University of Portsmouth

Development of a Liquid Chromatography Ion Trap Mass Spectrometer Method for Clinical Drugs of Abuse Testing with Automated On-Line Extraction Using Turbulent Flow Chromatography

By

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Abstract

Aims The method for the confirmation of drugs of abuse for addiction testing within King's College Hospital prior to 2008 was a labour intensive thin layer chromatography method. To replace this with a faster method more suited to future requirements, the laboratory bought a liquid chromatography system with ion trap mass spectrometric detection. The development of the routine analytical method and the implementation of this method within the laboratory using on-line solid phase extraction and Turboflow® sample preparation will allow the laboratory to operate successfully in the field of clinical drugs of abuse testing in the future.

Method Analyses are performed on an ion trap mass spectrometer with an electrospray ion source following reversed phase liquid chromatography, initially using on-line solid phase extraction with a Jasco XLC® series autosampler and pump and later a Thermo Turboflow® on-line extraction method with a CTC Combi-Pal® autosampler and Agilent 1100 series liquid chromatography system. Elution of drugs and metabolites is performed with a multi-step gradient of ammonium formate buffer and acetonitrile, followed by regeneration of the extraction and analytical columns to starting conditions. Detection is achieved with a Thermo LCQ Fleet ion trap mass spectrometer with a combination of full spectrum survey scans, dedicated product ion scans, neutral loss scans and data dependent product ion scans in two analysis segments. Total run time is only 20 minutes, allowing a throughput of around 65 samples per day.

Results The methods include the novel combination of the elimination of any hydrolysis step, on-line SPE or Turboflow extraction, detection of multiple drug groups, full spectrum analysis and library matching, the use of data dependent scans and ion trap mass spectrometry using MS^3 and neutral loss scans. The methods developed were validated using a departmental method validation protocol and accepted for routine use. Simultaneous detection of over fifty analytes has been found possible in a range of clinically relevant drug groups, including opiates, amphetamines, methadone, propoxyphene, cocaine, ketamine and their metabolites. The use of neutral loss scans and product ion scans of phase 2 drug metabolites permits the addition of previously unidentified drugs and metabolites to the method, allowing the laboratory's services to develop in line with requirements of the service. Quality is maintained through the use of standard operating procedures, staff training, quality control samples and external quality assessment.

Conclusion Drugs of abuse testing is key for treatment and monitoring of drug addiction. The introduction of modern mass spectrometry techniques has reduced the turnaround time of routine analysis for a range of drugs and metabolites and increased the range of drugs that can be analysed. The methods introduced have revolutionised testing at King's College Hospital and produced a method which is capable of evolving with the needs of the service to keep abreast of future requirements of the service.

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iv. Abbreviations

2D	Two Dimensional
3D	Three Dimensional
6MAM	6-Monoacetylmorphine
AGC	Analyser Gain Control
amu	Atomic Mass Unit
APCI	Atmospheric Pressure Chemical Ionisation
APPI	Atmospheric Pressure Photoionisation
C18	Eighteen Carbon Chain
C8	Eight Carbon Chain
CE	Capillary Electrophoresis
CI	Chemical Ionisation
CID	Collision Induced Dissociation
COSHH	Control of Substances Hazardous to Health
CPA	Clinical Pathology accreditation
CSA	Cambridge Scientific Abstracts
DDS	Data Dependent Scanning
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EI	Electron Impact Ionisation
EMIT	Enzyme Multiplied Immunoassay Technique
EQA	External Quality Assessment
ESI	Electrospray Ionisation
FAB	Fast Atom Bombardment
FID	Flame Ionisation Detector
FRCPath	Fellow of the Royal College of Pathologists
FRCS	Fellow of the Royal College of Surgeons
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
ICP	Inductively Coupled Plasma
i.d.	Internal Diameter

IDA	Information Dependent Acquisition
KCH	King's College Hospital
LC-MS	Liquid Chromatography-Mass Spectrometry
LLE	Liquid-Liquid Extraction
LX	Laminar Flow Injection
M3G	Morphine-3-Glucuronide
MALDI	Matrix Assisted Laser Desorption Ionisation
MAX	Mixed Anion Exchange
MCP	Microchannel Plate Detector
MCX	Mixed Cation Exchange
MDA	Methylenedioxyamphetamine
MDEA	Methylenedioxyethylamphetamine
MDMA	Methylenedioxymethamphetamine
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry or Mass Spectrum
MS/MS	Tandem Mass Spectrometry
MS2	Secondary Mass Spectrometry Scan
MS3	Tertiary Mass Spectrometry Scan
MTBE	Methyl *tert*-Butyl Ether
m/z	Mass to Charge Ratio
NEQAS	National External Quality Assessment Scheme
NHS	National Health Service
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
PFP	Pentafluorophenyl
pK_a	Dissociation Constant
Q	Quadrupole
QC	Quality Control
RSI	Reverse Search Index
SDS-PAGE	Sodiumdodecylsulphate – Polyacrylamide Gel Electrophoresis
SELDI	Surface Enhanced Laser Desorption Ionisation
SHLS	Synergy Health Laboratory Services
SI	Search Index
SIM	Selected Ion Monitoring

SLAM	South London and Maudsley NHS Trust
SLE	Supported Liquid Extraction
SOP	Standard Operating Procedure
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
SRM	Selected Reaction Monitoring
TIAFT	The International Association of Forensic Toxicologists
TLC	Thin Layer Chromatography
tMRM	Triggered Multiple Reaction Monitoring
TMS	Trimethylsilyl
TOF	Time of Flight
TX	Turboflow® Injection
UKAS	United Kingdom Accreditation Service
UV	Ultra Violet
UV-Vis	Ultra Violet and Visible Light

v. Acknowledgements

There are many people who have helped, either directly or indirectly in my path to completion of this thesis.

At King's College Hospital are the staff of the Toxicology Unit, principally Dr Andrew Marsh and Professor Bob Flanagan, who gave me the professional guidance on this project and who taught me most of the basics of toxicology, as well as being extremely important in my professional development.

Also in the Toxicology Unit are the staff who helped with the day-to-day operation of the department and helped in many small but significant ways, such as assisting with scheduling of LC-MS time, preparation of mobile phases and instrument maintenance.

Finally in the Toxicology Unit are Michelle Birch and Lewis Couchman, who continued the work that I started, including finishing off the final method validation after I left the department and turned this development method into one that is used on routine basis.

Other people at King's College Hospital were also valuable here, from Dr Caje Moniz (Head of Department) to Jim Keating (Laboratory Manager) and Roy Sherwood, Colin Stone, Mike Wright and those others who helped to make sure that this project could be approved, started and completed.

At Synergy Health Laboratory Services the staff have had no direct input into the project, but have given me support during the writing up stage, sometimes with words of encouragement and sometimes just by showing a genuine interest in how I am progressing. It may sound small, but it is little things like this that help in the final weeks of writing up.

Last but not least are the staff and students of the University of Portsmouth, from my tutor Professor Graham Mills, my supervisors Dr Karen Ball and Dr Lee Banting, the tutors during the taught course phase of the doctorate and also to the students, whose discussions and contributions helped make this course a success.

vi. Dedication

This thesis is dedicated to my wife Sharon, without whom I would never have completed this program of study. Friend, sounding board, motivator and much more, Sharon helped me at every stage, from the early morning trains to Portsmouth to the late nights writing up. Without Sharon I would not be where I am today. Thank you Sharon.

vii. Declaration

I declare that whilst studying for the Doctorate in Biomedical Science at the University of Portsmouth I have not been registered for any other award at another university. The work undertaken for this degree has not been submitted elsewhere for any other award. The work contained within this submission is my own work and, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgement has been made in the text.

Frank Richard Evers

September 2014

viii. Ethical approval

The project proposal for this work was submitted to the ethics committee of King's College Hospital for assessment.

Formal ethics committee approval is indicated if there is to be any medical intervention or adjustment of a patient's treatment, if samples are collected for the project of if there is direct patient involvement, such as collection of data from questionnaires or interviews.

The committee decided that this project did not meet the criteria for requiring ethical approval, as the above indications are not present for this project. The letter from the ethics committee confirming this is reproduced in Appendix A.3

Formal informed consent is required where a patient sample is to be tested outside any clinical requirements, where the results of the testing are used to inform treatment or a sample is required solely for the purposes of the study. This study only uses surplus clinical material from routine laboratory testing. This is in line with the guidance of the Association for Clinical Biochemistry and the Human Tissue act, which specify that surplus material may be used for method development, quality and audit purposes. On this basis, consent is not required to be sought for this work.

1. Introduction

Drugs and medicines have been encountered, in one form or another, for many hundreds of years. Cannabis has been found buried with mummies from around 2700 years ago (Russo, *et al.*, 2008) and evidence of coca leaf chewing has been found in mummies dating back at least 2760 years (Rivera, Aufderheide, Cartmell, Torres and Langsjoen, 2005). These drugs were usually in their unpurified natural form and were often used socially or in religious ceremonies, as well as having medicinal uses. Opium in particular was used recreationally and the opium dens in London were notorious in the 18th century. Britain even went to war twice with China in the middle of the 19th century over the supply of opium, the opium wars.

As chemistry advanced during the 19th century, it became possible to purify active ingredients of plants to produce concentrated drugs close to those we use today. Morphine was purified from opium in around 1804 by FWA Sertürner (Jurna, 2003) and the use became more widespread after the invention of the hypodermic syringe and needle in the 1850's, leading to increased use in minor surgery and in anaesthesia (Brownstein, 1993). The use of these new, purified drugs led to dependence and more severe acute effects than either alcohol or opium (Rogers, 2011).

In an effort to reduce the addictive qualities of morphine, German chemists working for Bayer tried chemically modifying the morphine molecule. Previously they had success chemically modifying sodium salicylate, the analgesic found in willow bark that caused stomach problems and produced the less toxic acetylsalicylate, or aspirin, that is still in widespread use (Sneader, 2000, Mueller and Scheidt, 1994). With morphine they produced diacetylmorphine, but unfortunately this drug was found to be more addictive than the morphine it was designed to replace.

German chemists continued work on alternative painkillers and in the mid-1940s developed methadone, a synthetic drug that targets the opiate receptors in the brain, giving the same kind of painkilling effect as morphine and heroin (Brownstein, 1993). Experiments in the early 1960s showed that methadone could be used as a pharmaceutical therapy to treat heroin addiction and since then methadone has become the preferred treatment for heroin addiction in the UK (Kreek, 2000).

Cannabis use increased significantly in the 1960s and advances in technology led to the development of the Enzyme Multiplied Immunoassay Technique (EMIT)

immunoassays for drugs of abuse (Rodgers, *et al.*, 1978). This was frequently partnered with another emerging technology, the gas chromatograph with mass spectrometric detection (GC-MS) (O'Connor and Rejent, 1981). In skilled hands, a GC-MS could identify a range of drugs with a high degree of confidence and this technique became the “gold standard” method (DuPont, 1990). Any pre-existing thin layer chromatography (TLC) and colour tests were crude by comparison and many legal guidelines now specify mass spectrometry for legally defensible quantitative results (European Workplace Drug Testing Society, 2002, GTFCh, 2009, Society of Forensic Toxicology / American Academy of Forensic Sciences, 2006).

GC-MS analysis of drugs requires a high degree of skill and understanding, as well as expensive equipment and this might not be sustained in smaller laboratories, such as those testing for drugs for clinical use (Bryson, 1996). With a lower burden of proof, the quicker and cheaper TLC methods survived in these clinical laboratories, sometimes supplemented with high performance liquid chromatography (HPLC) and gas chromatography with a flame ionisation detector (GC-FID) (Nováková and Maresová, 1997, Center for Substance Abuse Treatment. , 2005)

In 1989, the Electrospray Ionisation (ESI) source was developed (Fenn, Mann, Meng, Wong and Whitehouse, 1989), allowing an HPLC to be linked directly to mass spectrometry to give the Liquid Chromatograph – Mass Spectrometer (LC-MS). Since then, the cost and complexity of LC-MS systems has reduced, while the computing power and capability has significantly improved (Annesley, 2003) and LC-MS systems are now within the reach of clinical laboratories (Kang, 2012). With the introduction of a mass spectrometer to the laboratory at King’s College Hospital (KCH), a method needed to be developed that would be suitable for clinical drugs of abuse testing to replace the existing TLC method and allow the laboratory to develop for the future.

1.1. **Samples used for drug testing**

Almost any bodily fluid has been used for the detection of drugs for one application or another and in post mortem toxicology it is also common to test for drugs in different organs. For clinical toxicology only three body fluids are suitable for routine testing: blood, saliva and urine.

Blood testing is difficult with regular drug users, partly because the long term injecting of street drugs can often lead to damage to veins that makes regular venesection difficult and also the high prevalence of blood borne viruses in this population group poses an unnecessary risk to clinical and laboratory staff.

Saliva testing is technically demanding due to the low sample volumes generally available, the low levels of drugs present and the fact that saliva is actually a mixture of secretions of the parotid, submandibular and sublingual salivary glands. The saliva secreted from each of the glands has a different composition, with parotid saliva being mainly serous, submandibular saliva being mixed serous and mucoid and the sublingual glands being predominantly mucus. Additionally, the composition of saliva from a single gland can vary in pH or protein content depending on the rate of saliva flow and there is the constant difficulty of food residue in the mouth.

In contrast, large volumes of urine are easily available with cheap non-invasive sample collection procedures. Urine is one of the principal routes of drug elimination from the body and the concentration increase caused by the kidney's reabsorption of water increases the levels of drugs to a level that are high enough to permit colourimetric testing without the need for mass spectrometry, well within the capabilities of the TLC and HPLC methods available when drug testing began. A difficulty with using urine as a sample is that the drugs and their metabolites are frequently conjugated as part of phase two drug metabolism. Although not normally protein bound, this conjugation still requires a hydrolysis step for many methods of analysis. As a result, urine has been the main sample type for diagnostic testing and routine monitoring of Department of Addictions patients.

When considering the analysis of drugs, there are two principal differences between the matrices available, from urine to waste water in sewage and saliva or hair. These are i) the concentration of drugs in the sample and ii) the degree of metabolism or degradation from the drug that has been consumed. Beyond these, drug testing is a matter of eliminating the effects of the matrix (urine, blood, body tissue and so on) and analysing and detecting the drugs. When considering published work, this greatly increases the scope of methods that could be considered. In spite of this increase in scope, there are still surprisingly few papers that detail methods for multiple drugs. There are several possible reasons for this, including commercial

sensitivity, a focus on the interpretation of the results or the often simpler approach of using methods focussing on a single class of drugs.

A literature search in October 2009 using the Cambridge Scientific Abstracts Illumina database identified 77 papers using HPLC with mass spectrometric detection that detailed the methods used, some used for analyses of multiple matrices (see table 1.1). This list is similar to that in the review (Peters, 2011) of papers published since 2006, which reported 17 methods in urine, 16 in whole blood, serum or plasma and only seven in both oral fluid and hair with a further 5 methods for the identification of perinatal drug use.

Table 1.1 Methods returned from library search using CSA Illumina database October 2009. Search terms ("drugs of abuse" or "drug screen" or opiate or cocaine or "drug test") and ("oral fluid" or urine) and (LCMS or "mass spectromet*" or "tandem MS" or "quadrupole or "ion trap").

Matrix	Number of papers
Urine	33
Blood, serum or plasma	12
Other body fluids	5
Saliva or oral fluid	12
Body tissues	5
Hair	9
Sewage and wastewater	8
Other (e.g. food, particles)	4
Total papers	77

In recent years the innovations in immunoassay and mass spectrometric techniques have allowed saliva testing to become more popular. Saliva testing does not require toilet facilities, allows for directly observed collection without embarrassment and reduces the risk of adulteration of the sample and, together with several companies vying for business, has increased in popularity in nurse led and third sector organisations and clinics. However, South London and Maudsley NHS Trust is no different from many others and most drug testing performed through Bethlem Royal and Maudsley Hospitals uses urine samples.

1.2. **Drug metabolism and existing practice**

This method was intended to identify users of three principal drugs: heroin, methadone and cocaine. To be able to reliably detect these drugs, it is important to know the metabolism and interfering factors for these drugs.

Heroin, or diacetylmorphine, has a very short plasma half-life and is not detected in urine. Its primary metabolite is 6-monoacetylmorphine (6MAM), which also has a short half-life and is present in urine in low concentrations for up to twenty four hours after heroin use (Heroin, 2011). This is further metabolised to morphine, which is excreted in urine over the next 3-4 days. Phase 2 drug metabolism prepares the drug for elimination from the body by conjugation with glucuronic acid. As a result, most morphine is excreted as morphine-3-glucuronide, but around 5-7 % is also excreted unchanged, and small amounts may be excreted as morphine-6-glucuronide and normorphine (Morphine, 2011).

Heroin is rarely pure and usually contains around 10% acetylcodeine, produced from the acetylation of codeine found in the raw opium (O'Neal, Poklis and Licht, 1998). This also has a very short half-life and is primarily metabolised to codeine (Brenneisen, Hasler and Würsch, 2002). This can be excreted unchanged, conjugated to glucuronic acid or further metabolised to morphine. Heroin use can, therefore, be identified by the presence of morphine and codeine and their metabolites. However, codeine is a readily available medicine that can be obtained over the counter without prescription (Codeine/Paracetamol - Pain Medicines and Drugs - NHS Choices). As it then metabolises to morphine, the presence of codeine and morphine does not necessarily mean that the urine sample tested is that of a heroin user. In this patient group, a limited clinical history is almost always available and the presence of morphine and codeine is enough to confirm the use of heroin. In other population groups, the presence of 6MAM can prove heroin use, but a morphine concentration higher than codeine can also point to heroin use.

The other principal opiate, dihydrocodeine, is easier to identify as it is metabolically distinct from morphine and does not metabolise to either morphine or codeine. As for codeine, it is also excreted conjugated to glucuronic acid.

Most of the samples tested within this work arose from known heroin users, as the main clinical users are the community drug teams and the addictions division of South London and Maudsley NHS Trust (SLAM). Most of these patients will be taking methadone as a substitution therapy for heroin and detection of this will supplement the immunoassay results previously obtained. Methadone is mostly excreted unchanged in the urine, but there are also significant amounts of the primary metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP).

Another drug in widespread use is cocaine. Cocaine is generally snorted or smoked as freebase (crack), but in this population it is often taken mixed with heroin. The resulting mixed drug is known as a speedball, and as the two drugs are partially metabolised using the same enzymes, the two drugs act synergistically (Duvauchelle, Sapoznik and Kornetsky, 1998). Cocaine is partially excreted unchanged, but is predominantly excreted as the hydrolysis product, benzoylecgonine. There are two other alternative metabolites of cocaine: anhydroecgonine is produced in the heat of a crack pipe and cocaethylene is produced in the presence of alcohol (Herbst, Harris, Everhart, Mendelson, Jacob and Jones, 2011). Neither of these metabolites are detected in the current TLC method.

As well as replacing the existing TLC method, it would be a benefit if the method could replace the existing GC method for amphetamines. This method can identify different members of the amphetamine group, including amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), methylenedioxy-methylamphetamine (MDMA), known as ecstasy, methylenedioxyethylamphetamine (MDEA) and phentermine. All of these drugs are mostly excreted unchanged, although amphetamine is a minor metabolite of methamphetamine and MDA is a minor metabolite of ecstasy. Extension of the method to other amphetamine-like substances, such as the novel psychoactive substance mephedrone, would also benefit the laboratory.

1.3. **Sample extraction**

Sample extraction is the name given to any process where the analytes are removed from the original sample matrix and transferred to a matrix that is suitable for chromatography. For GC systems that require water free samples for derivatisation,

this would mean transferring the analytes into a non-polar organic phase. As well as making the sample compatible with the chromatography, extractions can also remove interferences and concentrate the sample (Flanagan, Taylor, Watson and Whelpton, 2007).

1.3.1. Dilute and shoot sample preparation

Although not technically an extraction, this is perhaps the simplest sample preparation procedure in widespread use. It is generally used for urine samples that do not contain significant amounts of proteins and is simply the dilution of the sample by a factor of 10-20 or more (Fitzgerald, *et al*., 2012). Dilute and shoot is not suitable for most GC and normal phase HPLC applications, as the polar solvent content (usually water) is too high for the chromatographic part of the system. In addition, the dilution generally reduces the signal intensity, limiting the technique to analytes at a concentration in the low µg/L range and above and with some systems, such as ion traps, much higher concentrations. Dilute and shoot is commonly used to reduce ion suppression in triple quadrupole instruments when other sample preparation techniques (such as protein precipitation or derivatisation) are not required. Ion suppression (see section 1.6.7.) may be increased by salts and dilution of the sample reduces the salt concentration and therefore reduces the ion suppression. If the reduction of ion suppression is greater than the signal reduction caused by the dilution, this method produces an increased signal/noise ratio.

Although dilute and shoot is a common method of sample introduction, the number of papers utilising it is quite small, with only five of the papers identified in the literature search (see page 5) using the technique, with four of these papers being from the Karolinska Institute in Stockholm, the fifth being from the Saskatchewan Disease Control Laboratory in Canada. The reason for the limited number of publications in this area is unknown, but is most likely to be due to publication bias, rather than being a true reflection of the prevalence of the technique. This could occur either through the potential authors not considering the methods worthy of publication, and therefore not submitting the methods to journals, or by the journals and peer reviewers not accepting submitted manuscripts for the same or similar reasons.

1.3.2. Liquid-liquid extraction

This extraction method relies on the fact that polar and non-polar solvents are more often than not immiscible. Liquid-liquid extraction (LLE) is perhaps the simplest and cheapest extraction technique, requiring no expensive consumables and only a small amount of organic solvent (Flanagan, Taylor, Watson and Whelpton, 2007). Proteins and salts remain in the aqueous phase and analytes can be extracted into the organic solvent.

The selectivity of LLE can be modified by selection of different solvents and by adjusting the pH. Charged analytes will remain in the aqueous phase, as only neutral molecules can transfer into the organic layer (Flanagan, Taylor, Watson and Whelpton, 2007). Adjusting the analyte pH to around 2 units above the pK_a (for basic drug extraction) or 2 units below (for acidic analytes) will ensure that almost all of the analyte will be extracted. Selectivity can be enhanced further by additional extractions, typically a back-extraction into an aqueous phase at a different pH, although every extra process reduces the recovery of the target analyte. Evaporation and reconstitution of the organic phase can also be used to concentrate the analytes into a solvent compatible with the chromatography.

Liquid-liquid extraction is not easy to automate, as the sample tubes need to be well mixed and centrifuged to ensure good analyte transfer and instruments capable of this are not in widespread use. A recent development is to use a supported liquid extraction (SLE) cartridge, in which the aqueous sample is added to tube containing a Fuller's earth based support (Flanagan, Taylor, Watson and Whelpton, 2007). The sample is absorbed and an immiscible organic solvent is added, which drains through the support, performing the liquid extraction in the process. As with a liquid-liquid extraction, the solvent may then be concentrated or further extracted as necessary. However, an automated back extraction using SLE is generally carried out using a LLE or drying step (Brubaker, n.d.) and consequently SLE is not easy to automate when back-extracting.

There is a little more literature available for liquid-liquid extraction than for dilute and shoot, with 10 papers identified using this extraction technique, although only two of these (Mueller, Weinmann, Dresen, Schreiber and Gergov, 2005 and Klette, Horn, Stout, Anderson, 2002) use a urine matrix. None of these techniques includes a

back-extraction or SLE, most of the authors preferring to dry the organic solvent and resuspend in the mobile phase.

1.3.3. Solid phase extraction

Solid phase extraction (SPE) uses a silica or polymeric HPLC column packing material packed in a syringe barrel. The solvents used are optimised for either complete retention or complete elution, rather than the partial retention found in HPLC applications. Generally a sample is loaded at an optimal pH for retention and then washed with an appropriate solvent (Flanagan, Taylor, Watson and Whelpton, 2007). This solvent is selected to remove unretained or weakly retained interfering substances before a further solvent washes off the analytes into a collection vessel. As with LLE, this can then be evaporated and reconstituted as required. One disadvantage of SPE is that the extraction columns are disposable, and cost significantly more than the solvents required for liquid extractions. However, with no organic-aqueous boundary to detect, SPE can be automated with instruments capable of sequential addition of solvents and buffers and collection of the final extract, either as standalone (offline) instruments or directly connected to the chromatographic system, or online SPE.

Solid phase extraction has become one of the principal techniques in analytical toxicology, with 41 published LC-MS methods covering a range of matrices. SPE is more robust than standard LLE, with analyte recovery not being dependent on efficient solvent mixing or selective removal of the organic phase and this may explain the more widespread use of this technique

1.3.4. Online solid phase extraction

There are two different on-line SPE methodologies, those that are a simple automated SPE with disposable cartridges and those with re-usable SPE cartridges. Perhaps one of the best examples of the former is the Gilson ASPEC® automated SPE system that can either be operated in off-line or on-line modes. A good example of a method using this extraction system is the extraction and analysis of 29 drugs of abuse from oral fluid (Badawi, Simonsen, Steentoft, Bernhoft and Linnet, 2009). From a technical point of view, this can be seen as a simple automation of an existing SPE system, rather than a different method principle.

The first applications of re-useable online SPE methods differ very little from 2D-HPLC methods, with a first HPLC column acting as the SPE cartridge and the second column acting as the separation column. One of the difficulties with this application is that two HPLC columns connected in series can lead to very high pump pressures. To overcome this, online SPE cartridges have a large particle and pore size, leading to lower back-pressures. By reducing the size of the column to less than 10mm, analytes become more concentrated in a band, with little of the band broadening that can be found with a standard HPLC column. By combining an online SPE cartridge with standard HPLC equipment, extractions may be automated far more simply and cheaply than with a dedicated extraction system, although an additional HPLC pump is usually needed to allow SPE cartridge regeneration while the analytical gradient is running. SPE cartridges are more expensive than disposable cartridges used in conventional SPE, but can be cost effective when re-used. This does of course introduce an extra regeneration step before the cartridge can be used for the subsequent sample.

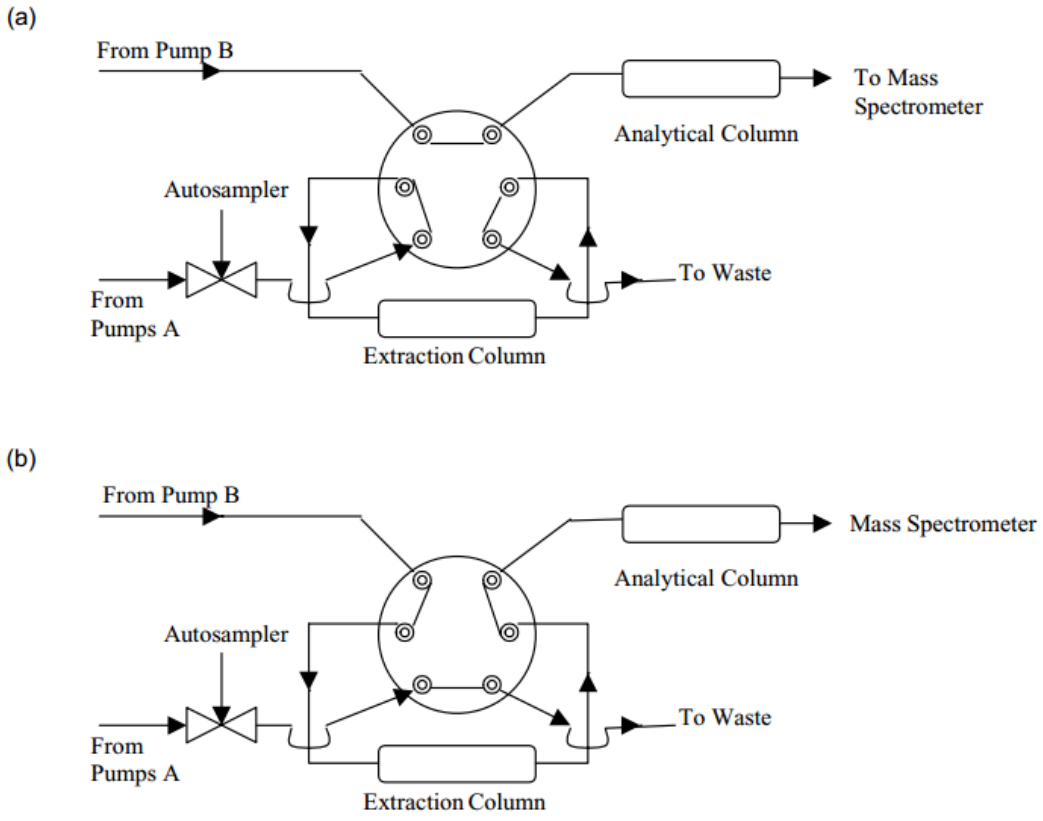


Figure 1.1 Plumbing arrangement of an online SPE system using 6-port valve (Xu, Turner, Meeker, Pursley, Arnold and Unger, 2003). a) Sample loading step with analytical column bypassed, b) Sample elution step. After sample eution, the valve is reset to position a) for extraction column regeneration

Different configurations have been tried with combinations of multi-port valves to suit the particular conditions in the laboratory. A six port configuration is shown (see Figure 1.1), but a method using an 8 port valve has been published by Callipo, Caruso, Foglia, Gubbiotti, Samperi and Laganà (2010) and a method using a 10 port valve has been published by Xu, Fan, Kim and El-Shourbagy (2006).

As a general rule, one HPLC pump is used to load the SPE cartridge while the other maintains a flow through the analytical column. The SPE cartridge is then switched to be in series with the analytical column for analyte elution and the SPE colmun is then regenerated prior to the next sample. Automated SPE is a technique that has not been widely published for the analysis of drugs of abuse. It may be that the equipment required for automation is not available in research laboratories and larger laboratories that have the equipment are less likely to publish their methods, perhaps due to commercial confidentiality for example.

A recent review of online sample extraction techniques covers this well, including variations such as stir bar sorptive extraction and the use of novel SPE materials, such as molecular imprinted polymers (Pan, Zhang, Zhang, Li, 2014)

1.3.5. **Solid phase micro-extraction**

Solid phase micro-extraction (SPME) works in a similar way to SPE, with a sorbent material to adsorb the analyte molecules. In SPME, the solid phase is typically coating a fibre contained within a hypodermic needle. The needle allows the septa of sample vials to be punctured, after which the fibre can be immersed in the sample. The relatively small sorbent size compared to the sample means that the sample needs to be agitated to ensure efficient extraction and this can take 20-30 minutes (Flanagan, Taylor, Watson and Whelpton, 2007). SPME can produce very clean extracts, but is ideally suited to headspace GC applications, where the fibre can absorb analytes in the vapour phase and then be placed directly in the GC inlet where the analytes can be eluted by thermal desorption (see Figure 1.2).

Variations of SPME have been tried and published, for example using an organic solvent soaked hollow fibre filled with an aqueous acceptor solution. Different pH solutions either side of the solvent membrane can help facilitate concentration of analytes by ion trapping (Yazdia and Es'haghi, 2005). This particular application is

particularly suited to water testing, as the low analyte concentration and long immersion time allow a good concentration step to facilitate trace analysis.

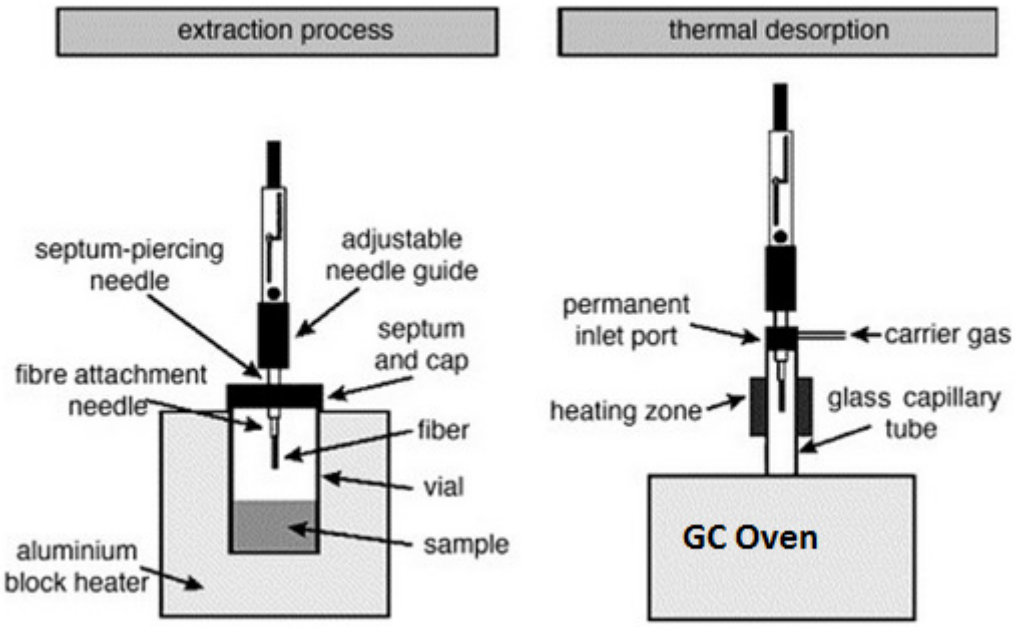


Figure 1.2 Diagram of solid phase microextraction with thermal desorption (Barié, Bücking and Rapp, 2006)

In the papers identified in the literature search (see p.5), none use SPME. It is most likely that the ease of interfacing SPME to gas chromatography strongly favours GC as an analysis method and the literature search specifically focussed on LC-MS.

1.3.6. Turbulent flow chromatography

Turboflow® is the trade name given to a system of online SPE that combines conventional SPE with a size exclusion column, technically known as turbulent flow chromatography (Couchman, 2012). The particles forming the solid phase contain pores in which analytes can bind to the polymeric or silica stationary phase. Larger molecules, such as proteins, have a much smaller surface area with which to interact and are retained less than the small molecule analytes for which the method is optimised. The extra functionality which makes the method particularly suitable for HPLC is that it is designed to operate at high flow rates.

A Turboflow® column is normally 0.5 mm diameter, much narrower than an HPLC column or an online SPE column (typically 2-5 mm). When operated at a flow above 2 mL/minute, the linear flow rate of the solvent through the column causes turbulence

within the spaces between the packing material, which is larger than the packing materials of HPLC (50 µm compared with 5-10 µm). This turbulence increases the efficiency with which the analytes adsorb to the stationary phase, allowing high recoveries compared to standard SPE.

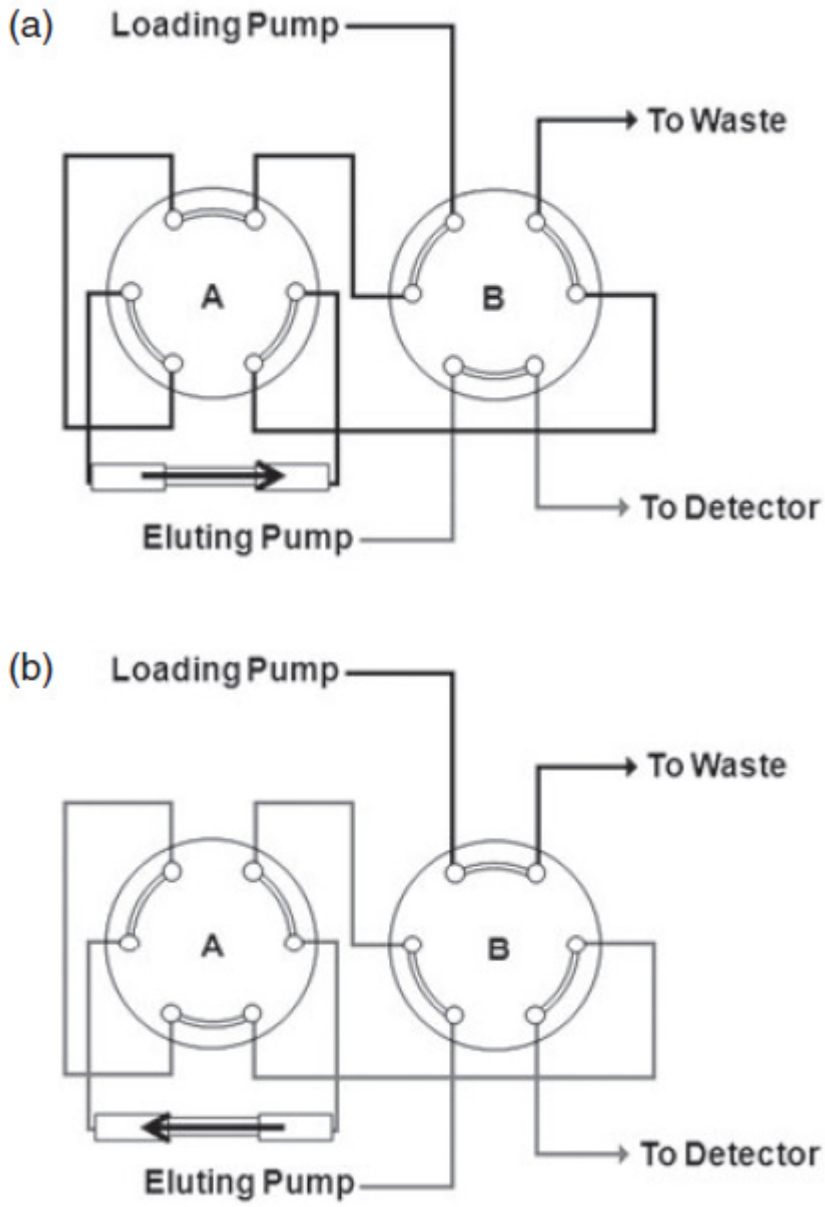


Figure 1.3 Plumbing of a Turboflow® System showing the valve arrangement (Couchman, 2012)

The Turboflow® system marketed by Thermo can be operated in either quick elute (see Figure 1.3) or Focus modes. Quick elute is generally used without an analytical column, although with careful solvent matching an analytical column may be used (Couchman, 2012). The focus mode is more versatile, with the elution solvent from the Turboflow® column being diluted with an aqueous mobile phase before loading

onto the analytical column. The relatively low organic strength is designed to be insufficient to elute the analytes on the analytical column, which are then focussed onto the head of the analytical column for later elution by an increase in the organic solvent strength.

There was little in the published literature about turbulent flow chromatography in 2009 and even less on its application to drug testing. Several of these were published in relation to drug discovery and were published as generic methods, not optimised for any particular drug target (e.g. Herman (2002) and Grant, Cameron and Mackenzie-McMurter (2002)). Methods have been published for 3 drugs, the antimycotic drugs ketoconazole (Ramos, Brignol, Bakhtiar, Ray, Mc Mahon and Tse, 2000) and terbinafine (Brignol, Bakhtiar, Dou, ajumdar and Tse, 2000) and the angiotensin II receptor antagonist telmisartan (Hempena, Gläsle-Schwarz, Kunz, Karst, 2006). Additionally a generic dual column method was published in 2002 (Grant, Cameron and Mackenzie-McMurter). None of these applications consider drugs of abuse or multiple classes of drugs. By 2014 there were more toxicology papers referring to Turboflow with 58 articles on the analysis of xenobiotics in serum, plasma or urine found in March 2014. It was also noted that several of these methods have been published on more than one occasion, for example at two different conferences (e.g. Duretz, Manchon, Guitton, Cohen and Moreau (2011) and Duretz, Cohen, Moreau, Guitton and Manchon (2011)) or as a poster and a journal article (e.g. Miles, Couchman, Spicer and Flanagan (2011), Couchman, Birch, Ireland, Corrigan, Wickramasinghe, Josephs, Spicer and Flanagan (2012)).

Of these 58 articles there are only four that relate to drugs of abuse:

Mueller, Duretz, Espourteille and Rentsch (2011) used a linear ion trap with an APCI source and dual Cyclone and C18XL Turboflow® columns to look for 356 drugs and metabolites in urine. An almost identical method was also published by this group (Mueller and Rentsch) in 2012 for 453 drugs in serum and plasma.

Yuan, Heideloff, Kozak and Wang (2012) used a triple quadrupole with an ESI source and dual Cyclone-MAX® and Cyclone-P® Turboflow® columns.

In 2013, two Turboflow® methods for drugs of abuse were published in the same paper by Schaefer, Peters, Schmidt and Ewald (2013), using a triple quadrupole in

MRM mode. One method was intended for opiates, amphetamines, cocaine and opioids while the other was intended for benzodiazepines. Both methods used an offline hydrolysis step, a Cyclone Max Turboflow column and a Hypersil Gold C18 2.1 x 50 mm analytical column. Use of a triple quadrupole allowed the method to be validated for quantitative analysis at the levels required for detection of drugs in apprehended drivers. The first method uses a solvent program very similar to the one outlined in the Thermo application note (Rezai, Kozak and Torchlin, 2007).

The methods of Mueller and Schaefer both use phenyl reversed phase columns, either PFP (Mueller) or phenyl/hexyl (Schaefer), and all 3 urine methods use an offline hydrolysis process. They also use two separate Turboflow® columns in series and the paper by Yuan states that this is the only way they could simultaneously extract and detect the cannabis metabolite, 11-nor-Δ9-tetrahydrocannabinol-carboxylic acid.

Mueller (2011) states that the offline hydrolysis is due to the poor Turboflow® retention of glucuronides of benzodiazepines and beta blockers.

While Turboflow has found some use within toxicology, there appear to be relatively few methods using other online extraction systems, although there are some, such as the Gilson ASPEC® automated SPE system described by Badawi, Simonsen, Steentoft, Bernhoft and Linnet (2009) or the Spark Symbiosis series of automated SPE instruments (Robandt, Bui, Scanella and Klette, 2010). There are also a few papers from groups using other small SPE cartridges similar to the Phenomenex cartridge used in the first part of this project, such as that by de Jager and Bailey (2011) using the Thermo Aquasil® C18 cartridge. Possibly the on-going consumable costs make these methods too expensive for most laboratories when the emphasis is on cost efficiency, especially in taxpayer funded institutions such as universities and the NHS.

1.4. **Chromatographic separation**

While it is possible to introduce a sample directly into a mass spectrometer, most clinical laboratory systems are hyphenated with a chromatography system. This allows the different components within a sample to be introduced sequentially and

therefore detected and identified individually. Both gas and liquid chromatography have been used for drug testing and each gives a different capability to the analysis.

1.4.1. Gas chromatography

Gas chromatography has been around since the 1950s and has been coupled with mass spectrometers since the 1960s. Gas chromatographs can be relatively simple instruments, comprising a separation column in a temperature controlled oven, a sample injection port and (usually) an autosampler.

The GC column is a tube usually made of glass, brass or steel and up to 3 m long, coiled to take up less space and fit in the oven. This column is packed with Fuller's Earth (or similar) that is coated in a liquid or stationary phase. Analytes must normally be introduced in a water-free medium, usually either an organic solvent or a vapour from a headspace injection vial. The heated injector port vaporises the solvent and analytes in gaseous phase are partitioned between the inert gas (usually hydrogen, helium or nitrogen) and their retention time is dependent on the partition coefficient of the analyte and on the temperature of the column. Often there is a temperature program that increases the temperature in a controlled manner, allowing some control over the elution times of the analytes.

1.4.1.1 *Capillary gas chromatography*

A most significant breakthrough came with the introduction of commercial capillary columns in the 1960's and 1970's (Scott, n.d.). These columns are usually between 5 and 30 meters long and less than 1 mm in internal diameter. The effect of this is that there are a very large number of theoretical plates in the column, meaning that chromatography peaks can be very pure, with typical peak widths of just a few seconds in a 10-20 minute run, sufficient to resolve *cis*- and *trans*- isomers of a compound. This means that there is a very high peak purity and spectra obtained in an electron impact source are highly reproducible, not only between different batches but also between different makes of instrument. The mass spectrum produced often has little noise and is likely to be very similar to a stored library spectrum. For drugs of abuse testing, this means that GC-MS quickly became the gold standard, a standard that has only recently been matched by liquid chromatography systems.

1.4.1.2 *Gas chromatography-mass spectrometry*

GC-MS is not without its disadvantages. As separation is achieved on the basis of volatility, GC-MS only works with volatile, heat stable substances. This puts a practical limit on the molecular mass of around 500 - 600 amu. Most drugs are of a relatively low mass, from 135 amu. (amphetamine) to 468 amu. (buprenorphine), well within the separating range of GC. However, some drugs of abuse are unstable at high temperatures, rendering them unsuitable for simple GC. Morphine, a very significant drug of abuse, has a molecular mass of 285, placing it well within the range of GC. However, morphine melts at about 250 °C and decomposes (Morphine, sulfate (pentahydrate), 2011), meaning that it cannot normally survive the GC separation to be detected. One common solution is to derivatise the morphine to both stabilise the structure and increase its volatility. Several of the most common derivatising agents add trimethylsilyl (TMS) functional groups in place of the hydrogen of the hydroxyl groups of the morphine. Although the mass is increased, the removal of hydrogen bonding hydroxyl groups actually increases the volatility of the drug. The molecule is also stabilised so that it can survive the temperatures encountered in the GC oven.

1.4.1.3 *Gas chromatography equipment*

As has already been mentioned, separation in a GC is mediated by temperature. A typical GC will begin with a low temperature of 100-150 °C and increase the temperature at 5 - 20 °C per minute until the final temperature is reached, often 300 - 400 °C. The system then needs to be cooled ready for the next sample. This puts a practical limit of around 3 - 6 samples per hour, although if operated isothermally it is possible to measure single drugs or single drug groups at a higher rate.

As shown (see Figure 1.4), the components of a GC system can be fairly simple, although autosamplers, column switching devices and cryotraps can all add to the complexity of a system.

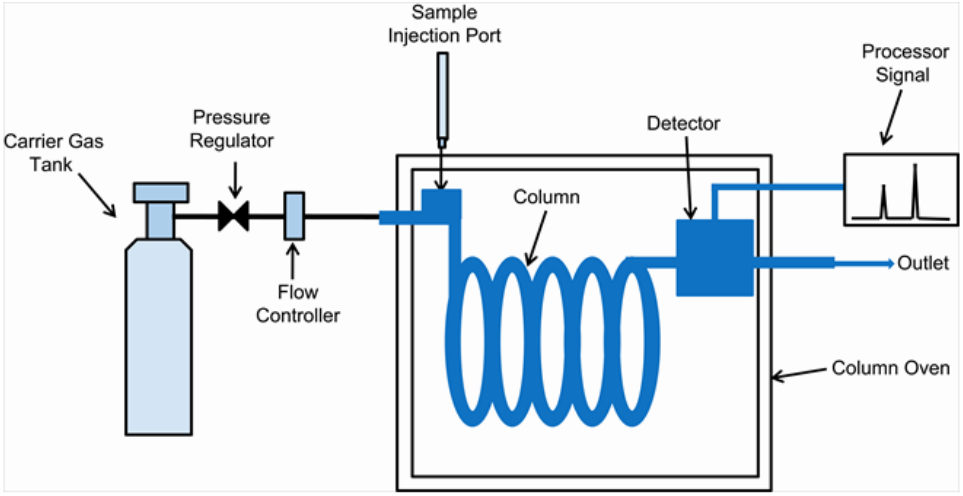


Figure 1.4 Schematic diagram of the main components of a gas chromatography system (Philadelphia College of Pharmacy, 2012)

Most GC systems cannot accept samples in an aqueous medium and the sample most commonly used for drug testing, urine, is water based. It is therefore necessary to extract the drugs from the aqueous sample into a solvent that is suitable for introducing into a GC. In practice the sample preparation procedures for drug testing must include an extraction and derivatisation before analysis, with analysis proceeding at the rate of a few samples per hour.

1.4.1.4 *Systematic toxicological analysis*

When screening a sample to try to identify whether there are any unexpected drugs present, capillary GC-MS with an electron impact source is unrivalled. Any peak identified can be compared with a drug library for a tentative identification and several different libraries are commercially available. Frequently the basic and acidic extracts of a sample are combined and analysed simultaneously.

This type of work is typically fairly low volume, well within the capability of these instruments running at only a few samples per hour, but is less good for clinical drugs of abuse testing where fast turnaround times are required and simpler methods are becoming increasingly important. One of the solutions to this is to use liquid chromatography.

1.4.2. High performance liquid chromatography

High performance (or high pressure) liquid chromatography (HPLC) was developed in the 1970s (Waters, n.d.) with ultraviolet or visible light detection (UV-Vis). A substantial advantage over GC is that the separation is usually performed at around room temperature in an aqueous solvent, meaning that derivatisation and extraction are not always required. This considerably simplifies the analytical procedure, but does of course come with a price. HPLC columns are much shorter and thicker than GC columns, usually being less than 25 cm long and 2-5 mm in diameter. If the column is much longer than this, the pressure needed to pump the solvent becomes very high, beyond the engineering standards required for simple pumps and connectors. These short columns generally have a much lower theoretical plate count than GC columns, so that the peaks obtained are not as sharp and defined, and separation is not as good. With a long gradient of increasing organic solvent composition, it may be possible to get good separation between different peaks, but the resolution of the system and the run time limits the number of peaks that can be identified with a UV detector. With a mass selective detector, this overlap is much less critical and as long as isobaric peaks do not overlap, a very high number of compounds may be identified. In an application note produced by Agilent (Zweigenbaum, Flanagan, Stone, Glauner and Zhao, 2009) over 600 different pesticides may be identified in a single 13 minute analysis.

1.4.2.1 *Components of a high performance liquid chromatograph system*

HPLC systems generally have more components than GC systems and are correspondingly more expensive. As well as the autosampler, a pump is required that is capable of pumping at the high pressures used in HPLC. Traditional systems had upper pressure limits of around 20,000 kPa, but modern systems can achieve pressures of 60,000 kPa. This is in contrast to GC systems where a column head pressure for a typical GC column may be up to 2,000 kPa and is usually less (Sigma Aldrich, 2010)

Most HPLC systems include a system for mixing two or more mobile phases. With a high pressure mixing system (see Figure 1.5), the two mobile phases are mixed on the high pressure side of the pumps. This gives a relatively small gradient delay volume, but requires a separate pump for each solvent. In contrast, a low pressure

mixing system will use a proportioning valve on the low pressure side of a single pump to achieve the same mixing of solvents. The gradient delay is generally longer than for high pressure mixing, but only a single HPLC pump is required.

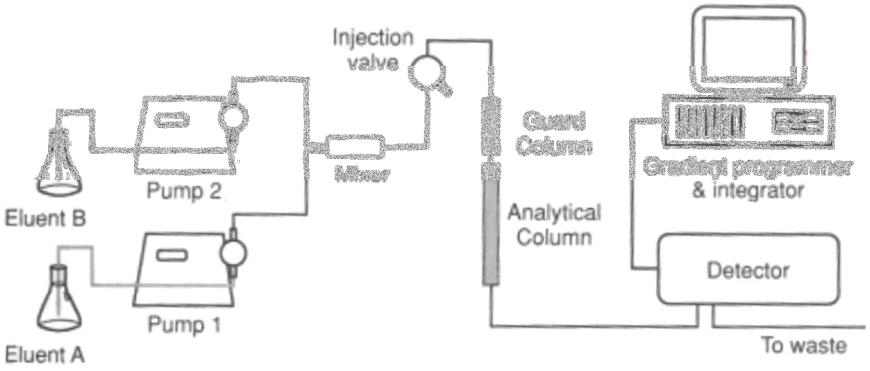


Figure 1.5 Diagram of the main components of a typical HPLC system (Flanagan, Taylor, Watson and Whelpton, 2007, p. 179)

Most systems will also have a column oven or column compartment to maintain the HPLC column at a set temperature. This increases the reproducibility of retention times and is important for any complex analysis. As for GC, there may be many additional components to the system, such as an autosampler, column switch or solvent degassers.

Another benefit of using HPLC is that a large number of different column and mobile phase combinations is possible, far more than for GC where the mobile phase is a non-reactive gas. Changes in column and mobile phase chemistry can change the retention characteristics of drugs completely, even allowing a reversal of the eluting order if required.

1.4.2.2 ***Normal phase chromatography***

The original HPLC methods used pure silica as a stationary phase and had a highly organic mobile phase. The mobile phase in normal phase HPLC has a low water content and interactions between the analytes and stationary phase are generally strongest for polar molecules (Lab-Training.Com, n.d.). A higher aqueous content of the mobile phase leads to less interaction between the analyte and column and this leads to a shorter retention time. The greater the polarity of the analyte, the greater the interactions between the analyte and stationary phase and the greater the

retention time, whereas non-polar analytes will elute relatively quickly. Normal phase HPLC requires the sample matrix to be largely organic and an extraction is usually required to maintain consistent retention times. Normal phase HPLC is not common in drugs of abuse testing.

In a literature search in 2009, only two papers were identified that used normal phase HPLC. Fitzgerald, Rivera and Herold (1999) used a reversed phase column and an anion exchange column as an online extraction system, with the normal phase column being used for the separation. In this way the high organic strength mobile phase used for the elution step does not hinder the separation in the following normal phase column. Naidong, Jiang, Newland, Coe, Lin and Lee (2000) used normal phase HPLC of hydromorphone following an acetonitrile precipitation of human plasma, the normal phase separation eliminating the need to dry or back-extract to recover the hydromorphone from the acetonitrile. These papers both represent an elegant solution to high organic content sample extracts, although most papers viewed use an alternative approach, mainly drying and resuspending the sample extract.

1.4.2.3 ***Reversed phase chromatography***

The advent of HPLC columns with alkyl groups attached enabled the silica to be protected from chemical reaction and allowed predominantly aqueous mobile phases to be used. Interactions between the analyte and column are generally through hydrophobic interactions, although for phases containing a phenyl moiety π bonding can also play a significant part (Sigma-Aldrich, 2013). Less polar analytes will be retained longer on a reversed phase HPLC column than polar analytes and a column with an eighteen carbon alkyl chain (C18) will give greater retention than an eight carbon chain (C8). For most common HPLC columns, a wide range of solvent conditions can be used, including gradients where the organic composition of a solvent is increased during the analytical run. Most reversed phase columns cannot cope with 100% aqueous solvents, as this may cause the alkyl chains to collapse (Przybyciel and Majors, 2002), and gradients typically start with above 10% organic component. There are some modern stationary phases that can operate in 100% water, such as some phenyl and cross-linked alkyl phases, which further increases

the range of conditions available to the chromatographer. Reversed phase columns are the most common in use in drug testing.

Of 72 published methods, two use normal phase and over half (41) of the others use a C18 column, the most commonly used reversed phase column. The remaining columns in use are predominantly phenyl type (17) or C8 columns (9).

One of the big advantages of HPLC over GC is the change in selectivity that can be achieved by altering the solvent conditions. Once a column is chosen, there are still several options that can significantly alter the retention characteristics of the system. One of these is the choice of the organic component of the separation, with the two most commonly used solvents being acetonitrile and methanol. Acetonitrile is the commonest solvent used in the methods identified above, with at least 50 methods using this as a solvent. This may be partly because acetonitrile does not strongly absorb UV radiation and has therefore become a very common HPLC solvent. Acetonitrile is also miscible with water and an increasing percentage of acetonitrile in a solvent leads to a lower pressure than water alone. It is not without its problems, as acetonitrile can cause polymer build up on HPLC check valves (Dolan, 2008) and is produced commercially as a waste product of the plastics industry. During the recession of 2008, acetonitrile prices increased by a factor of 10 (Kenward, 2010) and acetonitrile became in short supply.

Methanol has a similar solvent strength to acetonitrile and was used in at least 14 of the methods identified above. Although generally cheaper than acetonitrile (even before the price of acetonitrile increased in 2008), it is not without its problems, as a mixture of water and methanol gives a higher back-pressure than water alone. This means that flow rates must be lower when using methanol than when using acetonitrile, which can be a limiting factor when high throughput is desirable. However, methanol can reduce peak tailing compared to acetonitrile and can give a different selectivity, meaning that the choice of solvent must be a balancing act of several different factors.

Most other organic solvents in common use have their own problems when it comes to chromatography. Using a stronger solvent can affect the reproducibility of a separation, as a small variation in the solvent composition can have a more pronounced effect than a similar level of variation with either acetonitrile or methanol.

Other solvents are difficult to use due to their immiscibility (e.g. chloroform), toxicity (e.g. benzene) or cost (e.g. tax on ethanol) and as a result only acetonitrile and methanol are usually used as the principle organic solvents in HPLC.

1.4.2.4 *Ion exchange chromatography*

In addition to hydrophobic, hydrophilic and electrovalent (π bonding) interactions, retention can also be achieved by ionic interactions. Typically the HPLC column has a sulphonyl or amine group embedded, which are ionised except at very acidic and basic pH values respectively. Retention is achieved through interactions between ionic analytes and the stationary phase, with retention generally being dependent on the ionic strength (pK_a) of the mobile phase and analyte. Ion exchange HPLC can produce very selective retention (Morgan, Fisher, Evers and Flanagan, 2011), with neutral analytes being unretained. A strong ion exchange resin will be ionised under almost any pH condition, making it suitable for a wide range of analyses, and a weak ion exchange resin will become neutralised at a given pH, removing any ionic interactions and ionic retention.

Ion exchange chromatography can be combined with a reversed phase to produce a mixed mode interaction. This is usually achieved by attaching the ion exchanger to an alkyl chain, with retention being based on a mixture of ionic strength and hydrophobic interactions.

Most drugs of abuse are basic, with one or more amine groups that can become protonated under basic conditions. This makes cationic exchangers work well for many drugs, although some drugs, for example cannabis and ibuprofen, have a low pK_a and are more suited to anion exchange. However, ion exchange chromatography has not proved popular for multi-analyte drugs of abuse testing and ion exchange is far more common in the sample preparation steps of an analysis.

1.4.2.5 *Hydrophilic interaction liquid chromatography*

Hydrophilic interaction liquid chromatography (HILIC) is a recent extension to normal phase chromatography in which a pure silica stationary phase and an acetonitrile based mobile phase are used (Buszewski and Noga, 2012). A small amount of water in the mobile phase coats the silica to produce an aqueous stationary phase, similar to the waxy stationary phases in gas liquid chromatography. Retention is achieved

through differing hydrogen bonding strengths between the analyte and the aqueous layer on the stationary phase. It takes time for the water to form a stable layer and HILIC is not well suited to gradient chromatography. Since structures of drugs of abuse vary considerably, HILIC does not lend itself to multi-drug analysis, although it has been used for analysing single classes of drugs (Gheorghe, *et al.*, 2008).

1.4.2.6 ***Developments in high performance liquid chromatography***

Instrumentation for liquid chromatography is generally still recognisable from the first commercial HPLC systems 40 years ago. The computing power has increased so that pumps and autosamplers can generally be controlled from software and modern integration software is significantly easier to use than modular integrators. Gradients can be formed from high pressure or low pressure mixing with binary and quaternary pumps and autosamplers are increasingly sophisticated. However, where there has been the greatest effect on the chromatography is in the column. As well as the wide range of phases available, the structure of the particles that form the packing material has gone through several generations of development and these developments have led to a number of other significant changes that affect the separation.

For many years the only particle size available for an HPLC column was 5 µm diameter silica, which was generally available in a 4.6 mm internal diameter column that could vary in length between 5 and 25 cm. The van Deemter equation (see Equation 2, Appendix C) for resolution in HPLC balances the linear velocity of the eluent and the resolution of the separation. Eluent linear velocity itself is governed by the mobile phase flow rate and the internal column volume. Use of a narrower bore column will increase the eluent linear velocity and lead to increased chromatographic resolution. With increased resolution, the length of the column can be reduced, reducing the back-pressure on the pump and therefore allowing still higher flow rates. As a result, a 2.1 mm internal diameter (i.d.) operating at 0.2 mL/min would have a similar linear velocity as a 4.6 mm i.d. column running at 1 mL/min (see Equation 1, Appendix C). This has meant that around 75 % of columns used for drugs of abuse testing in the literature in the last 10 years are 2.1 mm internal diameter or less, with a median flow rate of 0.3 mL/min. Together with short HPLC columns (98 % of these are 150 mm long or less) analysis times and solvent usage has been cut dramatically

from the typical 20 – 30 minute runs at 1 mL/min or more that were previously common.

Another improvement in column technology is governed by the expanded van Deemter equation (see Equation 3, Appendix C) which shows that if the particle size is reduced, the plate height is also reduced, which means that the resolution is increased. Much of this increase is due to the increased surface area available with smaller particles, as the surface area of a sphere reduces with the square of the radius but the volume decreases with the cube of the radius. The 5 µm particles in common use ten years ago are now often replaced with smaller particles and 3 µm particles are common and, in the last few years, particles have become smaller still, with 1.8 µm particle columns now easily available commercially. This creates a technical challenge, as the spaces between the particles become smaller and the mobile phase does not pass though so easily. This leads to increased pressures and many modern liquid chromatography pumps are capable of pressures of 600 bar, compared to 150 - 200 bar that standard HPLC systems are capable of. These high pressures can cause the silica particles that form the stationary phase to be crushed and deformed and polymeric particles are now also available. As well as being more pressure resistant, polymeric particles can be more carefully controlled at the manufacturing stage so that the silanol groups that can affect some reversed phase columns are absent.

Although column size has reduced significantly, the reductions in particle size have been less dramatic, with only 40 % of these columns using particle sizes under 5 µm. This may be because the true benefit of smaller particles can only be achieved with increases in pressure, which necessitates new and expensive hardware. Use of a 5 µm particle can still give very good resolution without the expense of new HPLC pumps and autosamplers, particularly when combined with a narrow bore column.

An alternative to increasing the pressure capacity of the pump (and therefore the column fittings and autosampler) is to use particles that have a highly porous outer layer with an impermeable centre. This leads to an increased surface area, and therefore resolution, without the corresponding increase in pressure. These fused core or porous shell particles are available from several different manufacturers, but

are a recent development and have not found their way into the literature for drugs of abuse applications.

It follows that any technique that allows the use of a higher flow rate or analyte mass transfer (adsorption rate) can also increase the resolution of an HPLC system. Acetonitrile is often chosen as an HPLC solvent because it causes a reduction in back-pressure when mixed with an aqueous component in a gradient system, whereas a methanolic phase causes an increase in back-pressure. For a given pump or column, an acetonitrile gradient can therefore use a higher flow rate than a methanolic solvent without reaching the pump or column's pressure limits. Similarly, warming the solvent to above ambient temperature, typically to 40 – 60 °C, will reduce the viscosity of the mobile phase components and this will also reduce the back-pressure. Mass transfer is also more rapid at a higher temperature and the effects of a column oven are therefore more than simply a reduction in mobile phase velocity. Temperatures above this can be a hazard due to the flammability of organic solvents, so the benefits of the reduced viscosity are limited by safety. Some specialised applications use water superheated above 100 °C to improve the separation characteristics of a method. The superheated water can even allow separations to be performed in 100 % aqueous mobile phases. However, this needs specialised equipment and can only rarely be justified.

Drugs of abuse testing has taken advantage of many of these developments to improve the laboratory's ability to detect drugs. However, the use of a mass spectrometer partially negates the need for high quality separations, as the detector can be so highly specific. As a result, some of the more expensive or specialised options such as the smaller particle size columns have not been widely published.

Chromatography methods have not changed significantly in the 6 years since the project started, with short narrow-bore columns remaining the preferred choice. More efficient particles are increasingly available, such as fused core particles (Arbeláez, Borrull, Marcé, Pocurull, 2014). Acetonitrile is again readily available and the mobile phases used are similar to those found in 2008.

1.4.3. Capillary electrophoresis

Electrophoresis is the separation of ionic species according to their charge and their mass or structure. Widely used for the analysis of proteins in serum, modern electrophoresis uses a capillary column similar to those used in GC applications, although with a liquid mobile phase. A range of different separations can be produced based on the pH and composition of the mobile phase, including variations such as Capillary Micellar Electrokinetic Chromatography that allow the analysis of neutral components (Flanagan, Taylor, Watson and Whelpton, 2007).

Capillary electrophoresis is not commonly hyphenated with mass spectrometers and is not available for use at King's College Hospital. Although a significant analytical method, it will not be discussed further in this work.

1.4.4. Thin layer chromatography

Thin layer chromatography, as has been mentioned already, was the method of choice in early clinical toxicology laboratories. Modern TLC has changed significantly since the days of manually spread plates, and modern high performance TLC (HPTLC) systems can use very controlled conditions to produce very reproducible separations (Flanagan, Taylor, Watson and Whelpton, 2007). It is possible to then place a TLC plate into a mass spectrometer with a MALDI or SELDI source to use the mass spectrometer to "visualise" the plate in exactly the same way as a proteomics lab will identify the components of a 2D SDS-PAGE gel (Sodium dodecyl sulphate – polyacrylamide gel electrophoresis). However, this is not an efficient use of one of these expensive systems, which may be better utilised to identify complex mixtures of unknowns. As with capillary electrophoresis, discussion of these specialised techniques will not continue in this document.

1.5. Mass spectrometric detection

A wide variety of detectors can be used with chromatographic systems such as HPLC or GC. For HPLC, UV-visible spectroscopy is perhaps the most common detector, although specialist detectors such as electrochemical and fluorescent are also available. For GC, flame ionisation detectors, nitrogen-phosphate detectors and thermal conductivity detectors give either general detection or selective options for

detection. However, for toxicology there are few detectors that can beat the mass selective detector or mass spectrometer, which can be interfaced to either GC or HPLC systems, as well as to electrophoresis or thin layer chromatography if required.

The first mass spectrometers were standalone systems, with the sample introduced at one end and the result being produced subsequently. This technique is fine for the analysis of pure materials, mixtures and solids, but is not so useful when it comes to the analysis of complex mixtures such as may be found in biological fluids. To overcome this, an initial chromatographic separation is performed, followed by mass spectrometry of the chromatography product. These systems that consist of two different analytical techniques linked together are called hyphenated methods (Maurer, 2006). The most common arrangement is to have a chromatographic system, whether gas chromatography, liquid chromatography or capillary electrophoresis (CE), linked to a detector system, usually a mass spectrometer, although nuclear magnetic resonance (NMR) detectors have been used. However, the number of possible combinations is huge, with 2 separate chromatographic systems running in parallel being another option, for example GC-GC, or 2D-GC (Kolbrich, Lowe and Huestis, 2008).

1.6. **Mass spectrometry theory**

Mass spectrometry is the separation and detection of materials based on their relative molecular mass. This allows a highly selective method of detecting a substance, as the molecular mass will depend on the chemical structure of the molecule. Morphine has a molecular mass of 285 (National Center for Biotechnology Information, U.S. National Library of Medicine) and codeine has a molecular mass of 299 (National Center for Biotechnology Information, U.S. National Library of Medicine) (both rounded to whole numbers). It follows that a system able to separate and detect these two molecular masses can discriminate between the two drugs. Conversely, norcodeine, which also has a molecular mass of 285 is indistinguishable from morphine in a simple mass spectrometer, as their molecular formulae are identical (Fox, Twigger and Allen, 2009).

Light spectrometry is the measurement of the intensity of light after it has been passed through a prism to separate the different wavelengths. Early 20th century

spectrometers originally used a photographic plate to record the signal intensity and the wavelength would be determined by the position of the signal on the plate. In the same way, early mass spectrometers often recorded their output on a photographic plate, with the mass determined by the position on the plate and the degree of exposure representing signal intensity. This is the origin of the term “mass spectrometry” and a graph or plot of the relative signal intensity against the mass is still termed a “mass spectrum”. The similarities between light spectroscopy and mass spectrometry can be seen (see Figure 1.6), although the ion path in a mass spectrometer is generally curved.

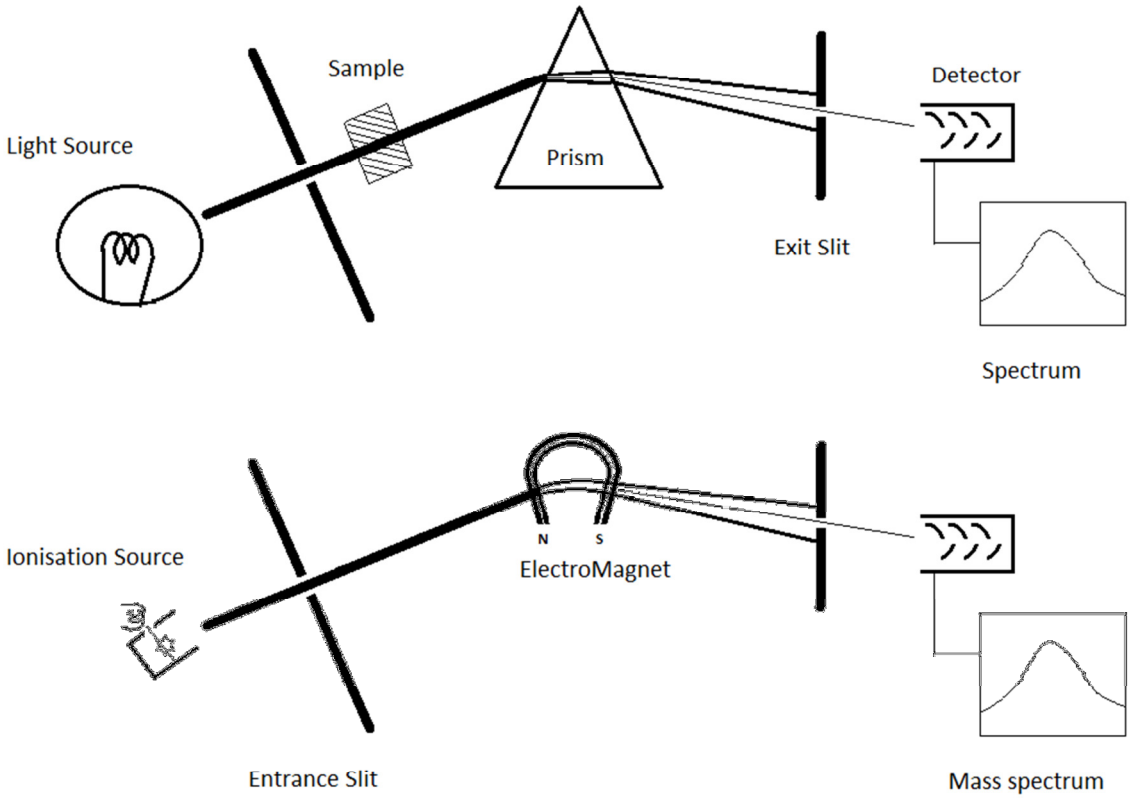


Figure 1.6 Comparison of the similarities between light and mass spectroscopy

1.6.1. Ions and molecules

A light spectrometer uses a prism to direct photons towards a photographic plate. Photons can pass easily through the glass. However molecules do not show this wave-like characteristic and their path would be blocked by any materials in their way. Mass spectrometers must therefore manipulate their analytes in a vacuum to avoid collisions with air molecules. This means that mass spectrometers need another way of manipulating their target analytes and most mass spectrometers use either electrostatic or magnetic interactions to control the movements of the materials to be detected. A good resource on the history of mass spectrometry and the principles behind the various different modes can be found at the Scripps Centre for Metabolomics (Scripps Cente for metabolomics, 2013).

Since most molecules are neutral, they will be unaffected by the electrostatic and magnetic forces and cannot be deflected, focussed or separated by the mass spectrometer. To avoid this molecules must be given a positive or a negative charge, which then allows them to be manipulated by the electrostatic or magnetic forces within the mass spectrometer.

Molecules can gain or lose one or more protons or electrons to become ions and these ions can be easily manoeuvred in the mass spectrometer by a series of shaped electrostatic or magnetic plates or lenses. The speed or direction of movement is determined by the molecular mass of the ion and the number of charges it has, a property known as the mass/charge ratio, or m/z. When a molecule has gained or lost a proton, the m/z of an ion will differ by one mass unit when singly charged. This ion is known as the molecular ion and is often denoted as [M+H] or [M-H]. The charge on the ion defines one of the basic parameters of a mass spectrometry method, either operating in positive mode or negative mode. (Scripps Centre for Metabolomics and Mass Spectrometry)

Most mass spectrometers are unable to tell the difference between a single charged ion and an ion that has both twice the mass and twice the number of charges. This is particularly problematic for large molecules such as proteins, but is less of an issue with small molecules such as drugs of abuse, which usually have a molecular mass in the range 100-500 atomic mass units (amu.). Smaller molecules are usually not

physically big enough to be stable with many charges and addition of a second charge will often cause the molecule to fragment (University of Pittsburgh, 2013).

1.6.2. Mass selection

There are a number of different technologies used by mass spectrometers to select and isolate the molecules being tested. The early magnetic sector instruments took up a large amount of space and were very expensive (Mass Spectrometry Facility, 2012). The modern sector type systems are smaller, but remain expensive and are not often linked to HPLC systems and will not be discussed further here.

There are four different mass selection technologies that are in widespread current use and each has its own benefits and limitations. The choice of mass spectrometer will be made largely dependent on the intended use and which of the technologies is most suitable.

1.6.2.1 *Quadrupole mass analyser*

In a quadrupole instrument, a set of four metal rods are arranged parallel to each other in a square formation (see Figure 1.7). The ions enter the space between the rods at one end and are subjected to the electromagnetic forces exerted by the rods. Opposite rods are arranged in opposing pairs, with one set having a radio frequency potential across them and the other pair a constant voltage. By rapidly varying the voltages applied, ions of a given mass or mass range can be made to stay in a stable trajectory within the rods and exit at the other end. Ions that do not fit the mass selection criteria have unstable trajectories and do not leave the ion channel at the exit.

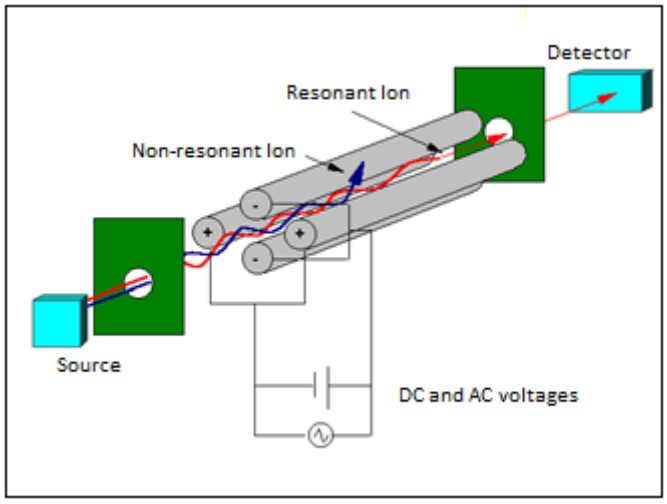


Figure 1.7 Schematic arrangement of a quadrupole mass spectrometer lens system (Tissue, 2000)

The size and geometry of the quadrupole rods defines the mass accuracy of the system (Gershman, Block, Rubin, Benna, Mahaffy and Zurbuchen, 2012), which is usually between 0.1 and 0.5 amu. Circular rods are common in cheaper systems and can provide good unit-mass resolution. More expensive and higher specification instruments have hyperbolic rods, which can give greater mass resolution. As ions are continually entering and exiting the quadrupole, the data collection rate can be quite high, leading to relatively low detection limits (Berger, Langlois, Oehme and Kallenborn, 2004). The selected mass can also be varied quickly so that a range of masses is scanned to produce a mass spectrum. Simple quadrupoles are also used in other instruments as non-selective mass guides, for example allowing all masses to be guided from a lens system to a mass filter (Mass Spectrometry Facility, 2012). Hexapoles and octopoles made from 6 or 8 rods operate on the same principles and are also common in modern instruments, but act only as transfer optics, not a mass filter (Pentek, 2011).

In terms of drugs of abuse testing, quadrupoles are now almost unused in LC-MS, although they still find use in GC-MS systems. In the survey of published drugs of abuse LC-MS methods (see table 1.1), only 6 of 68 systems identified used single quadrupole systems, the most recent being that described by Concheiro, Castro, Quintela, López-Rivadulla and Cruz (2006) that was developed and in use in 2004.

1.6.2.2 *Ion trap*

A three dimensional (3D) or quadrupole ion trap (see Figure 1.8) uses similar electrostatic interactions to a quadrupole, but the ions do not transit the length of the device as in a quadrupole and are instead trapped in the electromagnetic field. Usually the trap consists of two end cap electrodes and a ring electrode and when seen in cross section the similarity with a quadrupole cross section is apparent (see Figure 1.9). A mass or range of masses can be trapped within the space between the end caps and they are then ejected through holes in the end caps to reach the detector.

Figure 1.8 Schematic arrangement of the lenses of a quadrupole (3D) ion trap (O'Hair, 2006)

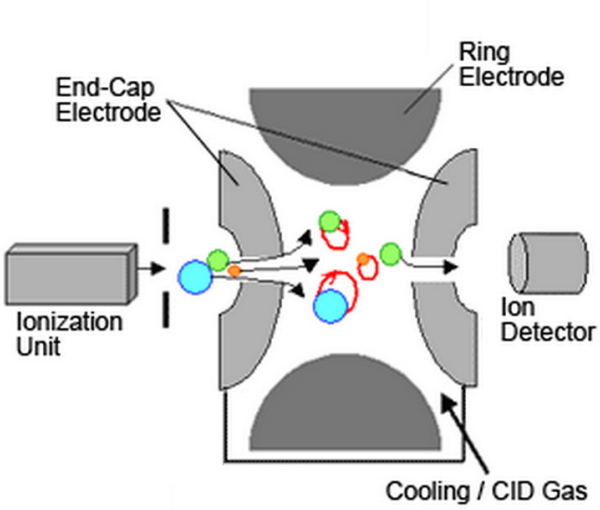


Figure 1.9 Sectional view of a quadrupole ion trap to show the similarity to a quadrupole (Shimadzu, 2014)

Two dimensional (2D) or linear ion traps (see Figure 1.10) consist of a quadrupole in which each rod is split into three sections. Collectively the middle sections act as the ring electrode and the end sections act as the endcap electrodes. In some models,

the ions are ejected through slits in the quadrupole rods (e.g. Thermo LXQ) and in some models the ions are ejected through the end of the quadrupole rods, in the same manner as if the trap was a standard quadrupole (e.g. AB SciEx Q-Trap). The advantage of a linear ion trap is that the cavity in which the ions are trapped is long and narrow, as opposed to a 3D ion trap where the cavity is of equal size in all three dimensions. This allows more ions to be confined simultaneously, giving an increase in mass resolution and limits of detection when compared to a 3D ion trap.

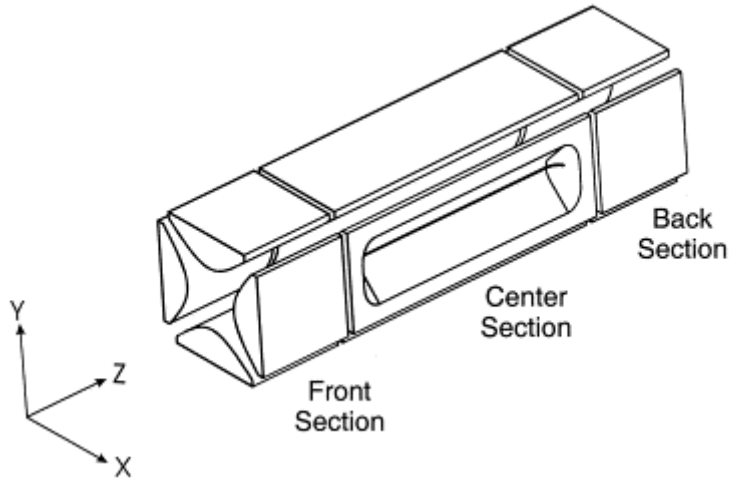


Figure 1.10 Diagram of a linear (2D) ion trap showing the ion exit slit in the centre section (Shwartz, Senko and Syka, 2002)

Ion traps often have a lower mass resolution than quadrupoles, although they can still achieve mass accuracy below unit mass. However, ion traps cannot allow a single mass to enter and exit continuously like a quadrupole and must work in discrete segments. As a consequence the data capture rate (and therefore limit of detection) will be poorer than for the equivalent specification quadrupole (Fitzgerald, O'Neal, hart, Poklis and Herold, 1997). However the rate at which ions are scanned out of the trap and detected is higher than for a quadrupole and an ion trap is therefore faster at producing a mass spectrum.

Ion traps are not common in toxicology testing and in the 68 methods referred to there are only 9 ion traps, all of them being Thermo's LCQ Deca or LCQ Advantage models. This is likely to be a result of the poor limit of detection of these systems compared to triple quadrupoles.

1.6.2.3 *Time of flight*

Time of flight (TOF) instruments (see Figure 1.11) release all ions simultaneously in discrete packets and accelerate them with the same energy along an ion path. Since the kinetic energy of the ions equals the mass divided by their velocity, the relative speeds at which the ions travel will be dependent only on their m/z ratio and they will arrive at the detector at different times, with the lighter ions arriving before the heavier ions. Often the ion guide will have the emitter and detector at the same end of the tube, with a reflector at the other end to reduce the space taken.

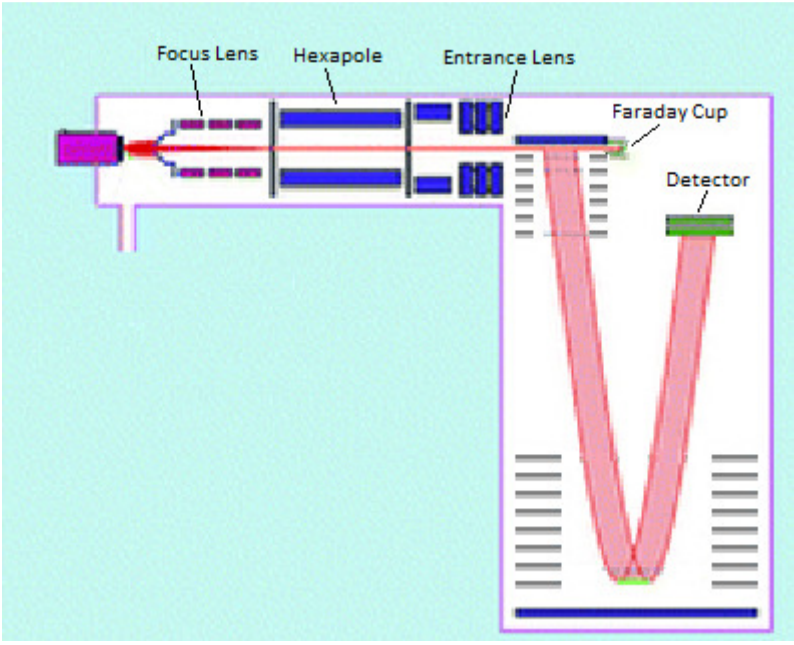


Figure 1.11 Schematic representation of the lens system of a time of flight mass spectrometer (Tong, Yu, Jin, Hi, Hang and Huang, 2009)

Mass resolutions can be very high (3 decimal places of mass accuracy) and comprehensive mass spectra can be obtained, but like an ion trap, data must be collected in discrete segments rather than continuously like a quadrupole. Only a single method was found for drugs of abuse using a time of flight detector (Pelander, Ristimaa, Rasanen, Vuori, Ojanperä, 2008)

1.6.2.4 *Orbitrap*

The orbitrap (see Figure 1.12) is a development of ion cyclotron resonance mass spectrometry that combines the mass separation and detection parts of a mass spectrometer in a single component. Most other mass spectrometers use a dynode for ion detection, in which the ions strike a target which emits electrons. These

electrons strike an electron multiplier, producing a signal that can be detected and processed. In the orbitrap, the movement of the ions around a spindle induces tiny currents in adjacent electrodes. These currents are amplified and subjected to a Fourier transform algorithm from which all masses present can be identified.

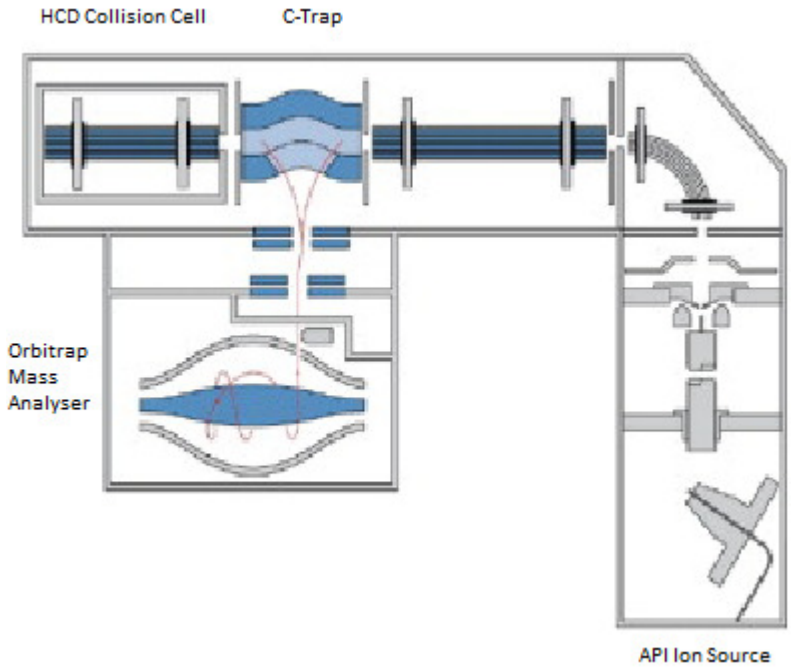


Figure 1.12 Diagrammatic representation of the lens system of an orbitrap mass spectrometer (Bateman, Kellman, Muenster, Papp and Taylor, 2009)

The orbitrap operates to better than four decimal places of mass accuracy, but has a data collection rate of one scan per second at this accuracy and a scan time of 100 milliseconds at its lowest resolution, around three decimal places of mass. Quadrupoles often have a data window of less than 10 milliseconds. These instruments were first marketed in 2005 by Thermo, but in the literature search mentioned there were no orbitrap papers listed. A more specific search identified the orbitrap as being used in doping control (Virus, Sobolevsky, Rodchenkov, 2008) and without the use of an HPLC system (Kauppila, Talaty, Kuuranne, Kotiaho, Kostiainen Cooks, 2007)

1.6.3. Tandem mass spectrometry

Collectively these techniques are known as mass spectrometry (MS) and they are suited to identifying masses of ion species present. Identification of the mass, known as the parent mass, is extremely useful and can allow the differentiation of two

different drugs, for example morphine (m/z = 286) and codeine (m/z = 300). However, parent mass spectra cannot tell the difference between two drugs of the same mass, or isobaric compounds, such as morphine and norcodeine (m/z = 286). These substances could be separated chromatographically in an LC-MS or GC-MS, or they could be split into fragments and these fragments subjected to mass spectrometry. This technique is known as tandem mass spectrometry (MS/MS or MS^2) and can allow a high degree of confidence in the identification of a molecule (Madeira and Florêncio, 2012).

Tandem mass spectrometers usually contain at least two mass selecting components, with some form of fragmentation mechanism between them. The commonest type of tandem mass spectrometer is the triple quadrupole, where the first and third quadrupoles act as mass filters and the second quadrupole is used as a collision cell. The collision cell is often a different design, such as a hexapole, but the instrument is still referred to as a triple quadrupole as the principle is the same. Triple quadrupoles can operate in selected reaction monitoring (SRM) mode where a given parent ion is fragmented and a single fragment ion detected (Madeira and Florêncio, 2012). Not much spectral information can be obtained, but this mode of operation gives one of the most sensitive modes of analysis and is well suited to the quantification of known analytes.

Tandem mass spectrometers will often have a quadrupole as the first mass filter (often abbreviated to Q), followed by a collision cell and then a different mass filter, either time of flight, an ion trap or an orbitrap. Each of these will give information rich spectra of the fragments of the parent ion and therefore very good identification criteria. Most combinations of spectrometer types have been tried and there are commercial quadrupole-traps, quadrupole-TOF, trap-trap and trap-orbitrap instruments. These hybrid designs are very good for the identification of unknown compounds and for identifying a wide range of analytes in a single sample.

These tandem mass spectrometers have been described as tandem “in space” (Madeira and Florêncio, 2012), as the two mass spectrometers are physically separated. It is also possible to use the same mass analyser to produce a tandem “in time” mass spectrometer (Madeira and Florêncio, 2012). In this case, an ion trap performs the initial mass spectrometry, but instead of ejecting the ions it fragments

them before detecting the products. The two mass spectrometry events are therefore separated in time rather than in space.

1.6.4. Instrumentation

Most of the instruments used for drugs of abuse analysis in these papers are triple quadrupoles (n = 44), although there are also 9 ion traps and one time-of-flight instruments. Quadrupoles are both sensitive and versatile, making them an ideal choice for these methods. However 8 of the methods use hybrid Q-trap or Q-TOF instruments. Triple quadrupoles are generally better at identifying a relatively small number of analytes and this can be seen by the 21 (48%) methods that are optimised for a single class of drugs or a only a few selected analytes. Ion traps and hybrid instruments are well suited to multi-analyte screening, and 14 (78%) of these methods are used for analysing a range of drugs from different classes.

Very few manufacturers make mass spectrometers for the clinical market and 60 of the instruments used are made by just three companies, Waters (including Micromass), Thermo (including Finnegan) and Applied Biosystems/SciEx. Bruker instruments are more common in inorganic chemistry and only returned a single method (Bones, Macka and Paull, 2007) and Shimadzu only produce a single quadrupole instrument and also returned a single method (Tatsuno, Nishikawa, Katag and Tsuchihashi, 1996). The remaining 7 instruments are all from Agilent.

Increasing performance and reducing costs in the mass spectrometer market have also changed how toxicology laboratories operate, with accurate mass Q-TOF or Q-Orbitrap (Q-Exactive®) systems becoming increasingly available. One of the principal advantages of these systems is that they are capable of storing the mass signal of all compounds within a sample. This means that it is easy to either re-analyse the data in the future to identify whether a drug or metabolite was present, or to search for a large number of poorly characterised substances such as legal highs. The Drug Control Centre at King's College, London, was the UK doping control laboratory for the 2012 London Olympics. Athletes have been told that their data may be reprocessed in the coming years to identify any user of new performance enhancing substances which have not yet been identified by the doping control agencies. In this way, the World Anti-Doping Agency hopes to avoid a repeat of the BALCO scandal, where the doping agent was unknown to the authorities until a syringe was handed to

them by an informant (What is the BALCO scandal?, 2011). By 2014 the hybrid instruments such as the Q-Trap or Q-TOF have come to dominate the published work, with at least 25 toxicology papers using these instruments.

There are several relevant reviews that cover the use of LC-MS in clinical and forensic toxicology and interested readers would do well to read those by Maurer (2004, 2005a, 2005b, 2007) and Peters (2011, 2012) and Maurer and Peters (2005)

1.6.5. Components of a mass spectrometer

1.6.5.1 *Sources*

The source is the part of the mass spectrometer into which the analyte is introduced and in which the analyte is normally ionised. In a standalone system, the sample is typically introduced to the mass spectrometer through a vacuum interlock. However, this method is not possible with hyphenated techniques, as there is a continual flow of carrier gas or HPLC eluent being delivered to the source. The excess liquid or gas would quickly destroy the vacuum and must be continually removed. This is a relatively minor task when the carrier gas is helium, but becomes more complicated when the mobile phase in the HPLC is water based.

1.6.5.2 *Hard and soft ionisation*

Analytes may be ionised in a number of ways, but they are usually separated into two classes, hard and soft ionisation. Hard ionisation usually involves the direct ionisation of the parent molecule and can cause the molecule to fragment. Often the molecular ion is not produced in significant quantities using hard ionisation techniques. Conversely soft ionisation techniques generally do not cause fragmentation of the molecular ion in the source, enabling the molecular ion mass to be easily identified. This is often achieved by the ionisation of the matrix in which the analyte is found, with this charge being transferred to the analyte by collisions between the molecules and ions (Scripps Cente for metabolomics, 2013).

1.6.5.3 *Electron impact ionisation*

The commonest hard ionisation technique is electron ionisation or electron impact (EI) ionisation, often used in GC-MS (Scripps Cente for metabolomics, 2013). The GC carrier gas containing the analytes is introduced into the source at high vacuum,

usually at right angles to the direction in which ions are to travel. An electron beam from a heated wire filament is directed across the source chamber, and the electrons strike the analytes, ejecting electrons or protons from the target molecules and causing ionisation at functional groups that are particularly prone to ionisation, for example phenyl rings and nitrogen atoms. The ions are then attracted to a series of lenses that accelerate them into the mass spectrometer (see Figure 1.13)

The amount of energy delivered by the electron beam is usually standardised at 70 electron volts (eV) and this is sufficient to fragment the molecule. Since the energy is directed at functional groups, the fragmentation patterns will be consistent and, for any given substance, the proportions in which the fragments are produced will be approximately constant. The masses and proportions of the fragments are constant and form the mass spectrum of an analyte.

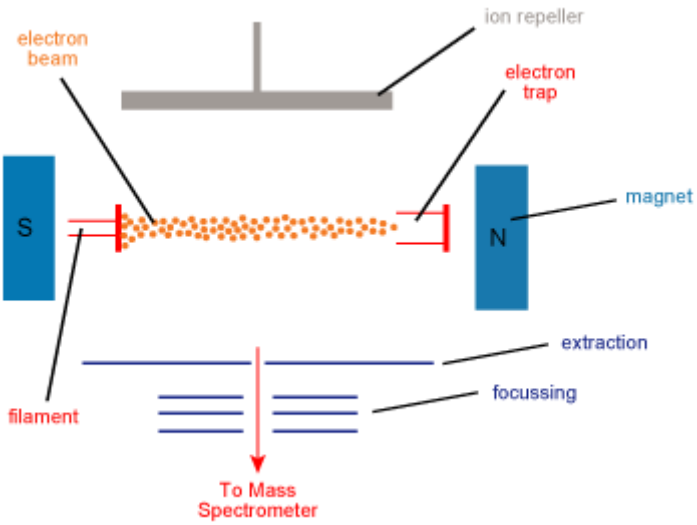


Figure 1.13 Schematic representation of an electron ionisation source (Gates, 2005)

1.6.5.4 *Chemical ionisation*

With soft ionisation, a carrier or other reagent is directly ionised and this transfers the charge to the analyte without causing fragmentation. The parent ion is produced in abundance and a mass spectrum produced at this stage will show only the molecular ion. In GC-MS this process is usually known as chemical ionisation (CI), typically using methane or ammonia as a reagent gas (Gates, 2005). The source configuration is very similar to EI, although the volume of the source is generally slightly smaller to allow the vacuum to be maintained. The reagent gas is introduced

directly into the source and the electron beam ionises the gas in preference to the analyte. Random collisions ensure that the charge is transferred to the analyte, which can then be accelerated into the mass spectrometer. Generally a GC-MS with CI needs a higher efficiency turbo pump to remove the reagent gas added to the vacuum chamber.

1.6.5.5 ***Electrospray ionisation***

In LC-MS, the hardest form of ionisation commonly used is electrospray ionisation (ESI) and the commonest source used in the papers identified, with at least 53 of the methods using electrospray ionisation. Electrospray ionisation is usually not hard enough to cause fragmentation, but can leave larger molecules with a number of charges. Since each extra charge reduces the m/z ratio, a single substance that has several charges can have the appearance of being several different substances.

An ESI source consists of a nebuliser pointing at the entrance to the mass spectrometer, although it is usually off-axis so that ions can be directed using electrostatic interactions. The HPLC eluent emerges from the tip of the nebuliser needle where the nebuliser gas evaporates it. The needle is also held at a high voltage, typically 3-4 kV, which causes the eluent droplets to ionise. A combination of the nebuliser and a process known as coulomb explosion causes the eluent to disperse into very fine, charged droplets before evaporating completely to produce gas phase ions which can be drawn into the mass spectrometer (see Figure 1.14). In the review by Peters (2011) 16 of the 17 methods for drugs in urine and 13 of 15 methods for drugs in blood use ESI, showing how this technique is almost the standard method for drug testing.

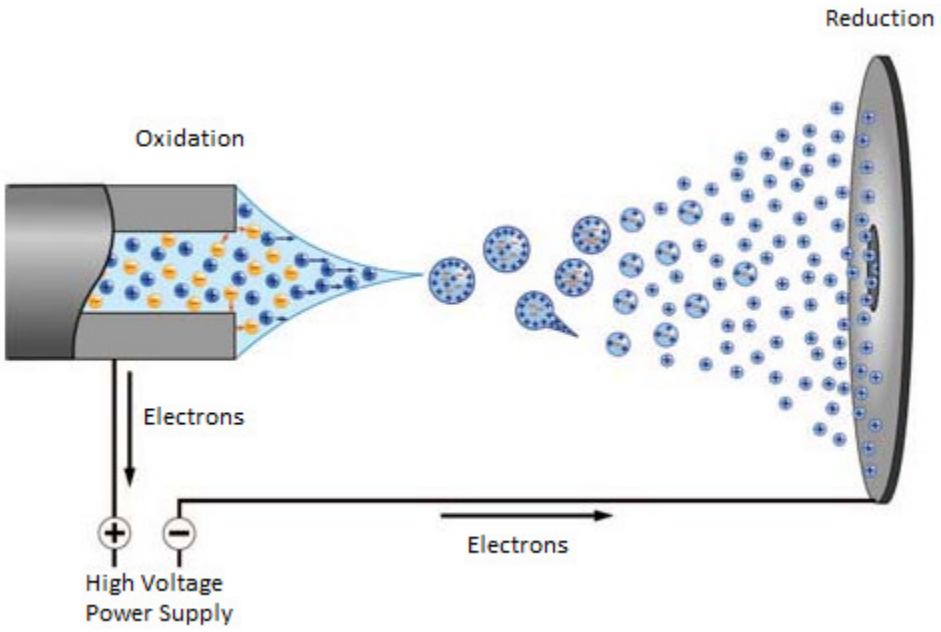


Figure 1.14 Image of an electrospray ionisation source showing coulomb explosion (Dahlin, 2008)

1.6.5.6 *Atmospheric pressure chemical ionisation*

The most direct equivalent to chemical ionisation for LC-MS is atmospheric pressure chemical ionisation (APCI) in which the HPLC eluent is evaporated by a heated nebuliser. Instead of the nebuliser needle being held at a high voltage, a separate needle is held near the end of the nebuliser. Instead of a reagent gas, the HPLC solvent acts as the reagent and, as for CI, the needle causes the reagent to be ionised, leading to soft ionisation (Takada, Sakairi and Koizumi, 1995). With an upper mass limit of around 1,000 amu, APCI is not as versatile as ESI and ESI is also better at ionising very highly polar substances. These limitations do not affect most of the relatively low mass drugs of abuse, yet the relative simplicity of ESI means that it dominates and only 8 of the papers identified specify the use of an APCI source.

1.6.5.7 *Other ionisation types*

There are a large number of ionisation types available for mass spectrometers and typically these will be optimised for specific analysis types. For example, matrix assisted laser desorption ionisation (MALDI) uses a laser to vaporise the substrate containing a sample. As for other soft ionisation types, the charge is then passed to the analyte (Tanaka, 2003). Similarly surface enhanced laser desorption ionisation (SELDI) uses a laser to create ions and both methods are ideally suited to

applications where a very small sample amount is available, either following a 2D electrophoretic separation or immobilisation by antibodies or other binding systems.

Fast Atom Bombardment (FAB) is similar to MALDI in that the ionisation is generated in the matrix. Instead of using a laser to ionise the matrix in FAB the matrix is ionised by bombardment with atoms, generally of argon or xenon. (Barber, Bordoli, Sedgwick and Tyler, 1981)

These techniques are all designed to work with solid or dried substrates and are not well suited to hyphenation with HPLC or GC systems. One that can be used is a variant on APCI known as atmospheric pressure photoionisation (APPI) which uses an intense ultra-violet (UV) light source to ionise the target molecules or the solvent. If ionisation efficiency is low, a dopant can be added to the sample. The dopant will ionise readily in UV light and therefore improve the ionisation efficiency (National High Magnetic Field Laboratory, 2013).

Although these sources are the main ones used for clinical and forensic biological testing, there are a large number of variants using almost any technique available for ionising molecules. These are generally optimised for particular applications, for example trace element analysis using inductively coupled plasma (ICP) sources, and are not generally relevant to drugs of abuse testing.

1.6.5.8 ***Vacuum pumps***

The ions in the mass spectrometer have to travel some distance from the source to the detector and if this space was at atmospheric pressure the ions would collide with the air molecules and never reach the detector. To overcome this, the flight path of the ions is kept at a vacuum and the strength of vacuum required depends on the length of the flight path. For example, a quadrupole with a path length of 10 cm will not need as high a vacuum as a triple quadrupole with a path length of 40 cm, or a TOF with a path length of 1 m. If the vacuum is insufficient, the number of ions reaching the detector will be reduced and the signal attenuated. (Scripps Cente for metabolomics, 2013)

Most systems will have a combination of pumps, usually consisting of a rough pump that is external to the instrument and one or more turbopumps inside the mass spectrometer. Turbopumps can produce the very low vacuums of 10^{-5} torr needed for

mass spectrometry, but must have a rough pump to reduce the pressure before they can work efficiently. The rough pump can pump down from atmospheric pressure to around 10^{-3} torr (Scripps Cente for metabolomics, 2013).

The pumping capacity of these vacuum pumps will also determine what type of hyphenated techniques are suitable. A capillary GC system will deliver typically a few millilitres of helium gas per minute to an EI source and the vacuum pump only needs to be sufficient to form and maintain a vacuum at this level. An identical instrument set up for CI will need a larger vacuum pump, as the pump will need to remove the reagent gas from the source as well as the carrier gas. To operate a similar source for an HPLC system would be impractical, as a few millilitres of solvent would expand to a litre or more of vapour even at room temperature (18 g of water will expand to 25 L at Standard temperature and pressure). To allow the use of HPLC, the solvent is evaporated at atmospheric pressure in ESI, APCI and APPI sources and the ions are formed in this atmospheric pressure region of the source (Scripps Centre for Metabolomics and Mass Spectrometry). A series of ion lenses then channels the ions into a narrow opening, often known as the capillary, that draws the ions into the low vacuum area of the mass spectrometer. A large vacuum pump is still required, but this now only needs to remove an atmospheric pressure gas flow, rather than a significant solvent vapour flow.

1.6.5.9 ***Detectors***

Photographic plates are no longer the method of choice for recording a mass spectrum. Instead electronic detectors, more sophisticated than the Faraday Cups used on the very early mass spectrographs, are generally used. Often these are crystals that will emit an electron when struck by an ion. These electrons are then amplified in an electron multiplier tube before striking an anode to generate a current. There are a number of different systems in use, for example the electron multiplier used in Thermo triple quadrupoles uses a series of dynodes, each held at a higher voltage than the last. Electrons therefore cascade from one dynode plate to the next until there are sufficient electrons to generate a signal at the anode that can be amplified electronically.

The systems produced by manufacturers such as Agilent use a continuous electron multiplier, where there is a single physical electrode that is cone or cup shaped. The

material the electrode is made of has a high resistance, so a potential applied at the front increases towards the anode, allowing electrons to be multiplied as they pass through the electron multiplier. When thought of as a continuous series of circular dynodes of increasing voltage, the similarities with a dynode series is easier to recognise (see Figure 1.15).

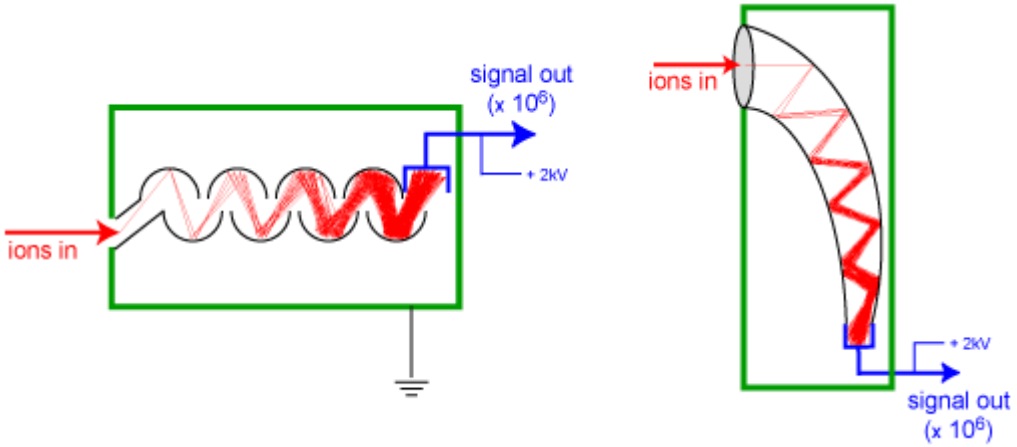


Figure 1.15 Comparison of the structures of discrete and continuous dynode electron multipliers (Gates, 2004)

In some systems, a microchannel plate detector (MCP) is used to detect and amplify the signal. This sort of detector is a flat plate that contains hundreds of microscopic channels running through the plate. Each microchannel is a miniature continuous dynode electron multiplier and often two or more microchannel plates are arranged in series. This design is particularly suited to applications where spatial resolution of the ions is required and in magnetic sector instruments this means that a range of m/z values can be recorded simultaneously, making the MCP detector analogous to a Diode Array Detector or photographic plate.

In some instruments, particularly MALDI-TOF systems, the initial dynode material is a scintillator that will emit a photon instead of an electron when struck by an ion. This photon is then detected in a photomultiplier tube, which is an electron multiplier with a scintillation detector ahead of the first dynode. Although the extra stage may appear to reduce the efficiency of the detector, this arrangement is several times more efficient than the MCP detector (Li, Tsai, Chen, Chen, Lee and Wang, 2007).

Orbitrap systems and ion cyclotron resonance mass spectrometers both measure the ions directly without the need to convert the ion into an electron for detection. In these systems, the ions are accelerated around a cavity or spindle and the

movement of the ions is analogous to an electric current. This current varies as the ion's energy dissipates and the changing current induces a measureable current in adjacent electrodes. This current can then be amplified electronically, although it must be subjected to a Fourier Transform algorithm to identify the masses detected (Perry, Cook and Noll, 2008).

1.6.6. Mass spectrometer scan types

Once the ions are inside the mass spectrometer, the ion may be measured directly and a signal recorded. For chemical ionisation and LC-MS, the output will be a molecular ion scan, normally referred to as a parent ion scan. For EI and other hard ionisation techniques, the output will be a mass spectrum comprising all of the fragments produced in the source. A variant of this is where a single ion is recorded, known as selected ion monitoring (SIM). For quadrupoles, this increases the sensitivity significantly, but for all other mass selectors the sensitivity gain is much less (ion traps) or absent (TOF and orbitrap). Several different SIM transitions can be monitored sequentially in a scan cycle, but the data obtained is much more limited than for full scans (Mellon, Self and Startin, 2000).

If using tandem mass spectrometry, there are more options depending on the settings of the first and second mass spectrometers and the collision cell and the type of source. It is easiest to understand using a triple quadrupole, although the same types of scan can be done with other systems (de Hoffman, 1996).

1.6.6.1 *Collision cells*

Collision cells are often quadrupoles or hexapoles, although ion traps and other designs of collision cell are available. Generally the collision cell is filled with a low pressure gas, which varies according to the instrument and manufacturer. This gas is frequently helium, argon or nitrogen. Collision induced dissociation (CID) is produced by the excitation of ions within the collision cell, giving an increased kinetic energy. The ions then hit the collision gas and break into fragments. The degree of fragmentation can be controlled by varying the collision gas pressure, the time of the excitation cycle or the amplitude of the excitation. Fragmentation is frequently optimised for each analyte to try to increase the amount of information obtained. For example, an ion trap instrument generally only excites a narrow target mass range.

On the Thermo's LCQ Fleet it is possible to use wideband activation, which increases the excitation window from 3 amu. to 20 amu. In this way, ions that preferentially lose water or a methyl group will be forced to fragment further, giving additional structural information. Similarly in some SciEx instruments the collision energy can be increased in stages to produce additional fragments and therefore a more characteristic mass spectrum.

1.6.6.2 *Product ion scan*

In a product ion scan, the first quadrupole is operated in SIM mode and the third quadrupole is set to scan (see Figure 1.16). This will produce a spectrum similar to that produced by EI, but since all of the fragments are derived from the parent ion, there will be less interference than in EI. This type of scan is possible with all tandem mass spectrometers, although the scan rate of a quadrupole or orbitrap mean that the cycle time is relatively long compared with an ion trap or TOF instrument. Product ion scans can give very good information about the parent ion, as the parent ion mass and all fragment ion masses are known. Since the fragments form an almost unique fingerprint of the analyte, this type of scan is very good for identifying unknown substances against a library of known spectra.

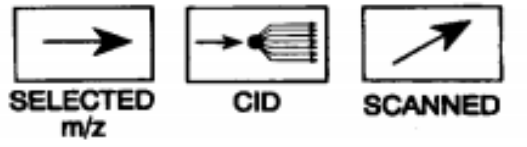


Figure 1.16 Diagrammatic representation of product ion scan (de Hoffman, 1996)

1.6.6.3 *Parent ion scan*

A parent ion scan is the opposite of the product ion in that the first quadrupole is set to scan and the third quadrupole is fixed on a particular mass, usually a lower mass than in the scan (see Figure 1.17). This scan is used to identify parent ions where the fragment is known, for example where there is a known side chain, adduct or derivative but the parent is unknown. Parent ion scans are only possible where the first mass analyser is continually scanning (tandem in space), meaning that it is not possible to do a parent ion scan in an ion trap or a TOF-TOF instrument. In an instrument that uses a full scan second mass analyser, such as an orbitrap, TOF or ion trap, the cycle time of the second mass analyser may reduce the scan speed of

the first mass analyser. Parent ion scans are therefore best performed with triple quadrupoles.

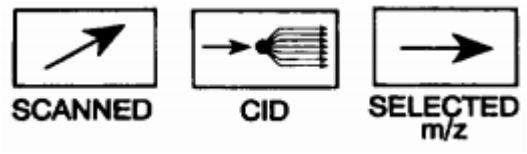


Figure 1.17 Diagrammatic representation of a parent ion scan (de Hoffman, 1996)

1.6.6.4 *Selected reaction monitoring*

One of the most common types of scan on a triple quadrupole is called selected reaction monitoring (SRM) where both quadrupoles are set to fixed values (see Figure 1.18). If several different SRM transitions are monitored sequentially, this is sometimes known as multiple reaction monitoring (MRM). In SRM, a known ion enters the collision cell and is fragmented. The fragments then enter the third quadrupole which is set to allow only a known fragment mass to pass. This is the tandem mass spectrometer equivalent of SIM and offers the greatest sensitivity of any hyphenated tandem technique. MALDI-TOF instruments can reach lower detection limits, but generally are not hyphenated with chromatographic systems and do not generally provide continuous data.

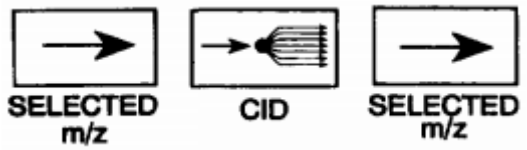


Figure 1.18 Diagrammatic representation of selected reaction monitoring (adapted from de Hoffman, 1996)

1.6.6.5 *Neutral loss scan*

In a neutral loss scan, the MS is operated in parent ion scan mode, but instead of monitoring a fixed fragment, the third quadrupole is also set to scan (see Figure 1.19). Both quadrupoles scan at the same rate, so that the mass difference between them remains constant. This technique only identifies parent ions that produce fragments in the same manner, losing the same uncharged mass. This is particularly useful in metabolic monitoring, as it allows the identification of known derivatives and conjugates: a signal will be generated when a conjugate, such as glucuronic acid, is removed from any parent ion in the collision cell.

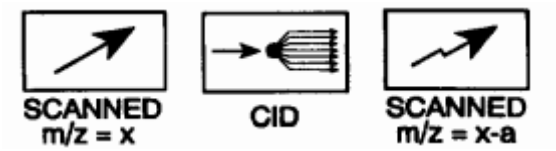


Figure 1.19 Diagrammatic representation of a neutral loss scan (de Hoffman, 1996)

A continuous neutral loss scan is not possible with an ion trap LC-MS or other tandem-in-time instrument (de Hoffman, 1996), although neutral losses may be identified by subtracting the mass fragments obtained from the parent ion. An advantage of this over a neutral loss scan is that a number of different neutral losses may be simultaneously identified.

1.6.6.6 *Multi-level mass spectrometry*

Systems that include an ion trap as one of their mass selectors also have another mode that can be used. In multi-level mass spectrometry MS^n, the fragments are retained in the ion trap after the collision and instead of being scanned out to produce a spectrum, most are ejected, retaining a single fragment. This fragment can then be fragmented further and its product used to produce an MS^3 spectrum (see Figure 1.20). This method can produce a very high degree of confidence in the identity of the initial molecular ion, at the expense of a loss in sensitivity. In some systems, this process can be theoretically repeated up to ten times to produce MS^{10}, although this is less common and is generally used for structure elucidation studies.

Figure 1.20 Diagrammatic representation of an MS^3 scan (adapted from de Hoffman, 1996)

1.6.7. Ion suppression

One of the difficulties with mass spectrometry is a phenomenon known as ion suppression. As already shown, analytes must be ionised to enter and be detected by the mass spectrometer. Anything that reduces the efficiency of the ionisation will therefore reduce the number of ions entering the mass spectrometer and therefore reduce the signal produced.

Ion suppression can happen in a number of ways. Perhaps the easiest to understand is the physical process of evaporation. In an LC-MS, the mobile phase must be evaporated to leave the analytes as gaseous phase ions. If not all of the mobile phase is evaporated, it follows that any analytes that remain in the liquid mobile phase will not be able to enter the mass spectrometer (Annesley, 2003). This is particularly noticeable when comparing highly aqueous mobile phases with organic phases. Water requires more heat to evaporate than, for example, acetonitrile, and analytes in an aqueous mobile phase will therefore have lower ionisation efficiencies than those in an organic solvent.

Another common method of ion suppression is found where there are different molecules present in the mobile phase. Phosphates are well known as being some of the worst salts when it comes to ion suppression and many methods use an extraction to remove phosphates (Debets, Mekes, Ritburg and Jacobs, 1995). The phosphate ion is very stable and will accept a charge in preference to analytes. Ion suppression by phosphates is a major problem with transferring HPLC methods to LC-MS, as phosphate buffers are very common with HPLC analysis.

There are a number of ways of reducing ion suppression and it is common to employ a mixture of techniques. Most ion suppressing substances, especially salts, can be removed by one of the sample preparation techniques described above, whether simple dilute and shoot or on-line SPE systems. Much ion suppression is evident on certain sections of a chromatogram, such as following the injection solvent front. Good HPLC separation and method development can eliminate this by adjusting the relative retention times of the analytes and ion suppression (Annesley, 2003). In some systems, an organic solvent is added to the column eluent prior to the MS source. This increases the volatility of the mobile phase and therefore the proportion of solute ions in the vapour phase. The reduction of ion suppression has been quoted as a reason for using APCI or APPI instead of electrospray for an analysis (Keski-Rahkonen, *et al.*, 2013).

1.6.8. **Data dependent scanning and survey scans**

Until the advent of powerful computers and software for controlling mass spectrometers, most mass spectrometer methods used the same acquisition parameters throughout a segment of an analytical run. This results in a lot of

background data without significant peak information. In complex methods, the number of MRM transitions either limit the number of analytes that can be analysed simultaneously within the same section of chromatogram or leads to shorter dwell times to try to fit more transitions per cycle. As computers became powerful enough to process analytical data during the acquisition, this allowed acquisition processes to be defined based on the results of a previous scan. Different manufacturers have different approaches to this, resulting in triggered MRM (tMRM) (Agilent), Information Dependent Acquisition (SciEx) and Data Dependent Scans (Thermo).

The Agilent tMRM system allows a series of additional MRM transitions to be performed if the signal of a specified transition exceeds a threshold. In this way, a more complete spectrum can be obtained of an analyte following its detection in a single MRM. Instead of acquiring full scan data throughout the run, the additional data is only collected when a defined peak is present.

The Thermo Data Dependent Scanning (DDS) is more flexible than this, allowing any defined scan to be triggered following rules in the acquisition software. This has led to the use of a "survey scan" where a full scan is performed without any fragmentation to identify molecular ions, followed by an MS^2 or other scan on any identified peaks. Further, the Thermo software can also trigger scan events based on the findings of an MS^2 or other scan, such as performing an MS^3 scan if an MS^2 signal exceeds a defined threshold.

The Information Dependent Acquisition (IDA) feature of modern SciEx systems operates in a similar way to Thermo's DDS, able to perform a MS survey scan and then trigger an MS^2 scan based on the results.

A library search in October 2013 (Web of Science, search terms Topic = toxicology AND ("information dependent acquisition" OR "data dependent scan" OR "survey scan") for toxicology papers quoting either Information Dependent Acquisition or Data Dependent Scanning or Survey Scan shows a total of only 40 references. Most of these papers are reviews and articles on new trends, showing that these new data processing methods are entering routine use.

One of these papers used a desorption electrospray ionisation source without chromatography (Nielen, Hooijerink, Claassen, van Engelen and van Beek, 2009)

and two papers described methods that were targeting a single drug and its metabolites. A further 14 papers were for multiple drug testing methods, using predominantly Information Dependent Acquisition from SciEx, six using the hybrid quadrupole-ion trap and five using triple quadrupoles. The remaining three papers used a SciEx quadrupole-TOF, an orbitrap and a Thermo LXQ ion trap.

The power of Data Dependent Scanning is that a relatively large number of analytes can be screened with a survey scan, allowing specific MS^2 scans to be targeted at potential peaks. This reduces the amount of uninformative data collected, allowing the instrument to collect more useful data. This can be seen by the fact that most of the papers are for at least 20 drugs, with one performing automated library searches for over 1000 analytes (Martínez Buenoa, Ulaszewskaa, Gomez, Hernando and Fernández-Albaa, 2012). None of these methods use on-line extraction, MS^3 scans and neutral loss experiments.

1.7. **Previous clinical method**

The previous method was developed by the author in 2002 and consisted of a solid phase extraction using Screen C SPE columns from Phenomenex. After an overnight hydrolysis with β-glucuronidase, sample (3 mL) was loaded onto an SPE column previously wetted with methanol and conditioned with an acetate buffer (pH 5). After drawing through under vacuum, the columns were washed with deionised water and phosphate buffer (pH 6.8) before being eluted with a dichloromethane:isopropanol:ammonia (70:28:2 v/v/v) solvent. The resulting eluent was dried down under a stream of air in a hotblock (60 ℃)and the residue resuspended in methanol before spotting 2 cm from the bottom of a 10 cm TLC plate. The developing solvent was ethyl acetate:methanol:ammonia mixture (85:15:2 v/v/v) and was equilibrated for at least 30 minutes before the plate was developed. After drying the plate was stained with a solution of hexa-chloroplatinate and potassium iodide (iodoplatinate stain) and the interpretation of the presence of drugs was made by an experienced senior clinical biochemist or biomedical scientist after keeping the plate in the dark overnight. An assessment of the sensitivity demonstrated a limit of detection of around 1500 ng/mL morphine and benzoylecgonine and around 500 ng/mL methadone and EDDP (unpublished laboratory data).

This method was suitable for use when used to assess the illicit drug use of patients from the addictions in-patient unit and community drug teams, but only when combined with an immunoassay primary screen. The poor limits of detection made this method unsuitable for diagnosis of illicit drug use in an unselected population and the three day turnaround is too slow for most acute medicine.

In addition to taking 3 days to produce a result, the TLC part of the method was technically demanding and depended significantly on the skill of the scientist applying the samples to the TLC plate. The interpretation was subjective and although repeatability was good, results would occasionally differ depending on the person reading and interpreting the plate, in particular on the presence or absence of small amounts of codeine.

It was also desirable to reduce the amount of solvents in the laboratory, especially the chlorinated solvents (dichloromethane). Dependent on the batch size, this method would generate around 50 – 100 mL of chlorinated solvents and 100 mL of non-chlorinated solvents each day. The iodoplatinate staining solution is also hazardous, with halogenated platinum salts causing skin sensitisation and being on the Health and Safety Executive's EH40 list of maximum exposure limits.

An advantage of the TLC approach was that any drug extracted by the SPE cartridges and reacting to the stain was visible on the plate. This meant that drugs such as propoxyphene could be easily identified without requiring additional processing or additional standard solutions. However, this also meant that there were a large number of unidentified spots on the TLC plate. Occasionally, the intensity and positioning of these spots prevented the accurate interpretation of compounds of interest, particularly when patients were on a number of antibiotics and antidepressants.

As other laboratories around the country improve their sensitivity, the external quality assessment scheme looks to meet their expectations. This includes the lowering of the scheme thresholds for the identification of drugs. At the outset of this project, the clinical threshold for the detection of morphine was 1500 ng/mL, but the current threshold for morphine is 300 ng/mL (LGC Standards Proficiency Testing, 2013). Without changing the method to improve its limit of detection, the laboratory would be receiving false negative reports for morphine.

The TLC part of this method had been in widespread use for many years when the laboratory was based at Bethlem Royal Hospital and there was limited scope for improvements. Further information could be obtained by the use of sequential staining steps and resolution could be improved by the use of an automated TLC applicator system and HPTLC. This would have increased either the time taken to produce a result, the cost of analysis, or both, and neither of these options are desirable in a laboratory that is looking to reduce its costs and improve the service it gives to its customers. As a result, it was decided that this method had reached the end of its usefulness.

In 2007, King's College Hospital acquired the toxicology laboratory at Bethlem and transferred it to new premises at KCH in 2008. A new consultant toxicologist was appointed and new areas of analysis considered. As part of this, it was decided to purchase two LC-MS systems, one for drugs of abuse and one for therapeutic drugs.

1.8. Aims and objectives

1.8.1. Aim

The principal aim of this project was to develop an automated LC-MS method for clinical drugs of abuse testing

1.8.2. Objectives

The following objectives were defined and agreed with the University of Portsmouth at the outset of the project.

- Develop a Turboflow® liquid chromatography method giving suitable extraction and separation of the opiate and amphetamine classes of drugs.
- Develop an ion trap MS procedure to reliably identify opiate and amphetamine class drugs
- Evaluate the LC-MS method developed for detection of synthetic opioids (e.g. methadone, buprenorphine) and cocaine metabolites, with other analytes that are required by the laboratory service

- Introduce the methodology into routine use within the laboratory, providing sufficient training to staff to allow continued operation and understanding
- Consult with the local drug addiction specialists to provide a drugs of abuse service that meets their current and future needs

In November 2009, a new job opportunity meant that the practical aspects and objectives of the project had to be reduced, specifically the final introduction of the method, staff training and evaluation in consultation with the local drugs of abuse services. The author left KCH in January 2010 before the method had been completed and discussions with the course leader and departmental supervisors resulted in the scope of the project being extended back to the originally developed online SPE method, including the introduction and evaluation of this method. The SPE method was an integral part of the development of the final Turboflow® method and demonstrates the full method development and improvement program, as well as the introduction of the method into routine laboratory processes.

1.8.3. Clinical justification

The work involved in a project such as this is significant and unless there is a real clinical justification for expenditure of time and resources, the project is a purely academic exercise. While this may be a worthy aim, this does not fall within the remit of the professional doctorate course, which has as a central aim that the work should be of real clinical value.

There are several reasons why development of these methods is justified for the Toxicology Unit at King's College Hospital:

- Anticipated reduction in time taken in obtaining a result, leading to more efficient use of staff time and resources. This should also lead to a reduction in turn-around time, leading to a better laboratory service and one that is more able to attract further work from other sources
- Reduction in the use of hazardous solvents, leading to reduced risk and reduced solvent disposal costs

- Increased range of analytes available for testing, with an increased accuracy and precision. Improvement to laboratory standards elsewhere would lead to the Toxicology Unit lagging behind other service providers if no improvements were made.

- Increased availability and use of up-to-date technology such as LC-MS and Turboflow® are of particular benefit to a laboratory in a teaching hospital which regularly accepts students and placements from other laboratories worldwide.

1.8.4. **Contribution to the knowledge base**

Both online extraction with LC-MS and Turboflow® are methods lacking literature in the clinical environment. Many of the published methods are for single drug groups, whereas most of the multi-drug methods use either dilute-and-shoot or offline extraction. The only Turboflow® methods identified were published after the completion of the method development and required an offline hydrolysis step. Combination of these two areas would be an important addition to the literature. While this method is aimed at clinical drugs of abuse in urine testing, the principles also hold true for almost any matrix and the results of the thesis could be of use to researchers and routine laboratories looking at tests in matrices such as saliva, blood, tissue homogenates and non-clinical matrices. A degree of additional sample preparation will be needed for matrices with a solid or particular component (such as whole blood or hair samples), but the principles hold true.

The uniqueness of a project is difficult to assess, partly due to the commercial sensitivity that many laboratories are required to observe. Where this project is likely to be unique is in the combination of the fully automated sample preparation featuring online SPE and Turboflow® extraction to an ion trap LC-MS, giving a very high degree of accuracy in the identification of drugs. The project is also likely to be unique in the use of neutral loss scans to identify and then produce an MS^3 spectrum of glucuronide metabolites in urine, an approach that is difficult with the more common triple-quadrupole and accurate mass instruments and has not been published using Turboflow® sample preparation.

2. Reagents and equipment

2.1. **Hardware**

The mass spectrometer used throughout was an ion trap mass spectrometer (LCQ Fleet) from Thermo Fisher Scientific (Hemel Hempstead, UK), with a nitrogen generator (Peak, Scotland) supplying the source gases and a helium cylinder (BOC Speciality Gases, Guildford, UK) for the ion trap. This is an entry level ion trap system with an Ion Max ion source that can be used in both ESI and APCI modes. It is controlled by Thermo's Xcalibur software, which also performs the data analysis and integration. An additional option was the ToxID (version 2.0) software from Thermo which is intended as an automated drug identification and reporting system.

Two different HPLC systems were used during the development process. Initially a Jasco XLC system was used (Great Dunmow, UK), comprising two high pressure pumps (XLC-3185PU), a mixer unit (XLC-2080-32MX), autosampler (XLC-3159AS), degasser (XLC-2080-54DG) and column oven (XLC-2067CO). This was controlled by EZChrom software (Agilent) running on the same PC as Xcalibur.

The second HPLC system used was the Turboflow® system from Thermo (Hemel Hempstead, UK). This comprised a CTC Combi PAL autosampler system with cooled sample trays and dual injector ports, an Agilent G1312A binary HPLC pump (Agilent, Wokingham, UK), an Agilent G1311A quaternary HPLC pump,G1379 degasser and the Turboflow® valve module. This system was controlled by Aria software (Thermo), again running on the same PC as Xcalibur.

2.2. **Consumables**

Table 2.1 Parts and consumables used during the method development

Supplier	Part	Part Number	Description
Thermo Fisher Scientific	HPLC column	25405-052130	50 x 2.1 mm 5 µm PFP
	Guard Cartridges	25405-012101 (Pack of 10)	Hypersil Gold PFP 5 µm
Phenomenex	Online SPE column	25405-052130	Strata X (50 x 2.1) online SPE column
	Membrane filters	Nylon 66 AFO-0504	0.45 µm HPLC solvent filters
Esslab	HPLC crimp vials	5181-3375	HPLC crimp vials
	Crimp caps	5818-1210	Crimp caps

Parts and consumables (see Table 2.1) were purchased from existing suppliers and all were paid for without any influence on this project either implied or offered. A variety of Turboflow® columns were tested, all from Thermo (see Table 2.2)

Table 2.2 Turboflow® Column Part Numbers

Designation	Part Number
C18	953280
C8	952819
Phenyl	953278
Cyclone	953288
Cyclone-P	952605
Cyclone MAX	952980
Cyclone MCX	953287

MCX: mixed cation exchange, MAX: mixed anion exchange

Pipettes and pipette tips were supplied by Esslab (Essex, UK)

Volumetric flasks, class B, obtained from VWR International (Leicester, UK)

Mobile phase filtration apparatus (vacuum pump, vacuum flask, sintered funnel) was used to filter all solvents and supplied by VWR International (Leicester, UK)

A glass 500 µL syringe (Hamilton, UK) was used for infusion of standard solutions to the LC-MS for tuning purposes.

2.3. **Reagents**

Deionised water was used from an ELIX35 deioniser (Millipore, UK).

Other reagents (see Table 2.3) were in general use within the laboratory.

Table 2.3 Reagents and Solvents used During the Method Development

Supplier	**Chemical**	**Part number**	
Fluka, Sigma-Aldrich Co. (Poole, UK)	Formic acid	94318	
	Ammonium formate	70221	
Rathburn (Walkerburn, Scotland)	Acetonitrile	RH1016	HPLC Grade
	Methanol	RH1019	HPLC Grade
VWR International (Leicester, UK)	Acetic acid		Concentrated (Glacial)

2.3.1. **Reagent preparation**

2.3.1.1 ***Ammonium formate***

Ammonium formate (1 mol/L) was prepared by dissolving of ammonium formate (10.61 g) in deionised water (~150 mL) and made up to 200 mL with deionised water in a volumetric flask, refrigerated for storage.

2.3.1.2 *Mobile phase A*

Mobile phase A was prepared by adding of formic acid (1 mL) and ammonium formate (10 mL, 1 mol/L) to deionised water (~900 mL), then made up to 1 L with deionised water in a 1 L volumetric flask. Formic acid must always be added to water to so that the weak dilution releases a small amount of heat which is insufficient to vaporise and splatter the acid. The mobile phase was filtered before use using the HPLC eluent filtration apparatus.

2.3.1.3 *Mobile phase B*

Mobile phase B was prepared by adding formic acid (1 mL) to acetonitrile (1 L) in a volumetric flask (1 L). The mobile phase was filtered before use using the HPLC eluent filtration apparatus.

2.4. Standards and controls

Reference standards (see Table 2.4) were all purchased from approved commercial suppliers.

Table 2.4 Reference standards and control materials used during method development

Supplier	Material	Part Number
LGC Standards (Teddington, UK) (LGC Promochem at the time of ordering)	1mL of 6-Acetylmorphine (1.0 mg/mL) in acetonitrile	CERA-009
	6-Acetylcodeine (1.0 mg/mL) in acetonitrile	CERA-053
	Codeine (1.0 mg/mL) in methanol	CERC-006
	Codeine-6-glucuronide (0.1 mg/mL) in water/methanol (80:20 v/v)	CERC-08
	Heroin (1.0 mg/mL) in acetonitrile	CERH-038
	Morphine (1.0 mg/mL) in methanol	CERM-005
	Morphine-3β-D-glucuronide (1.0 mg/mL) in methanol	CERM-031
	Morphine D_6 (1.0 mg/mL) in methanol	CERM-086

BioRad Urine Toxicology Controls (Negative, C2, C3 and C4) were used throughout the project. Drug levels in controls were determined gravimetrically by the supplier and quoted in the kit insert (see Table 2.5)

Table 2.5 Drug concentrations in QC materials

QC Material (Part Number)	Morphine-3-glucuronide (ng/mL free morphine)	Codeine	6-mono-acetylmorphine
C2 (442)	1500	1500	10
C3 (low opiate) (469)	375	375	10
C4 (444)	4000	4000	20

2.4.1. Preparation of standard solutions

Standard solutions were prepared by dilution of primary standard solution (1 mL) to 10 mL in methanol in volumetric glassware. Working standards were then prepared by addition of 1 mL of this intermediate standard to a 20 mL volumetric flask and filling to the mark with blank urine (BioRad Negative control). Working standards were transferred to 25 mL plastic bottles and stored refrigerated for up to 1 month.

2.5. LCQ Fleet overview

The LCQ Fleet is an entry level ion trap mass spectrometer, capable of operating in ESI or APCI modes. It uses the Ion Max-S source housing, which has an angled spray head directing the sample spray towards the ion transfer capillary. This is heated to around 300 ℃ and assists in the desolvation of the ions. The exit of this capillary is through the centre of the Tube Lens, which focusses the ions towards the skimmer. The capillary is set off-centre to the skimmer, so that any neutral species do

not enter the high vacuum area of the mass spectrometer. This area is maintained at approximately 1-2 torr and the skimmer is held at approximately 0 V, giving a voltage gradient of 3500 – 4000 V from the electrospray needle. Two Quadrupoles and an octopole then guide the ions into a Quadrupole Ion Trap, consisting of a ring electrode and two endcap electrodes. This is held at around 3 $x10^{-3}$ torr and -10 V. Ions exit the trap through a hole in the centre of the rear endcap electrode and are attracted to the dynode, typically held at -15,000 V.

A schematic diagram of the ion optics (see Figure 2.1) shows the ESI source to the left, ion capillary in yellow and the ion trap on the right between the two horizontal blue lines.

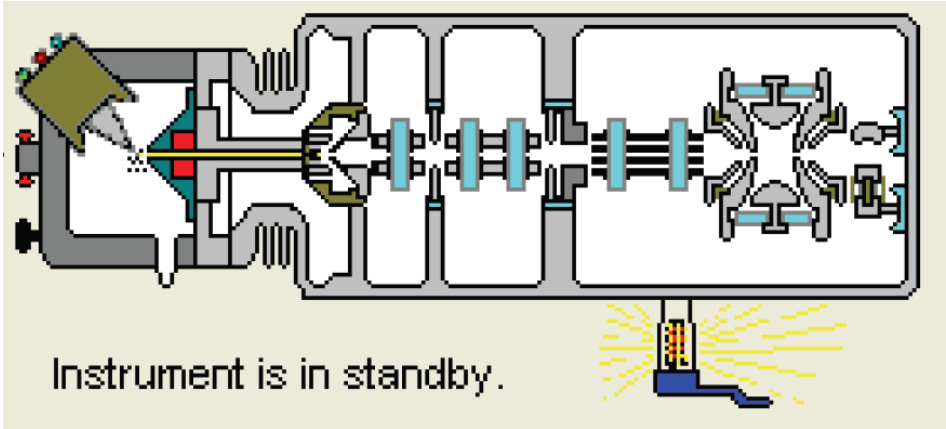


Figure 2.1 Schematic arrangement of the LCQ Fleet Ion optics (Xcalibur screenshot)

3. Method development

Development of a method from first principles requires significant time and routine working laboratories such as King's College Hospital often cannot justify this. It is therefore simpler to use a published method as a starting point and to modify it where it is unsuitable (see Table 3.1). As part of the procurement of the LC-MS, the laboratory received a Gold PFP column and a copy of Thermo Application note 409 (Rezai, Kozak and Torchlin, 2007). This application is intended as an unknown drug screen on an ion trap following offline solid phase extraction, using the PFP column supplied and an acetonitrile-formate buffer mobile phase. The method focusses predominantly on the Thermo LXQ LC-MS system, but also gives some details for the LCQ Fleet.

Table 3.1 HPLC solvent conditions from Thermo Application Note 409 (Rezai, Kozak and Torchlin, 2007)

Time	%A	%B	Notes
0	95	5	Sample injection
0.5	95	5	Gradient start
5.5	5	95	Gradient end
8.5	5	95	
8.6	95	5	Re-equilibrate
13	95	5	End run

The method was tried initially using aqueous standards without sample preparation to help validate the method. This worked well when the system had been allowed to equilibrate, but the retention times were wildly different from those quoted and when sequences of more than one sample were run, the second and subsequent samples gave no results. Investigation of the reasons for this identified the mixer as having a volume of 2 mL. With a flow rate of 200 µL/minute, this meant that the column was still full of acetonitrile when the second injection was made and the retention was virtually nil. Changing the mixer volume to 50 µL and ensuring equilibration between runs solved the reproducibility problem and produced retention times similar to those predicted by the application note.

3.1. Sample preparation

Having identified suitable LC conditions, the next step was to look at sample preparation. The simplest sample preparation methods for LC-MS are direct injection and dilute and shoot. Both were tried using a high control (BioRad C4) with very poor results.

3.1.1. Direct injection

Direct injection produced good results for some of the later eluting drugs, such as methadone, but was unable to detect even quite high levels (4000 ng/mL) of morphine, however much sample was injected up to the maximum 100 µL that the autosampler allows. Investigation of this identified ion suppression as having a very significant effect, with maximum ion suppression at the same time that morphine was expected. Most ion suppression in this matrix is due to salts in the urine, which are unretained on a PFP column. The retention of morphine glucuronide is very short and overlapped the area of ion suppression.

Since this method of sample preparation is quickest and cheapest, some effort was spent to try to reduce or eliminate the ion suppression. A solution of morphine was either injected as a sample or infused into the column outflow using the LCQ Fleet's integrated syringe driver and the ion intensity plotted as blank urine samples were injected. This gave a chromatogram trace showing the inverse of the proportion of ion suppression i.e. maximum signal at minimal ion suppression. The trace seen approximated the trace found when an LC column is overloaded and it was thought that by increasing the analyte retention it could be separated from the ion suppression. Analyte retention is governed by a number of factors and each of these was adjusted to try to remove ion suppression. A 100% aqueous mobile phase, not compatible with most reversed phase columns but compatible with the PFP column, increased the retention slightly, as did using a slightly lower flow rate. However, the greatest effect came from reducing the column temperature to 4 ℃, the minimum that the column compartment could cool to. Using drug free urine, the morphine glucuronide peak was delayed long enough to be mostly resolved from the ion suppression. However, further tests with more urine samples showed that the ion

suppression varied considerably from sample to sample and reliable reproducible results could not be obtained.

3.1.2. Dilute and shoot technique

Sample dilution (also known as dilute and shoot) is commonly used in mass spectrometry to reduce the effects of ion suppression. Both the signal and the ion suppression are reduced by the dilution, but the effect on the ion suppression is greater than the loss of signal, leading to an increased signal to noise ratio, and therefore increased sensitivity. This works well with triple quadrupole instruments, but is less so with ion traps which are less sensitive (see p.34). Although the signal to noise ratio is increased, the absolute signal intensity is still below that required for reliable detection of drugs near to the required threshold levels. The decision was therefore taken that some form of sample preparation was required.

3.1.3. Liquid-liquid extraction

Having established that some form of sample preparation is needed, the simplest to perform technically is a liquid-liquid extraction, or LLE. Liquid-liquid extraction relies on the immiscibility of the matrix (aqueous) with an organic solvent. Salts, proteins and ionised molecules are unable to cross from the ionic aqueous phase to the non-polar organic phase. Usually the organic phase is removed from the tube and concentrated, separating the analytes from the matrix.

Selectivity can be enhanced by adjusting the pH so that the analyte of interest is uncharged and therefore crosses into the organic phase. Improved selectivity can then be managed by isolating the organic phase in a fresh tube and adding an aqueous buffer with a different pH to the original extraction. The newly ionised metabolites cross back into the aqueous phase, leaving other non-polar analytes in the organic solvent. This back extraction process can give good selectivity, but works best when a single analyte group is to be isolated, for example it may extract amphetamines but not opiates.

A simple extraction in either chloroform or dichloromethane was used regularly in the laboratory prior to 2003, but there are some drawbacks which make it less suitable for regular routine use.

Many of the solvents that are immiscible with water are harmful, such as chloroform, dichloromethane and hexane. In addition, since they are not miscible with water, they cannot be disposed of down the drains and must be collected and disposed of via a chemical waste management company. Other suitable solvents, such as methyl *tert*-butyl ether (MTBE) are less hazardous than chloroform but may still cause difficulties with disposal.

One of the main reasons that LLE was decided against is that it is frequently difficult to automate, which was a significant part of the aim of the project. Automated instrumentation cannot easily identify the phase boundary of immiscible liquids, which means that pipetting of the organic phase containing the extracted analytes must be done manually. If the mobile phase is largely aqueous, the organic phase must also be evaporated before analysis, adding an additional step, and if the phase boundary is not clear a centrifugation step may be required.

A recent development has been the introduction of Supported Liquid Extraction (SLE) where a syringe barrel contains an absorbent support, such as Fuller's earth. The aqueous sample is completely absorbed on the sorbent and the organic phase is added to the top. On elution the analytes cross to the organic phase in the same way as in LLE and the organic phase is then collected and the solvent removed. However, as for SPE (see below), the aim of reducing hands on time for the analysis is not achieved.

3.1.4. **Solid phase extraction**

The logical sample preparation method to use would be off line solid phase extraction. This technique was being used for the existing routine method, so would have required minimal staff training and would have led to a simpler introduction process. However, part of the reason for moving away from the existing method was to reduce hands on time, increase automation and reduce the use of chlorinated solvents in the laboratory.

Phenomenex have developed a re-usable SPE cartridge that can be fitted into an HPLC system to facilitate online sample extraction. This cartridge contains 20 mg of a polymeric sorbent reversed phase with a large particle size of round 50 µm, generating very little back-pressure. This reversed phase SPE cartridge can be fitted

in series with an HPLC column and a divert valve. During the sample loading cycle, the SPE eluent is diverted to waste, removing the salts and proteins causing ion suppression. When the divert part of the cycle is complete, the valve can be switched and the remaining flow analysed on the mass spectrometer. Alternatively, the cartridge can be fitted on a loop in a 6-port 2-way valve in the same way that a sample loop is normally connected. As for the in-line arrangement, the sample is loaded while the flow is diverted to waste. When this cycle is complete, the valve is switched, reversing the flow through the SPE cartridge and onto the HPLC column. This arrangement has the advantage that any separation that occurs on the SPE cartridge is reversed when the flow direction is reversed.

3.2. Separation and detection method

The method supplied by Thermo for use with the application note is designed for the identification of unknown substances. The method has an extensive library supplied with it, but during testing the method was not able to reliably detect multiple drugs in a control sample. The system seemed to be identifying a lot of background noise as a signal and the instrument was focussing on this noise instead of the desired signal. The decision was therefore made to simplify the method to make it more specific to the application for which it was needed, drugs of abuse in urine.

The principal target ions for the method are the opiate drugs (morphine, codeine and dihydrocodeine) and their metabolites, principally the glucuronides. In addition, the methods that were to be replaced were capable of identifying cocaine, its principal metabolite benzoylecgonine, methadone and its principal metabolite, EDDP, and the amphetamine group substances amphetamine, methamphetamine and ecstasy (MDMA). It would therefore be desirable to be able to identify these drugs and their metabolites.

3.2.1. Chromatography method

Each of the required drugs was injected into the system as an aqueous standard or urine control, with the LC conditions varied to obtain an optimal separation. Drugs that are highly soluble, such as morphine-3-glucuronide (M3G) have very little retention, even in very weak mobile phases (low organic content). Conversely, methadone is highly retained in weak mobile phases and is also well retained in high

strength solvents (i.e. a high percentage of acetonitrile). So that M3G can be reasonably retained and the run time kept short for the methadone, a gradient separation is a necessity.

The gradient was developed by using a weak solvent in an isocratic run. Examination of the retention times identified a point where a suitable separation had been obtained beforehand and the following analytes could be accelerated while maintaining a degree of resolution. A variety of gradients were then tested, increasing the acetonitrile concentration at different rates to improve the separation of the analytes in the later part of the run. No simple solution was found to this, as if the gradient was increased too rapidly, the separation would be lost for the next analytes in the run (predominantly the amphetamines) but would be ideal for methadone and EDDP. Conversely, if the gradient was slow enough to maintain separation of the amphetamines, the run time for methadone was too long. A suitable compromise was reached by having a multi-step gradient, with an isocratic step after an initial gradient, followed by a step change to 100% acetonitrile for the methadone (see Table 3.2).

Table 3.2 Online SPE HPLC method flow parameters

Time (min)	Flow (mL/min)	%B	Divert Valve	Comment
0	0.1	5	To MS	Initial conditions to condition column
0.1	0.5	5	To Waste	Flush salts and unretained components to waste
1.5	0.2	5	To MS	Reverse the SPE cartridge and start to stabilise flow to ESI nozzle
12	0.2	40	To MS	Gradient to separate and elute analytes
12.5	0.5	100	To MS	Ramp to high organic content to elute highly retained components and regenerate SPE column
15.5	0.5	100	To MS	Regenerate SPE column
15.6	0.1	5	To MS	Return to baseline conditions
17	0.1	5	To MS	End equilibration

3.2.2. Tuning of the mass spectrometer lenses

To achieve optimum performance a mass spectrometer often must be tuned to the compounds under test. Two forms of tuning are required, the first is compound specific and identifies the collision energy required to fragment the ions optimally and the second is a common method that guides the ions from the source into the ion trap (see Figure 2.1).

Tuning is achieved by infusion of the drug into the source using the integral syringe driver of the LCQ Fleet and the tune program supplied. The parent and daughter ions are programmed into the tune program and the voltages of the various lenses and the fragmentation energy are varied in sequence to identify either the optimum ion focussing conditions or the optimum fragmentation energy. The values obtained can be manually transcribed for programming into the MS method, or the tune file can be saved for import into the MS control program.

The optimal collision energies are programmed into the analyte table on which data dependent scans are based, although a default collision voltage can be used which will be suitable for most analytes.

Different tune files can be loaded for each segment used in the method (see p.74) and the tune files should reflect the types of compounds under test. Small molecules such as most drugs do not require a very high voltage on the tube lens of the source to focus the ions into the first quadrupole. However, this voltage would be too low to focus larger molecules such as proteins and the signal obtained would be relatively weak. Conversely, the higher voltage required to focus the larger ions of proteins would create too much of a repulsive force which would dissipate small ions. Most drugs of abuse are in the 200 - 400 amu. range and use of the tune file created with morphine provides a suitable mid-range molecular mass for drug analysis.

3.2.3. Online solid phase extraction mass spectrometer acquisition method

Having decided on an LC method, the mass spectrometer method could be created. While the simplest method would be to test for every analyte at all stages of the analysis, this does not play to the strengths of an ion trap and the cycle time to test for every drug would be too long. This is because the time for a spectrum to be produced (an MS^2 scan) is up to 150 ms, and with at least fifteen analytes in the

initial method, the scan cycle time would be over 2 seconds. With peaks only 10-15 seconds wide, there would be insufficient data points for reliable peak identification.

Xcalibur software has a very powerful feature called data dependent scanning. This allows the results of one scan event to dictate the parameters of following scans. By using data dependent scanning it is possible to perform an initial survey scan using a full scan (i.e. no fragmentation) and use the results of this scan to decide which MS^2 scan or scans should be performed next. Various rules can be written that decide which scan should be performed next, based on the retention times, ions detected, ion intensity or previously detected ions.

A simple approach to an MS method would be to use the data from the Thermo Application note 409. Unfortunately this application does not give sufficient data to recreate the analysis. The retention times and parent ion masses are easy enough to identify by infusing pure drug solutions, but the details of the data dependent detection parameters are not given, reducing the usefulness of the application note.

The first MS method produced was a survey scan followed by a data dependent MS^2 scan on the most intense peak in the survey scan. However, this consistently missed most of the drugs on test, as there were frequently other species present, probably from the mobile phase, which were more intense. The solution was to define the ions that would be examined in the survey scan and to define the time windows in which they would be tested. In this way, all of the analytes should be detected in the survey scan and MS^2 scans performed.

This procedure works and peaks can be identified based on their MS^2 fragmentation patterns. However, not all peaks in a control material are identified and the peak for methadone is bisected by the EDDP peak, giving a significant proportion of missing data. The reason for this is that only the most intense peak in a time window is identified and weaker peaks that are not completely resolved will be masked by the more intense peaks.

While it is possible to define rules that prevent the same MS^2 scan from being performed in consecutive cycles, a simpler option is to perform several consecutive scans on not just the first but the second, third and fourth most intense peaks. In this way, all overlapping peaks in the defined analysis windows can be detected.

As an outline method, this works well for most drugs, but was shown to give poor results for two drugs in particular. Morphine, and its primary metabolite morphine-3-glucuronide, were frequently poorly identified in the survey scan, leading to inadequate peaks for reliable analysis. Part of the reason for this is that the ionisation efficiency of a metabolite is largely dependent on the environment in the source. In the highly aqueous environment at the start of the analytical run, the ionisation efficiency of morphine and morphine-3-glucuronide is poor and the extensive fragmentation of morphine gives a relatively low MS^2 signal.

The other problem analyte is 6-acetylmorphine (6MAM) which is present at significantly lower concentrations than any other drug or metabolite tested. The United Kingdom National External Quality Assessment Scheme (NEQAS) guidelines for clinical drugs of abuse testing specify 300 ng/mL as the cut off point for methadone and cocaine and 1500 ng/mL (Heathcontrol Drugs of Abuse in Urine Scheme Information) for opiates. Monoacetylmorphine is present in much lower concentrations and the threshold is 10 ng/mL, more than an order of magnitude lower. As 6MAM is unlikely to be one of the most intense ions present, defining this as a dependent scan is likely to result in missed results.

In the case of all three analytes, it was decided to dedicate a scan event to testing for these drugs in every set of scans. This ensured that these drugs are always tested, even in the absence of good survey scan data. The use of a survey scan, three dedicated MS^2 scans and several data dependent scans increased the scan cycle time considerably and also created a large amount of redundant data. Segmenting the run into two separate scan cycles allows dedicated MS^2 scans appropriate to the morphine and M3G in the first segment, from 1.5 minutes after sample injection to 2.5 minutes, and for 6MAM in the second segment.

One of the strengths of an ion trap compared to a triple quadrupole is the ability to perform MS^3 scans. This is particularly valuable in the case of the glucuronide metabolites. Part of the reason for this is that in an ion trap, all of the fragmentation energy is focussed on the parent ion mass. Smaller and larger masses are not affected by this fragmentation energy. This gives efficient fragmentation of the parent mass, but in the case of glucuronide metabolites, the only chemical bond broken is the link between the drug (or metabolite) and the glucuronic acid. All of the energy of

the fragmentation is absorbed by the weaker glucuronide linking bond and energy in the glucuronic acid or drug is dissipated. This means that there is a single fragment ion, which does not give reliable library search matches. Library searching works better when there are several fragments to match.

For the glucuronide metabolites, it is possible to isolate the fragment corresponding to the drug or metabolite, having lost the glucuronide moiety in the fragmentation process. By further fragmenting this ion, an MS^3 spectrum is obtained, which corresponds to the MS^2 spectrum of the parent drug. An extra dedicated scan event was defined for the morphine-3-glucuronide in the first segment, further improving the ability of the method to reliably detect morphine and its primary metabolite.

3.2.3.1 *Optimisation of mass spectrometer acquisition parameters*

There are a number of other MS settings which were not adjusted from the default settings at this time (see Table 3.3). They will produce perfectly adequate spectra and will not adversely affect the detection characteristics, but may be optimised. This optimisation is done for the Turboflow® method described later.

Table 3.3 Parameters not optimised at this time

Parameter	**Function**
Isolation width	The width of the mass window that the collision energy is applied to
Wideband Activation	Spreads the collision energy to cover ±20 mass units.
Default Charge State	Setting allows identification of multiply charged ions
Activation Q	The “Q value” offset at which the collision energy is applied
Activation Time	The time in milliseconds that the collision energy is applied
Collision energy	The amount of energy applied to induce fragmentation

3.2.4. Final online solid phase extraction mass spectrometer method

The final SPE method that was introduced into the laboratory was also published at the conference of The International Association of Forensic Toxicologists (TIAFT) in Geneva in 2009. It consisted of two segments (see Figure 3.1 and Figure 3.2).

Segment 1 consists of a survey scan followed by two data dependent scans and two dedicated scans to search for morphine glucuronide, which was also used to tune the lens system. The dependent scans looked for the most intense ions present in a list of ions, which can be found in the SPE method file (see Appendix F.3)

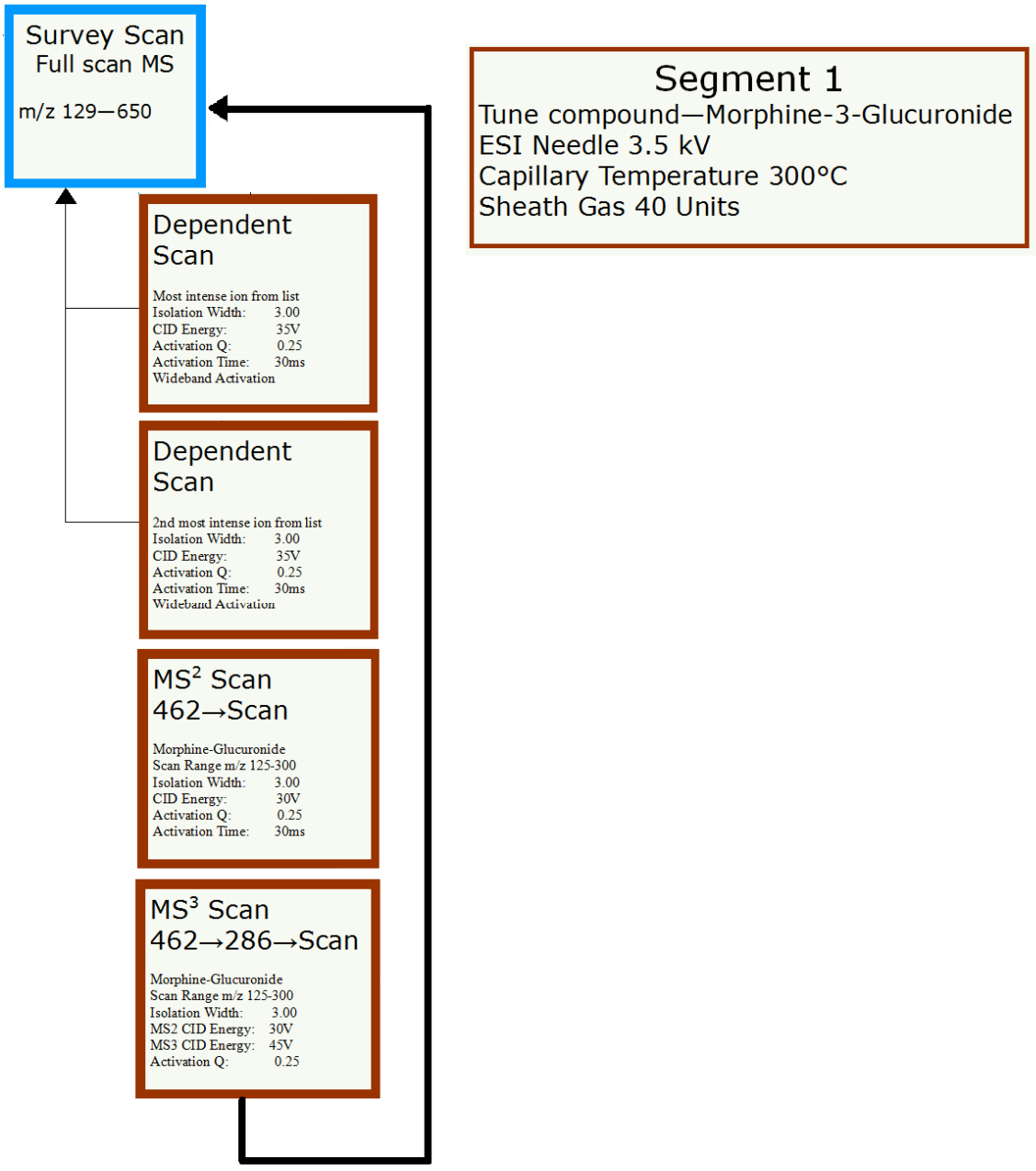


Figure 3.1 SPE Method Segment 1 scan parameters

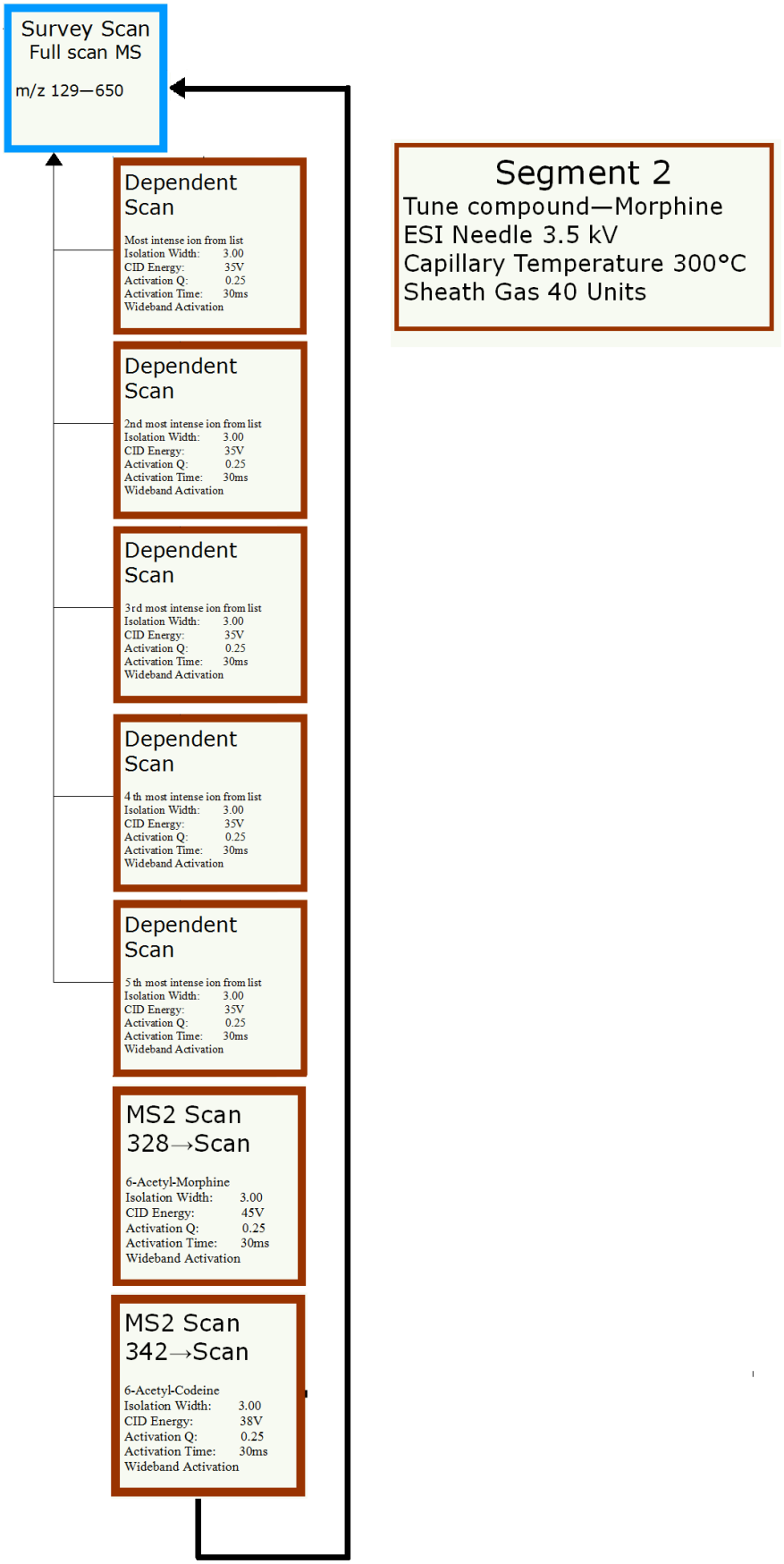


Figure 3.2 SPE Method Segment 2 scan parameters

Segment 2 used a total of 5 data dependent scans to search for up to five analytes, followed by two dedicated scans for 6-acetylmorphine and acetylcodeine. Morphine was used as a tune compound and the dependent scans looked for the most intense ions present in a list of ions (see Appendix F.3)

3.2.5. **Data processing**

ToxID® is an automated reporting system for use with Thermo mass spectrometers. A parameter file specifies some critical information, such as the parent ion mass, retention time, principal fragment ion mass and minimum library search scores. It also includes the report header information, such as the laboratory name and a link to the laboratory logo in bitmap format.

Following this is a list of all analytes that will be examined for reporting. Each analyte is listed with the molecular ion mass, the expected retention time and the minimum library match criteria to assign an analyte as positive.

This list of analytes needs to be maintained and updated as the method evolves, but the retention time can be taken from the expected retention time in the MS method.

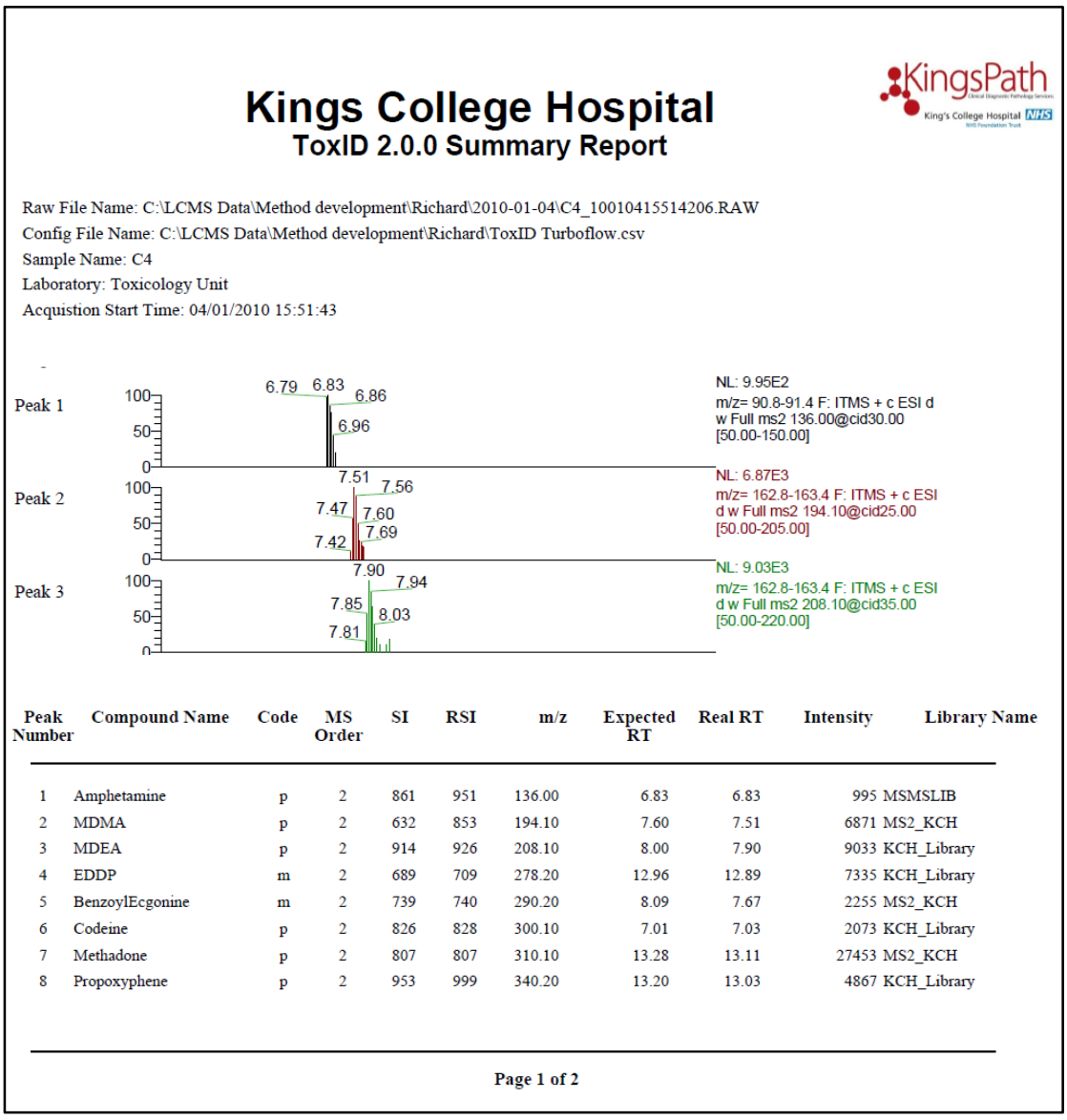
Two formats of report are available, a short format and a long format.

The short format report (see Figure 3.3) lists each identified analyte in a table with the library match results, retention time and peak intensity value and an image of the MS trace of each analyte

This report shows a large amount of information which allows closer examination of the data if necessary.

- The header shows the file name and path, the acquisition date and the sample ID
- Every trace lists the acquisition conditions in the description to the right, including the maximum signal intensity (shown as NL:), the m/z range scanned and the MS conditions (e.g. “ITMS + c ESI d w” shows an Ion Trap Mass Spectrometer operating in positive mode collecting centroid data with an electrospray source, with the data coming from a data dependent scan using wideband activation)

- Each trace shows every scan event that registered a signal matching the scan conditions and the principal product ion specified in the configuration file
- The table shows whether the analyte is a parent drug or metabolite (p or m), the MS order of the scan, the library search scores (search index(SI) and reverse search index (RSI)) and parent mass, expected and actual retention times and peak intensity, together with the library that the peak was matched with.



Kings College Hospital
ToxID 2.0.0 Summary Report

Raw File Name: C:\LCMS Data\Method development\Richard\2010-01-04\C4_10010415514206.RAW
Config File Name: C:\LCMS Data\Method development\Richard\ToxID Turboflow.csv
Sample Name: C4
Laboratory: Toxicology Unit
Acquistion Start Time: 04/01/2010 15:51:43

Peak Number	Compound Name	Code	MS Order	SI	RSI	m/z	Expected RT	Real RT	Intensity	Library Name
1	Amphetamine	p	2	861	951	136.00	6.83	6.83	995	MSMSLIB
2	MDMA	p	2	632	853	194.10	7.60	7.51	6871	MS2_KCH
3	MDEA	p	2	914	926	208.10	8.00	7.90	9033	KCH_Library
4	EDDP	m	2	689	709	278.20	12.96	12.89	7335	KCH_Library
5	BenzoylEcgonine	m	2	739	740	290.20	8.09	7.67	2255	MS2_KCH
6	Codeine	p	2	826	828	300.10	7.01	7.03	2073	KCH_Library
7	Methadone	p	2	807	807	310.10	13.28	13.11	27453	MS2_KCH
8	Propoxyphene	p	2	953	999	340.20	13.20	13.03	4867	KCH_Library

Page 1 of 2

Figure 3.3 Example of a ToxID short format report (traces 4-8 cropped)

All of this information is also given at the top of the ToxID long report, although in this case each analyte is listed on a separate page as shown for benzoylecgonine (see Figure 3.4). Each page includes the mass spectrum of the most intense scan in the

chromatogram laid alongside the matching library spectrum. There is also a “delta spectrum” displayed, which shows the library spectrum subtracted from the acquired spectrum. If the two spectra match well, the result will be a series of small peaks either side of the axis, representing the daily variability of fragmentation intensities. If there is a peak in either spectrum that is significantly different in relative intensity, this will show as a prominent peak in the delta spectrum. Examination of the delta spectrum gives a quick visual way to demonstrate that two spectra are equivalent.

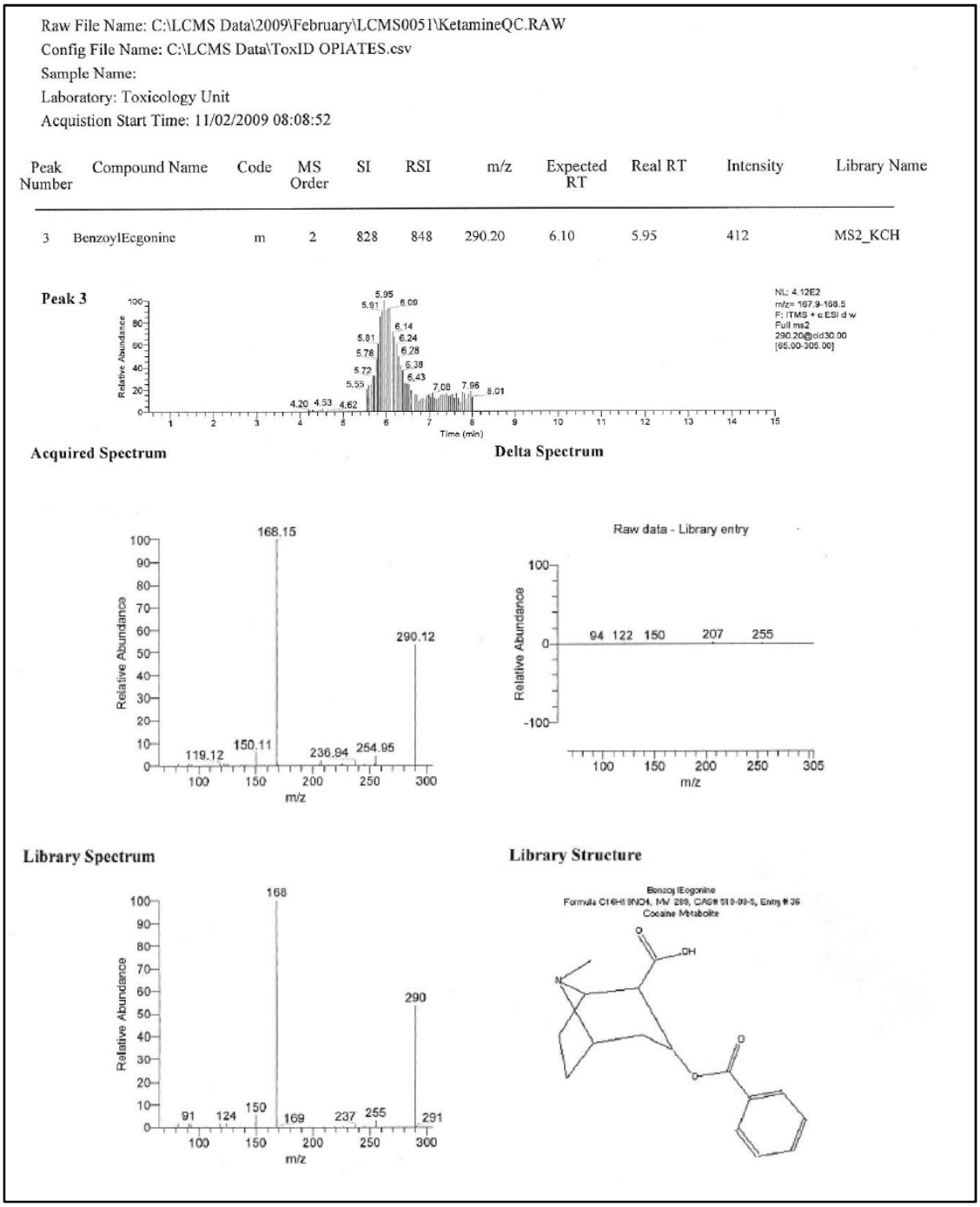


Figure 3.4 ToxID long format report for benzoylecgonine in a QC sample

Also on the long report is the chemical structure of the analyte (if this has been uploaded to the library), including the molecular formula, Chemical Abstracts Service number and the molecular weight.

Several different spectral libraries were supplied with the system, including the National Institute of Standards and Technology (NIST) and Maurer/Pfleger/Webber and ToxLib supplied by Thermo libraries. Since the spectrum acquired by each type of mass spectrometer and at different collision energies can vary, every analyte detected on this instrument was also saved as an in-house library.

By default, ToxID searches through all libraries in the system and consequently there may be several library hits for every analyte. To reduce this, only the in-house library was searched. Each analyte could therefore only be detected once.

A separate library is required for MS^3 spectra and this can be handled a little differently. Since the MS^3 spectrum of a glucuronide conjugate following loss of the glucuronide moiety is exactly the same as the MS^2 spectrum of the unconjugated drug, this was used as a simple way of adding MS^3 spectra. For each glucuronide or sulphate conjugate found, the MS^2 spectrum acquired on the LC-MS was saved in the MS^3 library under the name of the conjugate. If this analyte is present in sufficient quantity, this will cause it to be identified twice in the ToxID report, firstly as the MS^2 conjugate and secondly as the MS^3 spectrum.

3.2.6. **Method validation**

Method validation for this stage of the project consisted of several elements:

- Within and between batch precision (repeatability) for the principal analytes previously analysed by TLC and Gas chromatography (ie morphine, codeine, dihydrocodeine, amphetamine, methadone, EDDP and ecstasy) and also 6-monoacetylmorphine.
- Comparison of results of 100 patient samples with previously analysed data, and evaluation of any differences, and 9 external quality assessment (EQA) results
- Assessment of carryover and ion suppression.
- Identification of limits of detection of the principal drugs

A validation document was prepared following the laboratory policy for validations and submitted to laboratory management for authorisation.

Also prepared at this time and submitted as part of the validation was a framework for the robust identification of new analytes to the LC-MS method, an assessment of the costs of the method, staff training requirements, continued quality control of the method and changes required to the laboratory computer system prior to introduction of the method, this validation document is reproduced (see Appendix E.2).

3.3. Turboflow® method

After routine operation of the method for several months, a Turboflow® HPLC system became available for use. This was considered as possibly more reliable than the original SPE method and would solve several difficulties in the laboratory. The first difficulty that was regularly encountered was that some urine samples would block the SPE cartridge, even with its larger 50 µm particles. Sometimes this would happen with as few as 100 injections, less than 25% the normal lifetime of the SPE cartridges. Secondly, the detection limit for morphine and morphine glucuronide were around 1000 ng/mL, which was perfectly reasonable for clinical testing at the time. However, the EQA scheme for drugs of abuse was shortly going to move to a 400 ng/mL cutoff and the existing method would suddenly become unsuitable. Finally the expected benefits of the Turboflow® system had not been realised in another area of the laboratory and demonstrating successful operation was politically desirable. Work on developing a Turboflow® method started in September 2009.

3.3.1. Turboflow® column selection

There are a number of different Turboflow® columns available (see Table 3.4), similar in performance to the types of HPLC columns in common use. An application note produced by Thermo uses an anion exchange column for opiates, but there is no consensus about which column is most suitable for drugs of abuse.

Table 3.4 Turboflow® column phases

Designation	Solid Phase
C18	Silica reversed phase
C8	Silica reversed phase
Phenyl	Silica reversed phenyl phase
Cyclone	Polymeric reversed phase
Cyclone-P	Polymeric reversed phenyl phase
Cyclone MAX	Mixed reversed phase and anion exchange
Cyclone MCX	Mixed reversed phase and cation exchange

3.3.1.1 *Turboflow® development method*

To evaluate a Turboflow® column, the outflow was connected directly to the mass spectrometer, bypassing the analytical column. Sample was loaded in a solvent that enables maximum retention using the phase under test (normally 100% aqueous) and then eluted in the recommended elution solvent and followed with a strong elution solvent. This pattern of use should produce peaks in three areas of the MS trace:

1 Unretained analytes. Unretained analytes should elute at or shortly after the solvent front

2 Retained analytes. Retained analytes stick well to the Turboflow® column but are eluted easily from the column

3 Strongly retained analytes. These analytes stick well to the Turboflow® column, but are difficult to elute

The general method for testing the Turboflow® columns is shown (see Table 3.5).

Table 3.5 Gradient used for evaluating Turboflow® columns

Step	Time (s)	Flow (mL/min)	Solvent	Comment
1	0 – 60	0.5	Load	Retention solvent
2	60 – 90	0.5	Aqueous Elute	Elution with aqueous solvent only
3	90 – 120	0.5	Organic elute	Elution with weak organic solvent
4	120 – 150	0.5	Organic clean	Strong organic solvent
5	150 – 260	0.5	Regenerate	100% aqueous solvent

The flow was kept at 0.5 mL/min to ensure that turbulent flow was not happening and any retention effect was attributable to the column and solvent combination only. Acetonitrile was used as the organic solvent, as this gives lower back-pressure than methanol, and this is a very common mobile phase, allowing comparison with other methods.

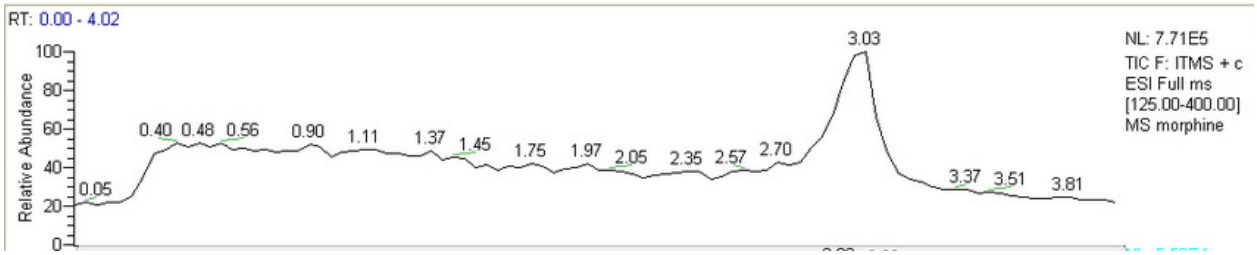


Figure 3.5 Optimisation of Turboflow® Cyclone MAX column retention: morphine retained on Turboflow® column for 3.03 minutes, demonstrating excessive retention

Morphine is strongly retained on an anion exchange column (see Figure 3.5). The sample was loaded in basic conditions and the loop elute step used an acidic mobile phase to attempt to elute the analytes. In this case, morphine was only eluted after the 100% organic elute step, meaning that the morphine was retained too strongly to wash off in normal analytical conditions.

All available Turboflow® columns except the C8 column were tested to see which gave the best performance. It was expected that since all of these drugs are basic

drugs, the MCX column would give the best performance and that reversed phase columns would allow moderate retention. While the reversed phase and MCX columns did give a high degree of retention, this was sometimes too strong to be eluted in 200µL of 100% acetonitrile.

The column selected for further development was the Cyclone MAX polymeric anion exchange column (see Table 3.6). This is counter-intuitive, since most of the drugs being tested do not easily form anions. Comparison of the elution profiles of the Cyclone MAX and Cyclone columns shows that they have very similar retention characteristics, but the Cyclone MAX allows a cleaner and fuller elution than the Cyclone column. The reason for this is probably that when eluting in acidic conditions, the stationary phase functional groups and analytes both become positively charged. This causes a repulsive force between the stationary phase and the analyte which is stronger than the reversed phase retention and the positively charged analytes enter the mobile phase. This is particularly advantageous for methadone, which is relatively non-polar and has a strong reversed phase retention and is not normally eluted in 200 µL of acetonitrile.

Table 3.6 Initial Turboflow® extraction method HPLC parameters

Start Time (s)	End Time (s)	pH	Elution pump solvent %B	Loading Pump Flow Rate (mL/min)	Elution Pump Flow Rate (mL/min)	Comment
0	60	9.5	0	2.0	0.2	Sample loading in turbulent flow conditions. Eluting pump maintains column flow
60	120	3.5	1	0.2	0	Elution from the Turboflow® column with the loop contents
120	360	3.5	50	1.0	0.2	Regeneration of the Turboflow® column
360	420	3.5	1	0.2	0.2	Filling the loop with elution solvent
420	600	9.5	0	0.2	0.2	Re-equilibrate Turboflow® column

As all samples are extracted through the same column, it is important to ensure that there is no carryover from one sample to the next. A sequence of four injections was performed with 5 analytes to assess carryover (see Table 3.7).

Table 3.7 Sample injection protocol for the assessment of carryover

Injection number	Injection type	Reason
1	Deionised Water	Prime sample loop, ensure there is no carryover from previous samples
2	Test sample	Urine sample spiked with analyte
3	Deionised water in Laminar flow	Laminar flow bypasses Turboflow® column, so any carryover identified is likely to come from the syringe or from the guard or analytical columns
4	Deionised water in Turboflow® injector	Any carryover identified must come from the injection port, Turboflow® column or the transfer valve

Carryover was assessed for amphetamine, morphine, morphine-3-glucuronide, cocaine and methadone. These were used to demonstrate carryover in the most clinically significant polar and non-polar analytes.

Carryover for methadone was estimated to be 1.47% at a level of 200,000 ng/mL in the Turboflow® injector system and the other analytes there was no carryover detected. In the formal validation document, carryover was assessed for amphetamine and benzoylecgonine and found to be not significant.

3.3.2. **Recovery**

Recovery can be defined as the amount of an analyte that is available for detection after the extraction. With a Turboflow® system, this is easy to estimate by injecting a sample first using the laminar flow injector and then with the Turboflow® injector. The

peak height or area of the Turboflow® injection as a percentage of the laminar injection gives the recovery.

Table 3.8 Assessment of recovery from the Turboflow® column

Analyte	Laminar Injection peak height	Turboflow® injection peak height	Recovery (%)
Morphine	6893	4495	65.2
Cocaine	122271	82630	71
Methadone	5359864	328227	73
Amphetamine	26550	24200	91
Morphine-3-glucuronide	44007	850	21

Poor recovery of morphine glucuronide (see Table 3.8) is likely to be due to the poor retention of this analyte on reversed phase columns. Calibration of the method should therefore be made using the Turboflow® injector, as calibration using the laminar flow injector is likely to give results only 65 – 90 % of actual for most analytes and only around 20 % of actual for morphine glucuronide.

3.3.3. Analytical column method

Experience with the SPE method suggested that a phenyl column was suitable, giving enhanced retention of drugs containing phenyl rings, which includes all of the drugs of interest. No change was therefore made to the analytical column, although a variety of different types of gradient were tested. The gradient was initially developed using laminar flow injection (LX). For conversion to the final Turboflow® injection (TX), the Turboflow® loading time (1 minute) needed to be added to the gradient and the loading pump flow during the transfer step needed to be made up from the eluting pump. Aqueous standards were used to develop the analytical method, as the Turboflow® sample preparation step was omitted.

As before, a gradient separation is required to enable sufficient retention of morphine-3-glucuronide and elution of methadone in a reasonable time. The

analytical gradient effectively starts at 60 seconds when the Turboflow® column is switched into the analytical system, as the morphine-3-glucuronide elutes in the 1% organic solvent at about 2 minutes.

A maximum run time of 20 minutes was chosen to try to ensure sufficient sample throughput. This leaves around 18 minutes to perform the separation before returning the analytical column to the starting conditions. Simple gradients that allowed methadone to elute in around 15 minutes did not give a good separation of amphetamine and cocaine and shallow gradients that allowed good separation of these left methadone being eluted far too late. Fortunately there is a big time gap between the main group of drugs (including all the amphetamine derivatives and cocainics) and methadone, allowing a gradient change to occur away from any peaks of interest. The relevance of this is that if the retention times change between runs, for example with decreasing column performance or slightly different mobile phase strength, this would have a disproportionate effect on the retention times of these peaks.

Table 3.9 Final Turboflow® extraction and HPLC Parameters

Step	Start Time (min)	Flow (mL/min)	%A	%B	Comment
1	0	0.2	100	0	Equilibration and Turboflow® loading
2	1	0	100	0	Flow of 0.2mL/min from Loading Pump
3	3	0.2	100	0	Morphine-glucuronide elution
4	4	0.2	85	15	Start of gradient
5	11	0.2	70	30	End of gradient
6	11.01	0.2	0	100	Step to elute methadone
7	16	0.2	0	100	Late eluting peaks
8	16.01	0.2	100	0	Return to Initial conditions
9	20	0.2	100	0	End of run

The optimum gradient starts after the elution of morphine glucuronide (which is eluted in 100% aqueous eluent) and after a step change to 15% organic rises gradually to 30% organic (see Table 3.9). At 11 minutes, the gradient conditions were changed to allow faster elution of methadone, with methadone being eluted in close to 100% acetonitrile. Although a range of gradient rates were tested to elute methadone, there was no degradation if the organic content was immediately increased to 100%.

3.3.4. **Mass spectrometer method**

Experience with SPE methodology and additional training on the capabilities of the ion trap system allowed a more comprehensive and targeted approach to developing the mass spectrometer method. This facilitated detection of a greater number of analytes at lower concentrations and also better quantitation and reduced target analyte list maintenance.

3.3.4.1 ***Scan parameters***

With an ion trap it is possible to keep the trap “open” for varying lengths of time and therefore to change the number of ions entering the trap. However, this has two adverse effects:

1 The increased collection time reduces the number of scan events and therefore reduces the number of data points across a peak. For confident analysis and quantitation, more data points are desirable.

2 As more ions enter the trap, the charge within the trap increases. Ions in the centre of the trap will be shielded from the full voltage of the trap by the ions around them and the practical effect of this is a decreased mass resolution.

To resolve this, the LCQ Fleet has a setting called the Analyser Gain Control (AGC). This defines a maximum time for the trap to acquire ions and monitors the number of ions entering the trap and closes the trap when a preset maximum is reached. It achieves this by performing a short scan event and counting the ions and then calculating the trap open time for the main scan event. This combination prevents trap overloading and also ensures that the scan time is not too long.

The ion count settings of the trap were maintained at the default settings, as any increase in the number of ions would reduce the resolution and any decrease could affect sensitivity or spectrum quality. However the maximum trap times were examined to see if there could be any performance improvement.

In practice, where a full scan was performed, the time that the trap was open was always limited by the maximum scan time, rather than the maximum ion limit. This was tested at 5, 10 and 15 ms and the signal intensity was similar for all times (see Table 3.10). For MS^2 scan events, the times tested were 50, 100 and 150 ms and the shorter ion time gave better results. This is partly because a shorter ion time leads to more consistent filling of the ion trap.

Table 3.10 Analyser gain control evaluation using 3 time settings using butalbital (m/z 312 at 6.5 minutes)

AGC Setting	MS response	Total scan time
50 ms	7.32×10^2	50 ms
100 ms	6.32×10^2	100 ms
150 ms	6.49×10^2	106 ms

As can be seen (Table 3.10), the greatest signal intensity was obtained with the shortest AGC setting. As a result, this setting was retained for all subsequent method development and for the final analytical method.

Another default setting of the MS is to use 3 microscans. The LCQ Fleet performs three separate scans within each scan event. These are then aggregated to give a single data point, the intention being to average the spectra to obtain a more reproducible mass spectrum. For a single scan event lasting 155 ms there will be four component scans (see Table 3.11). All 3 microscans use the same AGC settings defined by the AGC pre-scan. This uses up precious scan time and the number of microscans used and their contribution to spectrum quality were assessed.

Table 3.11 Typical scan component timings

AGC Scan	1st Microscan	2nd Microscan	3rd Microscan
5 ms	50 ms	50 ms	50 ms

All fragmentation in a mass spectrometer has a degree of variability. When looking at the ratio of intensities of 2 fragment ions, the European Union Guidelines on the interpretation of analytical results (The Commission of the European Communities, 2002) specify that 2 ions should have a ratio that varies by no more than 20% where the ions have similar intensities. By aggregating several microscans, the LCQ Fleet averages out this variability and the spectrum obtained should be more similar to stored library spectra.

A selection of analytes were tested using 1, 2 or 3 microscans and the performance evaluated.

Since the total response is the average of the responses of the microscans, the recorded response was similar for each of the drugs tested (amphetamine, ecstasy, benzoylecgonine, codeine, methadone, 6MAM and morphine-3-glucuronide).

The use of microscans is intended to improve spectrum quality, so the forward and reverse library match scores were also compared for the above drugs, and again there was no significant decrease in spectrum quality. A Search Index (SI) score of >600 and reverse search (RSI) of >700 was considered acceptable and this was obtained easily whenever there was a sufficient ion intensity to obtain a reasonable quality spectrum. This suggests that for this application, the use of a single microscan will not lead to a decrease in performance. However, unless an improvement can be demonstrated, there is no reason to reduce the number of microscans.

Additional microscans reduce the number of data points available, so an increased number of data points across a peak may be sufficient reason to reduce the number of microscans. Chromatograms were examined and the number of scan events across a peak were identified to see if there is an increase in data points.

Table 3.12 Assessment of number of data points for 3 analytes with 3 different microscan settings

Analyte	1 microscan	2 microscans	3 microscans
Amphetamine	5 data points	3 data points	3 data points
Morphine-3-glucuronide	9 data points	4 data points	3 data points
EDDP	18 data points	9 data points	5 data points

The reduced number of microscans does significantly increase the number of data points (see Table 3.12). For all subsequent analyses, a single microscan was used, meaning that more scan events can be included in the method, allowing a greater degree of analyte identification.

3.3.4.2 *Survey scans used for the Turboflow® method*

Experience of using survey scans during the SPE method development and operation allowed additional improvements to be made when it came to further MS method development. One of these improvements was to use the Turbo Scan feature of the ion trap to perform the survey scan faster. Turboscans reduce the time taken to perform the scan by increasing the rate at which ions are scanned out of the trap. While this is very much quicker than a normal scan, the overall effect is limited due to the trap filling time. However, the use of the turboscan does reduce the total analysis time, allowing more data points across a peak. On its own, this may not be a significant improvement, but the combined effect of several different improvements in the MS method reduce the total cycle time, allowing more drugs to be detected in a single analytical run.

The survey scans ran from m/z 125 to m/z 800 in all segments, covering the full range of expected parent ion masses.

3.3.4.3 *Scan segments used in the Turboflow® method*

In order to minimise unnecessary data acquisition, the MS method was split into 3 segments. Each segment can contain different scan events and be tuned using a different analyte.

Segment 1 lasted from the start of data acquisition for 6.6 minutes. Segment two lasts for only 1.45 minutes and segment 3 lasted for 8.45 minutes from 8.05 minutes until the end of the run. All three segments use the same parent mass and reject mass lists and the scan structures are the same across all segments. This means that the only practical difference between the segments is the dedicated scan event, set for morphine glucuronide in segment 1, 6-monoacetylmorphine in segment 2 and acetylcodeine in segment 3.

3.3.4.4 *MS^2 data dependent scans used in the Turboflow® method*

Experience with the initial SPE method allowed simplification of the data dependent scans for the Turboflow® MS method. The initial SPE method used a series of up to five MS^2 scans after the initial survey scan. Streamlining the technique allowed this to be reduced to a total of 5 scan events.

Following the survey scan, the most intense ion from the parent ion list was subjected to an MS^2 scan if the ion intensity was greater than 500 counts. This is the primary scan event in each segment, designed to detect any known drugs present.

Following this, a second data dependent scan was performed on the most intense ion present in the survey scan, excluding any masses listed in the reject mass list. While this scan event may be able to detect a wide variety of substances, this would require manual processing and manual library searching. The principal reason for this scan was to use the data acquired to search for neutral losses. If the most intense ion in the original survey scan is that of a glucuronide or sulphate conjugate, the MS^2 scan should allow the identification of the parent drug or metabolite using the principle of a neutral loss. For example, codeine (m/z 300) has a glucuronide metabolite with an m/z of 476. If codeine-glucuronide is identified as the most intense ion in the survey scan, the MS^2 scan should show a significant peak at m/z 476 – 176 = 300. This peak is then selected for an MS^3 scan in the fourth scan event of the cycle.

As previously discussed, using the most intense peak in a range for data dependent scans can lead to significant drugs being missed. A target compound list was therefore developed that lists all target analytes by their expected retention windows.

A full list of analytes and their target retention time windows can be found in Appendix F.4

Analytes and retention times were found by injection of aqueous standards of the drugs onto the analytical column and examining the survey scan data for the molecular ion peak. Once identified, the analyte was added to the mass list and the standard was re-injected to verify that the correct mass spectrum could be obtained. At this time, the mass spectrum of the analyte was acquired (if not already done so) and copied to the in-house mass spectrum library for library spectrum matching (see page 97 for ToxID spectrum matching).

Examination of the mass list shows that it is unlikely that more than 5 analytes will co-elute and this approach should not lead to any analytes being missed. However, the list should be periodically examined to ensure that any new drugs added to the list do not risk false negative results. A peak intensity threshold was also added so that dependent scans would not be triggered by background noise. The threshold was set at a level that was consistently above the baseline level, as assessed by a series of sample injections.

3.3.4.5 ***Dedicated scans used in the Turboflow® method***

As for the SPE method, a series of dedicated scans was set for critical analytes that may otherwise be missed.

The parameters were the same as for the SPE method, with dedicated scans for morphine and morphine glucuronide in the first segment, 6-monoacetyl morphine in the second segment and acetyl codeine in the 3rd segment.

Unlike for the SPE method, the 6-monoacetylmorphine scan was restricted to an isolation width of 1 mass unit. This was implemented to improve the mass spectra produced during these scans and therefore increase the specificity of the scan. Apart from this modification, no other parameters were modified during the method development stage, although following implementation it is understood that the 6-monoacetylmorphine parameters were amended slightly.

3.3.4.6 *Neutral loss scan in the Turboflow® method*

The reason for the second survey scan is that this enables neutral loss processing of data in subsequent MS^2 and MS^3 scans. Without defining the mass to be searched, a data dependent MS^2 scan is performed on the most intense peak within the mass range. The MS^2 scan is not intended to directly identify any drugs, but to look for any peak which loses 176 amu., corresponding to the loss of glucuronic acid. An MS^3 scan of this peak is then performed to try to identify the drug that formed the conjugate. Some of the advantages of operating in this fashion are that specific scans do not need performing for every different glucuronide and glucuronides of unspecified drugs can be identified. This leads to a simplification of the mass list in the analytical method, although for automatic reporting they would still need to be defined in ToxID.

Since an MS^3 scan would only be performed when a tentative glucuronide was identified, it is fairly quick and easy to look through all MS^3 scan events on Xcalibur software, although this is still a manual process, and library searches would need to be performed manually. If substances identified in this fashion are then added to the ToxID configuration file, this represents a powerful way of increasing the capabilities of the method.

Tramadol is found in urine conjugated to both glucuronic acid and also sulphate. Looking for glucuronic acid conjugates alone might lead to tramadol being missed and so a second mass was added to the neutral loss mass list, corresponding to the sulphate conjugate. In this way, the neutral loss scan looked for the most intense sulphate or glucuronide conjugate to perform an MS^3 scan on. The ToxID mass list needed to be updated and the library spectrum for the MS^3 sulphate conjugate added, but this change allows a further improvement in the capability of the developed method.

3.3.4.7 *Dynamic exclusion of masses in the Turboflow® method*

By specifying that an MS^2 scan needs to be done on the most intense peak in a scan, this leads to additional difficulties. Firstly, there may be peaks or a constant background of a scan that produce a large amount of unwanted data. These peaks may be from LC components (such as bleeding stationary phase) or plasticizers and

other substances that can enter the system. These were minimised by running some blank samples without drug. Any ion that had a significant background intensity was added to the reject mass list, so that this would never trigger an MS^2 scan. Also the general background signal was examined and the threshold for the MS^2 scan was set so that background signal would not trigger MS^2 scans.

Secondly, the presence of a large peak of one drug could be the most intense peak in the survey scan for some time and this could swamp the signal of any co-eluting drug, especially if the co-eluting drug is present in a lower amount.

To avoid this, a feature called dynamic exclusion was enabled. Dynamic exclusion causes a detected mass to be temporarily included in the reject mass list, so that subsequent scans do not look for the same substance continuously. After a defined time, the mass is removed from the dynamic exclusion list so that it may again be detected and subject to an MS^2 scan if it is still the most intense ion present.

There are two main settings for dynamic exclusion, the number of repeats and the exclusion time. The number of repeats defines how many times an ion must be detected before it can be added to the reject list and the exclusion time defines how long the ion remains on the reject list.

Initially the repeats was set to 5 and the exclusion time to 10 seconds. However, this typically leads to 5 consecutive MS^2 scans of the same mass, followed by a break of 10 seconds and then another 5 MS^2 scans. Chromatographic performance is poor, as a peak is identified just as the peak front arrives at the detector and is then either not detected again or is detected in 2 or 3 further blocks of data, as can be seen (see Figure 3.6).

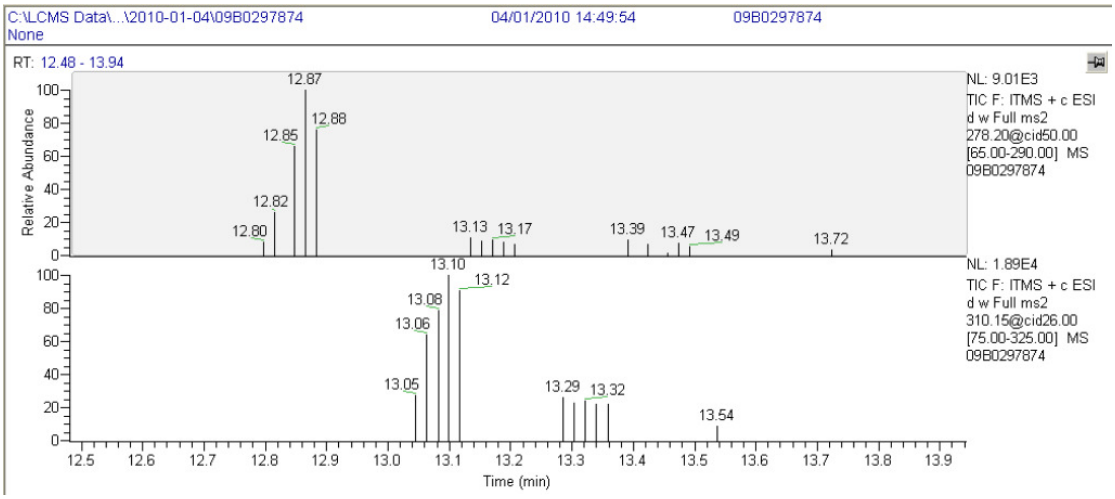


Figure 3.6 Chromatogram demonstrating the effect of poor Dynamic Exclusion settings (Upper trace: EDDP, lower trace: Methadone)

As well as making the chromatogram harder to read visually, this may mean that a peak is only detected at its start and any concentration information is not gathered. To rectify this, the repeats setting was changed to 1 and the duration to 2 seconds. This means that every time an MS^2 scan is performed, the same scan is disabled for a further 2 seconds. The chromatogram would show a data point every 2-3 seconds across the peak, allowing peak shape and intensity to be assessed, and would allow other substances to be identified in the intervening 2 seconds. The total cycle time varies depending on what else is being examined at any particular point of the run, but normally this allows at least 2-3 co-eluting peaks to be identified.

Dynamic exclusion can be set as a global setting and in this way the dynamic exclusion settings apply to the ions in the parent mass list, the neutral loss mass list and in all three MS segments.

3.3.5. ToxID configuration for the Turboflow® method

ToxID was set up in almost exactly the same way for the Turboflow® method as for the SPE method. The mass lists were different because the retention times were different, but otherwise there were no significant differences.

A number of additional drugs, principally benzodiazepines, were added to the configuration file to begin the process of identifying these drugs. The illicit heroin markers noscapine, desmethyl-papaverine, desmethyl-papaverine-glucuronide and

meconine were also added to the configuration file, following the work carried out by Tracey Gous (Gous, Sherwood, Evers and Blackburn, 2011).

The configuration file for ToxID can be found in Appendix F.2

3.3.6. Automated addition of internal standards

In the final phase of the project, experiments were performed to automatically add the deuterated internal standard to samples using the autosampler. A vial of internal standard was loaded into the autosampler tray and the needle programmed to draw up the internal standard before the sample. Drawing in this order reduces any risk of patient samples contaminating the stock of internal standard. The reverse is less likely to happen due to the much lower concentration of internal standard in most samples and that the needle will only visit a sample vial once on a normal run, rather than repeatedly as happens with the internal standard.

The method was set up to sample 40 µL of the internal standard, followed by a needle wash and a 10 µL air gap to further reduce the chances of contamination. The needle then visited the sample vial and drew 50 µL of sample before injecting all into the loading port of the HPLC.

This was demonstrated to work in theory, but the reduction in sample volume reduces the sensitivity of the method by 50 % and the normal sample volume of 100 µL was considered necessary. A 250 µL syringe and carrier were therefore ordered to finalise this part of the method development, but budget restraints in the National Health Service (NHS) in January 2010 meant that this could not be ordered in time to validate prior to leaving the Trust.

3.3.7. Method validation of the Turboflow® method

Method validation was performed as for the SPE method. As previously stated, the method was not complete at the time the author left the laboratory at King's College Hospital and the validation had to be completed by remaining staff. The validation document produced at this time can be found in Appendix E.7

4. Results and discussion

4.1. **Final online solid phase extraction method used for the routine analysis of samples**

The SPE method operated between December 2008 and August 2009 produced reliable results for the opiates, amphetamines, cocaine, methadone (see Figure 4.1) and was extended to several other analytes. It suffered a little from not having accurate quantitation, meaning that presence of an analyte could only be reported as present or absent, but the added certainty of the identity of a drug was a significant improvement over the TLC method. It was possible to compare results approximately by examining the ion intensity and score as +, ++ or +++ (or weak positive, positive, strong positive), but this was not often reported.

4.1.1. **Sensitivity and specificity**

Patient sample results from the SPE method were compared with results from samples analysed by TLC for Opiates or GC for amphetamines during October and November 2008, as well as a selection of methadone and cocaine positive samples (see table 4.1).

All results are qualitative by LCMS, TLC and GC and so cannot be processed using a Deming regression.

Table 4.1 Results of 100 samples analysed by both TLC, GC or immunoassay and LCMS

	Morphine	EDDP	Codeine	Methadone	Cocaine	Amphetamine	DHC
True POS	56	83	2	80	50	3	2
True NEG	39	17	93	20	48	97	98
False POS	4	0	5	0	2	0	0
False NEG	1	0	0	0	0	0	0
SENSITIVITY	98.2%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
SPECIFICITY	90.7%	100.0%	94.9%	100.0%	96.0%	100.0%	100.0%

The results of the TLC or GC are taken as “TRUE” and LCMS results are compared to these.

$$\text{Sensitivity} = \frac{\text{LCMS POSITIVES}}{\text{TLC POSITIVES}} \quad \text{or} \quad \frac{\text{True Positives}}{\text{(True POS + False NEG)}}.$$

$$\text{Specificity} = \frac{\text{LCMS NEGATIVES}}{\text{TLC NEGATIVES}} \quad \text{or} \quad \frac{\text{True Negatives}}{\text{(True NEG + False POS)}}.$$

Sensitivity and specificity relate the method being tested to the existing method, which is assumed to be correct. In this case the new method is more accurate than the existing method, as can be seen when the anomalous results are investigated.

4.1.1.1 ***False positive cocaine results***

These 2 samples had cocaine results of 204 and 238 ng/mL by immunoassay. Cocaine was present at levels detectable by the LCMS. This would be reported as negative by immunoassay, which uses a cutoff of 300 ng/mL to differentiate positive and negative results.

4.1.1.2 ***False positive codeine results***

Codeine is a common contaminant of heroin and the presence of codeine should not be unexpected in urine samples from heroin users. The limit of detection by LCMS is much lower than by TLC and in four of these samples, morphine (from heroin) was present. In the fifth sample, opiates were detected by immunoassay, but neither morphine nor codeine were detected by TLC.

4.1.1.3 ***False positive morphine results***

Three of these samples were opiate immunoassay positive, but no morphine was detected by TLC. The detection limit for morphine is slightly less for LCMS than TLC, but the level of interference is lower, enabling more certain identification of morphine.

The fourth sample was codeine positive, with only a weak morphine concentration. In most of the UK population, codeine can be metabolised to morphine, and this probably represents codeine use rather than heroin.

4.1.1.4 *False negative morphine result*

One sample found to be positive for morphine by LCMS was re-analysed by TLC and found to have no morphine. There was significant interference covering the morphine area on the plate and the original identification of a weak positive morphine was uncertain and made in conjunction with the patient history. This shows how the added certainty of identification by spectral library matching gives improved results compared to the existing method.

4.1.2. Precision

Precision was assessed by the analysis of quality control materials five times on the same batch (intra-batch precision) and on five consecutive batches (inter-batch precision) and determination of the coefficient of variation (%CV) (also known as residual standard deviation) of the response.

Five drugs from four drug groups were assessed at three levels to determine precision (see table 4.2).

Table 4.2 Reproducibility of analysis for the online solid phase extraction method

Drug	Level (ng/mL)	Intra-batch precision (%CV)	Inter-batch precision (%CV)	95% confidence limits (± ng/mL)
Morphine	1500	8.9	5.0	300
	225	24.7	5.0	111
	4000	17.7	15.6	1850
Codeine	1500	6.6	4.3	232
	225	15.7	14.0	93
	4000	12.7	18.7	1466
Amphetamine	375	2.5	15.5	114
	675	11.9	6.7	181
	1500	5.0	8.4	247
Cocaine metabolite	115	12.0	9.2	34
	185	6.7	5.9	32
	1000	4.5	10.4	204
Methadone	225	2.3	6.2	27
	375	3.9	6.0	44
	1000	1.9	14.9	292

The 95% confidence limits are 1.96 times the inter batch precision. Where the intra-batch precision is higher than the inter-batch precision, the precision values have been combined as the square root of the sum of the squares of the intra- and inter-batch precision values.

During routine operation, it also became evident that the online SPE cartridge sometimes became blocked, usually in the middle of an analytical batch and often overnight. Investigations suggested that this blocking was caused by precipitation of proteins in the SPE cartridge when exposed to acetonitrile. The life of SPE cartridges, intended to be around 500 – 1000 samples, was sometimes reduced to less than 100 samples, an unacceptable increase in costs and disruption to scheduled laboratory work. The aqueous equilibration step at the end of the run was therefore increased by one minute and after this was done there were no significant problems with SPE cartridge lifetime.

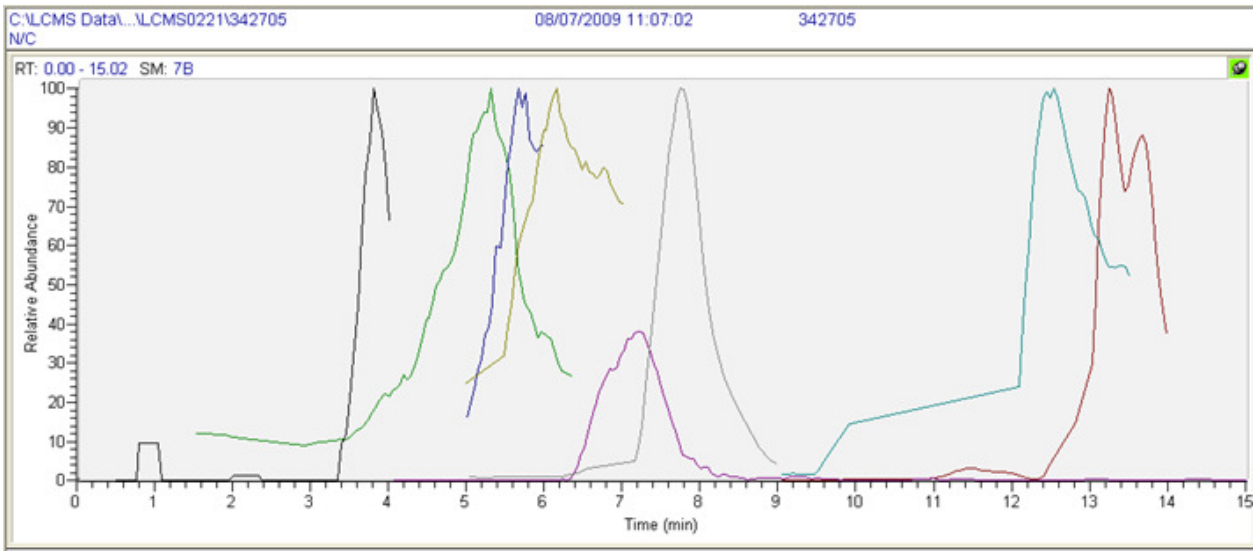


Figure 4.1 Results of a patient sample showing extracted ion chromatograms. (Black: morphine glucuronide, Green: morphine, Blue: codeine glucuronide, Olive: codeine, Purple: 6-MAM, Grey: benzoylecgonine, Light Blue: EDDP, Red: methadone)

Internal day-to-day performance of the method was monitored by the recording on worksheets of the results of the quality control samples (see Figure 4.2). Internal quality control results were very good and the method was able to give consistent qualitative results on a daily basis, during a working week.

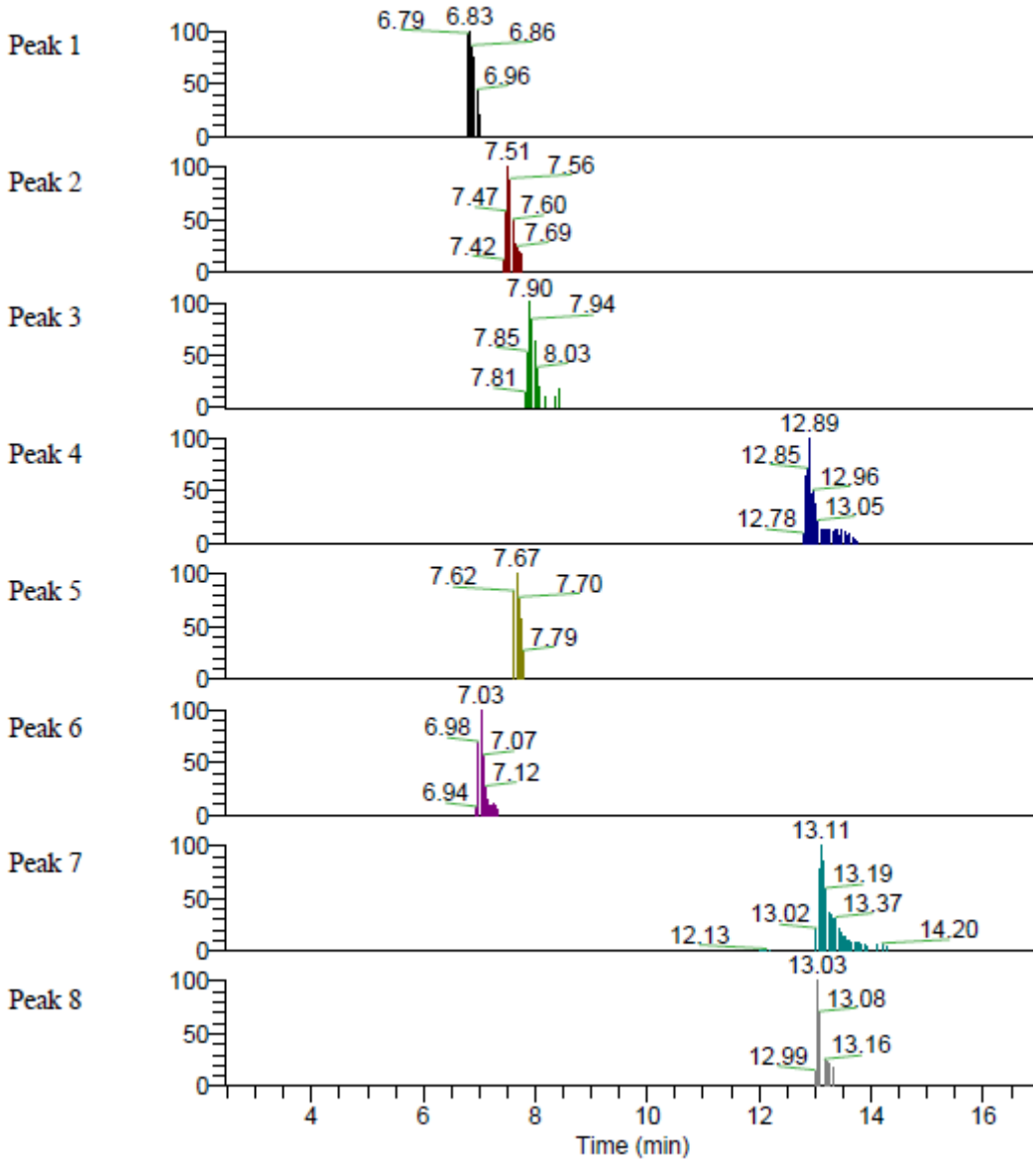


Figure 4.2 Quality control result printout, (1: Amphetamine, 2: Ecstasy, 3: MDEA, 4: EDDP, 5: Benzoylecgonine, 6: Codeine, 7: Methadone, 8: Propoxyphene)

Performance in the EQA (proficiency testing) scheme through the analysis of 12 separate samples throughout the year for all drugs within the laboratory's repertoire. In the first year of operation, the online SPE method did not result in any false positive or false negative results for the amphetamine group, benzoylecgonine, methadone or EDDP. There was 1 false negative result for 6-monoacetylmorphine and morphine (below the limit of quantification).

Several other modifications were made to the method during the first year of operation. A research trial that was underway in the Toxicology Unit was the identification of noscapine and papaverine metabolites. Noscapine and papaverine

are two alkaloids found in poppy seeds that survive the manufacture of heroin and can be detected in the urine of heroin users. They were being used in the Randomised Injectable Opioid Treatment Trial (RIOTT) to differentiate drug users prescribed diamorphine from those using heroin. The previous method in the laboratory was a GC-MS method and these analytes were added to the LC-MS method as part of an in house MSc project by Tracey Gous supervised by the author and the results of the trial were published (Strang, *et al.*, 2010). The initial identification of the spectrum of desmethyl-papaverine was made using the MS^n capability of the ion trap and published as a poster at the conference of The International Association of Forensic Toxicologists (TIAFT) in Geneva in 2009 (Evers, Marsh and Gous, 2009).

The other significant adjustment to the method was the addition of ketamine and its metabolite norketamine to the mass list. Ketamine is a sedative used by the ambulance service instead of opiates, as it does not depress the respiratory system in the way that opiates do. However, the reason for it being added to the LC-MS method was that it is in use as a recreational drug, particularly in the clubs of south London. The midwives in the obstetrics department at KCH were interested in knowing whether some of their mothers were taking ketamine. A ketamine standard was therefore obtained and the retention time and spectrum of these metabolites identified. Ketamine was therefore added to the repertoire of the Toxicology Unit, starting the expansion of the service from covering only the addictions department at SLAM to other service areas.

Another observation during the first year was that in most samples from heroin users the morphine glucuronide peak was adequate to produce an MS^3 library match in ToxID. However, in weaker samples, below around 2000 ng/mL, the glucuronide peak was present in the MS^2 spectrum but not in the MS^3 with sufficient peak intensity to provide a good library match. Given that these samples were from opiate users and had tested positive for opiates by immunoassay, this level of identification is sufficient to report a morphine positive result. However, the level of confidence is lower if the sample comes from a patient not known to be on opiates, as demonstrated by Bayesian probability.

Bayes theorem takes the pre-test probability and adjusts it based on the sensitivity and specificity of a test. If a known heroin user tests positive for opiates by immunoassay (with a specificity of around 85% in this population, Synergy Health unpublished data), it is highly likely that this is due to morphine, even without the confirmatory LC-MS test. However, in an unselected population, the specificity of the opiates immunoassay for morphine is only around 10% (due to the far higher prevalence of codeine). With a pre-test probability of only 10% (corresponding to the prevalence of morphine in the unselected population), the specificity of the confirmatory test must be much higher to report morphine as being positive.

As a result, the SPE method is more limited in its application and any identification of the presence of morphine should not be made solely on the MS^2 identification of morphine glucuronide, but *must* include either morphine (MS^2), morphine glucuronide (MS^3), a more intense codeine or codeine glucuronide peak or clinical details showing the expected presence of morphine. It was hoped that the improved results of the Turboflow® extraction would increase the MS^3 signal intensity sufficiently to allow the more widespread application of the LC-MS opiates method.

4.2. **Final Turboflow® method used for the routine analysis of samples**

The Turboflow® instrument methods are reproduced (see Appendix F.4 (Mass spectrometer acquisition method) and Appendix F.5 (Turboflow® HPLC method)).

The final protocol for drugs of abuse by Turboflow® was introduced into the laboratory on August 2nd 2010. The drugs included on this method were as shown (see Table 4.3).

Table 4.3 Analytes validated for analysis by Turboflow® 2010

Morphine	Dihydrocodeine glucuronide
Morphine glucuronide	Codeine
Codeine glucuronide	Methadone
6-Monoacetylmorphine	EDDP
Dihydrocodeine	Amphetamine
Benzoylecgonine	Methamphetamine
Acetylcodeine	Hydroxypapaverine glucuronide
Ketamine	Dihydroxypapaverine glucuronide
MDMA (Ecstasy)	Pholcodine
Hydroxypapaverine	Nortriptyline
Dihydroxypapaverine	Noscapine

Precision was formally recorded for only three analytes, morphine glucuronide, codeine and benzoylecgonine and is shown (see table 4.4).

Table 4.4 Reproducibility of analysis for Turboflow® method for three selected analytes

	Codeine	**Morphine glucuronide**	**Benzoylecgonine**
Within day reproducibility	12.34%	9.56%	15.59%
Between Day reproducibility	12.12%	16.33%	13.50%
95% confidence interval	±23.6%	±32.0%	±26.5%

In addition to reproducibility, around 100 samples were also analysed by the existing SPE method and the new Turboflow® method (see Table 4.5).

Table 4.5 Comparison of patient results using SPE and Turboflow® extraction

	Morphine glucuronide	Codeine	Methadone	Benzoylecgonine	Amphetamine
True POS	80	23	71	39	1
True NEG	20	77	29	61	99
False POS	1	11	9	1	1
False NEG	3	0	1	5	0
SENSITIVITY	96.39 %	100 %	98.61 %	88.64 %	50 %
SPECIFICITY	95.24 %	87.50 %	76.32 %	98.39 %	99 %

The anomalous results were investigated to identify why they differed from the original method (Birch and Marsh, 2010).

- The high number of false positives for codeine and methadone are caused by the fact that the SPE method was not very sensitive for these analytes. Therefore whilst it shows as a false positive, in fact they were all genuine positives that the SPE method did not detect.
- The few false negatives that have been found were likely caused by the analytes being present at such low concentrations that they were below the cutoff and would not have been reported as positives even with the SPE method.

These false negatives occurred where the drugs are identified as present, but below the reporting threshold. Even though the SPE method was able to identify the presence of the drug, the final report would have been negative and as such the Turboflow® method did not result in any false negative reports.

Limits of detection and linearity were not formally assessed at this time, contrary to the method validation plan that had been left with the laboratory (see Appendix E.6). Both authors of the validation document have left the Toxicology Unit and it is not recorded why the precision of other analytes was not assessed formally in the method validation.

4.3. Introduction into the laboratory

The Clinical Pathology Accreditation (CPA) Standards for the Medical Laboratory (Clinical Pathology Accreditation (UK) Ltd, 2010) were introduced in 1992 to set minimum standards for pathology laboratories and to improve patient safety. The standards include the requirements that methods are validated before introduction (Standard F1), that they are fully documented in Standard Operating Procedures (SOP) (Standard F2), that records are kept to enable the reconstruction of the assay conditions (Standard A9.4) and that staff performing the assays are trained in their performance (Standard F3.1). Documentary evidence is required for all of these activities and these shall also be assessed by an audit program to ensure that these standards are being adhered to (Standard H1.1). A series of documents therefore needs to be prepared to fulfil these standards for the LC-MS drugs of abuse assay, as well as formal procedures for the acceptance and introduction of the assay.

4.3.1. Documentation for the online solid phase extraction method

Initially the method was developed to replace the TLC opiate confirmation assay and the amphetamine GC-FID assay and also the confirmation of methadone and cocaine if required. As a first step in the process, a validation document was produced (see Appendix E.2) with an updated SOP (version 3.0 see Appendix E.1). The SOP was electronically authorised through the department's computerised document control system, QPulse, and the validation was submitted to the department's executive committee. The first live patient samples were analysed on December 1st 2008.

There are also a number of other documents that needed to be created or updated to maintain compliance with the CPA standards. The department's training scheme needed to be amended with the updated method (see Appendix E.3) and staff trained and a maintenance log needed to be prepared. Most analytical equipment performs best with a planned preventative maintenance schedule. In the case of the LC-MS this includes daily, weekly and monthly maintenance, as well as the manufacturer's annual service visit and any unscheduled maintenance. This maintenance log serves two purposes, firstly as a prompt that the operator to perform the maintenance, but

also as a record that the maintenance has been performed so that this activity can be audited.

The newly developed method is different to that in use prior to the change and as such the laboratory computer system requirements are different. These changes were first made in a backup copy of the laboratory computer system known as CERT and tested before introduction into the live database. There were four principal changes to the system at this time:

- Removal of the GC test code, a non-reportable free-text line to record the details of the GC analysis
- Renaming of the TLC test code to LCMS.
- For amphetamine screening sample positive results, the LCMS code replaced the GC test code.
- The worksheet for confirmation tests was amended to include all LCMS tests on one worksheet instead of requiring a TLC worksheet and a GC worksheet.

The SOP was updated in May 2009 when the Jasco HPLC was replaced with the Agilent and Turboflow® equipment, although at this stage the method was still the SPE method. This SOP is also reproduced (Appendix E.5) as SOP version 4.

4.3.2. Interfacing the hardware system to the laboratory computer system

Further modification to the validated SPE method during 2009 was the introduction of an analyser interface. Interfacing an analyser to the laboratory computer system can offer a number of benefits, including lower risk of transcription error and reduced level of data entry. While it is possible to set up a quantitation method on the LC-MS, which will produce numerical results that can be output to through an interface to the laboratory computer system, the reports produced by ToxID are in a PDF format and it is not as easy to import from these. However, it is possible to import the worksheet, which can save at least 5 – 10 minutes for every batch, representing at least 40 hours per year.

A data import method to allow simple data entry into the LC-MS was devised. This was based on a simple text file export from the LIMS system followed by some

formatting in Microsoft Excel (v.2003), which included addition of controls, worklist numbering and selection of the correct HPLC and MS method files. Screenshots of the Excel file can be found (see Appendix F.7 (EZChrom and Xcalibur) and Appendix F.8 (Aria)) together with the macros written in Excel.

4.3.3. Operation of the interface between the laboratory computer system and the LC-MS

The automation consisted of a formatted spreadsheet with 3 buttons at the top of a sheet formatted for printing. The first button (Full Worklist) runs a macro to import a text file previously exported from the Pathnet system. Lab numbers are inserted automatically into the correct positions on the worklist, together with the date of the worklist, operator and controls at the beginning and end of the worklist and after every 10 samples. The sheet is designed to be printed as a working record of samples to be prepared and to write results onto prior to transfer to the Pathnet laboratory computer system.

The second button (Edit Worklist) is used where the full list of outstanding samples is not to be included on the worklist, for example if there are additional samples to add or if the worklist is to be split into two. This works in exactly the same way as the first button except that the process is paused after the text file is imported from Pathnet to allow editing. A third button is used to resume the import process.

The final button is labelled Export Sequence and this copies the formatted export sheet onto a named text file that can be saved to a USB memory stick for transfer to the LC-MS computer for import into EZChrom and Xcalibur software.

When the Aria software was introduced, the import sequence became easier, as the Aria software passes sequence information automatically to the Xcalibur software. The sequence template was modified to match the Aria format, for example the sample vial identification and injection parameters, and the EXPORT macro edited to export a single .csv file.

The OPEN_WORKLIST macro was also simplified to remove the option of editing the worklist and also improve QC handling.

4.3.4. Maintaining the quality of laboratory results

In a modern clinical chemistry or toxicology laboratory, quality is an important and integral part of laboratory operations. One of the ultimate markers of laboratory quality is the accreditation that is awarded by the United Kingdom Accreditation Service (UKAS) under the banner of the CPA standards (Clinical Pathology Accreditation (UK) Ltd, 2010).

Among the required quality requirements that need to be implemented prior to introduction of a method are risk and control of substances hazardous to health (COSHH) assessments, a validation document and written Standard Operating Procedures. Copies of these documents can be found (see Appendix B and Appendix E). Ongoing assurance of the quality of results is monitored in a number of ways, with each activity assessing a different aspect of the testing.

The principal way in which quality is maintained is by the use of Quality Control (QC) samples. These are standards that are analysed as unknown samples on every batch and compared with previously established target ranges. Typically these ranges are based on the mean and 2 standard deviations of previous batches and the results are usually plotted as Levy-Jennings plots. However, with qualitative results this plot would be meaningless and results are recorded on the worksheet to demonstrate that they have been examined and assessed, but formal trending of the results does not occur. Batches are rejected if the QC sample results are unsuitable, with samples being rerun.

All results produced during the procedure are entered into the laboratory's computer system, initially Pathnet but later Clinisys. An additional step within the computer system is clinical authorisation. Authorisation involves a senior scientist examining the results in conjunction with other data available, often a set of previous results and a request form with clinical information. The purpose of this is to reduce the chances of incorrect results being released, but also to alert medical staff to any clinically relevant results, either by telephoning the ward or clinic or by the addition of a comment to the results.

Having established that an assay is performing consistently within the laboratory, additional samples are analysed and compared with the results of other laboratories.

Three of these proficiency testing or EQA samples are distributed by the scheme organiser every 3 months and analysed. Consensus results are only obtained after the results submission deadline. In this way, quality can be independently assessed and scored. Both QC and EQA results are needed to assure the laboratory that the results of every batch are accurate.

4.4. **Introduction of the Turboflow® method to routine use within the laboratory**

The author left King's College Hospital in January 2010 before the final details of the method were finalised. A list of outstanding work was given to the Toxicology Unit manager, with instructions for the completion of the validation (Appendix E.6).

Work in progress at this time included an assessment of the feasibility of automatically adding the internal standard as part of the autosampler program. Validation of the method was scheduled to occur after this assessment had been concluded. This assessment required a 250 µL autosampler syringe in place of the 100 µL syringe supplied with the system and delivery of this part was delayed. When delivered it was determined that an additional syringe holder was also required to enable the syringe to be held in place on the autosampler. An order for this part was placed as one of the last activities the author did in the laboratory. Laboratory financial constraints meant that the requisition for the larger syringe holder was rejected by laboratory management. As a result, the final project validation was completed by Toxicology Unit staff in June and July 2010, with some minor modifications to the author's intended protocol, particularly the offline addition of internal standard to the urine sample. The validation was completed in July 2010 and the method implemented in August 2010. This validation document and corresponding SOP are reproduced (see Appendix E.7 and Appendix E.8 respectively).

As for the SPE method, QC has been recorded simply as the presence or absence of the appropriate analytes in the QC samples. It was hoped that the peak intensity values would either be able to produce semi-quantitative numerical results, or at the very least would allow the peak intensity to be recorded and plotted. It has not been determined whether the analyte peak intensities are directly proportional to analyte

concentration. Should this be the case a semi-quantitative result may be obtained, allowing an assessment of between batch reproducibility and improving the ongoing assurance of the quality of the analysis.

4.5. **Evidence based medicine**

For centuries people have used naturally occurring substances to change the way that they perceive the world. Often this would be for spiritual reasons and the use of these substances would be generally small scale or low intensity. As these substances became more purified and more available, they were able to exert a greater effect on the users and would be taken for the enjoyment of the effects produced, rather than for the original spiritual reasons. As the strength of these drugs increased, they induced dependence in heavier users and led to addiction. The scale of use means that addiction psychiatry has become a branch of the medical profession that can be found in all areas of the country and community drugs teams can be found throughout the UK. As with all other areas of medicine, psychiatry has become increasingly evidence based and this evidence includes the use of laboratory diagnostic tests.

In order to assist a patient to overcome their addiction, there are several steps that need to be followed. Firstly the psychiatrist needs to identify the drug that the patient is addicted to and to quantify how much is being consumed. In most branches of medicine, this is fairly easy to achieve and at South London and Maudsley NHS Trust this is done by interviewing the patient. It is quite easy to interview a heroin user and determine that they inject daily, but the differing purity and combination of opiate drugs consumed can be difficult to convert to a single usage figure. For convenience, the addictions team often convert the amount of opiate drugs consumed into a common score based on the amount of money spent on drugs. Market forces mean that a lower grade heroin will be worth less on the street and will therefore contribute less to the overall cost of drugs.

Where addiction can suffer compared to other branches of medicine is that the addiction changes the user's perception of the world and users will often be inaccurate in their answers as they attempt to take advantage of the health services or to avoid their influence. For example, many users will enter treatment not because

they wish to cure their addiction but because this is the aim of somebody else, such as the legal system or a friend or relative. It is also common for users to seek treatment for reasons other than to give up their drug addiction. This is often for financial reasons, as drug addiction, particularly heroin addiction, can be expensive. Addicted patients will use the healthcare system to obtain free methadone, thereby reducing their spend on heroin and allowing them a degree of normal social functioning. In some cases, the aim is not to use the methadone to reduce the effects of addiction, but to sell to the supplier.

In order for a user to enter treatment, it is therefore necessary for the psychiatrist to have evidence that the patient in front of them is a heroin user. This is easily accomplished with the use of a laboratory test. The results of this test can be used in conjunction with the users stated drug consumption to help determine what prescription is appropriate.

Later on in treatment, laboratory tests will have a different focus. Detection of heroin metabolites can show the clinician that the patient is still using heroin (and may therefore need an increased methadone dose) and detection of methadone or buprenorphine can confirm that they are at least partially compliant with their prescribed treatment. In some cases, the addiction may cause the patient to deny, either to themself or to the psychiatrist, that they are still taking the drugs or that they have a drug problem.

When a patient is compliant with treatment or is abstaining, the urine drugs test allows this to be evidenced to the clinical team, allowing continued support and encouragement. Current trends in psychiatry include an increased use of contingency management, a process where users are given small rewards for the achievement of goals. This may include incentives such as cinema tickets for a given number of negative drug tests, or even a “gold star” for attending the clinic at the correct time and date. These rewards do not need to be expensive to help encourage a user with their treatment. Effective laboratory support is therefore a necessity for the implementation of some forms of contingency management.

As an inpatient on a detoxification and rehabilitation ward, it is important to medical services that the ward does stay drug free. It is hard enough to give up drugs with support and doubly so if there is somebody continuing to use drugs, the “bad apple”

in the barrel. Regular testing of the inpatients can provide the evidence that this is so and allow the early removal of patients who are breaking the rules and putting the rehabilitation of others at risk. The laboratory report can provide evidence of this to the ward staff and to the continuing user.

Of course there are other health effects of illicit drugs, such as the effect on the family or unborn child and all of these can benefit, at least in part, by the use of a urine drugs test in a clinical setting.

4.6. **Toxicology testing at King's College Hospital**

The method for testing for drugs of abuse, especially opiates, at Bethlem and Maudsley hospitals and then at King's College Hospital until 2008 was a thin layer chromatography method largely unchanged for 20 years. As the pattern of drug use changed and the expectations of the customers rose, especially with respect to turnaround times, the TLC method became out-dated and inadequate. Of the available technologies, LC-MS has proven itself to be fast and reliable and applicable to a wide range of situations and drugs. However, without a standardised method or equipment it is up to individual laboratories to determine the best conditions to use based in their own particular circumstances.

As time and cost pressures increase, there are several key areas where a laboratory can make improvements and the methods developed here were designed to take advantage of these improvements. Firstly, the degree of operator intervention required for the SPE method is significantly below that required for the original manual method, saving upwards of an hour a day of skilled operator's time. Secondly, the cost of consumables is reduced markedly, with the single SPE cartridge being re-used several hundred times instead of individual disposable cartridges. Thirdly, the elimination of the hydrolysis step has allowed typical turnaround times to be reduced from 3 days to 2 days. This improvement in result availability may not have any measureable effect in the laboratory, although the faster turnaround time may assist in gaining future work, but could lead to a measureable reduction in costs for the addictions services who use the laboratory, for example the faster response could lead to user being admitted to treatment quicker or removed from the ward earlier.

Other benefits not identified at the outset of the project include the identification by maternity services of mothers who are using ketamine, allowing intervention to improve infant survival or prognosis and the identification of “red heroin” as alprazolam, allowing better information to service users. The extension of the method has also allowed a colleague to obtain her master’s degree and has produced the results for a major trial into the use of injectable opioids.

The improvements due to the Turboflow® system are not as significant as those already achieved by the on-line SPE, but were still worth the time and effort spent on method development. The Turboflow® method further reduces staff hands-on time, although by a smaller margin and has improved the quality of results produced. It has also produced a framework that allows a wider range of drugs to be identified and allows a greater degree of quantification than the SPE method.

It could be argued that a simpler system could produce results that are as good or better than those produced by the methods developed here. For example, there are several manufacturer defined methods for the identification of drugs of abuse using a triple quadrupole that are less complicated than the method employed here. However, a triple quadrupole is generally significantly more expensive than an ion trap and does not allow the benefits of MS^3 identification of drugs and metabolites. Turboflow® and SPE sample extraction are both more expensive than the dilute-and-shoot methods that can be used with a triple quadrupole, but the sample clean up steps reduce the level of source contamination compared with dilute and shoot methods and are more extendible to other matrices.

Without the LC-MS, the drug testing element of the Toxicology Unit would have lagged behind the standards achieved in other NHS laboratories and could have compromised the long term future of this element of testing. Other LC-MS systems are available for use at King’s College Hospital, but all are used for other purposes and it would be difficult or impossible to guarantee access on a daily basis to allow timely reporting of drugs of abuse samples.

The procedure developed in 2010 remains in operation in the Toxicology Unit in May 2014, albeit with a few minor modifications, for example additional drugs, modified conditions for the detection of 6-monoacetylmorphine and a modification to improve

the limit of detection for morphine when the EQA scheme lowered the clinical cut-off to 300 ng/mL.

4.7. **Transferability to other laboratories and applications**

Ion trap LC-MS systems are uncommon in drugs of abuse testing laboratories and triple quadrupoles are much more common. The reason for this is that a triple quadrupole is more sensitive, allowing it to be used for a variety of other quantitative methods. For laboratories that need to search for unknown substances, a GC-MS is generally more popular because of the wide availability of mass spectral libraries and the historical widespread use of these systems, with off-the-shelf methods generally available from the manufacturers. Other more expensive LC-MS combinations such as the Q-Trap from Sciex or Q-TOF hybrids and Thermo's Orbitrap can perform as well in most areas and even remove the need for any sample preparation.

There are several advantages that this newly developed method gives compared to most other LC-MS systems:

1 Good quality mass spectra are obtained from all analytes. To obtain this type of data from a triple quadrupole requires fairly extensive parameter settings (e.g. tMRM from Agilent). Quadrupole-TOF instruments, quadrupole-traps and Orbitraps can acquire this data easily, but these instruments are generally more expensive than ion traps

2 Minimal sample preparation is required. Most methods require a sample dilution (dilute and shoot) or offline extraction, with or without a hydrolysis step. Hydrolysis typically adds at least an hour of incubation time prior to analysis

3 The use of MS^3 scans triggered from a neutral-loss scan allows the simple identification of glucuronide and sulphate conjugates, followed by library searching

While these advantages make this system well suited to the intended application, the rarity of this combination of Turboflow® and Ion Trap suggest that it is unlikely that this method will be employed exactly elsewhere. However, there are several

elements of the method that are readily transferrable to other laboratories using LC-MS.

The Turboflow® extraction and LC-MS method can be readily adapted for any mass spectrometer coupled to a Turboflow® system, whether this is an ion trap, triple quadrupole or an accurate mass system

The method files, consisting of the retention times and parent masses of the analytes, can be easily transferred to any other LC-MS system using the same column and solvent combination. Slight changes may need to be made, for example depending on the gradient delay volume for a particular system, but this change is easily within the capability of any experienced HPLC method developer.

Use of a CTC® autosampler for internal standard addition is unusual and presents a simple way of automating this analytical step, whether for drugs of abuse or any other similar type of application using internal standards. This can also be used to perform the dilution step of dilute-and-shoot methods used on triple quadrupoles and similar instruments. It is slightly more limiting in methods that require offline hydrolysis, as this will necessitate the accurate measurement of the sample volume and addition of a known volume of hydrolysis reagent.

The use of a neutral loss scan to identify glucuronide or sulphate metabolites is also unusual. It is not likely to be widely used in routine work as it is generally simpler to hydrolyse the conjugate and look for the parent drug. However, the use of a neutral loss scan could assist in the addition of new analytes to other LC-MS methods, or to improve the power of general unknown screens.

4.8. **Further work**

As a result of this work, a number of further items of research and development have been identified. These include significant whole pieces of work and also incremental method development actions that are essential for any laboratory to remain up to date.

4.8.1. Benzodiazepines

The benzodiazepine class of drugs contains around 30 common members and metabolites. In the SLAM addictions clinics diazepam and Librium® (chlordiazepoxide) are the most commonly prescribed benzodiazepines. However, other benzodiazepines are also available and in 2009 alprazolam was reported as being found as a component of illicit heroin. One sample of this was received at the laboratory and the mass spectrum obtained from an infusion of a solution of the substance demonstrated that this was alprazolam. Addition of the benzodiazepines and their metabolites to the method would allow this information to be communicated back to the community drugs teams on a patient level basis, allowing improved patient care.

This would also allow the addictions psychiatrists to start prescribing alternative benzodiazepines, such as nitrazepam or clonazepam, which have different metabolic pathways and are less prevalent through illicit channels. This would allow dose optimisation based on illicit benzodiazepine use as the metabolites of diazepam would be readily identifiable. A Turboflow® benzodiazepine method has been developed for use at King's College Hospital, but this has been developed on a triple quadrupole instrument separate from the general drugs of abuse confirmation method developed here.

4.8.2. Repertoire

As new drugs are detected using the described method, especially through the identification of glucuronide or sulphate metabolites, these drugs should be added to the mass list of the LC-MS method and also of ToxID, including addition to the in-house spectrum library.

The easiest way to advance with this is to constantly monitor a defined screen on the Xcalibur software that shows any MS^3 transitions. The trace is likely to be largely blank, with a few short areas where an MS^3 signal is detected, with this signal sometimes being only a few scan events long. Selection of the acquired MS^3 spectrum in these areas is easily achievable on the Xcalibur software and this can then be used to search any available MS spectral libraries. Any good library matches that have a parent ion mass that corresponds with the MS^2 product are then good

candidates for addition to the library. Additional confirmation may also be possible by searching the survey scan for a peak corresponding to the free drug parent ion. As already discussed, a drug needs to be present at an appreciable concentration to produce an MS^3 spectrum and it is therefore likely that such a drug will be identifiable, at least in low concentrations, in the survey scan.

It would also be prudent at this point to examine the metabolism of the identified drug and to examine the literature. If a potential glucuronide conjugate of a target is found using the above method, but the drug identified in the library does not form conjugates, it is then clear that the match is incorrect. Similarly if the drug is extensively and completely metabolised and not found in the urine, this would also suggest that the tentative match is incorrect. The literature search should also identify if there are any other metabolites that may be searched for in the survey scan to further increase confidence in the identification of the candidate drug.

Addition of the newly identified candidate parent ion into the MS^2 mass list, followed by a reinjection of the sample should then confirm the presence of the unconjugated drug by checking the resultant MS^2 trace against the spectral library. The increased signal intensity compared to the MS^3 signal is likely to lead to a more reproducible spectrum than that obtained in the original MS^3 trace and this can then be added to the in-house MS^2 and MS^3 libraries. In addition, the MS^2 trace of the conjugate can also be added to the in-house MS^2 library. Finally, the free and conjugated peaks need to be added to the ToxID configuration file to allow reporting.

In this way, it is possible to identify and save to the method new drugs and metabolites without necessarily having to obtain reference materials from a supplier. However, if there is no reference material and the sample in which the drug or metabolite is identified is not from a patient known to be taking the drug, this peak should always be treated with caution until an independent source of data can corroborate the candidate drug.

4.8.3. **Cannabis**

The Toxicology Unit at King' College Hospital does not have a validated method for the unequivocal identification of cannabis metabolites. An immunoassay method in use is very good (positive predictive value of the immunoassay is over 95% (SHLS

unpublished data)), but there are occasions where the immunoassay fails, for example in patients with HIV who are prescribed efavirenz (Atripla®, Sustiva®). A confirmation method for cannabis would be beneficial to KCH and would assist in the transferability of the method to other laboratories.

Cannabis is different from most of the other drugs of abuse in that the main urinary metabolite is acidic, whereas all the opiates, amphetamines, benzodiazepines and cocainics are basic drugs. This means that the retention characteristics on the Turboflow® column are likely to be different for cannabis. Cannabis metabolites should be investigated to determine whether they are retained on the Turboflow® column and what the elution and detection conditions are. Even if the described Turboflow® method does not allow the identification of cannabis metabolites, it should be possible to produce a method that is capable of performing this identification using an alternate Turboflow® column or solvent combination. It is likely that the level of cannabis metabolites normally found in urine is too low for this method to be suitable using the LCQ Fleet and this work has not been carried out.

4.8.4. **Other matrices**

An alternative matrix becoming popular, particularly with community drug teams, is saliva (also known as oral fluid or oral mucosal transudate). Currently no investigations into salivary drugs have been made using the described method. This would increase the repertoire of the laboratory and allow increased revenue, particularly as some of the local community drug teams have already switched to this testing strategy.

Similarly there is an increased interest in testing for drugs in hair, particularly from the family law sector. While this is outside the current remit of KCH, this is an area for examination. The Addictions Department of the Institute of Psychiatry have already expressed an interest in this area of research, as it allows a longer term picture of drug use to be built up. Currently the Addictions Department of SLAM continues to use urine as a primary drug testing sample and there has been no call for saliva methods to be developed. However, as with cannabis the levels of drugs normally found in saliva are generally too low for reliable detection with this ion trap method.

4.8.5. User feedback

One of the original aims of this project was to evaluate how this change in the service affected the treatment and prescribing habits of the community drug teams. Informal meetings with some of the key staff have given positive feedback to the better quality results obtained using the SPE method, but no formal assessment of these benefits has been made. There are two reasons why this element of the original project was not completed to the depth that would have been preferred:

- The author's decision to leave South London, preventing effective liaison with the users of the toxicology laboratory
- The transfer of the pathology division of King's College Hospital to a partially privately owned organisation meant that the focus of the toxicology unit manager and the laboratory manager was on internal organisational transition, rather than on obtaining formal feedback on a small aspect of the laboratory's repertoire

User satisfaction is still assessed as part of the laboratory's quality and accreditation requirements, but this is intended to examine the whole service rather than this particular aspect. As a result, there is no information available to allow the assessment of user satisfaction of this part of the pathology service in isolation.

5. Conclusion

The original stated aim of this project was to develop a Turboflow® method to detect drugs of abuse in a clinical population. This aim has been achieved and by reading through the pages of this thesis it is possible to understand some of the parameters that have been set or modified to enable a highly efficient method. The method developed is capable of detecting a wide range of drugs and metabolites with library spectrum matching for reliable identification. The lack of any hydrolysis step ensures simple sample preparation, and the combination of MS^2, MS^3 and neutral loss scans with data dependent scanning allow many drugs to be detected in the same sample. This combination of features has not been published by any other authors.

However, this method is not the end result but just the beginning. Without training for the staff operating the method routinely, without the ongoing quality control and ongoing method development, this method would have ceased operation when the author left the Toxicology Unit. Over four years later the basic method is still in operation, although as should be expected for routine methods, there have been some modifications along the way.

Turbulent flow chromatography is a powerful technique for simplifying sample preparation and ion trap mass spectrometers are able to give a very high degree of confidence in the identity of a substance. The combination of the two techniques has created a versatile method able to expand as the library data becomes more comprehensive without any need to alter the method details. This is particularly useful as laboratory methods come under close scrutiny from accreditation assessors and changes to methods come under increasing control.

Beyond the details of the method, the professional doctorate course has had a definite positive impact on the author and the completion of this course and thesis have changed the author's outlook from that of a laboratory technician to one of a healthcare scientist, looking beyond the walls of the laboratory and considering the impact and effects of his actions on the wider health economy.

6. Reflection

An important element of the Professional Doctorate at the University of Portsmouth is that it improves the professional development of the students. Part of this development is the use of educational techniques to assist the students to become experts in their chosen fields and to operate at a level expected from somebody with a doctoral qualification. Much has been made in the educational literature about reflective practice and a variety of different approaches and models have been discussed and tried. The professional doctorate will not have achieved its aim if the results of the course and the results of this thesis are shelved on completion and they do not lead to a change of behaviour. The following sections, written in the first person, demonstrate how the author has learned from the process, has changed his approach to work and has developed into a scientist able to perform to the very high standards expected from a doctoral candidate.

6.1. **Personal reflection**

I have been interested in “science” since before starting secondary school and it will have come as no surprise to those who knew me at school that I went on to work in a laboratory, study for an MSc and eventually start on this Professional Doctorate course. Perhaps some of the people who knew me would be surprised at the time between my first degree (completed in 1992) and registering for the doctorate (2005). Part of the reason for the delay was in finding the specific subject that I am passionate about. In 2001 I began working in toxicology and quickly knew that this was where I wanted to specialise. The 2004 course was already full when I enquired, so I began in October 2005.

One of the reasons for choosing this particular professional doctorate course was the politics of the NHS. In a laboratory there are 4 different groups of staff:

- the laboratory assistants who perform the more basic tasks and are often not qualified to degree level
- Biomedical scientists (such as myself) who typically perform complex analyses, are responsible for the implementation of quality within the laboratory and are generally responsible for the results produced

- Clinical Scientists who focus more on the interpretation of laboratory results, method development and communication of results to medical staff
- The medically qualified doctors who are generally in charge of the clinical governance of the laboratory, who will see their own patients and take ownership of more complex cases

Since the professionalisation of biomedical science over the last 20 years or so, the boundaries between clinical and biomedical science have become increasingly blurred. It is no longer gross misconduct for a biomedical scientist to authorise a result and biomedical scientists are now permitted to join the Royal College of Pathologists. Clinical scientists (who are relatively few in number) have strongly defended their position from biomedical scientists and it is not easy for a biomedical scientist to reach the top of their profession unless it is through management.

The professional doctorate course has allowed me to become an associate member of the Royal College of Pathologists, to be involved in research and to work at a level previously restricted to clinical scientists.

The course itself is open to medical professionals from the fields of nursing, pharmacy, physiotherapy and imaging and this crossover between professions has helped to break down the barriers often faced by laboratory staff, who too often do not fully consider the hospital outside the pathology department. The discussions between students have sometimes been lively and have helped me to obtain a fuller grasp of the wider NHS.

Two of the modules were what I considered typical doctoral level courses, namely the advanced research techniques and the publication and dissemination modules. They helped focus on the difference between a master's degree and a doctorate and have clearly been designed to assist in the completion of a thesis. However it is the first module that has perhaps had the greatest impact on my professional development.

6.2. **Professional review and development**

The module essentially asked "what is an expert" and "how do you get there" and asked students to develop a map of how this would be achieved. The Personal

Development Plan that I developed in 2006 gave a blueprint for my development that I broadly followed and this was revised towards the end of the taught element of the course in 2007.

I have had difficulty with the formal structure of some of the reflective practice models and work better with an unstructured approach. I have a good memory when it comes to toxicology and drugs of abuse and the notes I take of lectures, cases and from reading papers are very limited. As a result, when I am reviewing my notes it is not the detail that I focus on but the circumstances and application of the data, allowing me to extrapolate to other situations. An example of this is the combination of data from two papers, one on the differences in cocaine concentrations in the hair of light, moderate and heavy users and one on the differences in cocaine levels in hair from different parts of the body. When asked for an estimation of the level of cocaine use from body hair samples, I was able to assimilate these pieces of information to give an estimation of the cocaine use of an individual.

A colleague and I now compile a short document each month to review significant cases and to review articles and papers read. This document is circulated among all of the laboratory's senior staff for learning and comments and also saved to the department's document library for further learning opportunities. A similar document is also prepared when we attend conferences, so that all staff can receive the benefit of this company expenditure.

At the outset of the module I knew little about educational theory and we were encouraged to think about how we learn and therefore how we should teach different elements of our working practice. This has tied in well with two courses that I have attended since, the first on the role and responsibilities of a team leader and the second the Institute of Leadership and Management certificate in leadership and management. Both courses included self evaluations very similar to those done during the professional development module and I have been able to use all of these evaluations to further assist my personal development and understand how to get the best out of myself. Perhaps the biggest revelation was a statement in one of the evaluations that said that I am "disinterested in purely scholastic pursuits". At first I misread this as "not interested in knowledge", which is far from the truth – I enjoy reading New Scientist about subjects as diverse as particle physics and astronomy

as well as medical laboratory testing and I enjoy any science and nature based program on television such as Horizon. However, the real meaning of the statement was that I am not interested in knowledge *for the sake of education*. This simple phrase explains to me some of the reasons that it has taken over three years to complete the writing up of this thesis after leaving King's College Hospital. In my mind, the work for the project was complete, except for a few items that I could not achieve as I was no longer an employee, and the completion of the thesis was a *purely scholastic pursuit*. Even since this understanding, it has taken some time to really start writing properly, but as I write, the thesis is nearly complete and I am feeling satisfaction for a piece of work that I can be proud of. Writing this has also rekindled my interest in the work that I did and I have found the background reading interesting and informative. Perhaps the thesis has developed beyond a "purely scholastic pursuit" which is why I am now interested?

One of the principal aims of starting on the route of the professional doctorate was to enable me to reach the top of my profession and ultimately to allow me to achieve the status of consultant toxicologist. In pathology, the highest qualification that can be achieved is the Fellowship of the Royal College of Pathologists (FRCPath). Other titles and qualifications are either academic titles, such as "professor", or are of an equal but different standing as the FRCPath, such as FRCS (Fellowship of the Royal College of Surgeons), or are job titles, such as Consultant. As a result of this course I am now an associate of the Royal College of Pathologists and for acceptance to the fellowship I need to complete a doctoral level thesis and sit an oral exam with the college. It is intended that this thesis is the next step on the route to the fellowship.

6.3. **Advanced research techniques**

This module looked in some detail at the types of techniques generally found in doctoral level projects. Split into Quantitative methods and Qualitative methods, the two parts of the course were aimed at very different types of research. I am much more comfortable with quantitative research, the use of numbers to reach a conclusion. I find this intuitive and easy to understand and I received a high score for this part of the assignment. Although some of the specific techniques were new to me, such as the use of the SPSS® software, each part followed on simply from my previous knowledge.

It was different with the qualitative part of the module. Qualitative research remains a foreign language to me and while I may understand some of the background and general principles, the actual interpretation and analysis give me difficulty. The poor quality assignment I wrote was clearly a numerical persons attempt at qualitative research, with each individual phrase processed in excel almost as if it was a piece of numerical data. In trying to use a phenomenological approach to the data, one of the markers commented that I did not really pick out the meanings from the interviews. A personality assessment carried out through work has identified that I am not really sensitive to others feelings and the poor mark I received for this assignment reflects this.

However, this part of the unit has taught me about qualitative research and when discussing work with a social science researcher I was able to hold sensible conversations about the work without having to ask the most basic questions. I know that this area is not for me, but at least I understand that and know somebody I can refer to if this sort of data analysis is ever required.

6.4. **Publication and dissemination**

This module was one of the most enjoyable of the whole course and helped to give me the background to publication, for example an understanding of the whole process, and also of the effects of a journal's impact factor.

One of the main factors to come from the course was a realisation that much of the published work available is of relatively poor quality. While the aims and objectives of papers are frequently clearly stated, the completeness of the information is often lacking. When recently asked to interpret the results of a cocaine test done in leg hair of an Asian donor, I needed to assimilate data from several papers to reach a reasonable interpretation. Part of the reason for this was that there was one paper commenting on the difference between African and Caucasian hair (which incorporate cocaine differently), a second paper comparing cocaine levels in hair samples from different parts of the body and a third comparing relative levels of cocaine in hair according to the amount of drug used. While each paper was complete in itself, they were limited by the populations and therefore the subject data.

I have not written any papers since the start of this course, although I have made significant contributions to two papers and I have published 2 posters where I have been wholly or mainly responsible for the content and 2 posters where I have contributed. I want to publish more work and am awaiting a positive opportunity. A change of role within the laboratory has recently changed this and I have several papers or short communications that I aim to publish within the next 12 months. Without the understanding given during the lectures and particularly the assignment, I would not be contemplating publication. The assignment walked us through the publication process for two separate papers, one written by ourselves and one written by another student. By writing and commenting on other student's papers, and responding to the comments, it was possible to see the whole process from beginning to end. Although much work (and extensive printing) I believe that this assignment is a good introduction to publication and would recommend to the course leader that this element of the course is retained.

Although I have not published much written peer reviewed material I have written an article for Occupational Health magazine and have made a large number of oral presentations, both to scientific and non-scientific audiences. I really enjoy this aspect of my work and would like to do more of this. At the outset of the professional doctorate course, my public speaking skills were poor. I volunteered to give the first presentation in the second or third week of the course, not because I was confident but because I wanted to get it over and done with. I was then able to relax and watch how others performed and to learn from them. In the space of around 18 months, I went from a person reading from cue cards and gripping the table for support to somebody who can ad-lib a presentation to 100 people without nerves, walking around near the lectern and without any cues other than the contents of Powerpoint® slides. While presentation skills was not a directly taught element of the course, the presentations that were required and the way that this part of the course was structured made a very big difference to my improvement in presentation ability. The lecture I am required to give at the end of this course has, for me, been a very big target: I *will* perform well in this lecture.

6.5. **Project proposal**

The final part of the taught component of the course was the project proposal. Unlike others, my project was unencumbered by ethics. However, it was not always the case.

The original project that I wanted to pursue was a comparison of the results obtained, including clinical outcome, of drug users in treatment using either traditional laboratory drug testing or point of care drug tests. I worked on this project proposal for around a year before switching to this method development and this year taught me a lot. As well as some of the structure of community addiction services, I did learn about some of the ethics committee submission procedures and how to structure a project. It was more complex because the aim was to demonstrate equivalence, which is always much harder than to prove a difference. The reason that the project was finally dropped was that after promising initial discussions, the consultant with whom I was planning to work stopped returning my calls and e-mails. In consultation with my supervisors, my project was altered. Perhaps this taught me as much about the research and development process as if everything had worked right first time. As I write this, I am hoping again to work with the local addiction services and am coming up against some of the same problems. With what I learned last time and how I have developed professionally since, I am confident that I can make some progress here.

This perhaps summarises the whole course for me. It is not what I learned in the lectures that has hopefully earned me the title of Doctor. It is the process that I have gone through, the discussions that I have had along the way and how I have applied my knowledge that has made the difference. This thesis on its own is not a doctorate, it is simply one measureable objective along the pathway of professional development.

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8. Appendices

Appendices may be found on the CD accompanying this thesis.

Contents of Appendices CD