hair up to 6 cm in length strongly suggests chronic excessive alcohol consumption. Segmentation may provide additional information. If samples <3 cm or >6 cm in length are used, the results should be interpreted with caution.

It has been claimed that the same 'cut-off' concentrations can be used for non-head hair, but there is no robust evidence to support this assertion. The possibilities of a longer time period being represented by hair from parts of the body other than the head should be remembered, and there may be contributions from sweat, deodorants, skin care products, etc.

18.5.5.2 Fatty acid ethyl esters

The analysis of FAEEs alone is not recommended to assess abstinence from ethanol. It is now suggested that rather than using the sum of the ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate concentrations, ethyl palmitate cut-off concentrations of 0.12 mg kg^{-1} for a 0–3 cm proximal scalp hair segment or 0.15 mg kg^{-1} for a 0–6 cm proximal scalp hair segment should be used. A positive ethyl palmitate result combined with an EtG below 7 μg kg^{-1} result does not clearly disprove abstinence, but indicates the need for further monitoring.

For chronic excessive consumption testing, a cut-off concentration of 0.35 mg kg^{-1} for ethyl palmitate in scalp hair is considered strongly suggestive of chronic excessive alcohol consumption when measured in the 0–3 cm proximal segment. If the proximal 0–6 cm segment is used the proposed cut-off concentration is 0.45 mg kg^{-1} scalp hair. If other lengths of hair, or hair from other body sites are used; the results should be interpreted with caution.

18.5.6 Children

A review of two English language reports of hair concentrations of drugs in children living in an environment in which drugs are used, divided the subjects into three categories (i) neonates where hair was sampled at, or shortly after, birth; (ii) children believed to have been exposed passively to drugs in their environment; and (iii) children exposed as a result of either accidental ingestion, or deliberate administration by a caregiver (Wang & Drummer, 2015).

There were limited comparative data in all cases. On average, cocaine, codeine, 6-AM, and morphine showed higher concentrations in hair in category (i) as compared to children exposed passively (category ii). However, there was considerable overlap in the concentrations. Hair metamfetamine concentrations showed no significant difference between the two categories, although only one study reported hair concentrations in category (i). There was no difference in concentrations for those cases exposed passively (ii) or actively (iii) for codeine and methadone. There were insufficient data for other drugs and other comparisons.

Data comparison was confounded by the variability in extraction techniques employed as well as a by the variety of decontamination techniques used; some studies did not employ any decontamination whatsoever. Simply detecting drugs such as methadone or carbamazepine in the hair of children if they are living in a home where these drugs are available does not of itself prove deliberate drug administration (Kintz *et al*., 2010; Kintz, 2014). There may be a contribution of *in utero* exposure to hair drug concentrations in young children (Kintz, 2015).

A further report presented hair analysis data on 100 families with results for one or both parents and one to five children, 30 families with results only for both parents, and 11 families with results only for two to four children (Pragst *et al*., 2019). For parents with hair drug concentrations in the typical range of regular drug use, the drug was detected in the children's hair with the following frequency (%): methadone (66), 6-AM (64), cocaine (92), amfetamine (80), MDMA (43), and THC (67), with higher percentages for younger children. Despite variation in the data, clear trends were found: (i) the child:parent drug concentration ratio decreased with increasing age of the child and (ii) was higher for boys than for girls, emphasizing the environmental contribution to the results in the children.

18.6 Breath testing

Exhaled air is commonly used in alcohol testing and in monitoring exposure to carbon monoxide and other poisons such as volatile anaesthetics. The use of amfetamines, benzodiazepines, cannabinoids, cocaine, opioids, and a range of other non-volatile drugs and metabolites can also be detected in condensate from exhaled air (Beck *et al*., 2013; Trefz *et al*., 2017). In one study, drug testing using exhaled air provided as many positives as urine testing despite a shorter detection window (Beck, 2014).

As yet little is known as to the relationship between blood and exhaled air drug/metabolite concentrations. However, the possibility of testing for driving under the influence of drugs using exhaled breath condensate (EBC) collected with a filter collection device has been explored (Beck *et al*., 2019). However, laboratory sample pre-treatment and state-of-the-art laboratory-based MS analysis of the condensate was required (Box 18.6).

Box 18.6 Sample preparation of exhaled air condensate on an Empore disc C18 for methadone detection

- Cut the disc into 5 × 5 mm pieces using a scalpel and transfer to a 10 mL glass tube
- Add ISTD solution (100 µL of 100 µg L^{-1} [$^2H_3$]-methadone)
- Vortex mix
- Add 300 µL 2-propanol and mix
- Add 5 mL of methanol:ethyl acetate (1+4, v/v)
- Shake the mixture (1 h) in a thermostatic bath (37 °C)
- Centrifuge (15 min, 3000 *g*, 10 °C)
- Transfer the supernatant to a clean tube
- Repeat the extraction using 1 mL of methanol:ethyl acetate (1+4, v/v) and combine the two supernatants
- Add 10 % v/v aqueous formic acid (10 µL)
- Evaporate to dryness under a stream of nitrogen (40 °C)
- Dissolve the residue in 100 µL of methanol:ethyl acetate (1+1, v/v) prior to LC-MS/MS

18.6.1 Collection devices

The SensAbues sampling device (Figure 18.4) facilitates EBC collection in about 2 minutes. The mouth-piece is discarded and the housing containing the micro-particle filter is sealed prior to analysis. Testing for methadone, amfetamine, metamfetamine, 6-AM, morphine, BE, cocaine, diazepam, oxazepam, alprazolam, buprenorphine, and THC showed correct detection and identification based on LC-MS retention time and product ion ratios in SRM mode after solvent extraction of the filter. However, benzodiazepines showed poorer sensitivity than the other analytes.

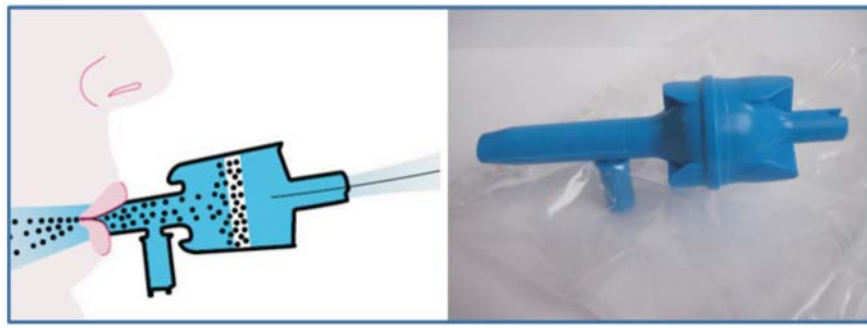


Figure 18.4 The SensAbues exhaled air condensate collection system (Beck *et al*., 2013–reproduced with permission of IOP Publishing)

18.7 Sweat testing

Sweat is produced by eccrine and apocrine glands originating in the dermis and terminating in secretory canals that flow into the skin surface and hair follicles. Collection of sweat has been suggested as a means of testing for substance misuse (De Giovanni & Fucci, 2013; Koster *et al*., 2014). Sweat may be collected either by forehead wipes, or via patches attached to the skin (Section 2.3.6). Sweat collection is non-invasive and commercially available sweat patches may be worn for an extended period of time (10–14 days or so). Use of sweat patches can detect drug use that occurred shortly before the patch was applied and whilst the device remains in contact with the skin. Problems with skin contamination have been investigated (Kidwell & Smith, 2001).

18.8 Summary

Testing for substance misuse is a complex area. The choice of matrix should be strongly related to the aim of testing because detection windows differ between different matrices. Moreover, other factors may influence the analytical results: external contamination, sample adulteration, and even the sampling devices themselves may influence the result. To overcome such problems, 'cut-offs' are advised by international organizations. Despite these 'cut-offs', above which one should report the result as positive, critical interpretation and knowledge of analytical drawbacks should be taken into account in every case.

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19 General Analytical Toxicology

19.1 Introduction

This chapter aims to give guidelines for laboratory methods performed to help diagnose or treat acute poisoning. Although testing for substance misuse, TDM, and the measurement of trace elements/toxic metals are clearly related areas, these topics are considered separately for ease of presentation (Chapters 18, 20, and 21, respectively). Notably, the use of so-called alternative specimens (oral fluid, head hair, sweat, etc.) is discussed as appropriate in Chapters 18 and 20.

Whilst immunoassay and other targeted procedures clearly have a part to play under certain circumstances, the selectivity, and hence sensitivity, identification power, and versatility of chromatographic methods, most notably GC and LC, in many cases linked to MS, has ensured that these procedures are the core techniques in most analytical toxicology laboratories.

If the clinical presentation or other information suggests that a targeted measurement should be conducted, then the choice of the procedure may be straightforward. Most laboratories will have validated procedures for measuring common analytes in commonly supplied specimens (plasma, whole blood, urine, oral fluid, vitreous humour) for specified circumstances (clinical management, forensic toxicology, etc.). A clear advantage with GC and/or LC methods in these circumstances is that by use of appropriate methodology, confirmation of analyte identity can be obtained at the same time as a quantitative result. Moreover, information on other compounds that may be present may also be obtained during the course of the analysis.

If, on the other hand, the agent(s) to be looked for are not known, then a more systematic approach (STA, Section 1.2.1) is required. Although TLC, GC, and/or LC are still in use for STA in some laboratories, most today use LC-DAD and MS coupled to GC and/or LC (Lee *et al*., 2018; Guitton *et al.,* 2019). GC-MS combines very good sensitivity with high selectivity and thus has found broad application in clinical and forensic toxicology, and in doping control (Maurer, 2002). Modern cost-effective bench-top quadrupole or ion trap instruments have brought GC-MS and even LC-MS/MS and LC-HRMS within the reach of most laboratories.

If a limited number of selected compounds are to be looked for, targeted GC-MS(/MS) or LC-MS(/MS) in either SIM or SRM mode can provide simple data evaluation (analyte peak at a given retention time) and high sensitivity because only ions characteristic of the suspected analyte(s) are looked for. This mode of operation is known as data dependent acquisition (DDA).

Alternatively, a comprehensive full-scan analysis, so-called ‘unknown screen’, can be performed in which all ions within a selected *m/z* range are fragmented and analyzed by GC- or LC-MS/MS. This mode of operation is known as data independent acquisition (DIA).

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

The drawbacks here are that the analysis will take longer and sensitivity is likely to be reduced. Moreover, in order to exclude the possibility of false negative results, the procedure(s) used should have been validated to ensure the detection of *all* specified analytes down to established minimum concentrations.

The ability of a given analytical method to identity a compound from a given set of test compounds is known as the identification power. One approach in quantifying identification power is the use of discriminating power (*DP*):

$$DP = 1 - \frac{2M}{N(N-1)} \tag{19.1}$$

where M is the number of pairs of compounds that are not resolved, and N is the number of compounds examined. As the identification power increases so the *DP* increases towards 1.0. The concept of discriminating power was introduced with the aim of quantifying the ability of paper chromatography, TLC, and GLC to give unequivocal identification of unknown analytes. However, given the resolution available with modern chromatographic systems and the selectivity offered in many cases by MS detection, the concept of discriminating power has become less useful.

A second approach to calculate identification power is the mean list length (*MLL*). A list length is defined as the number of feasible candidates for a particular analytical parameter, e.g. the retention index in a GC system (Section 7.3). The average of all list lengths gives the *MLL* for that set of compounds in that system. As with *DP*, *MLLs* can be calculated for a combination of systems. *MLLs* are >1.0, but will approach 1.0 as identification power increases. In both cases (*DP* and *MLL*), examination of a low number of test compounds will give an overestimation of the identification power of the method (Boone *et al*., 1999).

19.2 Gas chromatography

In developing GC methods, knowledge of the M_r and structure of the compound of interest is important even if a published method is to be followed. Information on any co-formulated or co-administered drugs may also be valuable. Instrument manufacturers may sometimes be able to give details of assay methods, current literature, and potential ISTDs for particular compounds.

19.2.1 Qualitative analyses

The role of capillary GC in STA has been discussed extensively and has been compared with LC-DAD, LC-MS, and other techniques (Maurer, 2004). GC retention data for many drugs and other poisons on a range of stationary phases of differing polarities are highly correlated because of the inter-relationship between M_r, volatility, and retention time that is dominant in GC. Experience with modern high-efficiency capillary GC columns has confirmed that a single low- or moderate-polarity column with temperature programming is satisfactory for most purposes if used with a selective sample preparation procedure and some form of selective detection, ideally MS. Polydimethylsiloxane (PDMS) phases such as SE-30/OV-1/OV-101 have the further advantage that retention index data (Section 7.3.2) for a large number of drugs and other compounds of interest generated on packed and capillary columns are available (Maurer *et al*., 2016a).

The introduction of ‘retention time locking’ (RTL) for capillary GC (Agilent) has prompted a re-evaluation of the use of retention index data in STA. The long-term precision of three retention

parameters, the absolute retention time, the relative retention time related to dibenzepin, and a retention index based on the alkylfluoroaniline series, were studied with 14 basic drugs on HP-5 (Agilent; Table 9.2) and DB-17 (J&W, equivalent to OV-17, Table 9.2) columns with and without the use of RTL. Using the constant flow mode, RTL gave better reproducibility with all three retention parameters when compared with the non-RTL method on both columns. RTL offered a significant advantage, not only within a single instrument, but also between methods, with RSDs of <0.1 % in relative retention time (Rasanen *et al*., 2003).

Phase 2 metabolites of drugs such as glucuronides and sulfates are very polar and as such cannot be analyzed directly by GC. Therefore, cleavage by either acidic, or enzymatic hydrolysis is necessary for most GC (and GC-MS) drug screening methods, particularly in urine (Section 4.4). Analytes with either low GC volatility such as morphine, or poor temperature stability such as some β-blockers that contain appropriate functional groups can be derivatized to facilitate GC analysis (Section 9.6). Derivatization with polyfluorinated reagents can improve not only GC properties significantly, but also the detector signal in the NCI mode (Habrdova *et al*., 2005). A further application is chiral derivatization, for example with (*S*)-heptafluorobutyrylprolyl chloride to form diastereomers that can be separated by conventional achiral GC with NCI-MS detection.

19.2.2 Quantitative analyses

Quantitative analyses can be performed using capillary GC provided that the injection is performed with quantitation in mind (Section 9.2.1). GC-NPD may prove a valuable adjunct to GC-MS not only in qualitative analyses, but also in quantitative work because NPD response may be less affected by co-eluting sample components. In addition, the NPD response gives an indication of the number of nitrogen (and/or phosphorus) atoms in the molecule, and analogues or other structurally related compounds can be employed as ISTDs more easily than with MS.

19.2.2.1 Ethanol and other volatiles

The simplest GC-FID method for the measurement of blood ethanol involved dilution of the sample (whole blood, plasma, or urine) (50 µL) with ISTD solution (0.16 g L^{-1} aqueous propanol, 500 µL) followed by vortex mixing (10 s) and direct injection of the resulting mixture onto a column packed with a molecular sieve such as Chromosorb 102 (Table 9.3). Other volatile solvents such as acetone, methanol, and 2-propanol can be resolved and measured if required. Modified carbon black (Carbopak, Table 9.3) materials can also be used.

Static isothermal HS sampling (Section 9.4) combined with temperature-programmed GC on a PDMS or other suitable capillary column and either FID, or dual detection (FID/MS) can be used to screen for not only ethanol, methanol, and 2-propanol, but also a wide range of other volatile compounds in biological fluids (Box 19.1; Figure 19.1). Tiscione *et al*. (2001) used a DB-ALC1 (J&W Agilent) fused silica capillary column (30 m × 0.32 mm i.d., 1.8 µm film) with a 1:1 split ratio to the FID and the MS (Figure 19.2). Alternatively, a split injection/dual column (PDMS-PEG) system can be used (Sharp, 2001). HS-GC-NPD can be used to measure acetonitrile.

In addition to static HS-GC, purge-and-trap extraction coupled to GC and sequential FTIR/FID has been used to detect and measure volatile analytes in blood (5 mL) using 3-propanone as ISTD (Ojanperä *et al*., 1998). A PoraPLOT Q capillary column was used. FTIR identification limits ranged from 0.01 mg L^{-1} for ethyl acetate, butanone, and sevoflurane to 24 mg L^{-1} for methanol, and generally allowed the detection of exposure to volatiles at

Box 19.1 HS-GC-FID of blood volatiles (Flanagan & Fisher, 2013–reproduced with permission of Elsevier)

- Sample preparation:
  - Add ISTDs ethylbenzene and 1,1,2-trichloroethane (25 and 10 mg L^{-1}, respectively) in outdated blood-bank whole blood (200 µL) to a glass septum vial
  - Add specimen (200 µL) and seal the vial
  - Incubate (65 °C, 15 min)
- Chromatographic conditions:
  - Column: 60 m × 0.53 mm i.d. SPB-1 (5 µm film)
  - Oven temperature: 40 °C (6 min), then to 80 °C at 5 °C min^{-1}, then to 200 °C at 10 °C min^{-1}
  - Injection: 300 µL of headspace

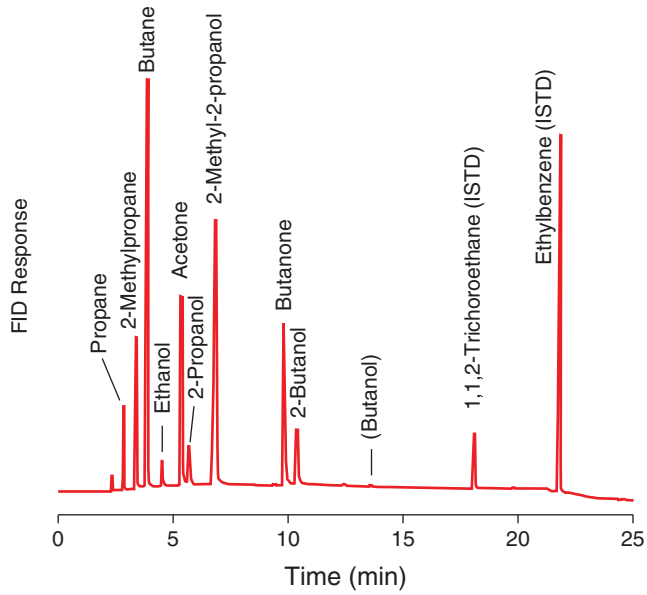

Figure 19.1 GC-FID of a whole blood sample obtained post-mortem from a patient who had inhaled the gas from a cigarette lighter refill

concentrations below those associated with acute toxicity. Using FID, the method was suitable for quantitative work. The throughput was five samples per day, the purge-and-trap stage being the limiting factor.

19.2.2.2 Carbon monoxide and cyanide

GC offers advantages over spectrophotometric methods for carbon monoxide analysis (Section 5.4.3.2), especially if badly decomposed post-mortem blood or tissues are to be analyzed. However, because the sensitivity for carbon monoxide by FID is very poor, either TCD or analyte reduction with hydrogen on a heated Ni catalyst to produce methane before FID have had to be used. This latter procedure introduces an additional step and requires non-standard apparatus.

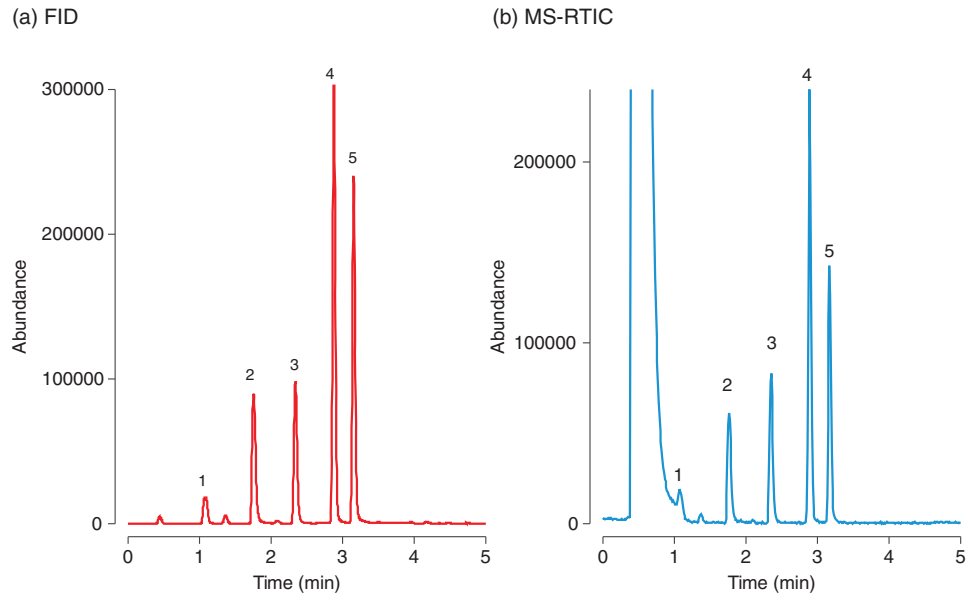

Figure 19.2 HS-GC of ethanol and other volatile compounds: (a) FID and (b) MS RTIC. Peaks: 1, methanol; 2, ethanol; 3, 2-propanol; 4, acetone; and 5, propanol (data kindly provided by Nicholas Tiscione)

With the development of the He-PDPID it is possible to measure carbon monoxide directly with good sensitivity (Section 9.2.2.5). Helium is generally used as the carrier gas and as the ionized species in the detector. Sample manipulation, assay calibration, calculation of % COHb, etc., are the same as with TCD.

Blood cyanide concentrations have been measured by GC-NPD using acetonitrile as ISTD after addition of either phosphoric acid, or glacial acetic acid to the sample in a HS vial (Calafat & Stanfill, 2002; Roda *et al*., 2018). Assay calibration was by addition of potassium cyanide solution to either alkalinized human blood, or deionized water. Note that cyanide ion is a metabolite of acetonitrile hence a different ISTD would be needed if acetonitrile had been ingested. As an alternative to GC-NPD, HS-GC-MS using $K^{13}C^{15}N$ or $[^2H_3]$-acetonitrile as ISTD has also been employed (Dumas *et al*., 2005; Murphy *et al*., 2006). Simultaneous measurement of cyanide and thiocyanate (Section 22.4.15.2) in plasma by GC-CI-MS has also been described (Bhandari *et al*., 2012).

19.3 Gas chromatography-mass spectrometry

With the exception of HS-GC-MS and pyrolysis GC-MS, analytes must be presented in an organic solvent and thus some form of sample preparation/analyte isolation procedure is required. A variety of LLE and SPE procedures have been described that can be used either as universal extraction methods for drug classes, or as highly selective procedures for a particular analyte. Kusano *et al*. (2019), for example, used a SPE procedure (Chapter 4) to target 63 drugs and pesticides in whole blood and Oenning *et al*. (2018) used disposable pipette extraction (DPX) to detect and identify 11 pesticides in urine.

GC-MS is mostly used in the EI mode (70 eV) to produce characteristic spectra (Section 13.3.1.1). This is important when screening for a very wide range of compounds when temperature programming is used in conjunction with large EI spectral libraries. General GC-MS libraries, such as the Wiley Registry of Mass Spectral Data and that produced by the National Institute for Standards and Technology (NIST) in conjunction with the US Environmental Protection Agency (EPA) and National Institutes of Health (NIH) are available complete with computer programs that facilitate comparison of spectra of compounds from sample analysis with those of known compounds.

Various algorithms are available to help in compound identification. There are also libraries specifically created for use in analytical toxicology (Maurer *et al*., 2016b; Rösner, 2018). If certain prerequisites (validated sample preparation procedure, defined ionization parameters, retention time matching, etc.) are fulfilled, such libraries facilitate compound identification even in the absence of reference compounds (Maurer, 2006). In addition, SIM mode can be used for targeted screening and sensitive quantification if appropriate calibration standards are available (Peters *et al*., 2003). Even though LC-MS can provide either complementary, or alternative methodology in many areas, GC-MS is required for the analysis of anaesthetic gases, solvents, and other volatile materials.

To monitor instrument performance, a solution of analytes covering a wide range of physicochemical properties and analyte retention times should be analyzed every working day (Figure 13.22). The quality acceptance criteria are that all compounds must be resolved and the peaks should be sharp and of sufficient abundance to be clearly identifiable. The peak area of underivatized morphine should be at least 10 % of the area of codeine. This criterion is important because morphine is the compound most likely to indicate poor GC performance (badly tailing peak). If these criteria are not fulfilled, the injection port liner should be removed, and the GC column should either be shortened by removing the top 10–20 cm, or replaced, and the ion source cleaned.

The use of 'blank' control samples in addition to assay calibrators is essential to monitor analyte carry-over. False-positive GC-MS results reported by proficiency test organizers are usually caused by carry-over.

19.3.1 Qualitative analysis

19.3.1.1 Targeted analysis

As discussed in Chapter 13, when using SIM it is usual to monitor two or more ions, one characteristic ion derived from the analyte and, if possible one or more 'qualifier' ions, and another ion characteristic of the ISTD, in separate channels to enhance the reliability of the analysis (Figure 19.3). SIM can also be used for analyte detection, identification, and simultaneous quantification after appropriate calibration. Typical analytes for such an application are THC and its main metabolites. Merged mass fragmentograms of extracted serum standard solutions containing THC, hydroxy-THC (OH-THC), THC-COOH and the corresponding deuterated ISTDs are shown in Figure 19.4. Identification criteria were the presence of the corresponding three monitoring ions at the defined retention time and the relative abundance of these ions. Selectivity testing should show that none of the selected ions interacts with matrix components. Commonly occurring ions such as *m/z* 44 $[CH_3NHCH_2]^+$, 58 $[(CH_3)_2NCH_2]^+$, or $[C_2H_4NHCH_3]^+$; 77 $[C_6H_5]^+$, 91 $[C_6H_5\text{-}CH_2]^+$, or 105 $[C_6H_5C{=}O]^+$; or $[C_6H_5\text{-}CH_2CH_2]^+$ should always be avoided when attempting compound identification (Section 13.8).

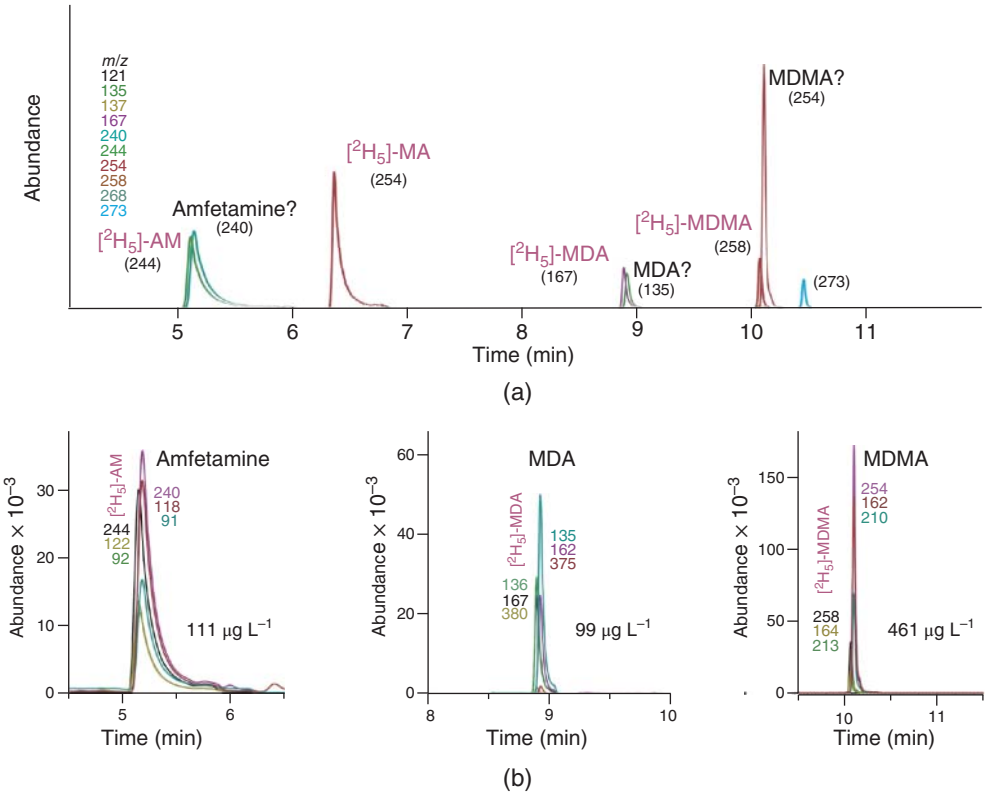

Figure 19.3 (a) Merged GC-NCI mass chromatograms of characteristic ions of heptafluorobutyric anhydride (HFBA) derivatives of amfetamine, metamfetamine (MA), MDA, MDMA, and [$^2H_5$]-labelled ISTDs obtained on analysis of a patient plasma sample. (b) Merged mass chromatograms of one target and two qualifier ions, respectively, of amfetamine, MDA, and MDMA (Peters *et al*., 2003–reproduced with permission of John Wiley & Sons)

A method for measuring stimulants including NPS in whole blood based on GC-CLND-APCI-QTOF-MS has been described (Mesihää *et al*., 2020). For the CLND, amfetamine, MDMA, and methylenedioxypyrovalerone were used as external calibrators for primary, secondary, and tertiary amines, respectively. After fortifying with 38 stimulants at three different concentrations, donor blood samples were analyzed by LLE at a basic pH followed by acylation with trifluoroacetic anhydride. All but three psychostimulants could be analyzed with a LLoQ of 0.05 mg L^{-1}. At the LLoQ, the CVs for between-day accuracy were 62–143 % (mean 93.5 %; median 88.5 %) and precision 7–22 % (mean, 16 %; median, 16 %).

Eleven post-mortem blood samples, containing 0.08–2.4 mg L^{-1} of amfetamine (n = 5), metamfetamine (n = 4), or MDMA (n = 4), were also analyzed and the results compared with an established GC-EI-MS method with appropriate calibration. The agreement between the two methods was 63–117 %. Regarding identification, the APCI source permitted detection of the intact precursor ion, or the respective acylation product, for all of the measured compounds. The GC-CLND-APCI-QTOF-MS method enabled quantitation of illicit psychostimulants in blood with reasonable accuracy without the need for reference standards for each analyte.

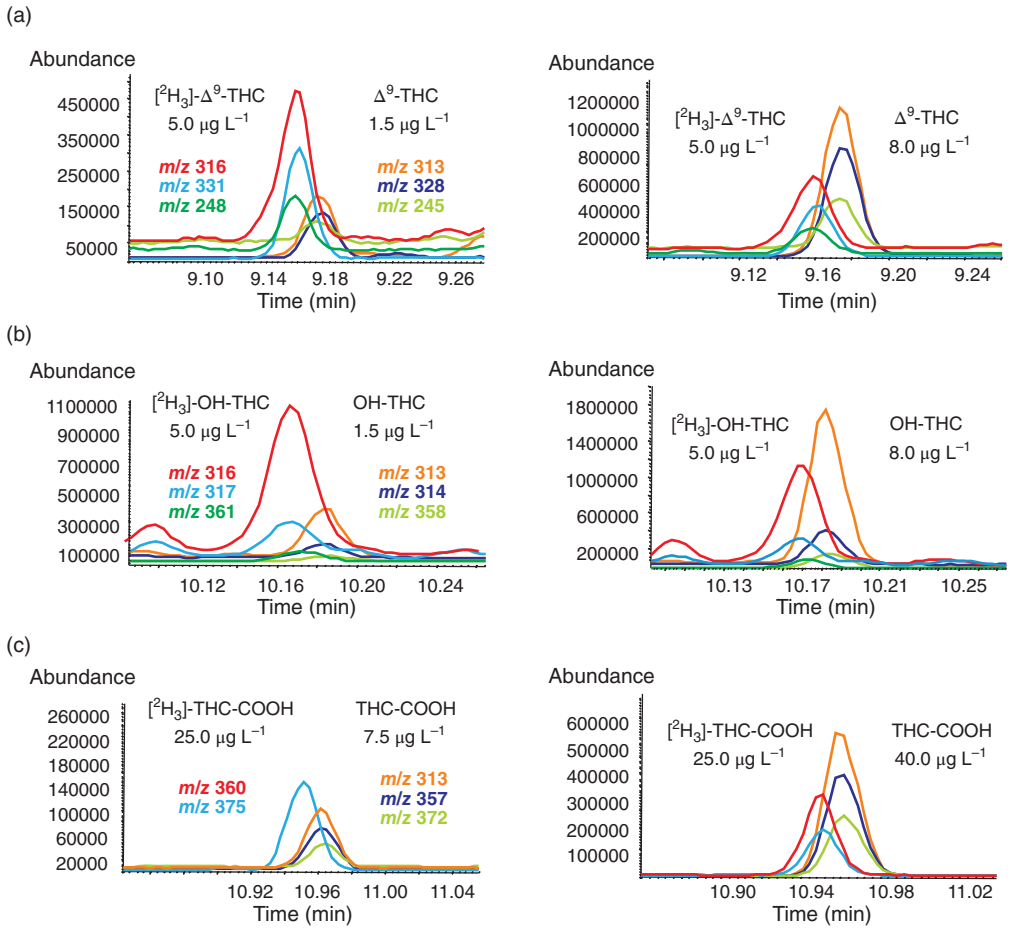

Figure 19.4 Merged mass fragmentograms of extracted serum standards containing (a) Δ^9-tetrahydrocannabinol, (b) 11-hydroxy-Δ^9-tetrahydrocannabinol and (c) 11-nor-9-carboxy-Δ^9-tetrahydrocannnabinol, at the given analyte concentrations. Redrawn from the data of Steinmeyer *et al*., 2002

19.3.1.2 Systematic toxicological analysis

Acid hydrolysis of a portion of a urine specimen and combining this with a portion of unhydrolyzed urine prior to extraction removes the risk of destroying acid-labile analytes (Table. 4.6) whilst enhancing the likelihood of detecting compounds that are principally excreted as conjugates (Box 19.2). The solvent mixture used has very high 'extracting power' and using pH 8–9 ensures that relatively water-soluble analytes and amphoteric compounds such as morphine and many metabolites are extracted (Section 4.2).

Derivatization with acetic anhydride (Box 19.2) to ensure reasonable GC behaviour requires a basic catalyst. Pyridine, although toxic, has the advantage over non-toxic catalysts such as *N*-methylimidazole in that it is volatile and can be removed by evaporation together with excess acetic anhydride prior to GC-MS. Acetate derivatives are preferred because derivative formation is rapid and quantitative, and the resulting mass spectra give valuable structural information.

Box 19.2 STA of urine by GC-MS: sample preparation

- Reflux portion of urine (2.5 mL, 15 min) with 12 mol L^{-1} hydrochloric acid (1 mL)
- Add 2.3 mol L^{-1} aqueous ammonium sulfate (2 mL) + 10 mol L^{-1} aqueous sodium hydroxide (1.5 mL) to give pH 8–9
- Add to unhydrolyzed portion of urine (2.5 mL) and extract with 5 mL DCM:2-propanol:ethyl acetate (1+1+3 v/v)
- Evaporate to dryness (reduced pressure, 70 °C) and add 0.1 mL acetic anhydride:pyridine (3+2 v/v) (5 min, microwave *ca*. 440 W)
- Evaporate to dryness (reduced pressure, 70 °C) and reconstitute in 100 µL methanol

However, a drawback is that acetylation of morphine and of codeine gives 6-AM and acetylcodeine, respectively, compounds that are important as indicators of use of diamorphine and of street heroin, respectively. Thus, in many cases a different derivatization reagent such as trifluoroacetic anhydride has to be used to avoid artefactual production of these products.

Many drugs, drug metabolites, and other compounds can be detected and identified if they are (i) extracted under the conditions applied, (ii) volatile under the GC conditions used, and (iii) if their mass spectra are in the reference libraries. In order to maximize the number of compounds that can be detected and identified, full-scan GC-MS should be used. However, evaluation of the 1000 or so mass spectra recorded during a typical analysis is not straightforward. Although the results could be compared spectrum by spectrum with a printed or electronic collection of reference spectra, searching for the presence of drug- or drug class-selective fragment ions can shorten the time taken for data analysis dramatically. The resulting reconstructed mass chromatograms indicate the possible presence of a corresponding drug, but confirmation of peak assignment needs comparison of the mass spectrum underlying the peak (after suitable background subtraction) with a suitable reference library. This procedure can be computerized.

A practical problem with MS is the amount of data produced in each analysis. If the instrument data evaluation software allows macros (a pre-programmed set of instructions) to be used, macros indicating the principal *m/z* peaks associated with a series of drugs and drug classes can be used in data evaluation (Maurer *et al.,* 2016a). For example, mass chromatograms with the ions *m/z* 157, 161, 174, 200, 216, and 230 (nominal mass) were used in the analysis of the designer drug 1-(3-trifluoromethylphenyl)piperazine (TFMPP) and its metabolites by GC-EI-MS after acid hydrolysis, LLE, and acetylation (Figure 19.5). The identities of the peaks in the mass chromatograms were confirmed by comparison of the underlying mass spectra with reference spectra. The mass spectrum underlying the peak at 8 min, the reference spectrum, the structure, and the library search 'hit list' are shown in Figure 19.6. Here, *m/z* 216 is the base peak representing the loss of (i) one acetyl (*m/z* 288) and (ii) one *N*-acetylmethylamine moiety of the hydroxylated metabolite shown.

This semi-automated approach can be further optimized by using open-access deconvolution software: AMDIS (Automated Mass Spectral Deconvolution and Identification System) (https://chemdata.nist.gov/mass-spc/amdis/downloads/). Use of AMDIS helps to process GC-MS data from the analysis of complex mixtures even in the presence of co-eluting compounds. To facilitate the use of AMDIS, the parameters for urine drug screening were optimized and validated using over 100 different authentic urine samples (Meyer *et al*., 2010). The protocol for urine screening using AMDIS has also been used for blood (Grapp *et al*., 2016). In both cases, a target library was generated from a larger library (Maurer *et al*., 2016b) by removing spectra that could not be present in the sample extracts, for example the presence of heptafluorobutyryl (HFB) derivatives in an acetylated extract.

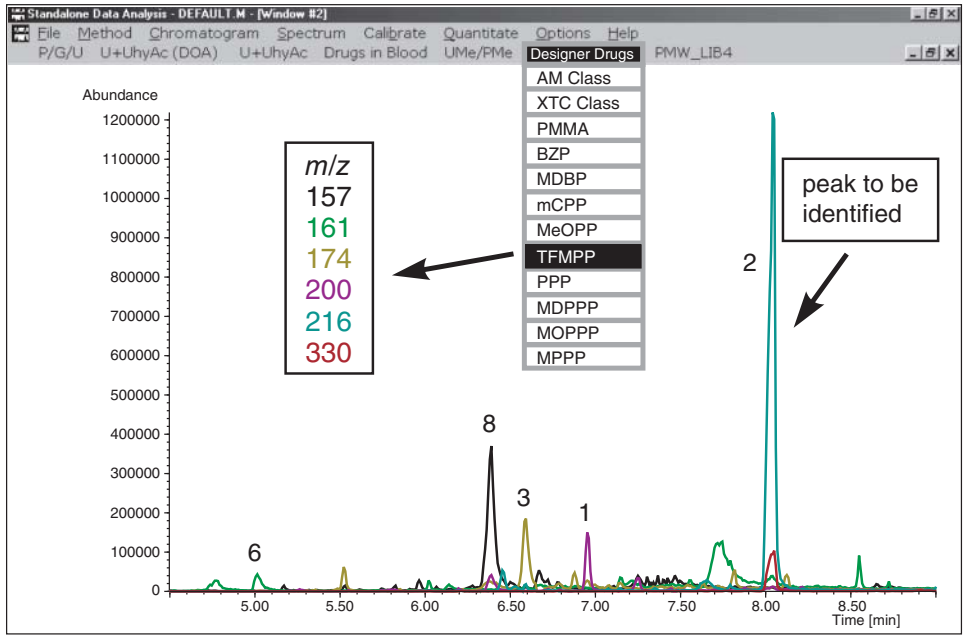

Figure 19.5 Reconstructed mass chromatograms of an acetylated extract of a 24-h rat urine sample after a dose of 1 mg kg^{-1} body weight TFMPP after searching for the substance-specific fragment ions (from Staack *et al*., 2003–reproduced with permission of John Wiley & Sons). The mass chromatograms were generated by use of the pull-down menus searching for the substance-specific fragment ions

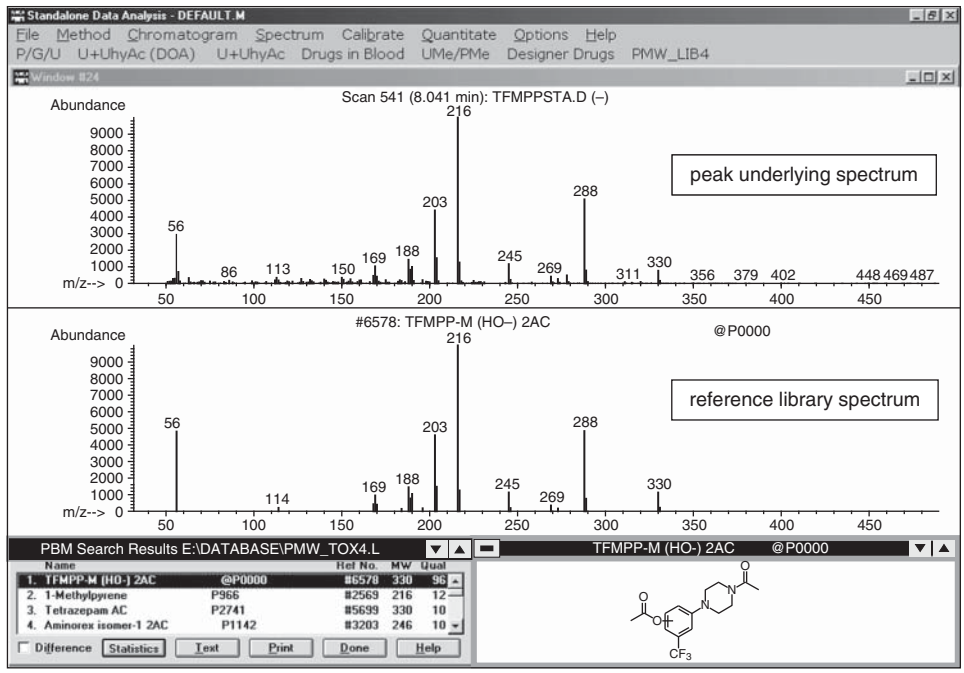

Figure 19.6 Mass spectrum of the peak at 8 min (Figure 19.5), the reference spectrum, the structure, and the library search 'hit list' (Maurer *et al*., 2016b–reproduced with permission of John Wiley & Sons)

19.3.2 Quantitative analysis

The analysis of plasma is inherently more complicated than that of urine because (i) plasma protein is present, (ii) analyte concentrations are often much lower than in urine, and (iii) quantitation is required if possible. In LLE, sodium sulfate may be added to increase the ionic strength of the aqueous phase thereby enhancing recovery of certain analytes (Section 4.2.2), whilst sequential neutral and basic extractions can be used to increase the range of compounds detected (Box 19.3).

Box 19.3 LLE for quantification in plasma by GC-MS or LC-MS

- To plasma (1 mL) add 0.1 mL methanolic [$^2H_3$]-trimipramine (10 mg L^{-1}) and 5 mL saturated aqueous sodium sulfate
- Extract with 5 mL diethyl ether:ethyl acetate (1+1) and centrifuge
- Transfer the organic phase to a pear-shaped flask and add 0.5 mL aqueous sodium hydroxide (1 mol L^{-1}) to the aqueous phase
- Re-extract the aqueous phase with 5 mL diethyl ether:ethyl acetate (1+1) and centrifuge
- Evaporate combined extracts to dryness and reconstitute in methanol (0.1 mL) or corresponding LC eluent prior to analysis

In emergency toxicology and/or if only a limited amount of sample is available, portions of a single extract can be analyzed by GC-MS, LC-MS, and LC-HRMS. Details are given in a number of publications. Thus, a general procedure for GC-MS without analyte derivatization was described by Meyer *et al*. (2014), use of this same sample preparation procedure prior to LC-MS/MS was detailed by Michely & Maurer (2018) and by Montenarh *et al*. (2015). Finally, its use with LC-MS^n and with LC-HRMS was documented by Caspar *et al*. (2019) and by Caspar *et al*. (2018), respectively. Use of MTBE rather than diethyl ether (Box 19.3) may be preferred on safety grounds.

SPE followed by derivatization, e.g. with HFBA, has been used for targeted screening and quantification of designer drugs by GC-MS (Peters *et al*., 2003) and for the LC-MS/MS of toxic alkaloids (Beyer *et al*., 2007). Use of a Confirm HCX, a mixed-mode (C_8 and SCX) SPE column (Biotage) has been found to give contaminant-free extracts even from haemolyzed blood (Box 19.4). Although assay development time is likely to have been greater than with LLE,

Box 19.4 SPE for quantification in plasma by GC-MS or LC-MS

- To plasma (0.5 mL) add 2 mL deionized water, 0.05 mL methanolic [^{2}H]-trimipramine (10 mg L^{-1}), and mix (rotary mixer, 15 s)
- Pre-condition SPE columns (Isolute Confirm HCX A 130 mg, 3 mL) with methanol (1 mL) and then deionized water (1 mL)
- Load sample mixture onto SPE column and wash sequentially with deionized water (1 mL), 10 mmol L^{-1} aqueous hydrochloric acid (1 mL), and methanol (2 mL)
- Dry under vacuum and elute into autosampler vials with 1.5 mL methanol:aqueous ammonium hydroxide (SG 0.88) (98+2)
- Evaporate to dryness under nitrogen (56 °C) and reconstitute in methanol (50 µL)

the established procedure is robust. However, the number of steps involved and the cost of the columns must be borne in mind if this approach is to be used.

In ethylene glycol (1,2-ethanediol) poisoning, accurate quantification is needed to guide treatment (Section 22.4.1.2). A simple and rapid thermal desorption GC-FID method for assay of ethylene glycol in whole blood without extraction and derivatization after addition of 1,2-butanediol (ISTD) has been described (Robson *et al.,* 2018). As an alternative, a range of glycols, glycolic acid (the principal plasma metabolite of ethylene glycol), and GHB can be measured by GC-MS after forming TMS derivatives (Figure 19.7). A 50 µL portion of sample was deproteinized and 20 µL of the diluted specimen were derivatized using bis-*N,O*-trimethylsilyl trifluoroacetamide and the catalyst dimethylformamide. After microwave-assisted derivatization, a portion was analyzed by GC-EI-MS in SIM mode.

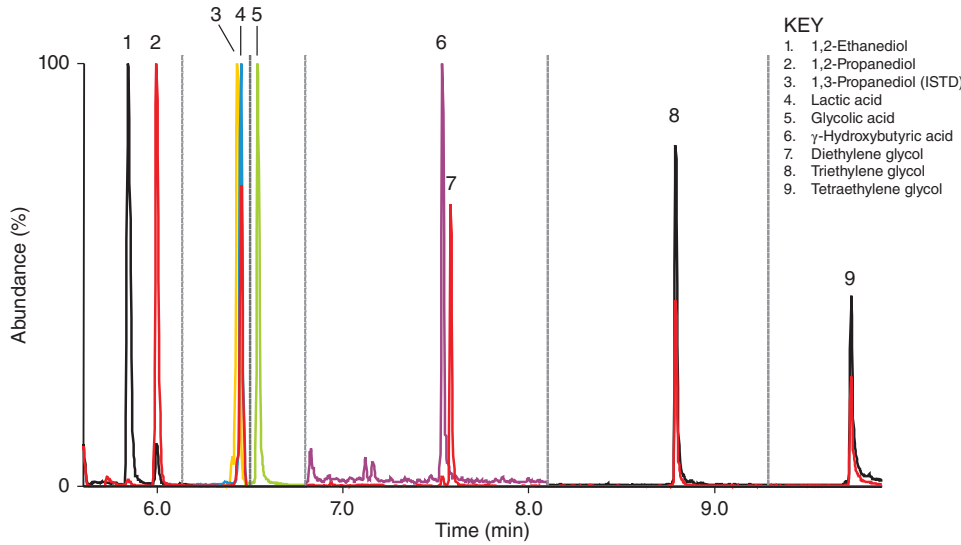

Figure 19.7 Mass fragmentograms of the quantifiers (*m/z*) of a plasma sample to which silylated analytes and 1,3-propanediol (ISTD) were added (Meyer *et al*. (2011)–reproduced with permission of Springer Nature). The monitored SIM masses for the given analytes in the windows indicated by dotted lines are given in Meyer *et al.* (2011)

All analytes were separated within 12 min. The LLoQs were of 0.05 and 0.01 g L^{-1} for glycols and for GHB, respectively. Calibration was linear from 0.05 to 1.0 g L^{-1} for glycols, and from 0.01 to 0.2 g L^{-1} for GHB. Validation criteria were shown to be in the required limits with the exception of lactic acid. The average analysis time from starting sample preparation until quantitative results was approximately 35 min.

Finally, as an alternative to six- or eight-point assay calibration (Section 3.2.5), a simple approach based on LLE (Box 19.3) with one-point calibration has been developed for emergency toxicology (Meyer *et al*., 2014). Bias and precision should be within ±30 % of IQCs at the upper (80 %) and lower (20 %) ends of the measurement range and accuracy, expressed as 95 % β-tolerance interval (Hubert *et al*., 2007), should be within ±50 % for low and for high QC concentrations (GTFCh, 2018). GC-MS using full-scan data acquisition facilitated the detection and measurement of 40 commonly encountered drugs in one analytical sequence.

Using single-point calibration, 35 drugs could be measured quickly, accurately, and reliably across the measurement range. Daily one-point calibration with calibrators stored for up to four weeks reduced the turn-around time to less than one hour.

19.4 Liquid chromatography

19.4.1 Qualitative analysis

Several LC-DAD systems for STA have been described. Compounds can be identified using 'retention indices' (calculated by interpolation between a series of reference drug markers, separate scales for acidic and for basic drugs) and spectral data. Such systems can be useful in performing targeted analyses under isocratic conditions once an analyte has been identified because they avoid the major problem of ion enhancement/ion suppression that can plague LC-MS.

DADs are now much improved with respect to wavelength accuracy and resolution, sensitivity, linearity, and operating software. UV spectra measured with up-to-date DADs from different manufacturers give good agreement, have the same quality as spectra measured by conventional UV spectrometers, and are highly reproducible. The calculation of similarity parameters by the DAD software includes the entire range of the spectrum and allows recognition of very small spectral differences (Pragst *et al*., 2001; Figure 19.8).

In a systematic study of 2682 toxicologically relevant substances, retention times relative to an ISTD and spectra in the range 195–380 nm were recorded using a 5 µm Lichrospher-RP 8 EC, 250 × 4 mm i.d. column with an acetonitrile:aqueous phosphate buffer (0.1 mol L^{-1}, pH 2.3) mixture in two different proportions – Eluent A: 37+63 [ISTD: 5-(4-methylphenyl)-5-phenylhydantoin] and Eluent B: 63+37 (ISTD: 4-phenylbenzophenone). There were 1650 different chromophores or chromophore combinations. In all, 1619 substances (60.4 %) were identified unambiguously by spectrum alone. The identification rate increased to 84.2 % by the combination of spectral data and relative retention time (Herzler *et al*., 2003; Pragst *et al*., 2004).

Therefore, LC-DAD in combination with defined sample preparation, LC retention data, and a good UV spectral database can be a useful supplementary technique in STA. Moreover, sample preparation may be simpler than with GC, although the chromatographic resolving power of temperature programmed GC-MS is lacking (Maurer, 2004). LC-DAD is sometimes useful in metabolite identification because in many cases such compounds have the same or very similar UV spectra to the parent substance and their retention times on reverse-phase columns may be altered in a manner characteristic of the expected metabolic product.

19.5 Liquid chromatography-mass spectrometry

LC-MS with single stage MS, MS/MS, or MS^n (Chapter 13) is widely used today in clinical and forensic toxicology. LC-MS/MS with either ESI, or APCI is used in targeted screening employing SRM, STA using full scan with either data-dependent, or data-independent MS/MS, and in quantitative work (Maurer, 2018).

As with GC-MS, instrument performance must be monitored daily using a solution of analytes covering a wide range of physicochemical properties and relevant retention times. The analyte concentrations, the injection volume, and the separation have to be adapted to the apparatus used (Figure 19.9). The quality criteria are as follows: all compounds must be resolved and the peaks must be of sufficient abundance to allow clear detection on the instrument used.

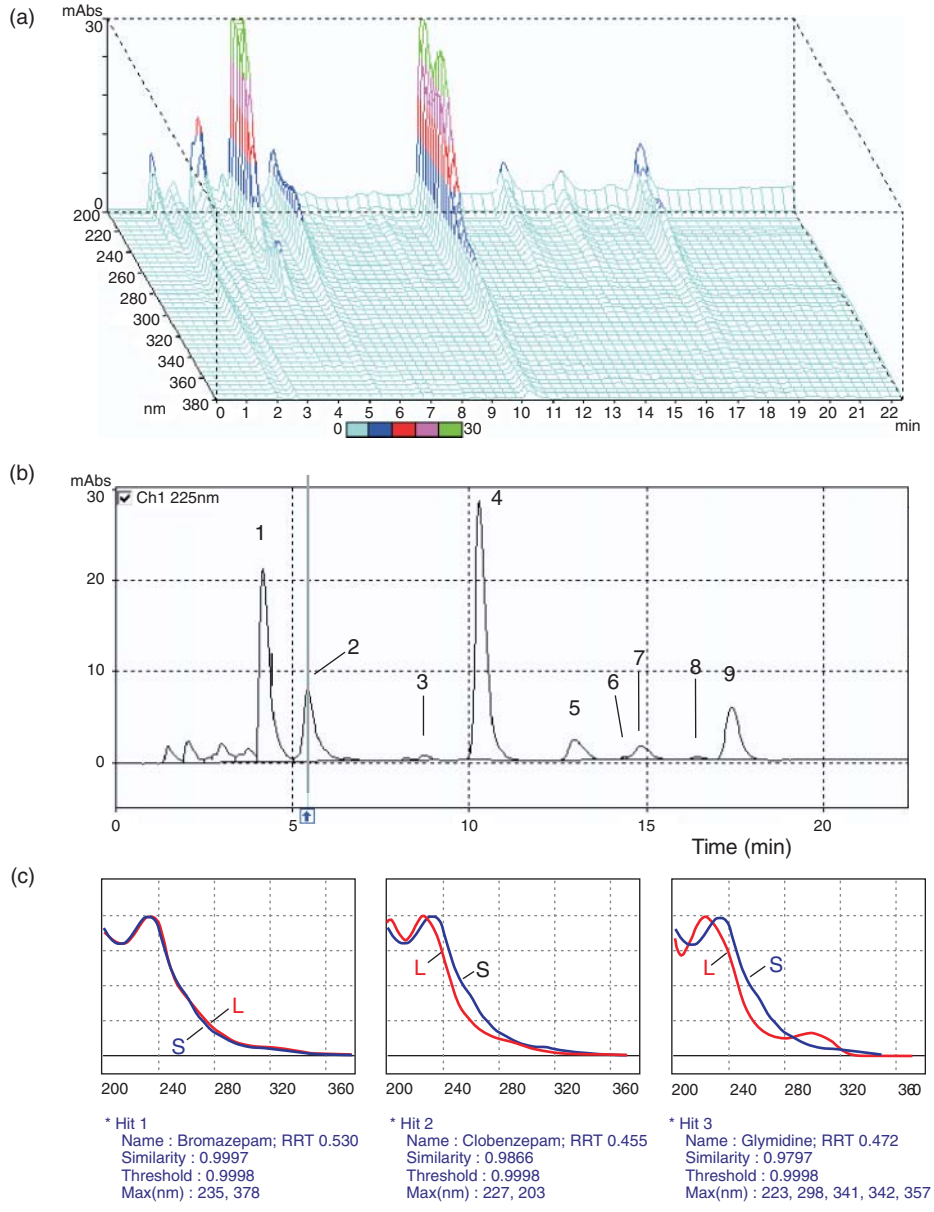

Figure 19.8 LC-DAD of a dichloromethane extract (pH 9) of a blood sample from a poisoned patient. (a) 3-D chromatogram. (b) Chromatogram (225 nm). Compounds (mg L^{-1}) identified by UV spectrum and retention time: peaks: 1 = tramadol (1.2), 2 = bromazepam (0.15), 3 = oxazepam (0.01), 4 = nordazepam (0.51), 5 = nortriptyline (0.07), 6 = nortrimipramine (0.01), 7 = amitriptyline (0.08), 8 = trimipramine (0.02), and 9 = diazepam (0.12). (c) Result of the library search of peak 2 (wavelength nm). A similarity index above 0.9990 is very strong evidence for identical spectra. S = sample spectrum, L = library spectra

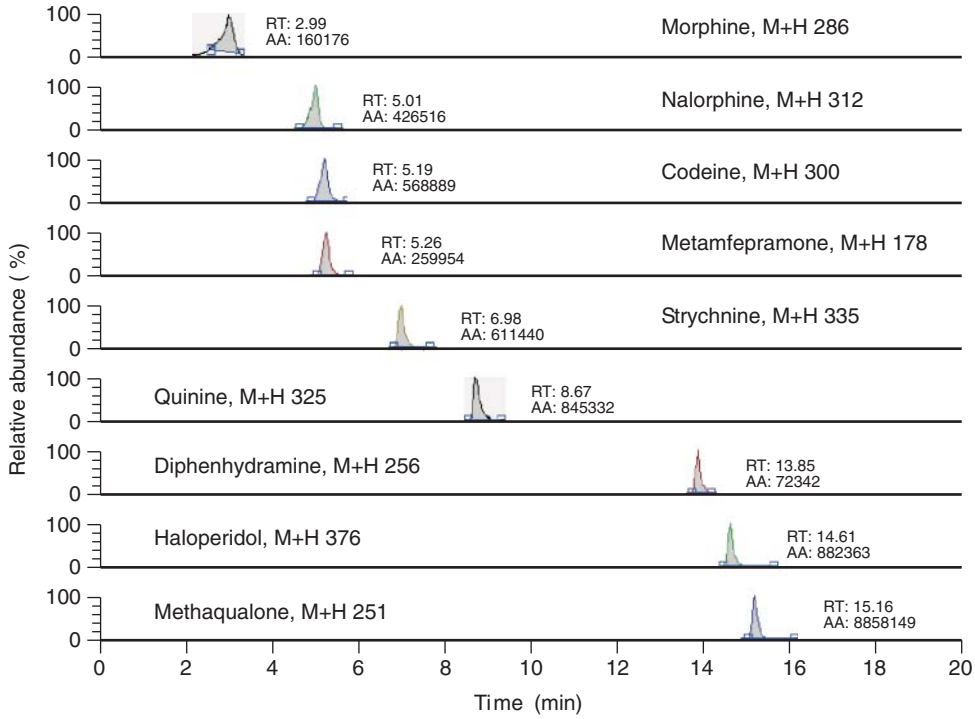

Figure 19.9 Typical LC-MS total ion chromatogram obtained on analysis of a methanolic solution of morphine, nalorphine, codeine, metamfepramone, strychnine, quinine, diphenhydramine, haloperidol, and methaqualone (all 5 mg L^{-1}). Instrument: linear ion trap, gradient elution, C18 column: 100 × 2.1 mm i.d., 1.9 µm aps, injection volume: 10 µL

Performance must be verified using calibrators and 'blank' samples. LC-MS/MS can be so sensitive that analyte carry-over leading to false positive results can be a major problem. Besides careful sample preparation, 'blank' sample extracts must be analyzed between samples. Testing for analyte carry over and carry under during method development and validation is mandatory. False-positive LC-MS results reported by EQA scheme organizers are usually caused by carry-over.

LC-MS(/MS) can provide the best selectivity amongst commonly used analytical techniques. However, selectivity increases with the number of ions monitored. For single stage MS in SIM mode a minimum of three diagnostic ions per analyte, preferably including the pseudomolecular ion, should be monitored. For MS/MS in SRM mode there is a minimum requirement to measure one precursor and two diagnostic transitions per analyte.

Interference in SRM can be caused not only by co-eluting analytes and matrix components, but also by contaminants in the sample, in sample containers, or in the reagents used. During selectivity testing such interferences can be detected and removed by optimization of the sample preparation procedure. A list of several hundred contaminants that may help to explain and thus eliminate interferences is available. Use of HRMS (Chapter 13) also helps in identifying contaminants because the accurate monoisotopic molar masses of many such compounds/fragments are known (Table 19.1). In SRM, problems (so-called 'cross-talk') can occur if two reactions

Table 19.1 Some potential interferences/contaminant ions in positive mode ESI (Keller *et al.*, 2008–reproduced with permission of Elsevier)

Monoisotopic mass (singly charged), u	Ion type	Formula for M or subunit or sequence	Compound or species	Possible origin
63.04406	$[A_1B+H]^+$	$[C_2H_4O]_nH_2O$	Polyethylene glycol	Plastics
77.05971	$[A_1B+H]^+$	$[C_3H_6O]_nH_2O$	Polypropylene glycol	Plastics
102.12773	$[M+H]^+$	$C_6H_{15}N$	Triethylamine	Buffer solution
122.08117	$[M+H]^+$	$C_4H_{11}NO_3$	TRIS	Buffer solution
149.02332	$[f+H]^+$	$C_8H_4O_3$	Phthalic anhydride	Phthalate plasticizer
163.03897	$[M-CH_3OH+H]^+$	$C_{10}H_{10}O_4$	Dimethyl phthalate	Plasticizer
171.00527	$[f+Na]^+$	$C_8H_4O_3$	Phthalic anhydride	Phthalate plasticizer
301.14103	$[M+Na]^+$	$C_{16}H_{22}O_4$	Dibutylphthalate	Plasticizer
315.25299	$[M+H]^+$	$C_{18}H_{34}O_4$	Dibutylsebacate	Plasticizer
317.11497	$[M+K]^+$	$C_{16}H_{22}O_4$	Dibutylphthalate	Plasticizer
371.10124	$[M+H]^+$	$[C_2H_6SiO]_5$	Polysiloxane	Followed by *m/z* 388.12779
371.22756	$[A_8B+H]^+$	$[C_2H_4O]_nH_2O$	Polyethylene glycol	Plastics
371.31559	$[M+H]^+$	$C_{22}H_{42}O_4$	Bis(2-Ethylhexyl) adipate	Plasticizer
371.31559	$[M+H]^+$	$C_{22}H_{42}O_4$	Dioctyl adipate	Plasticizer
375.25058	$[AB_3+Na]^+$	$[C_{15}H_{24}O]$ $[C_2H_4O]_n$	Triton	101 Detergents
388.12779	$[M+NH_4]^+$	$[C_2H_6SiO]_5$	Polysiloxane	See *m/z* 371.10124
389.25098	$[A_6B+Na]^+$	$[C_3H_6O]_nH_2O$	Polypropylene glycol	Polypropylene glycol
391.28429	$[M+H]^+$	$C_{24}H_{38}O_4$	Diisooctyl phthalate	Plasticizer
393.20951	$[A_8B+Na]^+$	$[C_2H_4O]_nH_2O$	Polyethylene glycol	Plasticizer
397.29485	$[AB_4+H]^+$	$[C_{15}H_{24}O]$ $[C_2H_4O]_n$	Triton	101 Detergents
405.22491	$[A_6B+K]^+$	$[C_3H_6O]_nH_2O$	Polypropylene glycol	Plastrics
405.26115	$[AB_4+Na]^+$	$[C_{14}H_{22}O]$ $[C_2H_4O]_n$	Triton	X-100, X-114, X-405, or X-45 Detergents
409.18344	$[A_8B+K]^+$	$[C_2H_4O]_nH_2O$	PEG	Polyethylene glycol
411.30810	$[AB_4+Na]^+$	$[C_{14}H_{28}O]$ $[C_2H_4O]_n$	Triton, reduced	X-100R, X-114R, X-405R, or X-45R Detergents
413.26623	$[M+Na]^+$	$C_{24}H_{38}O_4$	Diisooctyl phthalate	Plasticizer
429.24017	$[M+K]^+$	$C_{24}H_{38}O_4$	Diisooctyl phthalate	Plasticizer
454.29278	$[M+CH_3CN+Na]^+$	$C_{24}H_{38}O_4$	Diisooctyl phthalate	Plasticizer
610.18416	$[M+NH_4]^+$	$[C_2H_6SiO]_8$	Polysiloxane	Polysiloxane
667.17640	$[M+H]^+$	$[C_2H_6SiO]_9$	Polysiloxane	Polysiloxane
758.22175	$[M+NH_4]^+$	$[C_2H_6SiO]_{10}$	Polysiloxane	Polysiloxane
798.58785	$[M_2+NH_4]^+$	$C_{24}H_{38}O_4$	Diisooctyl phthalate	Plasticizer
803.54324	$[M_2+Na]^+$	$C_{24}H_{38}O_4$	Diisooctyl phthalate	Plasticizer
819.51718	$[M_2+K]^+$	$C_{24}H_{38}O_4$	Diisooctyl phthalate	Plasticizer

form the same *m/z* fragment ions from different precursor ions. If too short a scan time is used, fragment ions will not have emerged from the collision cell (Q2) before the next SRM fragmentation takes place, leading to 'ghost peaks' that can produce false positive results.

19.5.1 Qualitative analysis

19.5.1.1 Targeted analysis

Targeted screening for a series of drugs is possible using multiple SRMs in one analysis (Tang *et al.,* 2014). Such an approach can detect new analytes such as NPS by inserting identifying data as they appear in case work. As discussed for GC-MS, the combination of targeted screening followed by quantification is well established (Section 19.5.2.1).

19.5.1.2 Systematic toxicological analysis

For urine screening by LC-MS, a simple method has been described in which conjugates can be analyzed directly (Box 19.5). The extracts from this method can be applied to (i) urine screening by either LC-MS^n (Wissenbach *et al*., 2011a,b), or LC-HRMS/MS (Helfer *et al*., 2015), and (ii) targeted screening and quantification in plasma by LC-MS/MS (Remane *et al*., 2010a). Full details are provided in the respective papers.

Box 19.5 Sample preparation for LC-MS

- To urine (0.1 mL) add 0.5 mL acetonitrile **or** to plasma (0.5 mL) add 0.6 mL of butyl acetate:ethyl acetate (1+1, v/v)
- Rotary mix (2 min)
- Centrifuge (3 min, 10,000 *g*)
- Transfer 0.5 mL of the supernatant or extract to a glass vial
- Evaporate to dryness under a gentle stream of nitrogen (50 °C)
- Dissolve the residue in 0.05 mL of a mixture of eluents A (10 mmol L^{-1} aqueous ammonium formate + 0.1 % v/v formic acid, pH 3.4) and B (acetonitrile + 0.1 % v/v formic acid) (1+1 v/v)

Metabolite-based LC-MS^n urine drug screening using a linear ion trap and a reference library (Maurer *et al*., 2019) containing MS^2 and MS^3 spectra recorded in positive ESI mode and full scan information-dependent acquisition has been described (Wissenbach *et al*., 2011a; 2011b). This method has been transferred to a target screening system, Toxtyper (Kempf *et al*., 2014) to extend this to STA (Caspar *et al.,* 2019).

19.5.2 Quantitative analysis

19.5.2.1 Batch analysis

Multi-analyte LC-MS/MS for qualitative and quantitative analysis of over 130 antidepressant, antipsychotic, benzodiazepine, and hypnotic drugs in plasma after LLE (Box 19.4) has been

described (Remane *et al*., 2010a; 2011a; 2011b). After detailed ion suppression/ion enhancement studies APCI showed much smaller effects than ESI, although some analytes showed lower sensitivity (Remane *et al*., 2010b; 2010c).

19.5.2.2 Emergency toxicology

As a complement to GC-MS for emergency STA, a multi-analyte LC-APCI-MS/MS approach with a 5-min gradient for 45 drugs and active metabolites using the same LLE (Box 19.2) has been described (Michely & Maurer, 2018). The calibration ranges encompassed low therapeutic to large overdose concentrations. To shorten the turn-around time, one-point calibration was used. Matrix effects and ionization effects of co-eluting analytes were deemed acceptable, as were recoveries, precision, and selectivity. Thirty-two of 45 compounds fulfilled the criteria for measurement of the concentrations encountered in therapy, and 41 at overdose concentrations (±30 % SD). The reuse of the processed calibrator stored at 8 °C for up to 30 days was possible for 32 compounds only. In addition, analysis of authentic blood samples gave results comparable to those measured by fully validated TDM methods.

The Bruker Daltonik Toxtyper™ target screening system has been applied to the simultaneous (semi)quantification of 22 drugs and two active metabolites relevant to emergency toxicology in plasma (Caspar *et al.,* 2019). Samples were processed by LLE (Box 19.4) followed by a 1+4 dilution of the final extract. Plasma analyte concentrations were assessed using full-scan data and a stored five-point calibration graph. Assay calibration was linear over the ranges used and was stable for at least two months. The acceptance criteria recommended for emergency toxicology (GTFCh, 2018) were fulfilled for all compounds tested, except that bromazepam, lorazepam, oxycodone, and prothipendyl could only be measured reliably at concentrations above those attained in therapy. Similar results were obtained when using oral fluid (Plecko *et al.,* 2018).

19.6 Liquid chromatography-high resolution mass spectrometry

HRMS offers new opportunities in STA. Although HRMS can be used with GC (Pan *et al*., 2019), most published methods use LC-ESI in conjunction with either TOF, or OT mass analyzers (Maurer & Meyer, 2016; Meyer & Maurer, 2016; Maurer, 2018). Most instruments are hybrids with quadrupoles placed before the accurate mass analyzer (QTOF, Q Exactive) giving reproducible MS/MS spectra (Chapter 13). For the obligatory daily performance check, a methanolic solution of analytes covering a wide range of physicochemical properties and relevant retention times should be analyzed (Figure 19.10).

To ensure adequate instrument performance, all peaks in the test mixture must be resolved and of sufficient abundance to give clearly identifiable peaks. If these criteria are not fulfilled, the ion source should be cleaned and the instrument recalibrated. Furthermore, mass deviations should be monitored in positive ionization mode for metamfepramone, glibenclamide, and clarithromycin, and in negative ionization mode for pentobarbital and glibenclamide. All mass deviations (exact mass minus measured mass) should be lower than ±5 ppm for each precursor. If a deviation is greater, then the instrument should be recalibrated. In addition, performance and carry over within an analytical sequence should be monitored using control and ‘blank’ samples, respectively (Maurer *et al*., 2018).

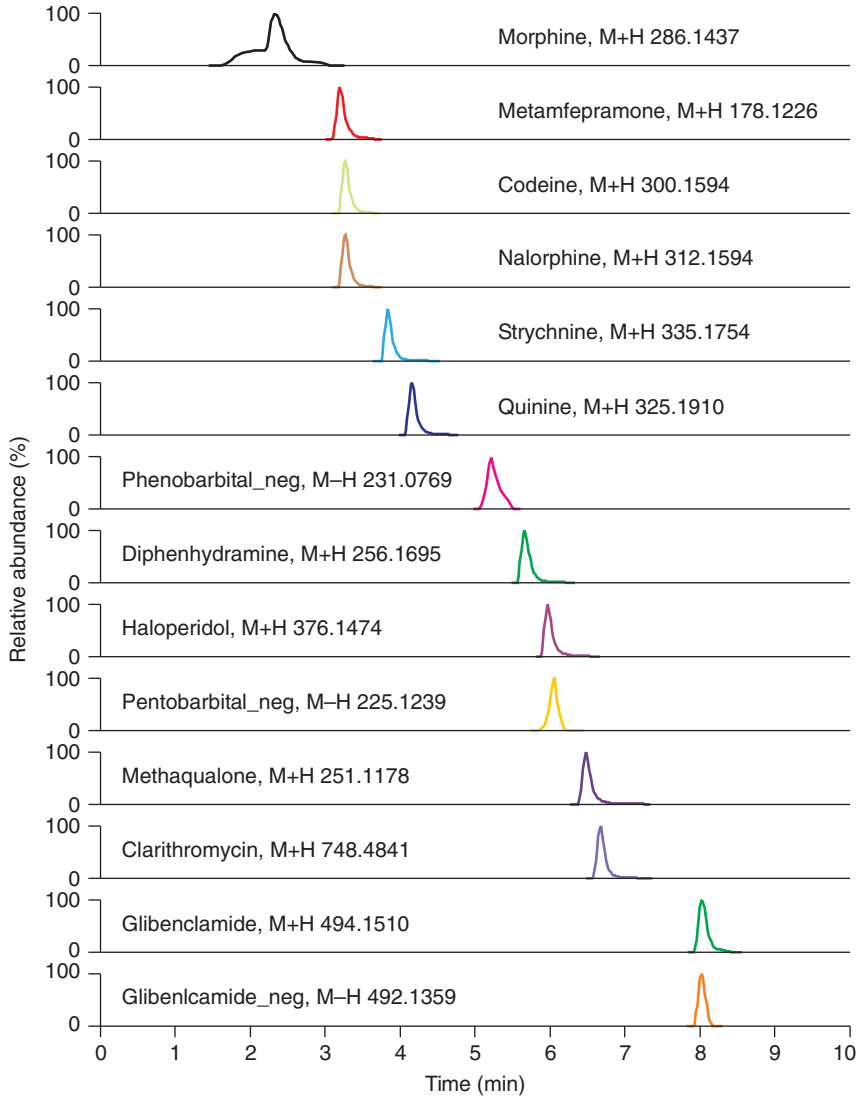

Figure 19.10 Extracted ion chromatograms of the precursors of test compounds (±5 ppm) (Maurer *et al.,* 2018). Injection: 10 µL of a solution containing phenobarbital and pentobarbital (500 µg L^{-1}), all other analytes (50 µg L^{-1}). Retention times (min): morphine (2.7), metamfepramone (3.2), codeine (3.3), nalorphine (3.4), strychnine (3.9), quinine (4.2), phenobarbital (5.3), diphenhydramine (5.7), haloperidol (5.9), pentobarbital (6.1), methaqualone (6.7), clarithromycin (6.8), and glibenclamide (8.0)

19.6.1 Qualitative analysis

Attempts have been made to develop non-targeted screening procedures using HRMS (Meyer & Maurer, 2016; Michely *et al*., 2018). However, most published methods are targeted procedures, but often have broader scope than low resolution MS methods using SRM.

19.6.1.1 Targeted analysis

LC-QTOF has been used to test urine for synthetic cannabinoid metabolites (Kronstrand *et al*., 2014). Primary MS identification was based on retention time, accurate mass measurement, and isotopic pattern. This was followed by a secondary MS/MS identification in which the spectrum obtained was matched with accurate mass CID spectra from an in-house library. Such an approach yields a flexible method that can be adjusted to measure NPS.

19.6.1.2 Systematic toxicological analysis

HRMS can be used for general screening by measuring the accurate (protonated or deprotonated) molar masses of the compounds detected. For analyte identification, the measured masses are compared with candidate lists according to increasing exact mass (Ojanperä *et al*., 2006). However, if more than one compound has the same exact mass false positives may occur. For example, the elemental compositions of morphine and of the pepper ingredient chavicine are identical, but their structures are quite different thus giving different ions when fragmented. In such cases, analyte fragmentation by either GC-EI-MS, or LC-MS with either in-source fragmentation (when using single-stage apparatus), or fragmentation in collision cells (when using conventional MS/MS, QTOF, or Q-Orbitrap equipment) can be used to facilitate compound identification (Table 19.2). However, morphine and norcodeine cannot be differentiated by fragmentation and thus must be differentiated by retention time.

To form fragments, in-source CID together with a reverse target database search has been developed and evaluated as an addendum to the LC-TOF screening method mentioned above (de Castro *et al*., 2012). Peak identification relies on protonated molecular accurate mass measurement, isotopic pattern fit, and retention time, as well as one to three qualifier ions for each analyte.

For LC-OT-HRMS/MS urine screening, the efficiency of (i) treatment with acetonitrile (Box 19.4), (ii) on-line extraction by turbulent flow chromatography, and (iii) 'dilute-and-shoot' has been compared (Helfer *et al.,* 2017). The total time taken for (i) acetonitrile treatment and extract evaporation was 10 min, (ii) for on-line extraction (plus time for initial urine centrifugation) was 6 min, and (iii) for the dilution approach 3 min. The LoDs for on-line extraction were comparable with those found after acetonitrile pre-treatment, but lower than for 'dilute-and-shoot'.

All three approaches were adequate for comprehensive urine screening. Analytes were separated within 10 min and detected using a Q Exactive MS in full scan mode with positive/negative ion switching, and subsequent DDA. Identification criteria were the presence of accurate precursor ion signal, isotope patterns, the five most intense fragment ions, and comparison with full HRMS/MS library spectra (Maurer *et al*., 2018).

The combined use of LC-QTOF-MS and LC-DAD in STA has been explored (Broecker *et al*., 2011). Separate portions of post-mortem blood samples were either extracted with dichloromethane, or treated with acetonitrile (1+4, v/v). The dried extracts were reconstituted in

Table 19.2 Some isobaric compounds and differentiating CID fragment ions (collision energy spread 35 V ±50 %) in a Q Exactive (Orbitrap) (Maurer *et al*., 2018–reproduced with permission of Wiley-VCH)

Analyte	Formula	$[M+H]^+$	Distinguishing Fragment	*m/z*
Metamfetamine	$C_{10}H_{15}N$	150.1277	C_9H_{11}	119.0855
Phentermine			$C_{10}H_{13}$	133.1012
Pseudoephedrine	$C_{10}H_{15}NO$	166.1226	$C_{10}H_{14}N$	148.1121
p-Methoxyamphetamine			$C_{10}H_{13}O$	149.0961
Oxcarbazepine	$C_{15}H_{12}N_2O_2$	253.0972	$C_{15}H_{10}NO_2$	236.0706
Phenytoin			$C_{14}H_{13}N_2O$	225.1028
Atomoxetine	$C_{17}H_{21}NO$	256.1696	$C_{10}H_{14}N$	148.1121
Diphenhydramine			$C_{13}H_{11}$	167.0855
Tramadol	$C_{16}H_{25}NO_2$	264.1958	C_8H_9O	121.0653
O-Desmethylvenlafaxine			C_7H_7O	107.0497
Amitriptyline	$C_{20}H_{23}N$	278.1903	$C_{15}H_{11}$	191.0855
EDDP			$C_{18}H_{19}N$	249.1512
Morphine	$C_{17}H_{19}NO_3$	286.1438	$C_{14}H_{13}O_3$	229.0865
Norcodeine			$C_{14}H_{13}O_3$	229.0865
Hydromorphone			$C_{14}H_{11}O_3$	227.07082
Chavicine			$C_{12}H_9O_3$	201.05517
Codeine	$C_{18}H_{21}NO_3$	300.1594	$C_{18}H_{20}NO_2$	282.1489
Hydrocodone			$C_{16}H_{17}O_3$	257.1172
Cocaine	$C_{17}H_{21}NO_4$	304.1543	$C_{10}H_{16}NO_2$	182.1176
Hyoscine (scopolamine)			$C_8H_{12}NO$	138.0913
Biperiden	$C_{21}H_{29}NO$	312.2322	$C_6H_{12}N$	98.0964
UR-144			$C_{14}H_{16}NO$	214.1226
Naloxone	$C_{19}H_{21}NO_4$	328.1543	$C_{19}H_{20}NO_3$	310.1438
6-AM			$C_{17}H_{18}NO_2$	268.1332
JWH-019	$C_{25}H_{25}NO$	356.2009	$C_{15}H_{18}NO$	228.1383
JWH-122			$C_{11}H_9$	141.0699

UR-144 = (1-pentylindol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone
JWH-019 = (1-hexylindol-3-yl)(naphthalen-1-yl)methanone
JWH-122 = (4-methyl-1-naphthalenyl)(1-pentyl-1H-indol-3-yl)-methanone

acetonitrile:0.1 % v/v aqueous formic acid (35+65) and portions of each extract were analyzed simultaneously on separate LC systems using the same autosampler. The ESI-QTOF-MS instrument was operated in data-dependent acquisition mode with switching between MS and MS/MS (cycle time 1.1 s). The full mass spectra and the CID fragment spectra of all essential $[M+H]^+$ ions were recorded. Libraries of accurate mass CID spectra (some 2500 substances) and of DAD-UV spectra (some 3300 substances) were used for compound identification. The sample preparation procedures proved to be suitable for both techniques and for a wide variety of substances of different polarity. The DAD data provided semi-quantitative estimation of the concentrations the drugs detected and identified.

19.6.2 Quantitative analysis

The use of HRMS gives selectivity at least as good as that of SIM in many cases and is simpler because analyte transitions do not have to be investigated. The assay of the non-vitamin K oral anticoagulants (NOACs) apixaban, dabigatran, edoxaban, and rivaroxaban in plasma gives an example of the use of LC-HRMS/MS for quantitative work (Box 19.6). The total analysis time was 6 min.

Box 19.6 Assay of apixaban, dabigatran, edoxaban, and rivaroxaban in plasma

- Samples, calibrators, and IQCs (100 µL) mixed with ISTD solution (50 µg L^{-1} both $[^{13}C_6]$-dabigatran and $[^{13}C_6]$-rivaroxaban in acetonitrile) (200 µL)
- After centrifugation (16,400 *g*, 4 min), supernatant (100 µL) injected onto a Cyclone-C18-P-XL Turboflow column
- Analytes focused onto an Accucore PhenylHexyl (2.1 × 100 mm, 2.6 µm aps) analytical column
- Elution: methanol + acetonitrile (1+1):aqueous ammonium acetate (10 mmol L^{-1}) gradient
- Data acquired using OT-HRMS in full-scan mode with DDA

Quantitation was based on extracted accurate-mass data [5 ppm window, external mass calibration (alternate days) using positive/negative ion calibration infusion solutions (Thermo Fisher Scientific)]. Calibration was linear (1–500 µg L^{-1} all analytes). Intra-assay precision (RSD) at 1 µg L^{-1} was 2.6, 4.2, 17.3, and 9.5 % for apixaban, dabigatran, edoxaban, and rivaroxaban, respectively. Mean recovery was 96–101 %. No ion suppression or enhancement was observed. The method could be used to identify a particular NOAC if information on the drug taken was lacking. The acquisition of full scan data allowed the retrospective identification of dabigatran metabolites (Figure 19.11).

19.7 Summary

STA consists of qualitative and quantitative analyses, often performed using MS linked to either GC, or LC together with other techniques as appropriate. For qualitative analysis using MS, either targeted screening using selected fragment ions formed by single or multiple stage MS, or comprehensive unknown full-scan approaches, which may be less sensitive, are used. Reference libraries and appropriate software aid compound identification.

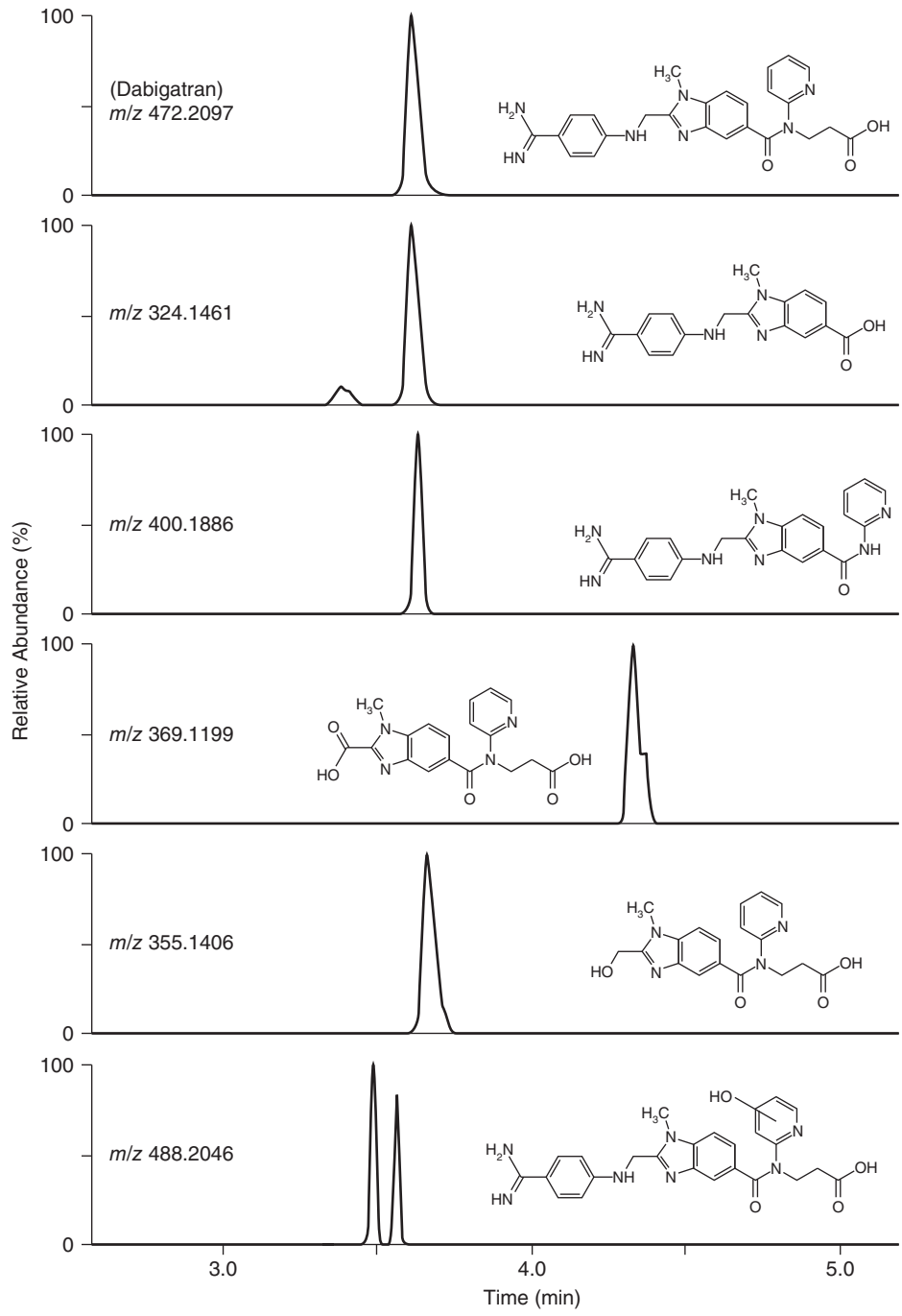

Figure 19.11 Identification of dabigatran metabolites in a sample from a 66-yr-old man prescribed 220 mg d^{-1} dabigatran etexilate (plasma dabigatran concentration 218 μg L^{-1}). Metabolite *m/z* values and structural formulae are shown on the chromatograms filtered by *m/z* (5 ppm window) from full-scan data (Gous *et al*., 2014–reproduced with permission from Wolters Kluwer Health, Inc)

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20 Therapeutic Drug Monitoring

20.1 Introduction

Measuring the concentrations of drugs given in therapy in an appropriate sample can be useful for compounds for which pharmacological effects cannot be assessed easily and for which the margin between adequate dosage and potentially toxic dosage is small. However, assays for a much wider range of compounds, and sometimes metabolites, may be requested to assess either adherence (compliance, concordance) to therapy, or to investigate and, if possible, prevent adverse effects of treatment (Box 20.1). For some drugs, the effects can be monitored directly (Box 20.2). However, drug assays may still be requested, for example for patients in whom antihypertensive therapy appears refractory to treatment and adherence is questioned (Aonuma *et al*., 2017).

Box 20.1 Indications for therapeutic drug monitoring

- Assess adherence
- Optimize dosage (maximize likelihood of therapeutic benefit)
- Minimize risk of dose-related adverse effects
- Investigate possible adverse effects or drug interactions
- Plan recommencement of chronic therapy after overdose, for example

Box 20.2 Biological effect monitoring

- Blood glucose – antidiabetic drugs (insulin, chlorpropamide)
- Blood lipids – hypolipidaemic agents
- Blood pressure – antihypertensive drugs
- Electrocardiogram – antiarrhythmic drugs
- Prothrombin time (International Normalized Ratio, INR) – warfarin
- Thyroid function tests - thyroxine

This chapter gives basic information to help provide a TDM service. Important points to consider are: (i) the time of sampling, (ii) the sampling site, (iii) the sample matrix, and (iv) the analytical method, including whether the analysis can be completed in a clinically relevant time

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scale. Knowledge of drug metabolism and PK is fundamental to advising on sample collection and in the interpretation of results. Knowledge of laboratory operations (quality management) is also important. TDM has much in common with assays of drugs and metabolites in clinical samples performed during drug development and evaluation (clinical trials). Hence, whatever method is used, assay validation and implementation criteria must meet FDA/CLSI standards (Section 3.1).

It is important to bear in mind the purpose for which the analysis has been requested when reporting and interpreting analytical results. Usually a range of concentrations that is associated with optimal therapeutic benefit and minimal risk of toxicity will have been defined (McCudden, 2018). Such a range may be referred to as a 'therapeutic range', 'reference range', 'target range', or 'therapeutic window' (Section 16.4.2). As an example, when lithium carbonate is used to treat bipolar disorder, serum lithium ion concentrations should be adjusted to ensure serum concentrations in a 12-hour post-dose sample fall in the range 0.6–1.0 mmol L^{-1} (Section 20.6.9.1). This is a target range and values outside of this range normally result in either therapeutic failure, or unacceptable toxicity. Note that some clinical laboratories set their own target ranges based on a combination of literature values and local experience.

With many drugs, the range of plasma concentrations associated with optimal therapeutic benefit is much less clearly defined, and interpretation of results has to be made in context. If a result is below the 'normally expected' range of plasma concentrations for a given dose, yet clinical benefit seems optimal there is sometimes no reason to alter the dose. However, where clinical benefit is difficult to assess, with anticonvulsants or antipsychotics, for example, there may be indications for adjusting dosage, clinical observation notwithstanding. There may also be indications for reducing dosage in the absence of clinically apparent features of toxicity. The classic examples are digoxin, where the features of toxicity may be confused with those of the disease (heart failure) being treated, and aminoglycoside antibiotics and some immunosuppressant drugs where toxicity may not be apparent until severe, possibly irreversible, tissue damage has occurred.

20.2 Sample collection

A standard TDM guideline is to sample blood from a vein remote from an infusion or injection site(s), either immediately before the next oral dose, or the in the morning after an evening dose (i.e. a trough sample). This allows time for absorption and distribution to tissues to be completed before sampling. Use of a trough sample alleviates the problem of identifying the time of the peak concentration and provides a reproducible reference time. Note that if poisoning is suspected, keeping to a specific sampling time post-dose is irrelevant because speed of diagnosis and treatment take priority.

With lithium and with digoxin at least six hours must be left between dosage and sampling. Peak blood lithium concentrations are normally reached 2–4 h after an oral dose, but equilibration of lithium across the blood–brain barrier is slow, and thus 6–12 h should be allowed between dosage and sampling to ensure that the serum lithium reflects the lithium concentration near to the site of action of the drug. (N.B. Serum lithium is usually measured to prevent interference from lithium in lithium heparin blood collection tubes.) Concentrations in samples collected 12 h post-dose are referred to as 'standardized 12 h' concentrations and represent trough concentrations if dosing is twice daily.

For some drugs, *AUC* may be a better indicator of 'exposure' to the drug and hence efficacy. However, measuring *AUC* within the dosage interval requires a number of samples to be

collected (Section 16.8). With ciclosporin, for example, using three samples collected at 0 (i.e. pre-dose), 1, and 3 hours post dose allowed accurate prediction of *AUC* in haematopoietic allogeneic stem cell transplant patents (Wilhelm *et al*., 2012).

An additional refinement for TDM of antimicrobials is the inclusion of minimum inhibitory concentration (MIC) for the pathogen under consideration. Thus, C_{max}/MIC, *AUC*/MIC or the time that the plasma concentration is above MIC may be used as criteria for accessing optimal treatment (Figure 20.1). *AUC*/MIC has been used for vancomycin, linezolid, and gentamicin. Peak/MIC has also been used for aminoglycosides, including gentamicin, whilst the time the concentration is above MIC has been used for β-lactam antibiotics (Eyler & Mueller, 2011).

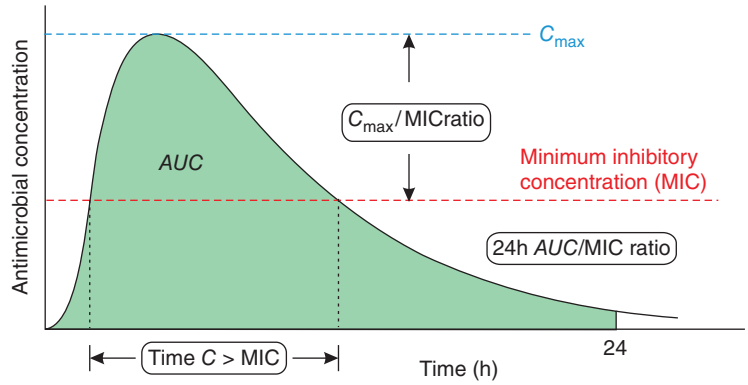

Figure 20.1 Schematic representation of parameters that have been used for TDM of antimicrobials in plasma

20.3 Sample types

20.3.1 Blood and blood fractions

Provided that the samples have been collected and stored correctly, there are usually no significant differences between the drug concentrations in plasma and serum (Handley *et al*., 2018). Serum has the advantage that if samples are to be frozen there is less precipitate (of fibrin) on thawing. Nevertheless, collection of plasma is convenient, and a heparinized or EDTA whole blood sample will give either whole blood, or plasma as appropriate. Blood collection tubes that contain a barrier gel should be used with caution, especially if basic drugs such as antidepressants or benzodiazepines are to be measured. Rapid decreases in the concentrations of posaconazole, sertraline, and citalopram when stored in gel-based tubes have been reported. Molecular descriptors such as log *P*, polar surface area, and protein binding may be good predictive markers of such problems (Steuer *et al*., 2016).

If a compound is not present to any extent within erythrocytes, use of lysed whole blood will result in an approximately two-fold dilution of the specimen. The immunosuppressants ciclosporin, sirolimus, and tacrolimus are special cases because redistribution between plasma and erythrocytes begins once the sample has been collected and so the use of haemolyzed whole blood is indicated for the measurement of these compounds (Section 20.6.8). Capillary blood, rather than venous whole blood, may be used if sample size is not a problem. However, care should be taken not to squeeze the site of collection to encourage the flow of blood because this may result in dilution of the sample with ECF.

20.3.1.1 Dried blood spots

The use of DBS, usually collected from a 'finger-prick' sample onto a purpose-designed card (Section 2.2.3.6), may present an alternative to conventional sampling for immunosuppressant and also for antiepileptic and antitubercular drug measurements (Vu *et al*., 2013; Hinchliffe *et al*., 2014; Zuur *et al*., 2016a; Velghe *et al*., 2019). DBS may be especially valuable where refrigerated transport and storage of samples is problematic and may reduce the total volume of blood required when taking serial samples. Analysis may also be simplified by use of surface analysis MS (for example DESI, paper spray, DART, MALDI, or LDTD-APCI). Using paper spray MS, a sample preparation and analysis time of approximately 30 s has been reported (Manicke *et al*., 2011).

Given these considerations, DBS have been widely advocated especially within the pharmaceutical industry. However, there are many problems in relating the results obtained to those obtained from plasma in addition to the obvious fact that finger-prick capillary blood is not plasma. Collection directly onto DBS cards means that taking an accurate volume of liquid blood for analysis is not possible. Moreover, analyte distribution on the collection card may be affected by chromatography of the analyte on the card during collection, the viscosity of the sample, which is related to haematocrit, which affects the amount of blood collected within a given area of the card, and so on (Klak *et al*., 2019). Analyte stability on the DBS card is a further consideration (Wagner *et al*., 2016). Guidelines for DBS method development and validation have been published (Capiau *et al*., 2019).

20.3.1.2 Volumetric microsampling devices

With these devices a fixed volume of capillary blood (10 or 20 µL) is taken and either used directly, or a fixed volume of plasma is separated with the aim of overcoming the area bias and homogeneity issues associated with DBS (Section 2.2.3.7). These devices simplify sample logistics management, and may allow sample collection in a patient's home, but of course come at a cost and are in reality aimed at use with sensitive analytical techniques such as either LC-MS/MS or LC-HRMS (Qu *et al*., 2017; Zwart *et al*., 2018).

20.3.2 Urine

TDM is normally aimed at providing quantitative as well as qualitative information, hence there has been little demand for urine testing simply to assess adherence. Moreover, urine testing may not be as simple as it sounds because other drugs or metabolites may interfere, or sensitivity may be less than with plasma in the case of drugs that are lipophilic and/or extensively metabolized prior to excretion. This being said, non-adherence to antihypertensive and related medication is a serious issue, with non-adherence rates of 20–30 % being revealed by LC-MS/MS of urine (Patel *et al.,* 2019; Richter *et al*., 2019; Wallbach *et al*., 2019). Similarly, when monitoring drug usage in patients under treatment with methadone or buprenorphine (opiate maintenance or withdrawal therapy), qualitative information usually suffices, and urine is the specimen of choice for several reasons. Firstly, the plasma methadone concentrations associated with clinical effect vary widely depending on the patients tolerance to opioids and hence there is no 'therapeutic range' as such, secondly urine is easier to collect than blood from patients many of whom have damaged veins, thirdly urine is less likely to be infective than blood, and finally it is important to monitor illicit drug use in these patients at the same time as monitoring adherence to either methadone, or buprenorphine (Chapter 18).

20.3.3 Oral fluid

Oral fluid consists principally of saliva, an ultrafiltrate of plasma, together with certain digestive enzymes and other components. There has been interest in the collection of oral fluid for TDM purposes because collection is non-invasive and salivary analyte concentrations are said to reflect non-protein bound ('free') plasma concentrations. However, reliable oral fluid collection requires a co-operative individual and even then it is not without problems. Oral fluid is viscous and thus is difficult to pipette. Some drugs, medical conditions, or anxiety, for example, can inhibit saliva secretion and so the specimen may not be available from all individuals at all times. Use of acidic solutions such as dilute citric acid to stimulate salivary flow alters saliva pH and thus is likely to alter the secretion rate of ionizable compounds.

Oral fluid is suitable for monitoring some antiepileptic drugs (Patsalos & Berry, 2013) and its use for neonates and infants for some 13 drugs has been reviewed (Hutchinson *et al*., 2018). Good correlations (r^2 >0.8) between plasma and saliva were found for neutral and weakly acidic drugs. However, for basic compounds such as amisulpride, and clozapine and norclozapine, oral fluid concentrations show no clear relation to plasma concentrations (Fisher *et al*., 2017; Neumann *et al*., 2018).

20.3.4 Keratinaceous samples

Analysis of keratinaceous sample (hair and nail) has been advocated to give a record of drug use, but the analytical procedures are complex and therefore expensive. The analyses are normally only used in a forensic context when it is though important to attempt to establish prior drug exposure (Section 22.3.2).

20.3.5 Other alternative matrices

Other samples that have been suggested for TDM purposes include tears, sweat, and interstitial fluid (ISF). The use of tears has the potential advantage that analyte concentrations may reflect those of the non-bound drug. However, collecting enough sample, particularly for assay of highly protein bound drugs, is problematic. Sweat is collected normally in specially designed patches worn on the skin and the sample would be collected over a period of time. In practice, sweat patches are only used for monitoring substance misuse (Section 18.7). Use of micro-needles to collect interstitial fluid (ISF), despite claims that the procedure is pain free, may not be widely accepted unless sampling can be directly coupled to the analysis (Kiang *et al*., 2017). However, at equilibrium, the analyte concentrations should be identical to the non-bound concentrations in plasma for those drugs (M_r <5000) that freely diffuse into ISF.

20.4 Analytical methods

In many instances, TDM is performed whilst the patient is visiting the clinic and the results are required in time for the clinician to make a decision as to whether drug dosage needs to be adjusted before the patient leaves. Therefore, it must be possible to perform the assay in the available time. POCT assays are rarely used for this purpose (Table 17.2), but immunoassays if available on random-access clinical chemistry analyzers may be ideal (Table 20.1). Many manufacturers offer similar panels.

As with all immunoassays there is the risk of interference and assay failure. Interference in a number of CEDIA TDM assays by a range of drugs has been reported, for example (Table 20.2).

Table 20.1 Some marketed EMIT TDM assays

Antiasthmatic	Theophylline, caffeine
Antidepressant (group specific)	Amitriptyline, nortriptyline, imipramine, desipramine
Antiepileptic	Phenytoin, phenobarbital, primidone, ethosuximide, carbamazepine, valproate
Antimicrobial	Amikacin, gentamicin, tobramycin, chloramphenicol
Antineoplastic	Methotrexate
Cardioactive	Digoxin, lidocaine, procainamide/NAPA, quinidine, disopyramide
Immunosuppressive	Ciclosporin, mycophenolic acid, tacrolimus

Table 20.2 Drug Interference in CEDIA TDM assays (Sonntag & Scholer, 2001–reproduced with permission of Sage Journals)

Analyte	Interfering compounds
Carbamazepine	Doxycycline, levodopa, methyldopa, metronidazole
Digitoxin	Rifampicin
Phenytoin	Doxycycline, ibuprofen, metronidazole, theophylline
Theophylline	Cefoxitin, doxycycline, levodopa, paracetamol, phenylbutazone, rifampicin
Tobramycin	Cefoxitin, doxycycline, levodopa, rifampicin, phenylbutazone
Valproate	Phenylbutazone

The Syva EMIT antidepressant assay was reported to cross-react with phenothiazines after overdosage, and quetiapine has been shown to give positive results in not only the EMIT tricyclic assay, but also with other immunoassays.

Chromatographic methods have formed the backbone of specialized TDM services for many years. MS detection is widely used (Adaway & Keevil, 2012; Maurer, 2018). LC-MS/MS is being used increasingly for TDM, particularly when it offers a fast turn-around and minimal sample preparation, and the ability to measure a number of different drugs and metabolites at the same time (Zuur *et al*., 2016b; Couchman *et al*., 2018). Direct on-line injection methods may reduce sample preparation time, improve reproducibility, and minimize sample manipulation and the potential for sample contamination.

Automated methods for LC include restricted access materials, turbulent flow chromatography (Couchman *et al*., 2012a, 2012b; Gous *et al*., 2014), on-line SPE methods such as MIPS, in tube SPME, and packed syringe microextraction for extraction of specific analytes from complex mixtures. Isotopic internal calibration, provided that appropriate isotopically labelled ISTDs are available, allows individual sample and IQC analysis by LC-MS/MS without the requirement for batch analysis, thus giving flexibility akin to random access clinical analyzers (Section 13.10.3). Automated sample preparation followed by FIA-MS/MS has also been described (Couchman *et al*., 2016).

20.5 Factors affecting interpretation of results

Detailed knowledge not only of the limitations of the analytical method(s) used, but also of the clinical pharmacology, toxicology, and PK of the compound(s) monitored is often important when interpreting analytical toxicology results in general. Patients may respond differently to a given dose of a particular drug, especially as regards behavioural effects. Further complicating factors may include the role of pharmacologically active or toxic metabolites, age, sex, smoking habit, and concomitant drug therapy (Table 20.3). The use of TDM in neonates presents special problems (Pauwels & Allegaert, 2016).

Table 20.3 Some factors that may affect interpretation of analytical toxicology results

Factor	Comment
Acidosis/alkalosis	Influences *V* of water soluble, ionizable analyte if pK_a is within two pH units of blood pH
Age	The very young and the elderly have lower metabolic capacity; the elderly tend to have lower hepatic blood flow, *V* and renal function; children have lower *V*
Burns	State of hydration; metabolic response to injury
Concomitant drug therapy (includes over-the-counter and alternative medicines)	Long term and recent: effect on absorption, protein binding, distribution, and/or clearance, contribution to overall pharmacological effects
Disease	Liver or renal disease may reduce metabolic capacity, plasma protein binding may be altered; decreased renal clearance affects drugs excreted in urine
Duration/intensity of exposure	Possible effect on tolerance, body burden, induction or inhibition of metabolism
Ethanol	Short- and long-term effects on clearance, potentiation of effect, etc.
Formulation	Sustained release, racemate, etc. If pesticide or antibiotic, vehicle may be more toxic than active ingredient
Genetics/hypersusceptibility	Acetylator status, cytochrome P450 polymorphisms, etc.
Haemolysis	Altered plasma:erythrocyte distribution
Hobbies	Access/exposure to unusual poisons
Infection/fever	Increased cellular permeability, changes in clearance mechanisms, increased plasma protein binding of basic drugs due to release of AAG into plasma
Mental state	Extremes of excitement or exhaustion may alter response to stimulant or depressant dugs
More than one drug prescribed	Potentiation/antagonism of effect(s), altered disposition/clearance
Nutrition	Possible change in plasma protein binding and/or disposition

Table 20.3 (*Continued*)

Factor	Comment
Occupation	Access/exposure to poisons in the workplace
Pregnancy	May alter drug disposition
Route of exposure	Many compounds exhibit higher acute toxicity if given either i.v. or by inhalation rather than orally
Sex	Males have greater body mass, but lower proportion of fat than females: affects distribution of some xenobiotics, notably ethanol
Shock	Absorption, distribution, metabolism, excretion of drugs may be reduced
Site of sampling	Especially important if patient undergoing an infusion, and in post-mortem work
Surgery/trauma	Absorption of orally administered drugs may be reduced; state of hydration; metabolic response to injury
Time of sampling relative to exposure and/or death	The longer the time between exposure and sampling (i) the more difficult poison detection (except perhaps with hair/nail) in clinical samples, (ii) the greater the potential for change if samples collected at the post-mortem examination
Treatment	May get displacement from binding sites including receptors; F_{ab} antibody fragments and chelating agents will increase the total plasma concentration even though they decrease the unbound concentration
Tolerance/smoking habit	Previous exposure may have produced pharmacological tolerance or cross-tolerance; induction of metabolism

20.6 Gazetteer

This section aims to give summary information on the main groups of compounds where TDM may play a part in patient management. Pre-dose sampling is assumed except where indicated.

20.6.1 Antiasthmatics

Normally, there is no indication for TDM of bronchodilators such as salbutamol (albuterol) given i.v., orally, or by inhalation. Clinical effect is usually assessed easily, and compounds of this nature are relatively non-toxic even in overdose. Plasma theophylline however, is monitored with the aim of reducing adverse effects when used as a bronchodilator (Table 20.4). Theophylline dose requirement is markedly affected by smoking habit. There is now evidence that theophylline has significant anti-inflammatory effects in chronic obstructive pulmonary disease at low concentrations. Theophylline can be methylated to caffeine by neonates, but not by young children or adults (Figure 20.2). Caffeine especially is used to treat neonatal apnoea (Gal, 2007).

Table 20.4 Antiasthmatic drug TDM assays

Drug	Reference range (mg L^{-1} plasma)
Caffeine	10–30 (neonatal apnoea)
Theophylline	8–20 (adults), 6–12 (neonatal apnoea)

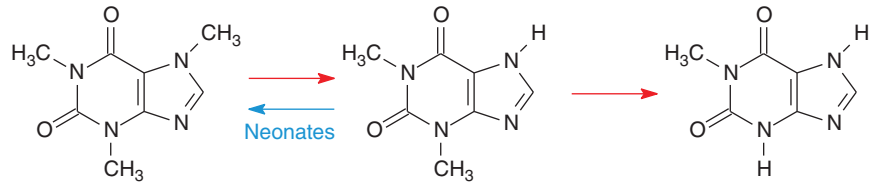

Figure 20.2 Metabolic pathways of methylxanthines

20.6.2 Anticoagulants

Anticoagulant therapy with vitamin K antagonists (VKAs), notably warfarin, and the effect of giving vitamin K to reverse anticoagulation, can be monitored easily by measuring the INR (Box 20.2). Non-vitamin K antagonist oral anticoagulants [NOACs, also known as direct-acting oral anticoagulants (DOACs); Table 20.5], in contrast, selectively inhibit coagulation serine proteases (Xa or IIa). They have a rapid onset of action, a relatively predictable pharmacokinetic profile, and relatively short $t_{½}$, making initiation, maintenance, and discontinuation of anticoagulant therapy considerably easier than with VKAs. However, reversal of anticoagulation is not as simple as with VKAs and the INR does not give an accurate indication of coagulation status. Whilst functional assays, for example, anti-factor Xa (AFXa) and direct thrombin inhibition (DTI), may be available to give a rapid assessment of anticoagulation status (Dias *et al.*, 2019), TDM of DOACs may also be useful (Gous *et al.*, 2014; Wiesen *et al.*, 2017; Lindahl *et al.*, 2018; Kuhn *et al.*, 2018).

Table 20.5 Non-vitamin K antagonist anticoagulant TDM assays

Drug	Reference range (μg L^{-1} plasma)
Apixaban	<300
Dabigatran (from dabigatran etexilate)	<200
Rivaroxaban	<240

Indications for NOAC TDM may include (i) after overdose (accidental or otherwise), (ii) in the elderly, especially those with comorbidities, (iii) when patients switch from an existing oral anticoagulant, (iv) in those at the extremes of body weight, (v) in patients with hepatic or renal impairment, (vi) in patients co-prescribed other medications where there is a risk of

drug–NOAC interactions such as with antiepileptic drugs, (vii) to assess adherence, and (viii) to assess anticoagulant activity before major surgery. Practical issues may be the use of citrate anticoagulant, which dilutes the sample, and the instability of edoxaban *in vitro*.

20.6.3 Antiepileptic drugs

TDM of antiepileptic (anticonvulsant) drugs is well established, in part because of its importance in phenytoin dose adjustment, and in part because combinations of drugs continue to be used in patients with epilepsy who do not respond to a single drug. This raises the possibility of metabolic drug–drug interactions. Carbamazepine, for example, induces the metabolism of some other drugs as well as its own metabolism (Patsalos *et al*., 2018) resulting in an up to three-fold increase in its rate of elimination, hence the time to steady-state may be 3 weeks or so ($t_{½}$ in adults: single dose 18–65 h, chronic dosing 5–25 h).

In newly diagnosed patients there is no clear evidence to support the use of TDM with the aim of reaching predefined target ranges in dose optimization with anticonvulsant monotherapy, although this does not reflect clinical practice with phenytoin especially. The relationship between plasma concentration and seizure control may not be well defined for certain drugs (for example valproate, vigabatrin), but nevertheless in general the risk of toxicity increases at higher doses/plasma concentrations (Table 20.6). Measurement of non-protein bound concentrations of phenytoin, carbamazepine, and valproate in certain situations may be helpful clinically. Oral fluid may be used (Patsalos & Berry, 2013).

Gabapentin, levetiracetam, pregabalin, topiramate, and vigabatrin are eliminated largely unchanged in urine hence plasma concentrations may be affected by alterations in renal function. Gabapentin shows dose-dependent bioavailability. Concomitant use of enzyme-inducing drugs can affect topiramate concentrations markedly. For the newer drugs that are largely metabolized prior to elimination (felbamate, lamotrigine, oxcarbazepine, tiagabine, and zonisamide), inter-patient variability in pharmacokinetics is just as important in dose adjustment as for many older antiepileptics (Iapadre *et al*., 2018; Patsalos *et al*., 2018)

Whilst developed for seizure control, many antiepileptic drugs have additional uses and in such cases the reference ranges established for use in epilepsy control may not apply. Carbamazepine, lamotrigine, and valproate are used in bipolar disorder, for example, and valproate is also used in acute mania when the plasma concentrations associated with efficacy may be somewhat higher than when the drug is used as an anticonvulsant (Taylor *et al*., 2018).

20.6.4 Anti-infectives

20.6.4.1 Antibiotics

TDM of aminoglycoside antibiotics such as gentamicin and tobramycin has been well established for many years. Interpretation is best provided in conjunction with microbiology laboratories. Peak (2 hours post-dose) concentrations of isoniazid, rifampicin, pyrazinamide, and ethambutol give information as regards effective oral dosage, but an additional sample at 6 hours may help differentiate between (i) delayed absorption, and (ii) generally poor absorption resulting in ineffective treatment, as well as giving further information such as an indication of $t_{½}$ (Alsultan & Peloquin, 2014; Balakrishnan & Shorten, 2016). The use of LC-MS/MS in the TDM of antitubercular drugs has been reviewed (Kuhlin *et al*., 2019). Across a wide variety of studies, a high proportion of patients undergoing first-line anti-tuberculosis treatment had 2 h drug concentrations below the accepted normal threshold (Mota *et al.,* 2016).

Table 20.6 Summary of antiepileptic TDM

Drug [metabolite]	Reference range (mg L^{-1} plasma)
Acetazolamide	2–12
Brivaracetam	0.2–2
Carbamazepine	1.5–12.0 (>7 bipolar disorder)
[Carbamazepine–10,11–epoxide]	[0.5–2.5][a]
Clobazam	<0.2 (clobazam), <2 (norclobazam)
Clonazepam	0.025–0.085 (children, may be lower in adults)[b]
Ethosuximide	40–100
Felbamate	20–110
Gabapentin	2.0–20
Lacosamide	10–20
Lamotrigine	1.0–15
Levetiracetam	<20
Methsuximide	10–40 (as normethsuximide)
Nitrazepam	0.05–0.15[b]
Oxcarbazepine	15–35 [as 10-hydroxycarbamazepine (licarbazepine)]
Perampanel	0.18–0.98
Phenobarbital	5–30 (15–30 neonatal seizures)
Phenytoin	7–20 (lower limit may be 5 or less)
Pregabalin	2–8
Primidone	<13 (also measure phenobarbital)
Rufinamide	30–40
Stiripentol	4–22
Sultiame	2.0–12
Topiramate	5–20
Tiagabine	<0.4 (achiral method)
Vigabatrin	5–35
Valproate	50–100 (anticonvulsant and in bipolar disorder), 55–125 (mania)
Zonisamide	15–40

[a]Concentrations normally 5–15 % of parent drug (10–30 % in patients co-prescribed valproate)
[b]Especial care needed in sample collection as degraded by light

Prompt, effective treatment minimizes the risk of developing drug resistance (Zander *et al*., 2019). Antimicrobial efficacy is dependent on both the concentration of drug in relation to the MIC and the time that this exposure is maintained (Figure 20.1). When the effect of concentration predominates over that of time, effective therapy is predicted from C_{max}/MIC. For the aminoglycosides the recommended ratio is 10–12, or *AUC*/MIC = 150 (Kuti, 2016).

β-Lactam antibiotics are classed as 'time dependent' and the criterion is the time the (non-bound) concentration is greater than the MIC. This is usually expressed as a percentage of the dosage interval. The target values vary between the carbapenems, penicillins, and cephalosporins, being 40, 50, and 50–70 %, respectively (Table 20.7). Plasma samples for the TDM of piperacillin, tazobactam, meropenem, and ceftazidime should be processed within 6 hours if kept at room temperature and within 3 days if kept at 4°C (Mortensen *et al*., 2019).

20.6.4.2 Antifungal drugs

Triazole antifungal drugs are used for the prophylaxis and treatment of invasive fungal disease in patients undergoing either haematopoietic stem cell transplantation (HSCT), or intensive chemotherapy. For chemotherapy and autologous HSCT patients triazole prophylaxis is mainly prescribed during periods of neutropenia, but for allogeneic HSCT prophylaxis is prescribed in early pre-engraftment neutropenia, followed by post engraftment acute and chronic graft versus host disease phases. The most frequently used triazoles in haematology practice are fluconazole, itraconazole, posaconazole, and voriconazole, although others are also available (Mueller *et al*., 2018; Schuster *et al*., 2019; Zheng & Wang, 2019).

Significant variations in triazole pharmacokinetics have been reported as a result of drug–drug interactions, and conditions affecting gastrointestinal absorption such as diarrhoea and mucositis. In addition, the absorption of posaconazole is affected by the food content of the stomach and gastric acidity at a time when patients may experience chemotherapy-induced nausea and vomiting and are receiving gastric acid-suppressive agents such as proton pump inhibitors or H_2-receptor blockers (Ceesay *et al*., 2016). Use of reduced graphene oxide (rGO) with a fluorescently labelled aptamer (Section 6.8) that can measure plasma posaconazole and other triazoles in a little over 1 hour using 100 µL of sample has been described (Wiedman *et al*., 2018).

The metabolism of itraconazole and voriconazole shows high inter-patient variability with no clearly predictable dose–plasma concentration relationship. Moreover, voriconazole exhibits dose-dependent (non-linear) pharmacokinetics at the plasma concentrations associated with effective therapy. It has been suggested that trough voriconazole plasma concentrations should be maintained below 4–6 mg L^{-1} to minimize the risk of visual disturbances, skin reactions, hepatotoxicity, and neurotoxicity (Table 20.7).

20.6.4.3 Antimalarials

Quinine was the first antimalarial drug. In non-infected subjects, plasma total quinine concentrations above 8 mg L^{-1} may be associated with clinical features of toxicity such as visual disturbance, leading in some cases to either permanent visual deficit or blindness. Quinine is normally 70–90 % bound to plasma protein, notably AAG. The plasma concentration of this latter protein is increased in severely infected patients (Section 16.10) and quinine protein binding is also increased (to 93 % or so). Hence higher plasma total quinine concentrations can be tolerated with no apparent toxicity (de Pablos-Martinez *et al*., 2016).

Chloroquine and hydroxychloroquine are chiral antimalarial drugs that again bind to AAG. Chloroquine is used as a malaria prophylactic at a single adult oral dose of 300 mg free-base weekly. A common cause of concern is that taking the drug daily leads to an enhanced risk of

Table 20.7 Some anti-infective drug TDM assays

Group	Drug	Reference range in an adult (mg L^{-1} plasma)[a]
Antifungal	Posaconazole,	Trough >0.5–0.7 (prophylaxis)
	Itraconazole (+ hydroxyitraconazole)	Trough >0.5 (prophylaxis)
	Voriconazole	1–4 to 6 (prophylaxis)
Antimalarials	Chloroquine	<0.05 (malaria prophylaxis), 0.2–0.3 (rheumatoid arthritis)
	Hydroxychloroquine	<0.05 (malaria prophylaxis), 0.4–0.5 (rheumatoid arthritis)
	Quinine	8–16[b]
Antimicrobial	Amikacin	Trough <10, peak 20–30
	Chloramphenicol	Trough <5, peak 10–20
	Ethambutol	<3 pre-dose, 2–6 post-dose
	Gentamicin	Trough <2, peak 5–10
	Isoniazid	3–6
	Kanamycin	Trough <8, peak <30
	Netilmicin	Trough <2, peak 5–10
	Pyrazinamide	20–60
	Rifabutin	0.3–0.9
	Rifampicin	8–24
	Sulfamethoxazole	Peak 100–120
	Teicoplanin	10–60 (20–60 *Staphylococcus aureus* infection)
	Tobramycin	Trough <2, peak 5–10
	Vancomycin	Trough 5–10, peak 20–40
Antiretroviral[c]	Amprenavir	Trough >0.4
	Atazanavir	Trough >0.15
	Efavirenz	Trough >1
	Indinavir	Trough >0.1
	Lopinavir/ritonavir	Trough >1
	Nelfinavir	Trough >0.8
	Nevirapine	Trough >3.4
	Ritonavir	Trough >2.1
	Saquinavir	Trough >0.1

[a] Achiral method
[b] Possibility of serious toxicity in non-infected subjects
[c] Suggested minimum target concentrations for patients with wild-type HIV

toxicity in the non-infected subject. These drugs are also used at higher doses as second-line agents to treat rheumatoid arthritis and hydroxychloroquine is also approved for the treatment of systemic lupus erythematosus (Mok, 2017). Desethylchloroquine and didesethylchloroquine are active metabolites of both drugs (Figure 20.3) and should be measured if TDM of these drugs is undertaken. Desethylhydroxychloroquine should also be measured in the case of hydroxychloroquine monitoring (Qu *et al*., 2017; Noé *et al*., 2019).

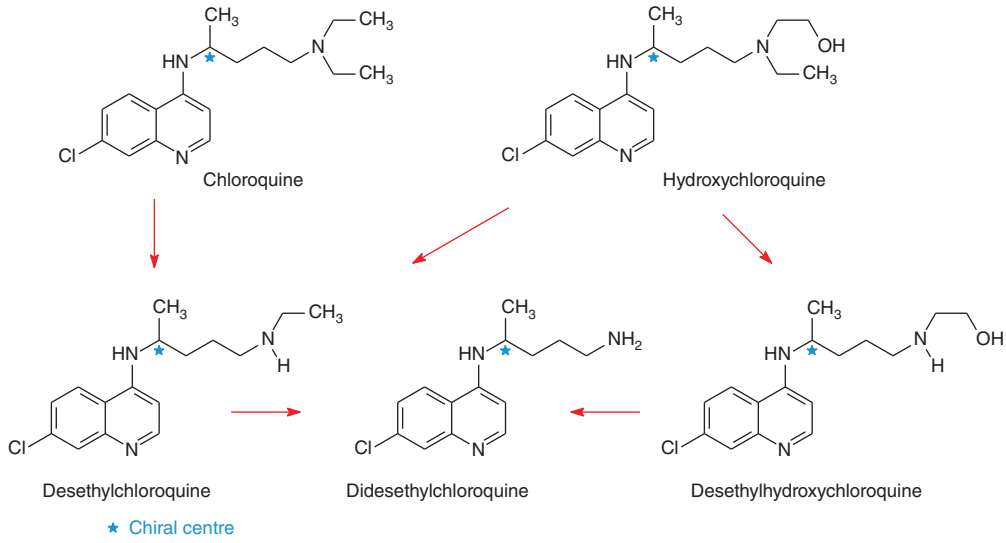

Figure 20.3 Metabolism of chloroquine and hydroxychloroquine

Chloroquine, hydroxychloroquine, quinine, and their metabolites are highly fluorescent, and thus LC with fluorescence detection presents a sensitive alternative to LC-MS/MS for the TDM of these drugs and not only simplifies ISTD choice, but also avoids ion enhancement/suppression issues (Noé *et al*., 2019).

20.6.4.4 Antiretroviral drugs

The observed wide inter-individual variation in antiretroviral pharmacokinetics led to the use of TDM with the aim of helping to optimize dosing of these drugs (Table 20.7). Although TDM can help assess adherence to treatment (Calcagno *et al*., 2017), the role of TDM in dose optimization with these drugs remains controversial (Punyawudho *et al*., 2016; Cusato *et al*., 2018; Waalewijn *et al*., 2019).

20.6.5 Anti-inflammatory drugs

Salicylates used to treat rheumatoid arthritis have been monitored traditionally, in part because of the availability of simple methodology. A target range of 250–300 mg L^{-1} was used. Monitoring of other analgesics and NSAIDs that have largely replaced salicylates in rheumatoid arthritis is rare and is mainly used to diagnose and, if possible, prevent toxicity.

20.6.5.1 Therapeutic antibodies

Infliximab is one of several monoclonal antibodies ('MAbs') administered i.v. that are used for treating chronic inflammatory diseases such as Crohn disease and various malignancies (Imamura, 2019; Papamichael & Cheifetz, 2019). LC-MS/MS can be used to measure a number of these compounds, including adalimumab, cetuximab, rituximab, secukinumab, tocilizumab, and trastuzumab in addition to infliximab (Jourdil *et al*., 2017; 2018; van der Gugten *et al*., 2019; Willeman *et al*., 2019).

TDM of infliximab has not been performed widely, but one study in outpatients with irritable bowel disease has shown large interindividual differences in infliximab trough concentrations (Warman *et al*., 2015). Overall, however, there was a significant association between remission and infliximab trough concentrations. Infliximab was not detected in a significant proportion of patients.

20.6.6 Antineoplastic drugs

20.6.6.1 Chemotherapeutic agents

TDM clearly has the potential to improve the clinical use of chemotherapeutic agents, most of which have very narrow therapeutic indices and highly variable between-patient pharmacokinetics. Plasma concentration–effect relationships have been established for 5-fluorouracil (1 g m^{-2} d^{-1}), 6-mercaptopurine (from azathioprine), and methotrexate (Table 20.8). Interpretation is complicated by the use of different agents simultaneously. *AUC* calculations may give more useful information than measurements at a single point in time (Goirand *et al*., 2018; Beumer *et al*., 2019). Relationships between plasma concentration and dose-limiting toxicities for epipodophyllotoxins, platinum-containing compounds, camptothecin, anthracyclines, and antimetabolites have also been described (Paci *et al*., 2014).

Table 20.8 Some chemotherapeutic drug TDM assays

Drug	Reference range in an adult (mg L^{-1} plasma)
5-Fluorouracil (5-FU)	<0.2 (1.5 μmol L^{-1}) – 120 h post-dose
6-Mercaptopurine (6-MP)	<0.2 (peak, maintenance doses)
Methotrexate	<1.0 (2.2 μmol L^{-1}) – 24 h post-dose; <0.45 (1 μmol L^{-1}) – 48 h post-dose

TPMT phenotyping is used to guide treatment with azathioprine to avoid life-threatening agranulocytosis (Section 15.7.4). Chouchana *et al*. (2012) have produced a therapeutic algorithm to facilitate dosage individualization for thiopurine, with starting doses based on TPMT status and thereafter dose adjustments according to five metabolite profiles.

20.6.6.2 Protein kinase inhibitors

Many of the protein kinase inhibitor (PKI) class of drugs that have revolutionized the treatment of certain cancers are tyrosine kinase inhibitors (TKIs). Others are mainly serine/threonine kinase inhibitors, including everolimus and vemurafenib. Imatinib, the first clinically useful

TKI, dramatically altered the treatment and prognosis of chronic myeloid leukaemia (CML) and gastrointestinal stromal tumours (GIST).

Since the introduction of imatinib, a number of TKIs have been developed (Table 20.9). Unlike many traditional anticancer therapies that are given i.v. TKIs are administered orally and their bioavailability is thus dependent on gastrointestinal absorption, first-pass metabolism and other factors such as genetics, drug–drug interactions, food intake, and smoking habit. Moreover, some TKIs are substrates of drug transporters and are extensively bound to plasma protein. Finally, some are capable of inhibiting their own transporters and metabolizing enzymes, making their disposition and metabolism at steady-state complex and unpredictable. As with most

Table 20.9 Some tyrosine kinase inhibitor TDM Candidates (Josephs *et al*., 2013–reproduced with permission of Wolters Kluwer Health, Inc.)

Drug	Reference range in an adult (mg L^{-1} plasma)	Notes
Axitinib[a]	0.01–0.1	Axitinib sulfoxide may indicate metabolic capacity or drug–drug interactions
Crizotinib	>.0.2[b]	*O*-Desalkylcrizotinib may indicate metabolic capacity or drug–drug interactions
Dasatinib	0.01–0.17	Two metabolites are active, but at low concentrations
Erlotinib	>0.5	Norerlotinib is pharmacologically active
Gefitinib	>0.5	Norgefitinib may indicate metabolic capacity or drug–drug interactions
Imatinib	>1 (CML and GIST)	Norimatinib may indicate metabolic capacity or drug–drug interactions
Lapatinib	>0.5[c]	Metabolite-induced hepatotoxicity may require the metabolites to be quantified
Nilotinib	>0.6[d]	–
Pazopanib	>20	Metabolites may indicate metabolic capacity or drug–drug interactions
Ruxolitinib	Not known	Two metabolites may display activity
Sorafenib	>3[e]	Sorafenib *N*-oxide may indicate metabolic capacity or drug–drug interactions
Sunitinib[a]	>0.05 (sunitinib + norsunitinib)	Norsunitinib active and considered in target range
Vandetanib	>4[f]	*N*-Desmethylvandetanib active metabolite
Vemurafenib	>40[g]	Two metabolites may display activity

[a] Displays light-dependent trans-/cis-isomerism, therefore total concentration reported
[b] Mean concentration attained from patients prescribed 250 mg twice daily
[c] Mean concentration in patients prescribed 1500 mg once daily
[d] C_{min} concentration applicable to quartile 1 from cytogenetic response
[e] Concentration attained in patients where dose has been increased due to poor response
[f] Minimum concentration attained in patients prescribed 300 mg d^{-1}
[g] Concentration attained in patients prescribed 960 mg twice daily

drugs there may be adverse effects, hence adherence is also a potential variable in assessing outcome (Cardoso *et al*., 2018). TDM may have a role in improving outcomes with this important group of drugs (Gao *et al*., 2012; Widmer *et al*., 2014).

20.6.6.3 Therapeutic antibodies

Interpatient PK variability observed with MAbs is comparable or slightly lower to that observed with TKIs. Despite data showing encouraging results with rituximab, cetuximab, and bevacizumab, as with MAbs used in treating inflammatory conditions (Section 20.6.5.1), overall TDM of these MAbs is not as yet supported by strong evidence (Widmer *et al*., 2014; Imamura, 2019).

20.6.7 Cardioactive drugs

20.6.7.1 Digoxin

TDM of digoxin is well established, although its clinical relevance is sometimes obscure (Aonuma *et al*., 2017). Immunoassays are used almost universally, but they have not been without problems. False immunoassay reports of 'digoxin' in patients with a variety of volume-expanded conditions, *viz*. diabetes, uraemia, essential hypertension, liver disease, and pre-eclampsia were attributed to the presence of digoxin-like immunoreactive substances, 'DLIS'.

The association of DLIS with volume expansion led to speculation that the interfering substance could be natriuretic hormones. Other structures proposed included non-esterified fatty acids, phospholipids, lysophospholipids, bile acids, bile salts, steroids, and endogenous ouabain. However, it is likely that 'DLIS' was simply a matrix effect in the assays used (Dasgupta, 2006). For example, an investigation of possible interference by DLIS on the Architect iDigoxin CMIA in serum samples from pregnant women, and patients with liver disease, renal insufficiency, critical illness, and kidney and liver transplant revealed no interference (Lampon *et al.,* 2012).

20.6.7.2 Other cardioactive drugs

TDM is useful with amiodarone to monitor adherence and to reduce the risk of toxicity, and to monitor adherence to sotalol and to other β-adrenoceptor blockers such as atenolol and propranolol (Table 20.10). Use of the calcium channel blockers verapamil and diltiazem is normally assessed by monitoring haemodynamic effects. Diltiazem and *N*-desmethyldiltiazem, desacetyldiltiazem and *N*-desmethyldesacetyldiltiazem, all of which may be pharmacologically active, are all unstable in plasma.

Perhexiline is used to treat hypertrophic cardiomyopathy. There is an increased risk of toxicity at plasma concentrations above the target range. An LC-MS/MS method for measuring perhexiline and its major hydroxylated metabolites in human plasma has been described (Camilleri *et al*., 2017). TDM is also useful in assessing flecainide dose requirement in children (Rahman *et al*., 2018). The use of LC-MS/MS for measuring milrinone and dobutamine in 20 μL neonatal and paediatric plasma samples at the concentrations attained in therapy has been described. Ammonium fluoride was added to the eluent to give added sensitivity in PCI and ascorbate was added to the samples to prevent dobutamine degradation (Takkis *et al*., 2019).

20.6.8 Immunosuppressants

TDM of ciclosporin, everolimus, mycophenolic acid (MPA, the active metabolite of mycophenolate mofetil), sirolimus, and tacrolimus is well established. TDM methods for their analysis

Table 20.10 Some cardioactive drug TDM assays (metabolites normally measured also shown)

Drug [metabolite]	Reference range in an adult (mg L^{-1} plasma)
Amiodarone	0.5–2
[Noramiodarone][a]	[0.5–2][b]
Atenolol	0.2-0.6
Digoxin	0.0008–0.002 (1.0–2.6 nmol L^{-1}) (6–12 h post-dose)[c]
Disopyramide	2.0–5.0
[Nordisopyramide]	[<5.0][b]
Flecainide	0.2–0.7
Lidocaine	1.5–5.0
Metoprolol	0.2–0.8
Mexiletine	1-2
Perhexiline	0.15–0.6
Procainamide [+ acecainide[d]]	10–30 (procainamide only, 4–8)
Propranolol	0.01–0.1
Quinidine	2.5–5.0
Sotalol	0.8–2.0 (β-blockade), 2.5-4.0 (antiarrhythmic)
Verapamil	0.1–0.2
[Norverapamil]	[0.1–0.2][b]

[a] *N*-Desethylamiodarone
[b] Ratio of metabolite to parent compound may be a guide to the duration of therapy and possibly to the likelihood of toxicity
[c] Assay may be unreliable in some patient groups (e.g. neonates, renal failure, hepatic failure)
[d] *N*-Acetylprocainamide

have been reviewed (Zhang & Zhang, 2018). All these drugs have narrow therapeutic windows and show considerable pharmacokinetic variability. TDM is essential to avoid adverse effects such as nephrotoxicity whilst maximizing efficacy. All is not straightforward, however, as some patients experience acute rejection episodes or post-operative complications despite blood concentrations within the reference range. As in the case of chemotherapeutic agents, *AUC* calculations may give more useful information than measurements at a single time point particularly for MPA, but collection of such samples in the out-patient setting is often impractical. Note that azathioprine (Section 20.6.6.1) is also used as an immunosuppressive drug.

Peak (2 h post-dose) sampling may be a better indicator of optimal ciclosporin dosage than trough or 4 h post-dose sampling, but this is disputed. Interpretation of either 'trough' or 'peak' results is complicated because (i) there may be considerable differences between the results obtained with immunoassay as compared with chromatographic methods, (ii) immunosuppressants are often used in combination to reduce the risk of toxicity from individual compounds hence the concentrations attained during optimal treatment are lower than when the drugs are used alone, and (iii) the amount of immunosuppression required for maintenance treatment varies widely depending on the engrafted organ. The guidelines given below (Table 20.11) are

Table 20.11 Some immunosuppressive drug TDM assays

Drug	Reference range in an adult (μg L^{-1})[a]
Ciclosporin (cyclosporine, cyclosporine A)	40–250 (trough, whole blood)[b]
Everolimus	5–7 (5–15 if initial response inadequate) (trough, whole blood)
Mycophenolic acid	2.5–4.0 (trough, plasma)
Sirolimus	0.003–0.015 (trough, whole blood)
Tacrolimus	0.001–0.012 (trough, whole blood)

[a] Single immunosuppressant, renal transplant patients
[b] Immunoassay may be unreliable in some patient groups (e.g. neonates, renal failure, hepatic failure)

those applicable to therapy with single immunosuppressants used after renal transplantation. Use of oral fluid for immunosuppressant TDM has been suggested (Paniagua-González *et al*., 2019), as has blood collection using a volumetric microsampling device (Zwart *et al*., 2018).

20.6.9 Psychoactive drugs

20.6.9.1 Lithium

When starting therapy to treat bipolar disorder, the target range for serum lithium ion concentrations is 0.6–0.8 mmol L^{-1}. If necessary, the dose can be increased to achieve 1 mmol L^{-1} even though mild adverse effects may occur at this concentration when lithium is given chronically. Mild to moderate toxicity is expected at 1.5–2 mmol L^{-1}, although patients in a manic state seem to have an increased tolerance to the drug. Severe toxicity, which may be irreversible, is likely above 2 mmol L^{-1}. Initial features of toxicity may include GI discomfort, nausea, vertigo, muscle weakness, and a dazed feeling. More common and persistent unwanted effects include fine hand tremor, fatigue, thirst, and polyuria. Progressive intoxication may manifest as confusion, disorientation, muscle twitching, hyperreflexia, nystagmus, seizures, diarrhoea, vomiting, and eventually coma and death.

Lithium is excreted primarily in urine and its renal clearance is proportional to the plasma concentration. Renal lithium clearance is, under ordinary circumstances, remarkably constant in individuals, but decreases with age and when sodium intake is lowered. Moreover, renal lithium excretion may vary greatly between individuals and lithium dosage must, therefore, be adjusted on the basis of the serum lithium concentration 6–12 hours post-dose. The serum lithium should then be monitored 3–4 monthly to ensure that dosage is still optimal.

20.6.9.2 Antidepressants

Whilst reference ranges for certain tricyclic antidepressants (TCAs) and their plasma metabolites (for example amitriptyline/nortriptyline and imipramine/desipramine) associated with effective treatment have been suggested, patients become tolerant to the adverse effects of TCAs and may show clinical improvement at higher plasma concentrations than those cited (Table 20.12).

In practice, TDM of TCAs and also of selective serotonin reuptake inhibitors (SSRIs) and other newer antidepressants such as venlafaxine has been concerned mainly with assessing

Table 20.12 Some psychoactive drug TDM assays

Drug [metabolite]	Reference range in an adult (mg L^{-1} plasma or serum)
Amisulpride	0.1–0.4
Amitriptyline [+ nortriptyline]	0.08–0.25
Aripiprazole	0.15–0.5[a]
Citalopram	0.07–0.50
Clomipramine [+ norclomipramine]	<1.0[b]
Clozapine	0.35–0.50[b, c]
Desipramine	0.08–0.16
Dothiepin [+ nordothiepin]	<0.50[b]
Doxepin [+ nordoxepin]	0.15–0.25
Fluoxetine	0.04–0.45
[Norfluoxetine]	[0.04–0.45][d]
Fluvoxamine	0.16–0.22
Imipramine [+ desipramine]	0.15–0.25
Lofepramine: see Desipramine	
Lithium	4–8 (0.6–1.0 mmol L^{-1}) (12 h post–dose)[e]
Mianserin [+ normianserin]	0.03–0.10
Nortriptyline	0.05–0.15
Olanzapine	0.02–0.04 (12 h post–dose)
Paliperidone	0.02–0.06[f]
Quetiapine	0.05-0.20[b]
Risperidone [+ total 9-hydroxyrisperidone]	0.02–0.06
Sertraline[g]	0.03–0.19
Sulpiride	0.3–0.5
Trazodone	0.8–1.6
Trimipramine [+ nortrimipramine]	<0.50[b]
Venlafaxine + *O*-desmethylvenlafaxine	<0.40
Ziprasidone	0.05–0.2

[a] Dehydroaripiprazole concentrations average 40 % of those of aripiprazole
[b] Adverse effects at higher concentrations may limit the dose that can be tolerated
[c] Upper limit not well defined. Norclozapine concentrations average 70 % of those of clozapine during normal therapy – norclozapine assay useful in monitoring adherence
[d] Ratio of metabolite to parent compound may be a guide to the duration of therapy and possibly to the likelihood of toxicity
[e] Upper limit may be higher in mania
[f] Total 9-hydroxyrisperidone
[g] Norsertraline only 10 % of the activity of sertraline

whether treatment failure is due to poor adherence, ultra-rapid metabolism, or drug–drug interactions leading to induction of metabolizing enzymes (Ostad Haji *et al*., 2012; Fiaturi & Greenblatt, 2018). TDM of SSRIs has been advocated in order to minimize the risk of drug–drug interactions due to inhibition of CYP2D6. Unlike some other SSRIs, citalopram has only a mild inhibitory effect on CYP2D6. Schoretsanitis *et al*. (2019) have suggested that plasma venlafaxine + *O*-desmethylvenlafaxine (active metabolite) concentrations >0.40 mg L^{-1} are not associated with an improved response.

20.6.9.3 Antipsychotics

Although of little benefit with established ('typical') antipsychotics such as chlorpromazine and haloperidol, TDM of newer (second generation or 'atypical') drugs, notably clozapine and to an extent olanzapine, can help by assessing adherence, guiding dose adjustment, and guarding against toxicity. In time, indications for TDM of other antipsychotics may become apparent (Schoretsanitis *et al*., 2018).

With clozapine, dose assessment is complicated because (i) there is a 50-fold inter-patient variation in the rate of clozapine metabolism, (ii) smoking can have a dramatic effect (on average 2-fold increase) on clozapine dose requirement, and (iii) the clinical features of clozapine overdosage can mimic those of the underlying disease. With olanzapine, a 12-hour post-dose plasma concentration of 20 μg L^{-1} seems to be needed to ensure a fair trial of the drug (Schoretsanitis *et al*., 2018). Olanzapine dose requirement is also affected by smoking.

20.7 Summary

For some drugs, notably some antiepileptics, anti-infectives, clozapine, digoxin, flecainide, some immunosuppressives, and lithium, TDM may be used to adjust the dose to individual need and to minimize the risk of dose-related toxicity. In the absence of other information, *V* and dose may be used to predict plasma concentrations, so that, for example, assay calibrators may be prepared over an appropriate concentration range. However, knowledge of how *CL* and distribution influence the time course of a substance in the body is essential to understanding the influence of age, sex, other genetic variables, disease, and other parameters on pharmacokinetics and hence clinical effect.

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21 Trace Elements and Toxic Metals

21.1 Introduction

Spectroscopy is the study of interactions between matter and electromagnetic radiation. When applied to quantitative analysis, the term spectrometry is used. Different types of spectroscopy are concerned with different regions of the electromagnetic spectrum (X-ray, UV, IR, etc.), the properties of the matter with which the interactions occur, for example molecular vibration, electron transitions, etc., and the physical interactions involved, i.e. scattering, absorption, or emission of radiation. An annual review of recent developments is published in the Journal of Analytical Atomic Spectrometry (Taylor *et al*., 2020).

With modern analytical techniques it is possible to measure individual elements at concentrations of parts per thousand million (μg L^{-1} or μg kg^{-1}) or less using microlitres or milligrams of sample. For many years, atomic absorption spectrometry (AAS) has been the technique of choice and can give accurate results quickly and without the need for very expensive equipment. It can be used to measure more than 60 elements, but can only be used to measure one element at any one time.

The field of trace element analysis changed radically with the development of ICP-MS. The detection limits and rate of sample analysis are equal to or better than those attainable with graphite furnace AAS, but in addition isotopes of most elements may be measured during the course of a single analysis. The situation changed yet again with the use of collision and dynamic reaction cells with ICP-MS. This technology eliminates many sources of interference and was accompanied by a considerable reduction in instrument cost.

Other important advances have included the use of techniques that separate different molecular conformations of an element (speciation) and *in vivo* analysis, initially involving neutron activation analysis (NAA), but now using X-ray fluorescence (XRF).

21.2 Sample collection and storage

By definition, trace elements are present at low concentrations in biological samples and many of the analytes that need to be measured occur naturally not only in such specimens, but also in the environment. Given that the analytical methods used are in the main inherently selective, the most important consideration in sample collection and storage is that of sample contamination. As a general rule, plastic containers are preferred to glass (Table 21.1). A 'blank' analysis guards against contamination from the sample tube going unrecognized. In the case of exhumations, samples of the surrounding soil should also be collected for analysis.

Fundamentals of Analytical Toxicology: Clinical and Forensic, Second Edition.
Robert J. Flanagan, Eva Cuypers, Hans H. Maurer and Robin Whelpton.

Table 21.1 Sample requirements for measurements of metals/trace elements

Element	Sample requirements	Comments
Aluminium	5 mL whole blood in plastic tube (no anticoagulant/separating beads[a]) 5 mL dialysate/supply water in plastic bottle rinsed several times with portions of the intended sample[a]	Use plastic and not glass tubes. Blood should not be separated
Antimony	5 mL EDTA whole blood 20 mL urine	Urine preferred
Arsenic	5 mL EDTA whole blood 20 mL urine	To diagnose chronic poisoning exclude seafood (shellfish, etc.) from diet for 5 days before sample collection if total arsenic is to be measured
Bismuth	5 mL EDTA whole blood	
Bromide	5 mL EDTA whole blood	Interpretation of the result depends on whether an organobromine or inorganic bromide is the source of exposure
Cadmium	2 mL EDTA whole blood[a] 10 mL urine[a]	Blood preferred
Chromium	2 mL EDTA whole blood[a] 20 mL urine (hard plastic bottle)[a]	Use of a plastic cannula to collect blood is advisable
Cobalt	2 mL EDTA whole blood[a] 20 mL urine (hard plastic bottle)[a]	Use of a plastic cannula to collect blood is advisable
Copper	2 mL EDTA or clotted whole blood, or 1 mL plasma/serum	If Wilson disease suspected, also send 10 mL urine
Fluoride	3 mL EDTA or clotted whole blood, or 1.5 mL plasma/serum, or 20 mL urine	
Iron	2 mL serum (no haemolysis)	To monitor use of a chelating agent, send 20 mL urine
Lead	2 mL EDTA whole blood	Blood should not be separated and free of clots
Lithium	5 mL clotted blood or 2 mL serum	Do not use lithium heparin anticoagulant
Manganese	1 mL EDTA whole blood or 0.5 mL plasma[a]	Use of a plastic cannula to collect blood is advisable
Mercury	5 mL EDTA whole blood 20 mL urine (hard plastic bottle)	Send samples promptly to avoid loss of mercury on storage
Nickel	20 mL urine	
Selenium	2 mL EDTA whole blood or 1 mL plasma/serum	
Silver	2 mL EDTA whole blood or 1 mL plasma	

Table 21.1 (*Continued*)

Element	Sample requirements	Comments
Strontium	2 mL EDTA whole blood or 1 mL plasma	
Thallium	5 mL EDTA whole blood 20 mL urine	
Vanadium	2 mL EDTA whole blood[a] 20 mL urine (hard plastic bottle)[a]	Use of a plastic cannula to collect blood is advisable
Zinc	1 mL plasma/serum (no haemolysis)	Do not use EDTA anticoagulant

[a]Send unused sample container from the same batch as used for sample collection to check for contamination

Anticoagulants and preservatives may be a source of contamination. EDTA is the anticoagulant of choice for the collection of whole blood because samples do not become viscous after a few days, unlike heparinized blood. However, if blood is allowed to clot and then sent to the laboratory unseparated it will not be suitable for iron and zinc assay because of leakage of these analytes from erythrocytes.

As regards sample containers, possible sources of contamination include rubber (found in some stoppers) and the O-rings inside certain tube closures. Some bottle lids contain a liner which may have a metal component. Vacuum blood collection systems represent another major source of contamination. Those containing clot activator and gel cell separation materials are likely to be unusable. There are sample collection systems marketed specifically for trace element analysis, but it is wise to check batches of such tubes for contamination before use with clinical samples. Needles may also be sources of contamination (with chromium, for example), and it may be necessary to collect blood through a plastic cannula after discarding the first few millilitres. Elements most susceptible to contamination from an exogenous source are aluminium, iron, manganese, and zinc, but nothing can be assumed to be unimportant.

Analyte concentrations are unlikely to alter on storage except in the case of mercury, but to avoid deterioration of the biological matrix, samples may be kept at 4 °C for short periods, and stored at –20 to –80 °C for longer-term preservation. In dilute solution, trace elements may be adsorbed onto the walls of a container. To prevent this from happening in urine, which normally contains little organic material to bind trace elements, it is advisable to acidify specimens as soon as possible after collection, for example by adding concentrated nitric acid to give a final concentration of 1 %, v/v.

Analysis of tissues other than blood is best performed using freshly collected material, although it is possible to examine specimens fixed in formalin and even embedded in wax. However, there are potential problems of both contamination and loss associated with fixing and embedding, hence careful monitoring for artefacts arising from pre-analytical treatment of the sample is essential.

21.3 Sample preparation

The aims of sample preparation are (i) to transform the specimen into a suitable form (usually a liquid) for introduction to the analytical instrument, with the analytes at concentrations that will produce a measurable response, and (ii) to reduce or eliminate possible interferences.

When instrument sampling systems include narrow-bore capillary tubing it is important that the transfer rates of samples and calibration solutions are equal. The high concentrations of cells, proteins, and other high M_r materials in samples such as serum and blood give rise to viscosity that is absent in aqueous calibration solutions. Therefore, assays may be subject to matrix interferences unless the viscosities of sample and calibration solution are matched. When the concentrations of analyte(s) are high, dilution with analyte-free water may be all that is needed. Alternatively, protein precipitation can be performed by addition of, for example, 10 % (w/v) trichloroacetic acid followed by centrifugation (Section 4.2.1).

A particularly useful approach is addition of a chelating agent followed by solvent extraction of the resulting complex. Interfering material is retained in the aqueous phase and, if the volume of solvent is less than that of the sample, analyte enrichment is also achieved. Various chelating agents and solvents have been employed, but a commonly used combination is ammonium pyrrolidine dithiocarbamate (APDC) and 4-methyl-2-pentanone (methyl isobutyl ketone, MIBK).

As with sample collection, the importance of avoiding contamination throughout the sample preparation procedure cannot be overemphasized. Equipment must be tested for cleanliness and reagents should be of the highest quality available. All reusable materials should be cleaned before use. Glassware should be first soaked in aqueous acid, for example 2–10 % v/v hydrochloric acid or nitric acid, and then rinsed with several volumes of purified water. Pure water has a resistivity of more than 18 MΩ cm and ideally should be used at all times. Blank samples should be taken through the entire procedure and IQC samples analyzed routinely. In addition to guarding against false high results, it is necessary to check for losses of analyte due to volatilization, adsorption onto container surfaces, or the precipitation of insoluble complexes.

21.3.1 Analysis of tissues

Solid specimens have to be digested to give an aqueous solution prior to analysis. Acid digestion is used most commonly. Methods with addition of nitric, perchloric, and hydrogen peroxide in varying combinations, sometimes with the inclusion of sulfuric acid, are widely employed and a number of possible procedures are available (Bazzi *et al*., 2005). Care must be exercised when using the potentially explosive perchloric acid.

Heating may be performed in conical flasks or beakers, or in tubes that fit an aluminium heating block. Usually, the heater temperature is gradually increased to around 250 °C, although higher temperatures may be required for some applications. Care is necessary to avoid loss of more volatile elements and mercury will almost certainly evaporate using this approach. Digestion is normally complete within 2–6 h. Microwave heating in sealed digestion vessels is used widely (Grinberg *et al*., 2005). Ovens have been specially designed for this purpose and include safety features to prevent damage from acid fumes and excessive pressure within digestion vessels. As many as 40 or more samples can be digested at the same time, often in less than 30 min and because each vessel is sealed there should be no loss of volatile elements.

In a different approach, sample and acid can be placed in a PTFE vessel (pressure bomb) within a stainless steel housing. The vessel is heated in either a microwave, or conventional oven and the sample is effectively destroyed after an hour or so, although the unit must be allowed to cool before it is opened. Pressure bombs are very expensive and for this reason most laboratories have a limited number, which may restrict sample throughput. Again, there should be no loss of volatile elements.

A second technique involves dissolution of the sample in a concentrated alkaline solution similar to those used to prepare tissue samples for liquid scintillation counting. Tetramethylammonium hydroxide (TMAH) is widely used. Samples are mixed with the reagent and heated for a few hours. Taking 50 mg of dried tissue and 2 mL of 50 g L^{-1} TMAH into a screw capped vial, complete solubilization is achieved by heating at 90 °C (2 h) with occasional shaking.

In another approach, sample destruction may be achieved by heating the sample in a muffle furnace, typically to 400–450 °C, followed by cooling and dissolution of the ash in 1 % v/v nitric acid. Loss of volatile elements must be considered, and this technique cannot be used if mercury is to be measured. These techniques for preparation of solid samples may also be used with liquid specimens, both to eliminate matrix interferences and to effect analyte enrichment.

21.3.2 Analyte enrichment

In the quest for lower LoDs, analyte enrichment has become important. Some examples have been mentioned above, but many methods have been developed for the specific purpose of concentrating the analyte. These involve trapping from a relatively large sample and subsequent elution into a smaller volume. The methods used include (i) adsorption onto materials such as charcoal or silica, (ii) use of ion-exchange resins either with, or without functionalized groups to trap specific elements, and (iii) size exclusion chromatography (SEC; Loreti & Bettmer, 2004). Enrichment in this way can be off-line, but many methods incorporate on-line sample processing.

Experience with ion-exchange chromatography and SEC led to an important development in sample preparation, speciation. In some circumstances, measurement of the total concentration of an element can be misleading hence it is necessary to measure particular species, for example Cr^{III} and Cr^{VI}, and As^{III}, monomethylarsonic acid, and dimethylarsinic acid to correctly evaluate the composition of the sample (Mandal *et al*., 2004). Methods for speciation involving chromatographic separation or differential solvent extraction are used routinely (Taylor *et al*., 2020).

21.4 Atomic spectrometry

Quantitative analytical atomic spectrometric techniques include AAS, AES, atomic fluorescence (AFS), inorganic MS, and XRF. AAS, AES, and AFS exploit interactions between UV/visible light and the outer shell electrons of free, gaseous, uncharged atoms. In XRF, high-energy particles collide with inner shell electrons of atoms to initiate transitions that culminate in the emission of X-ray photons. For inorganic MS, a magnetic field separates ionized analyte atoms according to their mass to charge (m/z) ratio.

21.4.1 General principles of optical emission spectroscopy

Uncharged atoms may exist at the most stable or *ground state* (E_0), having the lowest energy, or at any one of a series of *excited states* depending on how many electrons have been moved to higher energy levels, although it is usual to consider just the first transition (E'). This may be visualized in an energy level diagram (Figure 21.1). The energy levels, and the differences (ΔE) between them, are unique to each element.

The ΔE for movements of *outer shell electrons* in most elements corresponds to the energy equivalent to UV-visible radiation. The energy of a photon is characterized by:

$$E = h\nu \tag{21.1}$$

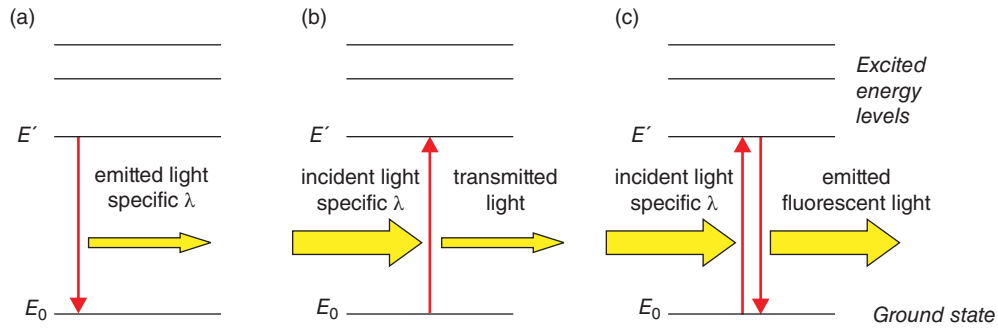

Figure 21.1 Energy level diagrams to show transitions associated with (a) AES, (b) AAS, and (c) AFS. The vertical arrows indicate absorption or emission of radiant energy (light)

where h is the Planck constant and ν is the frequency of the waveform corresponding to that photon. Furthermore, frequency and wavelength are related because:

$$\nu = \frac{c}{\lambda} \tag{21.2}$$

where c is the velocity of light and λ is the wavelength. Therefore,

$$E = \frac{hc}{\lambda} \tag{21.3}$$

and so a specific transition, ΔE, is associated with a unique wavelength.

Under appropriate conditions, outer shell electrons of vaporized, ground state atoms within the analytical system may be excited by heating. As these atoms return to the more stable ground state energy is lost. Some of this energy is emitted as light that can be measured. The intensity of the emitted light is proportional to the number of atoms present and the process is *atomic emission spectrometry* [AES; Figure 21.1(a)].

When light of a specific wavelength enters an analytical system, outer shell electrons of corresponding vaporized ground state atoms within the light path will be excited as energy is absorbed. Thus, the amount of light transmitted through the system to the detector will be attenuated. The loss of light is proportional to the number of atoms present and the method is known as *atomic absorption spectrometry* [AAS; Figure 21.1(b)].

Some of the radiant energy absorbed by ground state atoms can be emitted as light as the atom returns to the ground state. This emission is known as resonance fluorescence and is again proportional to the number of atoms in the light path. The technique is known as *atomic fluorescence spectrometry* [AFS; Figure 21.1(c)].

It follows from Equations (21.1)–(21.3) that the wavelengths of the absorbed and emitted light are the same, and are unique to any given element. This gives AAS, AES, and AFS great specificity, so that one element can be measured accurately even in the presence of an enormous excess of a chemically similar element.

21.4.2 Atomic absorption spectrometry

AAS can be used to measure more than 60 elements with instrumentation that is comparatively inexpensive and simple to operate. The method has sufficient sensitivity to measure many of these elements at the concentrations present in clinical specimens. The spectrometer consists

of a light source, atomizer, monochromator, detector, and readout/display. The essential feature of a good light source for AAS is to provide a high intensity, monochromatic output, which is achieved with hollow cathode or electrodeless discharge lamps. The monochromator, detector, and display are similar to those of other spectrometers.

The *atomizer* is any device that will generate ground state atoms as a vapour within the light path of the instrument. In the case of serum calcium, for example, the element is present bound to protein, complexed with phosphate, and as free Ca^{2+}. Atomization requires (i) removal of solvent (drying), (ii) separation from anionic or other components of the matrix to give Ca^{2+}, and (iii) reduction ($Ca^{2+} + 2e^- \rightarrow Ca^0$). The necessary energy is supplied as heat, from either a flame, or an electrically heated furnace.

21.4.2.1 Flame atomization

The typical arrangement involves a *pneumatic nebulizer*, mixing chamber, and an air-acetylene laminar flame with a 10 cm path length (Figure 21.2). Acetylene burns in air at approximately 2000 °C. A nitrous oxide–acetylene flame, which reaches approximately 3000 °C, is used for elements such as aluminium and chromium that form refractory oxides and have no effective atomization in an air–acetylene flame. A high-speed auxiliary air flow draws the sample solution continuously through the capillary due to the Venturi effect. The sample uptake rate through the nebulizer is usually about 5 mL min^{-1} and aspiration for several seconds is necessary to achieve a steady signal.

The sample emerges from the nebulizer as an aerosol with a wide range of droplet sizes, is mixed with the combustion gases, and transported to the flame for atomization. Only droplets less than 10 μm diameter actually enter the flame, with larger droplets falling to the sides of the chamber and running to waste. Thus, no more than about 15 % of the sample enters the flame. Hence, with the pneumatic nebulizer, the original sample undergoes dilution with the flame gases, a portion is lost in the mixing chamber, and there is considerable thermal expansion (i.e. further dilution) within the flame. In addition to dispersion of sample through the flame, there are losses of atoms as a result of the formation of oxides or other species at the flame margins.

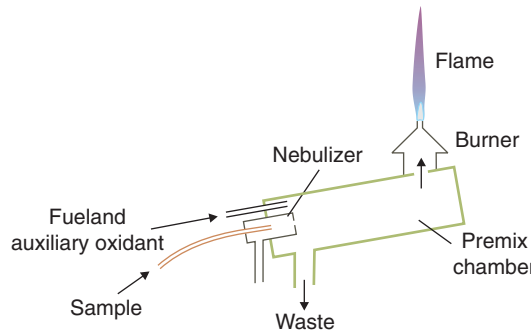

Figure 21.2 Pneumatic nebulizer for flame AAS

The advantages and disadvantages of the pneumatic nebulizer-flame atomization system are shown in Box 21.1. Because of its simplicity, speed, and freedom from interferences, this approach should be used whenever possible. The lowest analyte concentrations that can be measured are typically 1 mg L^{-1} or so.

Box 21.1 Advantages and disadvantages of pneumatic nebulization

Advantages
- Rapid
- Reproducible
- Few interferences
- Steady-state signal

Disadvantages
- Only about 15 % of the sample enters the flame
- Wide range of droplet sizes
- Low atomic density of sample in the flame
- Burner conditions impose limitations on the nebulizer

Improved sensitivity is obtained with devices that overcome the limitations of pneumatic nebulizers. Such devices include those that (i) trap atoms and give a greater density within the light path, (ii) bypass the nebulizer so that all of the sample is atomized, and (iii) introduce the sample as a single, rapid pulse rather than a continuous flow. Some employ a combination of these features. Devices used in a flame, e.g. the slotted quartz tube and Delves' cup, are most effective with more volatile elements such as zinc, cadmium, and lead. These three approaches to improved sensitivity also feature in other atomizers used in OES, AAS, AFS, and ICP-MS.

21.4.2.2 Electrothermal atomization

Most systems use an electrically heated graphite tube to vaporize the analyte and so this technique is often called *graphite furnace atomization*, although different materials are sometimes employed. Electrical contact is made to the ends of the tube and a voltage is applied. Resistance to the flow of current causes the temperature of the furnace to increase. A temperature program is used so that a solution placed inside the furnace is carefully dried, organic material is destroyed during an ashing step, and the analyte ions dissociated from anions. With a rapid increase in temperature, analyte ions are vaporized and reduced to ground state atoms prior to spectrophotometric detection. A further temperature increase ensures that the graphite tube is clean for the next analysis.

The atomization temperatures achieved by this technique can be up to 3000 °C, allowing refractory elements such as aluminium and chromium to be measured. Typically, only 10–50 μL of sample are needed for injection into the furnace and, because the entire sample is atomized within a small volume, a dense atom population is produced. The technique is, therefore, very sensitive and analyte concentrations of a few μg L^{-1} can be measured. However, electrothermal atomization AAS (ETAAS) is subject to greater potential interference than flame AAS (FAAS) and procedures to either eliminate, or compensate for such interference are necessary. Different forms of graphite (electrographite, pyrolitically coated graphite, and total pyrolytic graphite) are used and the design of the furnace and its mode of heating is optimized to promote atomization and reduce interferences.

21.4.2.3 Sources of error

Devices that involve flow of solutions, such as nebulizers or flow injection systems, will give inaccurate results if the samples and calibrators have different viscosities. If this occurs, internal standardization, standard addition, or the addition of reagents to equalize analyte and calibrant flow rates can be used to improve accuracy. Sample viscosity can be reduced by dilution with purified water, but this approach may give very low absorbance readings.

Different strategies to overcome this problem in the measurement of serum copper and zinc are outlined in Box 21.2. When the method includes several steps the possibility for contamination to occur, especially with zinc, is increased. Whichever sample preparation method is used, it is important to measure the aspiration rates of samples and calibration solutions through the nebulizer pre-mix chamber to ensure that they are equivalent.

Box 21.2 Actions to match sample and calibrator viscosity in the measurement of serum copper and zinc by FAAS

- Prepare the calibration solutions in 2 % v/v glycerol
- Add 1 part 10 % w/v trichloroacetic acid to 1 part serum or calibration solution. Mix well, centrifuge, and remove the supernatant
- Dilute the samples and calibrators with 5 % v/v aqueous butanol
- Dilute the mixture 5 to 10-fold in purified water

Chemical interactions may influence the rate of atomization. During FAAS, calcium bound to phosphate in serum is not entirely separated at 2000 °C and gives a lower result than an equivalent concentration in an aqueous calibrant. Addition of a releasing agent such as lanthanum (La^{3+}), which binds preferentially to phosphate, avoids this interference.

Problems may also occur within the graphite furnace. Components in the sample matrix can cause the analyte to become volatile and be lost during the ashing step, for example lead can be lost as lead chloride, $PbCl_2$. However, the most difficult problems are those that develop in the vapour phase as the samples are heated. Ground state atoms are vaporized into a rapidly changing thermal environment where the gas-phase temperature is lower than that of the tube wall and the ends of the tube are at a lower temperature than the centre. In consequence, vaporized atoms may condense on the cooler parts of the tube and then revaporize as the gas-phase temperature increases to the analyte appearance temperature, giving double peaks. This is because the molecular species formed give non-atomic absorption. Compensation for non-atomic absorption is provided by using chemical modifiers to stabilize the atoms and/or to promote destruction of the matrix at an earlier stage of heating (Table 21.2), by devices to establish isothermal atomization conditions, and by background correction (BC) techniques (Table 21.3).

Table 21.2 Chemical modifiers used in ETAAS

Modifier	Function
Nickel; ammonium phosphate; palladium; ruthenium	Form a thermostable complex with the analyte ions to allow a higher ash temperature to be used and so remove interfering species
Gaseous oxygen	Assists the ashing of the organic matrix. Mediates formation of atoms via activation of the graphite surface
Nitric acid; ammonium nitrate	Promote low temperature volatilization of halides to prevent analyte–chloride vapour phase interferences
Magnesium nitrate	Delays atomization so that isothermal conditions can be established inside the furnace

Table 21.3 Approaches to eliminate non-atomic absorption in ETAAS

Technique	Rationale	Examples
Chemical modifiers	Promote the destruction of sample matrix Delay atomization of analyte	Oxygen, magnesium nitrate, diammonium hydrogen orthophosphate
Isothermal atomization	Reduce vapour phase interactions by delaying atomization until furnace reaches constant temperature	L'Vov platform, graphite probe, novel furnaces
Background correction	Separately measure total and non-atomic absorption; difference = atomic absorption	Deuterium BC, Zeeman-effect BC, Smith-Hieftje BC

A typical method for the measurement of blood lead (Box 21.3) exploits each of these features. Matrix matched calibration solutions are prepared in a sample of blood with a low concentration of lead. Blood is diluted 15-fold with a phosphate/Triton solution and introduced into the graphite tube furnace of the AAS. The instrument is temperature programmed to dry, ash, and atomize samples at controlled rates. Oxygen is passed into the furnace during the ash phase to facilitate the destruction of organic material. Background correction is essential to compensate for the non-atomic absorption.

Box 21.3 Measurement of lead in blood by ETAAS

- Add whole blood (30 µL) to aqueous mixture (0.05 % v/v Triton X-100 + 0.5 % w/v diammonium hydrogen orthophosphate) (420 µL)
- Mix well and introduce to furnace
- Heating programme (instrument: Thermo 939)

Phase	Temp (°C)	Hold time (s)	Ramp (°C s^{-1})	Gas	Gas setting	Remarks
1	80	2	100	Ar	2	Drying
2	130	35	2	Ar	2	Drying
3	500	10	0	O_2	2	O_2 ashing
4	600	15	10	Ar	3	O_2 desorbed from furnace
5	1400	2	max	Ar	0	'Temp control' and 'Read'
6	2800	3	max	Ar	3	Cool

21.4.3 Atomic emission and atomic fluorescence spectrometry

21.4.3.1 Optical emission spectrometry

Flame atomic emission spectrometry (flame photometry) is convenient for the alkali metals at high concentrations, but OES is most useful with high temperature energy sources when multi-element analysis can be undertaken. The heat source for atomization and excitation to a

higher energy level can be a flame. Historical alternatives include arcs and sparks, but modern instruments use a plasma (argon or some other gas in an ionized state). The plasma is initiated by seeding from a high voltage spark to ionize the atoms:

$$Ar + e^- \rightleftharpoons Ar^+ + 2e^-$$

and is sustained with energy from an induction coil connected to a radiofrequency generator. This is known as an inductively coupled plasma (ICP).

Plasmas exist at temperatures of up to 10,000 °C and in the instrument have the appearance of a torch [Figure 21.3(a)]. Samples can be introduced via a nebulizer or, as for AAS, by hydride generation or cold vapour generation (Section 21.4.5), by electrothermal vaporization from a graphite atomizer, or by laser ablation of solid specimens. Optical systems direct the light emitted by excited atoms and ions either via a monochromator to a single detector, or to an array of monochromators and detectors positioned around the plasma.

With the first arrangement a sequential series of readings can be made with the monochromator driven to give each of the wavelengths of interest in turn. Simultaneous readings can be made with the second arrangement as each of the monochromators transmit light of different required wavelengths. A sequential reading instrument is less expensive than a simultaneous reading instrument, but more sample is required to take a series of readings. For most elements the analytical sensitivity for ICP-OES is either similar to, or greater than, that obtained with FAAS.

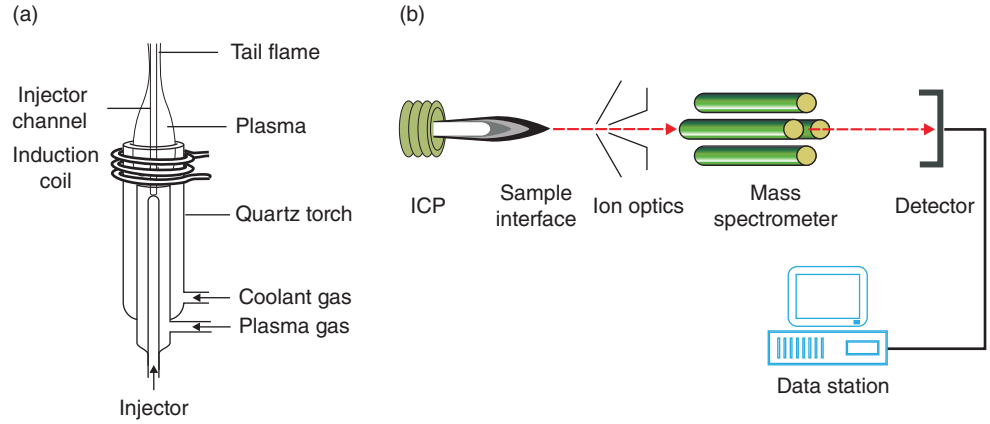

Figure 21.3 Inductively coupled plasma: (a) ICP torch (b) components of an ICP-mass spectrometer

At the high operating temperatures of the ICP many energy transitions take place, giving rise to the potential for spectral interferences when emission of light from different elements occurs at wavelengths that are too close to be separated by the monochromator. Most of these spectral interferences are known, so that when interference is suspected an alternative resonance line may be used for the measurement.

21.4.3.2 Atomic fluorescence spectrometry

Few commercial instruments for AFS are available and these are confined to the measurement of hydride forming elements and mercury (Section 21.4.5). The components of the instrument are similar to those for AAS. Effective atomic fluorescence requires intense, stable light sources

and these are difficult to construct reliably. Most success has been with electrodeless discharge lamps. The optical path of the emitted light is directed at 90° to that of the incident light so that only the emitted, fluorescent light reaches the detector. Very low detection limits can be achieved for the metalloid elements of Groups 4 to 6 of the periodic table.

21.4.4 Inductively coupled plasma-mass spectrometry

Because samples are taken to high temperatures any organic component is destroyed and some or all of the inorganic elements are ionized. When these ions are directed into a MS they may be separated by either an electric, or a magnetic field established by a quadrupole or some other mass filter. The ions, separated according to mass to charge (m/z) ratio, are detected and counted using an electron multiplier. This process is generally described as *atomic* or *inorganic MS* (Evans *et al*., 2020).

Various ion sources have been employed, but for clinical analyses most work uses ICP-MS (Goullé *et al*., 2014). Many elements can be measured quasi-simultaneously (ions are transmitted one mass at a time through quadrupole analyzers, but this happens extremely quickly, so effectively appears simultaneous) and detection limits are in the range of a few μg L^{-1} or less. Sensitivity is especially good for high atomic mass elements including actinides such as uranium; ICP-MS is often the method of choice for measuring these elements.

The other major feature of MS is the ability to measure isotopes selectively. The modules required for inorganic MS include sample introduction and ion generation, ion focusing, ion separation, ion detection, and data collection and display [Figure 21.3(b)].

21.4.4.1 Ion sources

As mentioned above there are a number of ion generation devices, but the most widely used is ICP. Samples are usually introduced to the plasma either via a nebulizer, or by chemical vaporization (Section 21.4.5). Electrothermal vaporization and vaporization by laser ablation of a solid sample may also be used (Sussulini *et al*., 2017).

21.4.4.2 Mass analyzers

The ICP torch is interfaced to the mass analyzer via two metallic cones (skimmer and sampler) through which ions are extracted into the ion focusing unit where a system of ion lenses direct ions to the analyzer. Several analyzer configurations are available. Those with a quadrupole system have limited resolution allowing the separation of species on the basis of m/z (Figure 21.4). However, to exploit the full potential of the technique, a high-resolution sector field mass analyzer is required. TOF-MS is particularly useful for the analysis of rapid, transient signals, such as those generated by electrothermal vaporization.

21.4.4.3 Interferences

ICP-MS is subject to spectral interference caused by the presence of either isotopes of other elements such as $^{156}Gd^{2+}$ on $^{78}Se^{+}$, or ions formed from matrix components and the plasma gas. Examples important for clinical analyses include $^{40}Ar^{+}$ on ^{40}Ca, $^{31}P^{16}O_2{}^{+}$ on ^{63}Cu, $^{40}Ar^{35}Cl$ on ^{75}As, and $^{40}Ar_2{}^{+}$ on $^{80}Se^{+}$. Sector field ICP-MS can spatially resolve most of these interferences, although there is a loss of sensitivity at high resolution, which may lead to problems with some applications such as the measurement of blood selenium (Bunch *et al*., 2017).

Use of collision cell technology (Section 13.2.4) facilitates interactions between polyatomic ions and the collision gas that cause the former to lose more kinetic energy than the analyte

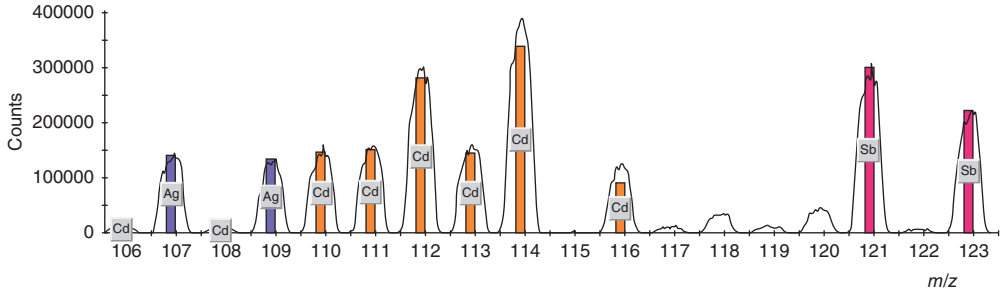

Figure 21.4 Example of ICP-MS mass spectrum (courtesy of Thermo-Fisher Scientific)

ion with which they interfere, greatly reducing spectral interferences. The use of hydrogen in a collision cell in a charge exchange reaction (CER) removes the interference of $^{156}Gd^{2+}$ on $^{78}Se^{+}$ (Harrington *et al*., 2014). Triple quadrupole ICP-MS instruments provide enhanced interference removal by allowing effective use of other reactive collision cell gases, such as oxygen and ammonia. Another approach involves separation of analyte ions from those involved in the formation of polyatomic species. Separation may be achieved by vaporization of the sample, for example by hydride generation or by electrothermal vaporization, or by prior use of a chromatographic or other separation step such as LLE. The addition of nitrogen, helium, or methane to the carrier gas or an organic solvent to the diluent can also reduce some of the argon-based interferences.

Non-spectral interferences associated with sample introduction and fluctuations in the ICP are effectively eliminated by using an ISTD. This should be an element not present in the original sample, not subject to spectral interferences, and with a mass and ionization energy close to those of the analyte(s). ISTDs often used with biological specimens are scandium, indium, and iridium for masses <80 u, 80–150 u, and > 150 u, respectively.

21.4.5 Vapour generation approaches

A family of chemical vapour generation techniques has been described (Tsalev, 1999), but most practical applications involve either volatile hydride generation, or the formation of mercury vapour.

21.4.5.1 Hydride generation

Elements such as arsenic, selenium, antimony, and bismuth, form gaseous hydrides, for example arsine (AsH_3). Using simple instrumentation, a reducing agent such as sodium borohydride is added to a reaction flask containing an acidified sample. Hydrogen is formed that reacts with the analyte, gaseous hydride is evolved, and transferred by a flow of inert gas to an ICP for AES or MS, or to a heated silica tube positioned in the light path for AAS or AFS (Lindberg *et al*., 2007).

The tube is heated by either an air–acetylene flame, or an electric current to a temperature that is sufficient to cause dissociation of the hydride and atomization of the analyte (Figure 21.5). There is no loss of specimen in the nebulizer, all the atoms enter the light path within a few seconds, and they are trapped within the silica tube, which retards their dispersion. Instrument development to give a continuous flow has simplified automation.

Typically, the hydride-forming element will be present in a sample as a range of species (e.g. As^{III}, As^{V}, monomethylarsonic acid, dimethylarsinic acid, arsenobetaine). The rate of hydride

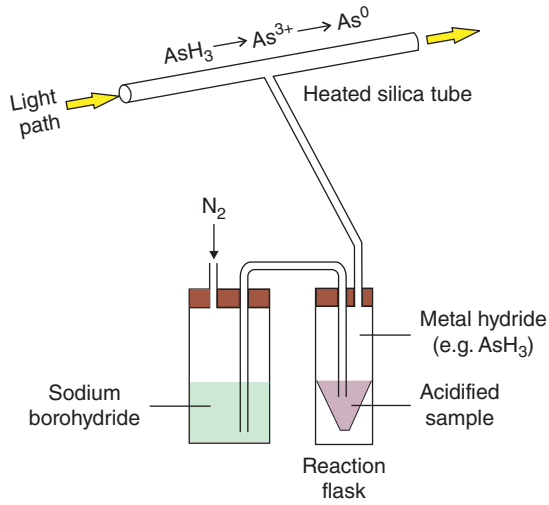

Figure 21.5 Hydride generation AAS

formation is dependent on the species so that to measure the total concentration of the element it is necessary to convert all of the different species present to the form that is the most reactive. This is usually achieved by heating with concentrated acids and then adding a reducing agent to give the reactive species (As^{III}, Se^{IV}, Sb^{III}, etc.).

Seafoods are a rich source of relatively non-toxic organoarsenic species such as arsenobetaine and urinary total arsenic concentrations will be high for several days following consumption of fish (Delafiori *et al*., 2016). It is possible to differentiate between dietary and non-dietary arsenic compounds by omitting the aggressive heating step and using a mild reducing agent such as an iodide salt or L-cysteine to convert any As^{III} metabolites to the hydride-forming species. The analysis will then reflect the exposure to toxic arsenic species.

Certain interferences are common to hydride generation whichever detector is used. If one hydride forming element is present in the sample in large amounts it will consume the reducing agent so that other elements may not be detected. High concentrations of transition element ions also inhibit hydride formation. If this is a problem, the use of masking agents, co-precipitation or chelation of the analyte followed by LLE of the chelate, can be used prior to the analysis.

21.4.5.2 Mercury vapour generation

Mercury forms a vapour at ambient temperatures and this property is the basis for cold vapour generation. A reducing agent such as tin(II) chloride is added to the sample solution to convert Hg^{2+} to elemental mercury (Hg^0). Agitation or bubbling of gas through the solution causes rapid vaporization of the atomic mercury, which is transferred to a flow-through cell placed in a light path (Figure 21.6).

To break the carbon–mercury bond in any organomercury compounds present, potassium permanganate may be added to acidified urine and the sample incubated at room temperature overnight. Excess permanganate is destroyed by addition of hydroxylamine hydrochloride. The reducing agent is added to the sample immediately before connection to the vapour generation accessory (Box 21.4). As with hydride generation, the LoD is a few nanograms, and instruments to perform both procedures have been developed.

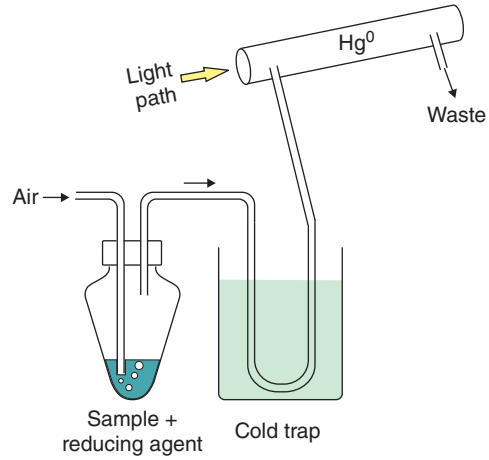

Figure 21.6 Cold vapour generation

Box 21.4 Measurement of mercury in urine by cold vapour generation AAS

- Place urine (1 mL) in a tube (4 °C)
- Add 0.2 mL concentrated sulfuric acid and 1.5 mL 6 % w/v aqueous potassium permanganate
- Stand overnight and then add 0.3 mL 20 % w/v hydroxylamine hydrochloride
- Add 1 mL 20 % w/v tin(II) chloride in 50 % v/v hydrochloric acid to reduce Hg^{2+} to Hg^0
- Connect to accessory and purge with air or inert gas to volatilize the analyte

21.4.6 X-Ray fluorescence

When high-energy photons, electrons, or protons strike a solid sample, an electron from the inner shells (K, L, or M) of a constituent atom may be displaced. The resulting orbital vacancy is filled by an outer shell electron and an X-ray photon is emitted. The energy (wavelength) of the emitted photon is equal to the difference in the energy levels involved in the electron transition. This phenomenon is known as *X-ray fluorescence* (XRF). The wavelength is characteristic of the element from which it originated, whilst the intensity of the emission is related to the concentration of the atoms in the sample (Potts *et al*., 2006). According to the type of spectrometer used to measure the emission, XRF is characterized as wavelength dispersive (WDXRF) or energy dispersive (EDXRF). Total reflection XRF (TXRF) is usually described as a separate technique, although it may be considered as a variation of EDXRF.

To perform an analysis, specimens are irradiated by high-energy photons, usually the polychromatic primary beam from an X-ray tube. However, the use of radioactive isotopes such as ^{244}Cm, ^{241}Am, ^{55}Fe, and ^{109}Cd as sources has clinical application in semi-portable instruments developed for *in vivo* XRF (Chettle, 2006).

The sample matrix can make a considerable contribution to signal intensity making calibration difficult, and requiring the use of reference materials, matrix matched standardization, and/or internal standardization. Fewer problems are encountered with samples prepared as very thin films, and in TXRF. In addition to the effect of the matrix, sensitivity is also influenced by wavelength, and lower atomic mass elements are more difficult to measure accurately.

High intensity X-rays are employed in WDXRF. The fluorescence energy is dispersed into individual spectral lines by reflection using an analyzer crystal. The diffracted beams are collimated and directed onto a photomultiplier tube. As with ICP-OES, spectrometers may operate sequentially, with a number of interchangeable crystals to permit the measurement of the full range of elements, or in a multichannel (simultaneous) mode usually preset for specific analytes. Detection limits for light elements (silicon and below) are 10–100 times lower than with EDXRF. Resolution is good, although less so at shorter wavelengths. Sequential instruments require long analysis times to measure several elements compared with simultaneous instruments or EDXRF technology.

For EDXRF, X-rays emitted from the sample are directed into a crystal detector. Pulses of current are generated with intensities that are proportional to the energy of the X-ray photons. The different energies associated with the various elements in the sample are sorted electronically. Compared with WDXRF, lower energy sources such as a low-power X-ray tube or a radioisotope (^{244}Cm, ^{241}Am, or ^{109}Cd) can be used. The detector has to be maintained in a vacuum at the temperature of liquid nitrogen (77 K). Analysis times are 10–30 times longer than with WDXRF, but because EDXRF is a truly multi-element technique, the total analysis time is not necessarily increased.

When a collimated beam of X-rays is directed against an optically flat surface at a shallow angle (*ca*. 5 minutes), total reflection will occur. This is the principle of TXRF in which the sample is exposed to both primary and total reflected beams, and is excited to fluoresce. Emitted radiation is detected and resolved as an energy dispersive spectrum. As there is effectively no absorption by the matrix, measurement and calibration are much simpler and sensitivities are greater than with other X-ray techniques. Useful applications of all types of XRF have been reviewed (Potts *et al*., 2006).

21.5 Colorimetry and fluorimetry

A number of compounds react with metal ions to give coloured products. In some such cases (Table 21.4), a quantitative assay may be developed taking advantage of the Beer–Lambert Law (Section 5.4.1). Applications to the analysis of clinical specimens are limited as sensitivity and selectivity are usually poor. Colorimetric measurement of calcium and magnesium in serum and urine, analytes that are present at concentrations of the order of mmol L^{-1} in these fluids, is routine. Measurement of zinc and copper in serum is also possible and commercial kits are available that can be used on modern clinical chemistry analyzers. However, there are interferences from the serum matrix and in EQA schemes the performance of these methods is poor.

Colorimetric methods for the measurement of iron(II) are available, but should be used with caution if chelating agents such as deferoxamine (desferrioxamine, DFO) have been given before the specimen was obtained, because the DFO–Fe complex will not react with the colour reagent. The LoD is 0.5 mg L^{-1}.

Some of the complexes formed between metals and dye compounds fluoresce. In addition, other compounds have been specially developed to exploit fluorimetric potential (Table 21.5). Fluorimetric assays generally have greater sensitivity and selectivity than colorimetric methods. Nevertheless, in practice only the measurement of selenium using 2,3-diaminonaphthylamine is of interest. This is as sensitive as the generally preferred AAS assays, but a much more complex sample preparation procedure involving acid digestion is needed. Hence, it is generally used only when there is no alternative. Use of a kinetic phosphorescence assay to measure uranium in biological samples has also been reported (Ejnik *et al*., 2000).

Table 21.4 Some compounds that react with metals to give coloured products

Reagent	Metal
Dithizone	Pb, Hg
2,2′-Bipyridyl	Fe
o-Cresolphthalein complexone	Ca, Mg
Methylthymol blue	Ca
Sodium diethyldithiocarbamate	Cu
2-Carboxy-2′-hydroxy-5′-sulfoformazylbenzene (Zincon)	Zn
1-(2-Pyridylazo)-2-naphthol (PAN)	Zn
3,3′-Diaminobenzidine	Se
Catechol violet	Al
Eriochrome cyanide R	Al

Table 21.5 Some compounds that react with metal ions to produce fluorescent products

Reagent	Metal
2,3-Diaminonaphthylamine	Se
3,3′-Diaminobenzidine	Se
2′,3,4′,5,7-Pentahydroxyflavone (Morin)	Be
8-Hydroxyquinoline	Mg
3-(2,4-Dihydroxyphenylazo)-2-hydroxy-5-chlorobenzenesulfonic acid (Lumogallion)	Al

21.6 Electrochemical methods

21.6.1 Anodic stripping voltammetry

ASV is ideally suited to the analysis of dilute solutions of heavy metals such as lead (Barón-Jaimez *et al.*, 2013). A reference electrode and a thin-film mercury graphite electrode are placed in the sample and a negative potential is applied to the mercury electrode, typically for periods of 2–30 s. This causes cations in the sample to concentrate ('plate-out') on the surface of the mercury electrode (the anode). The direction of the potential is then reversed to give an increasingly larger positive potential over 2–30 s. As the voltage reaches the half-wave potential of an element, lead (Pb^{2+}) for example, all such ions are discharged (stripped) from the anode thereby producing a current that can be measured (Figure 21.7)

The current produced is proportional to the number of ions appearing at that voltage and is compared with those given by calibration solutions. While ASV was not widely used in the analysis of biological specimens, a niche application developed, especially in the US, for the

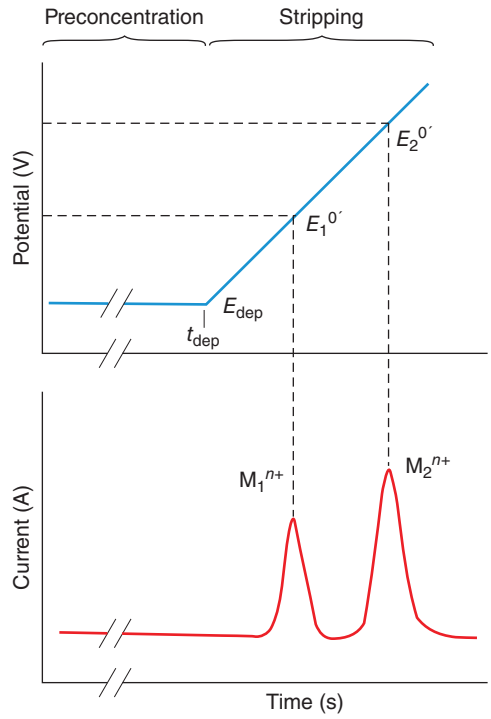

Figure 21.7 Schematic diagram of anodic stripping voltammetry. Metal ions plate-out on the anode (potential = E_{dep}) during the preconcentration step (t_{dep}). As the potential is increased the ions (M_1^{n+}, M_2^{n+}) are discharged at characteristic half-wave potentials ($E_1^{0\prime}$, $E_2^{0\prime}$)

measurement of lead in blood. A chelating agent is added to the sample to mobilize lead bound to red cells and protein, and the measurement performed using an instrument specifically designed for this application. The equipment was compact and could be set up in, for example, a medical examination room, but is no longer manufactured.

21.6.2 Ion-selective electrodes

Ion selective electrodes (ISEs), of which the pH meter is just one example, are widely used to measure major cations (Na^+, K^+) in biological specimens (Burnett *et al*., 2000). Electrodes for other metal ions and also for anions are available. Lithium (Li^+) is commonly measured in this way, for example, in plasma or serum and in whole blood. Use of lithium-free blood collection tubes is of course vital (Section 2.2.3.2). Fluoride (F^-) may be measured in biological samples, not only to assess possible exposure to this poison or compounds giving rise to F^- by metabolism, but also in medico-legal work to establish that enough fluoride has been added to a sample to ensure inhibition of microbial growth.

ISEs are examples of membrane electrodes in which the membrane has physical or chemical properties that allow movement of only one kind of ion between the internal filling solution and any test solutions. If the activities of the ions in the two solutions are not equal there is a tendency for ions to cross the membrane towards the lower activity solution, and the electric charge thus generated will oppose the migration of ions until equilibrium is established. The

actual number of ions involved is small and does not involve a gradual inter-diffusion between the two compartments. The potential represented by the movement of ions may be measured and is logarithmically related to the activity in the test solution. It is important to remember that it is *activity* and not concentration that is measured by an ISE.

The preparation of selectively permeable membranes has led to many applications. The membranes may be solid with a fixed ionic structure, e.g. glass that responds to Na^+, or a water-immiscible liquid containing dissolved material that will actively exchange the selected ions in solution. An effective calcium exchanger is a calcium salt of an alkyl phosphate dissolved in dioctylphenylphosphonate (Figure 21.8). The exchanger is prepared on a thin PVC layer to form the membrane.

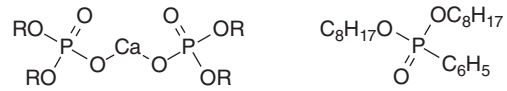

Figure 21.8 An example of an effective calcium exchanger

21.7 Catalytic methods

The concentration of an element can be measured from its catalytic role in a reaction, the rate of which is monitored using colorimetry or fluorimetry. The decolouration of ceric sulfate by arsenious acid, which requires iodine as catalyst, was once used to measure iodine in serum when investigating thyroid function. Most other examples of this type of reaction involve the oxidation of a substrate by hydrogen peroxide:

$H_2O_2 + KI \leftrightarrow I_3^-$	catalyzed by Mo
o-Dianisidine oxidation by H_2O_2	catalyzed by Cr^{VI}
Acid blue 45 oxidation by H_2O_2	catalyzed by Mn

Chemiluminescence for the measurement of metals is a special example of a catalytic technique. Oxidation of luminol by hydrogen peroxide at alkaline pH is accompanied by the emission of light (Section 6.6). Chromium, cobalt, copper, iron, and manganese have all been measured in this way. Such methods are very sensitive and can detect a few μg L^{-1} of analyte if interference from other metals can be excluded. However, these reactions have seldom been exploited in the analysis of biological samples.

21.8 Neutron activation analysis

NAA is sensitive and can be used to measure several elements simultaneously. However, because access to an atomic reactor is required, the technique is generally used only for special projects. In summary, the sample is bombarded with neutrons which generate a range of radioisotopes. As these isotopes decay the emitted radiation can be measured using conventional β- or γ-counters. Although characteristic emissions from individual radioisotopes impart some selectivity there may be interferences, especially from short-lived isotopes. Therefore, post activation ion-exchange chromatography is often employed to give acceptable results.

NAA is expensive because special equipment and highly trained operators are required, and in order to attain good detection limits irradiation times of several days may be needed. It is used, for example, in the characterization of reference materials when comparison with atomic spectrometric methods is to be performed. It may also be valuable in epidemiological and occupational toxicology studies (Spallholz *et al*., 2005), and in forensic work. An especial advantage in this latter area is that the sample is not consumed.

21.9 Chromatographic methods

21.9.1 Chromatography

In order to use chromatography to measure metal ions the problem that had to be addressed initially was that of detection using conventional LC and GC systems. It was found that chelates with, for example, trifluoroacetylacetonate could be measured by GC-ECD or LC-UV. However, sensitivity was inadequate for most applications. Nevertheless, work continued on the development of new chelating agents that afford improved detection limits (Hu *et al.,* 2005). A similar strategy has been used for ion chromatography. Although this latter technique is more usually associated with the measurement of anions, it is possible to measure metal ions using anion exchange columns, derivatization with a reagent such as 4-(2-pyridylazo)resorcinol, and colorimetric detection. Detection limits of 0.02–0.5 ng using a 50 µL aqueous sample have been reported. Other detection systems may also be used including OES and ICP-MS (Heitkemper *et al*., 2001).

At a time when ICP-MS was both expensive and subject to interferences, a novel approach was adopted in some laboratories in which a chelate of the element of interest [e.g. molybdenum with sodium bis(trifluoroethyl)dithiocarbamate] was prepared and analyzed by GC-MS. With this procedure any ions that might give rise to interferences in the MS were separated on the column, while the detector provided the necessary sensitivity. Subsequent developments with chromatography and ICP-MS also feature the use of a column to remove interferences, such as separation of As from Cl, which otherwise forms $^{40}Ar^{35}Cl$ and distorts the reading for ^{75}As.

21.9.2 Speciation

Measuring the total concentration of an element in a specimen usually provides the information that is required for most toxicological investigations, but there are situations when this is inadequate or misleading. The presence of non-toxic arsenic compounds in blood and urine, derived from seafood was mentioned above (Section 21.4.5.1), and measurement of arsenic species to differentiate between dietary and other sources of exposure may be needed. Another example is that of methylmercury and inorganic mercury where selective measurement of the mercury species present can give information as to the source of exposure.

Speciation analysis is also a feature of more fundamental work to investigate the metabolism of an element. Thus, speciation is an important topic for the analyst and features prominently in the recent literature, much of it involving use of chromatography to separate the analytes (Taylor *et al*., 2020). Detection may involve any analytical technique, but it is AAS, AES, AFS, and ICP-MS that are employed most commonly. Frequently, the chromatographic and detection systems are linked in series. Analyses involve any or all of the following steps: extraction (from tissue samples, for example), formation of a volatile derivative (for GC), chromatographic analysis, and measurement. The extraction procedure should extract all the element in the sample and no species transformation should occur.

21.10 Quality assessment

Unlike many organic compounds, inorganic analytes, with the notable exception of mercury, are generally stable in biological samples. In addition, calibration solutions can be prepared reliably and with high purity, and specialist reference laboratories are able to make measurements using, for example, isotope dilution analysis, that are traceable to international metrological standards. In turn, this means that certified reference materials are readily available for accuracy control and for method validation.

Apart from features such as inherent sensitivity, speed of operation, and cost of the analytical method used, contamination is the factor that has the greatest impact on the quality of results. Contamination can occur during (i) the collection and storage of specimens, (ii) sample preparation, and (iii) the spectrometric measurement itself. Scrupulous attention to cleanliness and to methodological detail is essential to obtain accurate and reproducible results. Data from EQA schemes indicate that whilst some routine laboratories can obtain good results, it is specialist trace element centres that tend to maintain the highest standards of performance.

21.11 Summary

The analytical toxicologist has a range of techniques available for the quantitative measurement of metal ions and other species in biological and related samples. Depending on the questions being asked the analyst can accurately measure a specific element at concentrations down to $\mu g\,kg^{-1}$ (ppb) or even less, or can produce a multi-element profile to indicate whether a metal is unexpectedly present or absent.

For many years AAS was the most important technique and whilst it continues to be used, ICP-MS is nowadays the method of choice. In addition, techniques are being developed and validated to identify and measure molecules in the sample with which the metal is associated. Because metals are ubiquitous in the environment it is vital to exercise extraordinary caution to ensure that there is no contamination at any stage from collection of the specimen to the generation of results.

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22 Clinical Interpretation of Analytical Results

22.1 Introduction

This concluding chapter gives some information to help in the interpretation of results as regards (i) clinical/emergency toxicology and 'brain-stem death' screening, and (ii) forensic toxicology. Clearly there is considerable overlap between, on the one hand, drugs monitored (usually in whole blood or plasma) to guide therapy, and on the other, testing for substance misuse, which traditionally has involved the analysis of urine.

PK considerations notwithstanding, the clinical interpretation of analytical results is a complex area. The aim of the analysis may be to help understand a clinical, or forensic scenario, or to provide evidence for the courts. Detailed knowledge, not only of the limitations of the analytical method(s) used, but also of the clinical pharmacology, toxicology, and PK of the compound(s) involved is usually important. Patients often respond differently to a given dose of a particular compound, especially as regards behavioural effects. Further complicating factors may include the role of pharmacologically active/toxic metabolites (Obach, 2013), and possible toxic effects of drugs on the liver (Real *et al*., 2019). Some compounds that are unstable in whole blood or plasma are listed in Table 2.9. Some other potentially important considerations are summarized in Table 20.1.

With regard to drugs, 'therapeutic', 'normal', 'normally expected', or 'target' plasma or serum concentrations for parent drug and metabolites often provide a basis for the interpretation of quantitative measurements as far as assessing the magnitude of exposure is concerned (Chapter 20). The interpretation of the concentrations of some substances may even be simplified by regulation. In the UK and in many other countries, breath and/or whole blood ethanol measurements in regard to driving a motor or other vehicle on a public road and blood lead measurements performed to assess occupational exposure provide examples.

In most cases, the interpretation of results in individual patients, which is often the responsibility of the laboratory or analyst providing the report, at least in the first instance, is dependent on the context in which the results are to be viewed. Reliable interpretation of analytical findings can be made only by comparing the results with information on analyte concentrations, clinical response and outcome, and the circumstances under which exposure occurred in other cases. It should be remembered that caffeine and nicotine, incidental findings in many analyses, are poisons in their own right (Chen *et al*., 2015; Jones, 2017). Cigarettes contain enough nicotine to cause serious toxicity if ingested by a young child; e-cigarette fluids are even more dangerous. Poisoning with fluorides may also be encountered, and thus caution may be required when adding sodium fluoride as sample preservative (Section 2.3.2).

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The investigation of poisoning may also be complicated by (i) the development of tolerance to the effect of drugs taken chronically, (ii) the lack of information on the blood concentrations of drugs, and in some instances of pharmacologically active metabolites, attained during chronic treatment, and (iii) that some drugs have idiosyncratic (not dose related) toxicity. Quinine, for example, even with only minute exposure from common beverages, can cause severe adverse reactions involving many organ systems (Liles *et al.*, 2016). In addition, people given psychoactive drugs may be at higher risk of self-harm, especially if either their condition has deteriorated, or they are not taking their prescribed medication. Furthermore, the underlying illness may carry a higher risk of sudden death than experienced in the general population.

In the case of illicit drugs there are the added complications of the method of administration and the purity of the preparation(s) used. Ethanol is often a complicating factor, and is of itself associated with many deaths either from direct toxicity alone and/or in combination with other drugs, or indirect effects such as inhalation of vomit or trauma. Ethanol is especially toxic in young children, and whilst traditionally associated with hypoglycaemia in this age group, hyperglycaemia has been reported in ethanol poisoning in a two-month-old child (Minera & Robinson, 2014).

Some of the information that should be available before attempting to interpret the results of toxicological investigations is listed below (Table 22.1). Clearly relevant circumstances such as the time since ingestion/exposure and the number of different poisons involved are important factors. The recent medical or occupational history, age, and state of health of the subject, amongst other factors, may all have a bearing on the interpretation of analytical results.

Table 22.1 Information important when interpreting the results of toxicological investigations

For each sample:	Name of patient or other form of identification including date of birth
	Nature of sample collected
	Time/date of sample
	Sampling site (if appropriate)
	Sample container used
	Sample preservation/transport/storage
	Name of person collecting sample
For each analytical report:	Time/date of analysis
	Nature of sample analyzed (plasma, whole blood, etc.)
	Unambiguous nomenclature (e.g. inorganic or total arsenic)
	Units used to report result(s)
	Name and accreditation status of laboratory and analyst performing the analysis and reporting result(s)
	Other drugs/poisons or groups of drugs looked for in the specimen or a related specimen
	Analytical method(s) used, and if appropriate the method identifiers
	Reference compound(s) used (source, purity, storage conditions, expiry date)

Proper interpretation is difficult in the absence of adequate background information, or if specimen collection has been inadequate. It may be particularly difficult to comment on the significance of a quantitative measurement carried out on a blood specimen collected from an unspecified site in a cadaver. Interpretation of findings can also be difficult if a prescribed drug is involved and the deceased may have acquired pharmacological tolerance to the drug through chronic exposure. The same applies particularly to users of drugs such as heroin or other opioids especially if the route of exposure is unknown. In the case of compounds encountered in the workplace, current biological monitoring guidance values may be available (HSE, 2018). However, after fatalities seemingly arising from deliberate misuse of toluene or other solvents, blood solvent concentrations may either be similar to, or lower than, those encountered after legitimate workplace exposure, for example.

Factors that must not be neglected are the selectivity and reliability of the analytical method. The results of immunoassays especially require confirmation using a method such as GC-MS or LC-MS if the data are to have any evidential value. Possible shortcomings in methodology as well as in sample collection, etc., must be taken into account when studying (older) published reports or compilations of analytical data to help guide interpretation, especially as regards rarely encountered poisons or unusual situations.

22.2 Clinical toxicology

The circumstances under which exposure to a drug or other substance may have occurred are crucial in assessing the potential involvement of the agent in an episode of poisoning, especially if harm may have occurred at lower than normal doses such as in anaphylaxis. On the other hand, patients can tolerate very large opioid analgesic doses with no ill effects if given the drug while undergoing mechanical ventilation. With inhalation anaesthetics, administration under controlled conditions is almost invariably safe even with the major stresses of surgery, but uncontrolled self-administration (volatile substance misuse, inhalant abuse, ‘glue sniffing’) by a healthy young adult may prove fatal. A given dose of chloroform, for example, is much more toxic if inhaled as a vapour rather than being ingested as a liquid, especially if accompanied by catecholamine release induced by excitation (Flanagan & Pounder, 2010).

The route of exposure can have a dramatic effect on toxicity. Metallic mercury is relatively non-toxic by ingestion, parenteral injection, or even i.v. injection, but is very toxic if the vapour is inhaled. Mercury salts and organomercurial compounds are generally toxic by all routes of exposure. Either i.v. injection of heroin, or inhalation of heroin vapour is far more dangerous than oral ingestion of an equivalent dose, possibly as a result of extensive pre-systemic metabolism following oral usage as well as other factors such as the risk of air embolism following an injection. Methadone has good oral bioavailability and is very toxic either by injection, or ingestion. This being said, the dose associated with serious toxicity from heroin or methadone is usually very much lower in someone who has not taken opioids recently because habituated individuals develop pharmacological tolerance to the adverse effects of the drugs on chronic exposure, and may in time require higher doses to achieve the desired euphoric effect. Tolerance, however, is quickly lost, the classic example being the individual who resumes a drug habit after a period of abstinence and suffers serious, often fatal, toxicity after their ‘normal’ dose.

Prior exposure does not always mean that tolerance will offer some protection to subsequent exposure. There is no evidence that chronic misuse of volatile substances offers protection against the risk of ‘sudden sniffing death’, for example. Indeed, with lithium salts, even a moderate overdose is likely to be much more dangerous in someone who has been taking the drug

long-term than in a naïve subject, possibly because lithium given chronically has accumulated in tissues. With some agents, notably halothane, a single exposure may cause no obvious adverse consequences, but subsequent use, even many years later, of either halothane, or a structurally related compound, may provoke massive toxicity because the initial exposure sensitized the individual via generation of antihalothane metabolite antibodies (Section 15.5.4.4).

Pharmacological tolerance acquired through chronic exposure is also a consideration when assessing likely toxicity after administration of antidepressant and antipsychotic drugs that are often encountered in self-poisoning episodes. In the case of clozapine, the daily dose may range up to 1600 mg in tolerant individuals, but 12.5 mg taken by mouth could kill a clozapine-naïve adult (Stanworth *et al*., 2012). Clozapine also provides an example of how metabolic tolerance acquired through smoking can have a major impact on the dose requirement; someone who starts smoking may lose the benefit of the drug within two or three days unless the dose is increased and, conversely, if a patient stops smoking then life-threatening toxicity (for example convulsions) may ensue within a few days unless the clozapine dose is reduced (Couchman *et al*., 2010). The dose requirement of olanzapine and of theophylline is similarly affected by smoking habit (Chui et al., 2019).

Genetic differences in drug response may arise from differences in pharmacodynamics (changes in receptor response) or in drug disposition. Most of the recorded gene polymorphisms relate to differences in the expression of drug metabolizing enzymes and transporters, and hence drug disposition (Musshoff *et al*., 2010; Neuvonen *et al*., 2011). If a compound is pharmacologically active, subjects who lack 'normal' drug metabolizing enzyme capacity may be more likely to suffer dose-related adverse effects, as has been suggested for venlafaxine (McAlpine *et al*., 2007), whereas those with multiple copies of the fully functional gene may show no response unless the dose is increased. The converse may be true for prodrugs.

An enhanced effect is of course to be expected if two or more drugs with similar properties are present in pharmacologically active amounts. Commonly encountered examples include the enhanced respiratory depressant effects of opioids such as dextropropoxyphene, methadone, and morphine in the presence of ethanol. There are more unusual interactions though. Transesterification of cocaine and of methylphenidate can lead to the production of active species (cocaethylene and ethylphenidate, respectively) with relatively long plasma half-lives if ethanol has been co-ingested. On the other hand, co-ingestion of ethanol usually protects against the toxicity of methanol in people who drink methylated spirits (ethanol denatured by adding a proportion of methanol) because ethanol blocks the toxic metabolism of methanol and there is usually time for the methanol to be excreted unchanged via the kidneys before the user fully eliminates the ethanol (Section 22.4.1.2).

Sometimes an antidote may be deliberately added to a pharmaceutical preparation. Buprenorphine, for example, is an orally active opioid with antagonist properties and is being used increasingly in opioid withdrawal programs. Co-formulation with the opioid antagonist naloxone aims to minimize the risk of diversion for intravenous administration because the user does not obtain the desired euphoric effect, but sublingual use of the drug is not affected because naloxone has low oral bioavailability.

Toxicity related to use of natural products is recognized increasingly (Lv *et al*., 2012; Wermuth *et al.,* 2018; Papsun *et al.,* 2019). Users of these preparations are at risk because herbal remedies, ethnic medicines, and other such preparations may contain undeclared drugs, inorganic poisons such as toxic metal salts, as well as toxic substances of natural origin (Murray *et al*., 2008; Byard, 2010; Teschke *et al*., 2012). Of course, the same considerations apply to illicit drugs. Severe parkinsonism in young people has occurred following injection of a manganese-containing street drug, due to the use of potassium permanganate in its manufacture

(Sikk *et al*., 2013). Counterfeit medicines that may contain undeclared drugs or other ingredients are another area of increasing concern (Gaudiano *et al*., 2016; Guo *et al*., 2016).

22.2.1 Pharmacokinetics and the interpretation of results

An understanding of the time course of a suspected poisoning or other episode is vital to interpretation of any toxicology result, because absorption, distribution, metabolism, and excretion of xenobiotics continues as long as the circulation is maintained, even if brain stem death has occurred. Results obtained from samples taken before absorption and/or distribution of an ingested drug are complete may be misleading as in the case of paracetamol and with sustained release preparations such as those containing diltiazem, lithium, morphine, oxycodone, or theophylline (Greene *et al*., 2018).

With poisons that can give rise to delayed toxicity such as ethylene glycol, methanol, paracetamol, and paraquat, clinical interpretation will be unreliable without some knowledge of the time of ingestion (Table 22.2). With paraquat, plasma measurements are at present only valuable in assessing the prognosis. In other cases of poisoning, continued metabolism after a period of hypoxia or anoxia, for example, may mean that the poison has been largely eliminated from the body before a sample is obtained. Features such as convulsions or vomiting after drug or alcohol withdrawal may occur in the absence of the drug involved and may prove fatal. Information on drugs given in treatment, including antidotes and compounds administered during procedures such as computerized tomography (CT), can be important when investigating such deaths.

Detection of tablet residues in stomach contents may point to recent ingestion, although in the case of drugs that decrease GI motility, drug residues may remain in the stomach for several days if the patient is treated in hospital. In post-mortem work, measurement of the amount of a poison in a sample of stomach contents may be helpful in establishing acute ingestion of an 'overdose' amount, especially if the total weight of stomach contents is known. Injection may be indicated by the presence of needle marks on the body. Injection, especially intrathecal (spinal) and i.v. injection, or inhalation, may be associated with serious toxicity even if blood concentrations are low as the poison may have been transported rapidly to the brain. After i.m. or i.p. injection, appreciable quantities of compound may remain at the injection site.

In humans, many anxiolytic and antidepressant drugs such as diazepam, imipramine, and amitriptyline are metabolized by *N*-demethylation. The secondary amine metabolites have similar values of V, but lower clearances than the parent drugs. Thus, these metabolites accumulate on chronic dosing to concentrations approaching or exceeding those of the drug administered. Thus drug:metabolite ratios for these compounds during chronic therapy are normally <1. Ratios >2 usually indicate that a large single dose has been administered recently. As an example, in 101 diazepam overdoses, the average diazepam:nordazepam ratio was 3 (Jatlow *et al*., 1979).

Probably the most important caveat after overdosage is that most pharmacokinetic parameters are derived from healthy volunteer studies (often males only) or from patients given therapeutic or sub-therapeutic doses. Continued absorption and non-linear kinetics following overdose may make the use of published pharmacokinetic parameters unreliable. For drugs that normally have a high oral bioavailability ($F > 0.9$) the proportion absorbed may be reduced after overdosage, but for drugs with low oral bioavailability, saturation of first-pass metabolism, for example, could markedly increase the proportion of the dose entering the systemic circulation. There may also be saturation of excretory mechanisms (hepatic metabolism, renal excretion) or inhibition by co-ingested compounds.

Table 22.2 Some compounds associated with delayed or irreversible toxicity

Compound/Group of compounds	Examples/Sources	Manifestation of toxicity
Acetylcholinesterase inhibitors	Insecticides, nerve agents	Respiratory failure
Amanitin	*Amanita phalloides* (Death cap)	Hepatorenal toxicity
Amfetamines including MDMA ('ecstasy')	–	Hepatic toxicity
Anticoagulants	Warfarin, other coumarins and indanediones Non-vitamin K antagonist oral anticoagulants: apixaban, dabigatran, rivaroxaban	Impaired haemostasis
Antimalarials	Chloroquine, quinine	Retinal and CNS toxicity
Antineoplastic agents	Cyclophosphamide, methotrexate	Systemic toxicity
Aspirin	–	Hepatic toxicity, impaired coagulation
Carbon monoxide	Automobile exhaust, central-heating boiler exhaust, smoke from all types of fires, etc.	Neuropsychiatric sequelae, e.g. parkinsonism; irreversible neurological damage
Chlorinated hydrocarbon solvents	Carbon tetrachloride, chloroform, 1,2-dichloropropane, 1,1,2,2-tetrachloroethane	Hepatorenal toxicity
Clozapine	–	Agranulocytosis, gastrointestinal hypomotility, bronchopneumonia
Diethylene glycol	Brake fluid	Hepatorenal toxicity
2-4-Dinitrophenol (2,4-DNP)	Dieting aid	Cardiovascular collapse
Ethylene glycol	Antifreeze	Renal failure, muscle damage, CNS toxicity
Halothane and related compounds	Inhalational anaesthetics	Hepatic toxicity
Herbal medications	Germander, greater celandine, kava, pennyroyal, valerian, etc.	Hepatic toxicity
Hexane, 2-hexanone	–	Peripheral neuropathy
Iron salts	Ferrous sulfate	Hepatic toxicity
Ketamine	–	Urological toxicity
Methadone	–	Respiratory depression
Methanol	Antifreeze, methylated spirit, synthetic alcoholic drinks	Retinal and CNS toxicity

Table 22.2 (*Continued*)

Compound/Group of compounds	Examples/Sources	Manifestation of toxicity
Monoamine oxidase inhibitors (MAOIs)	Phenelzine, tranylcypromine	Convulsions, cardiac arrest, cerebrovascular accident
Paracetamol	–	Hepatic and/or renal toxicity
Paraquat	–	Pneumotoxicity
Pemoline	–	Hepatic toxicity
Phenytoin	–	Hepatic toxicity
Phosgene	–	Pulmonary oedema
Phosphine	–	Pulmonary oedema
Sildenafil	–	Hepatotoxicity, retinal toxicity
Toxic metal salts	Cadmium, lead, mercury, thallium	Renal failure, CNS toxicity

In attempting to calculate either the time of dosing, or the size of a dose, the two most useful parameters are V and CL. If these are known, then $t_{½}$ can be calculated using the expression $t_{½} = (0.693 \times V)/CL$. The rate of drug elimination at any plasma concentration, C, is $C \times CL$, and clearance is a useful parameter for predicting steady-state concentrations on multiple dosing (Section 16.2.2.2). In addition, some appreciation of F is vital for interpretation of results after ingestion. If whole blood has been analyzed, then for drug that does not partition into erythrocytes, the plasma concentration may be calculated by assuming a haematocrit of 0.45–0.55. However, there will be a large discrepancy between blood and plasma concentrations for substances, hydroxychloroquine and lead for example, that are sequestered in erythrocytes.

22.3 Forensic toxicology

Forensic toxicology encompasses areas where toxicological analyses are performed to ascertain the most likely cause of a death, and when required the mode of death, be it either an assault involving the use of drugs or other poisons, or where an action has been influenced by drug use. Monitoring of either illicit, or performance-enhancing drugs in sport is a further important area, as is monitoring use of illicit/unauthorized drugs in the workplace, or on public roads.

22.3.1 Drug-facilitated assault

Any situation in which drugs or other poisons are given deliberately to another person or other animal without consent is an assault. This can include drugs given with homicidal intent as in euthanasia, for example, and drugs to facilitate either sexual assault, or robbery. It can also include medication errors.

'Date-rape' drugs include ethanol, benzodiazepines (such as diazepam, clonazepam, oxazepam, and temazepam), cannabis, cocaine, heroin, and amfetamine (UNODC, 2011; Bertol *et al*., 2018; Grela *et al*., 2018). More recently used drugs include flunitrazepam (Rohypnol, 'roofies'), γ-hydroxybutyrate (γ-hydroxybutyric acid, 4-hydroxybutanoic acid, GHB) and

its precursor γ-butyrolactone (GBL), hyoscine (scopolamine), and ketamine (Section 22.4.13). Sample collection as soon as possible after the reported incident is important. Qualitative identification in urine is often adequate except that with GHB quantitation is needed because it is an endogenous compound (Section 22.4.11).

In blood or plasma, most misused drugs can be detected at low mg L^{-1} concentrations for 1–2 d post-exposure. In urine the detection window after a single dose is 1.5–4 d. In chronic users, misused drugs can be detected in urine for approximately 1 week after use, sometimes longer in cocaine and cannabis users. In oral fluid, drugs of abuse can be detected at low mg L^{-1} concentrations for 5–48 h post-exposure. The detection 'window' for GHB is much shorter and may only be a few hours (UNODC, 2011). The short window of detection for some of these drugs linked to the reluctance of victims to come forward immediately works against the use of urine and oral fluid. Segmental hair analysis, on the other hand, has been used to detect drug administration several weeks post event, although there are many problems with this approach (Section 18.5.1).

22.3.2 Fabricated illness

Fabricated or induced illness (Munchausen Syndrome-by-Proxy) is a rare form of assault by a parent or carer, usually a child's biological mother, who either exaggerates, or deliberately causes features of illness in the child, or in rare cases in elderly or incapacitated dependents. Often covert administration of drugs or other poisons is involved. The practice may go undetected for years and can be especially difficult to diagnose even when suspicion has been aroused, in part because of the very wide range of substances that may be given (Table 22.3).

22.3.3 Post-mortem toxicology

The aim of post-mortem toxicology is either to help establish the cause of death, or to gain information on events immediately before death. If self-poisoning is suspected the diagnosis may be straightforward and all that may be required is confirmation of the agent(s) involved together with some indication of the amount ingested. If the cause of death is not immediately obvious, then possible poisoning or conditions such as alcoholic ketoacidosis should be considered. It may also be important to investigate whether death may have been a result of failure to adhere to prescribed therapy, for example with anticonvulsants, or prior use of psychoactive or incapacitating agents as with drug facilitated crime, hence sensitive and selective analytical methods are required.

It is not possible to look for every poison in every sample, even in the best equipped laboratory. Thus, it is important that those investigating fatalities understand what the laboratory can and cannot do. Herbal medicines, NPS, many volatile substances, and radioactive poisons such as ^{210}Po are areas where many laboratories may miss a poison (Byard, 2010; Lv *et al*., 2012; Teschke *et al*., 2012; Tettey & Crean, 2015; Jefferson *et al*., 2009). Of all the new misused drugs, the synthetic cannabinoids and opioids, notably fentanyl derivatives, and the *N*-benzyl substituted phenethylamines (so-called 'NBOMes') are emerging as amongst the most dangerous (Presley *et al*., 2016; Kronstrand *et al*., 2018; Elbardisy *et al*., 2019).

Some poisons, for example hydrogen sulfide and aluminium phosphide, are so reactive when in contact with tissues that any attempt to identify, let alone measure, the parent compound is futile (Jin *et al*., 2016). Other poisons may have been cleared from the circulation before toxicity either becomes manifest, or is recognized (Table 22.2). Some compounds, for example baclofen, carbon monoxide (measured as carboxyhaemoglobin saturation), digoxin, and lithium

Table 22.3 Some compounds/substances reported in malicious poisoning in children

Analgesics/antipyretics (paracetamol, salicylates)	Glafenine
Anticoagulants (warfarin)	Household products (oven cleaner, bleach)
Anticonvulsants (carbamazepine)	Inorganic salts (potassium chloride, sodium chloride, magnesium sulfate)
Antidepressants (amitriptyline, imipramine)	
Antidiabetics (insulin, phenformin, sulfonylureas)	Ipecac
Antihistamines (diphenhydramine)	Ketamine
Barbiturates (amylo/pheno/secobarbital)	Laxatives (bisacodyl, phenolphthalein, senna)
Benzodiazepines (clonazepam, diazepam, midazolam, temazepam)	Lidocaine
	Methanol
Carbon monoxide	Methaqualone
Chloral hydrate	Methotrexate
Chlorpyriphos	Opioids (codeine, methadone, propoxyphene)
Clonidine	Pancuronium
Clozapine	Paraquat
Cyanide	Pepper
Cyanoacrylate adhesive	Phenothiazines (alimemazine, chlorpromazine)
Digoxin	Phenylpropanolamine
Diuretics (furosemide, chlortalidone)	Pine oil
Doxylamine	Vitamin A
Ethanol	Water
Ethylene glycol	Xanthines (caffeine, theophylline)

(Table 1.7), may not be part of a normal laboratory 'screen' and thus the laboratory needs to be informed when they should be looked for. Particular problems may arise when the need to assess exposure to drugs or other poisons is only raised after the autopsy has been completed and the body has left the mortuary, or if there has been either extensive trauma, or decomposition.

The analysis of post-mortem specimens is especially challenging owing to the range and variable composition of the specimens that may be submitted, the wide variety of compounds and metabolites that may be encountered, and the invariably limited amount of sample available. If discovery of a body is delayed or death has occurred in a fire, for example, the extent of decomposition or charring can make not only specimen collection, but also the interpretation of qualitative let alone quantitative results even more difficult. Ensuring that the body is stored at 4 °C prior to the autopsy and that the autopsy is performed as soon as possible after death may minimize the risk of changes in blood analyte concentrations occurring before sampling (Table 22.4), but for some analytes post-mortem change occurs so rapidly that an analysis is often futile. Embalming or other similar procedures render most attempts at analysis futile. Contrast media injected prior to digital autopsy are another source of potentially confounding changes in the specimens sent for analysis (Palmiere *et al*., 2015).

Table 22.4 Factors influencing the likelihood of post-mortem change in blood analyte concentrations

Factor	Comment
Body storage temperature	The higher the temperature, the greater the potential for change
Headspace in specimen tube	Volatile analytes will equilibrate between sample and headspace; opening the tube when cold (4 °C) will minimize losses
Medical intervention	Attempted resuscitation may result in aspiration of stomach contents or movement of blood from central to peripheral sites after death, and possibly release of drug from traumatized tissue into blood
Nature of poison	Lipophilic compounds more likely to show increase in concentration than hydrophilic compounds; volatile or otherwise unstable compounds likely to show decrease; ethanol concentration may increase, or decrease depending on circumstance; depot injections may continue to release drug after death
Presence of poison in the airways, GI tract, or bladder	Post-mortem diffusion may alter concentrations in blood and in adjacent tissues (therefore sample liver from deep inside right lobe as this is furthest from stomach).
Position of body when found	May result in blood draining from central sites to peripheral sites
Site of sample collection	Central sites (heart, vena cava, or 'subclavian' blood) more likely to show changes than peripheral sites (e.g. therefore sample the femoral vein after appropriate isolation). Blood from the left ventricle of the heart is more likely to show change than blood from the right ventricle
Specimen preservation	Sodium fluoride needed to help stabilize certain analytes (e.g. ethanol, cocaine, 6-AM) – does not reverse any pre-collection changes
Time between death and specimen collection	A longer elapsed time gives more potential for changes as tissue pH decreases and autolysis proceeds
Transport of the body	May promote movement of blood from central site to peripheral sites
Volume of blood collected	A larger specimen volume less likely to be influenced by localized changes in blood composition, but increased risk of withdrawing blood from central sites such as the inferior vena cava if the femoral vein is being sampled and not isolated

The clinical interpretation of quantitative post-mortem toxicology results is fundamentally different to the interpretation of results obtained from samples obtained in life. Interpretation of post-mortem toxicology must take into consideration the clinical pharmacology of the agents in question, the age of the individual, the circumstances under which death occurred including the mechanics of the exposure and other factors such as whether prolonged resuscitation was attempted, how the body was stored prior to sampling, and how the samples were collected (Drummer, 2007; Ferner, 2008; Gruszecki *et al*., 2007; Byard, 2013). Further important considerations are the nature of the specimens sent for analysis, the stability of the analytes, and the suitability of the analytical methods employed (Skopp, 2010).

Blood obtained post-mortem is highly variable in its composition. There is always a degree of haemolysis and sedimentation of cells, clot formation, contamination with tissue fluid, or putrefaction/bacterial degradation may have occurred (Butzbach, 2010). Dehydration may

have resulted from exposure to heat during a fire, or dilution may have occurred in bodies recovered from water, a phenomenon perhaps more apparent in bodies recovered from fresh water than from sea water. Nevertheless, whole blood is commonly used in post-mortem toxicology because it is relatively simple to collect and is taken to be a relatively homogeneous liquid making it easier to dispense in the laboratory. In addition, there are often data on the plasma or serum (or sometimes whole blood) concentrations of many analytes measured during normal therapy in adults to provide at least some basis for the interpretation of results. However, the obvious comparison with plasma analyte concentrations measured in life must be performed with considerable caution, in part because the whole blood:plasma ratio may not be unity (Table 22.5).

Table 22.5 Whole blood:plasma ratios of some drugs and metabolites (data from Baselt, 2020)

Drug	Ratio blood:plasma	Drug	Ratio blood:plasma
Alprazolam	0.8	Ketamine	1.6–1.7
Amiodarone	0.6–0.8	Maprotiline	2.0–2.2
Amitriptyline	0.7–1.1	Methadone	0.7–0.8
Amphetamine	0.6–1.0[a]	Morphine	1.0–1.3
Chloroquine	2.5–5.9	Nordazepam	0.6
Chlorpromazine	0.8–1.5	Nortriptyline	1.2–1.7
Carbamazepine	0.6	Olanzapine	0.6
Cocaine	1.0	Oxazepam	0.7–1.1
Codeine	0.9	Paracetamol	1.1
Clozapine	0.8	Pentazocine	1.0–1.1
Norclozapine	1.0–1.3	Phenobarbital	0.8–0.9
Diazepam	0.6	Phenytoin	0.5–0.6
Dihydrocodeine	1.0	Promazine	0.7–0.8
Digoxin	0.5–0.6	Promethazine	0.6–0.7
Diphenhydramine	0.7–0.8	Quetiapine	0.6–0.7
Ethanol	0.7–0.9	Quinine	1.0
Fentanyl	0.8–1.0	Salicylate	0.8–1.2[a]
Norfentanyl	1.0–1.3	Temazepam	0.5
Fluoxetine	0.8–1.0	THC	0.5–0.6
Haloperidol	0.5–1.3	Tramadol	1.1
Reduced haloperidol	1.7–4.6	Valproate	0.5–0.6
Imipramine	1.0–1.2	Zopiclone	1.0

[a]Concentration dependent

Many tables of ‘therapeutic’ and ‘toxic’ blood concentration data have been published (e.g. Schulz *et al*., 2012), including compilations of post-mortem femoral blood concentrations observed in poisoning fatalities (Ketola & Ojanperä, 2019). Most such tables do not state the

criteria used to establish whether a death was indeed due to poisoning, the contribution of tolerance or disease, the site of blood sampling, and the interval between death and sampling. Moreover, simply relying on data from previous cases does not always recognize the possible magnitude, direction, and time-dependence of the changes in blood analyte concentrations that may take place after death (Saar *et al*., 2012). Unthinking reliance on tabulated 'toxic' or 'fatal' concentration data is likely to mislead (Kennedy, 2010; Jönsson *et al*., 2014), especially if, as is often the case, more than one poison is present (Jones *et al*., 2016). In the case of clozapine, for example, post-mortem blood concentrations of clozapine and norclozapine in clozapine-naïve subjects dying from acute clozapine poisoning are likely to be considerably less, perhaps 5–10-fold less, than in patients treated chronically with clozapine dying from causes unrelated to clozapine (Flanagan *et al*., 2005; Stanworth *et al*., 2012).

Blood concentrations of analytes with a relatively small V such as lithium may change minimally after death, although continued absorption from the gastrointestinal tract may occur post-mortem even with such compounds and this may be reflected in blood sampled from central sites such as the heart. Similarly, although unconjugated morphine concentrations in ventricular post-mortem blood are consistently higher than those at peripheral sites, there appears to be on average little change in morphine concentrations with time after death in blood specimens obtained from either central, or peripheral sites. On the other hand, marked increases in the concentrations of many lipophilic drugs with a relatively large V such as the tricyclic antidepressants when given chronically have been documented in central (heart, vena cava) as opposed to peripheral (femoral) blood (Han *et al*., 2012).

It is sometimes suggested that published values of V can be used to estimate the likelihood of post-mortem increase in blood concentration given the availability of information such as daily dose, etc. However, published values of V are themselves estimates. For clozapine, for example, literature values of V are 2.5–10 L kg^{-1}. For clozapine and norclozapine mean post-mortem increases in (presumed femoral) blood concentrations of these compounds of 480 and 360 %, respectively, have been observed in patients not thought to have died from clozapine poisoning (Flanagan *et al*., 2005). Similar findings have been reported with another centrally acting drug, fentanyl, in femoral blood (Olson *et al*., 2010). The dangers of using a concentration measured in post-mortem blood and a published value of V to attempt to estimate the peri-mortem plasma concentration or perhaps the dose taken are evident without even considering other variables such as the time course of exposure, the route of administration, and the fact that tissue equilibration may have been incomplete.

A further problem is that some analytes may be lost from, or even produced in, the body after death. Analysis of other fluids (urine, vitreous humour) and screening for other possible fermentation products is needed to confirm a blood ethanol result, especially if death involved extensive trauma or autolysis was advanced (Section 22.4.1.1). Analysis of urine and especially of vitreous humour may facilitate detection of 6-AM thereby giving definitive evidence of diamorphine use. Vitreous biochemistry may also be useful in some situations, such as assessing hyperglycaemia at the time of death, whilst β-hydroxybutyrate assay may be helpful in the investigation of alcohol-associated or diabetic ketosis (Belsey & Flanagan, 2016). Vitreous creatinine can help in interpreting blood concentrations of drugs or metabolites normally eliminated in urine, for example morphine conjugates when morphine has been given in palliative care. However, after death vitreous potassium increases and vitreous glucose falls rapidly such that measurement of these analytes is of no use in attempting to assess perimortem plasma potassium and hypoglycaemia, respectively.

Tolerance cannot be measured in retrospect, although hair or nail analysis can sometimes be employed in an attempt to assess exposure to toxic metals, illicit drug use or adherence to

prescribed medication in the weeks or months before death. Although hair is well preserved even after burial, analysis gives no information pertaining to acute poisoning and qualitative information on exposure is usually all that can be gleaned (Musshoff & Madea, 2007). Many factors such as differences in hair growth rate depending on anatomical region, age, sex, ethnicity, hair colour, and inter-individual variability in drug/metabolite incorporation taken together mean that interpretation of quantitative results, even in samples obtained in life, is not easy if the aim is to obtain a chronological record of exposure (Pragst & Balikova, 2006). Moreover, there are always the possibilities of atmospheric deposition of drugs on hair, of passive systemic exposure arising from, for example, absorption of drugs from contaminated surfaces via the skin and after death of diffusion of analyte into hair as autolysis proceeds. Note that laboratory procedures claimed to remove analyte from the hair surface prior to analysis of the hair matrix itself may simply redistribute analyte from the hair surface into the matrix, thus calling into question much of the literature on this topic (Section 18.5.1). External contamination can also affect the analysis of nail clippings (Krumbiegel *et al*., 2016).

Clearly, when there is the possibility that a patient or victim may have developed tolerance to a particular drug and/or that post-mortem changes in blood analyte concentrations may have occurred, the availability of additional information such as the results of an analysis of vitreous humour may be helpful. Analysis of bile and of tissue such as liver, however, really only provides qualitative information (Bévalot *et al*., 2016; Ferner & Aronson, 2018). Site-to-site variation in post-mortem drug concentrations has been reported within certain large organs such as the liver and also in muscle. Site-to-site variation in brain drug concentrations are also likely. Attempts to assess the dose from an isolated tissue measurement, in liver for example, can only be speculative.

Measurement of poison concentrations in a representative specimen of gastric contents can sometimes give an estimate of unabsorbed dose if the total volume of contents is known. However, simply detecting a basic drug in gastric contents does not prove recent ingestion, because ion-trapping of basic drugs that diffuse from blood into the stomach can occur, and in any event salivary excretion will have been almost inevitable.

Finally, the possible role of the ‘molecular autopsy’ in providing additional information to help interpret post-mortem toxicology data must be mentioned. In the case of ‘sudden cardiac deaths’ in those aged less than 40 years, the role of genetic testing has evolved as an important feature in both establishing an underlying diagnosis and in screening at-risk family relatives (Nunn & Lambiase, 2011; Semsarian & Hamilton, 2012). In cases where no definitive cause is identified at post-mortem, i.e. sudden unexpected death (also known as sudden arrhythmic death), genetic testing, including pharmacogenetic testing, may emerge as a useful adjunct in the investigation of the cause of death. Whilst the US FDA has required manufacturers to pinpoint relevant pharmacogenomic markers for certain drugs, including carisoprodol, citalopram, codeine, and risperidone, with the aim of identifying individuals who need lower or higher doses, or even a different drug (Agrawal & Rennert, 2012), more research into the cost/benefit of such an approach is needed before it can be advocated in routine case work (Sajantila *et al*., 2010).

22.4 Gazetteer

The last few years have seen dramatic expansion in the numbers of NPS available, especially novel ‘designer’ benzodiazepines, synthetic cannabinoids (‘spice’), novel opioids, and synthetic cathinones (‘bath salts’) and no more than a brief overview of the problems associated with these

ever-expanding groups of compounds can be attempted (Logan *et al*., 2017, Kronstrand *et al*., 2018; Welter-Luedeke & Maurer, 2019; Kraemer *et al*., 2019). In addition, new pharmaceuticals are introduced all the time bringing new problems for the analyst.

Methods for specific analytes and also on the interpretation of the analytical results can often be found in the literature and in secondary sources such as those listed in Table 22.6 or subsequent editions. Information on dose, structure, pK_a, and the occurrence of metabolites can often be obtained from these sources. Information and archives of data on newly reported NPS can be found at www.npsdiscovery.org and at www.unodc.org/tox.

Table 22.6 Analytical toxicology: basic reference sources

Author/editor	Title/Volume/Edition	Publication details
Baselt RC	*Disposition of Toxic Drugs and Chemicals in Man*. Edition 12	Seal Beach, CA: Biomedical Publications, 2020
Hathaway GJ, Proctor NH, eds.	*Proctor and Hughes' Chemical Hazards of the Workplace*. Edition 5	New York: Wiley, 2004
Karch SB, Drummer O	*Karch's Pathology of Drug Abuse*. Edition 5	Boca Raton: Taylor & Francis, 2015
Moffat AC, Osselton MD, Widdop B, Watts J, eds.	*Clarke's Isolation and Identification of Drugs*. Edition 4[a]	London: Pharmaceutical Press, 2011
Nelson LS, Hoffman RS, Howland MA, Lewin NA, Goldfrank LR, Smith SW, eds.	*Goldfrank's Toxicologic Emergencies*. Edition 11	New York: McGraw-Hill, 2019

[a]See also: Moffat AC, Osselton MD, Elliott SP, eds. *Clarke's Analysis of Drugs and Poisons,* [online]. London: Pharmaceutical Press (http://www.medicinescomplete.com/)

The volume by Baselt is the principal source of relevant information in analytical toxicology. Manufacturers of particular compounds may also be able to give details of assay methods, current literature, and potential ISTDs, although their own recommended methods may become 'fossilized' because of regulatory requirements.

22.4.1 Alcohols

22.4.1.1 Ethanol

Blood ethanol analysis is the commonest request in forensic toxicology, yet the interpretation of results is fraught with problems (Kugelberg & Jones, 2007). Some enzymatic methods for ethanol analysis used by clinical laboratories may give falsely elevated results in critically ill patients or post-mortem specimens. Analysis for alcohols should be performed using HS-GC (Section 19.2.2.1), which provides a reliable ethanol measurement and can be used to quantify acetone, methanol, 2-propanol, and other alcohols. However, ethanol can be lost from or generated in blood specimens if contaminated by bacteria or yeasts, or lost by evaporation if the sample tube has a large residual airspace or is not tightly sealed. There is also the possibility

of diffusion of ethanol from gastric residue or airways contaminated by vomitus to blood in central vessels such as the vena cava.

Deaths from acute ethanol poisoning in adults are usually associated with blood ethanol concentrations of the order of 3 g L^{-1}, although lower concentrations may be encountered if there are complications such as head injury, inhalation of vomit, or positional asphyxia. Where there is putrefaction or extensive injury to the body, or there is a delay of several days between death and the post-mortem examination, it is advisable to analyze blood taken from a peripheral vessel, urine, and vitreous humour. Use of skeletal muscle as an additional specimen for ethanol analysis has also been advocated, but it should only be used if no other specimen is available and the results must be interpreted with caution.

In blood, glucose and lactate provide substrates for microbial ethanol production. These compounds are not normally present in either vitreous humour, or urine, except in samples from diabetics, and vitreous humour is in any case well protected from bacterial infiltration after death. Thus, analysis of these latter fluids can provide important corroborative information, although this is not always conclusive. Ethanol distribution ratios (vitreous humour/femoral venous blood) show wide variation in post-mortem samples and therefore caution is needed when attempting to use vitreous humour to estimate the ethanol concentration in femoral venous blood. Dividing the vitreous humour concentration by two has been said to give a conservative estimate of the femoral blood concentration (Jones & Holmgren, 2001), but experience suggests this is too high a figure to use.

Measurement of butanol and other fermentation products, and performing microbiological analysis of the specimen, can also be undertaken to help assess any putrefactive changes. 2-Methyl-2-propanol (*tert*-butanol) has been recommended as an ISTD for GC ethanol assay as it is not likely to be produced by putrefaction. Microbiological investigation to help assess the likelihood of post-mortem changes in ethanol concentrations are performed rarely nowadays. However, ethyl glucuronide (Section 18.5.5) has been measured in post-mortem fluids and tissues, and may have a role in assessing post-mortem ethanol production (Vezzoli *et al*., 2015).

Addition of high concentrations of fluoride (at least 0.5 % w/v) will inhibit microbial metabolism and prevent any further change in a post-mortem blood, urine, or vitreous humour sample. However, the possible analytical consequences of adding fluoride, for example salting-out if using HS-GC and inhibition of enzymatic ethanol assays, must be remembered.

22.4.1.2 Ethylene glycol and methanol

Metabolism of ethylene glycol (1,2-ethanediol, most commonly found in radiator antifreeze) and of methanol (found in antifreeze, synthetic alcoholic drinks, hand swabs, etc.) gives a number of metabolites (Figure 22.1). Production of glycolic or formic acid leads to the acidosis and anion gap that characterizes poisoning with these compounds, whilst further metabolism of glycolate gives oxalate, which chelates calcium causing hypocalcaemia and deposition of calcium oxalate in tissues.

The initial features of poisoning with these agents and higher alcohols such as diethylene glycol, 1,2-propanediol (propylene glycol), and 2-propanol, are similar to those of ethanol and thus the diagnosis, and with it the opportunity for prompt antidotal therapy, may be missed (McQuade *et al*., 2014; Ng *et al*., 2018). Indeed, sometimes a diagnosis of ethylene glycol poisoning is only made in retrospect when glycolate is detected (see Section 19.3.2), often during screening for inborn errors of metabolism.

The analysis of toxic alcohols is not straightforward in practice, and may include the need to monitor blood ethanol when used as antidote. HS-GC-FID can be used for methanol, ethanol,

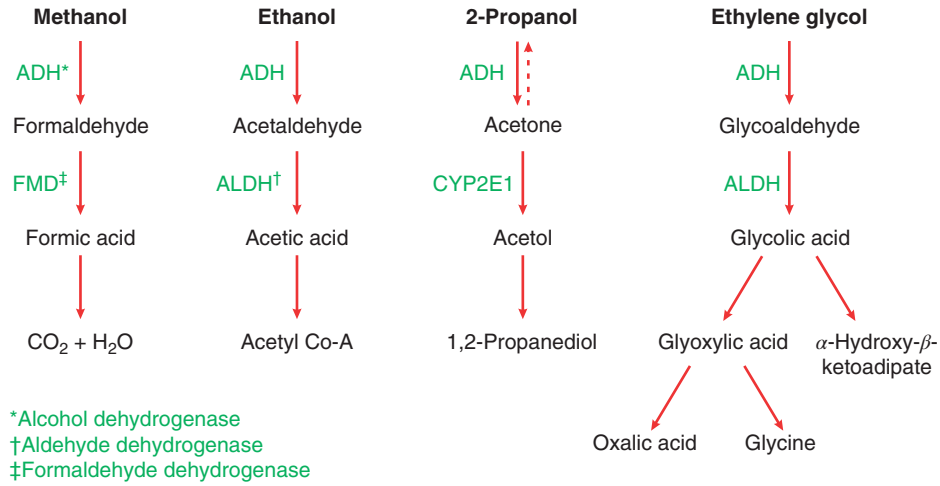


Figure 22.1 Mammalian metabolism of selected alcohols

2-propanol (Section 19.2.2.1), and GC-FID or GC-MS for glycols (Section 19.3.2). However, GC-MS of glycols may not be available on an emergency basis, hence an automated assay (Catachem) for ethylene glycol has been evaluated. The method has proved reliable in clinical use except that 1,2-propanediol interferes at higher concentrations (Robson *et al*., 2017). The analysis of metabolites such as formate and glycolate, and the detection of calcium oxalate crystals in urine (Section 2.3.3), are less useful because the aim of early diagnosis and treatment is to prevent production of toxic metabolites.

22.4.2 *Anabolic steroids*

Anabolic steroid detection and identification remain key issues in substance misuse testing, medical diagnostics, food safety control, and doping control. The use of image and performance enhancing drugs (IPEDs), notably anabolic steroids (Figure 22.2), has increased markedly over the last 20 years, especially in men. Although these compounds are of relatively low acute toxicity, they may have major unwanted effects such as acne, testicular atrophy, cardiomegaly,

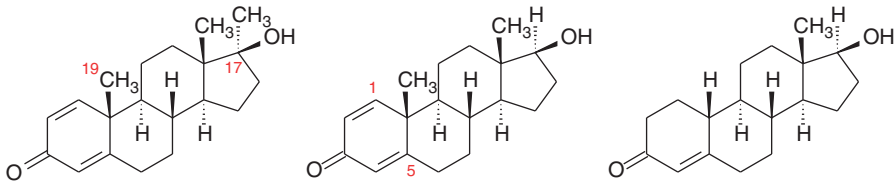


Figure 22.2 Some synthetic anabolic steroids

breast development in men, aggression, headache, and hallucinations. The long-term adverse health effects of the widespread use of these compounds are unknown (Horwitz *et al.*, 2019).

The misuse of unknown designer androgenic anabolic steroids is an important issue because these are the drugs of choice for doping in sport (Waller & McLeod, 2017; Anawalt, 2018). Analyses of anabolic agents, including designer steroids (Table 22.7), have been enhanced through the use of LC-MS/MS and GC-C-IRMS (Section 13.3.2). A knowledge of steroid metabolism is very important, especially when looking to detect use of the relatively new selective androgen receptor modulators (SARMs) (Thevis & Schänzer, 2018).

Table 22.7 Some steroids liable to misuse (alternative names in brackets)

Dihydrotestosterone and analogues	Estranes	Testosterone and analogues
Desoxymethyltestosterone (Madol, Pheraplex)	Dimethandrolone	Adrenosterone
Dihydrotestosterone (DHT)	Dimethyltrienolone	Boldenone
Drostanolone	Metribolone (methyltrenbolone)	4-Chlorodehydromethyl-testosterone (Turinabol)
Epiandrosterone	Mibolerone (dimethylnortestosterone)	4-Chlorotestosterone (Clostebol)
Mestanolone (Androstalone)	Nandrolone (19-nortestosterone)	Fluoxymesterone (Halotestin)
Mesterolone (Proviron)	Norbolethone (norboletone)	11-Ketotestosterone
Metenolone enanthate (Primobolan Depot)	Tetrahydrogestrinone (The Clear)	Metandienone (methandrostenolone, Dianabol)
Methasterone (methyldrostanolone, Superdrol)	Trenbolone	Methyltestosterone (Android, Metandren)
Methyl-1-testosterone	Trestolone (7α-methyl-19-nortestosterone)	Testosterone
Oxandrolone (Oxandrin, Anavar)		
Oxymetholone (Anadrol, Anapolon)		
Prostanozol		
Stanozolol (Winstrol)		
1-Testosterone (dihydroboldenone)		

Use of testosterone to enhance appearance and performance is widespread. I.m. injections of esters such as testosterone propionate and testosterone decanoate lead to long-lasting effects (weeks or months). Many strategies have been adopted to disguise use of this drug (Alquraini & Auchus, 2018). Although measurement of the urinary testosterone:epitestosterone ratio has aided detection of testosterone doping in the past, nowadays detecting testosterone administration relies on GC-C-IRMS (Piper & Thevis, 2017). A multidimensional GC-C-IRMS method for analyzing urinary steroids in doping control has been developed and validated. For every single individual, the $\delta^{13}C$ ($^{13}C/^{12}C$) values (‰) of the selected target compounds, i.e. testosterone

and/or its precursors/metabolites, are compared with those of endogenous reference compounds (Putz *et al*., 2018; de la Torre *et al*., 2019).

The control of the use of the designer anabolic steroid 19-nortestosterone relies on detection of the urinary metabolite 19-norandrosterone glucuronide. However, low concentrations of endogenous 19-norandrosterone can be found in urine. Thus, GC-C-IRMS should be used to establish an exogenous source of 19-norandrosterone, the use of other strategies proving inconclusive (Piper *et al*., 2016).

There are problems with relying on the use of GC-C-IRMS to prove the source of an analyte, however. The major diagnostic metabolite of nandrolone (19-norandrosterone) is also produced endogenously, hence GC-C-IRMS is necessary to help establish an exogenous source of the compound. However, of nine nandrolone-containing and five testosterone-containing preparations, 10 displayed δ^{13}C values within the range expected for synthetic steroids (less than –27 ‰), but four nandrolone preparations displayed δ^{13}C values that overlapped with the values considered to demonstrate an endogenous origin (range: –26 to –16 ‰; Brailsford *et al*., 2018).

22.4.3 Antidepressants

Deaths due tricyclic antidepressant (TCA) poisoning remain relatively common in the UK (Handley & Flanagan, 2014), which is of itself not surprising given the group of patients for which they are often prescribed. Be this as it may, the interpretation of post-mortem toxicology results poses similar problems to deaths involving antipsychotics and other drugs with a relatively large *V*, i.e. it is likely that a peripheral rather than a cardiac blood sample will more nearly reflect peri-mortem blood concentrations, but that post-mortem changes may complicate matters. Analysis of metabolites as well as the parent compound may help differentiate acute overdosage from chronic exposure.

The SSRIs are less toxic than TCAs in acute overdose, but nevertheless may be encountered after fatal self-poisoning (Handley & Flanagan, 2014). Venlafaxine is especially toxic in overdose, followed by citalopram. Citalopram is marketed as a racemate and the (*S*)-(+)- enantiomer is available separately (escitalopram). Chiral analysis may be helpful in deciding which drug has been taken after overdosage (Johansen, 2017).

22.4.4 Antidiabetic drugs

Although hypoglycaemia is an obvious adverse effect of antidiabetic agents such as chlorpropamide, gliclazide, glipizide, metformin, and insulin (Johansen & Christensen, 2018), it may occasionally develop during treatment with drugs used in everyday clinical practice, including NSAIDs, analgesics, antibacterials, antimalarials, antiarrhythmics, and antidepressants (Ben Salem *et al*., 2011). Blood and vitreous humour glucose concentrations generally fall rapidly after death and are an unreliable guide to ante-mortem glucose concentrations (Belsey & Flanagan, 2016).

22.4.4.1 Insulin and C-peptide

Insulin is initially synthesized as proinsulin, a form of insulin in which the α- and β-chains of insulin are linked to a third polypeptide chain (connecting peptide, C-peptide). Hydrolysis of proinsulin in the pancreas leads to secretion of insulin and C-peptide in equimolar amounts, and therefore in theory plasma insulin and C-peptide can be measured and the ratio of the two

analytes used as an indicator of endogenous insulin production when exogenous insulin, which lacks C-peptide, may have been given.

However, secretion of insulin and C-peptide is accompanied by the release of small amounts of proinsulins (proinsulin and other peptides related to proinsulin cleaved in a variety of positions). The first insulin assays, also called immunoreactive insulin (IRI) assays, were RIAs with polyclonal antisera (Section 6.2.3.1), which cross-reacted with proinsulins. With the development of monoclonal antibodies, two-site insulin assays were introduced, namely immunoenzymometric (IEMA), immunofluorometric (IFMA), and immunoradiometric assay (IRMA) methods. 'Insulin' values given by most IEMA/IRMA methods were around 20–40 % lower than corresponding IRI results. Secondly, insulin has a shorter $t_{½}$ than C-peptide. On the other hand, the $t_{½}$ of C-peptide is prolonged in renal impairment.

The insulin-degrading enzyme (IDE) (EC 3.4.24.56), specific for insulin, is widely distributed in tissues, including erythrocytes. However, haemolysis increases plasma insulin. Use of EDTA anticoagulant is also associated with an artefactual rise in plasma insulin. The presence of hepatic or renal insufficiency, or of anti-insulin or anti-proinsulin antibodies (analogous to the situation with digoxin assay if anti-digoxin F_{ab} antibody fragments have been given) may be further possible sources of error. The nature of the interference by anti-insulin antibodies in RIA depended on their affinity for insulin and the method used to separate bound from free radioligand, thus leading to either falsely high, or low results. In two-site immunoassays, the presence of anti-insulin antibodies could give rise to overestimation of plasma free insulin. The degree of overestimation depends on the comparative affinity of the autoantibodies and of the antibodies used in the assay.

If the affinity of the assay antibodies exceeded that of the autoantibodies, displacement of the autoantibody–insulin complex may have occurred, leading to over-estimation of the insulin concentration (Sapin, 2003). Measurement of free insulin required the removal of anti-insulin antibodies and antibody-bound insulin. Total insulin (free + bound) could be assayed by dissociating bound insulin by acidification before the antibodies were separated from the reaction mixture. Most methods used polyethylene glycol (PEG) to precipitate the antibodies. In two-site assays, other types of antibody may lead to falsely elevated results. These include anti-mouse antibodies (if mouse monoclonal antibodies are used) and autoantibodies such as rheumatoid factor. These types of interference are not specific to insulin assays, but apply to all two-site assays (Marks, 2002).

Plasma C-peptide is stable for 2–3 weeks at –20 °C and for up to 6 months at –80 °C, whereas plasma insulin is more stable (*ca*. 5 hours at room temperature, one week at 4 °C, several months at –20 °C). On the other hand, C-peptide is not hydrolyzed by insulin-degrading enzyme. Anti-insulin antibodies bind proinsulin via its insulin moiety and greatly retard its clearance from the circulation. Because of cross-reaction with proinsulin in some C-peptide immunoassays, proinsulin bound to anti-(pro)insulin antibodies can interfere.

If it is suspected that death has followed the injection of either an overdose of an insulin, or insulin administration to a non-diabetic, blood should be taken as soon as possible after death and serum separated and deep-frozen for subsequent analysis of insulin and C-peptide. This being said, meaningful interpretation of serum insulin concentrations is often not possible using immunoassays not only because of the problems inherent in such assays, but also because of the likelihood of analyte degradation post-mortem (Marks, 2015). The introduction of insulin analogues to clinical use is a further complicating factor.

22.4.4.2 Insulin analogues

Insulin analogues have modified amino acid sequences compared to native human insulin, and as such may not cross-react with insulin assays. The cross-reactivity of five analogues (lispro, aspart, glulisine, glargine, detemir) and two glargine metabolites (M1 and M2) with 16 commercial human insulin immunoassays as a function of concentration has been investigated (Heurtault *et al*., 2014). The cross-reactivity values for insulin analogues or glargine metabolites ranged from 0–264 %. Four assays were more specific to human insulin, resulting in negligible cross-reactivity with the analogues. However, none of the 16 assays was completely free of cross-reactivity with either analogues, or metabolites.

Similar results were obtained in a study of four analogues (aspart, glargine, glulisine, and lispro) on Immulite 2000 and Advia Centaur XP (Siemens Healthcare Diagnostics), and Elecsys Modular Analytics E170 (Roche) systems (Dayaldasani *et al*., 2015). In the Elecsys E170 assay, relevant cross-reactivity was only detected with insulin glargine, whereas in the other analyzers all analogues except glulisine showed significant interference.

In a study of 15 exogenous insulin preparations, the ability of 10 insulin immunoassays used in the UK to detect a single exogenous insulin at two different concentrations ranged from 0 to >140 % (Parfitt *et al*., 2015). Four assays had no cross-reactivity with any synthetic analogue. Two detected all insulin types (human, animal, and synthetic analogues), with the remainder having variable cross-reactivity (Table 22.8). It is important that laboratories are aware of the limitations of their insulin immunoassays to avoid missing a diagnosis of hypoglycaemia secondary to excessive exogenous insulin (Chemmanam *et al*., 2017).

Table 22.8 Cross-reactivity of 10 insulin immunoassays with 15 insulin analogues (Parfitt *et al*., 2015–reproduced with permission of Elsevier)

	Average percentage recovery (BOO and 1000 pmol/L)														
	Human insulin				1 amino acid difference			2 amino acid difference		3 amino acid difference				Complex insulins	
	Actrapid	Humulin S	Humulin I*	Insulatard*	Hypurin neutral porcine	Novorapid (aspart)	Hypurin isophane porcine*	Humalog (lispro)	Apidra (glulisine)	Lantus (glargine)	Hypurin neutral bovine	Hypurin isophane bovine*	Hypurin Protamine Zinc*	Levemir (determir)	Tresiba (degludec)
Mercodia Iso-Insulin	140	140	140	128	108	140	14	139	140	93	95	140	78	46	44
Abbott Architect	108	124	115	103	90	110	9	129	10	140	27	81	84	30	24
Siemens ADVIA Centaur Insulin	120	140	134	139	137	140	16	140	0	140	0	0	0	24	13
Diasorin Liaison XL Insulin	104	124	123	139	128	2	19	1	0	16	88	140	140	1	1
PE AutoDELFIA	93	115	111	123	140	1	23	1	1	10	55	115	121	1	1
Mercodia Insulin	95	113	113	96	116	0	9	0	0	7	62	140	95	0	0
Beckman Access Ultra Sensitive	79	103	94	79	67	108	6	98	2	124	26	138	41	1	3
Roche Elecsys Insulin	107	134	123	112	19	0	2	0	0	20	19	76	31	0	4
Siemens Immulite 2000	66	74	72	72	16	12	1	10	0	54	19	66	43	8	2
MSIA	140	140	139	140	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

<20	20–79	>80	Results truncated at > 140% recovery

22.4.4.3 LC-MS of insulin and insulin analogues

Protein and peptide identification and quantification have benefited from advances in LC-MS/MS (Bowers, 2009). LC-MS/MS of human insulin and C-peptide has been described (Taylor *et al*., 2016). Bovine insulin and [^{13}C/^{15}N]-C-peptide were used as ISTDs. Of the five

insulin analogues studied, only lispro caused significant interference in the insulin assay. There were no observed interferences for C-peptide. Following immunoaffinity extraction, human insulin and some analogues have been measured with both MALDI-TOF-MS and LC-MS (Nedelkov *et al*., 2018). Porcine insulin was used as the ISTD.

The use of exogenous insulin for performance enhancement in sport is difficult to detect. It has been suggested that the metabolite profile of circulating insulin is altered after s.c. insulin administration because of exposure to epidermal proteases (Thomas *et al*., 2015).

22.4.5 Antiepileptics

Analyses for antiepileptic (anticonvulsant) drugs may be requested not only in the diagnosis of acute poisoning, but also in the diagnosis of brain-stem death because drugs such as barbiturates or benzodiazepines may have been used in an attempt to control convulsions. Moreover, in the event of sudden unexpected death in epilepsy, an analysis may be requested with the aim of assessing adherence to antiepileptic medication in the days before death.

Drugs such as carbamazepine, lamotrigine, and sodium valproate are also used as mood stabilizers, and as such may be encountered when investigating deaths in people with mental health problems. Gabapentin and pregabalin are increasingly encountered in substance misuse-related deaths and may act to enhance the toxicity of opioids (Evoy *et al*., 2017; Handley *et al*., 2018).

22.4.6 Antipsychotics

Measurement and interpretation of the results of blood phenothiazine concentrations is often difficult because not only are some of these drugs, including chlorpromazine, unstable in aqueous solution, but metabolites of similarly poor stability and unknown pharmacological activity may be present. Haloperidol measurement is complicated by the presence of its tautomer ‘reduced haloperidol’ and a further metabolite 4-(4-chlorophenyl)-4-hydroxypiperidine (CPHP), which have to be taken into account when reporting blood haloperidol measurements.

With second generation or ‘atypical’ antipsychotic drugs, analyte stability is generally less of an issue although the sulfur-containing drug olanzapine is dramatically unstable in post-mortem blood albeit stable in plasma (Fisher *et al*., 2013b). However, the possible impact of factors such as tolerance, adverse reactions to the treatment (Handley *et al*., 2016), the risk of sudden death in schizophrenia, and the possibility of post-mortem changes (Figure 22.3; Flanagan *et al*., 2005), may make interpretation of post-mortem toxicology anything but straightforward.

22.4.7 Barbiturates

Once widely used as sedative and hypnotic agents, barbiturates such as amobarbital (amylobarbital), butobarbital, pentobarbital, and quinalbarbital (secobarbital), are no longer often prescribed in Western countries, although phenobarbital still finds use as an antiepileptic agent (Section 22.4.5). Their long-established use in ‘cutting’ (diluting) illicit drugs such as heroin has also declined substantially. Barbital (5,5-diethylbarbituric acid), however, is also still used in veterinary medicine and as a laboratory reagent and is a classic drug encountered after self-harm by laboratory employees. Barbiturates are highly toxic respiratory depressants when taken in overdose, and compounds such as pentobarbital, often sourced over the internet, and sometimes taken with an antiemetic, are still encountered in self-poisoning episodes (Druda *et al*., 2019; Solbeck *et al*., 2019).

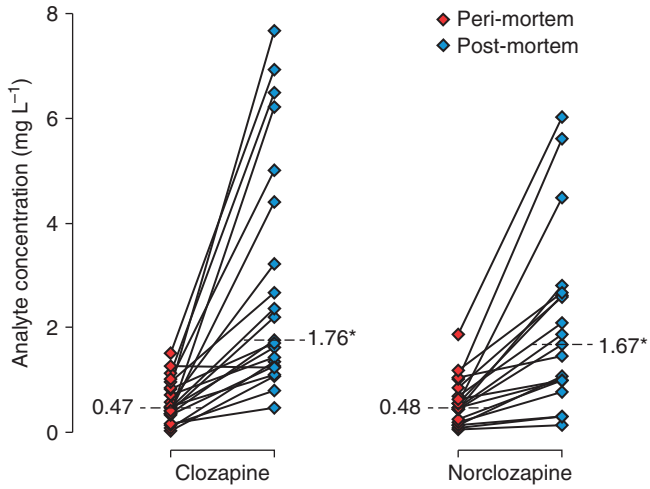


Figure 22.3 Median blood clozapine and norclozapine concentrations before and after death in patients dying from causes other than clozapine self-poisoning (*$p < 0.0001$, Wilcox ranked-pairs)

22.4.8 Benzodiazepines

Benzodiazepines are widely misused for their sedative and hypnotic effects, typically as part of a poly-drug use pattern. Amfetamine and cocaine users often take benzodiazepines to ameliorate the 'crash' during withdrawal, which may give rise to features such as anxiety, dysphoria, and insomnia. Opioid users commonly take benzodiazepines to enhance the 'high', with the use of diazepam being common (Vogel *et al*., 2013). Benzodiazepines licensed for use in countries outside of the UK have been sold and used as 'legal highs' within the UK, in particular phenazepam.

More recently, 'designer' benzodiazepines have become a rapidly growing class of drugs of abuse in their own right (Greenblatt & Greenblatt, 2019). The first designer benzodiazepines to become available online were diclazepam, flubromazepam, and pyrazolam (Figure 22.4). Most of these compounds were originally synthesized as drug candidates, but none are approved for medicinal use (Moosmann & Auwärter, 2018). Active metabolites of benzodiazepines have also been marketed as designer drugs, for example fonazepam (desmethylflunitrazepam) and nifoxipam (3-hydroxydesmethylflunitrazepam) are both active metabolites of flunitrazepam (Katselou *et al*., 2017).

22.4.9 Carbon monoxide

Unintentional carbon monoxide poisoning from, for example, an improperly ventilated gas fire, often affects more than one person. Children and pets are at especial risk. In most cases, the source of carbon monoxide will be obvious. Water-pipe smoking, for example, may give up to 50 % carboxyhaemoglobinaemia (Eichhorn *et al*., 2018). On the other hand, carbon monoxide can arise from unexpected sources such as dry wood stores (Fisher *et al*., 2013a). Carbon monoxide poisoning is also a well-known risk in piston-engined aircraft.

Measurement of blood carboxyhaemoglobin saturation (Table 22.9) may be helpful when investigating deaths in fires, for example. However, the analysis should be carried out

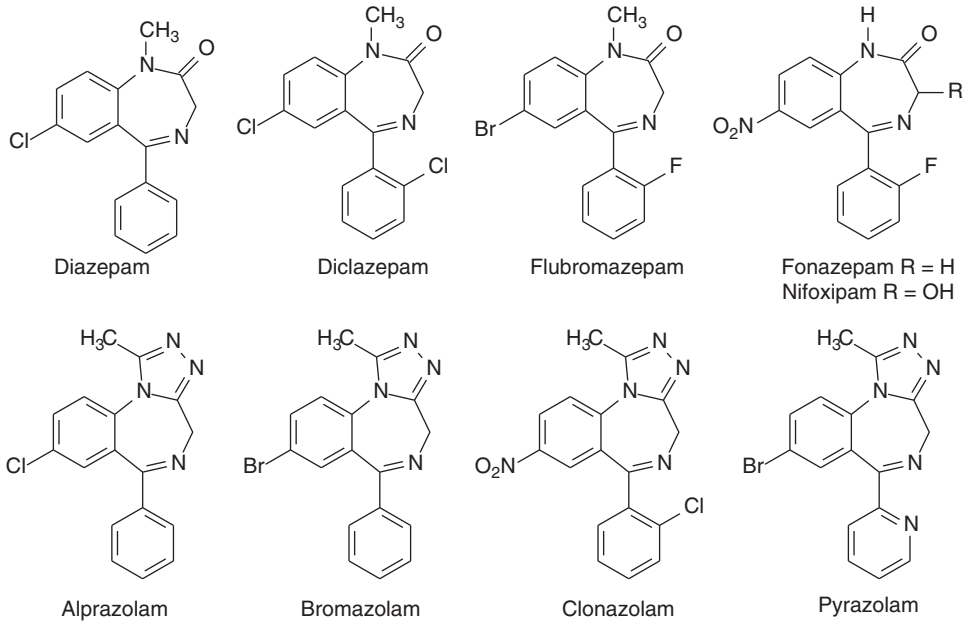


Figure 22.4 Comparison of the chemical structures of selected designer benzodiazepine drugs to licensed benzodiazepines (diazepam and alprazolam)

Table 22.9 Blood carboxyhaemoglobin saturation and clinical features of toxicity

Carboxyhaemoglobin saturation (%)	Clinical features
<1	Endogenous carbon monoxide production
3–8	Cigarette smokers
<15	Heavy (30–50 cigarettes/day) smokers
>20	Headache, weakness, dizziness, impaired vision, syncope, nausea, vomiting, diarrhoea (patients with heart disease are at especial risk).
>50	Coma, convulsions, bradycardia, hypotension, respiratory depression, death

without delay because there is a tendency for carbon monoxide to be lost on storage. Blood carboxyhaemoglobin is usually not raised in 'flaming' or 'flash' fires. Blood cyanide is of little value in fire investigations and tends to either decrease, or increase on storage. Moreover, the contribution of heat shock, other noxious gases, and/or oxygen deprivation to death is often unknown.

22.4.10 Cannabinoids

'Cannabis' contains Δ^9-tetrahydrocannabinol (Δ^9-THC, THC), Δ^8-tetrahydrocannabinol (Δ^8-THC), cannabidiol (CBD), and some 140 other cannabinoids in varying proportions.

A single dose of cannabis in an inexperienced user, or an overdose in a habitual user, can sometimes induce a variety of unpleasant effects including anxiety, panic, paranoia, and feelings of impending doom. The subject may become unusually pallid (a 'whitey'). In addition to difficulty in standing up and staggering, lack of eye coordination may be a further feature. The effects usually persist for only a few hours. Many of the toxic effects of cannabis can be confused with, and compounded by, co-ingestion of alcohol. Back-calculation of time of cannabis use from post-mortem blood concentrations of cannabinoids is not recommended because their plasma half-lives may be hours or days depending on when cannbinoids were last used (Hartman *et al*., 2016).

A complicating factor is the increasing availability of relatively high strength cannabis ('skunk') in the UK and elsewhere. This has been deliberately cultivated, usually indoors, to contain much larger amounts of THC, the principal active component of cannabis, and much lower amounts of CBD, than field-grown plants. CBD is non-intoxicating and has been found to offset the harmful effects of THC on memory impairment and psychosis (Freeman *et al*., 2019). Whereas standard cannabis can be expected to have a THC content of 2–8 % w/w, 'skunk' may contain over 20 % w/w and very little CBD. Epidemiological and experimental evidence demonstrates that cannabis with high THC concentrations and negligible CBD concentrations is associated with an increased risk of psychotic outcomes, an effect on spatial working memory and prose recall, and increased reports of the severity of cannabis dependence (Wilson *et al*., 2019).

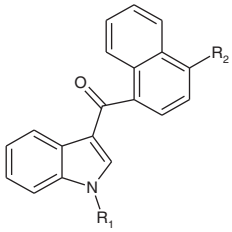
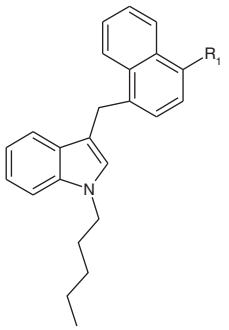
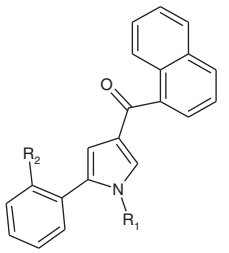
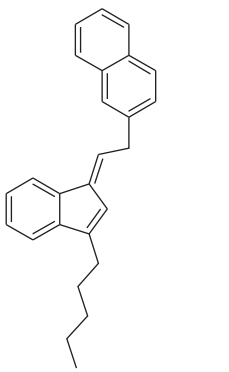
The likelihood of accidental overdosage is much higher when 'skunk' cannabis is involved. There are a number of reports of sudden unexpected death in young people associated with cannabis use alone (Dines *et al*., 2015; Noble *et al*., 2019; Drummer *et al*., 2019). Concentrated products such as resins and liquid concentrates, for example butane hash oil (BHO, also known as butane honey oil), made by extracting cannabis plant material with liquid butane), were associated with greater toxicity than other cannabis products. BHO and similar products typically contain some 80 % w/w THC. Distilled concentrates have been reported at up to 99.6 % w/w THC. Liquid cannabis preparations have been known to be injected, sometimes with fatal consequences (Gilbert *et al*., 2017). An LC-MS/MS assay for 11 cannabinoids and metabolites in plasma and in urine has been described (Klawitter *et al*., 2017).

22.4.10.1 Synthetic cannabinoids

Synthetic cannabinoids ('Spice', 'K2') are highly potent drugs that bind to the same receptors as THC and endogenous cannabinoids such as 2-arachidonoyl glycerol and arachidonoyl ethanolamide (anandamide) (Lu & Mackie, 2016). Many synthetic cannabinoids were originally synthesized as pharmacological probes for investigating the endogenous cannabinoid system and developing potential pharmacotherapies (Diao & Huestis, 2019). A huge range of synthetic cannabinoids is available (Abbate *et al*., 2018; Table 22.10). The metabolism of these compounds can be complex (Kavanagh *et al*., 2017; Grigoryev *et al*., 2019).

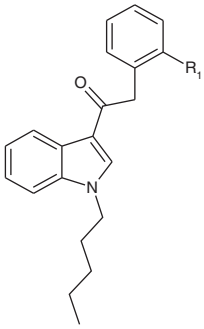
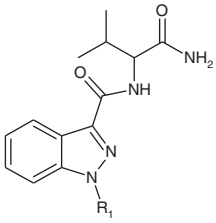
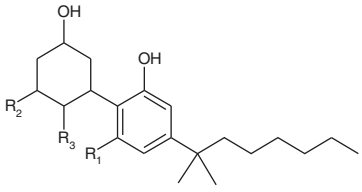
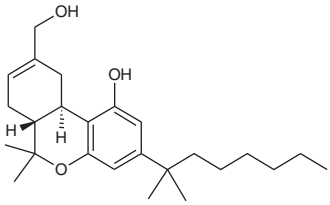
By April 2015, 858 synthetic cannabinoids had been scheduled in Japan (Uchiyama *et al*., 2015), and many more are likely to have been developed. Many are chiral. Considerable morbidity and mortality have been associated with the use of synthetic cannabinoids (Ozturk *et al*., 2019), with the risk of hospital admission estimated as 30 times higher than that associated with cannabis itself (Winstock *et al*., 2015). In a survey of 113 instances of synthetic cannabinoid use in the UK between 2014–18, 5F-ADB, AB-FUBINACA, MDMB-CHMICA, and 5F-AKB-48 were the agents encountered most commonly (Sharp *et al*., 2019). However, the popularity of a particular compound lasted for only about two years before it was superseded by a newer agent. During the study, 26 (23 %) subjects died (14 suicides).

Table 22.10 Chemical structures and names of selected synthetic cannabinoids

Class and Chemical Structure		Name
Naphthoylindoles		
	$R_1 = C_5H_{11}$, $R_2 = H$	JWH-018
	$R_1 = CH_3$, $R_2 = H$	JWH-070
	$R_1 = C_4H_9$, $R_2 = H$	JWH-073
	$R_1 = C_5H_{11}$, $R_2 = Cl$	JWH-398
	$R_1 = C_5H_{10}F$, $R_2 = H$	AM-2201
Naphthylmethylindoles		
	$R_1 = H$	JWH-175
	$R_1 = CH_3$	JWH-184
	$R_1 = O–CH_3$	JWH-185
Naphthoylpyrroles		
	$R_1 = H$, $R_2 = C_6H_{13}$	JWH-147
	$R_1 = F$, $R_2 = C_5H_{11}$	JWH-307
Naphthylmethylindenes		
		JWH-176

(*continued overleaf*)

Table 22.10 (*Continued*)

Class and Chemical Structure		Name
Phenylacetylindoles		
	$R_1 = H$	JWH-167
	$R_1 = O–CH_3$	JWH-250
	$R_1 = CH_3$	JWH-251
	$R_1 = F$	JWH-311
	$R_1 = Cl$	JWH-203
	$R_1 = Br$	JWH-249
Indazoles		
	$R_1 = CH_2$-Ph	AB-CHMINACA
	$R_1 = C_5H_{10}F$	5F-AB-PINACA
	$R_1 = CH_2$–PhF	AB-FUBINACA
Cyclohexylphenols		
	$R_1 = H, R_2 = H, R_3 = H$	CP 47,497
	$R_1 = OH, R_2 = (CH_3)_2, R_3 = H$	O-1871
	$R_1 = H, R_2 = H, R_3 = C_3H_6OH$	CP 55,940
Classical cannabinoids		
		HU-210

22.4.11 Cardioactive drugs

It has been known for many years that digoxin measurements in heart blood collected post-mortem may be misleading when attempting to assess peri-mortem plasma digoxin concentrations because of leakage of digoxin from heart muscle after death. Use of peripheral blood is no more reliable, although use of vitreous humour may be helpful. Amiodarone, noramiodarone, flecainide, and sotalol may also show post-mortem changes in blood concentration.

Spironolactone, canrenone, and potassium canrenoate cross-reacted in earlier Abbott digoxin immunoassays, as did various plant and other naturally occurring materials. Administration of antidigoxin F_{ab} antibody fragments has been used to reverse toxicity from cardiac glycosides present in plants such as *Apocynum cannabinum* (Indian hemp), *Digitalis purpurea* (Purple foxglove), *Nerium oleander* (Common or Pink Oleander), and *Thevetia peruviana* (Yellow Oleander). All of these substances may cross-react in polyclonal digoxin immunoassays (Dasgupta, 2006). However, monoclonal digoxin immunoassays may not cross-react with other cardioactive glycosides, and thus such assays should not be relied upon to confirm or refute exposure (Panesar *et al*., 2005).

22.4.12 Diuretics and laxatives

Clinical problems associated with diuretic and laxative misuse include electrolyte and acid/base changes that can involve the renal and cardiovascular systems, and may become life threatening. The teeth, gastrointestinal system, skin, and musculoskeletal system may be involved, with self-induced vomiting causing many complications (Roerig *et al*., 2010; Forney *et al*., 2016; MacDuff *et al*., 2016).

The diagnosis of diuretic/laxative misuse may not be easy, especially if these drugs are given covertly to young children. Chronic hypokalaemia of unknown aetiology suggests further investigation. Of 99 adult patients with chronic normotensive hypokalaemia (serum K^+ 2.8 $\pm$0.4 mmol L^{-1}, duration 4.1 $\pm$0.9 years), surreptitious use of laxatives ($n = 11$), or diuretics ($n = 12$) was found to be the cause of the problem in one out of every four cases. Unless the patient or carer admits to the covert administration of one of these agents, detection of the misused substance in urine is essential to establish the diagnosis.

Commonly used laxatives in the UK have included senna and its glycosides, phenolphthalein, danthron, rhein, aloin, and bisacodyl (Figure 22.5). The glycosides are activated by hydrolysis by GI tract bacteria and the aglycones and phenolphthalein undergo enterohepatic cycling (Section 15.6.2.1). Most are excreted as glucuronides in urine, hence sample hydrolysis is important to ensure detection. TLC was at one time the analytical method of choice, but can give poor results (Shelton *et al*., 2007). LC-MS/MS has been used to detect four laxative metabolites in urine after hydrolysis of conjugates, namely desacetylbisacodyl, aloe-emodin, emodin, and rhein. Deuterated ISTDs were used for desacetylbisacodyl and emodin assay, whilst biochanin A was used as ISTD for rhein and aloe-emodin (Middleberg & Homan, 2012). GC-MS after analyte derivatization may also be employed. Published methods for detecting diuretic misuse are mainly aimed at doping control, but can be adapted for clinical purposes (De Wilde *et al*., 2018)

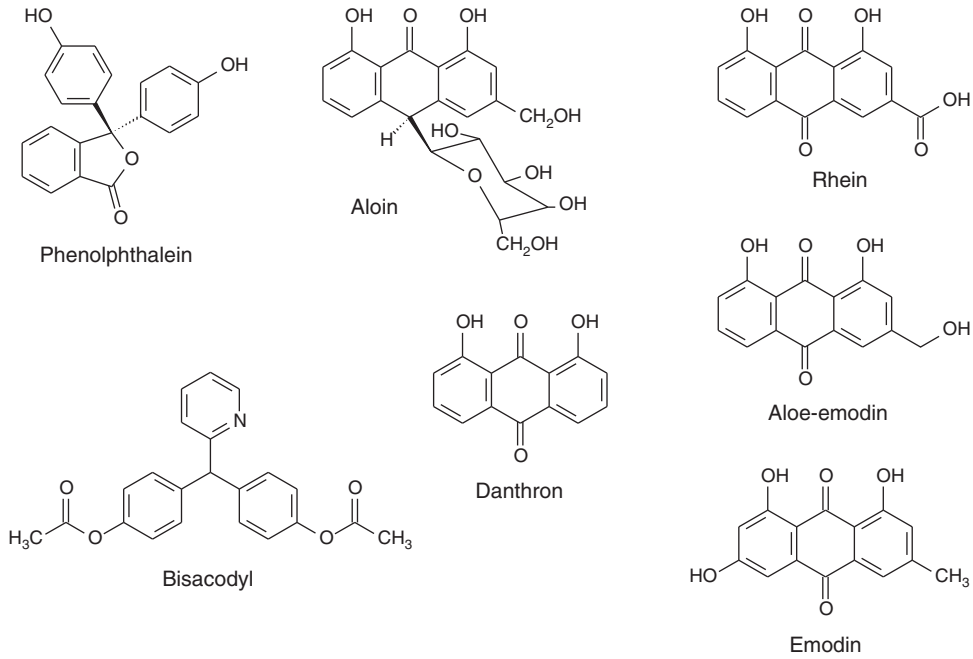


Figure 22.5 Some examples of laxatives

22.4.13 Hallucinogens

Serotonergic hallucinogens induce profound changes in perception and cognition. The characteristic effects of hallucinogens are mediated by 5-HT_{2A} receptor activation. The potential for *N*,*N*-diethyl lysergamide (lysergic acid diethylamide; LSD; Figure 5.8) to cause psychological disturbance and death from causes such as trauma and drowning has been long understood, but the drug itself is of relatively low acute toxicity (Nichols & Grob, 2018). LSD analogues such as *N*-ethyl-*N*-cyclopropyl lysergamide (ECPLA), *N*,*N*-methylpropyl lysergamide (LAMPA), and 1-propionyl-LSD (1P-LSD) may also be encountered (Grumann *et al*., 2019). LSD is unstable in biological samples unless the specimens are protected from light.

22.4.13.1 N-Benzylphenethylamines

Addition of an *N*-benzyl group to phenethylamine hallucinogens produces a marked increase in 5-HT_{2A}-binding affinity and hallucinogenic potency. *N*-Benzylphenethylamines (NBOMes) such as *N*-(2-methoxybenzyl)-2,5-dimethoxy-4-iodophenethylamine (25I-NBOMe) show very high affinity for the 5-HT_{2A} receptor. NBOMes can produce severe hallucinations at very low doses, the 4-iododerivatives, 25I-NBOMe, 25I-NBOH, and 25-NBMD [Figure 22.6(a)], being especially potent. Rhabdomyolysis is a relatively common complication of severe NBOMe toxicity, an effect that may be linked to NBOMe-induced seizures, hyperthermia, and vasoconstriction (Kyriakou *et al*., 2015; Halberstadt, 2017).

LC-MS/MS has been used to identify and quantify five 25-NBOMes (25B-NBOMe, 25C-NBOMe, 25D-NBOMe, 25H-NBOMe, 25I-NBOMe) in blood and urine (Morini *et al*., 2017). 25E-NBOMe was used as ISTD. LoD and LLoQ for the 25-NBOMes studied were 0.05 and 0.10 μg L^{-1}, respectively. 25C-NBOMe and 25H-NBOMe were present at concentrations

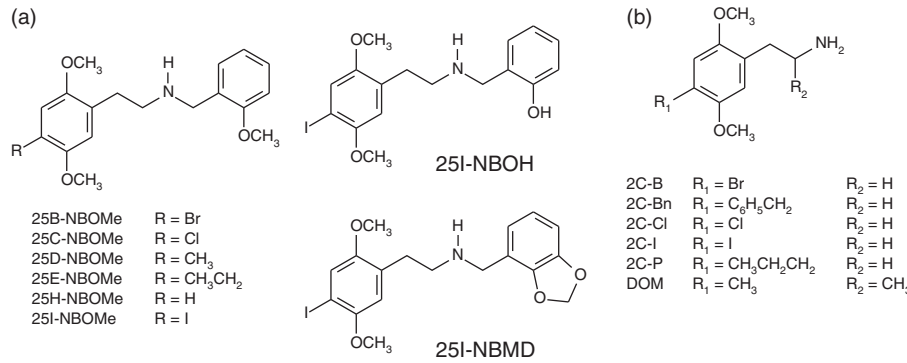


Figure 22.6 Selected examples of hallucinogens: (a) *N*-benzylphenylethylamines and (b) 2,5-dimethoxyphenylethyamines

of 2.80 and 0.29 μg L^{-1} in peripheral blood, 1.43 and 0.13 μg L^{-1} in central blood, and 0.94 and 0.14 μg L^{-1} in urine, respectively, in samples from a teenage male who looked agitated and drowned after jumping into a waterway. Cannabinoids were also present.

22.4.13.2 2,5-Dimethoxy-substituted phenethylamines

The 2,5-dimethoxy-substituted phenethylamines (2C-X) are hallucinogens that are structurally related to 2,5-dimethoxy-4-methylamfetamine (DOM) and include 2,5-dimethoxy-4-bromophenethylamine (2C-B) and 2,5-dimethoxy-4-iodophenethylamine (2C-I), and 2,5-dimethoxy-4-propylphenethylamine (2C-P) [Figure 22.6(b)]. 2C-B has similar properties to other dimethoxy-substituted hallucinogenic drugs (Papaseit *et al.,* 2018).

22.4.13.3 Mescaline and psilocybin

Mescaline (3,4,5-trimethoxyphenethylamine) and psilocybin are naturally occurring hallucinogens, found in the peyote cactus (amongst others) and several species of psilocybin mushroom, respectively. Psilocybin is a prodrug that is dephosphorylated to psilocin (4-hydroxy-*N*,*N*-dimethyltryptamine) in the body. Mescaline is one of the least potent hallucinogens, being some 4000 times less potent than LSD; a typical oral dose would be 200–400 mg. Over 90 % of the dose can be recovered from urine over the first 48 h. In a mescaline-associated fatality, death was as a result of trauma after a fall of some 600 ft. Post-mortem blood and urine concentrations were 9.7 and 1163 mg L^{-1}, respectively, and a sample of liver contained 70.8 mg g^{-1}. A total of 167 mg was recovered from stomach contents (Reynolds & Jindrich, 1985).

Fatalities associated with ingestion of psilocybin mushrooms are, as with those with mescaline, a result of trauma sustained during in a hallucinogenic experience, although the presence of phenethylamines may cause some cardiotoxicity (van Amsterdam *et al*., 2011). A LC-MS/MS method for quantification of psilocybin, psilocin, and mescaline in hair has been described with LLoQs of 0.04, 0.04, and 0.05 ng mg^{-1} of hair, respectively (Pichini *et al*., 2014).

22.4.14 γ-Hydroxybutyrate and γ-butyrylactone

GHB is a naturally occurring neurotransmitter. It has been used as a general anaesthetic, and as a treatment for narcolepsy and alcoholism. It is also misused as a recreational drug, as an

athletic performance enhancer, and as a 'date rape' drug. It is commonly used in the form of the sodium or potassium salt. Misuse of GHB and its precursor GBL, and of a related compound 1,4-butanediol (1,4-BD), has increased in the last 30 years (Schep *et al*., 2012). GHB overdoses have been associated with many deaths, with GHB concentrations in post-mortem blood averaging 0.5 g L^{-1}, but ranging up to 6.5 g L^{-1} (Corkery *et al*., 2015).

GHB is rapidly absorbed with peak plasma concentrations typically occurring within 1 hour of ingestion. It has a relatively small *V* and is rapidly eliminated with a $t_{½}$ of 20–60 min. Most of a dose is eliminated within 4–8 hours (Schep *et al*., 2012). Only some 1–5 % of a dose is excreted unchanged in urine. A urine cut-off of 10 mg L^{-1} has been suggested to differentiate endogenous GHB excretion from deliberate administration of either GHB, or GBL.

After death, GHB concentrations tend to increase, central blood and urinary cut-offs of 100 and 20 mg L^{-1}, respectively, being suggested to help diagnose use of GHB/GBL (Jones *et al*., 2018). GHB concentrations tend to be more stable in urine, vitreous humour, and CSF compared with blood because these sampling sites are protected from the spread of bacteria from the gut (Busardò & Jones, 2019). Because of the fact that GHB occurs naturally in the body, analysis of drinks and other material found at a scene may provide important evidence. However, GHB also occurs at low concentration in many non-alcoholic drinks (Elliott & Fais, 2017).

22.4.15 Inorganic anions

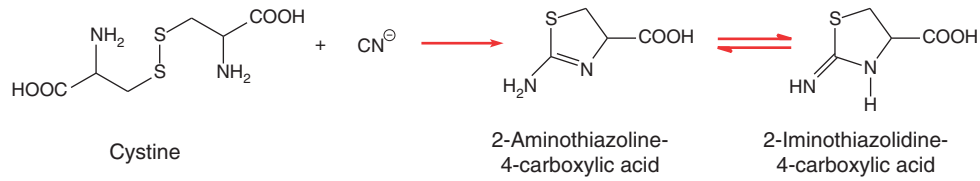
Fluoride measurement using an ion-selective electrode was discussed in Section 21.6.2. Although bromide ion is a metabolite of the anaesthetic halothane and bromide salts have been used as anticonvulsants, bromide assay is rarely required nowadays.

22.4.15.1 Azide

Sodium azide is widely used as a preservative in laboratory reagents and in biological samples, and as a source of gas in motor vehicle airbag inflation systems. It is extremely toxic. Poisoning may arise from accidental or deliberate ingestion in laboratory employees, for example, and as a result of internet purchases. Azide has been analyzed in blood and urine by GC-MS/MS after extraction combined with derivatization using pentafluorobenzyl bromide (Rojek *et al*., 2015). Azide concentrations of 0.18 and 6.50 mg L^{-1} were measured in blood and in urine, respectively, in a fatal case of azide self-poisoning. In other cases, azide has only been detected in stomach contents, bile, urine, and in tissues other than blood (Le Blanc-Louvry *et al*., 2012).

22.4.15.2 Cyanide and thiocyanate

Cyanide can arise from not only inorganic cyanides such as potassium cyanide, but also cyanogenic glycosides and other nitrile-containing compounds. In suspected cyanide poisoning *per se*, specimens of blood from more than one peripheral site, and stomach contents, should be collected. Identifying the source of cyanide exposure is important. If the source is unknown it may be useful to obtain a small specimen of brain (*ca*. 20 g) from a site deep within the brain to confirm the presence of cyanide. Blood and tissue specimens are best stored at 4 °C and should be analyzed as soon as possible after collection. Thiocyanate is a metabolite of cyanide and measurement of plasma thiocyanate has been used to assess cyanide exposure (Kirman *et al*., 2018). Simultaneous measurement of cyanide and thiocyanate in plasma by GC-CI-MS has also been described (Bhandari *et al*., 2012; Section 19.2.2.2). The minor cyanide metabolite 2-aminothiazoline-4-carboxylic acid (ATCA) has also been suggested as a marker for cyanide exposure (Figure 22.7; Li *et al*., 2019).



Cystine

2-Aminothiazoline-4-carboxylic acid

2-Iminothiazolidine-4-carboxylic acid

Figure 22.7 Interaction of cystine with cyanide

22.4.15.3 Nitrite

Inorganic nitrites may be used in deliberate self-poisoning, and nitrite is a metabolite of various organic nitrites such as glyceryl trinitrate and isobutyl nitrate. Methods for nitrite, and its metabolite nitrate, measurement in plasma using LC-UV after pre-column derivatization (Zhao *et al*., 2015) and for nitrite using ion chromatography with conductivity detection (Yan *et al*., 2016) have been described.

22.4.15.4 Phosphide

Phosphides such as aluminium phosphide and zinc phosphide are potent insecticides and rodenticides. As such they are widely available in many parts of the world. On contact with water or water vapour they hydrolyze to give highly toxic phosphine gas. Acute poisoning with these agents is often fatal – a mortality rate of 70 % has been quoted (Anand *et al*., 2011). Analysis of anything other than gastric contents or scene residues is unlikely to give evidence of exposure because of the rapid release of phosphine (Bumbrah *et al*., 2012), although measurement of aluminium and of zinc in blood may prove helpful in such cases.

22.4.15.5 Sulfide

Hydrogen sulfide is an extremely toxic gas and may be encountered during oil and natural gas production, in mines, in abattoirs, in sewers and wastewater treatment facilities, and in other confined spaces. The ability to smell hydrogen sulfide (‘rotten eggs’) is lost at higher hydrogen sulfide concentrations. Intentional hydrogen sulfide inhalation is usually accompanied by measures to seal the space in which the gas is released and is usually fatal (Bott & Dodd, 2013; Ruder *et al*., 2015).

As in cases where either cyanides or phosphides have been involved, there is a risk to first responders, pathologists, and others from emissions from samples such as stomach contents, let alone the risk of entering the contaminated atmosphere. GC-MS of sulfide and thiosulfate in post-mortem blood, urine, CSF, and pleural effusion after intentional and unintentioanal hydrogen sulfide poisoning has been described (Maebashi *et al*., 2011; Ventura Spagnolo *et al*., 2019). Measurement of the sulfide metabolites methyl and dimethyl sulfide may also be helpful.

22.4.16 Ketamine

Ketamine has a range of uses in human and in veterinary medicine including inducing and maintaining anaesthesia, chronic pain relief, sedation in intensive care, and treatment of depression. First used in the 1970s, it is on the WHO List of Essential Medicines. Ketamine is also misused for its hallucinogenic effects. Analogues of ketamine include other arylcyclohexylamines such as dechloro-*N*-ethylketamine, eticyclidine (PCE), 3-methoxyeticyclidine (3-MeO-PCE), 3-methoxyphencyclidine (3-MeO-PCP) 2-ketoeticyclidine, methoxetamine (MXE), tiletamine,

phencyclidine (PCP), and tenocyclidine (TCP). Acute poisoning with these compounds continues to be reported (Zidkova *et al*., 2017).

Ketamine is chiral. Metabolites such as norketamine and dehydronorketamine (Figure 22.8) are also chiral. (*R*)-(–)-Ketamine has been said to have greater antidepressant actions than (*S*)-(+)-ketamine, but without ketamine-related side-effects, which can include neurotoxicity, cognitive dysfunction, psychotomimetic effects, cardiovascular events, and uropathic effects (Morgan *et al*., 2012; Zhu *et al*., 2016). It seems likely the enantiomers act via different mechanisms. Be this as it may, (*S*)-(+)-ketamine (esketamine) has been available for use in analgesia and anaesthesia since the early 1990s (Trimmel *et al*., 2018), and is now being marketed as a low dose nasal spray for the rapid treatment of depression.

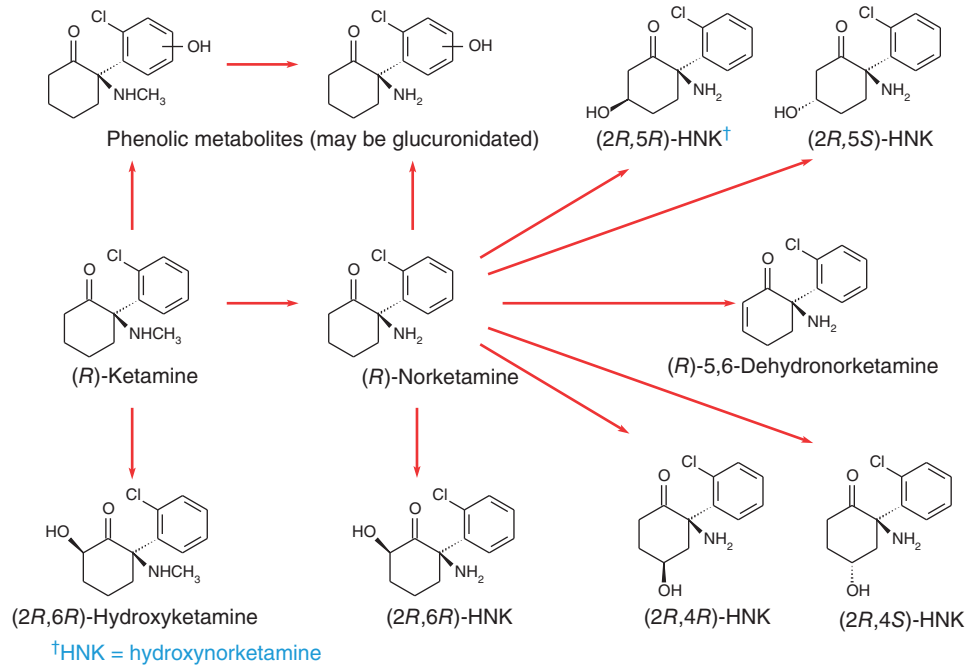


Figure 22.8 Selected metabolic pathways of (*R*)-ketamine

Ketamine may not feature in preliminary (immunoassay-based) urine or oral fluid substance misuse testing procedures unless requested specifically. Most laboratory-based methods use achiral methodology for ketamine and ketamine metabolites (Hu *et al*., 2019; Matey *et al*., 2019). The adverse events associated with ketamine, notably its effects on the urinary tract, which may include destruction of bladder function, usually occur after long term, high dose misuse, and may prove irreversible (Zhu *et al*., 2016).

22.4.17 Non-opioid analgesics

Aspirin is now encountered rarely after acute poisoning in higher-income countries. However, paracetamol poisoning is the most common cause of acute liver failure in the developed world and as such remains a common cause of hospital admission and death despite the availability of usually effective antidotal therapy (Wong & Graudins, 2017).

Serious toxicity may ensue if the patient either presents too late for antidotal therapy (Thusius *et al*., 2019), or has absorbed a very large amount of paracetamol (Marks *et al*., 2017). In the case of the former, use of the paracetamol urine test (Table 5.1) may be helpful in confirming recent paracetamol exposure if the plasma paracetamol has fallen below the LoD of the assay. In the case of the latter, very high (>1 g L^{-1}) plasma paracetamol concentrations may be associated with coma and profound metabolic acidosis, and the laboratory needs to be aware that samples may need to be diluted and re-assayed in the event that the assay is only calibrated to say 400 mg L^{-1}.

A further complication is that some patients may take several overdoses over a period of a few hours, so called 'staggered' overdoses (Craig *et al*., 2012). In such cases use of the nomogram of plasma paracetamol concentration against time of ingestion of the overdose used clinically to guide antidote administration (Wong & Graudins, 2017) is impractical, the plasma paracetamol results only serving to confirm exposure. In all cases, however, the laboratory needs to ensure that the units used to report the plasma paracetamol concentration are the same as those used in the nomogram.

22.4.18 Opioids

Opioid drugs act as neurotransmitters and neuromodulators at the three major subclasses of 'classical' opioid receptor: delta (δ), kappa (κ), and mu (μ). They are classified according to their receptor interaction into four groups: agonists, antagonists, partial agonists, and mixed agonist/antagonists (Table 22.11). In the US, prescription opioids are thought to have been responsible for many deaths in recent years (Gomes *et al*., 2018). However, assigning the cause

Table 22.11 Classification of opioid drugs

Agent Class	Examples	Action
Agonist	Codeine[a] Dihydrocodeine[a]	Activation of all receptor subclasses, though with different affinity
	Fentanyl	
	Morphine	
	Methadone	
	Oxycodone[a]	
	Tramadol[a]	
Antagonist	Naloxone Naltrexone	Devoid of agonist activity at all receptor subclasses; may displace an agonist from a receptor
Partial agonist	Buprenorphine	Partial agonist at μ-receptors; antagonist at δ- and κ-receptors
	Pentazocine	Partial agonist at μ-receptors; agonist at κ-receptors
Agonist/antagonist	Butorphanol	Partial agonist/antagonist at μ-receptors, partial agonist at κ-receptors
	Nalbuphine	Antagonist at μ-receptors, agonist at κ-receptors
	Nalorphine	Antagonist at μ-receptors, agonist at κ-receptors

[a]May act via metabolism to *O*-desmethyl metabolite

of death to acute opioid poisoning is often difficult because of the possible development of tolerance in people who have been taking such drugs chronically. The route of administration may also affect outcome, i.v. injection or inhalation of vapour being more likely to cause death than ingestion. The combination of opioids with ethanol may also enhance the risk of serious toxicity. Similarly, use of gabapentin and/or pregabalin (Section 22.4.5) may increase the risk of death through either reversal of opioid tolerance, or an additive effect of the drugs to depress respiration (Lyndon *et al*., 2017; Gomes *et al*., 2017).

22.4.18.1 Buprenorphine

Buprenorphine is given for analgesia and is also used to treat opioid addiction. Unfortunately, it can also be misused and has caused many deaths, often when used in combination with benzodiazepines and alcohol (Häkkinen *et al*., 2012). Co-formulation with naloxone has been employed to deter misuse of prescription drugs such as buprenorphine and oxycodone. When these preparations are taken as intended (orally or sublingually) they have the desired effect because naloxone has low oral bioavailability. However, when injected or insufflated ('snorted') the naloxone acts to block the effect of the opioid and induce symptoms of opioid withdrawal. LC-HRMS of urine can detect use of naloxone-containing preparations and can also detect sample adulteration with buprenorphine (Belsey *et al*., 2014).

22.4.18.2 Codeine/codeine analogues

Codeine has low analgesic potency and may act via metabolism to morphine. Dihydrocodeine and oxycodone, on the other hand, have much greater potency (Table 22.12) and oxycodone especially has been associated with many deaths in recent years (Handley & Flanagan, 2014; Pilgrim *et al*., 2015; Hedegaard *et al.,* 2018).

22.4.18.3 Diamorphine/morphine

The metabolism of diamorphine, the principal toxic component of street heroin, is complex and it is important to be clear about what has been measured in a blood, urine, or other biological sample as this affects the interpretation of the results. Diamorphine is metabolized very quickly to 6-acetylmorphine (6-AM; Figure 15.4), which has a $t_{½}$ of *ca*. 5–25 minutes and is sometimes detectable in biological samples. Detection of 6-AM provides clear evidence of diamorphine use as it is not produced from morphine. Morphine, the product of 6-AM metabolism, has a much longer $t_{½}$ (2–3 hours) than 6-AM, and is usually the compound that is detected after diamorphine administration. Morphine is available as a pharmaceutical preparation for oral or parenteral use in some countries, and is also the principal component of opium (Box 1.1).

Street heroin normally contains some acetylcodeine derived from codeine present in opium. Acetylcodeine is metabolized to codeine in the body, but the amounts present in blood after heroin use are usually insignificant as compared to the amounts of 6-AM and morphine. Morphine and codeine are metabolized principally by conjugation to give inactive derivatives (morphine-3-glucuronide and codeine-3-glucuronide) which are excreted in urine. A relatively small proportion of the dose of diamorphine/morphine is also metabolized to morphine-6-glucuronide which is pharmacologically active, but is only thought to contribute to the toxicity of morphine when the drug is given to patients in renal failure (Section 16.10).

Problems of establishing illicit opiate use are reflected in the number of different approaches that have been advocated. These include the blood morphine:codeine ratio and the presence of additional compounds derived from opium such as acetylcodeine, meconin, noscapine metabolites, papaverine metabolites, and reticuline (Dinis-Oliveira, 2019).

Table 22.12 Comparison of the potencies of fentanyl and selected derivatives relative to oral codeine and morphine (Adcock *et al*., 1988; Higashikawa & Suzuki, 2008; Suzuki & El-Haddad, 2017)

Compound	ED_{50}[a] (mg kg^{-1})	LD_{50}[a] (mg kg^{-1})	Potency ratio to morphine	Potency ratio to fentanyl
Acetylfentanyl	0.021	9.3	16	0.29
Acetyl-α-methylfentanyl	–	–	3.1	0.06
Alfentanil	–	–	–	0.1–0.2
α-Methylfentanyl	–	–	56.9	1.1
Butyrylfentanyl	0.047	–	1.5–7	0.03–0.13
Carfentanil	–	–	10,000	100
Codeine	18	–	0.1	0.002
Dihydrocodeine	–	–	0.1	0.002
Etorphine	–	–	1000–3000	19–56
Fentanyl	0.0061	62	54	–
4-Fluorofentanyl	–	–	15.7	0.29
β-Hydroxy-3-methylfentanyl	–	–	6300	28
Lofentanil	–	–	–	>100
3-Methylfentanyl	0.00058–0.0068	–	49–569	0.9–10.5
Morphine	2.3	–	1	0.02
Oxycodone	–	–	2	0.04
Remifentanil	–	–	–	2–20
Sufentanil	–	–	500–1000	5–10

[a] ED_{50} – median effective dose, LD_{50} – median lethal dose

Renal failure apart, the acute toxicity of diamorphine/morphine is very much dependent on (i) the dose, (ii) the subject's immediate past exposure to opioid agonists because people can tolerate exposure to much higher doses if they have been taking such drugs within the last few days, (iii) the method of administration, i.v. dosage being much more dangerous than inhalation of fumes or i.m. injection, (iv) the purity of any mixture injected, and (v) in the presence of other centrally acting drugs such as ethanol or methadone. Any interpretation of post-mortem morphine data must bear these factors in mind. Because the toxicity of morphine is largely due to depression of the respiratory centre, a patient maintained via mechanical ventilation may tolerate an otherwise fatal dose.

Traditionally immunoassays have been available that measure 'total' morphine (i.e. morphine plus metabolites, principally the inactive 3-glucuronide). The advantage of this is that, if used together with the 'free' (unconjugated) morphine result, an idea of the magnitude of the diamorphine/morphine dose can be obtained. If the free morphine is relatively high compared to the total morphine then this might suggest that death ensued quite quickly after diamorphine/morphine administration, whilst if the total morphine is high and the free morphine low,

this might suggest death was not due to morphine administration unless some secondary factor such as hypoxic brain damage had supervened.

Of course, there are many possible scenarios, but in general both free and total morphine should be measured. In addition, the measurements should be performed in blood and in urine, if available, and perhaps in other fluids because the results from these different specimens often provide complementary information to aid clinical interpretation. For example, the presence of low concentrations of free (unconjugated) morphine (<1 mg L^{-1}) in urine obtained post-mortem in a suspected heroin-related death strongly suggests that death occurred shortly after a heroin dose. Free morphine concentrations in ventricular post-mortem blood are consistently higher than those at peripheral sites, especially at concentrations >0.3 mg L^{-1}, but there appears to be little change with time between death and the blood sample being obtained from central or peripheral sites.

22.4.18.4 Fentanyl

Fentanyl is a potent opioid agonist used to treat severe pain and in combination with other drugs as an anaesthetic. It is given by injection, as a skin patch, as a nasal spray, or by buccal administration. It is especially dangerous to either heat the skin patch whilst it is attached to the skin because this delivers an overdose of drug quickly (Section 16.5), or to extract fentanyl from the patch and inject the resulting solution. Even used patches may still contain an appreciable amount of fentanyl.

22.4.18.5 Methadone

As with diamorphine and morphine, post-mortem blood methadone concentrations may be difficult to interpret especially if there is a history of prior use of methadone or other opioids, and/or other drugs are also present. Delayed toxicity, especially at night, is a feature of poisoning with this compound, which may further complicate the interpretation of results. Deaths from methadone in children and in subjects previously exposed, but who have lost tolerance to opioids, are not uncommon. Monitoring the main methadone metabolite, EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine), in urine not only provides evidence of adherence to methadone treatment, but also guards against non-adherent individuals adding methadone to 'blank' urine in order to obtain continued prescription of the drug with intent to supply to other persons.

22.4.18.6 Novel synthetic opioids

Synthetic opioids include MT-45, U-47700, AH-7921, and fentanyl derivatives (Armenian *et al*., 2018; Frisoni *et al*., 2018; Table 22.13). Both MT-45 and AH-7921 ('Doxylam') were originally synthesized in the 1970s by pharmaceutical companies. Both compounds are thought to have similar potency to morphine (Zawilska & Andrzejczak, 2015).

Misuse of many fentanyl derivatives has been reported (Helander *et al*., 2016; 2017; Kuczyńska *et al*., 2018). These drugs pose an especially serious problem because of their high potency (Table 22.12) and because they are often sold under the guise of heroin to unsuspecting users. Clusters of deaths as a result of use of fentanyl-contaminated heroin have been reported in Australia (Rodda *et al*., 2017), in the US (Bode *et al*., 2017), and in the UK (Rab *et al*., 2019). Acetylfentanyl has been sold as oxycodone (Stogner, 2014). Fentanyl and U-47700 have been sold as 'Norco' (hydrocodone and paracetamol) tablets (Armenian *et al*., 2017; Sutter *et al*., 2017). Most fentanyl analogues are metabolized extensively and thus identification and

Table 22.13 Some synthetic opioids that may be encountered when performing toxicological analyses

Common name(s)	Chemical Name	CAS no (free base)
Acetylfentanyl (AF, MCV4848, NIH10485)	*N*-[1-(2-Phenylethyl)-4-piperidyl]-*N*-phenylacetamide	3258-84-2
Acetylnorfentanyl	*N*-Phenyl-*N*-(piperidin-4-yl) acetamide	22352-82-5[a]
Acrylfentanyl (acryloylfentanyl)	*N*-Phenyl-*N*-[1-(2-phenylethyl) piperidin-4-yl]prop-2-enamide	82003-75-6
AH-7921	3,4-Dichloro-*N*-([1-(dimethylamino) cyclohexyl]methyl)benzamide	55154-30-8
Alfentanil	*N*-[1-[2-(4-Ethyl-5-oxotetrazol-1-yl) ethyl]-4-(methoxymethyl) piperidin-4-yl]-*N*-phenylpropanamide	71195-58-9
3-Allylfentanyl	*N*-phenyl-*N*-[1-(2-phenylethyl)-3-(prop-2-en-1-yl)piperidin-4-yl]propanamide	82208-84-2
Butyrylfentanyl	*N*-(1-Phenethylpiperidin-4-yl)-*N*-phenylbutyramide	1169-70-6
Butyrylnorfentanyl	*N*-Phenyl-*N*-4-piperidinyl-butanamide	–
Carfentanil	Methyl 1-(2-phenylethyl)-4-[phenyl (propanoyl)amino] piperidine-4-carboxylate	59708-52-0
4-Chlorofuranylfentanyl (4-chloro FuF)	*N*-(4-Chlorophenyl)-N-(1-phenethylpiperidin-4-yl)furan-2-carboxamide	–
4-Chloroisobutyrylfentanyl (4-Cl-iBF)	*N*-(4-Chlorophenyl)-*N*-(1-phenethylpiperidin-4-yl) isobutyramide	244195-34-4
Crotonylfentanyl	(2E)-*N*-Phenyl-*N*-[1-(2-phenylethyl)-4-piperidinyl]-2-butenamide	760930-59-4
Cyclohexylfentanyl	*N*-(1-Phenethylpiperidin-4-yl)-*N*-phenylcyclohexanecarboxamide	–
Cyclopentanoylfentanyl (CP-F)	*N*-(1-Phenethylpiperidin-4-yl)-*N*-phenylcyclopentanecarboxamide	–
Despropionylfentanyl (4-ANPP)	*N*-Phenyl-1-(2-phenylethyl) piperidin-4-amine	21409-26-7
2,2'-Difluorofentanyl	*N*-(1-(2-Fluorophenethyl)piperidin-4-yl)-*N*-(2-fluorophenyl)propionamide	–
N-Ethylcarfentanil	Methyl 1-ethyl-4-[(1-oxopropyl) phenylamino]-4-piperidinecarboxylate	–

(*continued overleaf*)

Table 22.13 (*Continued*)

Common name(s)	Chemical Name	CAS no (free base)
Fentanyl	*N*-Phenyl-*N*-[1-(2-phenylethyl) piperidin-4-yl]propanamide	437-38-7
Fentanyl isothiocyanate	*N*-{1-[2-(4-Isothiocyanatophenyl) ethyl]piperidin-4-yl}-*N*-phenylpropanamide	85951-63-9
2-Fluorobutyrylfentanyl	*N*-(2-Fluorophenyl)-*N*-(1-phenethylpiperidin-4-yl)butyramide	–
3-Fluorobutyrylfentanyl	*N*-(3-Fluorophenyl)-*N*-(1-phenethylpiperidin-4-yl)butyramide	
4-Fluorobutyrylfentanyl	*N*-(4-Fluorophenyl)-*N*-[1-phenethylpiperidin-4-yl)butyramide	244195-31-1
2-Fluorofentanyl (2-FF, *o*-FF)	*N*-(2-Fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl]-propanamide	–
3-Fluorofentanyl	*N*-(3-Fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl]-propanamide	–
4-Fluorofentanyl	*N*-(4-Fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl]-propanamide	90736-23-5
4-Fluoroisobutyrylfentanyl (4F-iBF, 4-FIBF, *p*-FIBF)	*N*-(4-Fluorophenyl)-*N*-(1-phenethylpiperidin-4-yl)isobutyramide	244195-32-2
2-Furanylethylfentanyl	*N*-[1-[2-(2-Furanyl)ethyl]-4-piperidinyl]-*N*-phenyl-propanamide	1443-49-8[a]
2-Furanylfentanyl (Fu-F)	*N*-Phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]furan-2-carboxamide	101345-66-8
3-Furanylfentanyl	*N*-Phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]furan-3-carboxamide	–
Hexanoylfentanyl	*N*-(1-Phenethylpiperidin-4-yl)-*N*-phenylhexanamide	–
Hydrocinnamoylfentanyl	*N*-(1-Phenethylpiperidin-4-yl)-*N*-phenyl-(3-phenyl)propionamide	–
β-Hydroxyfentanyl	*N*-[1-(2-Hydroxy-2-phenethyl) piperidin-4-*N*-phenylpropanamide	78995-10-5
ω-Hydroxyfentanyl	3-Hydroxy-*N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]henylpiper	83708-11-6
β-Hydroxythiofentanyl	*N*-[1-(2-Hydroxy-2-thiophen-2-ylethyl) piperidin-4-yl]-*N*-phenylpropanamide	1474-34-6
Isobutyrylfentanyl	2-Methyl-*N*-phenyl-*N*-[1-(2-phenylethyl) piperidin-4-yl]propanamide	119618-70-1
N-Isopropylcarfentanil	Methyl 1-isopropyl-4-[(1-oxopropyl) phenylamino]-4-piperidinecarboxylate	–

Table 22.13 (*Continued*)

Common name(s)	Chemical Name	CAS no (free base)
2-Isopropylfuranylfentanyl (2-isopropyl Fu-F)	*N*-(2-Isopropylphenyl)-*N*-(1-phenethylpiperidin-4-yl)furan-2-carboxamide	–
Lofentanil (3-methylcarfentanil)	Methyl (3*S*,4*R*)-1-(2-cyclohexylethyl)-4 –(cyclohexyl-propanoylamino)-3-methylpiperidine-4-carboxylate	61380-40-3
Loperamide	4-[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]-*N*,*N*-dimethyl-2,2-diphenylbutanamide	53179-11-6
Methoxyacetylfentanyl	2-Methoxy-*N*-(1-phenethylpiperidin-4-yl)-*N*-phenylacetamide	101345-67-9
4-Methoxybutyrylfentanyl (4-MeO-BF)	*N*-(4-Methoxyphenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl]-butanamide	2088842-68-4
4-Methoxyfentanyl	*N*-(4-Methoxyphenyl)-*N*-[1-(2-phenylethyl)piperidin-4-yl]propanamide	1688-41-1
2-Methoxyfuranylfentanyl (2-MeO-FuF)	*N*-(2-Methoxyphenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl]-2-furancarboxamide	101343-50-4
4-Methoxyfuranylfentanyl (4-MeO-FuF)	*N*-(4-Methoxyphenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl]-2-furancarboxamide	–
4-Methoxymethylfentanyl (R-30490)	*N*-[4-(Methoxymethyl)-1-(2-phenylethyl) piperidin-4-yl]-*N*-phenylpropanamide	60618-49-7
2-Methylacetylfentanyl	*N*-(1-Phenethylpiperidin-4-yl)-*N*-(*o*-tolyl)acetamide	–
3-Methylacetylfentanyl	*N*-(3-Methylphenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl]-acetamide	1443-51-2[a]
2-Methylacrylfentanyl	*N*-(1-Phenethylpiperidin-4-yl)-*N*-(*o*-tolyl) acrylamide	–
3-Methylbutyrylfentanyl (3-MBF)	*N*-[3-Methyl-1-(2-phenylethyl)piperidin-4-yl]-*N*-phenylbutanamide	97605-09-9
N-Methylcarfentanil (R-32395)	Methyl 1-methyl-4-[(1-oxopropyl) phenylamino]-4-piperidinecarboxylate	59708-50-8
α-Methylfentanyl	*N*-(1-(1-Methyl-2-phenylethyl)-4-piperidinyl)-N-phenylpropanamide	79704-88-4
2-Methylfentanyl	*N*-[2-Methyl-1-(2-phenethyl) piperidin-4-yl*]-N*-phenylpropanamide	1443-53-4[a]
3-Methylfentanyl (3-MF, mefentanyl)	*N*-[3-Methyl-1-(2-phenylethyl)piperidin-4-yl]-*N*-phenylpropanamide	42045-86-3
2-Methylfuranylfentanyl (2-methyl FuF)	*N*-(1-Phenethylpiperidin-4-yl)-*N*-(2-tolyl)furan-2-carboxamide	–

(*continued overleaf*)

Table 22.13 (*Continued*)

Common name(s)	Chemical Name	CAS no (free base)
2-Methylmethoxyacetyl-fentanyl	2-Methoxy-*N*-(*o*-tolyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl]-acetamide	–
N-Methylnorfentanyl	*N*-(1-Methyl-4-piperidyl)-*N*-phenyl-propanamide;	24775-71-1[a]
4-Methylphenethylacetylfentanyl	*N*-[1-[2-(4-Methylphenyl)ethyl]-4-piperidinyl]-*N*-phenylacetamide	1071703-95-1
3-Methylthiofentanyl	*N*-{3-Methyl-1-[2-(thiophen-2-yl)ethyl] piperidin-4-yl}-*N*-phenylpropanamide	86052-04-2
Mirfentanil	*N*-[1-(2-Phenylethyl)piperidin-4-yl]-*N*-(pyrazin-2-yl)furan-2-carboxamide	117523-47-4
MT-45	1-Cyclohexyl-4-(1,2-diphenylethyl) piperazine	52694-55-0
Norcarfentanil	Methyl 4-[(1-oxopropyl)phenylamino]-4-piperidinecarboxylate	61085-87-8[a]
Norfentanyl	*N*-Phenyl-*N*-(piperidin-4-yl)propionamide	1609-66-1
Norsufentanil (R30451)	*N*-[4-(Methoxymethyl)-4-piperidinyl]-*N*-phenylpropanamide	61086-18-8
Ocfentanil (A-3217)	*N*-(2-Fluorophenyl)-2-methoxy-*N*-[1-(2-phenylethyl)piperidin-4-yl]acetamide	101343-69-5
3-Phenylpropanoylfentanyl	*N*-Phenyl-*N*-[1-(2-phenylethyl)-4-piperidinyl]-benzenepropanamide	79279-02-0
N-Propylcarfentanil	Methyl 1-propyl-4-[(1-oxopropyl) phenylamino]-4-piperidinecarboxylate	–
Remifentanil	Methyl 3-{4-methoxycarbonyl-4-[(1-oxopropyl)phenylamino]-1-piperidine}propanoate	132875-61-7
Sufentanil (R30730)	*N*-[4-(Methoxymethyl)-1-(2-thiofuran-2-ylethyl)-4-piperidyl]-*N*-phenylpropanamide	56030-54-7
Tetrahydrofuranylfentanyl (THF-F)	*N*-Phenyl-*N*-[1-(2-phenylethyl) piperidin-4-yl]oxolane-2-carboxamide	–
Tetramethylcyclopropane-fentanyl (TMCP-F)	*N*-Phenyl-*N*-[1-(2-phenylethyl) piperidin-4-yl]-2,2,3,3-tetramethylcyclopropane-1-carboxamide	–
Thienylfentanyl	*N*-Phenyl-*N*-[1-(thiophen-2-ylmethyl) piperidin-4-yl]propanamide	117332-93-1[a]

Table 22.13 (*Continued*)

Common name(s)	Chemical Name	CAS no (free base)
Thiofentanyl	*N*-Phenyl-*N*-{1-[2-(thiophen-2-yl)ethyl] piperidin-4-yl}propanamide	1165-22-6
2-Thiofuranylfentanyl	*N*-(1-Phenethylpiperidin-4-yl)-*N*-phenylthiophene-2-carboxamide	–
Trefentanil (A-3665)	*N*-{1-[2-(4-Ethyl-5-oxo-4,5-dihydro-1H-tetrazol-1-yl)ethyl]-4-henylpiperidine-4-yl}-*N*-(2-fluorophenyl) propanamide	120656-74-8
U-47700	3,4-Dichloro-*N*-[(1*R*,2*R*)-2-(dimethylamino) cyclohexyl]-*N*-methylbenzamide	82657-23-6
U-50488	2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-[(1*R*,2*R*)-2-pyrrolidin-1-ylcyclohexyl] acetamide	67198-13-4
Valerylfentanyl	*N*-Phenyl-*N*-[1-(2-phenylethyl) piperidin-4-yl]pentanamide	122882-90-0

[a]Hydrochloride

detection of metabolites as opposed to the parent drug is important, particularly if there is a delay between exposure and sample collection (Allibe *et al*., 2018).

The extremely high potency of drugs such as carfentanil and lofentanil makes it imperative that strict safety precautions (first aid, including availability of naloxone and of staff trained in using it) are enforced when handling reference compounds and drug seizures that may contain these substances.

22.4.18.7 Tramadol

Tramadol is unusual in that as well as being an opioid it is also a serotonin–norepinephrine reuptake inhibitor (SNRI). Deaths can occur when tramadol is taken together with other SSRIs, MDMA, and related compounds (Pilgrim *et al*., 2011).

Tramadol and its *O*-desmethyl metabolite have two chiral centres (Figure 22.9). The drug is available as the racemate, (1*R*,2*R*/1*S*,2*S*)-tramadol. The *O*-demethylation catalyzed by CYP2D6 was shown to be enantioselective with preference of the (+)-enantiomer by comparing the pharmacokinetics of tramadol in CYP2D6 extensive and poor metabolizers (Pedersen *et al*., 2006). *O*-Desmethyltramadol (desmetramadol) is a much more potent μ-opioid agonist than tramadol itself, and whilst both (+)- and (−)-*O*-desmethyltramadol are inactive as serotonin reuptake inhibitors, (−)-*O*-desmethyltramadol retains activity as a norepinephrine reuptake inhibitor. *O*-Glucuronidation of the *O*-demethylated metabolites gives rise to diastereomeric products that are separable on achiral columns. The reaction favours the 1*S*,2*S* enantiomers (Overbeck & Blaschke, 1999). *N*-Demethylation of the parent drug is a minor pathway in humans.

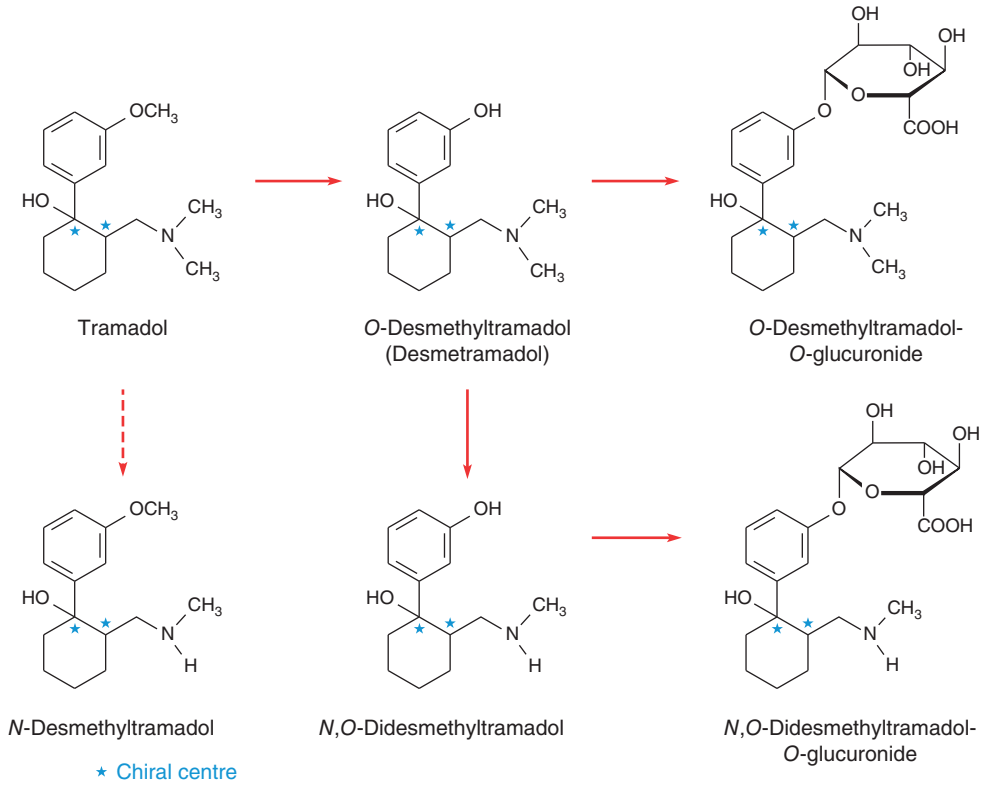


Figure 22.9 Metabolism of tramadol

22.4.19 *Organophosphorus compounds*

Some organophosphorus (OP) pesticides are used as herbicides and are relatively non-toxic to humans. However, most are insecticides that interfere with neurotransmission by inhibition of acetylcholinesterase and are very toxic in man. OPs are also used as chemical warfare agents (nerve agents, Table 22.14). OP nerve agents are very difficult to detect analytically in biological specimens from people who may have died as a result of exposure to these compounds.

OP pesticides often have a pungent smell (of garlic), and this property can be helpful in indicating a diagnosis. Many of these compounds are hydrolyzed in alkaline solution while some (for example azinphos-methyl, diazinon, and malathion) are also unstable in acid. Many are also unstable in blood (Ageda *et al*., 2006). LC-MS has been used to measure 10 OP insecticides (acephate, methidathion, dichlorvos, fenthion, EPN [*O*-ethyl *O*-(4-nitrophenyl) phenylphosphonothioate], diazinon, phenthoate, malathion, fenitrothion, and cyanophos) in serum at concentrations encountered after acute poisoning with these agents. However, of the compounds tested, dichlorvos and malathion degraded rapidly over 24 h at room temperature, whereas methidathion and diazinon remained relatively stable over 4 weeks at all temperatures studied (Inoue *et al*., 2007). A GC-MS method for measuring these compounds in post-mortem blood has also been described (Park *et al*., 2009). However, for clinical purposes measurement of either plasma cholinesterase, or more usually erythrocyte acetylcholinesterase are in reality the only procedures that help to assess the severity of exposure and progress with treatment (Section 6.9.3).

Table 22.14 Some organophosphorus compounds

Insecticides	
Azinphos-methyl	*O,O*-Dimethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4H)-yl)methyl]dithiophosphate
Diazinon	*O,O*-Diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl]phosphorothioate
Malathion	Diethyl-2-(dimethoxyphosphinothioylthio)succinate
Mevinphos	2-Methoxycarbonyl-1-methylvinyldimethylphosphate
Omethoate	*O,O*-Dimethyl *S*-methylcarbamoylmethylphosphorothioate
Parathion	*O,O*-Diethyl-*O*-4-nitrophenylphosphorothioate
Nerve agents	
Sarin	(*R,S*)-Propan-2-yl methylphosphonofluoridate
Soman	3,3-Dimethylbutan-2-yl methylphosphonofluoridate
Tabun	(*R,S*)-Ethyl *N,N*-Dimethylphosphoramidocyanidate
VX	Ethyl ({2-[bis(propan-2-yl)amino]ethyl}sulfanyl)(methyl)phosphinate

22.4.20 Phosphodiesterase 5 inhibitors

Sildenafil, tadalafil, vardenafil, and the newer avanafil, desmethyl carbodenafil, and udenafil, amongst others, selectively inhibit phosphodiesterase 5 (PDE5), which is cGMP-specific and responsible for the degradation of cGMP in the corpus cavernosum. These drugs are used primarily to treat erectile dysfunction, as well as some other conditions such as pulmonary hypertension. Analyses of these compounds may be requested during (i) the investigation of sudden unexpected deaths in males (Bakota *et al*., 2017), (ii) DFSA, and (iii) poisoning in childhood. They and various analogues are often found as undeclared constituents of foods, dietary supplements, counterfeit drugs, and herbal medicines (Lee *et al*., 2019).

22.4.21 Stimulants and related compounds

22.4.21.1 Amfetamine and metamfetamine

Amfetamine is used under various names in the treatment of attention deficit hyperactivity disorder (ADHD), narcolepsy, and obesity. It is widely misused. (*S*)-(+)-Amfetamine, the dextrorotatory enantiomer, possess CNS stimulant activity. (*R*)-(–)-Amfetamine is used as an antitussive in some countries, notably the US. Strictly, 'amfetamine' and 'metamfetamine' are the names of the respective racemates. Clearly, chiral analysis can be important under certain circumstances (Newmeyer *et al*., 2015). Immunoassays using antibodies raised against (*S*)-(+)-metamfetamine may show poor cross-reactivity with (*R*)-(–)-metamfetamine thus increasing the risk of false negatives if both compounds are being looked for.

Amfetamine and metamfetamine feature in many deaths, often in combination with ethanol and/or other drugs (Handley & Flanagan, 2014). Fatal toxicity with these compounds, as with cocaine and other stimulants, is seemingly unpredictable and bears no relation to post-mortem

blood concentrations. Deaths may be attributed to ventricular arrhythmia, major haemorrhage, or exacerbation of pre-existing heart disease. Fulminant hepatic failure may occur after overdosage. Note that many compounds can give rise to amfetamine *in vivo*, either directly, or by giving rise to metamfetamine (Table 22.15). Conversely, metamfetamine has been identified as a contaminant in proprietary amfetamine tablets.

Table 22.15 Compounds giving rise to amfetamine either directly, or via metamfetamine *in vivo*

Amfetamine	Metamfetamine
Amfetaminil	Benzfetamine
Clobenorex	Dimetamfetamine
Ethamfetamine	Fencamine
Fenethylline	Furfenorex
Fenoproporex	Selegiline
Lisdexamphetamine	
Mefenorex	
Metamfetamine	
Prenylamine	

22.4.21.2 Cocaine

Cocaine is a diester and is hydrolyzed rapidly *in vivo* and *in vitro* by plasma and tissue esterases, and in alkaline solution, respectively, to BE and EME (Figure 22.10). Hydrolysis of cocaine can continue after death, even in the brain. Addition of *ca*. 2 % w/v sodium fluoride to a peripheral blood sample and storage at –20 °C reduces plasma esterase activity, thereby increasing the likelihood that the measured blood cocaine concentration reflects the systemic cocaine concentration at the time of sampling. However, if there is significant delay before the post-mortem then interpretation of cocaine concentrations can be extremely difficult. It is important to measure BE independently.

Further factors that may be important in the interpretation of results are route of administration, the form of cocaine used (salt or free base, 'crack'), the duration and intensity of prior exposure, and of course the presence of other poisons. Some cocaine is metabolized by cholinesterase, and users may inhibit metabolism by taking cholinesterase inhibiters such as organophosphorus (OP) compounds – this may be a consideration in an otherwise unexplained death in cocaine users. Adulterated cocaine may be yet another factor – amfetamine, atropine, diltiazem, hydroxyzine, ketamine, levamisole, lidocaine, phenacetin, phentermine, and the trazodone and nefazodone metabolite 1-(3-chlorophenyl) piperazine (*m*-CPP) have been found in illicit cocaine, for example (Gameiro *et al*., 2019). It has been suggested that aminorex [(*R*,*S*)-5-phenyl-4,5-dihydro-1,3-oxazol-2-amine] is a minor metabolite of levamisole [(*S*)-(–)-tetramisole] in man, but aminorex could not be detected in urine samples from users of cocaine adulterated with levamisole (Eiden *et al*., 2015; Handley *et al*., 2019).

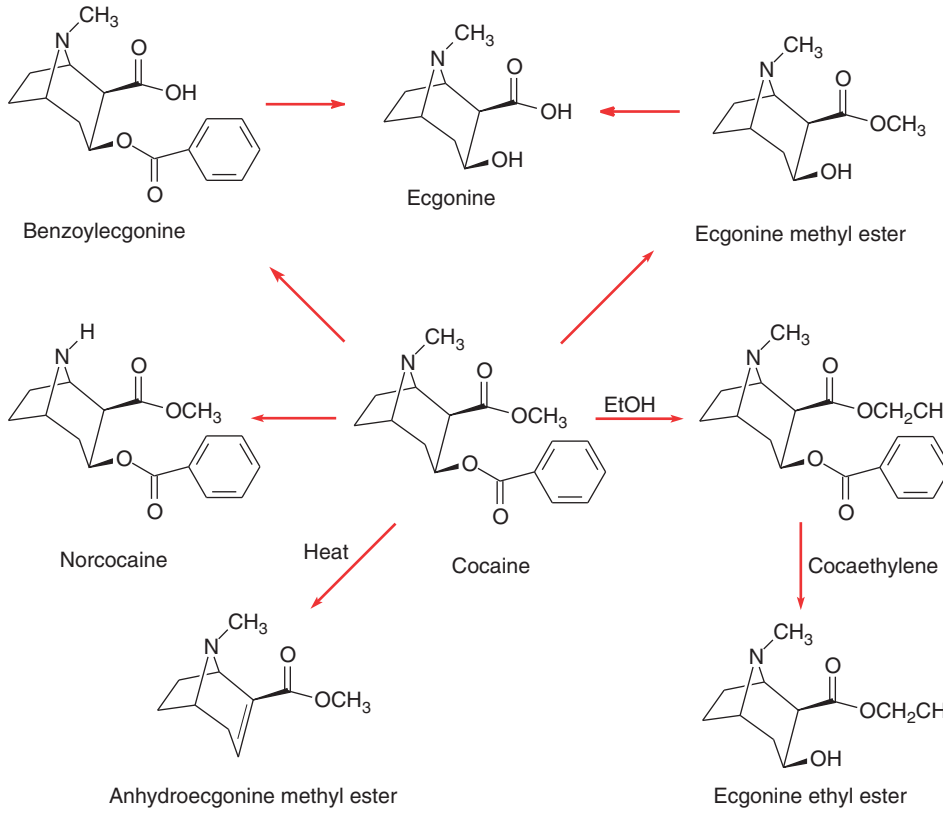


Figure 22.10 Some metabolites and decomposition products of cocaine

Saliva, sweat, and hair samples may contain relatively high amounts of cocaine whereas urine contains relatively small amounts of parent drug, but large proportions of BE and EME. Cocaine is often taken with alcohol and this can be confirmed by the presence of cocaethylene (a result of trans-esterification, Section 15.5.4.6, although cocaethylene may also be present in illicit cocaine if ethanol has been used in preparing the drug) and ecgonine ethyl ester. Cocaethylene has a longer $t_{½}$ than cocaine and is, if anything, more potent. Anhydroecgonine is a pyrolysis product formed when 'crack' cocaine is smoked, but may also be encountered as a GC artefact (Toennes *et al.*, 2003).

There is no clear site-dependent variation in post-mortem blood cocaine, BE, or cocaethylene concentrations, perhaps because of the competing processes of tissue release and degradation of the analytes. In some individuals the acute toxicity of cocaine seems to be dose unrelated. It has been suggested that measurement of brain cocaine is important in the investigation of some cocaine-related deaths, such as those preceded by a period of excited delirium. Histological assessment of cardiac muscle to exclude cocaine-induced myocarditis as a cause of death is also important, however.

22.4.21.3 MDMA and related compounds

Deaths related to methylenedioxymetamfetamine (MDMA, 'XTC', 'ecstasy') use are not clearly related to dose and are often associated with use of alcohol and/or other illicit drugs (Handley &

Flanagan, 2014). Hyperthermia and rhabdomyolysis leading to renal failure may occur. Deaths attributable to methylenedioxyamfetamine (MDA, 'adam'), methylenedioxyethylamfetamine (MDEA, 'eve'), and other related compounds are relatively rare. It is important to analyze not only blood, but also urine and stomach contents if available in suspected MDMA-related deaths and to look for other drugs as well as MDMA. In the UK recent samples of MDMA tablets (2016–8) tend to have higher MDMA content compared to earlier years (Couchman *et al*., 2019). Dramatic within-batch MDMA-content variability (up to 136 mg difference) and dissolution rate on standard *in vitro* testing was also found.

Mephedrone (4-methylmethcathinone, '4-MMC', 'Meow Meow', 'Miaow', 'White Magic', 'M-Cat', 'Bubble') was one of the first NPS to appear. It was portrayed as a 'legal' alternative to MDMA. Many novel stimulants have been developed since that time and continue to be sold (Welter-Luedeke & Maurer, 2016; Table 22.16). As with MDMA, many have hallucinogenic properties and many are chiral. The so-called 2C-FLY series contain methoxy moieties integrated in a 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b′]difuran core (Wagmann *et al*., 2019; Iwersen-Bergmann *et al*., 2019).

As well as structural alteration of illicit psychoactive drugs, alteration of drugs with stimulant properties has been reported. Methylphenidate is a psychostimulant prescribed as first-line treatment in ADHD, but may also be misused for its stimulant properties. When methylphenidate is taken with ethanol, (*S,S*)-ethylphenidate may be formed by enantioselective trans-esterification (Dinis-Oliveira, 2017). More recently, ethylphenidate has been marketed as a 'legal high'. Details of six other phenidate analogues sold as 'legal highs' have been published (Klare *et al*., 2017).

22.4.22 Trace elements/toxic metals

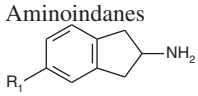
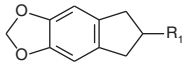
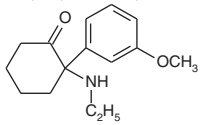
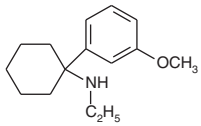
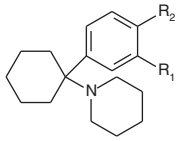
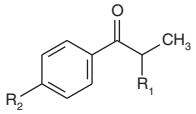
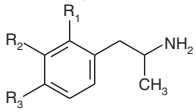
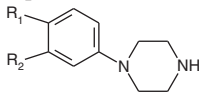
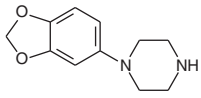
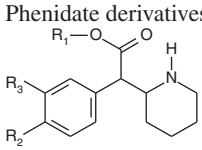
Ethnic cosmetics such as surma may contain from 0–80 % (w/w) elemental lead as either the oxide, or sulfide and such products are important causes of lead poisoning. Herbal or other types of traditional medicines may also contain toxic doses of salts of lead or other toxic metals (Posadzki *et al*., 2013). Lead-contaminated opium is a further problem (Alinejad *et al*., 2018). Reviews of arsenic and of selenium speciation are available (Marcinkowska & Barałkiewicz, 2016; Rekhi *et al*., 2017; Taylor *et al*., 2017).

Although cobalt has an essential role as a constituent of vitamin B_{12}, excessive exposure is associated with a number of adverse health effects (Leyssens *et al*., 2017). Selenium too has an essential role in the body, but excessive exposure is likewise associated with a range of toxic effects (Vinceti *et al*., 2018). Measurement of chromium, cobalt, and vanadium in the investigation of metallosis arising from the wear of metal-on-metal articulations in orthopaedic devices has also become an important area of study (Ring *et al*., 2016; Day *et al*., 2017; Georgi *et al*., 2017).

22.4.23 Volatile substances

It is especially important to consider all circumstantial and other evidence in cases of possible VSA-related sudden death, especially if legitimate exposure to solvent vapour is a possibility. Death may occur through (i) direct toxic effects on the heart (fatal arrhythmia), (ii) respiratory depression, which may be compounded by partial asphyxia associated with the use of a plastic bag, for example, or simply by displacement of oxygen by the inhaled vapour, (iii) asphyxia secondary to choking on inhaled vomit, and (iv) trauma whilst intoxicated. More rarely drowning in the bath whilst intoxicated has been reported. Sometimes toxicological analysis of blood

Table 22.16 Chemical structures and names of selected novel stimulants

Class and Chemical Structure		Name
Aminoindanes		
	$R_1 = H$	2-Aminoindane (2-AI)
	$R_1 = I$	5-Iodo-2-aminoindane (5-IAI)
	$R_1 = NH_2$	5,6-Methylenedioxy-2-aminoindane (MDAI)
	$R_1 = NHCH_3$	5,6-Methylenedioxy-*N*-methyl-2-aminoindane (MDMAI)
Arylcyclohexylamines		
		Methoxetamine
	$R_1 = OCH_3, R_2 = H$	3-Methoxyeticyclidine (3-MeO-PCE)
	$R_1 = H, R_2 = OCH_3$	
		3-Methoxyphencyclidine (3-MeO-PCP)
		4-Methoxyphencyclidine (4-MeO-PCP)
Cathinones		
	$R_1 = NH_2, R_2 = H$	Cathinone ('khat')
	$R_1 = NHCH_3, R_2 = H$	Methcathinone
	$R_1 = NHCH_3, R_2 = CH_3$	4-Methylmethcathinone (mephedrone, 4-MMC)
Phenethylamines	$R_1 = F, R_2 = H, R_3 = H$	2-Fluoroamfetamine (2-FA)
	$R_1 = H, R_2 = F, R_3 = H$	3-Fluoroamfetamine (3-FA)
	$R_1 = H, R_2 = H, R_3 = F$	4-Fluoroamfetamine (4-FA)
	$R_1 = OCH_3, R_2 = H, R_3 = H$	2-Methoxyamfetamine (2-MA)
	$R_1 = H, R_2 = OCH_3, R_3 = H$	3-Methoxyamfetamine (3-MA)
	$R_1 = H, R_2 = H, R_3 = OCH_3$	4-Methoxyamfetamine (*p*-methoxyamfetamine, PMA, 4-MA)
Piperazine derivatives		
	$R_1 = H, R_2 = H$	Benzylpiperazine (BZP)
	$R_1 = OCH_3, R_2 = H$	4-Methoxyphenylpiperazine (MeOPP)
	$R_1 = Cl, R_2 = H$	4-Chlorophenylpiperazine (pCPP)
	$R_1 = H, R_2 = Cl$	3-Chlorophenylpiperazine (mCPP)
		3,4-Methylenedioxybenzylpiperazine (MDBP)
Phenidate derivatives		
	$R_1 = C_2H_5, R_2 = H, R_3 = H$	Ethylphenidate
	$R_1 = CH_3, R_2 = CH_3, R_3 = H$	4-Methylmethylphenidate
	$R_1 = CH_3, R_2 = Cl, R_3 = Cl$	3,4-Dichloromethylphenidate
	$R_1 = C_2H_5, R_2 = Cl, R_3 = Cl$	3,4-Dichloroethylphenidate
	$R_1 = CH(CH_3)_2, R_2 = H, R_3 = H$	Isopropylphenidate

or other specimens may be the only evidence of poisoning, the scene having been 'tidied up' by relatives or carers.

Measurement of relatively non-volatile compounds such as toluene in blood can be performed isothermally by headspace GC (Section 19.2.2.1). In very general terms, blood concentrations of volatile substances such as toluene of 10–20 mg L^{-1} or more, are encountered after acute poisoning with these agents. In other words, the concentrations attained after acute poisoning are similar to those of inhalational anaesthetics during controlled anaesthesia, and are thus two or so orders of magnitude lower than those observed in poisoning with relatively water-soluble compounds such as ethanol (Flanagan *et al*., 1997; Wille & Lambert, 2004).

Many very volatile compounds can be detected in blood after deliberate exposure to these agents if simple precautions are taken (Section 2.3.1). The tube should be as full as possible and should only be opened when required for analysis, and then only when cold (4 °C). An anticoagulant (lithium heparin or EDTA) should be used. If the sample volume is limited it is advisable to select a container to match the volume of blood so that there is minimal headspace. Specimen storage at 2–8 °C is recommended and sodium fluoride (2 % w/v) should be added to minimize microbial metabolism. When investigating a suspected VSA-related fatality, analysis of tissues such as brain or lung may prove useful as relatively high concentrations of volatile compounds may be present when they are undetectable in blood.

When deliberately misused by inhalation to achieve intoxication, no particular compound seems any more, or any less, toxic than any other. Deliberate inhalation of the vapour of solvents such as toluene and trichloroethylene is rarely reported nowadays from Western countries, but misuse of substances such as nitrous oxide or liquified petroleum gas (LPG), a mixture of propane, butane, and isobutane ('butane') used as a fuel gas in cigarette lighter refills, for example, and as an aerosol propellant, is common. These substances are gases at normal room temperature, and thus an analysis can realistically only provide confirmation of exposure, quantification not being practicable even in blood unless special precautions are taken (El Balkhi *et al*., 2016). The presence of propane and butane metabolites, which may include 2-propanol, 2-butanol, *tert*-butanol, butanone, 2,3-butanediol, 3-hydroxy-2-butanone, and 2,3-butanedione, may indicate LPG inhalation for some time before death (Sasao *et al*., 2015; Figure 19.1)).

In line with other substance misuse-related deaths, most VSA-related deaths are unintentional, but some are suicides. Again, circumstantial evidence of intent, which may include measures to exclude access to oxygen, and an analysis for drugs and other poisons such as carbon monoxide is important when investigating such occurrences. Deliberate inhalation of asphyxiant gases that are available in cylinders such as argon, carbon dioxide, helium, and nitrogen may also be encountered. Numbers of such deaths are increasing in the UK as in other parts of the world (Handley & Flanagan, 2014).

Natural gas (usually largely methane) is non-intoxicant, but is likewise an asphyxiant gas. Suicide involving deliberate inhalation of natural gas and even of vapour from heated oil has also been reported (Maryam & Elham, 2010). As with LPG-related deaths, attempts to quantify asphyxiant gases in specimens obtained post-mortem are fraught with difficulty and of doubtful value, although the measurement of helium in human blood by GC-MS using ^{21}Ne as ISTD has been reported (Tsujita *et al*., 2019).

22.5 Sources of further information

Websites where further information on aspects of analytical toxicology and related areas may be obtained are listed below (Table 22.17). Assayfinder (www.assayfinder.com/) can be used to locate a UK provider of an unusual or rare assay.

Table 22.17 Some sources of information on analytical toxicology and related disciplines

American Academy of Clinical Toxicology (AACT)	www.clintox.org
American Association of Clinical Chemistry, TDM and Toxicology Division	www.aacc.org/community/divisions/tdm-and-toxicology
American Society of Health-System Pharmacists (AHFS)	www.ahfsdruginformation.com/ahfs-drug-information/
Association for Clinical Biochemistry and Laboratory Medicine (ACB)	www.acb.org.uk
European Association of Poisons Centres and Clinical Toxicologists (EAPCCT)	www.eapcct.org
European Monitoring Centre for Drugs and Drug Addiction (EMCDDA)	www.emcdda.europa.eu
European Network of Forensic Science Institutes (ENFSI)	enfsi.eu
French Society of Analytical Toxicology (SFTA)	www.sfta.org
Health & Safety Executive, Health and Safety Laboratory (HSE HSL)	www.hsl.gov.uk
International Association of Therapeutic Drug Monitoring & Clinical Toxicology (IATDM-CT)	www.iatdmct.org
International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)	www.ifcc.org
International Programme on Chemical Safety INTOX Programme (IPCS INTOX)	www.who.int/ipcs/poisons/en/
London Toxicology Group (LTG)	www.the-ltg.org/
Martindale: The Complete Drug Reference	about.medicinescomplete.com/publication/martindale-the-complete-drug-reference/
National Institute for Occupational Safety and Health (NIOSH)	www.cdc.gov/niosh
Royal Society of Chemistry – The Merck Index *Online*	www.rsc.org/merck-index
Society of Toxicological and Forensic Chemistry (GTFCh)	www.gtfch.org/cms/index.php/en/
Society of Forensic Toxicologists (SOFT)	www.soft-tox.org
The International Association of Forensic Toxicologists (TIAFT)	www.tiaft.org
Toxicological Society of Belgium and Luxembourg (BLT)	www.blt.be/
United Nations Office on Drugs and Crime (UNODC)	www.unodc.org
US Drug Enforcement Administration (DEA)	www.dea.gov

The standard National Library of Medicine (NLM) search engine for the Index Medicus (Medline) database of scientific journal citations and abstracts (Pubmed) is at www.ncbi.nlm.nih.gov/pubmed. There are many other NLM databases. As an example, chemical structures, synonyms, etc, can be found at: pubchem.ncbi.nlm.nih.gov.

A WHO sponsored link: http://www.intox.org/ is a valuable source of toxicological and poisons information. The UK NPIS primary poisons information database (free access to UK National Health Service personnel) is Toxbase (www.toxbase.org). The Clinical Evidence (BMJ Group) website (www.clinicalevidence.com/ceweb/resources/index.jsp) has a glossary and statistics modules amongst other features.

22.6 Summary

All the available evidence must be taken into account when investigating any death or other incident where poisoning is suspected. An overall knowledge of the circumstances, time course, clinical/post-mortem observations, possible poisons involved and their toxicology and metabolism is paramount. Toxicological analysis can provide objective evidence of exposure and of the magnitude of exposure. Factors which may assist in the interpretation of post-mortem blood toxicology results are the possible availability of (i) ante-mortem samples (whole blood or plasma/serum), and (ii) routine TDM data.

Even if such 'baseline' data are available, comparison with post-mortem data must be made in the knowledge of the possible effects of the time elapsed since death, the site of collection of the blood sample, and analyte stability, amongst other factors. Furthermore, TDM samples are frequently measured pre-dose ('trough' concentrations) whereas the samples for toxicological analysis are usually taken without regard to the time since the last dose. Measurement of major blood metabolites is important if only to ensure that they have not been quantified together with the parent drug, and the blood drug:metabolite ratio might indicate whether exposure was acute or chronic. Analysis of other fluids and/or tissues may provide further useful information.

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